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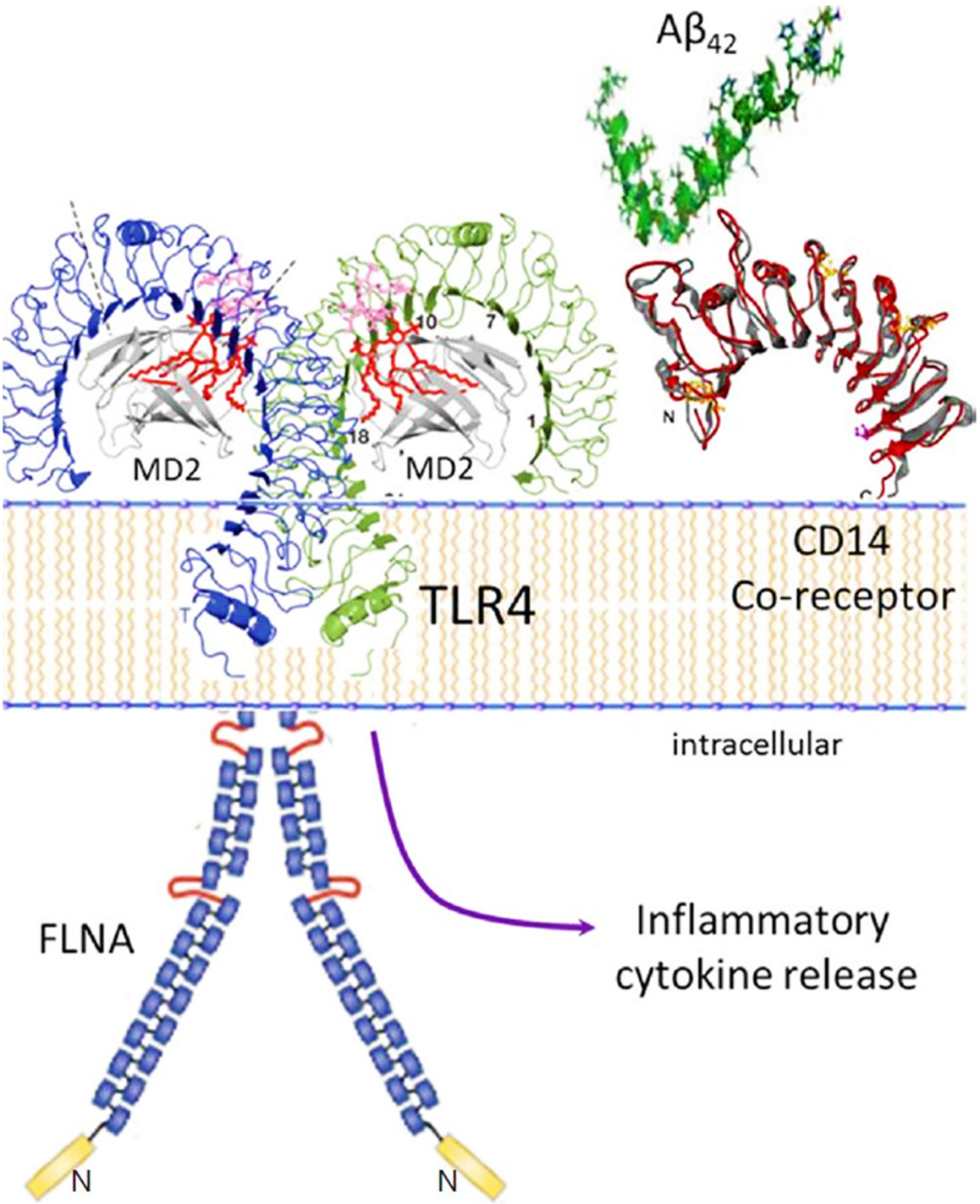

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CONTENTS

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Original Article **Open Access**

Meningeal carcinomatosis: a retrospective analysis of seventy-seven cases

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Meningeal carcinomatosis, cerebrospinal fluid, magnetic resonance imaging, therapy

ABSTRACT

Aim: Meningeal carcinomatosis is a special type of malignant tumor characterized by short survival and poor prognosis. In the present study, the authors aim to analyze the clinical, laboratory data and prognosis of meningeal carcinomatosis patients. **Methods:** The authors enrolled 77 cases of meningeal carcinomatosis from 2003 to 2013 in the First Hospital of Jilin University. The clinical data including age, gender, symptoms at onset, clinical manifestations, primary tumors and the laboratory data including cerebrospinal fluid (CSF), tumor markers as well as the imaging data were analyzed. The interval between the onset of primary tumor and the onset of central nervous symptoms, treatments and survival time were also analyzed. **Results:** The onset of meningeal carcinomatosis was usually acute (46.2%) or subacute (39.0%). The most frequent symptom at onset was intracranial hypertension (70.1%). Symptoms such as headache, vomit and high lumbar puncture intracranial pressure was observed in 56% of cases during the course of the disease. CSF abnormalities such as higher protein concentration (73.4%), more CSF pleocitosis (57.1%) and lower glucose levels (48.4%) were found in 95.3% of meningeal carcinomatosis patients. Non-contrast enhanced cerebral magnetic resonance imaging (MRI) showed that 13.2% patients had abnormal meningeal changes while in the enhancement scan 35.3% patients showed changes. The serum tumor markers increased in 84% of the patients. There were no differences regarding the mean survival between patients who received intrathecal chemotherapy and those who received brain radiotherapy or supportive treatment. **Conclusion:** The most common clinical manifestation of meningeal carcinomatosis is intracranial hypertension. The most common primary tumor is lung cancer, followed by gastric cancer and breast cancer. The linear enhancement of meningeal on the MRI scan is of great importance for the diagnosis of meningeal carcinomatosis.

Quick Response Code:

INTRODUCTION

Meningeal carcinomatosis (MC) is caused by the spread of cancer cells to the leptomeninges and by their dissemination within the cerebrospinal fluid (CSF).[1] MC prognosis is usually poor with a short survival.[2] In recent years, several studies focused on the early diagnosis of MC. CSF cytology combined with CSF tumor markers were reported to have great value for early diagnosis and for addressing the origin of meningeal carcinomatos.[3]

METHODS

Patient information

From 2003 to 2013, patients who admitted to Department of Neurology at the First Hospital of Jilin University and fulfilled the diagnostic criteria of MC were enrolled.[4] This retrospective study was approved by the ethics committee of the First Hospital of Jilin University, Changchun, China.

Clinical data

A total of 77 patients were enrolled in our study. We collected data about the gender, age of onset, type of onset, initial symptoms, clinical manifestations, physical examination, time and frequency of lumbar puncture, CSF pressure, routine biochemical and cytological examination results, CSF tumor markers, imaging examinations [head computed tomography, magnetic resonance imaging (MRI) scan and enhanced scan], serum tumor markers, primary tumors, the interval between primary tumor and central nervous symptoms, treatment methods and the survival time.

The above data was provided by the First Hospital of Jilin University Clinical Laboratory and Imaging Department.

Statistics

Statistical analysis was performed using SPSS version 17.0 software (SPSS, IBM, West Grove, PA, USA). Categorical data were presented as proportions, while continuous data were presented as means and standard deviations of means or interquartile ranges depending on the distribution of the data. Differences in proportions were tested by the Chi-square tests and differences in continuous variables were tested by student *t*-tests. For all statistical tests, $P < 0.05$ was considered to be significant.

RESULTS

The demographic data of MC patients

Among the 77 patients enrolled to the study, 35 were males and 42 were females. The median age at diagnosis was 55 years old (ranging from 2 to 76 years old).

Clinical manifestations and physical examination findings

In this patient cohort, there were 36 (46.2%) cases with acute onset, 30 (39%) cases with subacute onset and 11 (14.3%) cases with chronic onset. Most patients were admitted to hospital with increased intracranial pressure symptoms such as headache, nausea and vomit. Some patients showed cranial nerve or brain parenchyma damage symptoms and severe cases presented with unconsciousness or cerebralhernia. Physical examination revealed meningeal irritation, pyramidal signs, cranial nerve paralysis, *etc*. The main symptoms and signs are shown in Table 1.

Lumbar puncture and CSF routine test

Intracranial pressure, CSF cytology, tumor markers and immunoglobulin

The high lumbar puncture intracranial pressure was observed in 56% (32/57) of cases, and 21.1% of them showed over 400 mmH_2O. The distribution of the intracranial pressure is shown in Figure 1.

In the first CSF cytology examination, 82.2% (60/73)

Table 1: Symptoms and signs of meningeal carcinomatosis patients

CNS involvement symptoms and signs	Number of cases (%)	Cranial nerve involvement	Number of cases (%)	Spinal and PNS involvement	Number of cases (%)
Headache, nausea and vomiting	54 (70.1)	Abducens nerve palsy	10 (13.0)	Bilateral limbs weakness or paresthesia	12 (15.6)
Dizziness	21 (27.3)	Oculomotor nerve paralysis	10 (13.0)	Tendon reflexes diminish or disappear	11 (14.3)
Hyperspasmia	11 (14.3)	Facial paralysis	10 (13.0)	Neck and shoulder pain	5 (6.5)
Mental disorders	5 (6.5)	Double vision	7 (9.1)	Unsteady gait	3 (3.9)
Disturbance of consciousness	3 (3.9)	Hypoglossal nerve palsy	7 (9.1)	Rectal bladder dysfunction	1 (1.3)
Papilledema	4 (5.2)	Decreased vision	4 (5.2)	Saddle area sensory loss	1 (1.3)
Pyramidal signs	20 (26.0)	Hearing loss	4 (5.2)	Lasegue positive sign	1 (1.3)
Meningeal irritation	41 (53.2)	Pronunciation or dysphagia	4 (5.2)		

CNS: central nervous system; PNS: peripheral nervous system

cases showed abnormal cells; while in 8.2% (6/73) cases abnormal cells were found through a second time lumbar puncture; only in one case tumor cells were found in the third lumbar puncture. Cell morphology analysis revelaed that 14 cases showed adenocarcinoma cells. The remaining cases were characterized by an increase in cell size, irregular shape, cell body stain and darker cytoplasm. Cell membranes were incomplete and with protrusions. Nuclear cytoplasm ratio increased, and nucleus were centered or showed deviation, occasionally they were double-nucleated. In some cells, cytoplasmic vacuoles were observed near the membrane. Some mitotic cells could also be observed. The cytology results for different cancers are shown in Figure 2. Only 3 cases of the CSF tumor markers were checked, and all the results were abnormal. Patients with elevated IgG accounted for 72.7% (16/22).

CSF routine biochemical examination was found abnormal in 95.3% (61/64) patients. High protein accounted for 74% (46/62) (normal range 0.15-0.45 g/L), reduced glucose was 45% (27/60) (normal range 2.3-4.1 mmol/L), reduced chlorine accounted for 38% (23/60) (normal range 119-129 mmol/L), elevated white blood cell count was 59.7% (37/62) (normal range 0-8 × 10^6/L), which was given priority to mononuclear cells.

Tumor marker changes in MC patients

The rate of abnormal serum tumor markers was 84%. Sixty-eight percent (17/25) of the patients had increased carcinoembryonic antigen. In 44% (11/25) of the patients CYFRA21-1 increased. The rate of the increased CA125 was 32% (8/25). 32% (8/25) of the patients had increased CA199. In 28% (7/25) of the patients NSE increase was observed. The rate of the increased CA153 was 24% (6/25). There were 16% (4/25) of the patients who had increased CA724. Four percent (1/25) of the patients' alpha-fetoprotein increased. The rate of elevated blood sedimentation was 66.7% (10/15).

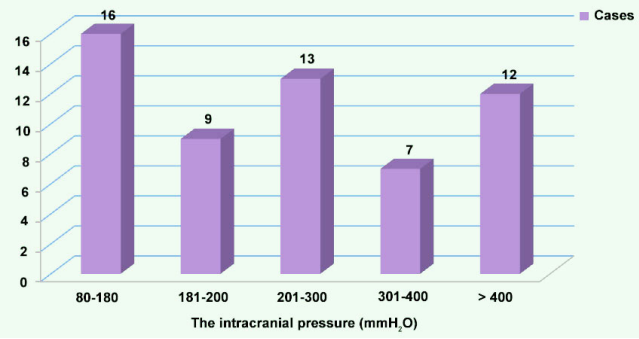

Figure 1: The distribution of the intracranial pressure

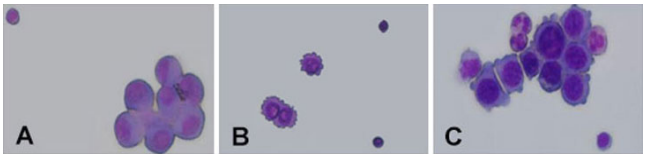

Figure 2: Cytology results for three types of cancer patient. (A) One case of lung cancer meningeal carcinomatosis cerebrospinal fluid cytology test result; (B) one case of breast cancer meningeal carcinomatosis cerebrospinal fluid cytology test result; (C) one case of gastric meningeal carcinomatosis cerebrospinal fluid cytology test result

Imaging findings in MC patients

Twenty-four cases had head computed tomography examinations. Among these, one of them showed that meninges thickened significantly. One case showed the expansion of the ventricles and hydrocephalus, the others had no significant abnormalities. Fifty-three cases had head MRI scan examinations and 17 (32.1%) of them were abnormal. A total of 12 (35.3%) cases had meningeal reinforcement in the 34 cases of enhanced scan.

Primary tumors

The most frequent primary tumor in our study was lung cancer (35/77, 58.3%), followed by gastric cancer (10/77, 16.7%), breast cancer (6/77, 10%), melanoma (3/77, 5%) and non-hodgkin's lymphoma (2/77, 3.3%). In addition, one patient presented with primary lesion in ovarian and one presented with colon cancer. In one case the primary tumor was nasopharyngeal carcinoma, and in another it was acute lymphocytic leukemia. Seventeen (22.1%) had no primary tumor. The interval from diagnosis of primary tumor to the onset of central nervous system (CNS) symptoms was also analyzed: 45 cases (58.4%) initially presented with CNS symptoms without history of tumor; 26% (20/77) patients developed CNS symptoms when the primary tumor had been diagnosed for no more than one year; 6.5% (5/77) patients experienced CNS symptoms at one to two years after the diagnosis of primary tumor; 9.1% (7/77) patients did not experience the CNS symptoms until the primary tumor has been diagnosed for more than two years.

Treatment and survival

Thirteen patients received intrathecal chemotherapy (with methotrexate or cytarabine or dexamethasone) and radiotherapy treatment and other 13 patients received only symptomatic and supportive treatment. The remaining patients were lost during the follow-up. The mean survival period in the radiotherapy and chemotherapy treatment group was 24.77 ± 22.80 weeks, whereas in the symptomatic and supportive treatment group it was 12.46 ± 18.00 weeks (P = 0.14). There was no statistically significant difference between the survivals of these two groups.

DISCUSSION

MC is a special type of metastatic carcinoma of the central nervous system with a incidence of 5-10% in all solid tumors.[5,6] As a gradual increase of incidence and a high mortality have been observed over time, early diagnosis is crucial. Due to its diverse clinical manifestations, it is difficult to make a diagnosis only with imaging examinations and CSF cytology is necessary to find abnormal cells.

At onset, the age is similar between MC and other tumors. In fact, MC can be found at any age, but it is more common in the elderly. Most patients present with acute or subacute onset, while a small number of patients with primary tumors may be in chronic onset. Since cancer cells can move into the CSF and infiltrate the dura and the brain parenchyma, clinical evidence of the disease could appear as symptoms of meninges and brain parenchyma damage,[7] cranial nerve palsy and spinal nerve root symptoms.[8] The most frequent initial symptom is intracranial hypertension such as headache, nausea and vomit. The main reason for headache is the increasing intracranial pressure caused by cancerous obstruction of CSF circulation pathway. Besides, the meninges irritation is partly responsible for headache.

In this study, we found 11 (14.3%) cases of convulsions, 5 (6.5%) of mental disorders such as illusion and personality change, 3 (3.9%) of conscious disorder and other CNS damaged symptoms. The common signs include 41 (53.2%) cases of meningeal stimulation and 20 (26%) cases of positive pyramidal tract, *etc*. The most common damaged nerves include abducens nerve, oculomotor nerve and facial nerve, and each type of damaged nerve accounts for 10 cases (13%) in our study. The other cranial nerves such as optic nerve, auditory nerve, hypoglossal nerve, glossopharyngeal nerve also can be involved, which was similar with what already described in the literature.[9] In this study, there were 12 (15.6%) patients with bilateral limbs weakness or paresthesia, 11 (14.3%) patients with tendon reflexes diminished or disappeared, 5 (6.5%) patients with the neck shoulder pain which is the effect of the infiltration of tumor cells and the irritation of the spinal nerve root. In addition, there was one case with rectal bladder dysfunction and saddle area sensory deficiency. The Kernig sign and the Lasegue sign may be positive in the physical examination.

The cerebral vascular endothelial permeability increases when the meninges are stimulated by tumor cells and produce various chemicals, at the same time the CSF circulation path may be blocked by tumor cells. All of above can cause increase in the intracranial pressure. This is confirmed by lumbar puncture measurement. Regular and biochemical tests were used in 62 cases. The protein level and white blood cell count in CSF were found elevated in 47 (47/62, 73.4%) cases and 36 (36/62, 57.1%) cases, respectively. The obstruction of CSF circulation path, the disruption of the blood-brain barrier and the increase of vascular endothelial permeability may cause the protein extravasation and diapedesis. Interestingly, 30 (30/62, 48.4%) cases showed a lower CSF glucose content than normal, which could be the result of glucose consumption caused by the proliferation and strong metabolism of tumor cells. In addition, as it is difficult for blood glucose to pass through the blood brain barrier, the CSF glucose lacks the timely supply from blood, which is another reason for the drop in glucose content.[10] In 26 (41.9%) cases lower CSF chloride value was observed. A lot of glycolysis produced a great amount of acid metabolites, facilitating the CSF chloride decline (as the chloride reduces easily in acid conditions). At the same time, loss of large amount of gastric acid after frequent vomiting is another reason for chloride reduction. So the overall abnormal rate of protein, glucose, chloride and white cells in CSF was high.

Seventy-three cases underwent CSF cytology examination. Tumor cells in the CSF were found by one lumbar puncture and two lumbar punctures in 60 cases (82.2%) and 6 cases (8.2%), respectively. Only in one case tumor cells were not found until the third time lumbar puncture. Enough samples combined with accurate assessment from clinical physicians would improve the diagnostic rate.[11] Only in 3 cases, CSF tumor markers were abnormal. It is difficult to diagnose the source of tumor cells on the basis of cytology morphology. We can combine cytology examination with CSF tumor markers to diagnose the properties and origin of tumor cells.[12]

It is difficult to diagnose MC through standard MRI scan. But we can find some indirect signs such as ventricular expansion, hydrocephalus, *etc*. There are a lot of dural blood capillaries in normal humans, but there is no blood brain barrier because lack of close connection between endothelial cells. Although the capillaries of leptomeninges are fewer than dura, the blood brain barrier exists because there are lots of close connection between endothelial cells.[13] The normal dura may be strengthened during head MRI enhanced scanning, but it does not show nodular or hybrid reinforcement. MC also destroys blood brain barrier, in fact the majority of patients shows dural abnormal reinforcement such as nodositas, line type and mixed type. A small number of patients have no

obvious reinforcement. The leptomeningeal abnormal reinforcement may be due to the direct infiltration of tumor cells from dura or from choroid plexus. Enhanced scan can discover more abnormal meninges, which play an important role in the auxiliary diagnosis of MC.

Serum tumor markers have important significance for the source of the tumor. Tumor markers can have different levels in different clinical periods and can reflect the dynamic changes, recurrence, metastasis, or treatment effect of the tumor. The abnormal rate of serum tumor markers is 84% in our study. The erythrocyte sedimentation rate, in some physiological conditions, such as anemia, menstrual period, after three months of pregnancy, infection, malignant tumor, tissue necrosis and other diseases, was found increased. The change is not specific, but has great value for malignant tumors fast development.

Most patients with MC have primary tumor. However, in some patients with CNS damage as the first symptom no primary tumor is found, or is found only after a period of time. In this study, primary tumor incidence in patients is consistent with the incidence of MC[4] in China and the most common is lung cancer.

Literature reported that untreated patients with MC survive for 4-6 weeks whereas patients undergoing regular treatment survive for 4-6 months.[14] In this study, the difference was not statistically significant when the survival of patients with radiotherapy and chemotherapy was compared with symptomatic treatment patients. Although patients with MC received intrathecal injection of chemotherapy and whole brain radiotherapy, the treatment did not improve survival. Because MC is considered the terminal stage of the disease, concurrent chemoradiation has no impact on patient outcome, but can improve clinical symptoms.[15]

In this study, the clinical manifestations of 77 patients are diverse, but most of them are characterized by increased intracranial pressure symptoms such as headache, nausea and vomit. Therefore, when patients have above symptoms, we should be vigilant about MC and improve the head MRI and enhanced MRI scan, if necessary. CSF cytological examination is the gold standard of the diagnosis of MC. Because CSF tumor marker has not been regarded as a standard criterion of diagnosis and due to the lack of a recognized standard reference value, its application is still restricted. However, since tumor markers have significant implications for primary tumor source, combining this with serum tumor markers examination would contribute to identify the source of tumor cells. Through the analysis of MC, we hope to improve the early diagnostic rate of the disease and help clinicians develop a detailed and suitable treatment plan for patients to increase benefit.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Informed consent was obtained from all individual participants included in the study.

Ethics approval
This study was approved by the ethics committee of the First Hospital of Jilin University.

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Original Article **Open Access**

Single low-dose lipopolysaccharide preconditioning: neuroprotective against axonal injury and modulates glial cells

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Dr. Brandon P. Lucke-Wold is completing the MD/PhD program, Master's in Clinical and Translational Science, and global health track at West Virginia University. He is also co-founder of SwifTag Systems, a biotechnology company specializing in efficient and cost-effective laboratory animal tagging, inventory, and tracking. He plans to pursue neurosurgery residency training following graduation from West Virginia University. He enjoys running and rock climbing as well as spending time with his wife and newborn daughter.

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Lipopolysaccharide preconditioning, oncostatin M receptor, diffuse axonal injury, gliosis, neuroprotection

ABSTRACT

Aim: Over 7 million traumatic brain injuries (TBI) are reported each year in the United States. However, treatments and neuroprotection following TBI are limited because secondary injury cascades are poorly understood. Lipopolysaccharide (LPS) administration before controlled cortical impact can contribute to neuroprotection. However, the underlying mechanisms and whether LPS preconditioning confers neuroprotection against closed-head injuries remains unclear. **Methods:** The authors hypothesized that preconditioning with a low dose of LPS (0.2 mg/kg) would regulate glial reactivity and protect against diffuse axonal injury induced by weight drop. LPS was administered 7 days prior to TBI. LPS administration reduced locomotion, which recovered completely by time of injury. **Results:** LPS preconditioning significantly reduced the post-injury gliosis response near the corpus callosum, possibly by downregulating the oncostatin M receptor. These novel findings demonstrate a protective role of LPS preconditioning against diffuse axonal injury. LPS preconditioning successfully prevented neurodegeneration near the corpus callosum, as measured by fluorojade B. **Conclusion:** Further work is required to elucidate whether LPS preconditioning confers long-term protection against behavioral deficits and to elucidate the biochemical mechanisms responsible for LPS-induced neuroprotective effects.

Quick Response Code:

INTRODUCTION

Endotoxin preconditioning with lipopolysaccharide (LPS) was previously shown to be neuroprotective in models of ischemic stroke and traumatic brain injury (TBI).[1] The neuroprotective mechanisms of LPS remain unclear, particularly with regards to vascular-mediated effects. Prior studies have studies this using contusion-based models of neurotrauma. The contusion model irreversibly injures a focal brain region, creating a gross structural void associated with significant vascular injury.[2] The mechanisms of LPS preconditioning are poorly understood and have not been studied in a model of diffuse axonal injury. There is emerging evidence that pro-inflammatory regulation is neuroprotective in models of neural injury,[3] particularly the regulation of pro-inflammatory cytokines and microglial phenotype changes. Microglia is pleiotropic and transition between continuum states. These states are termed M1 or M2 depending on the inciting events.[4] Microglia may have protective and destructive roles, depending on the transition state.[5] The ratio of microglial states can predict the subacute outcome following injury. M1 microglia are pro-inflammatory and can cause long-term deficits.[6] Although LPS has been shown to influence astrocyte phenotypes, it is not clear whether LPS preconditioning modulates microglial phenotypes following diffuse axonal injury.[7]

In this study, we investigated the neuroprotective effect of LPS preconditioning in a closed-head model of diffuse axonal injury for the first time. This model of TBI has increased clinical relevance due to axonal shearing seen with diffuse neurotrauma.[8] We hypothesized that LPS preconditioning would reduce inflammation and neurodegeneration following diffuse axonal injury. In addition, we predicted that LPS preconditioning would regulate the oncostatin M receptor (OSMR) in astrocytes and activate the M1 microglia phenotype after TBI. TBI promotes pro-inflammatory cascades and increases the expression of glial fibrillary acidic protein (GFAP);[2,9] however, a link between astrocyte regulation and the microglia phenotype after TBI has not been investigated. In this study, we explored the effect of LPS preconditioning on astrocytes and microglia and addressed the relationship between cytokine receptor expression, astrocyte reactivity, and microglial phenotype. Improving our understanding of the protective effects of LPS preconditioning may accelerate the identification of novel therapeutic targets that reduce damage after TBI in individuals at risk of concussion.

METHODS

Animals

All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of West Virginia University and were performed according to the principles of the Guide for the Care and Use of Laboratory Animals, published by the Institute of Laboratory Resources, National Research Council (National Institutes of Health publication 85-23-2985). Thirty-two male Sprague Dawley rats (Hilltop) at 3-4 months of age were used in this study. Animals were given standard rat chow and water *ad libitum*.

LPS preconditioning

Rats were pretreated with a single intraperitoneal injection of either 0.2 mg/kg LPS (Sigma-Aldrich) or 0.9% saline (equal volume) 7 days prior to TBI.

Locomotor behavior

After LPS injection, the development of sickness behavior was monitored using activity chambers as described by Godbout *et al*.[10] Locomotor activity was assessed using an automated activity monitoring system (San Diego Instruments, San Diego, CA) that recorded beam breaks in the x, y, and z planes. Animals were acclimated to the room for 1 h prior to testing. Testing chambers consisted of square Plexiglass housing and 16 × 16 photobeam arrays to detect lateral movements. An 8 × 8 array, located above the 16 × 16 array, detected rearing-associated movements. Activity was quantified over 30 min and the sum of fine, ambulatory, and rearing beam breaks was calculated to give the total number of beam breaks. These recordings were completed at 2, 4, 24, and 48 h post-injection.

TBI induction

Animals were divided into four groups: sham surgery (n = 8), sham surgery with LPS pretreatment (n = 8), impact-acceleration injury following saline injection (n = 8), and impact-acceleration injury with LPS pretreatment (n = 8). Anesthesia was induced and maintained using isoflurane (4% induction, 2% maintenance). Body temperature was controlled with a homeothermic heating blanket equipped with a rectal probe. Rats received an impact-acceleration injury as described previously.[11-13] Briefly, a 10-mm diameter and 3-mm thick stainless steel disk was affixed to the skull with cyanoacrylate between bregma and lambda. The animal was placed in a prone position on a foam bed with a metal disk directly beneath a 2-m tall Plexiglass tube. A 450-g weight was dropped from the top of the

tube, striking the metal disk. The disk was then removed while the rat was under anesthesia, the skull inspected, and the wound sutured. The animal was then returned to its cage, which was placed on a heating pad. Recovery from injury and anesthesia were monitored. No mortality was observed with the current injury parameters, and no gross lesions were apparent at the time of sacrifice indicating mild diffuse axonal injury.

Tissue preparation

Seven days after TBI, animals were anesthetized and perfused transcardially with physiological saline. Brains were removed and sectioned for histological analysis. The frontal cortex was selected for histological analysis. Tissue sections were placed in 4% paraformaldehyde for a minimum of 1 week. Following fixation, brains were processed using a Tissue-Tek VIP 5 Automatic Tissue Processor (Sakura Finatek, Torrence, CA). Processed tissues were paraffin-embedded with Tissue-Tek Tec 5 embedding system (Sakura Finatek, Torrence, CA) and sliced (6 μm) using a Leica RM2235 microtome (Leica Microscopes, Buffalo Grove, IL). Sections were mounted on glass slides and heat-fixed. Immediately prior to staining, tissues were de-paraffinized with xylene and alcohol washes.[14]

Fluoro-Jade B (FJB) staining

FJB staining was used to identify neural degeneration. For FJB labelling, slides were rehydrated through a series of alcohol and deionized (dH_2O) water rinses then incubated in 0.06% potassium permanganate for 10 min. Then, slides were rinsed for 2 min in dH_2O water and incubated with FJB in 0.1% acetic acid for 20 min. After staining, slides were washed three times in dH_2O.

GFAP staining

Tissues were incubated in rabbit anti-cow GFAP antibody (Dako, Glostrup, Denmark) at a dilution of 1:500 in 4% horse serum in Dulbecco's phosphate buffered (DPBS) overnight. Then, tissues were washed three times in DPBS and incubated in biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) diluted at 1:10,000 in 4% horse serum in DPBS for 4 h. Next, tissue was treated with avidin D-horeradish peroxidase (Vector Laboratories, Burlingame, CA) diluted at 1:1,000 in DPBS for 1 h. Sections were then stained in DAB chromogen solution (Vector Laboratories) for 5 min, then tissues were rinsed three times in DPBS and dried overnight.

M1 microglia staining

Tissues were incubated in mouse anti-rat CD68 antibody (AbD Serotec, Kidlington, UK) at a dilution of 1:100 in 4% horse serum in DPBS overnight. Sections were washed three times in DPBS and incubated in a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories) diluted at 1:10,000 in 4% horse serum in DPBS for 4 h. Following secondary antibody incubation, tissues were incubated in alkaline phosphatase (Life Technology, Carlsbad, CA) diluted at 1:100 in Tris-bovine serum albumin for 1 h. Then, tissues were rinsed three times in DPBS and incubated in Fast Blue BB salt (Santa Cruz Biotechnology, Santa Cruz, CA) for 5 min. Tissues were washed in xylene, mounted using an antifade agent, and cover slipped. The slides were sealed with acrylic and stored in the dark in a laboratory refrigerator.

GFAP and OSMR staining

Tissues were labelled with rabbit against anti-cow GFAP (DAKO) antibody at a dilution of 1:500 in 5% horse serum in PBS overnight at 4 °C. Next, sections were washed twice for 10 min each in PBS prior to application of Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Grand Island, NY) at a dilution of 1:100 in PBS for 3 h. Following secondary antibody incubation, slides were rinsed twice for 10 min each in PBS. Then, tissue was labelled with a goat anti-mouse OSMR antibody (LifeSpan Biosciences, Seattle, WA) at a dilution of 1:200 in 5% horse serum in PBS overnight at 4 °C. Following incubation, slides were rinsed twice for 10 min each in PBS prior before applying biotinylated anti-goat IgG (Vector Laboratories) at a dilution of 1:10,000 in 5% horse serum in PBS for 2 h. Next, slides were rinsed twice for 10 min each in PBS and incubated in Streptavidin Alexa Fluor 546 (Life Technology) at a dilution of 1:100 in PBS for 1 h. Slides were rinsed in PBS for 10 min and then coverslipped with Vectashield Mounting Media containing DAPI (Vector Laboratories). Finally, slides were sealed with acrylic and stored in the dark in a laboratory refrigerator at 4 °C. Images were acquired using a Zeiss Axio Imager 2 microscope and quantified using ImageJ with standard co-localization quantification techniques and the co-localization plugin established by Bolte *et al*.[15]

Histological quantification

Stereology and optical fractionation were used to quantify histological results as previously described.[16-18] Briefly, a region of interest encompassing the corpus callosum was drawn at low power using an Olympus AX70 microscope and StereoInvestigator software. The region encompassing the corpus callosum was chosen

because it undergoes robust biochemical changes following impact-acceleration TBI.[19] The software selected random 75-μm counting frames with a depth of 6 μm, and the object of interest was marked by an investigator blinded to treatment. The region of interest volume was previously identified, and the number of cells marked by the observer was quantified.

Statistical analysis

Data were analyzed using GraphPad Prism version 4.0. One-way ANOVA with *post-hoc* Tukey's test was used to compare histological findings across control and various experimental groups. Repeated measures two-way ANOVA was used to analyze the total activity data. An overlap coefficient of 0.6 or greater indicated strong co-localization, a coefficient between 0.4 and 0.6 indicated medium co-localization, and a coefficient < 0.4 indicated weak co-localization. A P value of < 0.05 was considered statistically significant for all studies.

RESULTS

LPS induces transient acute sickness behavior

LPS injection induces systemic sickness in rodents as evidenced by an acute reduction in activity.[10,20] Figure 1 shows a significant reduction in total activity after LPS administration compared with saline-treated animals based on time ($F_{3,66}$ = 8.14, P < 0.001), treatment ($F_{1,66}$ = 18.67, P < 0.001), and the time treatment interaction ($F_{3,66}$ = 22.92, P < 0.001) using two-way repeated measures ANOVA. Total activity was reduced by approximately 71% and 59% at 2 and 4 h post-injection, respectively. This was resolved within 24 h, indicating that sickness behavior was acute.

LPS preconditioning reduces neuronal degeneration and glial activation following TBI

Previous studies have demonstrated a neuroprotective effect of LPS preconditioning following controlled cortical impact injury with large vascular insult.[2] Figure 2 shows a significant difference in cortical FJB expression between experimental groups ($F_{3,32}$ = 59.79; P < 0.001). LPS preconditioning significantly reduced FJB levels following TBI according to one-way ANOVA with Tukey's *post-hoc* test (q = 8.50, P < 0.001). No difference was observed between sham-injured and LPS-treated animals (q = 0.13, P > 0.05), indicating that peripheral administration of LPS did not induce neurodegeneration.

GFAP expression in the cortex differed significantly between experimental groups ($F_{3,32}$ = 57.92; P < 0.001) [Figure 3]. LPS preconditioning significantly reduced GFAP levels after TBI according to one-way ANOVA and *post-hoc* Tukey's test (q = 8.70, P < 0.001). No difference was observed between sham-injured and LPS-treated animals (q = 2.89, P > 0.05), indicating that peripheral administration of LPS did not activate astrocytes.

LPS preconditioning reduces M1 microglia activation after TBI

To investigate the effect of LPS preconditioning on classically activated microglia, CD68 expression was quantified by stereology. Figure 4 shows a significant difference in CD68 expression between experimental groups ($F_{3,32}$ = 28.22; P < 0.001). The presence of M1 microglia (CD68 expression) was significantly reduced after TBI following LPS preconditioning compared with no LPS pretreatment according to one-way ANOVA with *post-hoc* Tukey's test (q = 9.77, P < 0.001). Importantly, no significant differences in CD68 expression were observed between injured animals with LPS preconditioning and sham-injured animals (q = 2.121, P > 0.05). These findings were consistent with IBA-1 staining for undifferentiated microglia, which showed a qualitative reduction in LPS pre-conditioned animals [Figure 5].

LPS preconditioning reduces OSMR expression in astrocytes after TBI

One of the primary mechanisms for regulating astrocyte activation is neuropoietic cytokine signaling through the gp130 receptor-signaling complex.[21] TBI associated with significant vascular injury upregulates members of the neuropoietic cytokine family, including

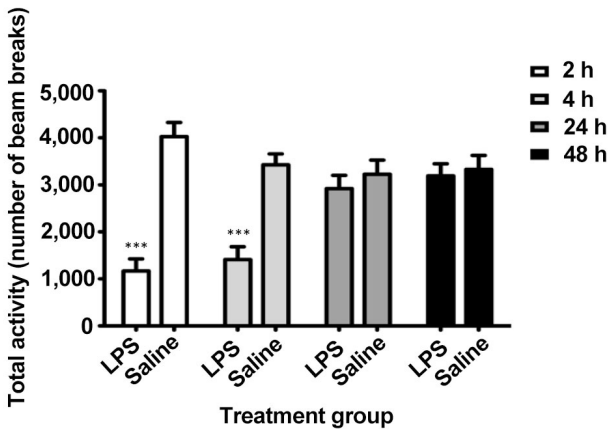

Figure 1: Total locomotor activity after LPS injection by number of beam breaks at 2, 4, 24, and 48 h after injection. Acute sickness was present at 2 and 4 h but resolved by 24 h. ***P < 0.001. LPS: lipopolysaccharide

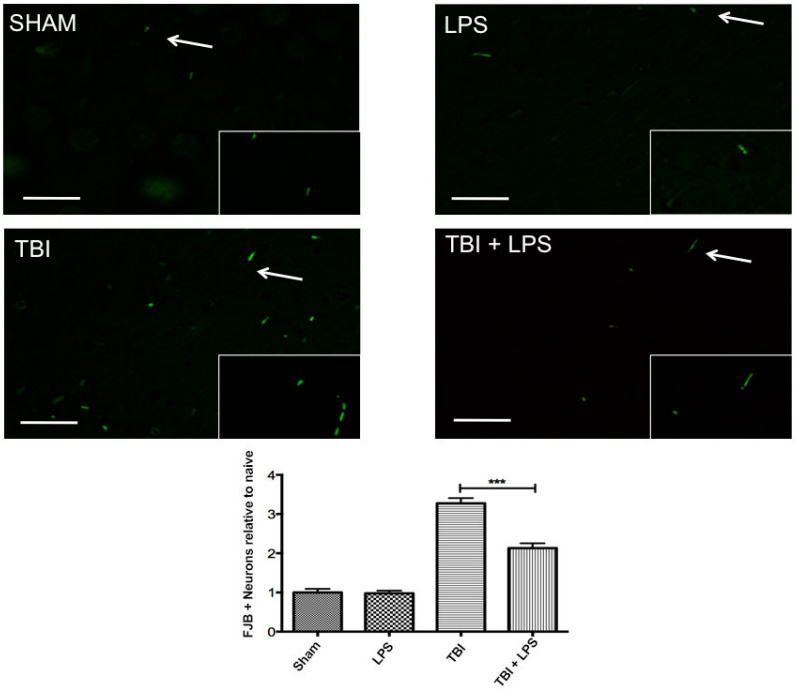

Figure 2: Neural degeneration increased following traumatic brain injury. Fluoro jade B significantly increased following traumatic brain injury but LPS preconditioning ameliorated this effect. Scale bar = 50 μm. ***P < 0.001. LPS: lipopolysaccharide; TBI: traumatic brain injuries; FJB: Fluoro-Jade B

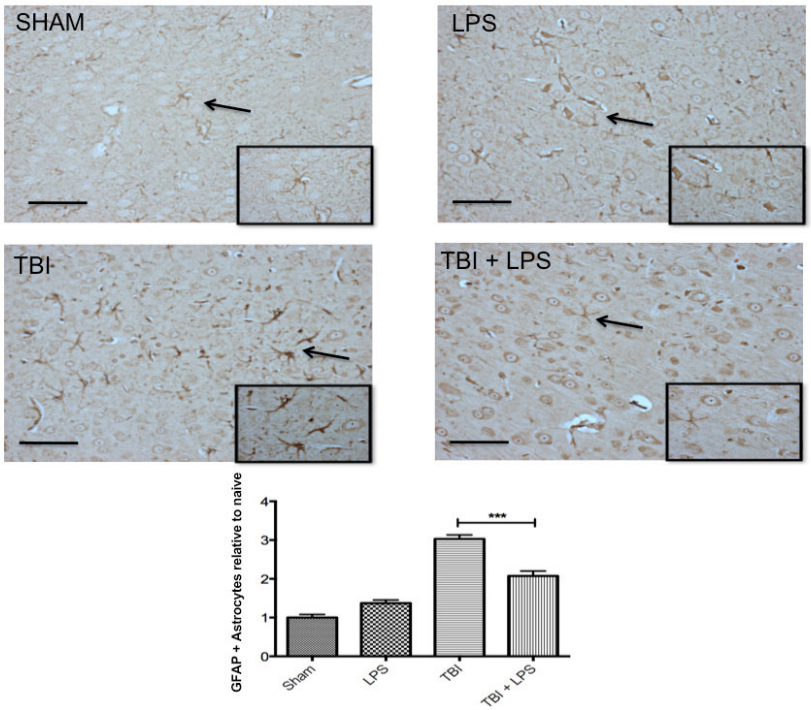

Figure 3: Astrocyte reactivity increased following TBI. GFAP increased significantly following TBI but LPS preconditioning ameliorated the effect. Scale bar = 50 μm. ***P < 0.001. LPS: lipopolysaccharide; TBI: traumatic brain injuries; GFAP: glial fibrillary acidic protein

OSMR.[22] We observed significant OSMR upregulation following diffuse axonal injury [Figure 6]. OSMR expression differed significantly between experimental groups ($F_{3,32}$ = 11.80; P < 0.05). OSMR expression was reduced after TBI in LPS pre-conditioned animals compared with no LPS pretreatment according to one-way ANOVA with *post-hoc* Tukey's test (q = 6.51, P < 0.05). No difference was observed between LPS-treated animals and sham-injured rats at this time point (q = 0.45, P > 0.05).

GFAP expression was quantified to measure astrocyte activation and was significantly different between experimental groups ($F_{3,32}$ = 6.30; P < 0.05) [Figure 6]. LPS preconditioning significantly reduced GFAP expression after TBI as shown by one-way ANOVA and *post-hoc* Tukey's test (q = 4.44; P < 0.05). Again, no difference was observed between LPS-treated and sham-injured rats at this time point (q = 0.45; P > 0.05). Importantly, there was a strong correlation between GFAP and OSMR expression evidenced by the yellow overlay (overlap coefficient r = 0.722), indicating a high degree of overlap within

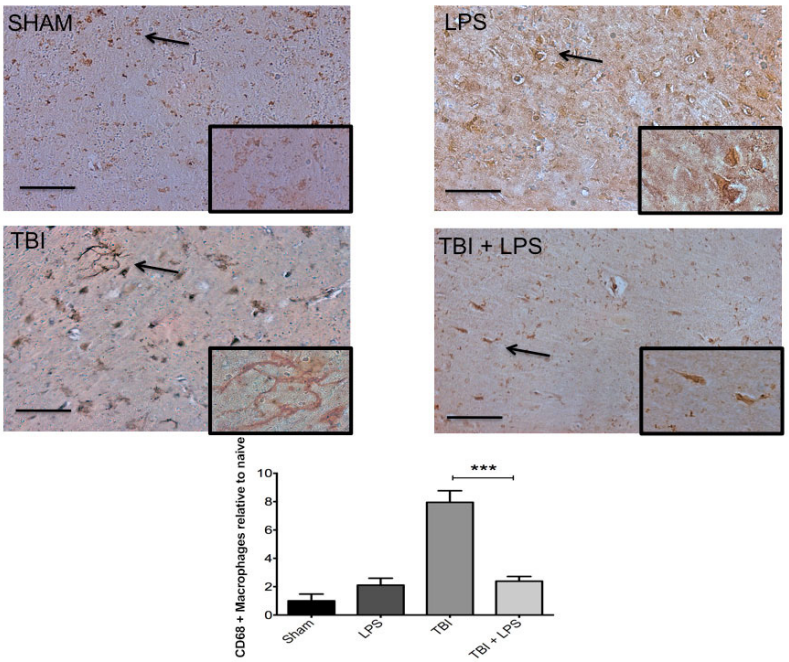

Figure 4: M1 microglia activation increased significantly following TBI. CD68 was significantly increased following TBI but LPS preconditioning ameliorated the effect. Scale bar = 50 μm. ***P < 0.001. LPS: lipopolysaccharide; TBI: traumatic brain injuries

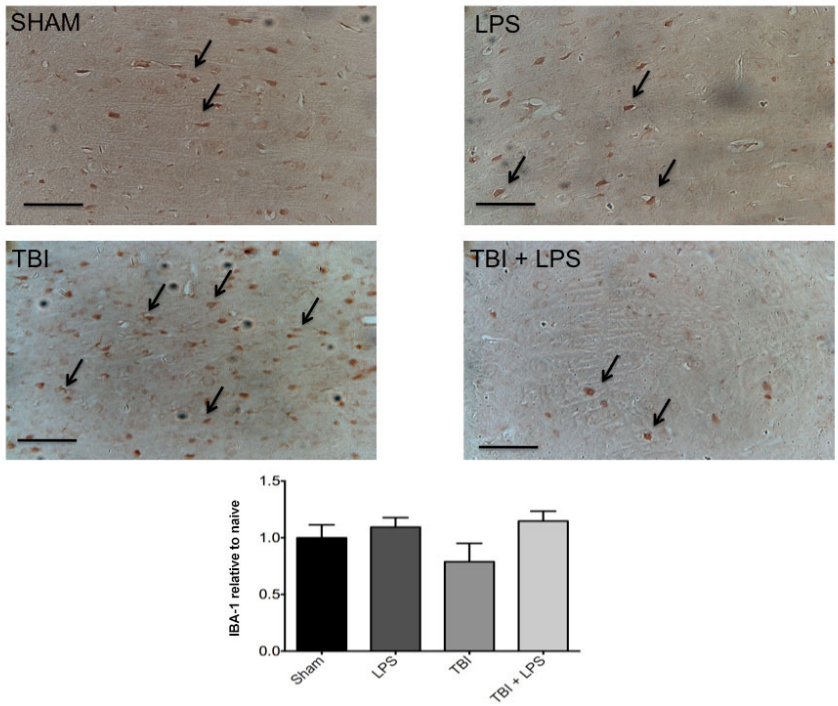

Figure 5: No significant differences were observed in IBA-1 microglia staining between groups. LPS: lipopolysaccharide; TBI: traumatic brain injuries

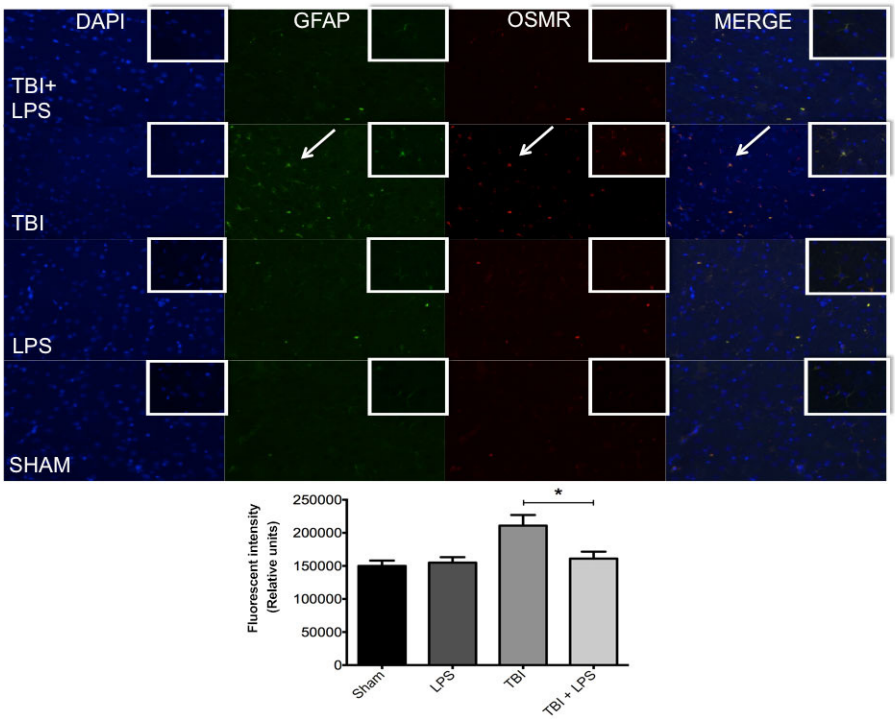

Figure 6: Colocalization of GFAP and OSMR with DAPI. GFAP (green) and OSMR (red) were significantly increased in the same cells (merged yellow) following TBI. LPS preconditioning prevented OSMR upregulation. *$P < 0.05$. LPS: lipopolysaccharide; TBI: traumatic brain injuries; GFAP: glial fibrillary acidic protein; OSMR: oncostatin M receptor

the same astrocyte. This was partially mitigated by LPS preconditioning (overlap coefficient $r = 0.478$).

DISCUSSION

Diffuse axonal injury is induced consistently by the weight drop method without producing a grossly visible lesion.[23] We have previously confirmed that the injury parameters used in this study induce diffuse axonal injury by measuring β-amyloid precursor protein expression.[24] The weight drop model is ideal for testing neuroprotective strategies because it induces consistent axonal damage and a characteristic progression of traumatic axonal injury in rodents.[25] Axonal injury reduces cerebral blood flow following neurotrauma.[26] Therefore, it is potentially worthwhile to investigate compounds that contribute to vascular preconditioning. Vascular preconditioning by heat activation reduces TBI severity and the extent of axonal damage by selectively activating hypoxia-inducible factor 1α.[27] Low-dose LPS pretreatment has also been used for successful vascular preconditioning in penetrating models of TBI.[28] One proposed mechanism for LPS is a reduction of inflammatory mediators before injury.[29] Inflammatory mediators can activate gliosis.[30]

In this study, we show for the first time that low-dose LPS preconditioning is protective in a closed-head model of diffuse axonal injury. LPS preconditioning has previously been shown to be protective in penetrating models, but there was significant vascular disruption in these models and they were generally more severe than our model of diffuse axonal injury. The findings of the present study are significant in that they demonstrate that LPS preconditioning regulates microglia and OSMR in a model of diffuse axonal injury. Furthermore, these protective effects are sustained at one week post-injury. Protection was established in a mild injury model with no mortality or gross pathological changes, indicating that LPS pretreatment may also protect against mild neurotrauma.

Longhi *et al*.[2] showed that LPS preconditioning alters IL-6 and OSM expression following TBI. In this study, we demonstrated that LPS may exert a neuroprotective effect against diffuse axonal injury through modulation of neurodegeneration and the gliosis response. This supports the notion that LPS induces neuroprotective effects originally proposed by Longhi *et al*.[2] We observed a transient acute sickness induced by LPS pretreatment. However, LPS preconditioning had the following effects, including: reduced FJB, OSMR, GFAP, and CD68 expression. Decreased FJB staining was indicative of reduced neurodegeneration. This has been demonstrated *in vitro* by Zhu *et al*.,[31] and our findings have now confirmed this *in vivo*. Increased

FJB staining has been associated with motor deficits following TBI.[32] In a future study, we plan to look at the role of LPS preconditioning in preventing motor deficits following TBI.

Reactive astrocytes can inhibit axonal regrowth after an axon is severed.[33] OSM activity increases in demyelinated areas, leading to an upregulation of OSMR. This upregulation indicates a loss of detrimental connectivity,[22] while decreased OSMR indicates preservation of myelin integrity and axonal tracts. Our findings show that LPS preconditioning significantly reduces OSMR levels [Figure 7]. Interestingly, GFAP expression was also downregulated, which may reflect an interaction between LPS and toll like receptor 4 (TLR4). Low-dose LPS administration can stimulate TLR4, which activates signaling cascades to suppress innate immunity and astrocyte activation. These cell-signaling events permit axonal regeneration without the threat of glial scar formation.[34] As long as myelin integrity is maintained, neurons can re-innervate most of their lost connections. The clinical use of LPS preconditioning is obviously limited because injury cannot be predicted, but targeting TLR4 pharmacologically might represent a reasonable strategy. Further work is needed to determine the exact interactions between low-dose LPS administration and TLR4.

In addition to effects on astrocytosis, LPS preconditioning has also been associated with microglial changes. LPS can promote the infiltration of macrophages into the brain, which helps resolve the microglia response after diffuse traumatic axonal injury.[35] Microglia is broadly grouped into pro-inflammatory M1 microglia and pro-survival anti-inflammatory M2 microglia.[36] The balance of M1 to M2 microglia is tightly controlled following injury.[37] M1 microglia can exacerbate axonal injury, thereby limiting functional recovery. We showed that LPS preconditioning selectively inhibited the M1 response. LPS caused an acute increase in pro-inflammatory markers, which may signal peripheral macrophages to cross brain vasculature by chemotaxis. Peripheral macrophages alter the inflammatory mileu of the brain while attenuating microglial activity.[38] In contrast, M2 microglia are largely unaffected due to the earlier peak of activation after injury, which we have shown using non-differentiated IBA-1 imaging.[39] The brain establishes functional recovery by shifting the balance away from M1 microglia.

LPS has been associated with inflammation. Suppressing inflammation limits the effect of LPS on human physiology.[40] LPS increases tissue necrosis factor α, interleukin 6, and interleukin 1 expression [Figure 6].[41] LPS preconditioning reduces neuronal loss and microglia activation in other injury models, such as global hypoxia.[42] In the present study, LPS preconditioning also significantly reduced neuronal degeneration following diffuse axonal injury. Chronic LPS-mediated inflammation is detrimental

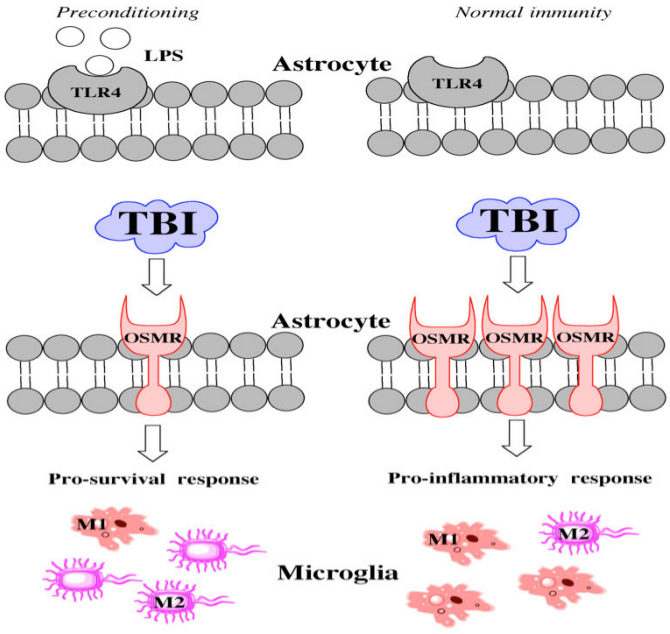

Figure 7: Hypothetical schematic showing the mechanism of LPS action. LPS: lipopolysaccharide; TBI: traumatic brain injuries; OSMR: oncostatin M receptor; TLR4: toll like receptor 4

to the brain, but acute LPS preconditioning provides "inflamaprepping" that primes the nervous system for response to injury.[43] Although inflamaprepping is not a universal to all TBIs, it offers a potential therapeutic advantage for a certain subset of individuals, namely soldiers and athletes. Soldiers and athletes are at increased risk of concussion and subconcussive injury. Prevent injury and enhancing recovery in these individuals is receiving increasing attention. Inflamaprepping with a systemically injected agent such as low-dose LPS is clinically feasible and may limit gliosis and subsequent glial scar formation by preparing the brain for trauma. Ultimately this would facilitate rapid axonal regeneration guided by preserved myelin tracts. In the current study, we have shown that inflamaprepping inhibits gliosis, downregulates the OSMR receptor, and shifts the microglia phenotype balance away from the pro-inflammatory M1 state, thereby decreasing neurodegeneration and promoting neuroprotection. The benefits of this neuroprotection on preventing behavioral decline will be investigated in a future study.

There are some limitations to the present study. Firstly, we did not assess post-injury behavior. Based on our histopathologic findings, we expect that LPS preconditioning prevents behavioral deficits following TBI, but needs to be verified in a future study. Secondly, we did not or examine glial marker expression later than 7 days after LPS treatment. This data would have indicated the current state of gliosis and inflammation at the time of injury.

In conclusion, we have shown for the first time that low-dose LPS preconditioning has protective effects in a diffuse axonal injury model. LPS preconditioning prevented both astrocyte and microglia activation through downregulation of the OSMR receptor. This protective effect was verified by reduced FJB staining, indicating decreased degeneration. Preconditioning and inflamaprepping may be viable targets for TBI treatment and may prevent long-term behavioral sequelae in patients. Future work will examine the long-term functional changes that lead to neurodegenerative disease progression and tauopathies. We will elucidate whether LPS preconditioning reduces tau hyperphosphorylation and improves behavior following repetitive injury. Mediating the gliosis response with LPS preconditioning may decrease neurodegeneration and slow the development of tauopathy.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patients were involved.

Ethical approval

Ethical approval was obtained prior to the commencement of the study.

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Case Report **Open Access**

Parasellar extra-axial cavernoma mimicking meningioma: a case report

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Extra-axial cavernoma, parasellar cavernoma, meningioma

ABSTRACT

Parasellar extra-axial cavernomas are rare lesions. The authors report a case of extra-axial cavernoma in a 50-year-old male patient, who presented with occipital headache and double vision. The magnetic resonance imaging showed an enhancing extra-axial dural-based mass in the left parasellar region invading cavernous sinus, hyper-intense on T2-weighted images, iso-intense on T1-weighted images and high relative cerebral blood velocity on magnetic resonance perfusion. The patient underwent a left pterional craniotomy and parasellar space occupying lesion was excised. Histopathology was suggestive of cavernous hemangioma.

INTRODUCTION

Cavernomas are benign. They usually have an intraparenchymal origin, but occasionally arise from the duramater. Extra-axial cavernous angiomas account for 0.4-2% of all intracranial vascular malformations. They usually occur in the middle cranial fossa, associated with the cavernous sinus. Here, we report a case of a 50-year-old male patient who presented with left occipital headache and diplopia due to a parasellar space occupying lesion mimicking a meningioma.

CASE REPORT

Case history

A 50-year-old man had a 1 month history of left occipital headache unresponsive to drug therapy with common analgesics. The intensity of this symptom increased over time. Later, it was associated with double vision. There was no history of seizures, vomiting and limb weakness. The patient was hemodynamically stable. His neurological examination was normal, except for left 6th and partial left 3rd nerve palsy.

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Imaging

The magnetic resonance imaging (MRI) showed an enhancing extra-axial dural-based mass in the left parasellar region invading cavernous sinus; the lesion size was 5.7 cm × 3.8 cm. It was hyper-intense on T2-weighted images [Figure 1A] with flow voids and iso-intense on T1-weighted images [Figure 1B]. There was marked homogenous enhancement in post-contrast study [Figure 1C]. MR perfusion showed high relative cerebral blood velocity [Figure 1D].

Surgery

The patient underwent a left extended pterional craniotomy with extradural clinoidectomy. Thorough devascularization of the extradural tumor based blood vessels was done. Dura was opened based on the middle meningeal artery and anterior sylvian fissure splitting was done. Enucleation and tumor debulking was done using ultrasonic aspirator, suction and bipolar cautery under microscopic assistance. Tumor was stiff, highly vascular, greyish white to red in colour and there was a clear arachnoid plane between the space occupying lesion and brain parenchyma. It was dura based on greater wing of sphenoid and left parasellar area. Excision of the tumor was almost total. The middle cerebral artery branches, 2nd, 3rd, 4th and 6th cranial nerves were identified and preserved.

Histopathology

Histopathology showed sections of a tumor mass composed of compactly aggregated vascular channels of varied sizes with thick hyalin walls and lined with endothelial cells. There were occasional foci of calcifications. But there were no other tissue element including meningothelial cells around the tumor mass or among the vascular channels. The findings were

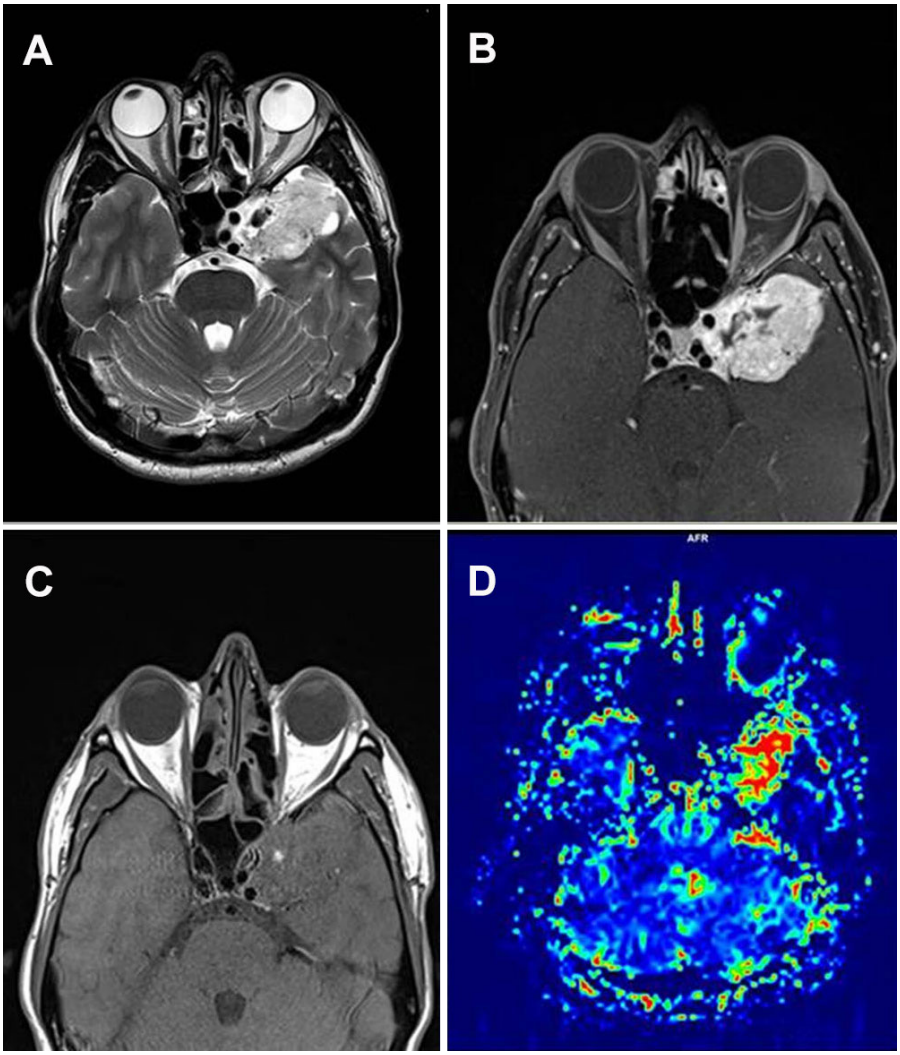

Figure 1: (A) High-resolution Axial T2 Fat sat sequences demonstrate hyperintense extra-axial lesion located in the left parasellar region, which extends to middle cranial fossa. The lesion partly encircles left distal ICA; (B) axial T1 W sequence shows iso-intense lesion; (C) post-contrast Axial T1 W sequences show marked homogenous enhancement; (D) perfusion map of CBV was obtained. High rCBV noted, demonstrating a highly vascular lesion. CBV: cerebral blood volume

suggestive of cavernous hemangioma [Figure 2].

Follow-up

The postoperative MRI brain with contrast did not show any residual hemangioma [Figure 3]. The patient was asymptomatic without any additional neurologic deficit.

DISCUSSION

Cavernous angiomas are vascular malformations. They are composed of enlarged sinusoidal vessels arranged in clusters, enclosed by a thin endothelial wall without interposed tissue within. They lack an elastic lamina, smooth muscles and are sometimes ossified or calcified. The term "cavernous angioma" has been used interchangeably with "cavernous hemangioma," "cavernous malformation," or "cavernoma". These lesions are vascular abnormalities rather than neoplastic processes.[1]

Cavernomas most commonly originates from the brain parenchyma. However, they also arise intraspinally, or from the dura.[2] Extra-axial dural-based cavernous malformations are extremely rare when compared to their intra-axial counterparts. In 1994, Lewis *et al*.[3] identified two types of dural cavernous angiomas.

(1) One present in the dura of the middle cranial fossa usually in the vicinity of the cavernous sinus.

(2) The second type consists of dural-based lesions such as the convexity, cerebral and cerebellar falx, the tentorium, posterior fossa, and the floor of the anterior fossa.[4]

The separation of the two types is important due to the differences in the patient population affected and the more aggressive clinical course of dural-based cavernous angiomas in the middle cranial fossa.

The malformations of the first group are more clinically aggressive because of their localization and vascular supply. In these cases, both preoperative radiation and embolization are recommended because they reduce intraoperative bleeding risk.

On the other hand, in the cases of the second group, neither radiation nor embolization is necessary to successfully remove cavernous hemangiomas outside the middle cranial fossa, since their vascular supply can be easily controlled through the surgical exposure.[5]

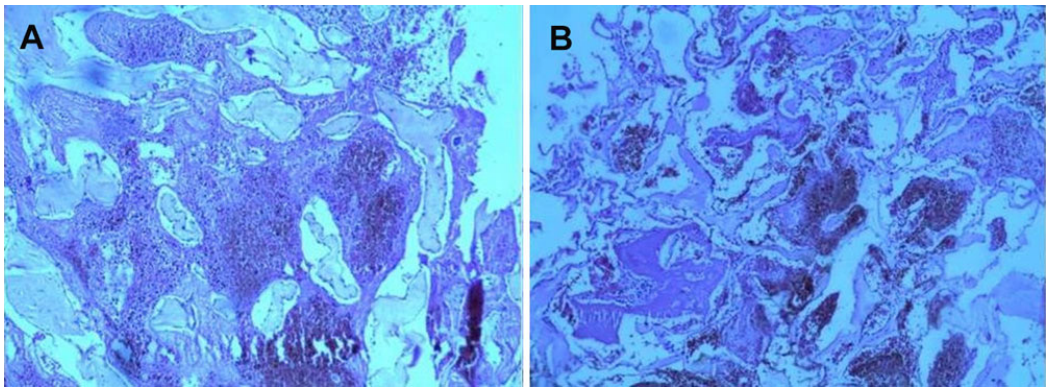

Figure 2: HE staining at low magnification (×10) showing (A) cavernous haemangioma and (B) dilated vascular channels with fibrous walls, devoid of intervening neuroglial tissue

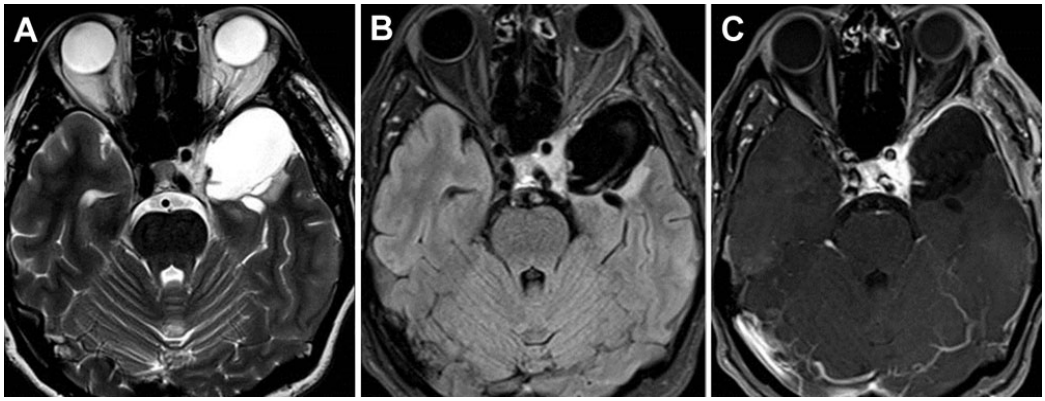

Figure 3: Postoperative magnetic resonance images. (A) Axial T2W sequences show fluid signal collection in the left parasellar region; (B) axial FLAIR reveals suppression of fluid signal; (C) axial post-contrast T1 shows no contrast enhancement, suggesting no residual lesion

Dural-based cavernomas are histologically similar to intraparenchymal cavernous angiomas. However, Rosso *et al*.[6] showed a completely different appearance on computed tomography (CT), MRI and angiography. This fact makes accurate preoperative diagnosis very difficult, as imaging findings can be varied and resemble meningiomas.

Cavernous dural hemangiomas can closely resemble meningiomas on CT and MRI in terms of signal characteristics, enhancement pattern, and localization.

The CT appearance of dural cavernous hemangiomas, like meningiomas, shows a well-defined extra-axial mass lesion on a broad dural base with regular contrast.[5] Occasional calcifications may be present.[5] The cerebral angiography may not be performed when CT or MRI findings are felt to be diagnostic, and the lesion is surgically accessible.[5]

On MRI, cavernomas are usually either isointense or hypointense on T1 sequences and mixed to hyper-intense on T2W sequences. Much like on CT, they also usually enhance on MRI. Meningiomas can also appear hyper-intense on T2 sequences, though they are usually hypo-intense. Hence, a T2 hyperintense lesion is far from specific for dural-based cavernoma outside the middle cranial fossa. Moreover, cavernomas can have dural tails like meningiomas, and may also cause significant perilesional edema.[7]

Angiography can show a tumor blush, hypervascularity or an avascular mass. Even angiography can be completely negative, or demonstrate pooling of contrast medium during the late venous phase.

In the present scenario, where medical and surgical sciences have advanced, stereotactic radiosurgery represents a therapeutic option. This seems to be crucial because dural cavernous hemangiomas are different from meningiomas in their clinical features such as surgical difficulty and sensitivity to radiosurgery. Although the treatment of choice is the total surgical removal, it is also possible to treat the lesion with radiosurgery after the histopathological confirmation in cases of a partial removal.

In conclusion, the occurrence of dural cavernomas may be suspected in the presence of extra-axial parasellar space occupying lesions especially if neuroradiological data suggest a meningioma. Intra-cavernous hemangioma give high signal intensity on T2W images, with a strong homogenous enhancement on contrast enhanced T1W images. It is important to keep this entity among the differentials when planning resection of a lesion that seems to be a meningioma. Cavernous angiomas bleed profusely during surgery, hence perioperative mortality is high as a result of uncontrollable bleeding. Preoperative angiogram and embolization are also recommended. The treatment of choice should always include surgery since it allows histopathological confirmation.

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Conflicts of interest
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Patient consent
Informed consent was obtained from the patient.

Ethics approval
The patient was treated within the standards of our institute and the report was approved.

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Guidelines **Open Access**

Treatment guidelines of chronic inflammatory demyelinating polyneuropathy in China

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Chronic inflammatory demyelinating polyradiculoneuropathy, or chronic inflammatory demyelinating polyneuropathy (CIDP) is an acquired immune-mediated inflammatory disorder at the peripheral nervous system, in which the progression is chronic and also remission relapse. In most cases, it is also associated with cerebrospinal fluid (CSF) protein-cell separation. Electrophysiologically, the peripheral nerve conduction velocity decreases, blocks and characterized as discrete abnormal waveform. Pathologically, there is also multifocal demyelination of myelinated fibers, nerve endometrial edema, inflammatory cell infiltration, *etc*.

CIDP can be classified as classical and variant types, the latter of which is rare, including pure motor, pure sensory, distal acquired demyelinating symmetric (DADS) neuropathy, multifocal acquired demyelinating sensory and motor (MADSAM, also known as Lewis-Sumner syndrome) neuropathy, *etc*.[1]

CLINICAL SYMPTOMS AND CLASSIFICATION

Classical CIDP

(1) This will onset at different age groups, and commonly found in 40-60 years old. There is no gender difference in onset rate; (2) no clear history of infection; (3) classifications: this can be classified into two sub-types: chronic progressive and remission relapsing. For young patients, there are more cases of remission relapsing subtype, who will have a better prognosis. For elderly patients, there are more cases of chronic progressive sub-type, who will have a worse prognosis;[2] (4) clinical symptoms: the symptoms onset chronically, which progress over eight weeks;[3] but 16% of the CIDP patients showed a subacute onset, the symptoms of which progress rapidly and reaches to a peak in 4-8 weeks. Additionally, patients are sensitive to glucocorticoid response. Patients are classified as CIDP tendency but not acute inflammatory demyelinating polyradiculoneurophy. CIDP symptoms confined to the peripheral nervous system, mainly including: (A)

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cranial nerves abnormalities: less than 10% of patients suffer from facial paralysis or opthalmoplegia.[2] In some cases, the cranial nerves innervated bulbar muscles are compromised. In even rare cases, there will be papilledema; (B) gravis: in most cases of CIDP of classical type, patients suffer from gravis, which extend to both proximal and distal extremities; (C) sensory disturbances: most patients also suffer from numbness of the limbs or occasionally pain. There is sometimes loss of glove- or sock-like pinprick and deep sensation. In severe cases, there is also severe sensory ataxia; (D) abnormal tendon reflexes: tendon reflexes are diminished or even disappeared. In some cases, there would be reduction or loss of tendon reflexes in normal muscle; (E) autonomic dysfunction: it is expressed as orthostatic hypotension, sphincter dysfunction, arrhythmia, *etc*.

Variant CIDP

(1) Pure motor CIDP: about 10-11%. There is only the numbness without any sensory symptoms; (2) pure sensory CIDP: about 8-17%. There are only sensory symptoms, e.g. sensory ataxia, numbness and pain. However, with the extension of the course, there may be symptoms of motion expressed; (3) DADS: numbness and/or sensory impairments limited in the distal extremities. DADS is onset slower that the classical CIDP, some of which is a category of IgM monoclonal obulinemia, a subtype of monoclonal gammopathy of unknown significance (MGUS), and associated with peripheral neuropathy. Steroid therapy does not produce any therapeutic effect. In contrast, CIDP without IgM monoclonal obulinemia is sensitive to steroid therapy; (4) MADSAM: there is limb asymmetrical sensorimotor peripheral neuropathy, which is clinically similar to multifocal motor neuropathy (MMN), but there is also evidence of sensory damages and no anti-ganglioside GM_1 antibody titer.[4]

ACCESSARY EXAMINATION

Electrophysiology

In the motor nerve conduction examination, there is peripheral nerve demyelination. There is also conduction block at the entrapment site or discrete abnormal waveform, which is useful for diagnosis for demyelination. Median, ulnar, tibial and common peroneal nerves are commonly diagnosed. Results of electrophysiological tests should be consistent with the clinical symptoms. The standardized criteria of electrophysiological diagnosis includes: (1) motor nerve conduction: there is at least one of two testing motor nerves with the following abnormalities. (A) Fifty percent or more prolongation of distal latencies; (B) velocity of motor nerve conduction is 30% or above lower than the lower limit; (C) twenty percent or more prolongation of F wave latency than the normal upper limit. When examining the amplitude of negative phase wave, which is lower than the lower limit by 20%, the F wave latency should be prolonged by 50% or more. In certain cases, there is also absence of F waves; (D) partial blockade of motor nerve conduction: when comparing the proximal and distal end of the normal segments of peripheral nerves, the former amplitude of negative phase of compound muscle action potential (CMAP) decreases by 50% or more. If the reduction is less than 20%, the reliability of this examination is not guaranteed; (E) discrete abnormal waveform: the duration of CMAP negative phase wave is widened for 30% or above. (2) Sensory nerve conduction: there could be delay of sensory nerve conduction and/or decrease of amplitude. (3) Electromyography with needle electrodes: although it is usually present as normal, there could be abnormal spontaneous potential, prolongation and elevation of amplitude of potential of motor units, and even loss of motor units when there is secondary axonal damage.[3]

CSF

There are about 80-90% of patients with CSF protein-cell separation, which is about 0.75-2.00 g/L (in some cases, it can be higher than 2.00 g/L).

Biopsy of sural nerve

In the situation that the electrophysiology test result is not consistent with the clinical symptoms and vasculitic peripheral neuropathy and hereditary neuropathy cannot be excluded, biopsy of sural nerve is necessary. The main pathological hallmarks include segmental demyelination of myelinated nerve fiber, axonal degeneration, proliferation of Schwann cell with onion-like formation, mononuclear cell infiltration, *etc*.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

Diagnosis

The current diagnosis of CIDP is in fashion of exclusion. One can be suspected as CIDP when the following criteria are fit: (1) chronic progression or remission relapse of CIDP associated symptoms over eight weeks; (2) numbness of proximal and distal extremities in different degrees in symmetrical manner; however, some are in asymmetrical pattern such as MADSAM. The reduction or loss of tendon reflexes with also depth paresthesia; (3) CSF protein-cell separation; (4) reduction and blockade of peripheral nerve conduction or discrete abnormal waveform; (5) excluded as other neuropathies; (6) improved by glucocorticoids.[4,5]

Differential diagnosis

(1) POEMS syndromes, or Crow-Fukase syndrome, demyelination based polyneuropathy, organomegaly of liver, spleen and lymph nodes, endocrine abnormalities such as diabetes, hypothyroidism and *etc*., M protein (mainly IgG type with elevation) and darkening skin. Systemic multi-system examination is needed for diagnosis; (2) MMN: it is a kind of motor specific asymmetrical chronic acquired dymelinating polyneuropathy (CADP), which usually onset in adult male. MMN is onset with asymmetrical numbness of distal of upper extremities, which spreads to the proximal ends of upper extremities and lower. However, in some cases, onset could be initiated at lower extremities. Most of the affected muscle distributes as in a mononeuropathy. However, electrophysiology indicates multifocal distribution of motor transmission blockade. MMN does not highly differ from classical CIDP, but it is similar to MADSAM. The major difference of these two conditions is as followed: MMN is not with any sensory related symptoms but anti-ganglioside IgM, GM_1, is found in serum. Intravenous immunoglobulin, IVIg, or cyclophosphamide (CTX), but not glucocorticoid, could improve the symptoms. For MADSAM, there is not anti-ganglioside IgM in serum but glucocorticoid is efficacious in treatment; (3) cancers causing peripheral neuropathy (Paraneoplastic syndromes): it is a non-metastatic peripheral neuropathy but it could onset before, synchronized and after the carcinogenesis. It is found usually in patients at middle or elder ages, which a progressive disorder not treatable by glucocorticoid. This can be diagnosed by comprehensive examination and identification of tumor; (4) MGUS associated with peripheral neuropathy: CADP could be seen in MGUS with unknown etiological reasons, mainly IgM type. Unlike to CIDP, MGUS associated peripheral neuropathy demonstrated more sensory symptoms than motor and more significant at the distal extremities. About 50% of patients are with positive result in the test of anti-myelin-associated glycoprotein antibody. This disorder cannot be efficiently treated by immunosuppressive or immunomodulatory agents, but rituximab is potent in the treatment. In some cases, the MGUS in IgG or IgA types are associated with CADP, which is with similar clinical symptoms and electrophysiology. The key diagnosis highly relies on the positive finding of M protein in immunofixation electrophoresis; (5) refsum disease: it is a genetic problem of motor and sensory peripheral neuropathy caused by the phytanic oxidase deficiency, which leads to deposition of phytanic acid. This is usually found in adolescents and adults with symptoms as peripheral neuropathy, ataxia, deafness, retinitis pigmentosa, scaling skins, *etc*. There is also significant increase of CSF protein, which leads to misdiagnosis of CIDP. The critical diagnostic criterion is the significant elevation of plasma level of phytanic acid.[2,6]

The diagnosis or confirmation of CIDP needs to be differential with other factors leading to chronic polyneuropathy, such as metabolism, drug interaction, toxicology, and connective tissue diseases. For adolescent patients, it is essential to eliminate the opportunities of different hereditary demyelinating peripheral neuropathy, such as Charcot-Marie-Tooth disease.

TREATMENT

Immunotherapy

(1) Glucocorticoid: this is usually the primary mediation for CIDP. For instance, intravenous administration of methylprednisolone (500-1,000 mg/day) for 3 to 5 consecutive days will be prescribed, followed by a gradual reduction of dose or oral administration of prednisone (morning) for 1-2 months, which will also be reduced in dose accordingly. Alternatively, intravenous administration of dexamethasone (10-20 mg/day) for 7 consecutive days will also be prescribed, shifted to 1 mg/kg prednisone (morning). After 1 to 2 months of treatment, dose could be reduced or changed to oral administration of prednisone (1 mg/kg for morning) for 1 to 2 months followed by a gradual reduction of dose. The mentioned oral therapy by prednisone could be reduced to 5-10 mg and last for more than 6 months; afterward, the treatment could be terminated according to the conditions. During the glucocorticoid therapy, supplementary calcium and potassium, and gastric mucosa protected may need to be considered; (2) intravenous immunoglobulin (IVIg): the treatment course consists of intravenous perfusion of 400 mg/kg Ig for 3 to 5 days, which is repeated once in a month for 3 months. If necessary, the treatment could be extended to months; (3) plasma exchange: for CIDP patients who are applicable with, plasma could be exchanged for 30 mL/kg each time. The course, once a month only, consists of 3 to 5 exchanges, each of which separated by 2 to 3 days. Cautiously, plasma exchange is only allowed 3 weeks after the treatment of IVIg; (4) other immunosuppressants: when the aforementioned approaches failed, or in condition of hormonal dependence or intolerance, other immunosuppressants, such as azathioprine, CTX, cyclosporine and methotrexate, could be considered. Azathioprine, clinically common for CIDP, could be prescribed in 1-3 mg/kg, orally administered in 2-3 times, with also the monitor of hepatic and renal functions and blood biochemistry.[5,7-9]

Neurotrophic approaches
Mainly vitamin B therapy: B1, B12 (methylcobalamin, adenosine cobalamin), B6, *etc*. could be included.

Symptomatic treatment
For neuropathic pain, carbamazepine, amitriptyline, tramadol, gabapentin and pregabalin, *etc*. could be prescribed.

Rehabilitation
When the CIDP condition is under controlled, early start of exercises of neurological rehabilitation can prevent muscle atrophy and contractures.

PROGNOSIS

The prognosis in remission relapsing type of CIDP is better than the chronic progressive type; 70-90% of patients can be improved well by immunotherapy, but not in a minor group. In some rare cases, immunotherapy is potent in treatment for a transient period, following by the dependence.

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Patient consent
There is no patient involved.

Ethics approval
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Topic: Infectious Disease of Central Nervous System **Open Access**

A review with comments on herpes simplex encephalitis in adults

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ABSTRACT

Herpes simplex encephalitis (HSE) can cause permanent injury to the brain parenchyma. As such, it is usually treated as a medical emergency for which correct immediate diagnosis and introduction of specific therapies are critical for survival and prognosis. Here, the authors review the current status of diagnosis and treatments and discuss unsolved issues surrounding therapeutic interventions. The authors also highlight the current expectations for future management of HSE.

INTRODUCTION

Herpes simplex encephalitis (HSE) is an acute infectious disease of the central nervous system (CNS) caused by herpes simplex virus (HSV). It typically occurs in the frontal and temporal lobes, causing hemorrhagic necrotic lesions of brain parenchyma. HSE is the most common cause of sporadic fatal viral encephalitis,[1] accounts for almost 20% of all cases of encephalitis,[2] and has an annual incidence of 1 in 250,000 to 500,000.[3]

In immunocompetent adults, more than 90% of HSE cases are due to HSV-1,[4] whereas HSV-2 is typically responsible for HSE in immunosuppressed individuals.[5] Unfortunately, this CNS disease is life-threatening, as it can also affect the brainstem preferentially or both hemispheres simultaneously[6] in addition to the frontal and temporal lobes, causing a series of clinical features including cognitive impairment, personality changes, seizures, aphasia, and focal weakness.[7,8]

HSE is associated with 70% mortality in untreated patients. In treated cases, there is also about 30% mortality and a high-incidence of severe and permanent neurological sequelae, such as memory impairment, personality and behavioral abnormalities, or seizures.[9,10] Because of its high morbidity and mortality, it has become a global public health problem with a huge economic impact on the whole society.

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With the development of the diagnosis, and antiviral therapies, the management of patients with HSE has improved rapidly in recent years. However, acyclovir resistance and a better understanding of pathogens and pathogenesis also represent new challenges.

Here, we review the current status of diagnosis and therapies for HSE and discuss current controversies and expectations for the treatment of this disease.

DIAGNOSIS

Current status of diagnosis

HSE is a medical emergency and correct and immediate diagnosis is fundamental for the prognosis and therapeutic interventions. At patient's first presentation a meticulous medical history and a careful neurological examination are critical. Peripheral blood count and cellular morphology, such as lymphocytosis, are also helpful in differential diagnosis. Cerebrospinal fluid (CSF) typically shows a lymphocytosis of 10-200/mm^3 (or more) and increased protein of 0.5-1.0 g/L or more.[7] During the early stage of the disease, electroencephalography (EEG) with the evidence of spike and slow wave localization to the temporal lobe might suggest HSE, however, EEG has a sensitivity of approximately 60% and a specificity of 80%.[11]

Virus isolation in cell culture, serological tests for specific antigen or antibody production and brain biopsy play a crucial role for the etiology and diagnosis of HSE. However, all have been now replaced by the detection of HSV using polymerase chain reaction (PCR) in the CSF, that shows a sensitivity of approximately 96%, and a specificity of approximately 99%.[12]

Neuroimaging is also importance in suspected HSE cases. Magnetic resonance imaging (MRI) is more specific and sensitive than computed tomography (CT), because of its non-ionizing radiation, multiplanar imaging capability, improved contrast of soft tissue, and high anatomic resolution.[13,14] Usually MRI shows the abnormalities characteristic of edema and/or enhancement in temporal and frontal lobes, the insular cortex, and the angular gyrus.[15-17] Nowadays, the use of DWI and FLAIR imaging is strongly encouraged,[18] as approximately 5% HSE patients show a normal MRI at presentation.[19] However, it is worth noting that the sensitivity of a new CT post-processing tool based on frequency-selective nonlinear blending (best-contrast CT) seems to be equal to that of DWI and FLAIR, as suggested in a recent study.[20]

Unresolved issues in diagnosis

PCR-based test in CSF has been established as a gold standard method for the diagnosis of HSE. However, there is evidence of pseudo-negative result influenced by the time of CSF sample collection: the PCR may be negative for HSV-1 during the first 3 days of the illness,[12] however, if the CSF is re-examined after a few days, the PCR may then become positive. The European consensus report[21] recommends repeating the CSF PCR routinely after 14 days of treatment, although this does not guarantee a positive result at 10-14 days after illness. In particular, it has been previously shown that PCR positive result most commonly occurs during the first week of infection, even in the case of concurrent treatment with acyclovir.[22] In light of these data, clinicians should give full consideration to the patient's clinical manifestations and curative effects to determine if the PCR test on CSF should be repeated.

THERAPY

Current status of therapy

Correct immediate introduction of specific therapies could reduce the extent of injury and impact on survival.

Antiviral therapy

Acyclovir (ACV), a guanosine analogue, targets viral DNA replication and is the most efficient drug for the treatment of HSE. The recommended dose is 10 mg/kg IV every 8 h for 14 days. In immunocompromised patients or children under 12 years, the treatment usually lasts for at least 21 days. Therapy should begin as soon as HSE is suspected, in fact treatment delays are usually associated with a significantly poorer disease outcome.[3] Renal toxicity, caused by crystallization of ACV in the kidneys, can be prevented by hydration and slow infusion rates, however kidney function should be monitored and any sign of renal impairment should be considered.[23]

Valacyclovir (VCV), an L-valyl ester prodrug of ACV, which is converted to ACV by the hepatic enzyme VCV hydrolase has been shown to have a better oral bioavailability than ACV. A recent study indicated that the administration of VCV at 1,000 mg three times daily result in adequate acyclovir concentrations in the CSF and could be considered an acceptable early treatment for suspected HSE in resource-limited settings.[24]

Corticosteroids

Corticosteroids as an adjunct treatment for HSE are still controversial. One study showed that corticosteroids increase patient benefit[25] but they are not routinely recommended[3,16,26] as a large prospective randomised trial is still needed. However, steroid administration is recommended in situations where HSE patients show severe cerebral edema that could result in severe brain

swelling, coning and death and lumbar puncture is to be avoided.[27]

Other therapies

Supporting therapies are also very important for HSE patients to prevent a variety of complications, such as respiration or cardiac failure, fluid balance disorders and deep vein thrombosis. For patients with increased intracranial pressure, neurointensive care unit management is essential. Clinicians in the UK recommended that the management of HSE should be a participatory process, which is co-produced by health professionals, patients, and their families.[28]

Unresolved issues in therapy

One important issue to consider is whether it is safe to stop acyclovir when the CSF PCR result is negative. The 2012 ABN guidelines recommend that aciclovir might be stopped in immunocompetent patients, if: (a) an alternative diagnosis has been made, or (b) HSV PCR in the CSF is negative on two occasions in a 24-48 h period and MRI is not characteristic for HSV encephalitis, or (c) HSV PCR in the CSF is negative once after 72 h from neurological symptoms appearance, with unaltered consciousness, normal MRI (performed after 72 h from symptoms appearance) and with white cell count in the CSF less than 5×10^6/L (B, III).[3]

Another issue to take into account is that the combination of acyclovir with other antiherpetic drugs is either synergistic or additive. A randomized controlled study by The National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group showed that, following standard treatment with intravenous ACV for PCR-confirmed HSE, an additional 3-month course of oral VCV therapy did not provide additional benefit.[29]

Up to date, there is a common belief that no other drugs can replace ACV for the treatment of HSE and drug combinations are not recommended.

PROSPECTS

Although ACV treatment in HSE is very effective, patient mortality is still approximately 14-19% and 45-60% of the survivors suffer from neuropsychological sequelae.[29] On the other hand, viral resistance is also a potential limitation of HSE therapy.

In this context, there is an urgent clinical need for new therapeutic methods which can result in better clinical outcomes for HSE patients and can prevent viral reactivation or infection. New approaches, such as TLR agonists,[30] IL-1 antagonists[31] or vaccines may be helpful in the future.

On the biological front, mechanisms of virus latent infection/recurrence and analysis of viral gene structure and function need to be further explored using advanced technologies. This might be accelerated by the development of molecular genetics approaches that could draw attention to the genetic conditions of susceptible populations.

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Conflicts of interest

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Patient consent

There is no patient involved.

Ethics approval

This article does not contain any studies with human participants or animals.

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Case Report **Open Access**

Reversible posterior leukoencephalopathy syndrome: single photon emission computerized tomography observations

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ABSTRACT

The authors report clinical correlations of single photon emission computerized tomography (SPECT) findings in reversible posterior leukoencephalopathy (RPL). These are observations that have not received wide attention in literature. A 31-year-old hypertensive gentleman, on discontinuing antihypertensive medications, presented with vomiting, headache, focal motor to bilateral tonic-clonic seizures, altered sensorium, right gaze palsy and right hemiparesis. Accelerated hypertension was noted and he improved well with antihypertensive and anticonvulsant therapy. While cranial magnetic resonance imaging (MRI) revealed extensive bilateral lesions, SPECT imaging revealed perfusion defects involving bilateral basal ganglia, left parieto-occipital, right cerebellar and right occipital regions, which corresponded with clinical deficits on examination. While MRI is the standard of care for the evaluation of RPL, this case suggests that SPECT abnormalities may be better localized to the pathogenic lesions. Furthermore, this may begin to explain the pathophysiology of injury in RPL.

INTRODUCTION

The syndrome of reversible posterior leukoencephalopathy (RPL) was first described by Hinchey *et al.*[1] who reported the clinical and radiological features in 15 patients. The clinical findings included headache, vomiting, confusion, seizures, cortical blindness and other visual abnormalities.[1] Computerized tomography (CT) and magnetic resonance imaging (MRI) studies in these patients revealed extensive bilateral white matter abnormalities suggestive of subcortical and cortical edema without

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infarction especially in the posterior regions of the cerebral hemispheres.[2] While such imaging features have now been well documented, data describing the functional imaging characteristics in this syndrome are rare.[3-6] The pathophysiology of RPL syndrome involves cerebral autoregulatory dysfunction. Acute rise of blood pressure beyond the upper limit of cerebral autoregulation leads to dilatation of arterioles dilate resulting in cerebral hyperperfusion in a pressure-passive manner. This leads to breakdown of the blood brain barrier resulting in extravasation of fluid and blood products into the brain parenchyma.[7] Early changes in neurological diseases can be identified by imaging regional blood flow which is possible using single photon emission computerized tomography (SPECT) imaging which is sensitive to local metabolism and has been utilized in conditions like dementias, epilepsy and traumatic brain injury.[8] SPECT studies may reveal focal areas of hypoperfusion that are discordant with findings of MRI or CT. For instance, in traumatic brain injuries functional imaging techniques may explain or predict postinjury neuropsychologic and cognitive deficits that are not explained by anatomic abnormalities detected by MRI or CT.[9] SPECT study may thus allow us to understand the clinical correlations of the abnormalities detected in routine imaging findings like MRI and such correlations pertaining to RPL syndrome are reported in this communication.

CASE REPORT

A 31-year-old male presented with history of headache associated with recurrent episodes of vomiting for the past 24 h. He developed 2 episodes of right focal motor to bilateral tonic-clonic seizures 3 h prior to admission. Following the seizures he remained in altered sensorium.

He had a prior medical history of medication-treated hypertension for the past 4 years. Renal biopsy was obtained 1 year prior to presentation and revealed focal segmental glomerular sclerosis with multifocal tubular atrophy. He was assessed by nephrologist for the same and was then placed on continuous ambulatory peritoneal dialysis along with antihypertensive therapy (amlodipine 5 mg/day and prasozin 5 mg/day). He was poorly compliant with his anti-hypertensive medicines and had discontinued them 1 week prior to this presentation.

On examination, his recorded vital parameters were: pulse: 142/min, regular and blood pressure: 240/180 mmHg. General physical and cardiorespiratory examinations were unremarkable. Neurologically he was drowsy, arousable, but not obeying commands. There was no word output. Optic fundi revealed no papilledema. There were features of grade 3 hypertensive retinopathy. There was right gaze palsy, right hemiplegia (grade 0/5 over the upper and lower limbs), bilaterally brisk deep tendon reflexes and extensor plantar responses.

Investigations revealed: blood urea 29 mg/dL, serum creatinine 1.6 mg/dL and uric acid 8.8 mg/dL. Urinalysis revealed albuminuria. Hemogram revealed leukocytosis (total leucocyte count 28,500 cells/cu.mm). Abdominal ultrasound (done 1 year ago) showed congenital pelviureteral junction obstruction in the right kidney with hydronephrotic sac, dilated renal pelvis and thinned renal parenchyma and the left kidney showed compensatory hypertrophy. MRI of the brain completed on day 2 of the illness revealed multiple lesions that were hypointense on T1-weighted images, hyperintense on T2-weighted and FLAIR images [Figure 1]. The lesions involved bilateral cerebellar hemispheres, brainstem, basal ganglia, posterior parieto occipital regions, corona radiata, centrum semiovale and splenium of corpus callosum with effacement of ventricular system and sulci. The lesions were moderately bright on diffusion weighted imaging (DWI with b value 600) and lower signal in ADC map. Anterior circulation was relatively spared. MR angiography revealed dolichoectasia with atheromatous dilatation of anterior and posterior circulation arteries without major vascular occlusion. Brain SPECT was performed on the 3rd day of illness after administration of 20 mCi of Technetium-99m ethyl cysteinate dimer (99mTc-ECD). Images were acquired with a gamma camera, 1 h after tracer administration. Acquired images were then reconstructed in transaxial, saggital and coronal axes. Images revealed perfusion defects involving bilateral basal ganglia, left parieto-occipital, right cerebellar and right occipital regions [Figure 2].

Intravenous sodium nitroprusside (0.25 μg/kg/min initial dose but then titrated up) was administered for hypertension. Intravenous fosphenytoin (20 mg/kg of phenytoin equivalent) was given followed by maintenance phenytoin (5 mg/kg/day) therapy. There were no further recurrences of seizures. His blood pressure gradually decreased and oral antihypertensive therapy was instituted. His mental status improved by the second day after admission and limb power was grade 1/5 on the 3rd day. The power of his motor movements rapidly improved subsequently and was grade 5/5 on the 5th day after admission and treatment. He remained asymptomatic since the 5th day after admission. At the time of discharge he was on a combination of prasozin 5 mg, clonidine 0.3 mg, labetolol 300 mg and amlodepine 10 mg/day for control of hypertension.

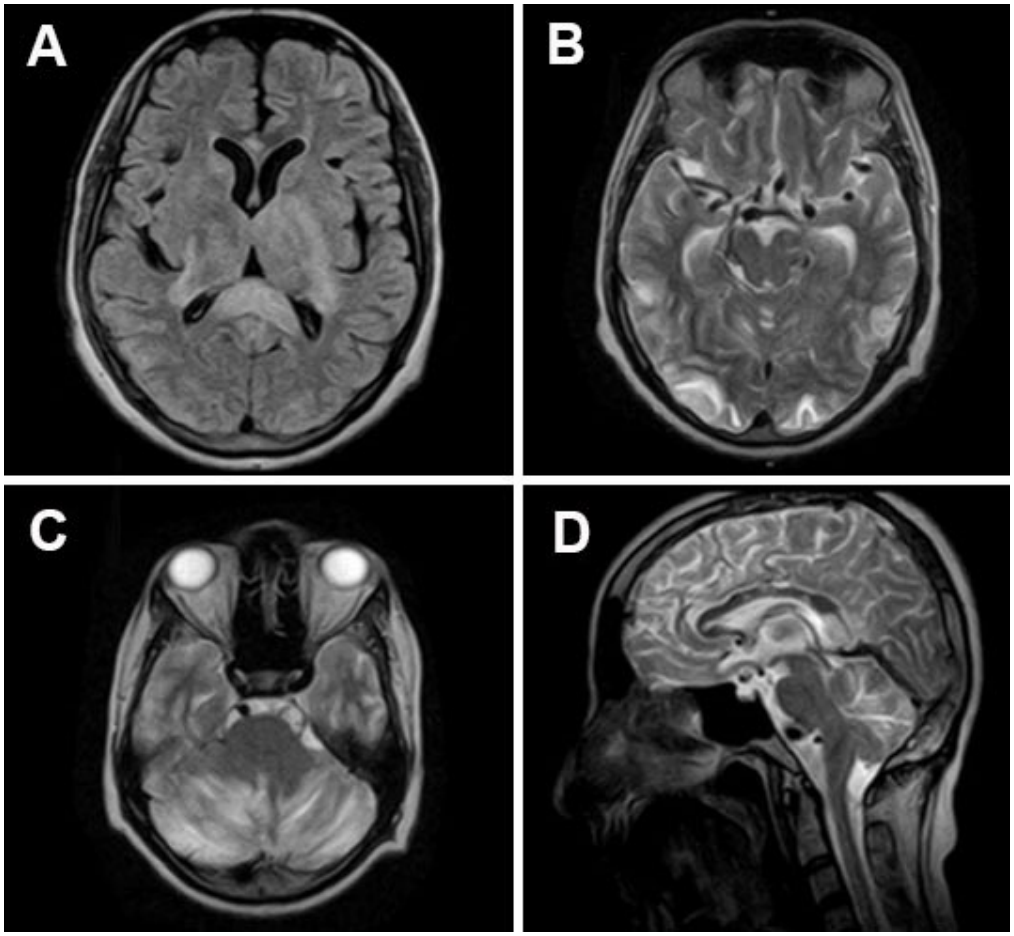

Figure 1: Magnetic resonance imaging in a patient with reversible posterior leukoencephalopathy. FLAIR axial (TR, 6160 ms; TE, 30 ms; TI, 1100 ms; FOV 23 cm × 23 cm; matrix 256 × 192; slice thickness, 5 mm with 1 mm gap) (A); TSE T2-weighted (TR, 4500 ms; TE, 118 ms; FOV 23 cm × 23 cm; matrix 256 × 192; slice thickness, 5 mm with 1 mm gap) axial (B and C) and sagittal images (D) are shown. Figure shows hyperintensities over both thalami, posterior limb of left internal capsule and corpus callosum on FLAIR axial image (A); hyperintense signals are seen over bilateral parieto-occipital cortices (B) and both cerebellar hemispheres (C) on T2 axial images; hyperintensities over corpus callosum and cerebellum are also seen in a T2 sagittal image (D)[16]

A repeat MRI done completed 2 weeks after the institution of treatment revealed resolution of the lesions over the cerebellar hemispheres, brainstem, basal ganglia and cortical regions. Follow up SPECT study could not be done.

DISCUSSION

This is a report of SPECT characteristics in a patient presenting with RPL syndrome. SPECT provides clinically useful information about brain perfusion and such data can especially be useful in conjunction with CT scan or MR imaging in diseases that cause flow abnormalities. The coupling between local metabolism and blood flow allows SPECT to provide indirect information about metabolism in focal areas of the brain.[10] Hinchey *et al*.[1] noted that while the radiological abnormalities were symmetric, the degree of involvement and the clinical manifestations were often asymmetric. Thus, in RPL syndrome where the defects are more functional than structural, SPECT abnormalities in the form of areas of hypoperfusion correlated well with the clinical abnormalities as was seen in this case. While MRI revealed bilateral parieto-occiptal and cerebellar hemispheric changes, SPECT images revealed predominant left hemispheric changes that correlated with the clinical picture of right hemiparesis and right gaze palsy and the bilateral basal ganglia lesions correlated with the bipyramidal signs.

Central to the pathogenesis of RPL syndrome is the failure of cerebral autoregulation and endothelial dysfunction. When the upper limit of autoregulation is exceeded arterioles dilate resulting in hyperperfusion that leads to breakdown of blood-brain barrier with focal transudation of fluid and petechial hemorrhages.[1] Alternatively, in severe cases, it has been postulated that disordered cerebral autoregulation may lead to reactive focal vasoconstriction, thereby resulting in local hypoperfusion, cytotoxic edema, and cerebral infarction.[11] Endothelial dysfunction has also been

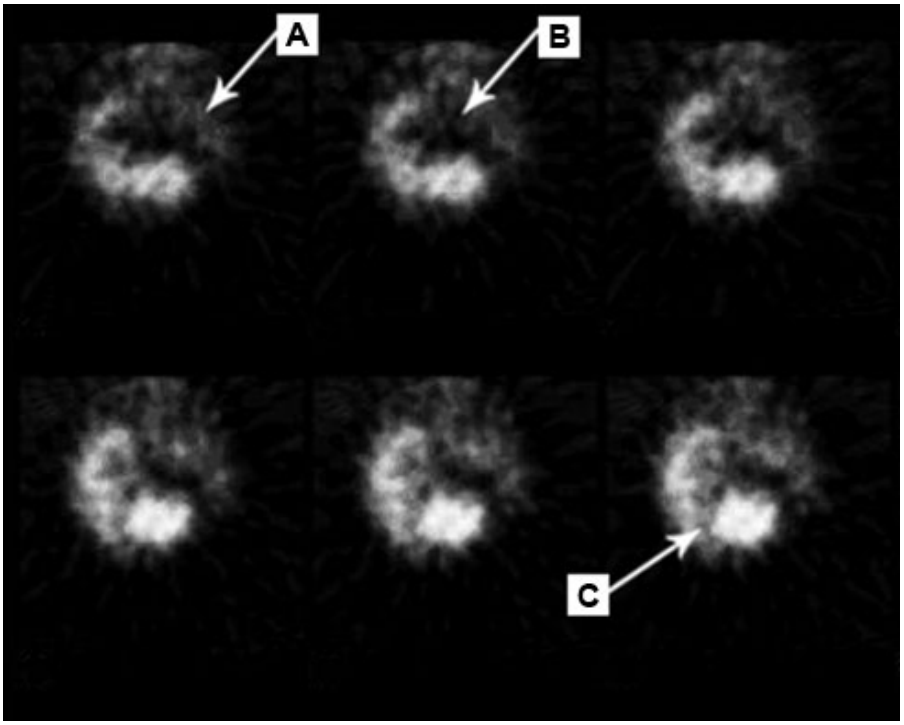

Figure 2: Brain single photon emission computerized tomography images obtained after administration of 20 mCi of 99mTc-ECD in a patient with reversible posterior leukoencephalopathy. Perfusion defects are seen involving the left fronto-parietal region (A), bilateral basal ganglia (B) and right cerebellum (C)

implicated in the pathophysiology of RPL syndrome, especially in cases associated with preeclampsia or cytotoxic therapies.[1] Vasogenic edema related to this is the most common abnormality on neuroimaging in patients with RPL syndrome and involves the cortical and predominantly subcortical white matter in the posterior portions of the cerebral hemispheres, especially bilaterally in the parieto-occipital regions usually sparing the calcarine and paramedian occipital-lobe structures.[1] The latter feature helps to differentiate the syndrome from top-of-the basilar artery syndrome with involvement of posterior cerebral artery territories bilaterally. This differentiating feature was seen in our patient as well. In addition there was also involvement of bilateral cerebellar hemispheres, brainstem, basal ganglia, centrum semiovale, corona radiata, and splenium of corpus callosum. Involvement of the last two areas has not reported by earlier authors. However, clinical abnormalities correlating with such extensive radiological involvement were lacking in our patient.

Perfusion defects were seen over the right cerebellar region on SPECT imaging. Hypometabolism and hypoperfusion of the cerebellar cortex contralateral to the side of infarct usually occurs during the first two months after infarction and is referred to as crossed cerebellar diaschisis. Even relatively small lesions with mild metabolic depression also have been noted to produce contralateral cerebellar hypometabolism. It is thought to be due to interruption of cortico-ponto-cerebellar connections due to the infarct which then causes deafferentation and transneural metabolic depression of the contralateral cerebellar hemisphere.[12] It could be proposed that the right cerebellar perfusion defects seen on SPECT were an early manifestation of this phenomenon although the interval between the onset of the disease and the time of SPECT is short in our patient. While MRI revealed bilateral cerebellar lesions, only SPECT revealed abnormalities corresponding to the left cerebral hemispheric involvement with corresponding right cerebellar hemispheric changes. Thus, SPECT is a useful tool in understanding the pathophysiological abnormalities underlying the disease. It has been known that post-traumatic psychosis is associated with perfusion defects in frontal lobes even when the routine imaging studies like MRI and CT scan are normal.[9] Thus SPECT better localizes areas of clinical injury and may prove to be a useful tool in understanding the pathophysiological abnormalities underlying RPL syndrome.

The advantage of SPECT is its ability to detect ischemic tissue before irreversible damage occurs.[13] For instance, it has been shown that SPECT is a sensitive indicator of perfusion and is considered superior to anatomic imaging modalities such as CT or MRI in detecting acute ischemic stroke in the first few hours following the events. Immediately after

acute stroke, a focal or regional area of hypoperfusion or no perfusion will be seen which is larger than the lesion that is later seen on CT or MRI. Brain perfusion SPECT patterns may predict the outcome of stroke patients and thus help in the selection of candidates for fibrinolytic therapy as well as decompressive hemicraniectomy.[14,15] Functional imaging studies like perfusion and diffusion weighted MR imaging and MR spectroscopy could also be utilized to detect ischemic changes.[16-19] Hyperintense signals are seen in areas of both vasogenic edema and infarction on routine T2 weighted images. However, on diffusion weighted imaging, vasogenic areas demonstrate increased diffusion and cytotoxic edema seen in infarcted areas demonstrate decreased diffusion. In our patient repeat MR imaging revealed resolution of the lesions suggesting reversible ischemia rather than established infarct. Thus it is understandable that functional studies like SPECT may enhance the understanding of the extent and nature of the lesions typical of the syndrome. Follow up SPECT study however could not be done in the patient.

In conclusion, SPECT findings in our patient with RPL syndrome correlated positively with the presented clinical deficits: advantage of SPECT study over routine MRI is its good correlation with clinical abnormalities. MRI abnormalities, in contrast to SPECT findings, are extensive and poorly correlate with clinical deficits. Thus SPECT may be an invaluable tool to understand the morphological and functional changes in RPL syndrome.

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Patient consent was obtained from the patient.

Ethics approval
Data collection in our study involving the patient is consistent with the ethical standards of the institution's ethics committee.

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Prenatal zinc supplementation to lipopolysaccharide infected female rats prevents neurochemical, behavioral and biochemical deficits produced in infants

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ABSTRACT

Aim: Recent research revealed an association between maternal infection i.e. lipopolysaccharide (LPS) exposure during pregnancy and increased risk for central nervous system disorders being passed onto the off-spring. Therefore, the present study was designed to investigate the effect of LPS infection during d14-17 of pregnancy (equivalent to third trimester in humans) on neurochemical, neurobehavioral abnormalities, biochemical as well as histopathological parameters in male/female pups. Also, the effect of zinc supplementation throughout pregnancy to female rats in ameliorating LPS induced neurodegenerative effects caused in pups were evaluated. **Methods:** Pregnant female rats were administered single dose of LPS (200 µg/kg) intraperitoneal on d14-17 of their pregnancy. Zinc supplementation was given throughout pregnancy (75 mg $ZnSO_4$/L) in drinking water. **Results:** LPS injection to pregnant female rats significantly altered the levels of neurotransmitters (dopamine, serotonin and norepinephrine) in pups. Also, marked deterioration of motor behavior parameters (actophotometer, rotarod) as well as cognitive decline (plus maze and active avoidance) has been observed in male as well as female pups. Whereas, supplementation with zinc limited the alterations in behavioral parameters as well as significantly improved the level of neurotransmitters in prenatally exposed pups of both genders. However, levels of malondialdehyde and nitric oxide formed as well as antioxidant defense system including reduced glutathione, superoxide dismutase and catalase were found to be excessively compromised in female pups when compared to male pups. **Conclusion:** Hence, the study indicated LPS mediated toxicity in prenatally exposed pups is gender specific and zinc supplementation during pregnancy was found to attenuate LPS induced toxicity in pups.

INTRODUCTION

Lipopolysaccharide (LPS) is a toxic component of cell walls in gram-negative bacteria and is widely present in the digestive tract of humans and animals.[1] As revealed by recent studies humans often get exposed to LPS

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as it is present suspended in the air as a component of the air pollutant PM2.5 or as part of house dust and aerosols generated from contaminated water.[2,3] These PM2.5 i.e. particulate matter less than 2.5 μm originate from several sources like oil refineries, metal processing facilities, tailpipe and brake emissions, residential fuel combustion, power plants, and wild fires. Furthermore, occupational exposure to LPS is common for people in agricultural settings or in textile mills as suggested by previous reports.[4] It has also been reported that gastrointestinal distress and excess alcohol intake are known to increase uptake of LPS from gastrointestinal tract into blood.[5,6]

Further, high levels of LPS have also been detected in women with bacterial vaginosis.[7] In humans, gram-negative bacterial infections are a recognized cause of embryo loss and preterm labor.[8] Mimicking maternal infection by exposing the pregnant rodents to LPS at early gestational stages resulted in embryonic resorption and fetal death.[9,10] LPS exposure at middle gestational stages caused teratogenesis, fetal death and preterm delivery.[11-13] In addition, several studies showed that maternal LPS exposure at late gestational stages led to fetal death, growth restriction, skeletal development retardation, and preterm labor.[14-18]

Numerous reports demonstrate that inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), have been associated with LPS-induced adverse developmental outcomes. Indeed, several studies showed that maternal LPS exposure during pregnancy significantly increased the level of proinflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain.[19,20] Another study found that the expression of TNF-α, IL-1β and IL-6 is also much higher in brains with periventricular leukomalacia (PVL) than in those without PVL.[21] Mimicking intrauterine infection and inflammation by LPS exposure during pregnancy significantly increased the levels of TNF-α, interleukin-1β (IL-1β) and IL-6 in maternal serum, fetal liver and amniotic fluid, TNF-α and IL-10 in fetal brain in rodents.[18] Several studies showed that maternal exposure of LPS (120 μg/kg) intraperitoneal in mouse at late gestational stages markedly impaired the learning abilities and social behavioral performance in adulthood.[22-24]

Nutritional deficits may also cause neurodevelopmental disorders, such as spina bifida, which is common, and anencephaly, which is rare. Both disorders are neural tube defects with malformation and dysfunction of the nervous system and its supporting structures, leading to serious physical disability. Folic acid (vitamin B) supplementation in early pregnancy, aimed to prevent neural tube defects, may also reduce mental health problems in children.[25] Iodine deficiency in the pregnant mother subsequently causes hypothyroxinemia i.e. low maternal free thyroxine (T_4) and results in damage to the developing brain, which is further aggravated by cretinism in the fetus.

Zinc is also one of an essential element in diet of pregnant females as maternal zinc deficiency affects fetal growth and development, complications of pregnancy, labor and delivery and maternal and infant health.[26] Zinc is a structural constituent essential for cell growth, development, and differentiation.[27] Increasing evidence demonstrates that zinc has an anti-inflammatory effect.[28] A recent study found that subcutaneous injection with zinc sulfate alleviated LPS-induced neurodevelopmental damage in fetal brain.[29,30] Zinc is capable of inhibiting LPS or IL-1β-induced nitric oxide (NO) formation as well as NO formation by NO synthase (NOS).[31] Many studies reported that Zn^{2+} inhibits LPS-induced TNF-α production by inactivating nuclear factor-κB (NF-κB) genes which is mediated by protein kinase A.[32,33] Another mechanism reported that Zn^{2+} increased the intracellular levels of cGMP due to reduced enzyme activity of phosphodiestrase-1 (PDE-1), PDE-3, PDE-4 in cellular lysate and inhibits the LPS-induced TNF-α and IL-1β.[34] The development of anxiety has been previously reported in zinc-deficient rats.[35,36] Zinc can act as a critical neural messenger in healthy and diseased states of the brain through its ability to regulate N-methyl-D-aspartate receptor activity which have central importance in cognitive functions (learning and memory).[37] Recent studies showed that $ZnSO_4$ supplementation during pregnancy protects against LPS-induced fetal growth restriction and demise through its anti-inflammatory effect.[28]

Nevertheless, the molecular mechanism of zinc-mediated protection against LPS induced developmental toxicity remains elusive. Present study was designed to investigate the gender biased effects of LPS injection during d14-17 of pregnancy on the neurobehavioral, biochemical and histopathological parameters in off-springs. Also, the neuroprotective potentials of zinc supplementation in ameliorating these LPS induced alterations have been established.

METHODS

Animals

Healthy Sprague-Dawley rats between 5-7 weeks age were procured at the central animal house of Panjab University, Chandigarh, India. They were acclimatized in the department animal house for two weeks in polypropylene cages in hygienic conditions. They were

provided standard animal feed and water *ad libitum* throughout the treatment period. All procedures were done in accordance with ethical guidelines laid down by the Ethics Committee on the Use of Experimental Animals of the Panjab University and in general according to the NIH guidelines (Rule No. 23-85, as revised in 1985).

Chemicals

All the chemicals were purchased from Sigma-Aldrich (St. Louis, USA) and Sisco Research laboratories Pvt., Ltd. (Mumbai, India). Zinc sulfate was purchased from HiMedia (Mumbai, India).

Experimental design

Rats were preferred for this experiment due to: (1) shorter gestation length (20-22 days), shorter estrous cycle (4-5 days), litter size of about 7-9, weaning age of about 21 days and relatively short period age of sexual maturity (7-8 weeks). Rat pregnancies are more size consistent, rats can bred quickly after parturition and rat brains show early sexual dimorphism.[38] At the commencement of the study 12 female rats of 12-14 weeks of age were randomly distributed into four cages with 1 male of 14-16 weeks of age into each cage.[38] Pregnant female rats were then divided into four cages and were grouped as follows:

Group A (control): pregnant females were injected intraperitoneal with single dose of normal saline (200 µL) at day 14 of pregnancy; Group B (LPS treated): pregnant females were injected with single dose of LPS (200 µg/kg) intraperitoneal at day 14 of pregnancy; Group C (LPS + zinc supplemented): pregnant females were injected single dose of LPS (200 µg/kg) intraperitoneal at day 14 of pregnancy. Animals were also administered with $ZnSO_4$ (75 mg/L) dissolved in drinking water throughout their gestation period; Group D (zinc supplemented): pregnant females were supplemented with $ZnSO_4$ (75 mg/L) throughout their gestation period.

Neurobehavioral analysis

Elevated plus maze test

Elevated plus maze (EPM) test is a learning task which measures spatial long-term memory and evaluates the cognitive behavior.[39] Apparatus used for the EPM test consists of 2 open arms (40 cm × 6 cm) and 2 closed arms of the same size connected with a central platform (6 cm × 6 cm). The maze was placed 50 cm above the ground. Rats were allowed to move freely in this apparatus for 90 min of training. After a few hours, trained pups were again allowed to move freely for the same time and time spent in the open arms and closed arms were analyzed. The apparatus was cleaned with 25% ethanol after testing the pups.

Active avoidance test

Memory impairments can be observed in Active Avoidance Test.[40] Apparatus used for this test consisted of 2 chambers, which are separated by a partition. One of these chambers is enlightened, where the animal has to be kept. After 10 s, the buzzer is kept on and after 10 s the shock is given. If the animal jumps to the other side of the compartment as soon as the buzzer is set on, it means the animal has avoided the test. But in case, if the animal jumps to the other compartment aftershock or does not jump is termed as escapism. The number of escaped trials/2 min/animal were noted and analyzed statistically.

Actophotometer (total locomotor activity)

Total locomotor activity (ambulation's and rearing) was measured by using a computerized Actophotometer (IMCORP, Ambala, India). An array of 16 infrared emitter/detector pairs measured animal activity along a single axis of motion, the digital data being displayed on the front panel meters as ambulatory movements. Rats were allowed to acclimatize to the observation chamber for a period of 2 min. The activity was monitored continuously for a period of 5 min. Locomotion was expressed in terms of total photo beam counts per 5 min period per animal.[41]

Rotarod

The effect of LPS on the muscle performance was evaluated using a Rota-rod. Animals were given 2 initial training trails of 300 s each, approximately 10 min apart, to maintain posture on a Rota-rod which is 3 cm in diameter and rotating at a constant 25 revolution per minute. After the initial training trials, a baseline trial of 120 s was conducted. The time each animal remained on the rotarod was recorded; animals not falling off the rotarod were given a maximum score of 120 s.[42]

Estimation of neurotransmitter levels (epinephrine, nor-epinephrine, dopamine and serotonin)

Biogenic amines dopamine, norepinephrine and serotonin were measured by high performance liquid chromatography (HPLC) with electrochemical detector.[43] Water standard system consisting of a high pressure isocratic pump. A 20 µL sample injector valve, C18 reverse phase column and electrochemical device were used. Data was recorded and analyzed with the help of Empower software. Mobile phase consisting of 10 mmol/L sodium citrate, 32 mmol/L NaH_2PO_4, 0.025 mmol/L EDTA, and 0.77 mmol/L 1-heptane sulphonic acid was used. pH of mobile phase was adjusted to 4.5 with the help of orthophosphoric acid. Electrochemical conditions for the experiment were +0.800 V, sensitivity ranges from 5-50 nA.

Separation was carried out at a flow of 1 mL/min. Samples (20 µL) were injected manually. On the day of experiment frozen samples of midbrain were homogenized in the homogenizing solution containing 0.1 mol/L perchloric acid. After that samples were centrifuged at 12,000 *g* for 5 min. The supernatant was further filtered through 0.25 micron nylon filters before injecting in the HPLC injection pump. Data was recorded and analyzed with the help of Empower software.[43]

Biochemical estimations

All the tissues (10% w/v) were homogenized in 10 mmol/L PBS, pH = 7.4. Homogenate was made using mechanically driven Teflon Potter-Elvejhem type homogenizer for total disruption of cells. Homogenate was centrifuged first at 10,000 *g* for 30 min at 40 °C. Pellet was discarded and supernatant was used for various biochemical estimations.

NO

NO was estimated by the method of Raddassi *et al*.[44] The level of NO was estimated as nitrite, a NO metabolite and remains stored in tissues as Nitrates (NO^{3-}) or Nitrite (NO^{2-}). Thus, NO concentration can be estimated by measuring concentrations of NO^{3-} and NO^{2-} in combination. The simplest technique is the monitoring of reduction of NO^{3-} to NO^{2-} by nitrate reductase or metallic catalyst, followed by the calorimetric Griess Reaction to measure NO^{2-} levels (nitrite levels). A standard curve was made with serial dilutions of sodium nitrite by making its volume to 100 µL; add 100 µL of Griess reagent in wells of ELISA reader plate. Instead of sodium nitrite 100 µL of homogenate was added along with 100 µL of Griess reagent in subsequent wells of ELISA plate. The colorimetric reaction was allowed to proceed for 10 min at room temperature in dark, and optical density was measured at 550 nm using ELISA reader.

Glutathione reduced

Estimation of glutathione reduced (GSH) was performed in the tissue homogenate by the method of Moron *et al*.[34] The assay was performed by mixing 100 µL of 2.5% homogenate and trichloroacetic acid (TCA). The precipitated proteins were separated by centrifugation at 2,000 *g* for 15 min; 0.1 mL supernatant was diluted to 1 mL with 0.2 mol/L phosphate buffer (pH = 8.0). Further, 2 mL of freshly prepared 0.6 mmol/L 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in buffer was added. In this method, DTNB is reduced by -SH groups to form 1 mole of 2-nitro-5-mercaptabenzoic acid per mole of SH. The nitro mercaptobenzoic acid anion released has intense yellow color and can be used to measure -SH groups at 412 nm.

Lipid peroxidation (MDA) assay

Lipid peroxide formation was assayed by the method of Wills.[45] An aliquot containing 0.5 mL of tissue homogenate (10% w/v), diluted to 1.0 mL using 0.1 mL Tris-HCl buffer (pH = 7.4) was shaken. Samples were incubated at 37 °C for 2 h with constant shaking. After incubation, 1 mL of ice cold 10% TCA was added and mixed it thoroughly; the reaction mixture was centrifuged at 800 *g* for 10 min. One mL of 0.67% thiobarbituric acid (TBA) was added to 1 mL of supernatant and color developed at 100 °C for 10 min. Samples were cooled and diluted with 1 mL double distilled water. The absorbance was read at 532 nm. The amount of Malondialdehyde formed was calculated on the basis of molar extinction coefficient of MDA-TBA chromophore (1.56×10^5 M^{-1} cm^{-1}) and results were expressed as nmoles of MDA/mg protein.

Catalase

The enzymatic activity of catalase was estimated by UV spectrophotometer method described by Luck.[46] H_2O_2 was used as substrate. The UV absorption of H_2O_2 solution is measured at 240 nm on decomposition of H_2O_2 with catalase. The amount of H_2O_2 decomposed was calculated on the basis of molar extinction coefficient of H_2O_2 (39.4 M^{-1} cm^{-1}) and results were expressed as µ moles of H_2O_2 decomposed/min/mg protein.

Superoxide dismutase

Superoxide dismutase (SOD) estimation was assayed by the method of Kono.[47] The principle of SOD activity assay was based on the inhibition of nitrobluetetrazolium (NBT) reduction using the following reagents. Solution A: EDTA (0.1 mmol/L) containing 50 mmol/L sodium carbonate, pH 10.0; solution B: NBT (90 mmol/L) in solution A; solution C: Triton-X (0.6%) in solution A; solution D: hydroxylamine hydrochloride (20 mmol/L) pH 6.0. The reaction mixture contained 1.3 mL of solution A, 0.5 mL of solution B and 0.1 mL of solution C. The reaction is initiated by the addition of 0.1 mL of solution D to the reaction mixture and the rate of reduction of NBT in the absence of enzyme source was recorded at 560 nm for 3 min, which is considered as reference. Following this an appropriate amount of the enzyme source (PMS 20-50 µL) was added and the rate of reduction was noted for 3 min at 560 nm. Percentage inhibition in the rate of NBT reduction is calculated and one unit of the enzyme that is the inverse of the amount of the protein (mg) required to inhibiting the reduction rate by 50%. The results are expressed as IU/mg of protein.

Histopathology

Histology of brain tissues was done by the method

of Humanson.[48] All brain tissue sections were fixed in 10% neutral buffered formalin. The tissues were processed and embedded in paraffin and sectioned at 4 µm thickness. The sections were stained with hematoxylin and eosin (HE). The dried stained slides were seen under the Leica microscope. Images were captured at 10× and 40× and auto corrected in Microsoft office 2010 to enhance image clarity.

Statistical analysis

Statistical analysis was done by one way ANOVA followed by LSD with *post-hoc* multiple pairwise comparisons between genders and different groups to estimate whether the differences between the mean values of groups are statistically significant or not. Results were taken significant at $P \leq 0.05$.

RESULTS

Prenatal zinc supplementation improves LPS induced neurobehavioral deficits in pups

Total locomotor activity was assessed in prenatally LPS treated male and female pups on actophotometer. Prenatal LPS exposure significantly ($P < 0.05$) decreased total locomotor activity in male (31.59%) as well as female (30.17%) pups when compared to control pups. Zinc supplememtaion to female rats throughout pregnancy improved locomotor activity in case of female pups (21.60%) [Figure 1]. In case of rotarod test a significantly ($P < 0.05$) decreased mean fall off time was observed in (54.30%) male and (65.93%) female pups in comparison to control pups. Zinc supplementation however significanty increased the mean fall off time in pups of both sexes.

Spatial memory was assessed in prenatally LPS treated male and female pups using a EPM as well as active avoidance test as shown in Figure 2. Time spent in closed arm was significantly ($P < 0.05$) decreased in case of prenatally LPS treated male pups (66.23%) and female pups (6-fold) when compared to control pups. However, following zinc supplementation time spent in the closed arm was significantly ($P < 0.05$) increased in male (14.84%) and female (47.26%) pups when compared to LPS treated pups.

Prenatally LPS exposed pups of both genders showed significant ($P < 0.05$) increase (2-fold) in the number of escaped trials when compared to control pups. However, number of escaped trials were significantly ($P < 0.05$) decreased in male (35.29%) and female (41.67%) pups following zinc supplementation to LPS treated mothers when compared to prenatally LPS treated pups.

Effect of prenatal zinc supplementation on neurotransmitter levels in pups

Present study relates the deficit in locomotor activity and short term memory with neurotransmitter levels which were measured using HPLC as shown in Table 1. Prenatal LPS exposure significantly ($P < 0.05$) decreased the level of dopamine in male (38.37%) and female (41.70%) pups when compared to control pups. In case of pups from zinc supplemented mothers a significant ($P < 0.05$) improvement in dopamine level was observed (male 52.2%) and (female 13.37%) when compared to prenatally LPS treated pups.

Prenatal LPS exposure significantly ($P < 0.05$) increased the level of norepinephrine (NE) in male (78.21%) and female (24.42%) pups when compared to control pups. However, NE levels were found to decrease significantly ($P < 0.05$) in male (27.98%) as well as female pups (14.42%) following zinc supplementation to LPS treated mothers when compared to prenatally LPS treated pups.

Prenatal LPS exposure also significantly ($P < 0.05$) decreased the level of 5-HT in male (30.58%) and female (55.76%) pups when compared to control pups. Whereas, 5-HT levels were significantly ($P < 0.05$) improved with (14.40%) increase in male and female (60.86%) pups following zinc supplementation to LPS treated mothers when compared to prenatally LPS treated pups.

Effect of prenatal zinc supplementation on oxidative stress markers (NO levels, MDA level (lipid peroxidation) and antioxidant enzymes (GSH, SOD and catalase)

As seen from Table 2 a significant ($P < 0.05$) elevation

Table 1: Effect of prenatal zinc supplementation on neurotransmitter levels in mid brain of prenatally LPS treated male and female pups

Group	Dopamine (ng/mg tissue)		Serotonin (ng/mg tissue)		Norepinephrine	
	Male	Female	Male	Female	Male	Female
Control	0.645 ± 0.050	0.885 ± 0.040	0.340 ± 0.030	0.260 ± 0.007	3.81 ± 0.08	5.69 ± 0.13
LPS	0.398 ± 0.030*	0.516 ± 0.060*	0.236 ± 0.000*	0.115 ± 0.005*	6.79 ± 0.08*	7.07 ± 0.12*
LPS + $ZnSO_4$	0.605 ± 0.050#	0.585 ± 0.030#	0.270 ± 0.010#	0.185 ± 0.005#	4.89 ± 0.08#	6.05 ± 0.11#
$ZnSO_4$	0.748 ± 0.020*#	0.901 ± 0.010#	0.313 ± 0.010*#	0.245 ± 0.005#	3.08 ± 0.08*#	5.05 ± 0.11*#

Values are expressed as mean + SD; n = 5/group. *$P < 0.05$ *vs.* control group, #$P < 0.05$ *vs.* prenatally LPS treated group. LPS: lipopolysaccharide

Table 2: Effect of prenatal zinc supplementation on NO levels, lipid peroxidation and antioxidant defense system in mid brain of prenatally LPS treated male and female pups

Group	NO (nmoles/mg protein)	LPO (nmoles of MDA/mg protein)	GSH (μmoles/ mg protein)	SOD (I.U)	Catalase (nmoles of H_2O_2 hydrolysed/mg protein/min
Male					
Control	0.389 ± 0.051	40.11 ± 7.64	31.91 ± 0.60	0.349 ± 0.054	0.700 ± 0.120
LPS	0.430 ± 0.020	50.03 ± 5.54	12.26 ± 1.09*	0.269 ± 0.041	0.540 ± 0.062
LPS + $ZnSO_4$	0.314 ± 0.038#	45.41 ± 7.60#	19.69 ± 1.68#	0.290 ± 0.035	0.610 ± 0.082
$ZnSO_4$	0.357 ± 0.054#	36.50 ± 9.76	31.25 ± 1.34#	0.340 ± 0.033	0.800 ± 0.091#
Female					
Control	0.299 ± 0.028	39.95 ± 7.57	29.77 ± 1.04	0.607 ± 0.013	1.000 + 0.170
LPS	0.438 ± 0.022*	55.13 ± 4.79*	12.01 ± 0.70*	0.407 ± 0.050*	0.670 + 0.083*
LPS + $ZnSO_4$	0.321 ± 0.066#	41.14 ± 6.50#	20.84 ± 0.68#	0.470 ± 0.050	0.750 + 0.110
$ZnSO_4$	0.299 ± 0.038#	27.36 ± 4.29#	29.37 ± 0.90#	0.716 ± 0.110*#	1.160 + 0.180#

Values are expressed as mean + SD; n = 5/group. *P < 0.05 *vs.* control group, #P < 0.05 *vs.* prenatally LPS treated group. LPS: lipopolysaccharide; NO: nitric oxide; LPO: lipid peroxidation; GSH: reduced glutathione; SOD: superoxide dismutase

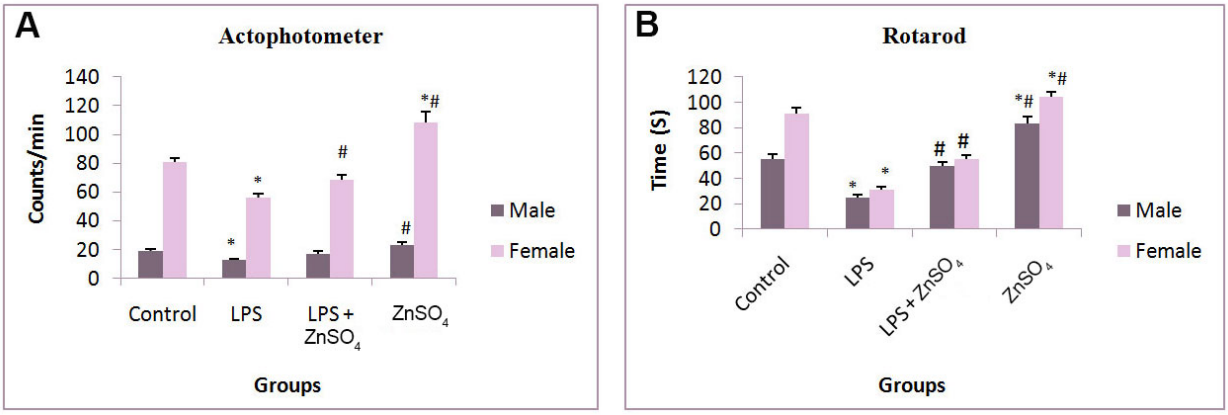


Figure 1: Effect of prenatal zinc supplementation in (A) actophotometer and (B) rotarod on prenatally LPS treated male pups and female pups. Values are expressed as mean + SD; n = 5/group, *P < 0.05 *vs.* control group, #P < 0.05 *vs.* prenatally LPS treated group. LPS: lipopolysaccharide

in NO levels were observed only in prenatally LPS treated female pups (46.49%) when compared to control pups. However, with zinc supplementation to LPS treated pregnant rats significantly (P < 0.05) decreased NO levels in female (26.71%) pups when compared to prenatally LPS treated pups. Prenatally LPS exposed female pups showed significant (P < 0.05) increase in lipid peroxidation when compared to control pups (37.99%). However, significant (P < 0.05) decrease in MDA levels were observed in case of female pups from zinc supplemented mothers (25.37%). Similarly, prenatal LPS exposure significantly (P < 0.05) decreased the GSH levels in male (61.57%) and female (59.65%) pups when compared to control pups. However, GSH levels were found to be significantly (P < 0.05) increased in male (60.60%) and female (73.52%) pups following zinc supplementation to their LPS treated mothers when compared to prenatally LPS treated pups. Prenatal LPS exposure significantly (P < 0.05) decreased the enzymatic activity of Catalase (33%) and SOD (32.94%) in only female pups when compared to control pups. Whereas, pups following zinc supplementation to their LPS treated mothers showed non-significant results when compared to prenatally LPS treated pups.

Histopathological studies

Coronal section from both male and female pups from prenatally exposed female rats were prepared to analyse LPS induced alterations in hippocmapus and cortex region. Figure 3 shows the histo-architecture of hippocampus and Figure 4 cortex of all the groups for both prenatally LPS exposed (A) male and (B) female pups respectively. Necrotic cells with inflammatory infilterate were seen in histological slides of hippocampus and cortex area in prenatally LPS exposed both male and female pups. Cystic lesions were also observed in the cortex of prenatally LPS exposed male pups. Decrease in the number of necrotic cells was observed in hippocampus as well as cotex of zinc supplemented group for both male and female pups.

DISCUSSION

In the current study, the effects of prenatal zinc

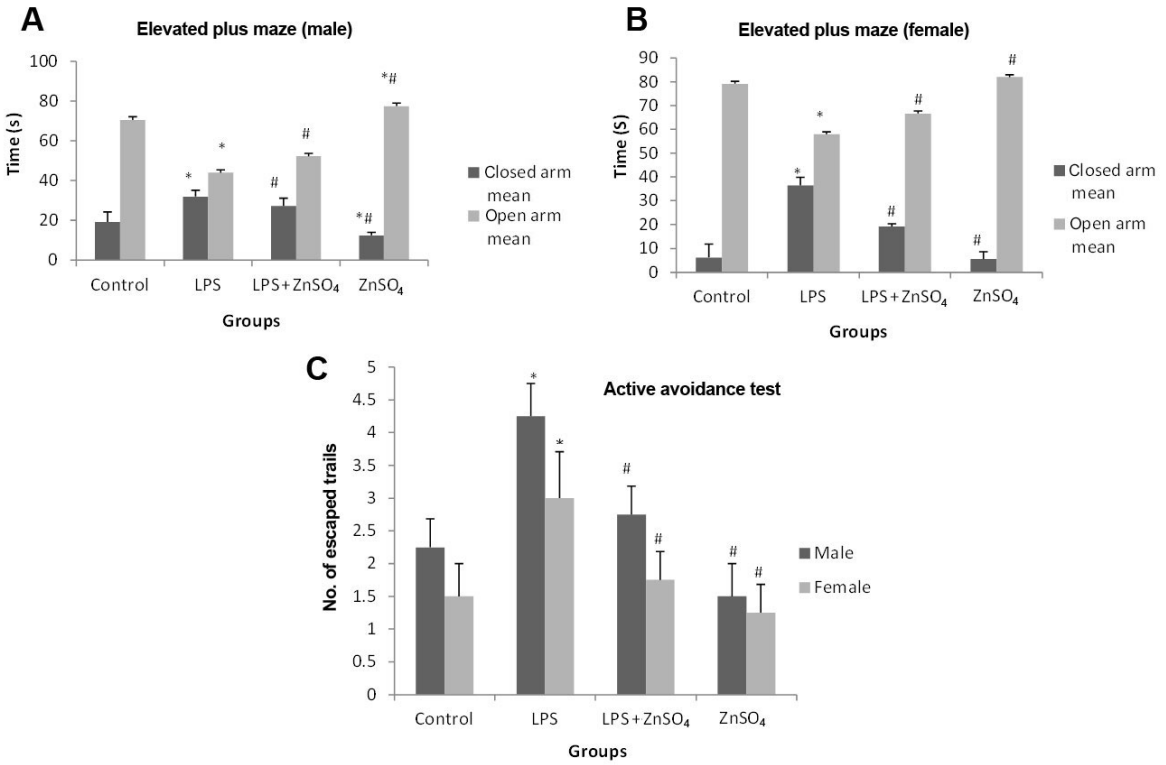
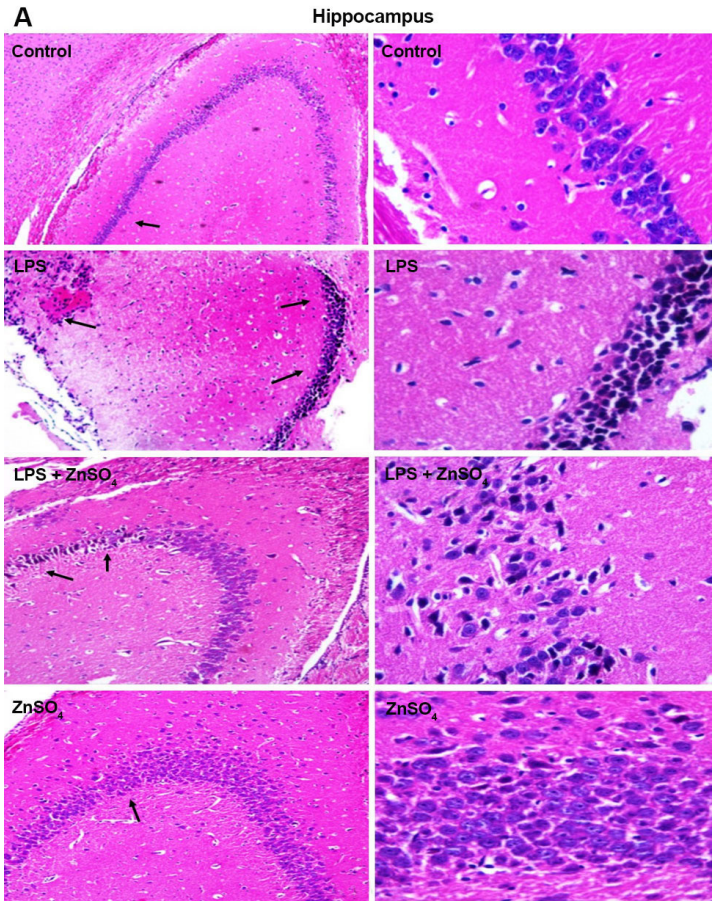


Figure 2: Effect of prenatal zinc supplementation on elevated plus maze in prenatally LPS treated (A) male pups, (B) female pups and in (C) active avoidance test on both the genders. Values are expressed as mean + SD; n = 5/group, $^{*}P < 0.05$ *vs.* control group, $^{\#}P < 0.05$ *vs.* prenatally LPS treated group. LPS: lipopolysaccharide



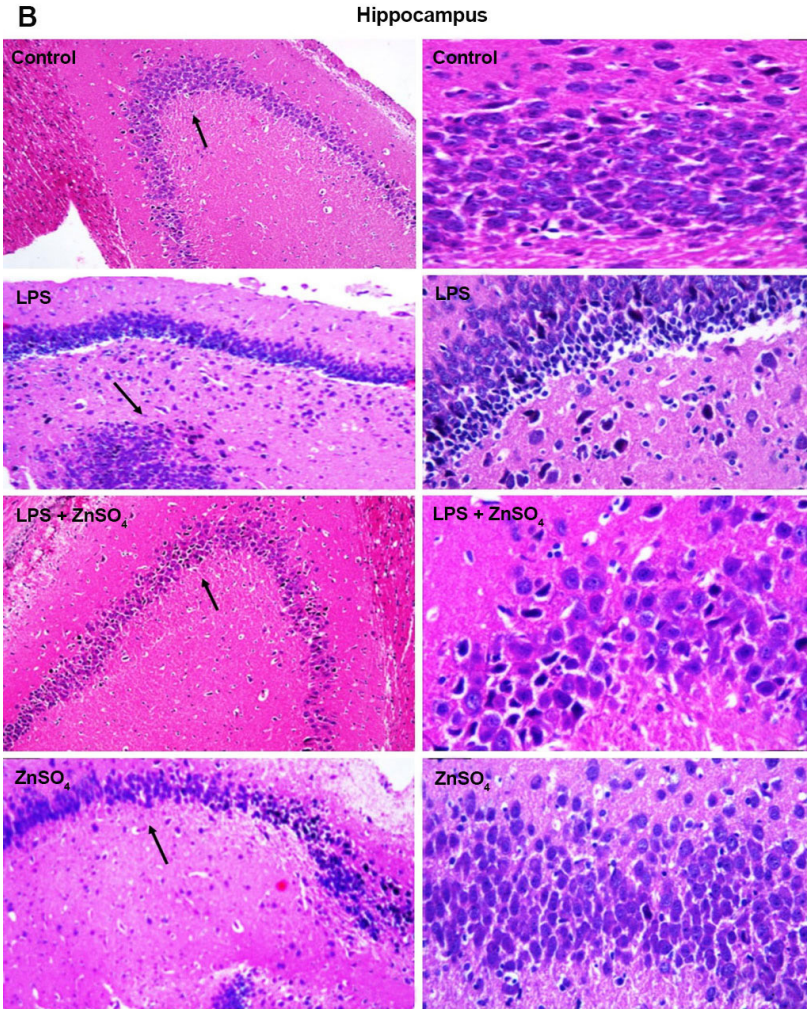


Figure 3: Representative figures are HE stained coronal sections of the brain showing the effect of prenatal zinc supplementation in hippocampal region of prenatally LPS treated (A) male and (B) female pups. Left side: ×10; right side: ×40. LPS: lipopolysaccharide

supplementation against LPS-induced endotoxemia during third trimester of pregnancy were investigated. It was observed that LPS-induced endotoxicity has major effects on female pups as compared to male pups. Although behavioral impairments were seen in both prenatally exposed male and female pups. Importantly prenatal zinc supplementation prevented these effects in female pups. Many studies have reported behavioral impairments in new born as well as adult off-spring due to early, mid or late gestational LPS exposure. According to Taweel *et al.*,[49] it was shown that gestational exposure of LPS has an inhibitory effect on righting reflex, rotating reflex and cliff avoidance activity in mice off-spring (PND21). Also, zinc supplementation during pregnancy significantly improved reduced motor activity and responsiveness in rats and monkeys.[50] Similarly, in the present experiment with zinc supplementation to mothers throughout pregnancy showed improvement in total locomotor activity as well as rotarod behavior.

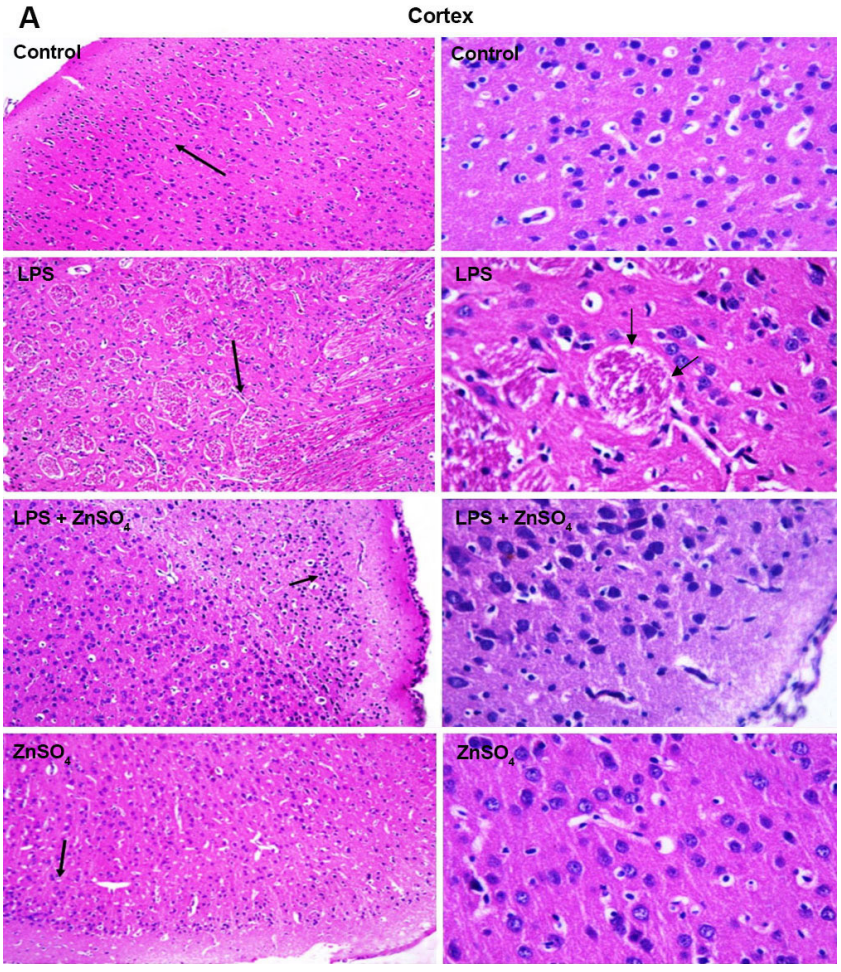
Prenatally LPS treated male and female pups spent maximum time in open arm on EPM and they escaped maximum number of trails in active avoidance test performed in the present study. This aberrant behavior can be due to loss of memory. Several reports demonstrated different memory based tests like nest-seeking, cliff avoidance and morris water maze *etc*. on prenatally LPS treated pups and pups showed impairment in behavior.[51,52] Maternal zinc supplementation prevents aberrant behavior in object recognition task in mice off spring following early gestational exposure of LPS.[53] In accordance with the previous reports in case of present study also an improvement in memory following maternal zinc supplementation was observed.

Loss of total locomotor activity and short term memory were further correlated with decrease in dopamine (DA) and serotonin (5-HT) levels. As per previous reports a long term reduction of DA and 5-HT along with other neurophysiological changes were observed

due to early gestational exposure of LPS in rats.[54] Also, a decrease in number of TH positive cells was observed in rat off-spring following LPS exposure.[55] In the case of our study a significant decline in the levels of neurotransmitters DA, 5-HT and NE were observed following LPS exposure which was further found to be improved in pups of zinc-supplemented mothers. This improvement in case of DA level was more significant in case of male pups as compared to female pups. This improvement in DA level in both sex pups could be related to the property that DA receptor regulating factor is a zinc finger transcription factor.[56]

A decrease in 5-HT levels in prenatally LPS exposed male and female pups could be related to decreased expression of serotonin transporter (5-HTT) was observed in somatosensory cortex using positron emission tomography in rabbit kits on post-natal day 1 when their mothers were injected with 20 μg/kg LPS intra-uterine on E28.[57] On the contrary, significant augmentation in 5HT content was observed in frontal cortex of prenatally LPS (1 mg/mL every second day from E7 until full-term) exposed female pups (PND21). Similarly, 5-HT levels were found to be significantly increased in co-supplemented group of both male and female pups in the current study. Also, studies have reported synergism of zinc and essential fatty acids in regulating DA, NE and possibly serotonin activity as zinc is essential for conversion of dietary pyridoxine to its active form, pyridoxal phosphate, and pyridoxine is necessary for conversion of tryptophan to 5-HT.[58]

In the present study, a significant increase in levels of NE in pups of both genders from LPS treated mothers was observed. The results are supported by the report that LPS has direct effect on hypothalamus to stimulate the efflux of NE and this effect was probably due to IL-1β.[59] NE negatively regulates the expression of pro-inflammatory cytokine expression at least to TNF-α which could contribute to the observed anti-inflammatory properties of NE.[60] However, NE levels were significantly decreased in co-supplemented pups. Further, the female pups from LPS treated mothers showed significant increase in NO levels and lipid peroxidation as depicted from increased MDA levels. However, in case of female pups from zinc supplemented mothers showed significant decrease in NO levels. It has been reported that Zn^{2+} inhibits



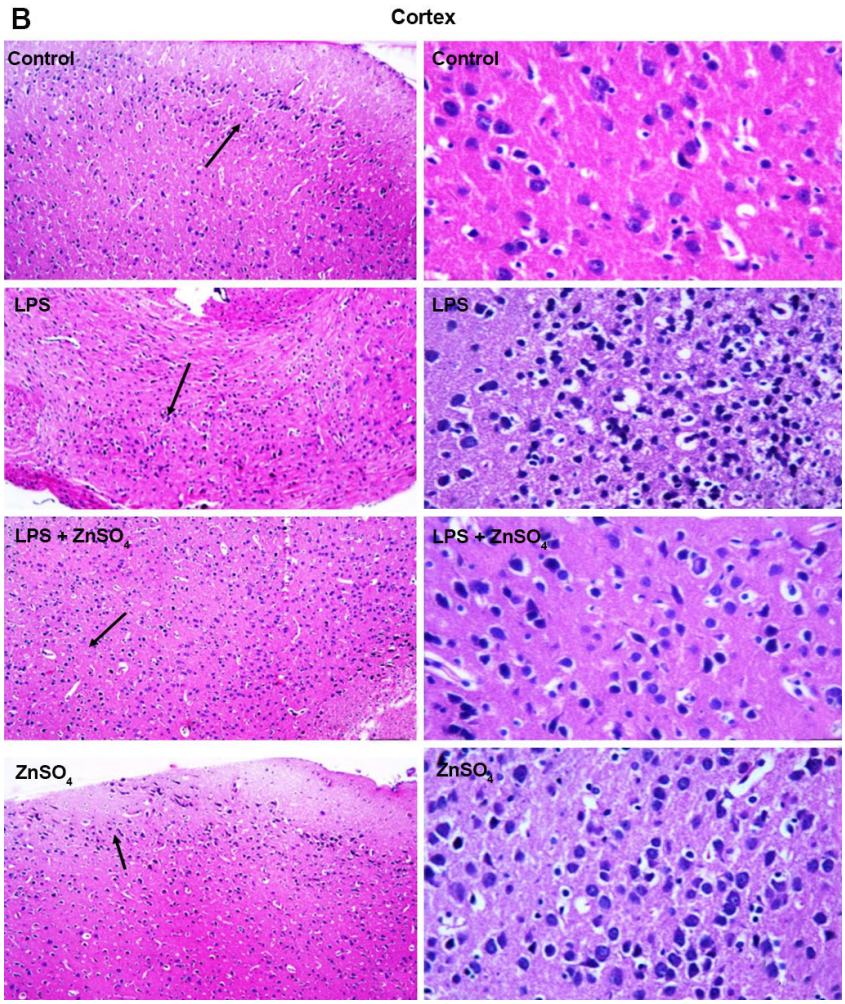


Figure 4: Representative figures are HE stained coronal sections of the brain showing the effect of prenatal zinc supplementation on cortex of prenatally LPS treated (A) male pups and (B) female pups. Left side: ×10; right side: ×40. LPS: lipopolysaccharide

production of inducible NO synthase to produce NO by (1) interfering with calcium-activated colmudulin function; (2) inhibiting NADPH dependent cytochrome P-450; and (3) by binding to glutathione and other thiols to decrease nitric oxide synthase activity.[31]

Significant reduction in GSH levels were observed in both prenatally LPS treated male and female pups. Whereas in case of pups from zinc supplemented mothers showed significantly increased GSH levels. Glutathione system is important for cellular defense against ROS. O^{2-} and NO radicals, although they cannot react directly with GSH, can oxidize GSH after undergoing intracellular redox reactions NO can react with O^{2-} by radical-radical interaction forming peroxynitrite ($ONOO^-$), thus clearing and scavenging O^{2-} in a diffusion limited rate.[61-63] $ONOO^-$ is a strong oxidizing agent, a reactive nitrogen species which oxidizes GSH rapidly to GSSG and depletes intracellular store of GSH. Similar results for glutathione system have been previously reported as decrease in GSH levels of maternal and fetal liver at 3 h, 6 h and 16 h after LPS injection to pregnant mice was observed.[64] Also, a significant decrease in levels of GSH in prenatally LPS treated mice pups and 4-month-old rats were reported.[65,66] Studies showed therapeutic efficacy of N-acetylcysteine, a potent anti-oxidant and precursor of glutathione to attenuate LPS-induced white matter injury and hypomyelination in the developing rat brain.[67] Zinc was found to be effective in decreasing oxidative stress via metallothionein by regulating the secretion of pro-inflammatory cytokines[68] and these metallothionein's are strong scavengers of free radicals.[69,70] In the present study only prenatally LPS treated female pups showed significant decrease in the activity of catalase and SOD enzyme. However prenatal zinc supplementation was unable to restore the activity of both the enzymes.

Female pups were found to be more prone to ill effects of prenatal LPS exposure as compared to male pups. As suggested by Paris *et al.*,[71] more of anxiety

levels in prenatally IL-1β exposed female pups were observed due to decrease in progesterone turnover to its metabolites in hippocampus which was not the same in case of male pups. However, this could also be related to differential increase of NE levels in case of pups of both sexes as observed in the present study. Results from histopathological studies were also in line with the above discussed results. Therefore, it can be suggested that LPS exposure prenatally effects pups and is gender specific and the same is the case with Zinc supplementation.

Authors' contributions

Study planning and experimental design: B. Nehru, N. Sharma
Experimental work and preparation of Tables and Figures: P. Arora, N. Sharma
Evaluation of the results: B. Nehru
Manuscript writing and communication: N. Sharma

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient involved.

Ethics approval

Ethical approval was obtained prior to the commencement of the study.

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Topic: Stroke **Open Access**

Cerebral ischemia at early postoperative period of direct revascularization for moyamoya disease: a case report and literature review

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ABSTRACT

Hypoperfusion and hyperperfusion could be causes of early postoperative complications that lead to neurological deterioration in patients with moyamoya diseases (MMD) after superficial temporal artery (STA) and middle cerebral artery (MCA) anastomosis. Here, the authors described a case of child-onset bilateral MMD that manifested transient cerebral ischemia in the contralateral hemisphere after left STA-MCA bypass in young adulthood. A new onset of cerebral ischemia in the contralateral hemisphere and transient neurological deterioration suggested the fragile hemodynamics of MMD during early perioperative period. Serial evaluation of postoperative cerebral hemodynamics and perfusion might facilitate targeted management in patients with unstable or advanced MMD.

Quick Response Code:

INTRODUCTION

Moyamoya disease (MMD) is one of the major causes of stroke in children and adults. It is characterized by progressive stenosis or occlusion of terminal portion of internal carotid arteries and development of fragile collateral vessels (moyamoya vessels).[1] Revascularization surgery is a recommended therapy in these patients.[2] Although the long-term outcome of direct revascularization through superficial temporal artery (STA)-middle cerebral artery (MCA) bypass is generally favorable, early postoperative neurological events are still frequently reported and contribute to neurological deterioration.[3] The change of perioperative cerebral perfusion is suggested to be dynamic, therefore to identify the underlying mechanisms and risk factors might improve the postoperative management, especially in patients with advanced MMD and unstable hemodynamics. In MMD with unilateral STA-MCA bypass, symptomatic cerebral ischemia in the contralateral hemisphere occurs in about 3-14% of patients.[4-7]

We herein presented a case of childhood-onset bilateral MMD, which developed transient cerebral ischemia in the contralateral hemisphere after STA-MCA bypass in her young adulthood. Transient weakness of the left extremities and acute cerebral infarctions in the contralateral hemisphere were observed from postoperative Day 1 to Day 6. The neurological deficit improved after intravenous infusion of fluid and free radical scavenger. There were no subsequent neurological events, and the preoperative neurological deficit significantly improved during the follow-up period. The mechanism and management of the neurological events in the early postoperative period were further discussed with a literature review.

CASE REPORT

A 21-year-old female with 5-year history of paroxysmal limbs weakness presented to our hospital with a reoccurrence of symptoms and slurred speech 15 days ago. There was no family history of MMD. Digital subtraction angiography (DSA) revealed extensive stenosis of the bilateral terminal portions of the internal carotid arteries (ICAs) and abundant moyamoya vessels on both sides, leading to a diagnosis of MMD. The bilateral anterior cerebral artery (ACA) and the right posterior cerebral artery (PCA) were not identified. The left PCA was also involved with stenosis and collaterals of vascular network. Collaterals from the posterior circulation formed between right occipital and parietal lobe [Figure 1]. Magnetic resonance imaging (MRI) showed multiple infarct lesions in the bilateral watershed. Perfusion computed tomography (CT) showed a reduced cerebral blood flow of the bilateral hemisphere with a mild domain on the left side [Figure 2].

Considering the patient had barylalia, a left STA-MCA anastomosis was first performed between the parietal branch of the STA and MCA (M4 segment) supplying the frontal and parietal lobe. The intraoperative blood pressure (BP) remained between 120-140 mmHg. We followed our postoperative protocols with fluid infusion, a prophylactic anti-epileptic drug, aspirin, and statins. The patient did not display any neurological deficits after surgery until she appeared restless and delirious 8 h afterwards. We suspected hyperperfusion syndrome after revascularization, while the perfusion CT was not available at night. Consequently, the patient was managed with blood pressure control (systolic blood pressure between 110-130 mmHg) and intravenous fluid infusion. On the 1st day after surgery, she appeared to have barylalia and retrograde amnesia, thus intravenous edaravone (60 mg/day) was added. On the 2nd day after surgery, the retrograde amnesia completely resolved, while left limb weakness appeared with muscle strength of Grade 3. Postoperative imaging on the 3rd and 6th day after surgery revealed an improved perfusion of left hemisphere, but identified a *de novo* lesion between the right temporal and occipital cortex with high intensity on T2 weighted image (T2WI) and diffusion weighted image (DWI) and decreased cerebral blood flow (CBF), which was in the contralateral hemisphere of STA-MCA anastomosis [Figure 3]. Fluid infusion ensuring euvolemia, and edaravone were continued with systolic blood pressure between 130-140 mmHg. The patient's left limb weakness was completely resolved by the 6th day after surgery. She was discharged 13 days after surgery with the barylalia relieved and no other neurological deficits during the latest follow-up period of 2 months.

DISCUSSION

Extracranial-intracranial direct anastomotic bypass is recognized as a treatment for ischemic MMD with favorable long-term outcome.[1] In the acute postoperative period of STA-MCA bypass, however, transient neurological events or stroke can be observed despite the improvement of cerebral perfusion at the site of anastomosis.[1,3] Rapid, increased local perfusion and perioperative hemodynamic fluctuations after bypass surgery might increase the risk of abnormal perfusion in the adjacent area or remote regions, especially in patients with advanced stage, bilateral, or unstable MMD.[8-10]

In MMD with unilateral STA-MCA bypass, symptomatic cerebral ischemia in the contralateral hemisphere more commonly occurs in patients with advanced stage (Suzuki stage 4 to 6), PCA involvement and postoperative hypotension on postoperative Day (POD) 1 and Day 2.[7] Advanced Suzuki stage and PCA involvement indicate the progression of MMD.[11] In these patients, cerebral hemodynamics might be unstable and more susceptible to cerebral infarction after fluctuations of perfusion during the perioperative period.[7] The patient in this case had a bilateral advanced stage of MMD (stage 4 for the left and stage 3 for the right) with right PCA involvement [Figure 1]. Although the unilateral direct revascularization might directly influence the blood flow of the contralateral hemisphere through collaterals, the collaterals from the contralateral side, in the present case, were poorly developed and were mainly around the midline, which might not explain the ischemia of the remote convexity [Figure 1]. Retrospective analysis of DSA suggested that the right PCA collaterals to the watershed between parietal and occipital lobes were not well developed. Preoperative perfusion CT also indicated impaired perfusion in the aforementioned area [Figures 1 and 2]. These angioarchitecture and perfusion features might be the underlying factors contributing to the focal ischemic deterioration after stress, including surgery and delirium. Therefore, intensive perioperative care should be recommended for these MMD patients with high risk factors [Table 1].[12] Real-time monitoring of the cerebral blood flow (CBF) showed that the regional CBF after revascularization followed a variable pattern with significant decreases between 12 h and 24 h after surgery and at 36 h after surgery.[13] This CBF pattern correlated with the onset of a transient neurological event in our case and suggested a careful blood pressure management during the early postoperative period. In addition, rather than arbitrarily maintaining the blood pressure between certain parameters, the blood pressure management should be referenced to the preoperative level to avoid a rapid decrease from the baseline.[7] Recent data suggested that prophylactic

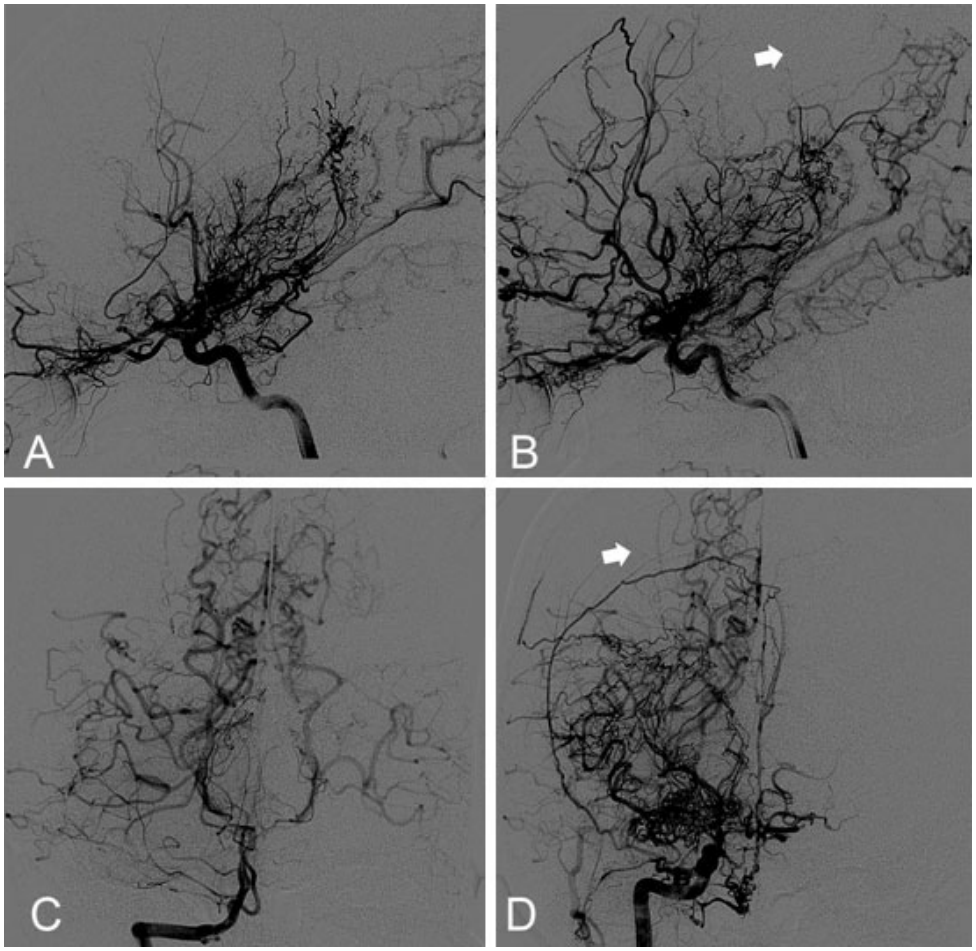

Figure 1: Preoperative angiography. Left ICA (A) and right ICA (B) angiography revealed a bilateral moyamoya disease. Vertebral angiography showed stenosis at proximal segment of right PCA and pooly developed left PCA, suggesting posterior circulation involvement (C). The anterior-posterior (B) and coronal (D) view of right ICA showed a watershed between parietal and occipital lobe, where there was lack of moyamoya collaterals from both anterior and posterior circulation (white arrow). ICA: internal carotid artery; PCA: posterior cerebral artery

Table 1: Pathology and management of perioperative focal neurological deficit after direct revascularization for moyamoya disease

	Pathogenesis	Risk factor	Presentation	Diagnosis	Management
Hyperperfusion	Rapid focal CBF increase, impaired cerebral auto-regulation	Left anastomosis Elder age at operation	Incidence: 15-38% Symptom: BP dependent deterioration of FND or seizure; delayed ICH (3.3%); evident between POD 2 to 6	CBF analysis (SPECT, PET, CTP, ASL or BOLD-fMRI); CBF increase at the site of anastomosis; neuroimaging; DWI: absence of acute ischemia; FLAIR: *de novo* hyperintense ivy or belt sign	POD 0; sBP 110-130 mmHg, Edaravone (30 mg), Minocycline (100 mg); POD 1-2 sBP < 140-150 mmHg if no HP; normotensive later Edaravone (30 mg), Minocycline (200 mg)
Hypoperfusion	Susceptible region insufficiently supplied by abnormal vascular network or collaterals	Preoperative: advanced stage MMD, unstable MMD with rapid stenosis progression or repeated ischemic stroke, PCA involvement, Pre-OP cerebral infarction Intra-or postoperative: hypotension (compared with pre-op BP), hypocapnia, hypovolemia, hematocrit reduction	Incidence: 4-59%, Asian might be higher than Caucasian Symptom: FND with two peak of onset at POD 0.5-1 and POD 1.5-2, cerebral infarction (10-15%)	CBF analysis (SPECT, PET, CTP, ASL or BOLD-fMRI); CBF decrease in susceptible region; neuroimaging; DWI: acute cerebral ischemia	PO 6 hour to 1 day; careful BP and volume management (no rapid decrease compared with pre-op BP); POD 1-2; early CBF evaluation; edaravone during POD 0-7 might be helpful
	Retrograde blood from STA-MCA bypass conflict with anterograde blood from proximal MCA or collaterals	Stenosis of collaterals or anterograde feeding arteries		CBF analysis (SPECT, PET, CTP, ASL or BOLD-fMRI); CBF decrease in shifted watershed, CBF increase at the site of anastomosis; neuroimaging; DSA: pre-OP collaterals or proximal MCA feeding the MCA territory and correlating with the new hypoperfusion lesion; MRI: acute cerebral ischemia in shifted watershed (usually fed by other branches of collaterals or MCA away from the site of anastomosis) w or w/o hyperperfusion sign due to bypass flow	
Normal perfusion	Vasogenic edema, ischemia/reperfusion injury may damage BBB with increased vascular permeability	NA	Incidence: 1-2%; FND or asymptomatic; no seizure found	CBF analysis (SPECT, PET, CTP, ASL or BOLD-fMRI); absence of hyperperfusion or hypoperfusion; Neuroimaging; DWI: absence of hyperintensity; ADC: ADC value significantly increase, suggesting vasogenic edema rather than cytotoxic edema	POD 0 to 7; sBP < 130 mmHg; minocycline (200 mg/day); edaravone

ADC: apparent diffusion coefficient; ASL: arterial spin labeling; BBB: blood-brain-barrier; BP: blood pressure; CBF: cerebral blood flow; CTP: CT perfusion; DWI: diffusion weighted image; FLAIR: fluid attenuated inversion recovery; FND: focal neurological deficit; ICH: intracranial hemorrhage; MMD: moyamoya disease; MRI: magnetic resonance imaging; NA: not applicable; PCA: posterior cerebral artery; PET: positron emission tomography; PO: postoperative; POD: postoperative day; SPECT: single-photon emission computed tomography

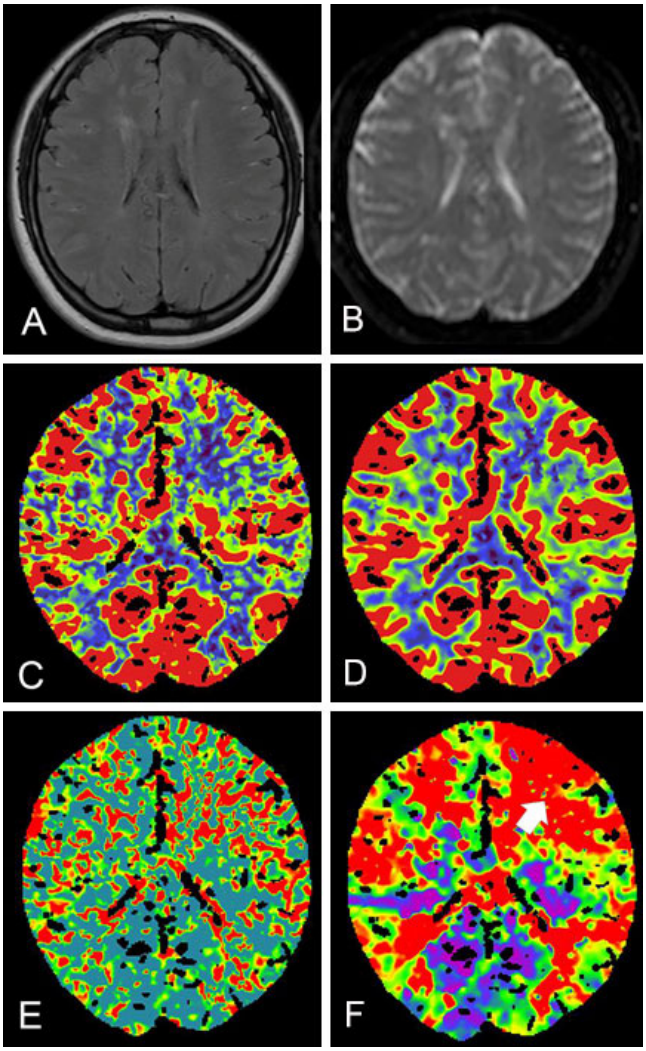

Figure 2: Preoperative magnetic resonance image and perfusion CT. No acute cerebral ischemic lesion on preoperative FLAIR (A), DWI (B), perfusion CT revealed mild reduced CBF (C), and CBV (D) along with increased MTT (E) and TTP (F) at left frontal lobe (white arrow). CT: computed tomography; CBF: cerebral blood flow; CBV: cerebral blood volume; DWI: diffusion weighted image; FLAIR: fluid attenuated inversion recovery; MTT: mean transit time; TTP: time to peak

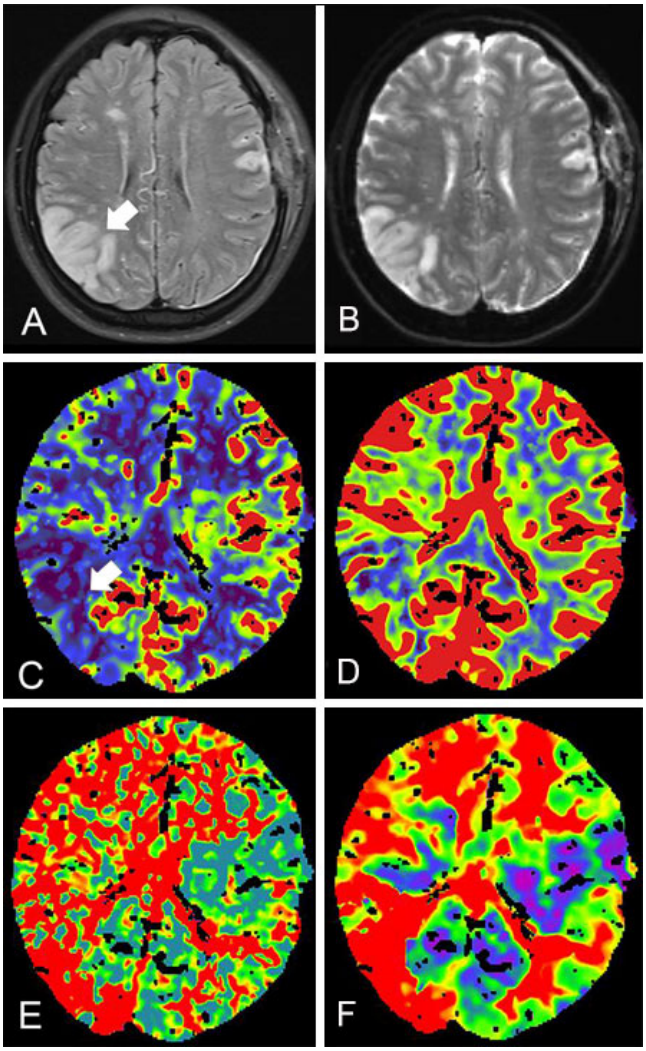

Figure 3: Postoperative magnetic resonance image and perfusion CT. MR image at postoperative Day 6 revealed a ischemic lesion (white arrow) of hyperintensity between the right parietal and occipital lobe on FLAIR (A) and DWI (B). Perfusion CT at postoperative Day 3 showed CBF (C) and CBV (D) reduced at the corresponding site. Regional MTT (E) and TTP (F) increased significantly. CT: computed tomography; CBF: cerebral blood flow; CBV: cerebral blood volume; DWI: diffusion weighted image; FLAIR: fluid attenuated inversion recovery; MTT: mean transit time; TTP: time to peak

mild blood pressure lowering might reduce the risk of hyperperfusion without increasing the incidence of ischemic stroke,[14] although its validation still needs to be verified in other independent cohorts and high risk subgroups.

Both hyperperfusion and hypoperfusion have been identified as reasons for transient neurological deficits [Table 1].[6,8,13] Postoperative cerebral perfusion status might fluctuate from initial hyperperfusion after bypass surgery to local and transient hypoperfusion before it normalizes to a new level.[13] Moreover, the perfusion at different region might also be distinct during the same period of time.[10] CBF evaluation at the early postoperative period (POD 1 to 3) might be recommended to tailor the perioperative management based on the perfusion status.[15] The DWI and fluid attenuated inversion recovery (FLAIR) sequence could also be helpful to identify hyperperfusion and acute ischemia with typical radiological features.[15-17] In our case, we suspected that the transient delirium might have been associated with hyperperufusion syndrome, so we followed our previous protocol with CBF analysis and neuroimaging on the third postoperative day. However, in this case, the patient's neurological event occurred within 2 days after surgery, and the imaging beyond that time did not provide a timely evaluation for tailoring management prior to the formation of the new cerebral infarction. Considering the time pattern of postoperative fluctuation in cerebral perfusion, an early

combination of CBF analysis and neuroimaging within 2 days after surgery might be reasonable [Table 1].[13,15]

Although the positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are the most extensively used quantitative methods for cerebral hemodynamics assessment, the use of radionuclides and the high cost of the examination limit the serial perioperative PET or SPECT scans. Therefore, the alternative noninvasive methods without contrast agent, such as blood-oxygen-level dependent functional MRI (BOLD-fMRI) or arterial spin labeling MRI (ASL-MRI), might be of great clinical importance. Previous studies suggested that the delayed and reduced magnitude of the BOLD response might indicate the severity of compromised hemodynamics in patients with cerebral ischemic diseases.[18,19] A most recent report observed BOLD responses to specific stimulation in patients with MMD before and after revascularization, and found a predicator for postoperative outcomes, suggesting the feasibility of using BOLD-fMRI for individualized assessment of cerebral hemodynamic impairment and hemodynamic improvement after revascularization in patients with MMD.[20] ASL is another promising technology for cerebral hemodynamics assessment in perioperative management of MMD.[21] Several studies have demonstrated the advantage and accuracy of ASL in cerebral blood flow evaluation.[22,23] Recent advances in multiple-parameter ASL (multiple inversion time-pulse or post labeling delay) might allow for more precise assessment of cerebral hemodynamics.[24-26] Future studies in neuroimaging will provide better radiological tools for evaluation of cerebral blood perfusion.

Free radical scavenger and anti-inflammatory medicine might also be helpful in reducing the risk of perioperative neurological events.[15] A recent case-control study suggested MMD patients using edaravone (a free radical scavenger) had a decreased frequency of transient neurological event after direct revascularization surgery.[27] We added ederavone in this case as recommended in some protocols, and the neurological function markedly improved without disability, despite a cerebral infarction. Minocycline, a matrix metalloproteinase-9 inhibitor, might also ameliorate the reperfusion injury at the site of anasotmosis and the remote area after blood pressure reduction and has been used in patients with MMD to prevent both hyperperfusion and cerebral infarction at the remote area.[9,28,29] Further studies might be feasible to unravel the therapeutic effect of these medicines in larger populations.

Postoperative agitating delirium would increase the cerebral oxygen consumption, which might increase the risk of cerebral ischemia during the early postoperative period of MMD. We did not find any evidence opposing sedation in patients with MMD. However, most sedative agents have a potential side effect of hypotension. Although these agents might be used during anesthesia, intraoperative hypotension was common and did not independently increase the risk of postoperative ischemic complications.[7,30] In contrast, postoperative hypotension was a significant risk factor for postoperative ischemic events.[7] Therefore, further studies might be conducted to discuss the sedation in MMD patients with postoperative, agitating delirium.

Postoperative transient neurological events were reported to occur in 40-60% of revascularization surgery for MMD,[10] and 4-14% of patients exhibited postoperative cerebral infarction.[10,31] This report is congruent with previous observations that early postoperative neurological deficits usually resolve within 7 days, while irreversible cerebral infarction may persist in some patients with or without neurological deficits.[12] The incidence of transient neurological events varies among different reports, and it seems that studies from Asian countries showed a higher rate of postoperative stroke than those from America.[31,32] The association between perioperative neurological events and permanent neurological deficits should be further examined in future studies.

Authors' contributions

The definition of intellectual content: X.L. Chen, L. Ma
Literature review: X.L. Chen, L. Ma
Manuscript preparation and editing: X.L. Chen, L. Ma, J.L. Lu
Data acquisition: Y. Chen, J.L. Lu
Concept contribution: X. Ye, R. Wang, Y.L. Zhao
Definition of intellectual content: X. Ye, R. Wang, Y.L. Zhao
Manuscript review: X. Ye, R. Wang, Y.L. Zhao

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patient.

Ethics approval

The treatment and data collection in our study involving the patient is consistent with the ethical standards of the Institutional Review Board.

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Topic: Infectious Disease of Central Nervous System **Open Access**

Developing an international consensus guidance for myasthenia gravis using RAND/UCLA appropriateness method

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ABSTRACT

Aim: Myasthenia gravis (MG) is a rare and heterogeneous disease for which there is no generally accepted standard of care. Thus, it is critical that MG experts develop consensus guidelines based on their practice and disease management to assist clinicians and provide advice for insurance companies, health organizations and institutional review boards. **Methods:** An international treatment guidance was developed based on national guidelines established in the US, Denmark, Norway, Germany, Japan, Netherlands, United Kingdom and Europe. The RAND/UCLA appropriateness method (RAM) was applied to reach consensus among 15 worldly renowned experts and experienced clinicians. **Results:** This paper introduced the RAM procedure with its principles and applications and conducted a brief review of the resulting 2016 international consensus guidance for MG in comparison to clinical experience and management of Chinese MG patients. **Conclusion:** The 2016 international consensus guidance is a major contribution to the treatment and management of MG, providing an up-to-date expert consensus to assist clinicians around the world, especially those with limited experience and/or practice in countries/regions that have limited resources to develop local treatment guidelines. It is also an important contribution showing how RAM can help to develop consensus guidance for treatment of rare diseases based on scientific findings and expert experience.

Quick Response Code:

INTRODUCTION

Myasthenia gravis (MG) is a neuromuscular transmission disorder. The incidence of MG ranges between 0.3-2.8 per 100,000, affecting more than 700,000 people worldwide.[1] The prognosis for patients with MG has been improved tremendously in recent years due to the increasing use of immunomodulating treatments. However, there is no optimal treatment approach for all patients due to disease heterogeneity, thus an internationally recognized standard of care for MG is still missing.

The MG symptoms and the "morning light evening heavy" characters were first described as early as 1672 by British clinician Thomas Willis,[2] whereas the cause of the disease remained a mystery until the 1960s. At that time, MG was described as the result of antibodies binding to the neuromuscular junction, most commonly against the acetylcholine receptor.[3] However, up to date management of MG still remains a great challenge.[4]

There are many reasons behind the need to develop an international consensus guideline for MG management. Firstly, early expert treatments can significantly improve the prognosis of a MG patient, as some physicians have not seen enough MG patients to be familiar with all its cardinal features. This is due to MG low incidence and heterogeneity. Secondly, uncontrolled clinical trials may be potentially biased, while the few successful randomized controlled trials (RCTs) cannot be generalized to assess the effectiveness and safety of multi-regimens, and to select the best treatment for each patient. Thus, it is critically important that experts share their knowledge and competence to improve the management of MG. Such expert-developed guidelines not only will help the clinician, but will also represent a unique resource for third-party payers such as insurance companies, governmental health organizations and institutional review boards.

METHODS

In October 2013, a Task Force of the Myasthenia Gravis Foundation of America (MGFA) assembled a panel of 15 internationally recognized MG experts, chaired by Donald Sanders of the Duke University and Gil Wolfe of State University of New York at Buffalo, and moderated by Pushpa Narayanaswami of Harvard University. The main goal of this panel was to develop treatment guidance statements based on formalized consensus. The guideline development employed the RAND/UCLA Appropriateness Method (RAM),[5] which was established and refined by RAND and the University of California, Los Angeles (UCLA) in the 1980s.

The first meeting was held in February 2013 in Durham, North Carolina, to make decisions on cardinal definitions that were going to be instrumental for subsequent guidance treatment statements: goals of treatment, minimal manifestations, remission, ocular MG, impending and manifest myasthenic crises and refractory MG. Definitions without consensus were modified upon the panelists' suggestions and shared with the panel for subsequent voting rounds.

The first draft of the MG guidance treatment statements was prepared by the two executive chairmen and the Harvard University service providers, based on recent publications and guidelines from the US, Denmark, Norway, Germany, Japan, Netherlands, United Kingdom and Europe.[6-11] The following three assumptions were agreed *a priori*: (1) treatment costs and availability would not be considered; (2) clinical examination is performed by experienced physicians for the evaluation of neuromuscular disease; (3) the MGFA Clinical Classification refers to the state of the patient at the time of evaluation. Guidance statements were developed for the following seven topics: MG symptomatic and immunosuppressive (IS) treatment, IV immunoglobulin (IVIg) and plasma exchange (PLEX), impending and manifest myasthenic crisis, thymectomy, juvenile MG (JMG), muscle-specific tyrosine kinases (MuSK) antibody-positive MG and MG in pregnancy.

The consensus guidance statements were refined using a quantitative evaluation system of the RAM program. Initial and revised statements were voted and commented on anonymously at least three times during the process. The facilitator (Dr. Pushpa Narayanaswami) was the only person who made announcements about the statements and collected votes and feedbacks. Dr. Narayanaswami was not allowed to vote or participate in discussions and feedbacks to ensure the maximum objectivity of the process. The chairmen gathered the votes anonymously and revised the statements, seeking for ultimate consensus. A second face-to-face meeting and panel discussion was held in March 2014 after the first round of vote. The second and third round of votes were solicited after statements revision to reflect all panelists' comments and experiences. After all three rounds, consensus was reached on all definitions and guidance statements. So far, this represents the first official international MG treatment guidance and as such it was published in its final form on June 29, 2016

in the journal of *Neurology*.[1]

RAM was developed to combine the best available scientific evidence, even when the randomized controlled trials (the golden standard in evidence-based medicine) are not available or cannot provide enough detailed guidance for everyday clinical practice. The RAM is based on the collective judgment of experts with the common objective to release statements regarding the appropriateness of following a procedure, using the multiple-rounds Delphi polling sessions to assess the treatment rationality.[5]

The procedure of RAM

There were 2 major concepts during the RAM: appropriateness and agreement. The median rating at each round is the appropriateness score and the summary of the appropriateness scores for each recommendation is its agreement score. Appropriateness ratings are collected from each panelist to quantitatively assess the relative harm or benefit of a particular intervention. Each recommendation is rated on a 9 point scale: 1-3 are extremely inappropriate to inappropriate (i.e. risks > benefit); 4-6 are uncertain (i.e. risks ≈ benefit); 7-9 are appropriate to extremely appropriate (i.e. benefit > risks) [Table 1].

The detailed procedure of RAM is listed in the flow chart of Figure 1.

Disagreements and uncertainty ratings assist in determining "grey" areas for future research. Panel consensus is NOT forced. Rather, the degree of agreement is used to define the strength of the recommendations. Agreement: ≤ 3/13 panelists or ≤ 4/14 panelists rate the recommendation outside the 3 point region containing the median score of appropriateness. Disagreement: ≥ 4/13 panelists or ≥ 5/14 panelists rate the recommendation in the 1-3 region and the same number in the 7-9 region of appropriateness.

Therefore, if the appropriate scores on a particular recommendation have a median of 7-9, the recommendation is considered to be appropriate without disagreement, instead, if the appropriate scores have a median of 4-6, it could be either uncertain (if the actual scores are within 4-6) or disagreement (if some scores are in 7-9 region and others are in 1-3 region), and the recommendation must be revised for future consensus. Similarly, if the appropriate scores have a median of 1-3, the recommendation is considered to be inappropriate without disagreement.

It is also possible to reach an agreement about a recommendation being appropriate, inappropriate or even uncertain. Facilitator manages voting but does not vote. Cost and availability considerations are NOT used at this stage and all options are assumed to be affordable and freely available.

RESULTS

The application of RAM

RAM is useful for rare cases with low morbidity, lack of RCT clinical research, pooling available research evidence and expert experience to draw up a guideline,

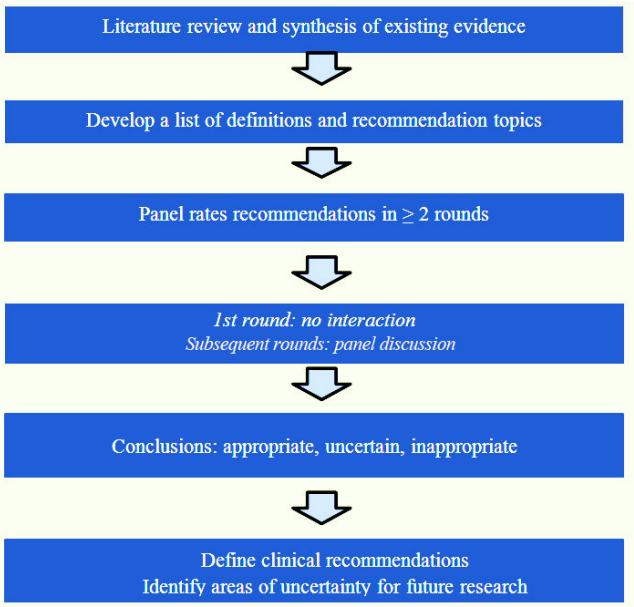

Figure 1: Flow chart of the RAND/UCLA rationality approach to develop a consensus (adopted from Dr. Sanders' presentation with permission)

Table 1: RAND/UCLA appropriateness method rationality and its meaning of the 9-point score (adopted from[5])

Inappropriate	1	Extremely inappropriate (risk greatly exceed benefits)
	2	Moderately inappropriate
	3	Slightly inappropriate
Uncertain	4	May be inappropriate
	5	Uncertain/equivocal (benefit and risk about equal)
	6	May be appropriate (expected health benefits to an average patient exceed the expected health risks by a sufficiently wide margin to make intervention worthwhile and the intervention is superior to alternatives, including no intervention)
Appropriate	7	Slightly appropriate
	8	Moderately appropriate
	9	Extremely appropriate (benefit greatly exceed risks)

solicit the expert panel's opinion and quantify the level of approval. However, if this does not meet the stated objectives, the recommendations of the consolidated expert group will be revised and the above steps will be repeated so that the views will be recognized to the maximum benefit.

The advantages of group decisions are obvious. A group is less likely than an individual to draw a wrong conclusion. If panelists are properly chosen, they can represent a wide range of knowledge and experience. Their interaction stimulates debate and consideration of many opinions that may challenge previously well-accepted ideas and stimulate new ones.

However, formal consensus has its own pitfalls: (1) only one person can speak at a time, limiting the number of ideas expressed and discussed; (2) a social pressure might induce to agree with the majority or a "powerful" voice in public; (3) the desire to reach agreement may override concerns about the accuracy of the result and may result in premature closure without consideration of all possible alternatives.

When uncertainty and differences of opinions exist, the RAM process of summarizing judgements helps to identify areas of agreement and establish areas of disagreement. The combination of face-to-face discussion at early stage and the solicitation of anonymous votes and comments handled by a non-voting facilitator are effective in maximizing the input of all experts' knowledge and experience.

The case of developing MG international consensus guidance using RAM

More than two years passed between the initial appointment of the MGFA Task Force to develop treatment guidance for MG in October 2013 and the final acceptance of the publication of the international consensus guidance in July 2016. At the beginning, all definitions obtained consensus easily and all guidance statements were eventually agreed upon as being appropriate by the panel by the time of publication. However, not all topics took the same effort to reach consensus. Here are some examples of extreme cases during the RAM process.

Example 1 – easy consensus

The panelists easily reached consensus on statements about immunotherapy.

Round 1 statement: "If high steroid doses are needed chronically to achieve or maintain an adequate response, a steroid-sparing agent should be added, typically along with the steroid, to permit subsequent reduction of the steroid dose to the lowest necessary to maintain an adequate response."

Round 1 votes: median 9, appropriate. Range 6-9. Agreement: yes.

Consensus had been achieved; however, based on panel input and discussion, the statements were modified and re-voted.

Round 2 statement: "A non-steroid IS agent should be used alone when steroids are contraindicated or refused. A non-steroid IS should be used initially in conjunction with steroids when the risk of steroid side effects is high based on medical co-morbidities. A non-steroid IS should be added to steroids when: (a) steroid side effects, deemed significant by the patient or the treating physician, develop; (b) response to an adequate trial of steroids is inadequate; (c) symptoms relapse upon steroid taper."

Round 2 votes: median 9, appropriate. Range 8-9. Agreement: yes.

Final statement on the publication: "A non-steroid IS agent should be used alone when corticosteroids are contraindicated or refused. A nonsteroidal IS agent should be used initially in conjunction with corticosteroids when the risk of steroid side effects is high based on medical comorbidities. A nonsteroidal IS agent should be added to corticosteroids when: (a) steroid side effects, deemed significant by the patient or the treating physician, develop; (b) response to an adequate trial of corticosteroids is inadequate; or (c) the corticosteroid dose cannot be reduced due to symptom relapse."

Example 2 – difficult consensus

Considerable effort was needed to reach consensus on statements about thymectomy in childhood MG.

Round 1 statement: "In children and adolescents aged 5-10 years, thymectomy should be considered only after failure of symptomatic therapy and immunotherapy."

Round 1 votes: median 6, range 1-9, uncertain/equivocal.

Round 2 statements (modified based on discussion): "(A) in patients under 15 years of age, thymectomy should be considered in generalized MG after unsatisfactory response to AChEs and immunotherapy; (B) there is wide consensus that thymectomy is indicated in peri-pubertal and post-pubertal children with moderate to severe AChR-ab+ MG; (C) published reports also suggest that early thymectomy (within the first 12 months of onset of symptoms) is more effective

than delayed thymectomy; (D) for seronegative children, there is always a risk that some will have a CMS and not immune-mediated JMG; (E) evaluation at a centre specializing in childhood neuromuscular diseases should be considered before recommending thymectomy in young patients with seronegative MG."

Round 2 votes: median 8, range 2-9, 4 panelists rated 2-4, and the rest in the 7-9 range. There was still a disagreement, no consensus.

Round 3 statements (modified based on discussion): "(A) the value of thymectomy in the treatment of pre-pubertal MG patients is unclear, but thymectomy should be considered in children with generalized AChR$^-$ab$^+$ MG either if: the response to AChE inhibitor and immunosuppressive is unsatisfactory, or If there is a need/desire to avoid potential complications of immunosuppressive therapy; (B) for children diagnosed as seronegative GMG, the possibility of a congenital myasthenic syndrome or other neuromuscular condition should be entertained, and evaluation at a center specializing in neuromuscular diseases is of value prior to thymectomy."

Round 3 votes: median 8, range 7-9, appropriate with consensus.

Final statement on the publication: "(A) the value of thymectomy in the treatment of pre-pubertal patients with MG is unclear, but thymectomy should be considered in children with generalized AChR antibody-positive MG. (a) if the response to pyridostigmine and IS therapy is unsatisfactory; or (b) in order to avoid potential complications of IS therapy. (B) For children diagnosed as seronegative generalized MG, the possibility of a congenital myasthenic syndrome or other neuromuscular condition should be entertained, and evaluation at a center specializing in neuromuscular diseases is of value prior to thymectomy."

DISCUSSION

Preliminary definitions

Among the preliminary definitions compiled for the 2016 International Consensus Guidance for MG, for the first time two concepts are given clear definitions and provide highly valuable guidance to the clinical practice of treating MG patients.

The first concept is impending myasthenic crisis. It is defined as "Rapid clinical worsening of MG that, in the opinion of the treating physician, could lead to crisis in the short term (days to weeks)." In the past, crisis in MG is only referred to as manifest myasthenic crisis, defined as "MGFA Class V Worsening of myasthenic weakness requiring intubation or non-invasive ventilation to avoid intubation". The concept of "impending myasthenic crisis" will raise the awareness of the physicians who can take proactive approach to intervene before crisis actually takes place.[12]

Another concept is refractory MG. It is defined as "PIS is unchanged or worse after corticosteroids and at least 2 other IS agents, used in adequate doses for an adequate duration, with persistent symptoms or side effects that limit functional, as defined patient and physician." Refractory MG has been the focus of several discussions,[13,14] although without a specific definition until the 2016 Guidance. The definition of refractory MG could be furthered developed and improved, however the one currently approved provides a common denominator for MG specialists.

Guideline topics

The consensus guidance treatment statements were developed around the following seven major topics: symptomatic and IS treatment of MG, IVIg and PLEX, impending and manifest crisis, thymectomy in MG, juvenile MG, MG with MuSK antibodies and MG in pregnancy. The following four topics require further discussions.

Symptomatic and IS treatment of MG

The statement on the use pyridostigmine is straightforward and relatively easy to reach consensus. It is almost always the first choice in treating MG patients. However, when pyridostigmine is not readily available due to various social-economical reasons (for example in recent months in mainland China), physician may directly prescribe nonsteroidal IS agents.

We totally agree with the statements on the use of IS treatment, especially statement 5 on IS agent dosage and duration of treatment. It is highly desirable to prescribe a low dose of corticosteroids and dosage adjustments should not be made too frequently and abruptly ("no more frequently than every 3-6 months") based on our decades of clinical experience in China, although there are very different views and approaches regarding dosage and duration of treatment among Asian physicians.[8,15,16]

IVIg and PLEX

Although the guidance was developed with *a priori* agreement of not considering treatment costs and availability, it is worth noting that IVIg and PLEX are not covered by the Chinese insurance system. Since they are both expensive procedures (about $4,400-$7,300/IVIg and $1,500/PLEX), their applications have been limited.

Statement 1 mentioned that PLEX and IVIg are used as short-term treatment in MG patients with life-threatening signs. Our clinical experiences suggest that PLEX and IVIg alone are not sufficient in manifest MG crisis cases.[17,18] There are many complications during MG crisis (such as pneumonia, pneumothorax, heart failure and renal failure *etc*.) when PLEX and IVIg are not appropriate. Instead, a comprehensive treatment plan should be designed for each individual MG patient undergoing crisis.

Statement 2 mentioned that the choice between PLEX and IVIg depends on conditions of individual patient such as sepsis and renal failure. In addition, our clinical experiences suggest that IVIg is safer than PLEX to patients with cardiovascular disorders.[17]

Thymectomy

We totally agree with the statements about pre-pubertal patients with generalized AchR antibody-positive MG and all patients with MG with thymoma. Our research has shown that thymectomy on juvenal generalized MG patients did not affect their growth.[19,20]

Statement 5 mentioned that less invasive thymectomy approaches such as endoscopic and robotic approaches appear to yield similar results to more aggressive approaches and show a good track record for safety in experienced center. However, based on our experiences, endoscopic and robotic approaches to thymectomy are much less desirable for thymoma than extended thymectomy.[21-23]

MG with MuSK antibodies

In Chinese or Asian population in general, MG patients with MuSK antibodies are rarely seen. However, due to the lack of universal testing kits, the actual percentage is yet to be determined. As far as we know up to date, the testing center for MG patients in the US does not provide testing kits for other countries. Over a dozen of Chinese MG patients were found to be double seropositive using testing kits made in China (a surprisingly much higher positive rate than using testing kits made in UK) while only two of them had thymoma. This was in marked contrast to experts' experiences (personal communication with Dr. Donald Sanders). Testing kits made in Germany would not be available to Chinese patients until 2017. An internationally recognized standard testing kits for MuSK antibodies is yet to be developed.

In summary, the 2016 international consensus guidance is a major contribution to the treatment and management of MG. It provides an up-to-date expert consensus to guide clinicians throughout the world, especially to those who have limited experiences or practice in countries or regions that have limited resources to develop local treatment guidelines. More importantly, it is an extraordinary example of how the RAND/UCLA appropriate methods can help to develop a consensus guidance for treatment of rare diseases, bringing together the best of existing scientific results with the experience of specialists around the world.

Authors' contributions

Conceived the manuscript: W.B. Liu, W. Fang
Wrote the first draft in Chinese: W.B. Liu
Translated the first draft into English: H. Ran
Developed the conceptual structure and revised the manuscript: W. Fang
Participated in the clinical work and approved the final draft: C.Y. Ou, L. Qiu, Z.D. Huang, Z.Q. Lin, Y.K. Li, X.X. Liu, H. Huang

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient data involved.

Ethics approval

There is no ethics issue in this paper.

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Case Report Open Access

Two cases of Guillain-Barré syndrome after cerebral hemorrhage or head trauma

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ABSTRACT

Guillain-Barré syndrome (GBS) is an uncommon disease involving widespread peripheral nerve inflammatory demyelination which results in ascending symmetrical limb paralysis and areflexia. Approximately 2/3 of cases occurred following a simple, trivial antecedent infection. In northern China, diarrhea caused by *Campylobacter jejuni* is the most common etiology of GBS. This article presents 2 cases - post cerebral hemorrhage and post head traumatic GBS. Both patients suffered from acute motor axonal neuropathy, a main subtype of GBS, 14 days after cerebral hemorrhage or head trauma without any antecedent infection. The possible pathophysiological mechanisms are discussed in the article, and the importance of increasing the awareness of early diagnosis, as well as early treatment with intravenous immunoglobulin and supportive care, in this special pathogenic GBS is emphasized.

INTRODUCTION

Guillain-Barré syndrome (GBS) is an acute inflammatory polyneuropathy that primarily damages human spinal nerve roots and peripheral nerves. One of the possible pathological changes is lymphocyte and macrophage infiltration around the small blood vessels of peripheral nerves, which causes demyelination.[1] As far as we know, various factors can result in GBS. Almost 2/3 of GBS patients suffer from bacterial or viral infections within 1-4 weeks before onset. *Campylobacter jejuni* (*C. jejuni*) is the most common pathogen of the prodromal infection especially in northern China. The most common pathogenesis of GBS is mainly

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based on hypothetical mechanisms deduced from molecular modeling of prodromal infections, caused by pathogens such as *C. jejuni*, which can activate the body's immune system to produce antibodies, resulting in peripheral nerve demyelination or axonal injury. In particular, serum anti-ganglioside antibodies in the acute phase are detectable in approxiately 60% of GBS patients.[2]

In recent years, weakness of extremities and weakened, or even loss of, tendon reflexes have been reported in some patients with traumatic brain injury, cerebral hemorrhage, spinal cord injury, brachial plexus injury, or pelvic fracture fixation.[3-6] In this article, we provide detailed clinical data of two patients who were diagnosed with GBS following brain hemorrhage or craniocerebral injury. The diagnosis of our 2 patients is based on the diagnostic criteria of GBS published in 2014, which was modified according to the criteria published in 1990.[7]

CASE REPORT

Case 1

A 33-year-old male suffered from right basal ganglia hemorrhage (moderate volume) and gradually regained his muscle strength (left arm 3/5, left leg 2/5, right limbs 5/5) (the Medical Research Council grading system) after receiving conservative treatment. The patient received conservative treatment immediately after brain hemorrhage including neurotrophic treatment, mannitol, antihypertensive treatment and so on, without any treatment with gangliosides injections, during which there was no evidence of infection such as fever, cough, or elevation of white blood cells. However, fourteen days later, the muscle strength of both lower limbs decreased sharply to 0/5, the left arm 1/5, and the right arm 2/5, accompanied with hypomyotonia and the absence of tendon reflexes, without other pathological signs of the nervous system. A reexamined cranial computed tomography (CT) scan showed there were no new lesions, but his muscle weakness worsened rapidly. Besides, cranial magnetic resonance imaging (MRI) was performed on him, demonstrating moderate volume hemorrhage in the right basal ganglia region and no signs of new hemorrhage. MRI of the cervical vertebra showed mild disc (C2-C7) herniation. Unfortunately, his muscle strength continued to decrease, and five days later, all extremities decreased to 0/5 and respiratory failure occurred. A nerve conduction study (NCS) indicated: compound muscle action potential (CMAP) amplitude of the right median nerve and the common peroneal nerve on both sides were not elicited; CMAP of the right ulnar nerve significantly decreased; F wave was not evoked in both upper and lower limbs; and the motor nerve conduction velocity (NCV) was close to normal. The patient was transferred to the intensive care unit (ICU) right away, due to the respiratory problems, and a ventilator was used to keep him alive. We collected a cerebrospinal fluid (CSF) sample, and the analysis showed a protein concentration of 0.56 g/L, a normal cell count (4×10^6/L) and the pressure of CSF was 170 mmH_2O. The result of the serum anti-ganglioside antibody test was negative 17 days after the occurrence of muscle weakness. According to the diagnostic criteria, he was diagnosed with acute motor axonal neuropathy (AMAN), a subtype of GBS.

Intravenous immunoglobulin (IVIg) 0.4 g/kg per day was given to him 5 days after the occurrence of quadriplegia for a consecutive 5 days of treatment. However, no significant improvement was observed. After 7 days, the same dose of gamma globulin was administered for another 5 days and rehabilitation treatment was continued. A month later, the ventilator was gradually discontinued and the patient regained some muscle strength (right arm 3/5, left arm 2/5, and both legs 1/5). Meanwhile the rehabilitation treatment continued. A 4-month follow-up revealed that he could walk slowly with some support by arm.

Case 2

A 41-year-old male with a left frontal contusion, laceration, and subdural hematoma, due to a motorcycle accident, was included into the case analysis. At first, the patient's conditions had improved obviously after conservative treatment. However, 2 weeks later, he suffered from sudden quadriplegia (muscle strength 0/5 for all limbs) along with areflexia, without any evidence of antecedent infections and without treatments with gangliosides injections. Another brain CT scan was performed immediately, but no obvious changes were observed. Then the patient was transferred to the ICU because of respiratory failure, and a ventilator was used. NCS showed that: CMAP of the right median nerve and sural nerve were not elicited; CMAP of the common peroneal nerve on both sides were significantly decreased; F waves were not evoked in either upper or lower limbs; NCV was close to normal. Lumbar puncture showed that the CSF pressure was 190 mmH_2O, protein concentration was 1.87 g/L, and total nucleated cells were 2.0×10^6/L. Diagnosis of GBS was definite and he was treated with IVIg (0.4 g/kg per day) for 5 days. During the treatment, his respiratory function recovered slightly, but with no improvement in muscle strength in any limb. Over another 5-day course of IVIg, his muscle strength recovered to 2/5 in both arms but remained 0/5 in the lower limbs. Six months later, the patient could gradually stand up with

some support and hold light objects.

DISCUSSION

We reported two patients with severe GBS following cerebral hemorrhage or head trauma. In the two cases above, typical clinical manifestations were the sudden occurrence of flaccid tetraplegia, areflexia, hypomyotonia, and respiratory failure, without any sensory dysfunction. NCS of both patients showed decreased CMAP and prolonged distal F wave latencies, indicating motor axon injury, which are common electrophysiological features of AMAN. In addition, the result of the CSF tests indicated albumino-cytologic dissociation, and IVIg proved to be efficient in both patients.

The most common subtypes of GBS are acute inflammatory demyelinating polyradiculoneuropathy (AIDP) and AMAN. In contrast to North America and Europe, where more than 90% of the patients are classified as AIDP, approximately 65% of patients in China suffer from AMAN, a subtype of GBS with only motor fiber damage.[8] Analogous to our cases, the features of nerve conduction studies demonstrated decreased motor amplitudes with an absence of F waves.

The exact pathogenesis of GBS following brain trauma and hemorrhage is not completely clear. Tan *et al*.[6] performed sural nerve biopsy and found phagocytosis of myelin sheath debris in the endoneurium. Tan suggested that head trauma and surgery had elevated serum and CSF myelin basic protein levels, which led to immune system activation to produce anti-myelin antibodies that cause demyelination. Moreover, relevant literature indicates that blood-brain barrier (BBB) damage plays an important role in post-traumatic GBS pathogenesis.[9,10] Disintegration of the BBB during neurotrauma leads to the accumulation of localized T lymphocytes and macrophages, which may induce the transformation of microglial cells in the nervous system into antigen presenting cells.[3,11,12] Activated microglia can present post traumatic neuronal debris to the immune system and stimulate B cells to produce antibodies against the myelin sheath, causing demyelination, especially in the peripheral nervous system.[13] The explanation of GBS following head trauma or hemorrhage may be that some substances originating in the central nervous system, usually being weakly immunogenic, are transported through the disrupted BBB to the peripheral nervous system where they cause demyelination or axonal damage.

It has been reported that anti-ganglioside antibodies are elevated in patients with post traumatic head injury.[3] Some studies have shown that nearly 80% of AMAN patients are anti-ganglioside antibody positive, especially within the first week of onset.[4] The best treatment for GBS is IVIg or plasma exchange, both of which aim to remove antibodies. However, serum anti-ganglioside antibodies were negative in case 1. We postulate 2 possible explanations: one is that the antibody level had decreased sharply after 2 courses of IVIg when it was obtained 17 days after onset; the other is the patient suffered from anti-ganglioside antibody negative GBS. The 2 patients reported showed significant recovery due to prompt diagnosis and treatment.

In clinical practice, if a sudden bilateral limb weakness occurs after traumatic injury or hemorrhage, which cannot be explained by findings of imaging and routine laboratory examinations, GBS should be considered.[14] Early examination of the nervous system, EMG, CSF test, and search for anti-ganglioside antibodies can support the diagnosis of peripheral nerve demyelinating disease. For patients diagnosed with GBS, gamma globulin should be given as soon as possible, if there are no contraindications, otherwise plasma exchange can be used.[15] Despite the rare reports of GBS following head trauma or brain hemorrhage, we find that it is not as rare as we thought in clinical practice. GBS is not a common disease and it is easy to be ignored or misdiagnosed, especially following other severe situations like trauma and brain hemorrhage. Thus, it is of great significance to enhance the awareness of early diagnosis and early treatment of this special pathogenic GBS, which will increase the survival rate and improve the quality of patients' life.

Authors' contributions

Collection of study cases and problem analysis: H. Jia
Writing of the paper and critical revision of the article: H. Jia, B. Li
Revised article: H. Jia, Y. Tian, Y.M. Wu

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There are no conflicts of interest.

Patient consent

Consent was approved by patients in both cases.

Ethics approval

Data collection in our study involving the patient is consistent with the ethical standards of the institution's ethics committee.

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Short Communication **Open Access**

Trends in neurology fellowship training

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ABSTRACT

Aim: A need for Neurologists exists in the USA. The majority of Neurology residency graduates go on to additional subspecialty training. **Methods:** Data from the Accreditation Council for Graduate Medical Education from 2001-2014 and the United Council for Neurologic Subspecialties from was analyzed for trends in the number of Neurology subspecialty training programs and their composition. **Results:** There has been an overall trend of growth in the number of accredited Neurology subspecialty training programs and fellows. These trends vary between specific subspecialties. **Conclusion:** The authors provide an overview of the contemporary state of Neurology subspecialty training in the USA. A clearer understanding of subspecialty training allows for anticipation of workforce surpluses and deficits.

INTRODUCTION

Since the foundation of the American Board of Psychiatry and Neurology (ABPN) in 1934, the number of accredited Neurology specialties and subspecialties along with the associated certifications has risen substantially. There has been a steady increase in both the number of programs and overall number of residents. Since the accreditation of the 1st ABPN fellowship, Clinical Neurophysiology, in 1989, five more subspecialties have been accredited by the Accreditation Council for Graduate Medical Education (ACGME) in the past 25 years. Physicians now have opportunities to become certified in Child Neurology, Neuromuscular Physiology, Neurodevelopmental Neurology, Vascular Neurology, and Endovascular Surgical Neuroradiology in addition to Clinical Neurophysiology. More recently the United Council for Neurologic Subspecialties (UCNS) has also accredited Behavioral Neurology and Neuropsychiatry (2004), Clinical Neuromuscular Pathology (2005), Headache Medicine (2005), Neuro-oncology (2005), Neurocritical Care (2005), Neuroimaging (2006), Autonomic Disorders (2007), Geriatric Neurology (2007), and

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Neural Repair and Rehabilitation (2010)[1] [Table 1]. There are also a number of subspecialties (Movement Disorders, Neurohospitalist) which are not accredited by either the ACGME or UCNS.[2,3]

Over 2,200 graduating medical students matched in Neurology Residencies in 2014. If current trends persist, the majority will seek additional subspecialty training and certification.[4-6] The establishment and existence of these numerous subspecialties may not only be reflective of, but may also help drive the subspecialization of neurologists.[1] There is interest in providing earlier subspecialty exposure to grow the number of trainees pursuing training in specific subspecialties.[7] There are also ongoing efforts to augment residents abilities and interest in pursuing careers in academic medicine,[8] many of which include subspecialty training in neurology fellowships. All of this is occurring within the context of changes in the healthcare environment and their subsequent effect on neurology resident education.[9]

Our study attempts to provide a comprehensive descriptive overview of the current state as well as a clear and easily understandable picture of the trends in Neurology graduate education training. An understanding of the current and future workforce in Neurology will be essential for the estimation of future geographic and specialty skill surpluses and deficits in the workforce. This understanding will be of value in the decision making regarding how to best address those deficits.

METHODS

Data was collected from the 2001-2002 to 2013-2014 editions of the ACGME Data Resource Book.[10] Accredited United States and international programs must verify their statistics and update them yearly. UCNS specialties and subspecialties data were obtained from their website.[11] We analyzed these yearly reports focusing on the changes taking place not only in the number of programs and residents, but also individual breakdowns by gender, specialty, and program.

RESULTS

In alignment with the increasing number of physicians pursuing careers in neurology, the number of programs for neurology subspecialty fellowship training has shown a steady increase over the course of the past thirteen years. The increase in the number of specific subspecialty fellowship programs is mirrored by an increase in fellows enrolling in training programs in those subspecialties. The number of overall fellows per program has remained fairly steady. This is indicative of subspecialty training programs remaining a relatively stable size. The largest increases each year in total number of training programs is found in Child Neurology, Neuromuscular Medicine, and Vascular Neurology. Child Neurology has shown a 175% increase during the 13-year period of the study with an average increase of 13.46% each year. Neuromuscular Medicine, having only been accredited for 9 years, has also shown rapid growth, increasing 600% over the 9 years of its accreditation, averaging a 66.67% increase each year. Vascular Neurology, having been accredited for 11 years, has grown 860%, averaging 78.18% more fellows each year. Interestingly, while all 3 subspecialties have shown dramatic increases in fellows, the changes in their respective number of programs have differing trends. Child Neurology has

Table 1: Accredited neurology fellowships

Subspecialty	Year approved	Accrediting body
Child Neurology	1934	ABPN
Clinical Neurophysiology	1989	ABPN
Pain Medicine	1998	ABPN
Neurodevelopmental Disabilities	1999	ABPN
Vascular Neurology	2003	ABPN
Behavioral Neurology and Neuropsychiatry	2004	UCNS
Neuromuscular Medicine	2005	ABPN
Sleep Medicine	2005	ABPN
Clinical Neuromuscular Pathology	2005	UCNS
Headache Medicine	2005	UCNS
Neuro-Oncology	2005	UCNS
Neuroimaging	2005	UCNS
Neurocritical Care	2005	UCNS
Hospice and Palliative Care	2006	ABPN
Autonomic Disorders	2007	UCNS
Geriatric Neurology	2007	UCNS
Neural Repair and Rehabilitation	2010	UCNS
Epilepsy	2011	ABPN
Brain Injury Medicine	2011	ABPN

ABPN: American Board of Psychiatry and Neurology; UCNS: United Council of Neurologic Subspecialties

increased its programs by only 10.6% in 13 years, with Clinical Neurophysiology increasing at 443% in 9 years, and Vascular Neurology. This most likely represents a maturation of the subspecialty and the slowed growth rate one would expect with this.

The existence of gender differences amongst Neurology subspecialty trainees can be noted. Neurodevelopmental Disabilities (89%) and to a lesser degree Child Neurology (65%) demonstrate a disproportionate number of female trainees while Endovascular Surgical Neuroradiology (0%) and Vascular Neurology (26%) have a disproportionate number of male trainees. While in some subspecialties the proportion of female trainees remains relatively stable, Neurodevelopmental Disabilities (89%), Child Neurology (65%), and Clinical Neurophysiology (51%) all have a growing percentage of female trainees. It is unclear what exact effect the initial growth in the proportion of female Neurology residents peaking in 2009-2010 at ~47%, and the subsequent decline will have on subspecialty career choices [Figure 1].

DISCUSSION

The number of physicians in the United States becomes larger and more diverse with each new class of graduating medical students. This influx of new physicians benefits the medical community with the supplementation of new physicians to an aging society and growing healthcare needs. Additionally these trainees will replace physicians lost to attrition via retirement or death. With over 120 specializations for new medical graduates to choose from,[12] medical trainees have a broad range of potential career choices. With a predicted growth in the shortage of clinical neurologists, understanding the current career training choices made by Neurology trainees and the trends which these choices are following will be important for addressing the shortfalls predicted in the number of clinical neurologists in the USA.[13,14] The changes occurring in neurology residents and fellows over the past decade have been gradual and unique to each subspecialty.[15] In addition to the well-established subspecialties there are residents seeking additional training in less codified, but still important fields such as therapeutic development.[15]

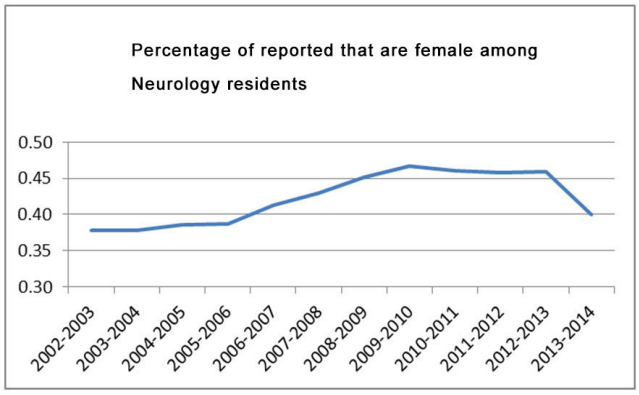

Figure 1: Trend in percentage of female neurology residents over time

Viewed broadly, there is an evident steady increase in the number of Neurology susbspecialty training programs and fellows. When looked at on a subspecialty by subspecialty basis the trends become more complicated. Some subspecialties such as Endovascular Surgical Neuroradiology have a limited number of accredited programs and fellows that interpretation of trends is not feasible. The situation for Endovascular Surgical Neuroradiology is particularly complicated as there are multiple training paths which can be followed to reach this endpoint and there are a number of subspecialists, often situated in separate departments, including neurology, neurosurgery, and neuroradiology who care for these patients and have overlapping skill sets. The effects of the training background on patient outcomes have not been thoroughly studied. Other recently accredited subspecialties such as Neurovascular and Clinical Neuromuscular have demonstrated robust growth rates since inception (650% per year and 344.4% per year, respectively). Other subspecialties, such as Neurodevelopmental have shown little growth in the number of training programs (growth rate of 75% per year) and a steady number of new fellows. These trends are most likely substantially affected by real or perceived work-force demand, as has been previously implied for some subspecialties.[16,17] Other factors which likely influence career choice in neurologic subspecialties include, but are not limited to, lifestyle factors such as salary, work hours, and duration and rigor of training as well as the factors which can influence success in an academic career.[18] Our study does not address the underlying factors which influence these trends, but merely describes them. This knowledge helps facilitate strategic planning on how to best assist supply meeting demand. While the details of how to do so are beyond the scope of this study, they can include various incentives to increased recruitment in subspecialties facing deficits in clinicians.

Authors' contributions

Developed the concept of the study: T.S. Hodgson, R.V. Lukas

Analyzed the data: J.S.A. Williams, T.S. Hodgson, R.V. Lukas

Composed the manuscript: J.S.A. Williams, R.V. Lukas, T.S. Hodgson

Revised the manuscript for content and style: J.S.A. Williams, F.D. Goldenberg, R.V. Lukas

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Patient consent
There is no patient data involved.

Ethics approval
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Review Open Access

Neuroimaging of corpus callosum in central nervous system demyelinating disorders

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ABSTRACT

Corpus callosum (CC) is the largest white matter structure in the brain, consisting of 200-250 million contralateral axonal projections. It is the major commissural pathway connecting the hemispheres of human brain. The pathology of CC includes wide variety of entities that arise from different causes such as congenital, inflammatory, tumoral, degenerative, infectious, *etc*. This study reviews the most reliable neuroimaging data of human CC in central nervous system (CNS) demyelinating diseases to facilitate the understanding of different pathological entities of the CC and their role in anticipation of probable prognostic findings. After a brief description of normal anatomy and functions of CC, this review examines the most valuable findings obtained using conventional and functional magnetic resonance imaging. It also demonstrates the most well organized findings of how CC features influence prognostic factors of demyelinating disorders, which could have a great value for choosing proper therapy methods. The authors also provided a brief review of other demyelinating disorders which are primarily caused by other pathological factors other than autoimmunity. As a conclusion, the authors showed the importance of CC as an critical part of the brain, which should be explored by different methods of imaging, correspondent to clinical evaluation of CNS demyelinating disorder to widen our knowledge on pathology and clinical patterns of such disorders.

INTRODUCTION

Corpus callosum (CC) is the largest fiber bundle that connects cortical and subcortical regions of the brain. It also interconnects both cerebral hemispheres, promoting functional integration of sensory and motor functions. The CC is composed of many different fiber types, with varied thickness and myelin sheath variations. The callosal tracts are frequently affected in patients with multiple sclerosis (MS) and other demyelinating disorders such as acute disseminated encephalomyelitis (ADEM), Devic's neuromyelitis optica, marchiafava bignami (MBD), *etc*. Magnetic resonance imaging (MRI) is the gold standard imaging

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of demyelinating lesions of callosal tract, however there are microstructural pathologic alterations in the CC that cannot be assessed with conventional MRI. Furthermore, in some demyelinating diseases including MS, normal appearing white matter (NAWM) has been reported to have noticeable histological abnormality.[1] Advanced MRI techniques such as diffusion tensor imaging (DTI) and MR spectroscopy have been employed to evaluate changes in NAWM and histological changes in active lesional areas.[2,3] The DTI is an advanced MR imaging technique that provides quantitative information regarding structural features of central nervous system (CNS). It allows the calculation of many parameters, including: mean diffusivity, which is a directionally averaged measurement of the apparent diffusion coefficient (ADC) and fractional anisotropy (FA), which summarizes the orientation-based dependence of diffusivity.[4] Many authors have investigated pathologic features of CC in different demyelinating diseases through various imaging methods, especially MR imaging. They mostly focused on functional MRI, DTI, MR spectroscopy in recent years of research. This study aimed to provide a short review of different pathologic features of CC in neuroimaging of demyelinating disorders of CNS, which are mostly auto-inflammatory types. It also shows the role of these features in prognosis of these disorders to help further discrimination and comparison between such diseases.[5,6] Cognitive dysfunction is one of the major factors affecting the social functioning and quality of life of patients suffering from demyelinating disorders especially MS patients and It is one of the most important factors which can be closely related to neuroimaging features of MS. This can play a key-role to help determine the outcome of the disease and also their response to possible routine therapies.[7-11] In this article, we review different aspects of imaging and involvement patterns of CC in various CNS demyelinating disorders to determine more helpful ways of discrimination of such diseases and also, to demonstrate the role of white matter lesions (particularly in CC) in evaluation of prognosis of these demyelinating disorders.[12-16] To our knowledge there was no article previously, discussing and comparing CC features in different CNS demyelinating disorders similar to ours.

NORMAL CC

Anatomy

The CC is a prominent band of compact white matter composed of transversely oriented nerve fibers by which every part of one hemisphere is connected with corresponding part of the other hemisphere. It comprises four parts: (1) the reflected anterior portion, or the rostrum; (2) the genu, or the anterior bulbar end; (3) the splenium, or the posterior rounded end; (4) the body, which lies between the genu and the splenium. The anterior half of human CC (genu, rostrum, body) contains fibers interconnecting frontal association cortical areas and the isthmus mostly contains primary motor, somatosensory and auditory fibers. In the splenium, primary visual, association temporooccipital and parietal commissural fibers are mixed, forming a single segment with the hippocampal commissure through which parahippocampal fibers cross. Large diameter fibers are densest in the isthmus and the posterior splenium, whereas small fibers are more numerous in the genu and anterior splenuim. The MR imaging characteristics of CC are similar to those of white matter: high signal intensity on T1 weighted images and low signal intensity relative to gray matter on both T2 weighted and proton density-weighted images. Measurement in the mid-sagital plane has been subject to extensive investigations. Normal MR imaging features of CC were reported by Okamoto *et al*.[17] They showed focal thinning is seen superiorly at the junction of body and splenium in 25% of healthy individuals and considered to be a normal variation. On axial sections parallel to canthomeatal line at the high ventricular level, the body of CC is sectioned longitudinally, displaying a broad band of white matter bordered by the upper outer corners of lateral ventricles.

Function

The function of the CC has been investigated for centuries, suggesting it's correlation with intelligence. The earliest studies during the 16th century believed it to be the "seat of the soul". It took until the 18th century for Franz Joseph Gall and Johann Spurzheim, dissecting alcohol fixed brains, to describe bundles of axons passing through the callosal white matter and connecting the 2 hemisphere. It's known functions include: (1) inter-hemispheric exchange of information; (2) facilitation of some cortical activities; (3) inhibition of some cortical functions and integration of inputs reaching one or both hemispheres.

One of the latest contributions in assessment of physiology and function of the CC in different analytical studies is functional MRI.[18-20]

CC IN MULTIPLE SCLEROSIS

MRI

MS is the most common autoimmune disease of the CNS and it's characterized by inflammation, demyelination and degenerative changes. The CC

is one of the common sites of brain involved in MS patients. In MS, we can observe characteristic macroscopic changes in conventional MRI, including: (1) CC atrophy; (2) MS plaques; (3) signal changes within callososeptal interface.[6] MS plaques mostly occur in the body of the CC, as suggested by some studies.[21-25] Diffuse atrophy of the CC is believed to be a part of general cerebral atrophy in long standing cases or it can be caused by wallerian degeneration and loss of axons within the CC.[26] According to the study by Chen *et al*.,[6] MRI findings can have a big role in discrimination between MS and other demyelinating disorders, in particular MS and Neuromyelitis optica. In this study in which sagittal T2 flair images with 2 mm thickness were obtained from 23 patients with neuromyelitis optica (NMO), 42 patients with MS and 27 controls, results showed that subcallosal dash-dot sign was much more common in opticospinal MS patients than NMO. Contrary to the subcallosal dash-dot sign, subcallosal striations had no meaningful difference between MS and other two groups.[6] According to some studies, MS lesions of CC found to be small, at the lower border of CC next to the septum pellucidum and crossed the midline asymmetrically. On the other hand, ADEM causes individually large, asymmetric lesions and involvements in marchiafava bignami disease were large, often symmetrically in the midline of the splenium and did not reach the edge of the CC.[5,27] Central CC volume along with medulla oblongata volume can help discriminate between different subtypes of MS. Various subtypes of MS affect different neuroanatomical regions of the CC differently. Most of the patients with secondary progressive MS had central CC with the volume of less than 55 mm, while patients with primary progressive MS had more CC volume centrally.[28]

Functional MRI

Although MRI is the gold standard imaging for evaluation of MS brain lesions, more recent MR techniques helped in exploring axonal loss, wallerian degeneration and microscopic changes in detail. DTI is promising technique for detecting structural changes in MS lesions and revealing microscopic changes in NAWM and Normal appearing gray matter (NAGM). Using water diffusion as a basis to construct anatomic details, DTI offers the potential to identify structural and functional adaptations before gross anatomical changes. Most important DTI parameters are Mean diffusion (MD) and FA. There are also some other parameters in the matter of directional diffusivity such as: radial and axial diffusivity, which have been used by many recent studies.[15,25,26,28] Such parameters interestingly found to be helpful in detecting microscopic and structural changes in lesional and NAWM. Most of recent studies showed increased ADC and reduced FA in both pathologic and NAWM.[29-33] Sigal *et al*.[11] used DTI to investigate CC integrity in MS patients and age matched controls. In agreement with other studies,[15,26,28,31] MS patients had significant reduction in CC 's FA compared with control group in all sub-region of CC, indicating axonal loss and dysfunction in callosal fibers. Most recent studies agree on the fact that in MS patients all MRI indices and parameters are altered pathologically especially in the CC structure.[34] In the study by Farber *et al*.[35] ADC was found to be helpful as a great parameter for differentiation between MS and other demyelinating diseases, as ADC was found to be significantly elevated in CC in MS patients compared to control group and patients with ADEM disease.[35] A study by Ozturk *et al*.[13] showed all MRI indices were diffusely abnormal in the CC. In this study both FA and magnetization transfer ratio (MTR) were decreased and mean and directional diffusivity were increased, but it is to be said that MTR and FA had greatest difference between disease and control group. By spatially tract profile analysis they localized the most abnormal segments in the body and isthmus, with relative sparing of the rostrum and genu.[13]

CC as a prognosis indicator

Quantitative MRI abnormalities in the CC partially account for cognitive and upper/lower extremity dysfunction in MS and ultimately the prognosis. Since cognitive disability is particularly difficult to measure at bedside, and because cognitive and non-cognitive disability may proceed at different rates, the ability to associate cognitive impairment with imaging data may be useful for monitoring patients and assessment of response to therapy in clinical trials. Previous studies in MS have shown significant correlation between cognitive status and CC microstructure.[11,36-38] There has been also studies about the relationship between CC involvement's patterns and prognosis of MS.[7-11] Most of them showed that the damage to white matter network especially CC contributes to the reduced processing speed in task specific abilities.[39] A significant increase in CC's MD was observed in relapsing remitting MS, even in benign form.[40-44] Moreover, patterns of tract FA reduction for cognitive test, including localization of lesions in the body and splenium of the CC, only partially overlapped with T2 lesions, supporting that NAWM abnormality contributes to cognitive dysfunction. In the study by Rimkus *et al*.[45] results showed correlation between mean diffusion and radial diffusivity, and expended disability status scale (EDSS), suggesting possible relationship between callosal demyelination and sensory motor dysfunction. The cognitive dysfunction was concomitant with DTI changes in CC. MS group of patients showed decreased

FA and increased MD compared to the matched control group, which can be interpreted as loss of complexity in the white matter tracts in the initial pathological process. As a conclusion, the microstructural changes of CC can be helpful in determination of prognosis in MS patients. They also found that macroscopic changes of CC had no direct association with cognitive dysfunction in such patients.[45] There were also some studies like study by Natarajan *et al*.[46] in which they found that the most abnormal DTI indices were present in secondary progressive MS patients. According to a longitudinal cohort study in 2012 CC atrophy of 6 months could predict clinically definite MS within 2 years. It showed that faster decrease in CC cross sectional area and higher T2 lesion volume indicated a poorer prognosis.[35] DTI directional diffusivity may offer the potential to monitor therapeutic options and further understanding of the disease process and prognosis. In a DTI study looking at longitudinal changes in brain tissues in a group of patients with MS receiving Natalizumab therapy, the authors found increased FA, decreased radial diffusivity and no change in axial diffusivity in gadolinium enhancing lesions over the course of therapy. On the other hand, in normal appearing brain tissue, CC's FA and axial diffusivity demonstrated further decline over time, while no significant change in radial diffusivity was observed.[47] Hence, the decline in axial diffusivity may suggest involvement of axonal loss and degeneration in normal appearing brain tissue at the early stage before active lesions develop, possibly attributing to poorer prognosis and progressive disability often observed in MS patients despite treatment. Many recent studies agree on the fact that longitudinal changes are most rapid in CC area of the brain in MS disorder in different types of imaging.[16,19,35,43]

CC IN ACUTE DISSEMINATED ENCEPHALOMYELITIS

MRI

Acute disseminated encephalomyelitis is an uncommon immune mediated inflammatory demyelinating disease of the CNS. It is usually a monophasic illness, which may occur after vaccination, viral infection, in association with rheumatic fever, or with unrecognized antecedent disorder. The clinical picture is widespread CNS disturbance, including: drowsiness, coma, multifocal neurological signs and seizure due to involvement of the brain, spinal cord and optic nerves. Radiologic findings of ADEM are usually not pathognomonic. As a result, the differentiation of diagnosis is always difficult. Pathologic findings of ADEM are usually due to: (1) vascular damage; (2) circulating immune complex deposition; (3) complement activation and some other mechanisms yet to be investigated in further studies.[48,49] This changes lead to an alteration of the blood brain barrier, which becomes visible by contrast enhancement in CT and MRI. Increasing T1 and T2 times are also observed and many studies agree on the fact that contrast enhancement are dominantly present in acute phase of the disease and fade out as the acute stage is passed. These studies also showed that the probable underlying causes for hyper intense lesions of brain and spinal cord may be edema and demyelination.[48,50,51] The most important common features of white matter involvement is symmetric periventricular and callosal hyper intense lesions on T2 weighted and flair images as a result of edema and demyelination. As a common finding, many studies showed that most of the ADEM patients (78%) have absolute or relative periventricular sparing which was found to be a typical characteristic of ADEM in MRI. Some studies suggested that MRI involvement of CC in ADEM patients can be multiple or single, especially in the splenium of the CC. They also showed that all callosal lesions can be enhanced or not, in MRI (with Gd). Generally, the greatest difference between MS and ADEM is that, the likelihood of CC involvement in MS is far more common than ADEM (~60% *vs*. ~15%).[5,52]

Functional MRI

ADEM predominantly affects white matter. It was reported that functional MRI parameters (axial and radial diffusivity) can estimate the extent of myelin injury in the CNS white matter. Although first studies on patients of ADEM showed elevated ADC and reduced FA, Further exploration of directional diffusivity revealed unchanged axial diffusivity and markedly increased radial diffusivity, suggestive of demyelination. These findings were consistent with findings of study by Petzold *et al*.,[53] which showed patients of ADEM having elevated ADC in sub-acute phase and reduced ADC in acute phase. Many other studies agree on this DTI changes in patients with ADEM disorder. Tillema *et al*.[5] did a retrospective DTI study looking at non-lesional white matter changes within central fibers of the CC's genu and internal capsule in pediatric MS and ADEM. They found lower FA values, increased radial diffusivity and no difference in axial diffusivity in patients with ADEM while in patients with MS results were significantly different (decreased FA and increased radial diffusivity).[5]

CC as a prognosis indicator

Patients with ADEM are usually presented with variable neurologic signs especially after an infectious episode. MRI is the technique of choice to show these lesions

and also to illustrate possible prognostic criteria in imaging aspect of such diseases. In some studies, it has been suggested that diffusion weighted imaging (DWI) of CC may not be helpful in determination of prognosis in patients of ADEM, on the other hand, Donmez *et al*.[51] suggested that brainstem involvement in ADEM disease may have an influence on the prognosis of the disorder, correspondent to the studies showing beneficial use of ADC parameters in prediction of motor disabilities. Combined use of clinical and radiologic findings are needed to predict the chance of relapse in patients suffering from ADEM. Patients with large demyelinating lesions may have more degree of disabilities evaluated by EDSS but they have an excellent response to therapy. It was showed that size of the lesions is not a direct indicator of poor prognosis. According to what we found in different studies on "ADEM prognosis" there is no certainty on how useful are MRI features to predict outcome of ADEM and there are yet more studies to explore this area of research.

CC IN DEVIC'S NEUROMYELITIS OPTICA

MRI

Neuromyelitis optica is a CNS demyelinating disease causing acute transverse myelitis with bilateral optic neuropathy. Paraplegia and blindness are possible complications. There is no definite imaging criteria to distinguish NMO from other demyelinating disorders such as MS and ADEM.[54,55] Irrelative of what is found in CC that will be discussed further in this article general distinguishing findings of MRI in such diseases are: midbrain lesions in the ventral part with poorly defined margins for ADEM *vs*. Medulla lesions in the dorsal part with poorly defined shape for NMO, and pons lesions with well-defined shape for MS (as the most common sites of involvement). CC involvement is more common in MS in comparison to NMO but there are also some involvement pattern differences to be pointed out. CC lesions in NMO are mostly evident in acute phase of disease and they have generally some similar characteristics. They are usually multiple, edematous and heterogeneous in intensity, while in chronic stage, lesions shrink and disappear. In MS, lesions are small, non-edematous, and the intensity is homogenous in the acute phase and they are more commonly located at lower margin of CC.[56-59] As Chen *et al*.[6] showed in their study, subcallosal dash dot sign was helpful as it was more common in patient with MS than in the NMO. In another study by Makino *et al*.,[36] it was showed that involvement of splenium of CC in NMO patients was more common than the involvement of the same area in patients with MS (57% *vs*. 27%). The lesions in NMO also tended to spread from the lower to upper parts of CC. They also found out that lesions in NMO were much more heterogeneous than in MS.[36]

Functional MRI

As we previously pointed out, functional MRI and DTI method of imaging are more helpful in exploration of microstructural and functional changes, especially in NAWM in demyelinating disorders from the aspects of discrimination and diagnosis. According to the study by Kimura *et al*.[58] in patients with NMO, damage to extensive regions of NAWM has been observed. To investigate this possibility that microstructural alterations are present in these WM tracts, DTI should be applied. According to this study findings FA was decreased in splenium of CC and left optic radiate. In another study focused on DTI features of NAWM in NMO patients which was performed by Jeantroux *et al*.,[59] it was showed that ADC was increased and FA was decreased in NMO patients in posterior limb of internal capsule and optic radiation and spinal cord NAWM. FA had the best correlation with EDSS. FA was lower in spinal cord lesions. In contrast there was no difference between two groups, neither in the anterior limb of internal capsule nor in the CC. These results suggest that NAWM outside the tracts mentioned above remained normal, showing that infralesional abnormality is not usually seen in NMO in contrast to the MS disease.[59] These findings are consistent with the findings by Sun *et al*.,[60] in which they found the similar results suggesting that DTI parameters (mean diffusivity and lambda1) were unchanged in CC region. This field of study needs further investigations yet to determine the distinguishing patterns and parameters in NAWM, especially callosal region in NMO patients.[61,62]

CC as a prognosis indicator

A combination of biomarkers, neuroimaging data and clinical symptoms are needed to predict prognosis of NMO. It is difficult to consider callosal tract features of neuroimaging as the only indicator of disease outcome. In many studies DTI parameters, especially FA, showed to have the closest correlation with EDSS. As a result, it can be helpful in measuring disease outcome and disability. He *et al*.[63] showed decreased FA and increased ADC of CC, especially during the acute phase of the disease, plays an important role in the anticipation of cognitive dysfunction and clinical outcome. The researchers have compared regional measures of patients with stable and acute NMO with healthy patients. Both acute and stable NMO patients had a higher average FA in regions of interest of the thalamus and putamen. Acute NMO patients had significantly higher average MDs than controls in the genu of the CC and optic radiation, and significantly

lower average MDs in medulla oblongata, internal capsule and thalamus.[64]

CC IN SUSAC SYNDROME

MRI

Susac's syndrome (SS) is a clinical triad of encephalopathy, branch retinal artery occlusion and sensorineural hearing loss. It is sometimes misdiagnosed because of similarities with ADEM and MS. Typical triad is not commonly seen.[65] Brain is the main target organ for SS, which makes MRI the best diagnostic test. Susac syndrome mostly causes micro-infarction in both gray and white matter. This can cause hyperintense lesions at any area of the brain, including CC. Micro-infarctions in SS have two specific patterns on MRI: "snowball lesions", in central parts of CC, and "string of pearls", which is commonly seen in internal capsule.[22,66] Raets *et al*.[67] showed that the combination of typical central callosal lesions with string of pearls is pathognomonic for SS. Encephalopathic SS always involves CC. Snow ball lesions evolve to central callosal holes in the course of the disease. A pathognomonic change in post-encephalopathic phase of SS disease is linear defects in central part of CC called "smokes".[67] As reported, CC involvement plays a critical role in early diagnosis of SS and demands aggressive therapy. In severely affected patients, atrophy of cerebellum is usually seen during sub-acute and chronic phase. Correspondent to what stated previously, Mateen *et al*.[68] demonstrated a series of cases of SS with 79% involvement rate of CC. CC can be of significant help to distinguish SS from other demyelination disorders of CNS. They showed CC involvement in SS is typically in the central part of the CC.

Functional MRI

Kleffner *et al*.[69] showed the most specific finding of DTI in SS patients, was 25% reduction of FA in genu of Corpus callosum.Reduced fractional anisotropy in the prefrontal areas of the brain was also observed; while in MS patients, it was mainly seen in the body, rostrum and splenium of CC. DTI is considered a useful method to detect microstructural damage based on FA.[69] FA reflects the spatial directionality of water diffusion, which is decreased in white matter damage; contrary to normal diffusion in conventional MRI of CC and prefrontal area. This puts more emphasis on how essential are new methods of Imaging in neuroimaging exploration of CC disorders.

CC as a prognosis indicator

In spite of lack of knowledge on this specific subject, some studies pointed out that FA reduction and involvement of genu of the CC can be useful to predict the outcome of the disorder. The more decrease in FA, the more complication is expected in the course of the disease. There were also some evidences showing that serial DTI parameters can play a role in prediction of outcome and prognosis of the disease but, there are many aspects to be studied and explored yet in this field of research.[60,63,70-72]

OTHER DEMYELINATING DISORDERS

Marchiafava bignami

MBD disease is a rare form of toxic demyelination of CC associated with chronic alcohol consumption. Several MRI findings have shown lesions not only in the CC but also in the hemispheric white matter. General pattern of CC pathology in MBD is hyperintensity on flair imaging and sometimes hemispheric white matter, reflecting damage to myelin and vasogenic edema of the CC and extracallosal projections.[27] Because of pathological variety of this disorder there is no certain sequence and pattern of pathology of CC in MBD. Some studies showed that involvement of the CC in MBD was initially in the genu, without significant association with DTI changes of CC and NAWM. Afterward, there would be some changes in the splenium of CC.[73] The most probable reason why DTI changes were not associated with initial changes in the genu of CC is that the changes were mostly due to vasogenic edema and that the lesion then converted into cytotoxic edema process. Serial MRI and DTI method of evaluation in MBD cases have been found to be significantly helpful and that's why there will be a great need for further studies on this method of evaluation for such demyelinating disorders.[27] It suffices to briefly review MBD data because there is still significant potential for further new neuroimaging methods for such a rare disease and our main goal in this review is demyelinating diseases with autoimmune nature rather than the disorders caused by environmental factors.

Infectious demyelinating disorders

Many CNS disorders can cause secondary demyelination as an early or late complication. One major category of CNS diseases, are infective ones. To show the importance of CC in CNS involvement of such disease, a brief review is presented. Infective demyelinating diseases, such as subacute sclerosing panencephalitis (SSPE), streptococcus meningitis, Lyme disease, *etc*. can involve CC with various patterns. For instance, in SSPE lesions are mostly asymmetric, bilateral and T2-hyperintense and involve the temporal and parietal lobes in the acute stage. No specific pattern of involvement regarding the CC is reported to date, although some case reports

Table 1: Summary of changes in corpus callosum in different demyelinating diseases and their role in determination of prognosis

	MS	ADEM	NMO	Susac	MBD
MRI	All parts of CC (mostly body) plaques and then possible atrophy/ small, at the lower border of CC crossing the midline asymmetrically	Mostly in splenium of CC (widespread lesions in brain, mostly sparing periventricular area)	Multiple heterogeneouslesions/ less common involvement of CC/mostly no lesions in chronic stage	Snowball lesions in central CC/linear defects called "smoke" in central CC during post-encephalic phase	Acutely involves genu and then splenium of CC/ large, often symmetrical, in the midline of the splenium, does not reach the edge of the CC
fMRI	Significant increase in ADC (MD)/ decreased FA	Increased ADC (AD and RD) in sub-acute phase but decreased ADC in acute phase/ decreased FA	Mostly unchanged parameters in CC. If seen: increased ADC/decreased FA in CC and optic radiation	Decreased FA in genu of CC	Mostly no significant changes
Prognosis	Increased MD/ decreased FA = more disability	Increased ADC = more disability	Increased ADC and decreased FA are helpful in acute phase	FA change and genu involvement = more disability	No certain evidences are available

MS: multiple sclerosis; ADEM: acute disseminated encephalomyelitis; MBD: marchiafava bignami; MRI: magnetic resonance imaging; CC: corpus callosum; FA: fractional anisotropy; ADC: apparent diffusion coefficient; MD: mean diffusion; NMO: neuromyelitis optica; AD: axial diffusivity; RD: radial diffusivity

presented splenium involvement.[74] Streptococcus meningitis caused by group B streptococcus results in brain infarction as it's main mechanism of action. It disrupts blood supply to thalamus, periventricular white matter and basal ganglia. Although, there have been case reports demonstrating involvement of callosal splenium in this disorder but to our knowledge no solid study investigated changes of inter-hemispheric fibers in detail. Borrelia burgdorferi is the pathogen causing Lyme disease, which is commonly mistaken for MS. General patterns of CC lesions are seen in Lyme disease. Fluid-attenuated inversion recovery and T2 weighted MRI are the most efficient ones to explore such lesions.[75]

CC can be demyelinated in many other disorders which are not primarily demyelinating. Neurodegenerative diseases are one of the most important instances to cause CC demyelination as a long term complication, However because CC damage is not the distinguishing feature of such disorders, it is out of the scope of this study to evaluate this group of CNS disorders.

CONCLUSION

As summarized in the Table 1, this study demonstrates that neuroimaging of white matter, especially callosal area of brain, plays an important role in distinguishing many demyelinating diseases from one another. We should focus on many new neuroimaging methods, such as DTI, fMRI, *etc*. to investigate more possible ways of further evaluation and pattern comparison in such disorders. Additional data are clearly needed if we are to gain further insight into callosal pathological pattern's map and to establish practical ways for application of CC differences in diagnosis of various CNS demyelinating disorders.

Authors' contributions

Analysis and interpretation of data: A. Neshatfar
Study conception and design: M. Etemadifar
Revision and data collection consultant: A.A. Zamani
Data collection: M. Salari

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Conflicts of interest

There are no conflicts of interest.

Patient consent

There is no patient data involved.

Ethics approval

Not applicable.

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Case Report **Open Access**

A case report of anti-*N*-methyl-d-aspartate receptor autoimmune encephalitis with sensory attack. Is limbic encephalitis only "limbic"?

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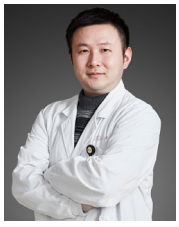

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ABSTRACT

To emphasize the early diagnosis and treatment of anti-*N*-methyl-d-aspartate-receptor (NMDAR) autoimmune encephalitis, a rare clinical condition, teratoma-related, anti-NMDAR encephalitis should be suspected if young patients present with psychiatric, movement, and sensory symptoms. Early diagnosis and treatment can decrease the mortality and disability rate.

Quick Response Code:

INTRODUCTION

Limbic encephalitis is believed to be a disorder affecting the medial temporal lobe of the brain. The underlying cause can be either of autoimmune origin or viral infection. *N*-methyl-d-aspartate-receptor (NMDAR) antibodies, voltage-gated potassium channel antibodies, and glutamic acid decarboxylase receptor antibodies are the most common causes of autoimmune limbic encephalitis. Although considered as limbic encephalitis, however, these diseases are not restricted to the limbic system. Here, we present a case of anti-NMDAR encephalitis with teratoma. This patient showed not only "limbic symptoms" but also sensory disturbances and extrapyramidal symptoms, which suggested that more extensive lesions, including cortical and subcortical regions, might have been involved.

CASE REPORT

A 15-year-old Chinese female presented with a 1 month worsening of mania and memory problems. She also reported mild fever, generalized fatigue, anorexia, right hand abnormal involuntary movement, and paresthenia on the right side of her body. She denied any infectious history before symptoms started. She also denied any headache, vertigo, nausea, or blurry vision. Neurological examination showed an irritable patient with impaired short-term memory, echolalia, echoapraxia, stereotype movement in her right hand, and oral-facial dyskinesias.

On admission, lumbar puncture found 8 white blood cells (per mm^3), glucose 40 mg/dL, and protein 40 mg/dL. Serum human immunodeficiency virus antibody, syphilis rapid plasma reagin, antinuclear antibody, antibodies to extractable nuclear antigens, antineutrophil cytoplasmic antibody, anti-double stranded DNA, thyroid peroxidase antibody, and the venereal disease research laboratory test were negative. Electroencephalography (EEG) showed slowing of the normal background frequency. Cranial magnetic resonance imaging (MRI) showed no obvious abnormal signal changes, including in the limbic system. Viral encephalitis was first suspected, therefore intravenous high doses of steroids, acyclovir, and glycerol were administered. After treatment initiation, the patient's symptoms deteriorated, with worsening consciousness, intermittent and alternating mania attacks, abulia, echolalia, echoapraxia, 2 episodes of generalized seizures, and persistent right side paresthenia. Serum anti-neuromyelitis optica (NMO)/aquaporin-4 (AQP4) antibody was negative. Blood gas failed to show any hypoventilation. Repeated lumbar puncture showed no significant change in cell count, glucose, or protein level. Herpes simplex virus (HSV) and cytomegalovirus (CMV) polymerase chain reactions were negative in cerebrospinal fluid (CSF). The immunoglobulin G (IgG) index was 0.5. CSF oligoclonal banding was also negative. Repeated 3T MRI also failed to show any positive findings [Figure 1A]. An fluorodeoxyglucose (FDG)-positron emission tomography (PET) scan indicated hypo-metabolism in the right temporal and bilateral occipital lobes [Figure 1B]. NMDAR antibody testing was performed using a standardized laboratory assay.[1] The result revealed positive anti-NMDAR antibodies both in serum and CSF [Figure 1G]. Abdominal computed tomography, with 5 mm slice thickness, and pelvic MRI were performed and revealed a 5.1 cm × 7 cm fat intensity cystic lesion in the rectouterine pouch, which indicated the possibility of a teratoma [Figure 1C and D]. Teratoma-associated anti-NMDA receptor encephalitis was then suspected, and intravenous immunoglobulin (IVIG) (0.4 g/kg per day) was administrated for 5 days. Valproic acid was used to control seizure attacks. The patient's symptoms improved 7 days after IVIG infusion, which was characterized by memory and consciousness improvement. Twenty days after admission to our hospital, the patient underwent a laparoscopic operation for complete teratoma resection [Figure 1E and F]. Immunochemistry staining revealed a 4.8 cm × 6.9 cm cystic tumor with fat, hair, teeth, and brain tissue components. The pathological diagnosis was mature cystic teratoma containing brain tissue. Using immunochemistry staining, we found that the brain tissue contained NMDA NR1/NR2 subunit receptor positive neurons [Figure 1H]. Patient CSF samples were screened for NMDAR IgG antibodies by immunofluorescence using human embryonic kidney (HEK) 293 cells transfected with the NR1 subunit of the NMDAR complex (Euroimmun, Germany) [Figure 1G]. Non-transfected HEK 293 cells served as a negative control for nonspecific fluorescence [Figure 1I and J].

The patient was discharged 1 month after admission. At that time, her symptoms significantly improved. EEG monitoring was performed 2 months after discharge, which revealed a normal pattern. Valproic acid was gradually tapered down. Six months after discharge, this patient was free of all medications. Her cognitive function was fully recovered and her psychiatric symptom, involuntary movement, sensory disturbance, and oral-facial dyskinesias disappeared. Cranial MRI was repeated 1 year after discharge, and no brain atrophy was observed.

DISCUSSION

Anti-NMDAR encephalitis is a type of limbic encephalitis that is typically found in young women with teratomas.[2] This kind of encephalitis is usually subacute at onset with significant psychiatric symptoms, including agitation, mania, hallucination, aggression as well as cognitive dysfunction.[3] Some patients will develop echolalia, echoapraxia, involuntary movements, such as stereotype, central hypoventilation, and autonomic instability, which have been considered more specific characteristics for helping in diagnosis.[4] Although extreme delta brush on an EEG can be another specific diagnostic marker, most patients, including this one, only present with nonspecific background change or diffuse slow waves, especially at the early stage of disease.[5] Usually, bilateral medial temporal lobe signal change on MRI scans raise suspicions for limbic encephalitis.[6] However, normal MRI results cannot exclude the diagnosis. Our patient underwent 3T cranial MRI scans twice, and no obvious change was found, including in the limbic system. It is known that, although NMDARs are more concentrated in the hippocampal area, they also can be found in many other areas of the brain, including sensory and association cortex and subcortical regions.[7] The widespread distribution of the receptor in cortical regions could explain the diffuse slow waves on the EEGs and the persistent sensory symptoms seen in our patient. Oral-facial dyskinesias indicated basal ganglion involvement. Although most anti-NMDAR encephalitis is limbic, some patients may have more extensive lesions, including cortical and subcortical; thus, limbic encephalitis is not always only limbic. Likewise, an FDG-PET scan of our patient showed hypo-metabolism in multiple brain regions. In addition, not all patients have positive MRI findings, especially at the early stage of disease, and we speculated that MRI scanning may not always be reliable for early diagnosis and differentiation. The differential diagnosis of anti-NMDAR encephalitis, excluded HSV encephalitis, CMV encephalitis, Hashimoto's encephalopathy, systemic lupus erythematosus encephalopathy, antiphospholipid antibody syndrome, Sjögren's syndrome, and primary central nervous angiitis.[8] We also tested for anti-AQP4 to exclude its co-occurrence with anti-NMDAR.[9,10] This patient was steroid unresponsive, since a high dose of intravenous administration of steroids failed to improve her symptoms. After IVIG infusion and tumor resection, she recovered to normal status in a short period of time, and we gradually tapered down all her medications. This patient did not show any relapse 1 year after discharge. Although most studies indicated recovery was a slow process for anti-NMDAR encephalitis, our experience in patient with teratoma and receiving tumor resection, had good prognosis and fast recovery time. In addition, these patients are not suggested to continue long-time immunosuppressant.

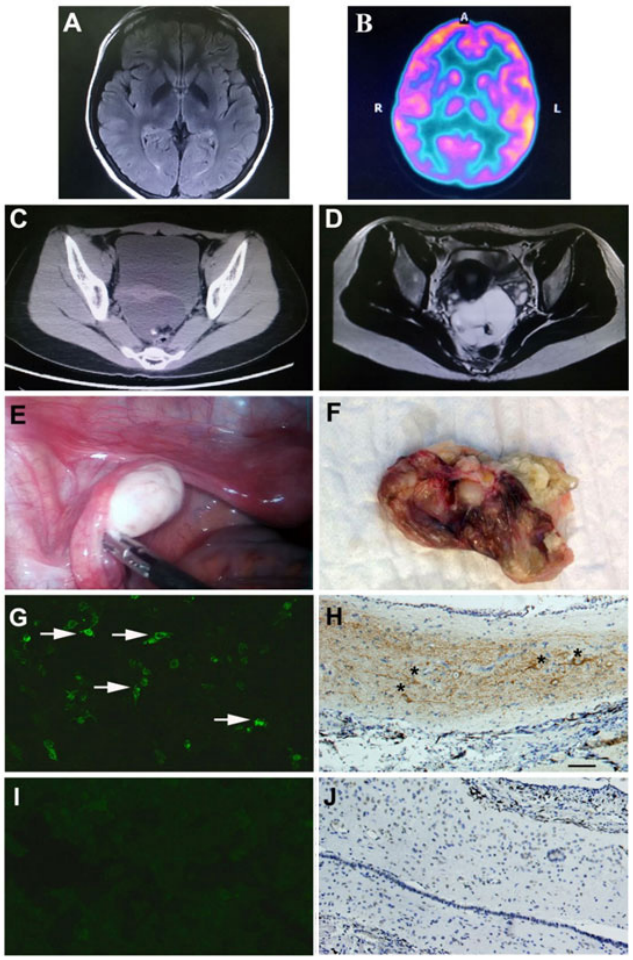

Figure 1: (A) Normal cranial MRI scan; (B) FDG-PET scan showed hypo-metabolism in the right temporal and bilateral occipital lobes; (C and D) pelvic computed tomography and MRI revealed a cystic lesion; (E and F) ovarian teratoma was resected during laparoscopy. The tumor consisted of bone, teeth and hair; (G) positive HEK 293 cells with anti-NMDAR antibodies using patient's cerebrospinal fluid (white arrows indicate the positive cells); (H) positive immunostaining of teratoma using NMDA NR1 receptor antibody (scale bar = 100 μm, black asterisks indicate the NR1 positive cells). The negative control of NMDAR immunostaining in HEK 293 cells (I) and in teratoma tissue (J). MRI: magnetic resonance imaging; NMDAR: *N*-methyl-d-aspartate-receptor; HEK: human embryonic kidney; FDG: fluorodeoxyglucose; PET: positron emission tomography

Anti-NMDAR encephalitis is a rare clinical condition and may associate with ovarian teratoma. This kind of autoimmune limbic encephalitis may extend to cortical and subcortical regions. Cranial MRI is not reliable for early diagnosis. Patients with teratoma usually have good prognosis after mass resection.

Authors' contributions

Conception, diagnosis and design: S. Chen
Manuscript preparation: X.J. Zhang

Data collection and assembly of data: M.S. Yao, X.H. Luan
Pathology diagnosis: F. Yuan
Manuscript revision: J. Liu, S.F. Chen, C.F. Jia
Final approval of manuscript: S.D. Chen

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patient.

Ethics approval

Data collection in our study involving the patient is consistent with the ethical standards of the institution's ethics committee.

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Topic: Stroke **Open Access**

When friend turns foe: central and peripheral neuroinflammation in central nervous system injury

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ABSTRACT

Injury to the central nervous system (CNS) is common, and though it has been well studied, many aspects of traumatic brain injury (TBI) and stroke are poorly understood. TBI and stroke are two pathologic events that can cause severe, immediate impact to the neurostructure and function of the CNS, which has been recognized recently to be exacerbated by the body's own immune response. Although the brain damage induced by the initial trauma is most likely unsalvageable, the secondary immunologic deterioration of neural tissue gives ample opportunity for therapeutic strategists seeking to mitigate TBI's secondary detrimental effects. The purpose of this paper is to highlight the cell death mechanisms associated with CNS injury with special emphasis on inflammation. The authors discuss sources of inflammation, and introduce the role of the spleen in the systemic response to inflammation after CNS injury.

Quick Response Code:

INTRODUCTION

Prevalence and incidence of stroke and TBI

Presently, there are over 500 defined neurological disorders caused by trauma, infections, degeneration, autoimmune disorders, structural defects, tumors, or strokes of the brain and spinal cord.[1] Of these disorders, which are characterized by progressive neuronal degeneration and adverse physical and cognitive impairments, stroke and traumatic brain injury (TBI) both have profound impacts on the American population and worldwide. According to the American Stroke Association, stroke is the 5th leading cause of death in the United States, killing 130,000 every year, and is the leading cause of preventable long-term disability. Healthcare services and medications for stroke alone cost a total of over $34 billion per year nationwide.[2] Stroke patients commonly suffer paralysis and other motor dysfunctions, which require extensive therapy.[3] Similarly, TBI accounted for approximately 2.4 million emergency department visits, hospitalizations, or deaths in 2010, and an estimated 5.3 million people are currently living with TBI-related disabilities in the United States.[4] Common features of TBI include bruising, torn tissues, bleeding, and physical damage to the brain resulting in long-term complications or death. Among other effects, characteristic symptoms of mild TBI include fatigue or lethargy, a change in sleep patterns, behavioral or mood changes, and trouble with memory, concentration, attention, or thinking, which are clear clinical manifestations of a neurological disorder.[4]

CNS inflammation in stroke and TBI

The CNS was previously believed to be an immune-privileged site, but a large body of research from the past few decades reveals a complex interplay between glial cells and systemic leukocytes in neuroinflammation. Neuroinflammation is a pathological hallmark of many neurological disorders. Following onset of both stroke and TBI, an acute inflammatory response is mounted to counter initial mechanical damage to brain tissue. Resident microglia become activated and carry out neuroprotective roles via secretion of pro-inflammatory cytokines.[5,6] However, infiltration of peripheral leukocytes through the compromised blood-brain barrier (BBB) coupled with chronically activated microglia propagates chronic inflammation and maintains a toxic environment that cyclically contributes to secondary axonal death.[7] Thus, sequestration of the neuroinflammatory response has been the target of recent therapeutic investigation to attenuate neurological damage. Our laboratory's long-understanding neuroinflammation in preclinical models of stroke and TBI is the main theme of this review paper.

Therapeutic effects of acute neuroinflammation

It is important to note that CNS inflammation is not entirely detrimental. Acute central inflammation following stroke or TBI is deemed to be "neuroprotective".[3,4] Activated microglia and CNS-specific T cells, for example, help maintain neurogenesis and spatial learning abilities in the adult brain.[8] Ziv *et al*.[8] described how a protective immune response that intends to eliminate danger and minimize tissue (neuronal) loss must be "regulated and shaped by a well-balanced innate-adaptive dialogue" between microglia and systemic T-lymphocytes.

Acute inflammation in stroke

In the acute inflammatory stage associated with stroke, potent pro-inflammatory cytokines TNF-α, interleukin 6 (IL-6), and IL-1β are upregulated in the cerebrospinal fluid (CSF) and blood in humans.[9] There is evidence that microglia 1 (M1) activated microglia locally produce TNF-a and IL-1, while IL-6 is also produced by neurons.[9] Additionally, Beschorner *et al*.[10] demonstrated abundant expression of cluster of differentiation 14 (CD14) by ischemia-activated microglia. Since CD14 is key pattern recognition receptor of the innate immune system also found on peripheral monocytes involved in cellular activation, this implicates resident microglial contribution to acute ischemic inflammation.[10] Investigations therefore aim to shift classically activated M1 microglia to the alternatively activated M2 phenotype, which secretes anti-inflammatory cytokines and neurotrophic factors that may contribute to neuroregeneration.[6]

Acute inflammation in TBI

The TBI brain experiences a short, endogenous pro-cell-survival stage in acute neuroinflammation, though this is not sufficient for long-term neuroprotection against chronic neuroinflammation.[11] Primary damage caused by TBI is mechanical, including neuronal injury and disruption of the BBB.[11] Following are two stages of immune response similar to that of stroke: an acute "neuroprotective" stage and a chronic "neurodegenerative" stage.[6] Microglial cells become activated into their pro-inflammatory states,[6] while some afford neuroprotective/regenerative capabilities to combat such damage, but only acutely.[6] Giunta *et al*.[5] showed that microglia promoted widespread cellular proliferation and focal neurogenesis in the dentate gyrus of the hippocampus. However, this protection appears to be insufficient as activated microglia secreting pro-inflammatory cytokines prove to have a more powerful role in acute inflammation and beyond; chronically activated microglia were found in TBI

patients up to two decades after the initial traumatic insult.[5,11] Neuroinflammation in the TBI brain appears to be more widespread. In TBI mouse model, there was significant upregulation of activated microglial cells in both gray and white matter not only at the TBI impacted cortical site but also at proximal adjacent ipsilateral areas and distal areas.[12]

Detrimental effects of chronic neuroinflammation

Chronic neuroinflammation is mediated by both central and peripheral sources.[6,13] If this delicate innate-adaptive, central-peripheral immune dialogue between central microglia and systemic lymphocytes is not properly regulated,[8] the resulting regulatory imbalance sustains a harsh environment by chronic neuroinflammation and causes secondary cell death and adverse neurological deficits. In stroke and TBI, physical trauma to the BBB activates an innate immune response, but the consequences of such mechanical damage extend beyond if not properly mitigated. Peripheral immune cells infiltrate the brain via the compromised BBB and thus exacerbate any existing central neuroinflammation.

Stroke and chronic neuroinflammation

The chronic "degenerative" stage of stroke involves BBB disruption associated with infarction of the parenchyma and cerebral vasculature.[14] At this point, invasion of immune cells and serum proteins through the damaged endothelial cell barrier precipitates adverse physiological consequences such as propagation of neuroinflammation, increased cerebral pressure and increased cellular death,[7] thus, the initial brain damage caused by stroke is exacerbated by this secondary BBB destruction. Ischemic stroke was found to induce an autoimmune response to neuronal antigens that can possibly potentiate or ameliorate long-term neuroinflammation.[15]

TBI and chronic neuroinflammation

Similarly, peripheral immune cells enter the TBI brain through the damaged BBB, continuing to release pro-inflammatory cytokines, attract more immune cells, and activate microglia, rendering a cycle of extended inflammation in the brain. A decrease in hippocampal neurons and decline in cell proliferation in the ipsilateral subventricular zone and the subgranular zoneconsistent with TBI pathology was also observed, indicating the deleterious effects of chronic inflammation.[12] Low graft survival of stem cells has been documented in the TBI brain during investigational cell therapy treatments, which may be attributed to the harsh environment caused by this secondary neuroinflammatory response.[16] Secondary neuronal damage caused by chronic inflammation of activated microglial cells appears to be the link between TBI and Alzheimer's disease (AD) neuropathology.[5] Several neuropathological hallmarks of Alzheimer's were observed in brains of chronic TBI patients, namelyamyloid-beta (AB) plaques and neurofibrillary tangles.[17] AB42 aggregation has been attributed to aging microglia's reduced phagocytic capacity and therefore decline in microglial clearance of AB plaques.[5] Further, post mortem analysis of TBI patient brains showed senile AB plaques present across all age groups, including children, suggesting that TBI is indeed the cause of AD in these patients.[5] Chronic inflammation and subsequent neuronal degeneration makes patients vulnerable to neurological deficits. In addition to Alzheimer's disease, TBI is strongly associated with several other neurologic disorders 6 months or more after injury.[18] Uryu *et al*.[19] characterized multiple proteins implicated in neurodegenerative diseases in post-mortem TBI brains, formed within 4 h to 5 weeks of injury. AB plaques co-accumulated with amyloid precursor protein, beta-secretase, and presenilin 1 and the presence of alpha synuclein was confirmed all within the axonal bulbs.[19] Alpha-synuclein is a presynaptic neuronal protein that aggregates to form toxic protofibrils which are then released from dying neurons to contribute to pathogenesis and also accumulated in the CSF following TBI in infants and children.[20,21] Synucleinopathy additionally links TBI and Alzheimer's to Parkinson's disease (PD). PD displays the same active contribution of reactive microglia to loss of dopaminergic neurons.[4] This "reactive gliosis" upregulates pro-inflammatory cytokines in both the brain and CSF of PD patients.[4] Finally, microglia are the first to respond to a traumatic spinal cord injury and were found to remain activated for at least 6 months post-injury in humans.[22] Intraspinal neurons and astrocytes contribute by producing pro-inflammatory cytokine IL-1B.[22]

The next section of this paper discusses the sources of inflammation after TBI and stroke, and how inflammation contributes to the pathogenesis of these disorders.

SOURCES OF INFLAMMATION: CENTRAL AND PERIPHERAL

Central and peripheral sources of neuroinflammation

There are both central and peripheral sources contributing to neuroinflammation of neurological disorders [Figure 1]. Traditionally, chronically activated microglia has been the targets of therapeutic treatment

but research suggests another viable option. Stem cells were shown to preferentially migrate to the spleen following ischemic stroke,[3] and splenectomies following stroke or TBI reduced neuronal damage.[23-26] This supports the concept of an existing dialogue between the local CNS and systemic immune system, because the spleen is the primary source of systemic inflammation, it has been the focus of investigation in the "brain-spleen inflammatory coupling" associated with stroke, TBI, and other neurological disorders.[18] Therefore, sequestration of inflammation to the spleen in order to attenuate chronic neuroinflammation and improve the efficacy of stem cell therapy provides a promising therapeutic approach to stroke and TBI treatment.

We have chosen to separate the body's response into two categories, central inflammation and peripheral inflammation, in an attempt to show the peripheral immune response's contribution to the cognitive decline following TBI and stroke, and elucidate the potential for research into novel therapies. By central inflammation we are referring to the role of resident cells of the CNS in inflammation, and by peripheral inflammation we are referring to the contribution of the systemic immune response to neuroinflammation after traumatic brain injury or stroke.

Central source of neuroinflammation

Traditionally, the immune system is thought to be non-existent in the CNS. Accumulating evidence now suggests that the CNS has its own immune system, and in addition, the peripheral immune system may play a role in neuroinflammation.[27-29] As mentioned in the previous section, the negative outcomes of stroke and TBI are exacerbated by the body's reaction to the injury. The body's inflammatory response, which protects against infection, also induces a chronic state of deterioration in the CNS, exacerbating the neurological deficits caused by the initial injury.

Glia

When talking about the CNS and immunity, it is important to highlight the role of glia. Glia are non-neuronal cells that maintain homeostasis; two common glia are astrocytes and microglia. Astrocytes make up the blood brain barrier, which separates the CNS from the rest of the body, including the peripheral immune system. To prevent entry of peripheral immune cells and counter otherwise widespread cerebral inflammation, the BBB forms a physical boundary with a specialized microvasculature consisting of endothelial cells connected by adherent and tight junctions.[30] This allows control of cerebral homeostasis via selective transport of molecules and cells.[31] When the BBB is compromised and microglia are activated, inflammation of the brain ensues.

Microglia

Microglias are the innate immune cells of the CNS. They are cells of myeloid lineage that populate the CNS during embryogenesis, and thus act similarly to

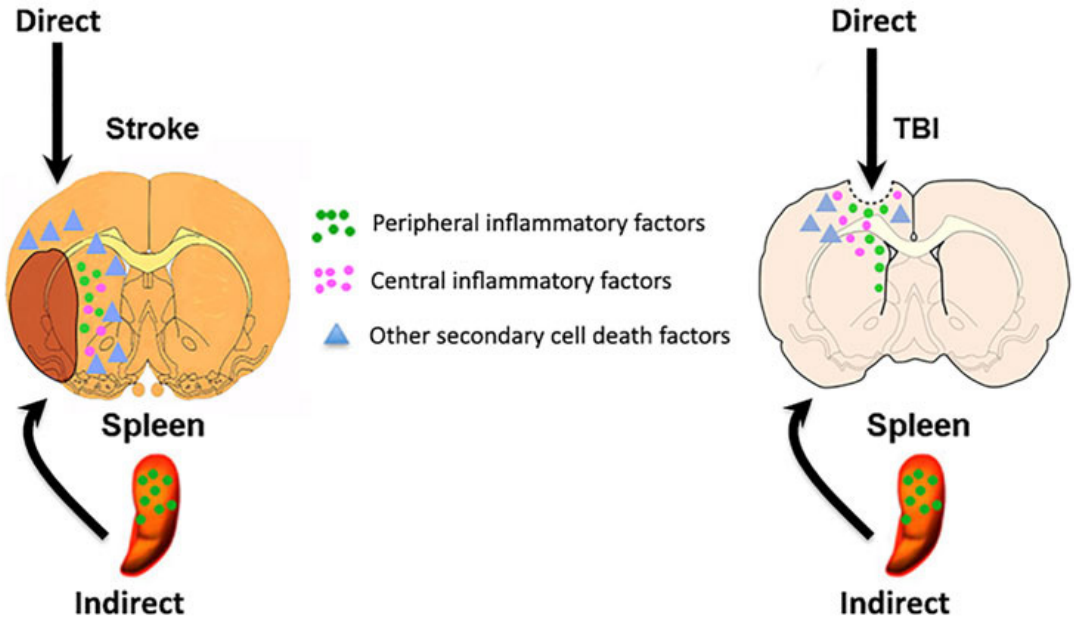

Figure 1: Central and peripheral sources of inflammation. Following CNS injury, such as stroke and TBI, the traditional concept entails a robust inflammatory response within the brain, but equally compelling recent evidence has demonstrated an active inflammatory response, especially from the spleen, contributing to the progression of the disease. Together with other secondary cell death factors, both central and peripheral inflammation exacerbate CNS injury. CNS: central nervous system; TBI: traumatic brain injury

the peripherally acting cells of the myeloid lineage: macrophages and dendritic cells.[32] Resident microglia are capable of clearing foreign pathogens or mediating a local, innate immune response in the brain. Microglia can be observed along a continuum of three idealized phenotypic states: resting, activated non-phagocytic (antigen presenting cell like), and activated phagocytic, depending on their level of activation, which is dictated by the amount and type of cytokines in the surrounding microenvironment.[33-35] Microglia sense the microenvironment, and mount a protective immune response after TBI, however the response is excessive and prolonged, and ends up leading to further degeneration instead of regeneration and repair.[11] Like macrophages, microglia can polarize into two subcategories, M1 or M2.[6] M1 is predominantly pro-inflammatory, and secretes high levels of pro-inflammatory cytokines like IL-1, IL12, and IFN-γ, and low levels of anti inflammatory cytokines like IL-10.[6] M2 is typically anti-inflammatory, and acts to clear debris and promote regeneration.[3] Pro-inflammatory M1 macrophages predominate after CNS injury.[6,11]

Central immune cells contribute to diffuse axonal injury

As mentioned in the previous section, current research shows that neuroinflammation is a major source of secondary cell death after TBI and stroke.[36-38] The major players in neuroinflammation are immune cells, microglia, cytokines, and chemokines that altogether exacerbate neuronal cell death after initial injury,[39] and lead to a phenomenon known as diffuse axonal injury, which leads to extensive lesions of cerebral white matter over a widespread area, outside of the initial lesion.[27,40] Insult to the CNS, either TBI or stroke, generates a neuroprotective immune response to prevent infection and stimulate neuronal repair. After injury to the CNS, neurons, astrocytes, and microglial cells all respond to play a role in the inflammatory response that ensues.[27] Glutamate release after TBI causes hyperactivity of neurons, leading to prolonged levels of intracellular calcium, and eventually cell death, this is known as excitotoxicity.[28] Both astrocytes and microglia contribute to the inflammatory response by producing chemokines; chemokines then attract monocytes to the site of injury.[27]

Microglial cell function and CNS injury

Microglia has several distinctive properties that allow them to participate in the pathological neurodegenerative processes after CNS injury. Upon activation, microglia undergo morphological changes, proliferation, and expression of major histocompatibility complex (MHC) class II molecules.[35] They are capable of phagocytosis of damaged and fragmented neuronal elements, antigen presentation to T lymphocytes, and production of soluble factors that at sufficient concentrations can induce further tissue injury and gliosis.[6,35] Pro-inflammatory cytokines, such as these released after neuronal injury, are strong activators of microglia.[41-43] Once activated, microglia produce more pro-inflammatory cytokines such as IL-1β, which ultimately leads to an extensive chronic proinflammatory state in the CNS.[11,44]

Both TBI and stroke is characterized by an initial insult to the CNS, compromising the blood brain barrier and causing microglial activation.[6] Prolonged microglial activation leads to a chronic inflammatory response that causes excitotoxicity, oxidative stress, mitochondrial dysfunction, blood brain barrier disruption, and inflammation.[45-49] Inflammation activates microglia, which then release more pro-inflammatory cytokines such as TNFα and IL-1β which cause upregulation of cell adhesion molecules in the surrounding vasculature and lead to a further increase in blood brain barrier permeability, and allows systemic involvement in neuroinflammation.[6,11,44,50]

M1 microglia afford neurodegenerative effects

As mentioned before, microglia can polarize into either M1 or M2 when activated. After CNS injury, both types of microglia are present, but type M1 tends to predominate and persist, leading to neurodegeneration instead of repair.[6,11] The capacity of the microglia to drive the response to CNS injury towards either further damage or repair exemplifies its role as a key player in central immunity, and is the reason it has become the target in studying the cognitive decline due to neuroinflammation after traumatic brain injury and stroke.

Pathological processes involving microglia

In addition to recruiting other immune cells to the site of injury, microglia contributes directly to neuronal damage through several pathological processes. When highly activated, microglia are capable of phagocytosis.[35] In the case of CNS damage, activated microglia phagocytose neuronal elements. Activated microglia also produce reactive oxygen species and reactive nitrogen species.[6] These are highly reactive molecules that increase the oxidative stress, and lead to destruction of neuronal cell membranes through lipoperoxidation.[51] Cell membranes allow the cell to maintain homeostasis; once the membrane is compromised the cell can die.

Chronic activation of microglia

The secondary inflammatory damage after insult to the CNS can be observed as cognitive decline days

and even up to years after initial injury.[11] In animal models it has been shown that microglia can be active for up to one year after TBI.[6,52-55] Post mortem studies have shown activated microglia up to 17 years after TBI in adult humans.[6,55] The observed cognitive decline, in conjunction with the presence of activated microglia after injury suggests a persistent chronic inflammatory stage mediated by microglia that exacerbates TBI and stroke pathology.

Peripheral source of neuroinflammation

The immune system consists of a network of cells, tissues, and organs that coordinate to protect the body from foreign pathogens, and promote tissue healing and regeneration. When the body is injured, cell death leads to leakage of nuclear or cytosolic proteins or protein fragments into the extracellular space. These intracellular fragments are pro-inflammatory signals called damage associated molecular patterns (DAMPS). DAMPS are recognized by pattern recognition receptors on dendritic cells, macrophages, and other cells such as vascular cells, epithelial cells, and fibroblasts, and elicit a pro-inflammatory response from these cells.[56] Once an immune response is mounted, it can either persist as chronic inflammation, or move towards resolution and tissue healing.

Peripheral immune cells are recruited after CNS insult

The inflammatory response activates the complement system to recruit immune cells to the intrathecal compartment;[48] neutrophils, monocytes and lymphocytes all cross the blood brain barrier and chemotax towards the site of injury.[57] Once these cells have reached the site of injury, they are activated to secrete free radicals, pro-inflammatory cytokines, prostaglandins and other inflammatory mediators, resulting in recruitment of more immune cells and microglia to the site of injury.[57,58] A great deal of research has been done on microglia, highlighting it as the key player in coordinating the immune response after an insult to the CNS.

Systemic immune response

The role of resident immune cells in the CNS after TBI is only part of the story. To get a full picture of the inflammatory response to TBI we must also look at the peripheral immune system. Multi-organ damage following TBI can lead to a more robust immune response in the brain,[28] highlighting the possibility that systemic inflammation could play a role in neuroinflammation. Because the BBB is compromised in a CNS injury, circulating inflammatory cells and cytokines can access the brain and contribute to the pathogenesis of TBI.[28,59] Leakage of chemokines and other inflammatory molecules through the compromised blood brain barrier into systemic circulation can attract peripheral immune cells to the site of injury.[28] This can potentially lead to an overactive inflammatory response known as systemic immune response syndrome.[28] Negative feedback to systemic inflammation is provided by the hypothalamus-pituitary-adrenal axis and sympathetic nervous system efferents.[28] In TBI, an imbalance between systemic immune response and negative feedback can lead to either excessive organ damage or susceptibility to infections and lack of regeneration.[28]

Role of peripheral chemokines

Cytokines and chemokines are very important to the pathogenesis of TBI. Although their exact role is unclear, data suggests that cytokines play a pivotal role in the body's response to TBI. After insult to the CNS, upregulation of the following cytokines occurs: TNFα, IL-1β, IL-2, IL-6, IL-8, IL-4, IL-18.[60-62] One important peripherally secreted chemokine chemokine (C-C motif) ligand 20 (CCL20) is upregulated after TBI, and interacts with CC chemokine receptor 6 (CCR6) to induce chemotaxis of T cells, B cells, and dendritic cells.[28] These cells can be found in the spleen, and are known to contribute to the pathogenesis of TBI.[28] Other peripheral cells are found at the site of injury, and contribute to the inflammatory process. It is known that the concentration of neutrophils peaks around 3-24 h after injury, and the concentration of monocytes peaks around 1-2 days after spinal cord injury.[63]

Synergistic central and peripheral inflammation

In the case of chronic neuroinflammation, both central and peripheral sources of inflammation work together to create a hyperactive immune response that ultimately leads to further damage rather than repair of neural tissue.[27,28]

It is well known that CCL20 acts as a chemokine for CCR6 expressing cells. In an experimental autoimmune encephalomyelitis (EAE) model, which is an animal model for brain inflammation similar to multiple sclerosis in humans, researchers have observed that CCL20 acts as a ligand for CCR6, allowing homing of lymphocytes, and other leukocytes to neural tissue.[64] In this specific case, it allows trafficking of Th17 or Th1 CD4+ Th cells, which release pro-inflammatory cytokines that can cause chronic inflammation.[64,65] CCL20 expression in the choroid plexus allows passage of CCR6+ Th cells to enter the CNS in the uninflamed brain, which then

allows a CCR6 independent pathway of recruitment of T cells to the brain parenchyma in the EAE model.[64] CCL20 expression is upregulated by proinflammatory cytokines IL6, and IL17.[65]

In a lateral fluid percussion model of TBI, Das shows that CCL20 expression is upregulated in the thymus and spleen 24 h after TBI, and upregulated in the cortex and hippocampus 48 h after TBI.[27] Based on the evidence obtained from the EAE model, this suggests a mechanism for peripheral involvement in neuroinflammation.[27,28,64] The fact that CCL20 is expressed in the spleen and thymus after TBI, before it is expressed in the brain, and brain CCL20 expression is reduced in rat's who's spleens have been removed suggests a peripheral mechanism of activation for CCL20 expression in the CNS.[27] It also speaks to the role that CCL20 plays in neuroinflammation after TBI. In other words, CCL20 upregulation in the spleen and thymus after TBI could indicate a peripheral signal that drives neuronal degeneration.[27]

In stroke, we see a similar peripheral involvement in chronic inflammation after insult. Nguyen and colleagues characterized the cytokine profiles in mice after ischemic CNS infarct, and showed a polarized T cell response based on the type of mouse used.[66] C57BL/6 mice had a Th1 polarized response, and BALB/c mice had a Th2 polarized response.[66] This suggests that the chronic inflammatory response in stroke patients could follow different courses, depending on the individual afflicted.[66]

In all of these instances, peripheral involvement in neuroinflammation acts in addition to the central inflammation perpetuated by microglia and other inflammatory mediators. In summary, injury to the CNS leads to a peripheral and central response that act together to cause inflammation, which eventually leads to a chronic inflammatory state that causes neural degeneration rather than repair and resolution after insult.

Although a strong connection between CNS injury and the immune system has been shown, little research has been directed at exploring the role of the thymus in TBI. Recent studies have shown upregulation of CCL20 in the thymus after TBI.[27] Other studies have shown that the liver may play a role in exacerbating the neuronal degeneration after TBI (Campbell *et al*.[67]). Depletion of hepatic Kupffer Cells reduced ED-1 positive macrophage and neutrophil migration into an IL-1β injected brain.[67] As a reservoir of peripheral immune cells, the spleen has been shown to play a major role in traumatic brain injury.[28]

ROLE OF THE SPLEEN AS A MAJOR PERIPHERAL INFLAMMATORY CONTRIBUTOR TO CNS INJURY

Function of the spleen

It has been shown that the spleen initiates an immune response that exacerbates the pathology of stroke and TBI, however the connection between brain injury and a splenic response has yet to be fully elucidated. The spleen has several functions in the body. It is a major lymphatic organ that lies within the peritoneal cavity; it actively monitors the body's circulation and filters blood.[68,69] In humans, the spleen plays a role in the mononuclear phagocyte system, recycles iron from old red blood cells, and mounts a defense against blood borne pathogens.[69,70]

The spleen as a reservoir of systemic immune cells

The spleen is also a reservoir of platelets, peripheral macrophages, and other immune cells.[28,70,71] Scientists used to think that the majority of monocytes patrolled the circulation, and irreversibly differentiated into macrophages and dendritic cells upon extravasation and tissue entry.[70] It is now known that the spleen actually acts as a reservoir for undifferentiated monocytes, and monocytes in the spleen outnumber monocytes patrolling the circulation.[68,70] This means that a majority of undifferentiated monocytes reside in the spleen, waiting to be deployed. Monocytes, distinct from macrophages and dendritic cells, cluster in the cords of the subcapsular red pulp of the spleen.[70]

Splenic immune cells home to injuries throughout the body

Spleen has the ability to rapidly deploy this cohort of undifferentiated monocytes.[68,70,72] Splenic monocytes have been shown to exit the spleen and accumulate in the heart after myocardial infarction to participate in immunological processes such as wound healing.[70] In the context of CNS injury, several aspects of the splenic response have been observed. One study has shown that the number of T cells in the spleen decreases 1-2 days after traumatic brain injury.[72] In a study that induced middle cerebral artery occlusion (MCAO) on mice, researchers observed both splenic contraction and a reduction in the number of splenic cells after stroke was induced.[68] In that same experiment, splenic contraction coincided with a decrease in monocytes in the spleen, and a concurrent increase in the same subsets of monocytes in the ischemic brain.[68]

Spleen and CNS injuries

Since research has shown that the immunologic response to TBI and stroke can in fact exacerbate the

damage from the initial injury, and further research has suggested that the spleen plays a role in mounting an immune response to the injured CNS, researchers tried knocking out the function of the spleen to observe the effect on TBI and stroke. Ajmo *et al*.[73] showed that removal of the spleen two weeks before permanent MCAO significantly reduced the infarction volume. In another study, researchers showed that removal of the spleen just before temporary MCAO caused a reduction in the accumulation of monocytes in the brain, but did not significantly change the infarct size.[68] In addition, splenectomy in rats immediately after traumatic brain injury reduced circulating levels of pro-inflammatory cytokines, decreased mortality, and increased cognitive functioning.[74] Furthermore, it has been shown that splenectomy immediately after mild TBI in rats attenuated CCL20 chemokine expression and neurodegeneration in the brain.[27]

The spleen and cognitive deficits

Although splenectomy is probably not advisable in human patients that have received a traumatic brain injury or stroke, these studies highlight the importance of the spleen in CNS injury. The splenectomy studies, in conjunction with the studies that show a loss of immune cells from the spleen and the appearance of the same subset of cells in infarcted brain tissue after stroke, lead researchers to believe that the spleen is bolstering the immune response in CNS injury. This data suggests that the spleen plays a role in the secondary wave of neurodegeneration after TBI and stroke, leading to more severe cognitive deficits.

Blood flow and microglial cytokines

Quantifying blood flow to the spleen after injury is important to understand the role of the spleen as a mediator in the immune process. Several ways of measuring blood flow to the spleen have been described. The control of blood supply to the spleen involves several aspects. It has been shown that IL-1 increases splenic blood flow by affecting the sympathetic vasoconstrictor tonus. In order for the spleen to remain perfused, resident macrophages must produce IL-1β to counteract noradrenergic vasoconstriction.[75] Sympathetic tone reduces perfusion, whereas inflammatory mediators such as IL-1β increase perfusion.

Blood flow after CNS insult and spleen

Blood flow to the spleen after TBI shows a biphasic hemodynamic pattern. In a study by Yuan *et al*.,[76] blood flow measurements were taken at 5 min, 15 min, 30 min, and 60 min after injury. Fluid percussion brain injury produced an immediate systemic hypertension followed by hypotension and low cardiac output. Immediately after TBI, blood flow to all organs either remained the same or increased for 30 min, then gradually decreased.[76] Blood flow to the kidney and spleen were decreased the most after TBI, which was attributed to sympathetic activity because of the high amount of sympathetic vasoconstrictor fibers running to those organs.[76] The resulting hyperactive sympathetic response, similar to what happens after TBI, is characterized by a widespread vasoconstriction that is also selective; flow is decreased through kidneys and splanchnic organs such as the spleen but not decreased to the heart.[76] It will be interesting if a similar phenomenon characterized by blood flow alterations in the spleen accompanies stroke.

Brain-spleen inflammatory coupling in CNS injuries

Lastly, it has been shown that immune cells in the spleen respond to cholinergic input. Studies have shown that there is a correlation between brain injury and autonomic release of pro-inflammatory cytokines from splenic macrophages. In a concept known as “brain-spleen inflammatory coupling”, researchers have hypothesized that the changes in autonomic input after CNS injury lead to systemic responses, including a response from the spleen. In increase in pro-inflammatory cytokines in the brain after CNS injury stimulates the posterior hypothalamus to increase sympathetic tone, leading to catecholamine release from the adrenal glands and peripheral vasoconstriction.[71] It has been shown that macrophages in the liver respond to adrenergic/cholinergic input, and thus can respond to changes in autonomic tone.[71,73,77] The body responds to CNS injury by increasing sympathetic tone, and immune cells in the spleen respond to this adrenergic input by producing large amounts of the pro-inflammatory cytokines TNF-α and IL-1β.[71,78] It is hypothesized that this systemic inflammatory response to TBI and stroke exacerbates TBI pathology.

Whereas elevated sympathetic tone increases the pro-inflammatory response from the spleen, increased parasympathetic tone has been shown to decrease the pro-inflammatory response from the spleen.[71] Macrophages in the spleen express a nicotinic catecholamine receptor α7nAChR which responds to parasympathetic input by reducing production of the pro-inflammatory cytokine TNFα.[71,79] Selectively activating this receptor after stroke in rats was shown to reduce infarct size and improve survival.[80] Other studies have shown improved neurological outcomes in animal models for stroke by either direct or indirect stimulation of this receptor.[71] This evidence suggests that

sympathetic and parasympathetic tone affect the splenic response to CNS injury, and targeting this response has the potential for novel therapeutic strategies.

A PARADIGM-SHIFT IN OUR UNDERSTANDING OF CNS INFLAMMATION

Many neurological disorders, including traditionally considered acute injuries such as stroke and TBI, have now been recognized as being plagued by neuroinflammation, which significantly contributes to the disease progression and is associated with secondary cell death reminiscent of chronic neurodegeneration. A worsening prognosis of stroke and TBI has implicated massive inflammation, arising not just from the injured but equally robustly detected from the peripheral organs, specifically the spleen. By investigating the sources and mechanisms of neuroinflammation, in particular the role of spleen-mediated inflammatory response, novel cell death pathways as well as innovative therapeutic targets are identified for interrupting this inflammatory process and providing avenues for ameliorating the secondary cell death of stroke and TBI. Recognizing that both the CNS and the peripheral immune system play important roles in the inflammatory process is a key to deciphering the cellular, molecular, and genetic pathways of neuroinflammation, and broadens our scope for developing new anti-inflammation-based treatments. As a major secondary lymphoid organ, the spleen intimately participates in the peripheral immune response that can exacerbate neuroinflammation and the subsequent chronic neurodegeneration. Accordingly, when contemplating with an anti-inflammatory strategy for stroke and TBI, an in-depth examination of this accumulating preclinical evidence suggesting the involvement of central and systemic sources of inflammation will be critical to a better understanding of the pathology and treatment of the secondary cell death that closely approximates the disease progression of stroke and TBI.

Authors' contributions

Conceptualized the topic: C.V. Borlongan
Drafted, wrote, and approved the final version of this paper: P. Marcet, N. Santos, C.V. Borlongan

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Conflicts of interest

C.V. Borlongan has patents and patent applications relating to stem cell therapy for stroke and TBI.

Patient consent

There is no patient data involved.

Ethics approval

Not applicable.

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Case Report **Open Access**

Miller-Fischer syndrome after etanercept

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Dr. Elena Grebenciucova is currently a multiple sclerosis fellow at the University of Pennsylvania. Her interests lie in the field of neuroimmunology and neuro-infectious disorders, and her research interests include learning why some patients on anti-tumor necrosis factor alfa agents develop demyelinating events, while others do not.

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ABSTRACT

The authors describe a case of Miller-Fischer syndrome, a rare demyelinating syndrome, preceded by a viral prodrome and three doses of etanercept, an anti-tumor necrosis factor α (anti-TNFα) agent. Anti-TNFα agents are associated with an induction of episodes of demyelination and may unmask multiple sclerosis in those who are immunogenetically predisposed.

INTRODUCTION

Miller-Fischer syndrome (MFS) is a rare self-limiting demyelinating disorder that is considered to be a variant of Guillain-Barre syndrome (GBS). MFS typically affects cranial nerves first and then descends, classically resulting in a clinical triad of ophthalmoplegia, areflexia, and ataxia.[1] An antibody against the ganglioside Q1b (GQ1b) is 90% specific and 85-90% sensitive.[2] Cases of demyelination, both central and peripheral, have been described in patients treated with anti-tumor necrosis factor α (anti-TNFα) agents. This case describes a patient with MFS preceded by both a viral illness and the use of the anti-TNFα agent etanercept.

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CASE REPORT

A 54-year-old male with history of psoriatic arthritis presented for an evaluation of the acute onset of ocular pain and double vision. He had recently started Etanercept, an anti-TNFα antibody. Five days after the 1st infusion, he developed rhinitis and a cough, which resolved in several days. He proceeded with his weekly 2nd and 3rd infusions, but 2 days after the 3rd infusion he developed blurred vision in the right eye and pain with eye movement. Two days later, he developed double vision and ocular pain when attempting upgaze, followed by headache and pain with eye movement in all directions.

Three days after the onset of his symptoms, he was evaluated by an ophthalmologist who noted incomplete adduction of the left eye on far right gaze. Five days later, a neuro-ophthalmologic examination showed bilateral eye adduction weakness with an upgaze palsy, along with a mild left eye ptosis and miosis. Direct and consensual reaction of pupils was intact. There was no afferent pupillary defect or disc edema.

The rest of the neurological examination was significant for areflexia, while cerebellar exam was normal.

A contrast-enhanced magnetic resonance imaging (MRI) of the brain was performed 3 days after onset of symptoms, and no midbrain or pontine lesions were noted. There was no optic nerve enhancement noted on orbital imaging. Acetyl-choline receptor and muscle specific kinase antibodies were both negative.

He underwent another MRI two weeks after the onset of symptoms, and both MRI of the brain and orbits with and without contrast were again unremarkable. At this point, MFS was considered, and a GQ1b antibody was tested, which was positive with a titer of 1:12,800. One month after the onset of his symptoms, he was essentially asymptomatic, and his neurologic examination normalized, other than residual areflexia.

DISCUSSION

MFS is a rare self-limiting demyelinating syndrome that is considered to be a variant of GBS. Annual incidence is estimated to be one case per million.[3] A clinical triad of ophthalmoplegia, arreflexia, and ataxia is the classic presentation.

An antibody to the neuronal GQ1b (or in some cases GT1a) is highly sensitive and specific for the diagnosis of MFS. About 60% of cases are preceded by a viral or in some cases bacterial illness. The disease occurs more commonly in males than females and is treated with intravenous immunoglobulins or plasmaphoresis, although according to the Cochrane review, patients who were not treated with either therapy had similar outcomes at 6 months.[4] However, the data are difficult to interpret due to the low incidence of the disease and consequent lack of randomized placebo-controlled trials. In most cases, the course of the disease is self-limiting and the outcomes are favorable. In our patient, the onset of MFS was preceded by a viral prodrome, but also occurred in the context of an anti-TNFα agent.

The use of anti TNFα agents in the treatment of various rheumatological disorders such as sarcoidosis, rheumatoid arthritis, and psoriasis has been increasingly recognized as causal to cases of demyelination, some of which remain monophasic, with a minority transitioning into multiple sclerosis. Due to their anti-inflammatory activity, anti-TNFα agents have been previously investigated in multiple sclerosis, however a randomized placebo-controlled trial of anti-TNFα agent in multiple sclerosis showed worsening of the disease activity in the anti-TNFα group.[5]

TNFα is a cytokine that binds to TNF receptor 1 (TNFR1) or 2 (TNFR2). TNFR1 binding results in the inflammatory effects of TNF, while TNFR2 binding contributes to the maintenance of immune tolerance. TNFα binding to TNFR2 in conjunction with IL-2 result in T regulatory cells proliferation, increase in forkhead box P3 expression and an increased immunosuppressive activity.[6] Thus, inhibiting TNF can alter the balance between the effector and regulatory T cells, potentially leading to dysregulation of immune tolerance and allowing an increased activity of autoreactive T cells. As a result of this imbalance, humoral immunity can be further activated resulting in the auto-reactive antibody generation.[7] It is possible that in the immunogenetically susceptible people, the use of anti-TNFα agents may result in an episode of demyelination or unmask the predilection for multiple sclerosis. The detailed pathophysiological mechanism has not been fully elucidated.

Cases of GBS have also been associated with the use of anti-TNFα agents.

Besides our case, a review of the literature uncovered three prior cases of MFS in association with the use of anti-TNFα blockers [Table 1].[8-10] The underlying mechanism of MFS in association with anti-TNFα blockers is likewise unclear. In our case, given a preceding viral illness, it becomes of interest whether the anti-TNFα agent further contributed to the development of the syndrome or if it had any

Table 1: Literature cases of MFS in association with anti-TNFα agents

Reference	Patient	Anti TNF agent	Underlying condition	Duration of treatment	Symptoms at onset	GQ1b titre	Treatment	Outcomes
Kurmann *et al.*[9]	77 years/ female	Adalimumab	Rheumatoid arthritis	2 infusions	Ataxia, areflexia, nystagmus	Negative	Steroids/ azathioprine	Gradual recovery over 1 year
Shin *et al.*[8]	56 years/ male	Infliximab	Rheumatoid arthritis	10 infusions	Ataxia, nystagmus	< 1:100	Steroids/ IVIG	Independent ambulation at 6 months
Ratnarajan *et al.*[10]	43 years/ female	Infliximab	Ulcerative colitis	2 infusions	Ophthalmoplegia	1:6,400	No treatment	Full resolution at 10 weeks
Our patient	56 years/ male	Etanercept	Psoriatic arthritis	3 infusions	Ophthalmoplegia, areflexia	1:128,000	No treatment	Full recovery 1 month

MFS: Miller-Fischer syndrome; TNF: tumor necrosis factor; GQ1b: ganglioside Q1b; IVIG: intravenous immunogloblin

contributing effect on the course or severity of the disease.

This case illustrates the importance of considering MFS in patients presenting with ophthalmoplegia (even without ataxia) during use of an anti-TNFα agent. Further studies are necessary to understand the pathophysiological mechanisms of the aberrant immune responses leading to MFS, and the immunogenotypes of patients that may be at higher risk for immune dysregulation.

Authors' contributions

Analyzed the data and drafted the manuscript: E. Grebenciucova, J.H. Pula

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Conflicts of interest

There are no conflicts of interest.

Patient consent

According to the requirements of the affiliation, this case report does not require patient consent.

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According to the requirements of the affiliation, this case report does not require ethics approval.

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Review **Open Access**

The association between human cytomegalovirus and glioblastomas: a review

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Dr. Michael J. Strong recently graduated from the MD/PhD program at Tulane University School of Medicine in New Orleans. He will be starting residency training in neurological surgery at the University of Michigan in Ann Arbor. His dissertation project utilized next generation sequencing and bioinformatics to investigate oncogenic pathogens. He has authored 30+ peer-reviewed publications. He has received numerous awards including the Alpha Omega Alpha Student Research Fellowship, the Campagna Scholarship in Neurological Surgery, the American Association of Neurological Surgeons Young Neurosurgeons Committee Mission Fellowship, and the National Institutes of Health National Research Service Award F30 Predoctoral Fellowship.

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ABSTRACT

Human cytomegalovirus (HCMV) was reported in glioblastoma multiforme (GBM) over a decade ago and this finding has the potential to increase our understanding of the disease and it offers an alternative tumor-specific therapeutic target. Due of this promise, there is a fair amount of time, energy and money being directed towards understanding and utilizing this connection for eventual therapeutic purposes. Nevertheless, the association between GBM and HCMV remains controversial. Several studies have reported conflicting results, further undermining the potential clinical value of this association. In this review, the authors will discuss the latest developments on this evolving issue. Specifically, the results of the latest studies, both positive and negative, will be discussed. Furthermore, potential theories to explain discrepancies reported in the literature will be proposed. Clinical implications including potential targets for anti-HCMV therapy and the latest developments in anti-HCMV therapy will be presented. Finally, solutions to remedy this controversial issue in neuro-oncology will be offered.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant primary brain tumor in adults. An estimated 26,070 new cases of primary malignant brain and central nervous system (CNS) tumors are expected

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to be diagnosed in the United States in 2017.[1] Nearly everyone diagnosed with GBM succumbs to the disease, which has a median survival of 12-15 months even with aggressive treatment.[2] Despite years of research, there has been minimal improvement in the overall survival rate. Reports of human cytomegalovirus (HCMV) in GBM 15 years ago by Cobbs *et al*.[3] raised hopes for potential viral targeted therapeutic options for this disease. Recently, anti-HCMV immunotherapy based clinical trials have been established to assess efficacy in treating this disease. At the basic research level, efforts are being made to investigate the oncogenic potential of individual HCMV genes to understand how HCMV might contribute to GBM. Despite these continuing efforts and the time lapsed since the discovery of HCMV in GBM, the association remains controversial. This review serves to highlight the latest developments in this association and its clinical validity as a therapeutic target for primary brain tumors.

ENVIRONMENTAL ETIOLOGIES OF GBM

Although several studies have investigated risk factors for brain tumors, our knowledge of their etiology is limited. The only clear environmental risk factor that has been identified for glial neoplasms is ionizing radiation.[4] The relationship between viruses and the development of primary brain tumors is complex and unclear. While the majority of efforts have been focused on studying HCMV, other viruses such as polyomaviruses JC and BK have been implicated in brain tumors.[5,6] JC and BK viruses typically are asymptomatic infections that predominately present in immune suppressed individuals. Disease states from polyomavirus infections are broad and range from BK virus-related nephropathy[7] to JC virus-related progressive multifocal leukoencephalopathy.[8] The propensity for the CNS characteristic of these viruses has led to attempts to develop better screening methods to clarify this relationship.[9]

HCMV AND GBM ASSOCIATION

Although an association between HCMV and GBM was first reported in 2002,[3] there is still a high degree of inconsistency in the literature regarding the detection of viral agents in CNS tumors. Further, the recent debate between Cinatl and Cobbs labs as to the presence of HCMV in GBM continues to fuel this ongoing controversy.[10-13] The initial concept of oncomodulation was developed by Cinatl *et al*.[14] in 1996. In their study, they proposed that HCMV could increase tumor malignancy by infecting tumor cells and affecting either directly or indirectly cofactors for tumor genesis.[14] To determine whether HCMV was actually associated with GBM, they developed a standardized viral detection protocol. However, in a recent paper, Baumgarten *et al*.[10] demonstrated negative results for HCMV in their GBM cohort despite demonstrating positive staining in their control samples. In a paper addressing this, Cobbs *et al*.[11] stated that Baumgarten *et al*.[10] did not use the carefully optimized protocol established in his lab,[15] which is crucial to detect low level HCMV infection in GBM. In response, Cinatl *et al*.[12] stated that in observing similar staining in their glioma samples from HCMV seropositive and seronegative patients, they reached out to the 3 groups reporting positive results. In one group, the data could not be reproduced. The other two groups agreed to stain samples from Cinatl's lab, however, found no difference in staining in the glioma samples observed between the HCMV seropositive and seronegative patients. Despite demonstrating negative results, this data was not available to publish. Moving forward, both groups agree that a standard protocol for detecting HCMV in GBM samples needs to be established and agreed upon.

Several hypotheses have been proposed to help explain discrepancies reported in the literature including geographic differences, differences in seropositivity, the use of different cohorts, and differences in protocols and experimental conditions used for traditional detection methods, such as polymerase chain reaction (PCR), *in situ* hybridization (ISH), and immunohistochemistry (IHC) assays, which can lead to differences in sensitivities for detecting low levels of viral gene expression.

Although differences in HCMV seropositivity have been investigated, there is currently no clear association between HCMV seropositivity and incidence of GBM. HCMV seroprevalance is lower in Whites than in Blacks and Hispanics; however, GBM incidence is higher.[16] Additionally, HCMV seroprevalance is higher in women than men, while the incidence of GBM is higher in men.[16]

As a way to consolidate the data regarding the detection of HCMV in CNS tumors, a symposium was convened in Washington, DC on April 17, 2011. At this symposium, oncologists and virologists studying this very relationship had the opportunity to discuss data addressing this topic. At the conclusion of this symposium, a summary paper was published reporting the consensus position in 4 major areas including the existence of HCMV in gliomas, the role of HCMV in gliomas, HCMV as a therapeutic target, and key future investigative directions.[17] Based on

the data presented at the symposium and discussions with experts at that time, it was concluded that HCMV sequences and viral gene expression exist in many high-grade gliomas and that *in vitro* studies suggest that HCMV can modulate key signaling cellular pathways in glioblastomas.[17]

Currently, HCMV is not considered to be a classic oncogenic virus because it has not been demonstrated to possess acute transforming activity.[18] Instead, it is believed that HCMV contributes to GBM pathogenesis through oncomodulation of host cellular pathways. This notion of HCMV modulating host cellular pathways stems from evidence generated in other model systems. Specifically, studies performed in a mouse model have shown that persistent infection of endothelial cells by CMV, defined as the expression of viral genes without evidence of cytopathogenic effect on host cells, resulted in the production of inflammatory cytokines and renin, which led to the development of hypertension.[19] Applying this evidence to GBM, one hypothesis proposes that persistent infection with HCMV could lead to production of inflammatory cytokines that may contribute to pathogenesis through disruption of the cell cycle.[17]

Of the estimated 173 open reading frames present in the HCMV genome,[20] only a few of the gene products, such as IE1, pp71, glycoprotein B, and US28, have been detected in GBMs. Forced expression of HCMV IE1 was shown to increase stemness properties (e.g. self-renewal) and proliferation of glioma stem-like cells *in vivo*.[21] Follow-up studies demonstrated that IE1 promotes the tumor phenotype in these settings through inactivation of the p53 and Rb tumor suppressor proteins and through activation of the PI3-K/AKT signaling pathway.[22] Long-term HCMV infected glioma cultures demonstrated upregulation of key signaling mediators such as SOX2, STAT3, BMX and IL-6.[23] In addition, infection of GBM cells with HCMV led to upregulation of CD133 and other stem cell-like factors such as Notch1, Sox2, Oct4, and Nestin.[24] HCMV infection of GBM cells has also led to tumor proliferation through an upregulation of a proteoglycan, endocan, which has been shown to be involved in several cellular processes including angiogenesis.[25] Previously, overexpression of HCMV pp71 was shown to induce a pro-inflammatory response via activation of NFKB signaling in adult neural precursor cells.[26] HCMV glycoprotein B has been shown to mediate viral cellular entry via the receptor tyrosine kinase PDGFR-alpha resulting in activation of the PI3-K/AKT signaling pathway.[27] Another key HCMV product that is implicated in GBM development is the chemokine receptor US28. HCMV US28 regulates several cellular pathways including STAT3, VEGF, and e-NOS signaling which promotes GBM pathogenesis by regulating angiogenesis, invasion, and immune evasion.[28,29] While these experiments show that these viral genes have the potential to be oncogenic, the question as to whether HCMV is association with GBM remains unclear.

The current line of thinking in the association between HCMV and GBM revolves around four potential hypotheses.[30] The first is that HCMV is causal; however, there is no evidence to date to support this concept in humans. The best evidence we have at this time for HCMV causing GBMs is in a mouse model.[31] Researchers at Brigham and Women's Hospital developed a mouse CMV-infected GBM mouse model using mut3 mice, which spontaneously develop grade III and IV astrocytomas. They demonstrated that MCMV-infected mut3 mice had decreased overall survival compared to naive mut3 mice.[31] The second hypothesis is that HCMV may be oncomodulatory, thereby enhancing tumor progression by a specific mechanism or a combination of mechanisms, which were detailed in the previous section.[17,18,20,22-24,26-29,32,33] The third hypothesis is that HCMV may be a bystander with little effect on tumor growth, and the HCMV antigens are expressed because of the highly immunosuppressive tumor microenvironment observed in GBM. There is no direct evidence to date to support this concept. The last hypothesis is that these observations are merely an experimental artifact. Several recent publications have outlined possible scenarios where detection of HCMV may be due to experimental artifact including cross-reactivity of antibodies,[34] the concentration of antibodies,[35] or presence of expression vector genetic material in sequencing datasets.[36]

HCMV DETECTION IN GBM

Several studies have investigated the association between HCMV and GBM in an attempt to resolve this controversial issue. Various detection approaches have been utilized by several investigators including traditional techniques (e.g. PCR, ISH, and IHC) and next generation sequencing (NGS) technology in an attempt to detect the presence of HCMV in GBM cells [Table 1].

The most recent studies that have reported a positive association utilized traditional detection methods. One study looked for the presence of HCMV antigens pp65 or IE1-72 in 25 pediatric GBM patients. The study showed a 66.7% detection rate in the samples for either pp65 or IE1-72.[58] The authors of this study also

Table 1: Studies evaluating presence of HCMV in gliomas

Authors	Number of samples analyzed	Detection method	Analyte
Cobbs *et al.*,[3] 2002	27/27 gliomas FFPE	IHC for IE1-72, pp65, p52	Protein
	10/10 gliomas	ISH biotinylated oligonucleotide probe specific for HCMV early gene mRNA ISH digoxigenin-labeled HCMV total genome DNA probe	RNA, DNA
	7/9 gliomas	Nested PCR for gB (UL55)	DNA
	2/2 GBM	EM-IHC anti-pp65 mAb and gold particles	Viral particles
Lau *et al.*,[37] 2005	0/17 gliomas FFPE 0/2 ODG FFPE 0/3 ependymomas FFPE	IHC for HCMV cocktail, pp65	Protein
	0/17 gliomas FFPE 0/2 ODG FFPE 0/3 ependymomas FFPE	ISH digoxigenin-labeled probe specific for HCMV early gene mRNA ISH digoxigenin-labeled probe specific for HCMV DNA (pp65 and pp150)	RNA, DNA
	0/17 gliomas FFPE 0/2 ODG FFPE 0/3 ependymomas FFPE	PCR for gB (UL55)	DNA
Sabatier *et al.*,[38] 2005	9/116 CNS tumors (15 ependymomas, 81 GBM, 20 ODG) TMA 1/25 gliomas fresh frozen	IHC for IE1, p52 ISH biotinylated HCMV DNA probe	DNA, Protein
Poltermann *et al.*,[39] 2006	0/73 CNS tumors (38 gliomas, 29 meningiomas, 6 ACNs) FFPE	IHC for IE, EA, pp65	Protein
	0/77 (40 gliomas, 31 meningiomas, 6 ACNs)	Nested PCR for IE1 and gB (UL55)	DNA
Mitchell *et al.*,[40] 2008	42/45 GBMs FFPE	IHC for IE1-72	Protein
	30/33 GBMs FFPE	IHC for pp65	
	16/16 GBMs FFPE	ISH FITC-conjugated 40-mer probes for HCVM IE1 ISH biotinylated HCMV total genome DNA probe	DNA
	16/17 GBM primary cultures	IHC for IE1, pp65 gB, and pp28	
	21/34 GBMs fresh frozen 13/17 GBM primary cultures	PCR for gB (UL55)	DNA
Scheurer *et al.*,[41] 2008	21/21 GBM FFPE 9/12 AA FFPE 14/17 LGG FFPE	IHC for IE1-72 and ISH fluorescein-labeled oligonucleotide total HCMV genome DNA probe mixture	Protein, DNA
Slinger *et al.*,[29] 2010	20/21 gliomas FFPE	IHC for IEA, US28	Protein
Lucas *et al.*,[42] 2011	25/49 GBMs FFPE	IHC for pp65	Protein
	8/49 GBMs FFPE	IHC for IE1	
Ranganathan *et al.*,[43] 2012	75/75 GBM FFPE	PCR using 12 HCMV primer pairs	DNA
	12/12 GBM fresh frozen	PCR using 19 HCMV primer pairs	
Rahbar *et al.*,[44] 2012	79/80 GBMs FFPE	IHC for IEA	Protein
	76/80 GBMs FFPE	IHC for LA	
	6/6 selected GBM FFPE	ISH HCMV-DNA total genome fluorescein labeled probes	DNA
Bhattacharjee *et al.*,[45] 2012	16/17 gliomas fresh frozen	Nested PCR for IE	DNA
	9/12 gliomas fresh frozen	WB for pp65, IE1-72, gB	Protein
Fonseca *et al.*,[46] 2012	27/75 gliomas fresh frozen	PCR for pp65	DNA
Khoury *et al.*,[47] 2013	0/168 GBMs 0/47 LGGs	RNA-seq	RNA
Tang *et al.*,[48] 2013	0/167 GBMs	RNA-seq	RNA
Rahbar *et al.*,[49] 2013	74/75 GBMs FFPE	IHC for IEA	Protein
	70/75 GBMs FFPE	IHC for LA	
	19/19 selected GBM FFPE	ISH CMV DNA probe	DNA
	5/5 GBM primary cultures	PCR for IE	DNA
Ding *et al.*,[50] 2014	51/67 gliomas FFPE	IHC for IE1-72	Protein
	44/67 gliomas FFPE	IHC for pp65	
	35/67 gliomas	Nested PCR for gB (UL55)	DNA
Dos Santos *et al.*,[51] 2014	21/22 GBMs fresh frozen	PCR for pp65	DNA
	20/22 GBMs fresh frozen	hemi-nested PCR for gB (UL55)	
Cimino *et al.*,[52] 2014	0/21 gliomas	Unmapped reads from targeted cancer gene panel	DNA

Continued...

Authors	Number of samples analyzed	Detection method	Analyte
Cosset *et al.*,[53] 2014	0/20 GBMs	Semi-qPCR for CMV Nested PCR for gB	RNA, DNA
	0/5 GBMs	RNA-seq	RNA
Yamashita *et al.*,[34] 2014	0/59 GBMs (40 fresh-frozen and 19 FFPE)	PCR for gB and IE1	DNA
	0 (confirmed)/5 GBMs (false-positive staining on WB confirmed by LC/MS/MS analysis)	WB for IE1/2 and pp28	Protein
	10/10 GBMs	IHC for pp28	Protein
	7/10 GBMs	IHC for IE1/2	
	5/10 GBMs	IHC for pp65	
	0/10 GBMs	IHC for UL44	
	0/10 GBMs	FISH HCMV BAC DNA	DNA
Solomon *et al.*,[54] 2014	0/68 GBM TMA	IHC for HCMV cocktail	Protein
Baumgarten *et al.*,[10] 2014	0/91 GBMs FFPE	IHC for p52, pp65, IEA	Protein
	0/10 GBMs	PCR for HCMV loci	DNA
Libard *et al.*,[55] 2014	363/417 extra- and intra-axial brain tumors (61/68 GBMs) TMA	IHC for pp65	Protein
Ahani *et al.*,[56] 2014	0/8 non-glioma tumor tissue 0/2 PA 1/3 AA 4/7 OA 12/16 GBMs	PCR (HCMV detection kit)	DNA
Tang *et al.*,[57] 2015	0/34 GBM	WGS	DNA
Wakefiled *et al.*,[58] 2015	14/24 peds GBMs	IHC for IE1-72	DNA, Protein
	12/24 peds GBMs	IHC for pp65	
	13/16 peds GBMs	ISH for HCMV DNA probe cocktail	
Bianchi *et al.*,[59] 2015	30/43 GBMs fresh frozen 8/14 ODG 17/20 meningiomas	IF for IE1 and LA	Protein
	2/6 IE1 IF-positive GBMs	IHC for IE1	Protein
	17/34 GBMs 5/14 ODG 6/13 meningiomas	Nested PCR for gB	DNA
Shamran *et al.*,[60] 2015	33/36 GBM FFPE	IHC for IE1-72	DNA, Protein
	28/36 GBM FFPE	IHC for pp65	
	26/36 GBM FFPE	IHC for LA	
	10/10 selected GBM samples	Nested PCR for IE1	
	0/30 meningioma FFPE	IHC for IE1-72, pp65, and late antigen	
Holdhoff *et al.*,[35] 2016	0/25 GBMs fresh-frozen	qPCR for US17	DNA, RNA, Protein
	0/70 HGG TMA	IHC for pp65	
	0/20 GBMs FFPE	IHC and CISH for IE1/2 and pp65	
	3/18 HCMV DNA plasma samples 8/15 serum HCMV IgG		
	0/18 GBMs FFPE	IHC, CISH, PCR	
Strong *et al.*,[36] 2016	0/157 GBM 0/13 recurrent GBM 0/514 LGGs 0/17 recurrent LGGs 0/92 MRI-guided GBM biopsies 0/9 glioma stem-like cell cultures	RNA-seq datasets	RNA
	0/51 GBM 0/10 recurrent GBM 0/64 meningioma	WGS datasets	DNA
Lin *et al.*,[61] 2016	0/19 GBM FFPE, 0/20 GBM OCT, 0/6 GBM fresh frozen	ddPCR for HCMV UL55	DNA
	4/19 GBM FFPE, 0/20 GBM OCT, 0/6 GBM fresh frozen	ddPCR for EBV LMP1	
	3/19 GBM FFPE, 3/20 GBM OCT, 0/6 GBM fresh frozen	ddPCR for HHV-6 U57	
Taha *et al.*,[62] 2016	0/32 GBMs	IHC for HCMV and PCR for UL34, UL80.5	DNA, Protein
Stangherlin *et al.*,[63] 2016	38/52 GBMs	PCR for UL83	DNA, Protein
	30/52 GBMs	IHC for HCMV nuclear protein	
	19/52 GBMs	ISH for early 2.7 RNA	
Xing *et al.*,[25] 2016	52/79 glioma	IHC for pp65	DNA, Protein
	43/79 glioma	ISH for pp65 DNA	

FFPE: formalin-fixed paraffin-embedded; IHC: immunohistochemistry; ISH: *in situ* hybridization; HCMV: human cytomegalovirus; PCR: polymerase chain reaction; GBM: glioblastoma multiforme; EM: electron microscopy; ODG: oligodendroglioma; CNS: central nervous system; TMA: tissue microarray; ACN: acoustic neuromas; AA: anaplastic astrocytoma; LGG: low-grade glioma; WB: western blot; LA: HCMV late antigen; FISH: fluorescence *in situ* hybridization; IF: immunofluorescence; PA: pilocystic astrocytoma; OA: oligoastrocytoma; WGS: whole genome sequencing; CISH: chromogenic *in situ* hybridization; ddPCR: digital droplet PCR; OCT: optimal cutting temperature; HGG: high-grade glioma; IEA: immediate-early antigen

performed ISH using a HCMV DNA probe cocktail and found that 81% of samples analyzed demonstrated HCMV specific staining.[58] Another study utilized multiple detection assays to test for the presence of HCMV. The targets for these assays were IE1-72, pp65, and late antigen. A total of 36 formalin-fixed paraffin-embedded (FFPE) GBM samples were tested across each assay with varying rates of detection. A total of 33 out of the 36 samples (91.6%) stained positive for IE1-72. The other two HCMV antigens, pp65 and late antigen, stained positive in 28/36 (77.7%) and 26/36 (72.2%), respectively.[60]

On the other hand, several recent studies have reported no association between HCMV and GBM. One study utilized several approaches including a prospective and retrospective analysis to detect the presence of HCMV in tissue, plasma, and serum of high-grade glioma (HGG) patients.[35] The authors of this study retrospectively analyzed 25 fresh frozen tissues from GBM patients using PCR, tissue microarrays from 70 HGG patients using IHC, and 20 FFPE GBM tissues using IHC and CISH targeted at IE1/2 and pp65. All tissue analyzed for the presence of HCMV were found to be negative irrespective of method used.[35] The prospective arm of the study contained 18 patients with newly diagnosed HGG. From these patients, a total of 11 FFPE whole sections, 38 plasma samples, and 15 serum samples were analyzed. Tissue samples were analyzed for HCMV using real-time PCR, CISH, and IHC under the same protocols as the retrospective arm. Utilizing these different detection methods there was no evidence of HCMV in the 11 FFPE samples. Eight of 15 patients were seropositive for HCMV. Of the 38 plasma samples that were collected HCMV was detected in low levels in 3 samples at baseline and only one in follow up.[35]

Another study took a comprehensive approach to analyze a wide variety of samples using NGS to detect the presence of viral genomes in the samples.[36] This analysis was performed through the publically available sequencing datasets from the Cancer Genome Atlas (TCGA). The samples tested included 157 RNA-seq datasets from primary GBM, 13 recurrent GBM, 514 low-grade gliomas, 17 recurrent low-grade gliomas, and 5 normal brain, and whole genome sequencing (WGS) datasets from 51 primary GBM, 10 recurrent GBM, and 20 normal matched blood samples. In addition, 92 RNA-seq datasets from MRI-guided biopsies and 9 glioma stem-like cell cultures were analyzed. Finally, 64 DNA-seq datasets from 11 meningiomas and their corresponding blood control samples were also analyzed. The authors of this study reported no strong evidence of HCMV in any of the samples. A few samples were found to contain low levels of viral reads but upon closer inspection the authors report that these reads likely represented artifact or non-pathological infections. Finally, evidence of human papillomavirus (HPV) and hepatitis B were found in a few LGG samples, however, the authors of this study state that these findings need further evidence to substantiate this association.[36]

Lastly, another study used droplet digital PCR (ddPCR) to detect the presence of HCMV, human herpes virus 6 (HHV-6), and Epstein-Barr virus (EBV) in brain tissue samples.[61] A total of 112 brain tissue samples comprising 45 glioblastoma, 12 astrocytoma grade III, 2 astrocytoma grade II, 4 astrocytoma grade I, and 49 controls were included in this study. The study reported that neither HCMV nor HHV-6A was detected in any of the astrocytoma samples. A higher frequency of positivity was observed for EBV and HHV-6B compared to controls.[61]

A few recent studies may shed light onto why there is such discrepancy observed in the literature. A study by Yamashita *et al*.[34] attempted to detect HCMV in GBM samples through a wide range of detection methods. They were unable to detect the presence of HCMV in many of the samples. Interestingly, the authors noted that the HCMV positivity demonstrated in a few samples were actually the HCMV antibodies binding to non-viral human proteins such as human albumin and myelin basic protein.[34] This finding suggests a previously unknown cross-reactivity among HCMV antibodies. Another study by Holdhoff *et al*.[35] assessed whether altering experimental conditions could generate false positive results. The authors of this study demonstrated positive staining in previously negative control fibroblast cells by using higher concentrations of the primary HCMV monoclonal antibody. Similarly, varying antibody concentrations in brain tumor FFPE samples demonstrated false positive staining. Taken together, the authors of this study suggest that false positive staining can be readily achieved simply by using high antibody concentrations even with antibodies that are designed to be specific.[35]

THERAPEUTIC TARGETS FOR HCMV

The potential for novel breakthroughs in treating patients with GBM has led to a search for HCMV specific targets. Currently, there are two main approaches that are being pursued; one focuses on anti-viral therapy and the other focuses on HCMV directed immunotherapy.

The main approach to anti-viral therapy revolves around the use of valganciclovir[64-68] and GTPases.[69] The rationale for using valganciclovir stems from the hypothesis that it will suppress HCMV replication in HCMV-positive GBM cells leading to the suppression of virus-mediated tumor promoting mechanisms. Recently, researchers at Vanderbilt University investigated the use of combination therapy using bevacizumab and valganciclovir in treating recurrent GBM, which demonstrated a trend toward improved survival in those patients.[67] Finally, valganciclovir may target other viruses besides HCMV, which have unclear roles in tumorigenesis. Despite these advantages, valganciclovir suppresses viral replication, but does not eradicate the virus. Therefore, short-term treatment of valganciclovir would not be ideal in treating glioma patients as the benefits of tumor suppression only last during treatment, necessitating long-term treatment to maintain the suppressive effects. Further, this therapy would not be suitable for GBMs where there is no HCMV present as the tumor cells would not be targeted.

The investigation of Rho GTPases and their contribution to tumor progression is another area that is under investigation as a potential treatment option.[69] The rho GTPase family is known to play a crucial role in cytoskeleton organization, cell movement, and division. Three proteins within this family that are frequently altered in tumors include RhoA & RhoC, which are typically overexpressed, while RhoB is usually downregulated.[70-72] Using a naive GBM cell line, a GBM cell line that stably expresses HCMV IE1, and shRNA technology to knockdown the Rho GTPases, it was determined that HCMV infection and Rho isoform states affect cell morphology and influence proliferation rate and motility of human GBM cells.[69]

The other approach to treating HCMV associated GBM involves the use of HCMV directed immunotherapy. The idea of HCMV in GBM has led to potential immunotherapy targets for treatment of GBM.[73,74] A study conducted by Nair *et al*.[75] evaluated whether T cells stimulated by HCMV pp65 RNA-transfected dendritic cells target and eliminate GBM tumor cells. The authors of this study concluded that HCMV-specific T cells can effectively target GBM tumor cells and their results support the rationale for the development of HCMV-directed immunotherapy in patients with GBM.[75] As a result of this association and the potential therapeutic options, several groups are exploring novel approaches to developing GBM-directed immunotherapy and vaccines.[74] Potential HCMV proteins that are being investigated for the development of immunotherapy targets include immediate early 1 (IE1), phosphoprotein 65 (pp65), and glycoprotein B (gB).[74]

CLINICAL IMPLICATIONS

HCMV is a ubiquitous virus infecting nearly the entire world population. With all the attention aimed at targeting HCMV in GBM cells, the validity of HCMV as a clinical target is being explored. As of February 2017, there are 13 clinical trials being conducted in the United States evaluating anti-HCMV therapy for GBM patients registered on clinicaltrials.gov [Table 2]. Of these studies, two were terminated because of poor patient accrual. The first was a study sponsored by Penn State University entitled, “A Phase I-II study of allogeneic CMV specific cytotoxic T lymphocytes (CTL) for patients with refractory glioblastoma multiforme (GBM).[76]” The goal of this study was to evaluate the safety and efficacy of the administration of partially matched, allogenic HCMV specific cytotoxic T cells for patients with GBM who failed primary therapy. The other study entitled, “Phase I/II administration of CMV (cytomegalovirus)-specific cytotoxic T cells in patients with glioblastoma multiforme (COGLI)” was sponsored by Baylor College of Medicine.[77] The goal of this study was to determine the maximum tolerated dose of HCMV-specific T cells administered in combination with temozolomide (TMZ) in patients with newly diagnosed GBM. Additional goals of this study included identifying potential side effects and assessing the efficacy of this therapy for the treatment of GBM. Additionally, one study sponsored by the University of Florida entitled, “Peptide targets for glioblastoma against novel cytomegalovirus antigens,” was withdrawn prior to enrollment of participants by the principal investigator.[78] The goal of this study was to identify the optimal and safe HCMV peptide specific vaccine regimen in combination with TMZ for patients with newly diagnosed GBM.

There are 8 studies currently active and/or recruiting patients. A phase I clinical trial sponsored by Baylor College of Medicine entitled “Administration of HER2 chimeric antigen receptor expressing CMV-specific cytotoxic T cells in patients with glioblastoma multiforme (HERT-GBM)” is being conducted to determine the largest safe dose of HER2-CD28 CMV-T cells, to identify the potential side effects of this therapy, and to evaluate its efficacy.[84] As of September 2016, this study is listed as ongoing but not recruiting participants. Another phase I/II clinical trial entitled “A Phase I/II clinical trial of autologous cytomegalovirus (CMV)-specific cytotoxic T cells for glioblastoma (GBM) patients” is being sponsored by

Table 2: Clinical trials evaluating anti-human cytomegalovirus therapy

Studies [ClinicalTrials.gov Identifier]	Sponsor	Status	Completion date
Autologous CMV-specific cytotoxic T cells for GBM patients [NCT02661282][79]	M.D. Anderson Cancer Center	Currently recruiting participants	2020**
Vaccine therapy for the treatment of newly diagnosed GBM (ATTAC-II) [NCT02465268][80]	University of Florida	Currently recruiting participants	2024**
Peptide targets for glioblastoma against novel cytomegalovirus antigens (PERFORMANCE) [NCT02864368][81]	Duke University Medical Center	Currently recruiting participants	2018**
DC migration study for newly-diagnosed GBM (ELEVATE) [NCT02366728][82]	Duke University Medical Center	Currently recruiting participants	2020**
Nivolumab with DC vaccines for recurrent brain tumors (AVERT) [NCT02529072][83]	Duke University Medical Center	Currently recruiting participants	2019**
CMV-specific cytotoxic T lymphocytes expressing CAR targeting HER2 in patients with GBM (HERT-GBM) [NCT01109095][84]	Baylor College of Medicine	Ongoing, but not recruiting participants	2028**
Basiliximab in treating patients with newly diagnosed GBM undergoing targeted immunotherapy and temozolomide-caused lymphopenia (REGULATe) [NCT00626483][85]	Duke University Medical Center	Ongoing, but not recruiting participants	2018**
Vaccine therapy in treating patients with newly diagnosed GBM (ATTAC) [NCT00639639][86]	Duke University Medical Center	Ongoing, but not recruiting participants	2018**
Evaluation of recovery from drug-induced lymphopenia using cytomegalovirus-specific T-cell adoptive transfer (ERaDICATe) [NCT00693095][87]	Duke University Medical Center	Completed	2015
Peptide vaccine for glioblastoma against cytomegalovirus antigens (PERFORMANCE) [NCT01854099][78]	University of Florida	Withdrawn	2014
Efficacy and safety of Valcyte® as an add-on therapy in patients with malignant glioblastoma and CMV infection [NCT00400322][88]	Karolinska University Hospital	Unknown*	
A study using allogenic-CMV specific cells for GBM [NCT00990496][76]	Penn State University	Terminated	2011
Administration of CMV-specific cytotoxic T cells in patients with GBM (COGLI) [NCT01205334][77]	Baylor College of Medicine	Terminated	2012

*Results of this study have been published in the *International Journal of Cancer*;[65] **estimated completion date; CMV: cytomegalovirus; GBM: glioblastoma multiforme; DC: dendritic cell

M.D. Anderson Cancer Center and as of January 2017 is recruiting participants.[79] The goals of this combined Phase I/II study is to determine the highest tolerable dose of HCMV cytotoxic T lymphocytes (CTLs) that can be administered in combination with TMZ to patients with GBM. The goal of the phase II component of this study is to identify if HCMV CTLs, when combined with TMZ, is effective in controlling GBM and whether this combination is safe for patients. There is a phase II clinical trial being sponsored by the University of Florida entitled "A Phase II randomized, blinded, and placebo-controlled trial of CMV RNA-pulsed dendritic cells with tetanus-diphtheria toxoid vaccine in patients with newly-diagnosed glioblastoma" that is currently recruiting participants as of January 2017.[80] The goal of this study is to evaluate whether administration of HCMV pp65-dendritic cells is effective, as defined by a change in mean overall survival of GBM patients, if given in conjunction with stronger routine chemotherapy.

Several Phase I and II clinical trials are being sponsored by Duke University Medical Center. One study, entitled "Peptide targets for glioblastoma against novel cytomegalovirus antigens", is currently recruiting participants as of December 2016.[81] The purpose of this phase I clinical trial is to determine side effects related to the peptide-CMV vaccine and assess the safety of a combination approach using the peptide-CMV vaccine and TMZ in patients with newly diagnosed GBM who underwent a complete or partial surgical resection. In addition, another goal of this study is to identify the TMZ regimen schedule that yields the highest number of T cells secreting interferon-gamma in response to the peptide-CMV vaccine. Another study, entitled "Evaluation of overcoming limited migration and enhancing cytomegalovirus-specific dendritic cell vaccines with adjuvant TEtanus pre-conditioning in patients with newly-diagnosed glioblastoma", is also currently recruiting participants as of June 2016.[82] This phase II clinical trial will determine the impact of pre-conditioning with Tetanus-Diphtheria (Td) toxoid on human CMV pp65-lysosomal-associated membrane protein (LAMP) mRNA-pulsed autologous dendritic cells (DCs). This study will also assess the impact of pre-conditioning with Td toxoid and/or basiliximab

on overall survival. A phase I clinical trial entitled "AVeRT: Anti-PD-1 monoclonal antibody (Nivolumab) in combination with DC vaccines for the treatment of recurrent Grade III and Grade IV brain tumors" is being conducted to assess the safety of giving DC vaccines with nivolumab, an anti-PD-1 monoclonal antibody, to patients with high grade gliomas.[83] Overall survival and progression-free survival will also be evaluated. This study is currently recruiting participants as of December 2016. Two additional studies sponsored by Duke University Medical Center are both ongoing but are not currently recruiting participants as of July 2016. One of these studies, entitled "Regulatory T-cell inhibition with Basiliximab (Simulect®) during recovery from therapeutic temozolomide-induced lymphopenia during antitumor immunotherapy targeted against cytomegalovirus in patients with newly-diagnosed glioblastoma multiforme", is a phase I clinical trial and will evaluate whether basiliximab, a monoclonal antibody to the IL-2 receptor of T cells. In addition, the study in determine whether basiliximab inhibits the functionality and numeric recovery of T-regulatory cells in GBM patients with TMZ-induced lymphopenia who are undergoing targeted immunotherapy using CMV pp65-LAMP mRNA-loaded dendritic cells and GM-CSF.[85] The other study from Duke is also a phase I clinical trial. The study entitled "Anti-tumor immunotherapy targeted against cytomegalovirus in patients with newly-diagnosed glioblastoma multiforme during recovery from therapeutic temozolomide-induced lymphopenia" is to determine the feasibility and safety of vaccinating with HCMV pp65-LAMP mRNA-loaded dendritic cells, with or without autologous lymphocyte transfer, in patients with newly diagnosed GBM who previously had TMZ-induced lymphopenia.[86]

A few additional studies investigating the use of HCMV as a novel target for GBM therapy have recently been completed. The phase I clinical trial entitled "Evaluation of recovery from drug-induced lymphopenia using cytomegalovirus-specific T-cell adoptive transfer" sponsored by Duke University Medical Center is listed as completed on clinicaltrials.gov.[87] In this study, the investigators assessed if administering HCMV-specific DCs to adult patients with newly diagnosed GBM with TMZ-induced lymphopenia enhanced the T-cell response and whether this therapy was safe for patients. Another study entitled "A randomized double blind controlled proof of concept study of the efficacy and safety of Valcyte® as an add-on therapy in patients with malignant glioblastoma with successful surgical resection of at least 90% of the initial tumor and CMV infection demonstrated histologically and immunohistochemically," was sponsored by Karolinska University Hospital in Sweden and has a current status that is listed as "unknown".[88] Despite the status on clinicaltrials.gov, results from this study have been published in the *International Journal of Cancer* and will be presented in more detail below.

While we await the results of these studies, there have been two published clinical trials reporting differences in clinical efficacy. The first study was conducted by Stragliotto *et al*.[65] in Sweden and consisted of 42 patients randomized 1:1 to valganciclovir or placebo in addition to standard therapy. The primary endpoint of the study was tumor volume at 3 and 6 months assessed by neuroimaging. Secondary endpoints included progression free survival and overall survival at 6, 12, 18, and 24 months. Authors of this study concluded that valganciclovir is safe and well tolerated in patients receiving both temozolomide and radiation therapy. However, primary and secondary endpoints were similar between the two groups, with trends but no significant differences observed. Despite demonstrating no significant differences, the authors, in a retrospective analysis of the same cohort with inclusion of additional patients on valganciclovir for compassionate reasons, found that the rate of survival of valganciclovir treated patients at 2 years was 62% compared to 18% in historical controls with similar demographics.[64] The interpretation of this data has called into question whether the quoted survival rate is misleading.[89]

Another study conducted by Mitchell *et al*.[90] at Duke University consisted of 12 patients randomized to pre-treatment with mature dendritic cells (DC) or tetanus toxoid (Td), to help stimulate the immune system, with HCMV-specific dendritic cell vaccine in addition to standard therapy. The main objective of this study was not to test the validity of HCMV as a tumor specific target since the authors have previous shown the presence of HCMV antigens in GBM. As such, the authors surmise that HCMV is a viable target for immunotherapy. Therefore, the authors of this study wanted to evaluate methods for enhancing the efficacy of HCMV-specific dendritic cell vaccines. In so doing, six patients were pretreated with mature dendritic cells, while the other 6 patients were pretreated with tetanus toxoid. Primary endpoint was DC accumulation and migration. Secondary endpoints included progression free and overall survival. The authors demonstrated that there was greater DC migration in Td-pretreated patients than in those treated with mature DCs. Also, Td-pretreated patients showed a significant increase in both progression-free survival and overall survival compared to DC-pretreated patients. The authors concluded that pre-conditioning with Td may

represent a viable strategy to improve anti-tumor immunotherapy.[90]

CONCLUSION

The notion as to how HCMV is associated with GBM is still unclear as there are discrepancies in the detection of HCMV. Theories as to why there are differences observed have been postulated and include the potential cross reactivity of HCMV antibodies used in traditional detection methods in addition to the variety of experimental conditions used. In light of the continued inconsistencies and ongoing debate, a standard detection protocol likely implementing multiple detection methods needs to be developed and interrogated in multiple institutions in order to remedy this controversial issue. Until this major endeavor is undertaken, the medical and scientific communities should be cognizant of this controversy. Although there is relative low risk in some of the experimental treatments discussed (e.g. valganciclovir), physicians and scientists should exercise caution when using anti-HCMV therapy until we have an updated consensus as to whether HCMV is associated with GBM.

Authors' contributions

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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patients were involved.

Ethics approval

Not applicable.

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Minireview **Open Access**

How are necroptosis, immune dysfunction, and motoneuron death connected in amyotrophic lateral sclerosis?

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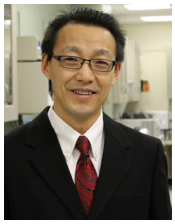

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amyotrophic lateral sclerosis

ABSTRACT

Abnormal immune response/inflammation is present in patients of amyotrophic lateral sclerosis (ALS). Autoimmune-related inflammation has been thought to be involved in the pathogenesis of ALS. However, how the abnormal immune responses are initiated, what specific immune cells and how these immune cells are involved in this disease have not been well understood. This is partly owing to two facts of ALS: late diagnosis and chronic nature. The late diagnosis makes it difficult to conclude whether the abnormal immune responses/inflammation is the cause or result of the disease. The chronic nature makes it difficult to determine the best timing for the detection of such autoimmune responses. To resolve these two challenges for research, the authors introduced motor nerve injury (facial nerve axotomy, FNA) into a pre-symptomatic mouse ALS model (8-week-old *SOD1*^{G93A} mice), which induces a readily detectable immune response in a predictable time period (3-14 days). The authors found that pre-symptomatic *SOD1*^{G93A} mice showed a higher basal level of T cell activation and Th17 cells than WT mice, which can be further increased by FNA. However, why these pro-inflammatory Th lymphocyte

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subsets are preferentially elicited in ALS has been elusive. Recently, several studies support that the programmed necrosis (necroptosis), a new type of cell death, is present in ALS. Because necroptosis results in the release of pro-inflammatory stimuli, we speculate that initial motoneuron (MN) necroptosis may be the cause of abnormal immune responses in the development of ALS, and subsequently, inflammation/immune response serve as an amplifier to cause more MN death. Here, the authors reviewed recent studies concerning the type of MN death, the inflammation/immune responses and the research strategies for ALS. With the available evidences from the literature, the authors present a hypothesized working model to indicate the possible connections among necroptosis, immune responses and MN death in the development of ALS and suggest the future studies for searching the potential therapy for ALS.

AMYOTROPHIC LATERAL SCLEROSIS AND RESEARCH MODEL

Amyotrophic lateral sclerosis (ALS) is a fatal motor neuron (motoneuron, MN) disease affecting approximately 30,000 people in the United States and 70,000 people worldwide.[1] Clinically, ALS attacks MNs in the spinal cord, brain stem, and motor cortex,[2] resulting in a loss of muscle tone, generalized weakness, spasticity, paralysis, and consequent fatality. The etiology of ALS is not fully understood, but genetic analysis has led to the identification of approximately 30 gene mutations.[3] The common gene mutations include *Cu/Zn superoxide dismutase 1* (*mSOD1*),[4] *TDP-43*,[5,6] repeat expansion of *C9orf72* gene,[7,8] and many other mutations.[3] The proposed pathogenic mechanisms include protein aggregation and toxicity, abnormal RNA processing, glutamate-mediated excitotoxicity, and neuroinflammation.[1,3] *mSOD1* is the first identified gene mutation in familial ALS (present in 20% of fALS); animal models have been established for this specific variant.[9] *SOD1^{G93A}* mice carry the human mutant *SOD1^{G93A}* gene and demonstrate an ALS-like disease course. These mice are currently the most widely used animal model to examine the behavioral, cellular, and molecular aspects of ALS.

MN DEATH TYPES IN ALS

The pathological hallmark of ALS is progressive loss of MN and motor function. The study for MN death and underlying mechanisms has been the major focus of ALS research in the past decades. Due to the limited availability for human specimen from living ALS patients, most of our knowledge regarding MN death in ALS has been derived from ALS animal model. The previously defined cell death types in ALS include apoptosis, autophagy, and necrosis. Apoptosis has been considered the main type of cell death for a long time.[10-13] In the past decade, several new types of cell death have been defined and characterized: such as parthanatos, inflammasome (NLRP3)-mediated pyroptosis, ferroptosis, pyronecrosis, mitochondrial permeability transition-dependent necrosis. As recently reviewed by Morrice *et al.*,[14] accumulating evidence indicates that multiple types of cell death may be involved in MN death in ALS.[15,16]

NECROPTOSIS AND ITS IMPLICATIONS IN ALS

Apoptosis is a well defined type of programmed cell death with the involvement of caspase activation. MN apoptosis has been considered as the primary cell death in ALS. Necroptosis is one novel type of cell death and it is also a type of programmed cell death but independent of caspase activation. It is stimulated by TNF and related inflammatory factors such as TRAIL, FasL and ligands for TLRs.[17-20] Activated Caspase-8 induces apoptosis and simultaneously represses necroptosis by cleaving RIP1 and RIP3.[21,22] Inhibition of Caspase-8 can convert apoptosis to RIP1/RIP3-mediated necroptosis.[23,24] Necroptosis is considered a pathologic form of cell death which is involved in viral infection, ischemia and reperfusion injury, liver fibrosis and neurodegenerative diseases.[25] Necroptosis can be inhibited by the blocking RIP1/RIP3 interaction using a small molecule inhibitor of RIP1 kinase, Necrostatin-1 (Nec1) or genetic deletion of *RIP3*. *Rip3$^{-/-}$* mice develop and grow normally but are resistant to virus-mediated cellular necrosis,[26] ethanol-induced liver injury,[27] and ischemia-reperfusion injury.[28] Recent studies have demonstrated that necroptosis in the nervous system is involved in a variety of neurological disorders, such as multiple sclerosis (MS),[29] traumatic brain injury,[30] and cerebrovascular disease.[31] Re *et al.*[32] first revealed that MN necroptosis also occurs in ALS. MN could be induced to undergo necroptosis by astrocytes with mutant *SOD1^{G93A}* or astrocytes from ALS patients. Because intracellular components released during necroptosis stimulate inflammation and immune responses, it is thought that necroptosis contributes to the development/progression of neurodegenerative diseases. Recently, Cirulli *et al.*[33] compared the whole exome sequences between ALS and control, and found that the non-canonical IκB kinase family TANK-binding kinase 1 (*TBK1*) was a gene associated with ALS by interacting with ALS-related proteins, optineurin (OPTN) and p62. Furthermore, Ito *et al.*[34] demonstrated that OPTN inhibited dysmyelination and axonal degeneration through suppressing receptor-interacting kinase 1 (RIPK1) and the downstream

necroptotic pathway with the involvement of RIPK3, and mixed lineage kinase domain-like protein (MLKL). Taken together, it appears that necroptosis plays a critical role in connecting certain gene mutations with the development of ALS.

While necroptosis itself results in MN loss directly, it also activates the immune system and results in a secondary inflammatory response. We think that inflammation/immune responses subsequently amplify MN degeneration. During this process, the innate immune responses may act as the first line of reactions to clean up the debris of dead cells with the involvement of vascular reaction, infiltration of neutrophil and macrophage, as well as the pro-inflammatory cytokine production. Later, the adaptive immune components (T and B cells) get involved in after certain MN-derived antigens (not clearly defined thus far) are processed and presented to T cells. This notion is supported by our recent finding of the roles of necroptosis in autoimmune bone marrow failure. We found that necroptosis of a small portion of stem cell/progenitors with gene mutation resulted in autoimmune responses, which caused a rapid depletion of bone marrow cells, which could be prevented by blocking necroptotic pathway via deletion of *rip3*.[35] We speculate that similar roles of necroptosis may also exist in ALS: a small portion MN that die of necroptosis may cause the autoimmune responses, in which the MN-specific antigens are released and presented to $CD4^+$ T cells. These T cells may preferentially develop into pro-inflammatory subsets and further amplify the inflammation/immune responses, and cause more MN death. Below, we continue discussing the dysfunction of immune system that has been identified in ALS.

IMMUNE DYSFUNCTION IN ALS

In the past few decades, accumulating evidence indicates that immune system abnormalities and inflammation contribute to the development of ALS.[36] Macrophage, T cell, and mast cell infiltration have been observed in ALS CNS tissues.[37] Because of the large amount of inflammatory cells infiltrating the CNS during disease progression, it was postulated that, similar to MS, ALS may also be in part an autoimmune disease.[38,39] Several anti-inflammatory and/or immuno-compromising drugs also support the contribution of inflammatory components in the pathogenesis of ALS. While the gene mutation and the resultant protein in MN may mediate a direct toxic effect for neuron, dysfunctional microglial cells and astrocytes with gene mutations may also contribute to neuronal death.[40-44]

While the glial cell-mediated neurotoxicity is relatively well documented, elucidating how other immune cells are involved in regulation of glial cell function and MN death remains a challenging project. First, the immune regulation in ALS appears to be more complicated than our understanding for it on the basis of the knowledge acquired from other conditions. For examples, it has been well known that NF-κB is a factor that mediated the signals of most inflammatory factors.[45] TNF-α, a classical inflammatory cytokine that activates NF-κB and significantly increased in ALS mice and patients. TNF-α is one of the best known factors mediating necroptosis,[17,18,46] but knockout of TNF-α in $SOD1^{G93A}$ mice did not block ALS disease in $SOD1^{G93A}$ mice.[47] In contrast, inhibition of NF-κB can slow down disease progression,[47] suggesting that other inflammatory cytokines, other than TNF-α, might also activate NF-κB and contribute to MN death. Second, the transgenic animal model of ALS is our current major tool to study this disease. However, findings in animal model may not be applicable for patient. For example, inhibition of microglial activation with *Minocycline* significantly delayed the onset of motor neuron degeneration and slowed down disease progression in ALS mice,[48] its effect in ALS patients remains in question.[49] Furthermore, the mutual inhibitory effect among immune components, such as the counteracting functions between Th17 and Treg cells, as well as between Th1 and Th2 cells (this topic will be further discussed in the below section of ENVIRONMENT-DEPENDENT FUNCTION OF $CD4^+$ T CELLS IN ALS), make it more complicated to use a general immunosuppressant to achieve therapeutic effect. For example, *Sulindac*, a nonsteroidal anti-inflammatory drug (NSAID) and inhibitor of pro-inflammatory COX-1 & COX-2, significantly increases the survival, and preservation of spinal cord motoneurons.[50] However, a large number of immunosuppressive approaches, such as cyclosporine, cyclophosphamide, and total lymphoid removal failed to benefit ALS patients.[51,52] The previously published data for both beneficial and detrimental effects of immune components imply that immune system has dual roles in ALS.

NECROPTOSIS AND IMMUNE DYSFUNCTION

Recently, Re *et al*.[32] and Ito *et al*.[34] found that necroptosis may be involved in the MN death. This finding provides an important connection between neurodegeneration and previously observed abnormal inflammation/immune response in ALS. Unlike apoptosis that does not elicit immune responses, necroptosis results in the releases of MN-specific antigens (MN-Ag) and other pro-inflammatory stimuli, which are also called Damage-Associated

Molecular Patterns (DAMPs), such as mitochondria, ATP and alarmins.[46] In addition, Frakes *et al*.[45] found that NF-κB-activation determines the pro-inflammatory phenotype of microglial cells, which in turn contributes to neuronal death. Two questions concerning how immune cells are involved in the regulation of glial cell function remains to be answered. First, what are the specific autoimmune cells involved in the interaction of glial cell and MN? and second, how should we dissect the dual functions of immune system in ALS? As aforementioned, the general immunosuppressants, which have inhibitory effects on overall immune system, appear to have minimal therapeutic effect, presumably due to the simultaneous inhibition of immune components that have beneficial effects.[51,52] Consistent with this notion, knockout of $CD4^+$ T cells in *SOD1*^{G93A} mice resulted in exacerbation of ALS-like symptoms and a decreased life span.[53,54] Collectively, it appears that $CD4^+$ T cell-mediated regulation determines the direction and nature of immune responses in ALS, and an ideal immune-based therapy for ALS should be able to inhibit the "detrimental" effects of immune cell without simultaneously comprising the "beneficial" functions. Therefore, it is necessary to identify the specific subsets of immune cells and to elucidate how they are involved in the pathogenesis of ALS.

ENVIRONMENT-DEPENDENT FUNCTION OF $CD4^+$ T CELLS IN ALS

In ALS patients, it was found that the total number of lymphocyte and naïve $CD4^+$ T cells ($CD45RA^+$) was decreased in late-stage and there was a significant $CD4^+$ T cell infiltration in the spinal cord and brain. In addition, T cells were present in ALS patient cerebrospinal fluid with a predominant Th1 phenotype. $CD4^+$ T cells have different subsets with distinct functions, which are determined by the cytokines they produce. IFN-γ-producing Th1 and IL-17-producing Th17 (both produce TNF-α) promote inflammation; In contrast, IL-4 producing Th2 and IL-10-producing T regulatory cell (Treg) inhibit inflammation. Other and our groups have showed that $CD4^+$ T cells mediate anti-inflammatory effect after nerve injury as well as in ALS disease; however it is in context- and subset-dependent manner.[53-56] It is generally thought that beneficial effects of $CD4^+$ T cells are mainly mediated by its anti-neuroinflammatory subsets, such as Treg and Th2 cells[56,57] and that detrimental effects are mediated by pro-inflammatory $CD4^+$ T subsets, such asTh1 and Th17 cells. The differential development of $CD4^+$ T subset is determined by both $CD4^+$ T cells and their cytokine environment. To determine whether the "detrimental" Th cells development in ALS was due to the defect of $CD4^+$ T cells with *SOD1*^{G93A} mutation or due to the special cytokine microenvironment in *SOD1*^{G93A} mice, we isolated $CD4^+$ T cells in the spleen from WT and *SOD1*^{G93A} mice and adoptively transferred to immune-deficient mice *Rag2*$^{-/-}$ ("normal" microenvironment) or *SOD1*^{G93A} (inflammatory environment) and studied if they can support motoneuron survival after facial nerve axotomy (FNA). Facial motoneuron (FMN) survival post 4-week operation in each group were counted and calculated as percentage of FMN number on axotomized side compared to uninjured side. *Rag2*$^{-/-}$ mice lacks B and T cells and FNA induced a significant FMN loss, which could be prevented by adoptive transfer of either WT whole splenocytes or purified $CD4^+$ T cell. *SOD1*^{G93A} mice also showed a significant FMN loss than WT mice after FNA. However, such loss could only be rescued by WT whole splenocytes but not purified WT $CD4^+$ T cells, suggesting that microenvironment determines the beneficial function of $CD4^+$ T cells and that microenrivronment in *SOD1*^{G93A} mouse does not support the development of beneficial $CD4^+$ T subsets. Therefore, we compared the FNA-induced FMN loss between *Rag2*$^{-/-}$ mice that received $SOD1^{G93A}$ whole splenocytes and *Rag2*$^{-/-}$ mice that received purified *SOD1*^{G93A} $CD4^+$ T cells. As expected, we found that the purified *SOD1*^{G93A} $CD4^+$ T cells still could support FMN survival but whole splenocytes did not.[58] Collectively, these data suggest that microenvironment in *SOD1*^{G93A} mice might have a condition that primes $CD4^+$ T cell into detrimental subsets.

TH17 CELLS, NERVE INJURY AND ALS

Th17 cells are a subset of $CD4^+$ T cells that play an important role in promoting inflammation, including angiogenesis and the recruitment of multiple types of immune cells to injury sites.[59] Th17 cells are crucial for the development of certain autoimmune diseases, exhibiting neurodestructive effects in neuroinflammatory diseases.[60] In ALS patients, elevated IL-17 and Th17-related cytokines (IL-6, TNF-α, IL-1 and IL-23)[61-63] have been observed. However, the contribution of Th17 cells in promoting ALS development has not been established yet because of two challenges: late diagnosis and chronic disease. Late diagnosis makes it difficult to conclude whether the abnormal immune responses/inflammation is the cause or result of the disease. The chronic nature of ALS makes it difficult to determine the best timing for the detection of such autoimmune responses. To resolve these two challenges, we have applied motor nerve injury (facial nerve axotomy, FNA) into a pre-symptomatic mouse ALS model (8-week old *SOD1*^{G93A}) mice, which introduces nerve injury and

MN death by surgery in an acute manner. Because of the chronic nature of ALS disease, no peak time of immune response has been defined previously. Without a peak time, it is challenging to characterize the subtle change of immune parameters. Our previous studies for immune responses following nerve injury indicated that $CD4^+$ T cell responses can be detected by intracellular staining for the cytokine production from the day 3 to day 14 post-axotomy.[64] Such axotomy model allows us to design the best measuring time point for the research and can be utilized as a model tool for further study of immune response in ALS mouse.

While more and more genetic risks (gene mutation) for ALS are being defined, the environmental risk factors for ALS are relatively poorly defined. One defined environmental risk is related to occupations, such as military veterans, varsity athletes, and professional football players.[65-67] Because these populations share the same risk which distinguished them from other populations, that is, they have increased chance of motor nerve injury, we reasoned that motor nerve injury might trigger the development and/or accelerate the progression of ALS. Therefore, we conducted a study to determine the immune responses after nerve injury in mouse ALS model.[68] Without axotomy, *SOD1*^{G93A} mice showed a higher level of Th17 cells in term of both frequency and absolute number, when compared to WT mice. Th17 cell frequency and total number can be further increased by nerve injury. We speculate that nerve injury might promote autoimmune responses, especially in those individuals with a genetic susceptibility. Collectively, these data suggest that motor neuron death, autoimmune responses, and disease progression may mutually promote each other.

Regarding further study for the roles of Th17 cells in mouse ALS model, the FNA in ALS mice may be a useful tool. First, FNA is a well-established paradigm for the study of MN survival, functional recovery and immune response after nerve injury.[69] After FNA, neuronal death occurs by two potential mechanisms: necrosis directly resulting from the disruption of cell integrity, and apoptosis resulting from the stimulation of excitatory neuronal transmitters and oxidative stress mediators. These death mechanisms have been also proposed for the MN death in ALS.[1] Additionally, ALS MN degeneration exhibits axonal "die-back" pathology, in which degeneration occurs at the neuromuscular junction causing the axons to withdraw from target muscle in a "die-back" manner to the cell bodies in the CNS. This process is very similar to axotomy-induced axonal damage and neuronal degeneration.[70,71] Furthermore, FNA can induce $CD4^+$ T cell responses in both WT and *SOD1*^{G93A} mice. Lastly and importantly, as we mentioned in the earlier section of "TH17 CELLS, NERVE INJURY AND ALS", FNA can "artificially" create an acute "onset" of neurodegeneration and provide a predictable time frame during which to study the acute immune response in mice.[64,72] Using this model, we found that FNA induced greater and prolonged immune responses in draining lymph nodes of *SOD1*^{G93A} mice than WT mice,[68] suggesting Th17 cell could be a potential therapeutic target for ALS treatment.

A HYPOTHESIZED WORKING MODEL OF ALS PATHOGENESIS AND FUTURE DIRECTIONS

Based on the literature and our data, we reason that there is a cascade in the ALS disease development/progression. As illustrated in Figure 1, we hypothesize that motor nerve injury triggers MN necroptosis (❶), which induces Th17 cell responses (❷), and Th17 cell-mediated autoimmune reactions (❸), leading to more MN death and promoting rapid disease progression. Specifically, ❶ environmental insult including physical damage or Wallerian-like degeneration, such as physical injury, toxin, radiation and/or intrinsic genetic defect cause the necroptosis of a small portion of MNs. During necroptosis, dying MNs release MN-specific antigens (MN-Ag) as well as pro-inflammatory stimuli, such as ATP, mitochondria, and alarmins;[18] ❷ MN-Ag is processed and presented to naive T cells in draining lymph node, where naïve $CD4^+$ T cells are primed into a Th17 cell phenotype in the presence of Th17-promoting cytokines [TGF-β, IL-6, IL-21 (murine) and IL-1 (human) and IL-23]; ❸ MN-Ag-specific Th17 cells are potentially recruited into the CNS, where they further promote more MN death through two possible mechanisms: (1) amplifying non-

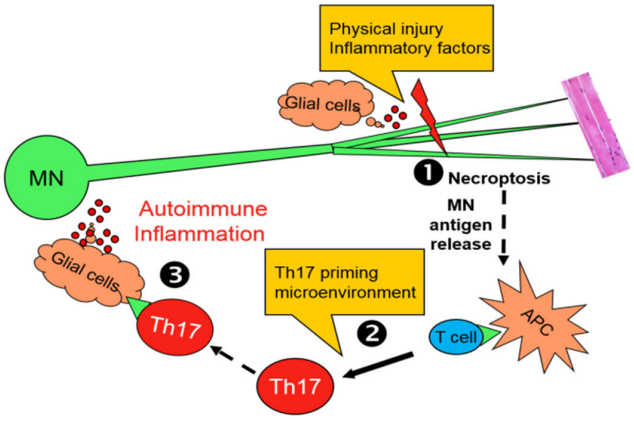

Figure 1: A working model of MN injury, necroptosis, and Th17 cell responses, and their roles in the development and progression of amyotrophic lateral sclerosis. MN: motoneuron; APC: antigen-presenting cell

specific inflammation by enhancing angiogenesis and recruiting neutrophil and other innate immune cells and cause stand-by damage to MNs; (2) interacting with infiltrating macrophage and/or glial cells (microglial cells and astrocytes) and initiating an autoimmune response specifically attacking MN. We think these two mechanisms may be present at the same time and synergically cause extensive MN necroptosis in a short period and lead to rapid disease progression after disease onset.

Although the overall model is supported by the published data, details in each step are still unclear. First, the specific MN antigens involved in ALS have not been clearly defined yet. Second, how the Th17-promoting cytokines are preferentially up-regulated in ALS is not clear. Third, whether pro-inflammatory cells are the cause or the results of MN death remains to be elucidated. Fourth, it is unknown whether the immune cells directly act on MN or indirectly sensitize the MN body to be more vulnerable to insults. Last, regarding whether the necroptotic pathway is a therapeutic target, Ito *et al*.[53] showed that blocking necroptosis through knockout of *Rip3* or using Rip1 inhibitor (Nec-1s) delayed the disease onset for approximately 5 days, but whether blocking necroptosis can slow down the disease progression after its onset remain elusive. It is suggested that necroptosis may play a role only in the initiation stage. After the onset, the disease progression may mainly mediated by the immune dysfunction, and the immune system should be concurrently targeted during ALS treatment.

Authors' contributions

Performed the literature search and drafted the manuscript: J.F. Liu, O.X. Zheng, J.G. Xin
Revised and edited the manuscript: H.H. Chen, J.J. Xin
Designed and supervised this study: J.J. Xin

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Case Report **Open Access**

Thymoma-associated panencephalitis: a newly emerging paraneoplastic neurologic syndrome

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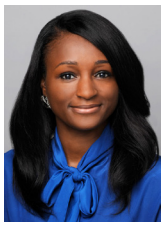

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ABSTRACT

Recently, a few case reports of thymoma-associated panencephalitis (TAPE) have brought to light a disease entity that has not been fully characterized. Literature review of TAPE reveals an array of associated neuronal antibodies, with varied responses to thymomectomy with or without immunotherapy. This report describes a case of TAPE and proposes that the $GABA_A$ receptor antibody is a potential target antigen driving the immune process in this disease entity. Treatment-wise, early thymomectomy consistently improves the overall course of disease. Further study of such cases will be critical in clarifying the mechanisms of disease, improving early diagnosis, and developing targeted approaches to treatment.

INTRODUCTION

Thymomas are often associated with paraneoplastic neurologic syndromes (PNS) as they have the propensity to express different autoantibodies against neuronal antigens, many of which clinical pathogenicity have not been established.[1,2] Quite rarely, thymomas have been associated with limbic encephalitis, a well-characterized neurological syndrome that presents with memory impairment, behavioral changes, and

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seizures.[3] The target antigen when it manifests in thymomas is largely unknown, however, some cases have reported antibodies to α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid receptor, leucine-rich glioma inactivated 1 protein (LGI1), contactin associated protein 2 (Caspr2), and glutamic acid decarboxylase (GAD).[4] A new entity has been described in a few cases of paraneoplastic encephalitis associated with thymomas, with lesions extending beyond the mesial temporal lobe structures.

CASE REPORT

A 35-year-old man presented with a 2-week history of seizures, poor appetite, generalized headaches and nausea. Neurologic assessment revealed poor attention, orientation and memory loss, without clinical seizures. Computed tomography head showed multiple cortical hypodensities, prompting an magnetic resonance imaging (MRI) brain, which revealed multiple foci of cortical hyperintensity involving the medial left frontal cortex, the right dorsal aspect of the insula and adjacent right temporal cortex as well as the posterior left temporal cortical medullary junction and the medial posterior left temporal cortex and lateral cortex [Figure 1]. Electroencephalogram revealed hyperexcitability over the right temporal region and frequent focal seizures and he was empirically treated with IV acyclovir, lacosamide and levetiracetam. Cerebral spinal fluid (CSF) analysis revealed protein of 0.51 g/L, glucose of 3.5 mmol/L, 3 × 10^{-6}/L red blood cells and 9 × 10^{-6}/L white blood cells (76% lymphocytes, 18% monocytes, 6% neutrophils). CSF and serum studies were also positive for neuronal antibodies to GAD [3.93 nmol/L in the CSF (normal < 0.02 nmol/L), > 250.0 kIU/L in the serum (reference range 0-5 kIU/L)], VGKC-complex antibodies [159 pmol/L in the CSF (reference range 0-31 pmol/L), 98 pmol/L in the serum (reference range 0-31 pmol/L)], CRMP5 (reflex titer < 1:240; positive western blot), and AchR [binding antibody 17.5 nmol/L in the CSF (normal < 0.02 nmol/L), and 142.8 nmol/L in the serum (reference range 0.0-0.4 nmol/L)]. Additionally, serum studies revealed presence of systemic antibodies to thyroperoxidase (37.8 kIU/L, reference range 0-5.5 kIU/L), thyroglobulin (372.2 kIU/L, reference range 0-5 kIU/L), Ro/SSA (6.8 AI, reference range < 1 AI), and an ANA titer of 80 (reference range 0-40) with a nucleolar pattern. A paraneoplastic syndrome was suspected, and full body imaging revealed a large multilobulated right anterior mediastinal mass, interfacing multiple right-sided mediastinal structures and invading the

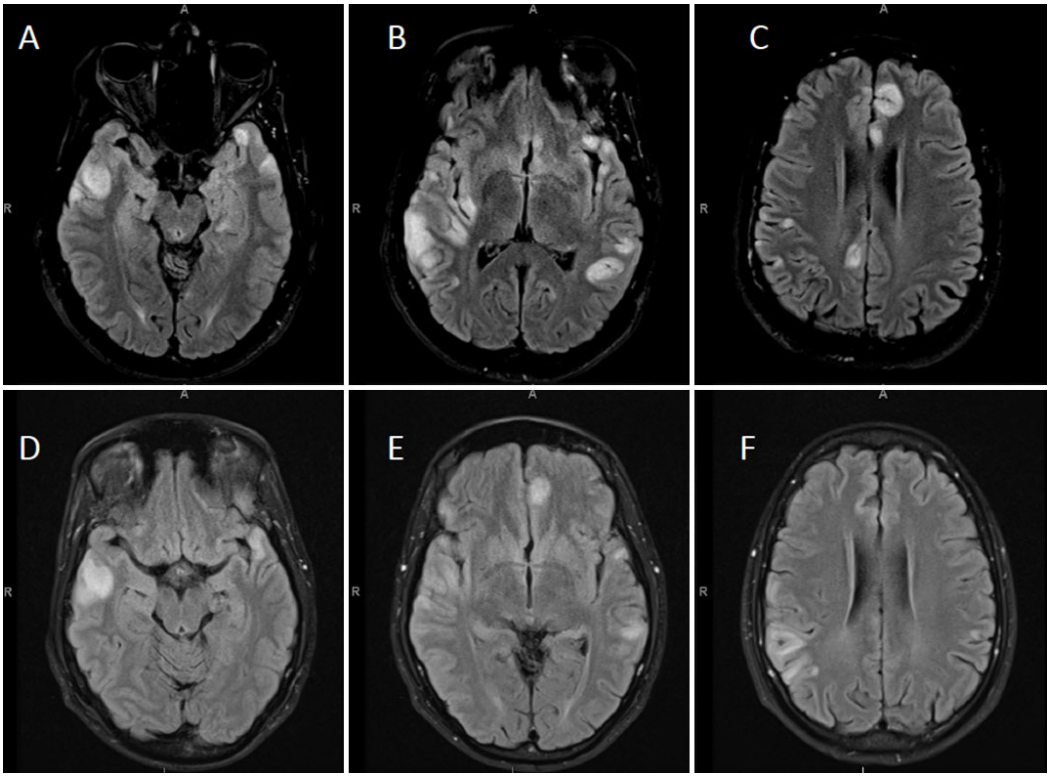

Figure 1: Initial and follow-up magnetic resonance imaging brain scans showing multiple cortically-based signal abnormalities. Images A-C are axial fluid-attenuated inversion recovery images showing multifocal elevated cortical T2/FLAIR signal intensity with associated swelling with involvement of the adjacent subcortical white matter from patient's initial presentation. There was no contrast enhancement, and there was no definite involvement of the deep gray structures, brainstem, or cerebellum. Images D-F are follow up images approximately two weeks later that reveal overall improvement, for instance, previously seen abnormality along the medial left frontal lobe, and in the posterior right insular lesion improved

bilateral lung apices. This mass was later confirmed to be a metastatic type B2, stage III thymoma via biopsy and after resection [Figure 2]. The patient was treated with intravenous methylprednisolone (1 g/day for 10 days) followed by 0.4 g/kg daily immunoglobulin (IVIG) for total of 4 days. He had significant improvement in his mental status with the first dose of steroids. Repeat imaging 2 weeks after initial presentation showed improvement in the cortical lesions, specifically, decrease in the abnormal fluid-attenuated inversion recovery (FLAIR) hyperintensities [Figure 1]. Further treatment included chemotherapy with cisplatin, doxorubicin, and cyclophosphamide given the invasive nature of the thymoma. Prior to thymomectomy, his course was complicated by gastrointestinal dysmotility syndrome thought to be a result of his paraneoplastic syndrome. He underwent a successful thymomectomy with complete resection approximately 3 months after initial presentation, and continued to improve thereafter.

DISCUSSION

Based on literature review, our patient is one of the very few reported cases of thymoma-associated panencephalitis (TAPE). These cases highlight the similar neurological symptoms at presentation -- seizures, confusion, and memory loss being the most common -- with a constellation of positive antibodies and different responses to treatment [Table 1]. The similar presentations and MRI findings in these patients support TAPE as a distinct disease entity and autoimmune disorder. Our case was unique as our patient was found to have multiple neuronal and non-neuronal antibody responses and had a drastic improvement in his clinical symptoms with just immunotherapy, unlike other cases that showed improvement after thymomectomy and/or extensive immunotherapy. In order to further characterize TAPE as a paraneoplastic syndrome, antibody associations need to be elicited.

In reviewing the prior reported cases, these patients can have a variety of autoantibodies as seen in Table 1, the most common being AchR, present in 9 of 16 cases, with 5 having symptoms of myasthenia gravis (MG). This is not surprising as up to 37% of patients with thymic tumors but without MG have been reported to have positive antibody titers to AchR.[5] Voltage-gated potassium channel (VGKC) was the 2nd most common antibody seen in 5 patients. It has been proven that the antibodies to VGKC actually target the associated proteins LGI1 and Caspr2, and not the channel itself, therefore rendering the term, “antibodies to VGKC” obsolete.[6] Antibodies to LGI1 and Caspr2 were present in 3 and 1 of the patients, respectively. Both CRMP-5 and GAD were occasionally present among the cases. The presence of GAD antibodies in thymoma is associated with stiff person syndrome and unlikely to manifest as encephalitis.[1] CRMP-5 has been shown to be a marker of immune response initiated by thymomas in different PNS, and therefore, may not be neuropathogenic.[7] Antibodies against Hu, Ri, and Yo were the least common, absent in 50% of the patients. Review of associated antibodies in these previously reported cases does not show a clear pattern, and it is unclear which antibodies are actually pathologic and which ones are incidental. Of significant importance, Petit-Pedrol *et al*.[8] recently reported 6 patients who presented very similarly to

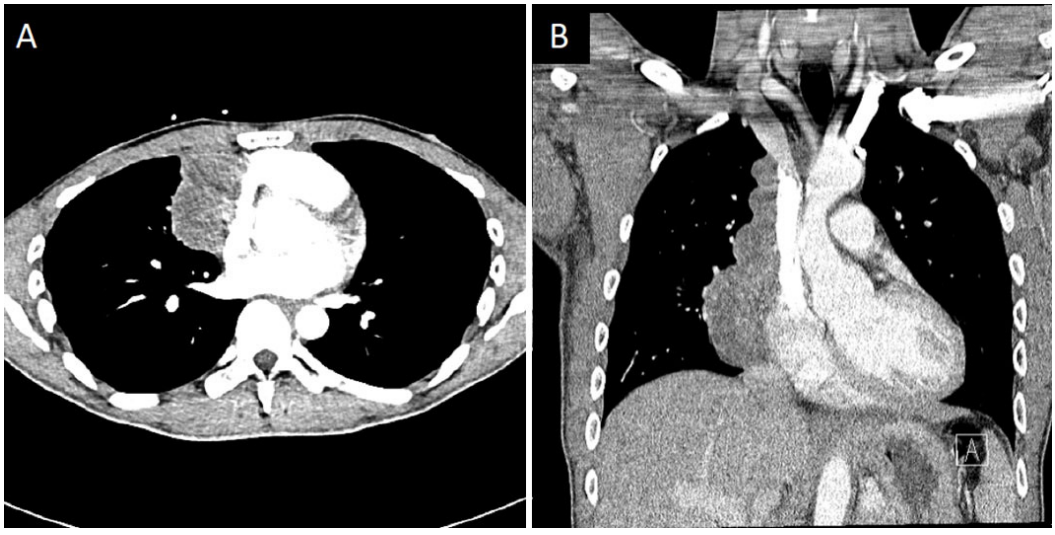

Figure 2: Computed tomography chest showing large right mediastinal mass. Contrast-enhanced computed tomography images (A: axial; B: coronal), showing a large multilobulated right anterior mediastinal mass measuring 6.0 cm × 4.7 cm in transverse dimension by approximately 13 cm in craniocaudal dimension. There was found to be mass effect with mild compression of the right atrium and superior vena cava (though patent). Thymoma was suspected, and proven later on biopsy

Table 1: Literature review of cases of thymoma-associated panencephalitis

Paper	Year	Age	Gender	Neurological symptoms	Antibodies (+)	Antibodies (-)	Cell surface *vs.* onconeuronal Ab	Treatment	Improvement
This case		35	M	Seizures, confusion, agitation, decreased to no verbal response	GAD, VGKC-complex, AchR (binding), CRMP5	Hu, Ri, Yo, VGCC, NMDA, GABA-B, AMPA, ANNA-3, anti-glial nuclear, PCA-2, PCA-Tr, amphiphysin, AchR (ganglionic neuronal)	Cell surface/ onconeuronal	Steroids, IVIG, chemotherapy, thymomectomy	1 day after steroids
Reginold *et al.*[11]	2016	47	M	Psychosis, seizures, ocular weakness	-	Hu, Ri, Yo	-	Steroids, thymomectomy	Over 4 weeks after steroids and thymomectomy
Simabukuro *et al.*[12]	2015	45	F	MG; 8 years later, memory loss, behavioral changes	GABA-A, LGI1, AchR	GABA-B, AMPA, NMDA, Caspr2, GlyR, mGLUR5, mGLUR1, GAD	Cell surface	Steroids, PLEX, thymomectomy	Improved after initial treatment, with recurrence 3 months later and improvement after removal of thymic met
Aragaki *et al.*[13]	2015	66	F	Leg cramping/inability to walk, seizures	-	AchR	-	Thymomectomy	6 days after thymomectomy and doing fairly well 7 months later
Aysal *et al.*[14]	2013	43	M	Seizures, MG (bulbar symptoms)	AchR	VGKC-complex	Cell surface	IVIG, pyridostigmine	MG symptoms improved after IVIG, then seizures improved after thymomectomy; MRI normal 2 years later and patient doing well
Suh *et al.*[15]	2013	42	F	Memory loss, voice change, ptosis, agitation, drowsiness	AchR	Hu, Ri, Yo	Cell surface	Thymomectomy	Improvement in mental status 10 days after thymomectomy and almost complete recovery 3 months later; asymptomatic 2 years later
Miyazaki *et al.*[16]	2012	46	M	MG; 4 years later, aphasia, seizures, delirium, visual hallucinations	AchR, GABA-A, LGI1	-	Cell surface	Thymomectomy, steroids, IVIG	Status epilepticus resolved 2 weeks after treatment, but cognitive impairment and psychological symptoms remained
Erkmen *et al.*[17]	2011	61	F	Seizures	LGI1	-	Cell surface	IVIG, steroids, thymomectomy	Over 4 weeks prior to thymomectomy
Werry *et al.*[18]	2009	32	M	Vertigo, diplopia, nystagmus, left hand clumsiness, olfactory disturbances, gait ataxia, myoclonic jerking, memory loss, hallucinations, anxiety; 1yr later, MG.	CRMP5; later AchR	VGKC-complex, Hu, Ri, Yo, Ma-2, amphiphysin	Cell surface/ onconeuronal	Thymomectomy, steroids, IVIG, PLEX	No improvement after thymomectomy, steroids, IVIG, and partial PLEX until 4 months later; 1 year later, developed MG, which completely resolved with immunosuppression

Continued...

Paper	Year	Age	Gender	Neurological symptoms	Antibodies (+)	Antibodies (-)	Cell surface *vs.* onconeuronal Ab	Treatment	Improvement
Rizzardi *et al.*[19]	2009	55	F	Seizures, aphasia	-	AchR, Hu, Ri, Yo	-	Thymomectomy	Total remission of symptoms 1 week after thymomectomy
Hammoud *et al.*[20]	2009	43	F	MG; 4yrs later, seizure, confusion, aphasia	AchR (binding, modulating), striational Ab, VGKC-complex	"Rest of paraneoplastic profile, including CRMP-5"	Cell surface	Steroids, IVIG, 1 cycle of chemotherapy	Some improvement of speech and cognitive function, but died 2 months later from mets
Okita *et al.*[21]	2007	33	F	Seizure; later, incontinence, confusion, decreased verbal response, apallial syndrome, RLE weakness; later, decreased consciousness and apallial syndrome	AchR	Hu, Ri, Yo, CRMP5, Ma-2, Tr, amphiphysin, VGKC-complex	Cell surface	Thymomectomy, steroids	Had thymomectomy several years prior to 3 separate presentations with ELE, 1 of which did not require treatment; the other 2 responded fairly well to steroids
Ohshita *et al.*[22]	2006	59	F	Memory impairment, apathy	GABA-A, Caspr2	AchR, Hu, Yo	Cell surface	Thymomectomy, chemotherapy	Improved initially with no treatment, but with recurrence, partial improvement of mental status 2 months after chemo
Ances *et al.*[23]	2005	38	M	Seizure, confusion, agitation; later, spasms and rigidity	GAD, neuropil of hippocampus	VGKC-complex	Onconeuronal	Thymomectomy, radiation therapy, steroids, IVIG, PLEX	Within 3 weeks after thymomectomy, radiation and steroid; developed spasms and rigidity after prednisone taper, not responsive to PLEX or IVIG, but improved with steroids only
Vernino *et al.*[24]	2002	34	F	Vertigo, tinnitus, vomiting; later, leg weakness and numbness, tingling of toes and fingers, R visual disturbance, R facial twitching, aphasia	Hu, VGKC-complex	AchR	Cell surface/ onconeuronal	Thymomectomy	Improved after thymomectomy
Rickman *et al.*[25]	2000	55	M	Dysarthria, seizure, confusion, short term memory impairment, word finding difficulty; later, unsteady gait, limb ataxia, expressive aphasia	AchR (binding, modulating), striational Ab, CRMP5	Hu, Ri, Yo, PCA-2, amphiphysin, VGKC-complex, VGCC, neuronal AchR	Cell surface/ onconeuronal	Thymomectomy, steroids, PLEX	Worsened after thymomectomy, with no response to steroids, but responded to PLEX; however, suddenly deteriorated later and died

GAD: glutamic acid decarboxylase; VGKC: voltage gated potassium channel; AchR: acetylcholine receptor; CRMP-5: collapsing response mediator protein 5; VGCC: voltage gated calcium channel; NMDAR: N-methyl-D-aspartate receptor; GABA-A-R: gamma-amino-butyric acid type A receptor; GABA-B-R: gamma-amino-butyric acid type B receptor; AMPAR: α-amino-3-hydroxy-5- methylisoxazole -4-proprionic acid receptor; LGI1: leucine-rich, glioma inactivated 1; Caspr2: contactin associated protein 2; GlyR: glycine receptor; mGLUR5: metabotropic glutamate receptor 5; mGLUR1: metabotropic glutamate receptor 1; ANNA-3: anti-neuronal nuclear antibody 3; PCA-1, 2, Tr: purkinje cell antibody 1, 2, Tr; PLEX: plasmapheresis; IVIG: immunoglobulin; ELE: extralimbic encephalitis; MG: myasthenia gravis

our patient (behavioral/cognitive changes, seizures, multifocal T2/FLAIR lesions) with high titer CSF and serum antibodies to $GABA_AR$. These patients did not have evidence of thymoma, however, did have other evidence of immune dysregulation, including the presence of other antibodies.[8] Most importantly, the $GABA_AR$ antibody was shown to have pathogenic effects on the antigen, specifically, downregulation of receptors in cultured neuronsand patients usually responded to therapy.[8,9] Furthermore, Ohkawa *et al*.[9] retested the sera of 2 patients previously with TAPE via a nonbiased proteomic method and identified antibodies to $GABA_AR$. Our patient was not tested for this antibody, however, his clinical findings, brain imaging, and response to therapy are suggestive of $GABA_AR$ associated panencephalitis.

One of the setbacks in managing TAPE is early recognition as there are so few cases reported. PNS associated with antineuronal cell surface antibodies are often highly responsive to treatment, while those with onconeuronal antibodies have a generally poor response.[10] Review of the cases shows responses to treatments differed without any clear correlation to antibody types. Every patient received thymomectomy with partial or full response followed by a combination of steroids, plasmapheresis and/or IVIG. This suggests that early surgical removal of tumors with or without immunotherapy could provide a good chance of recovery from disease. Albeit, the presence of both cell-surface and onconeuronal antibodies in our patient may indicate a poor prognosis despite his initial rapid improvement.

Authors' contributions

Concept, literature search, preparation, editing and revision of manuscript: L. Nwabuobi
Patient data and imaging acquisition, editing and revision of manuscript: J. Pellinen
Supervision and revision of manuscript: T. Wisniewski

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Verbal consent was obtained from the patient.

Ethics approval

Not applicable.

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Analysis of clinical data of viral encephalitis patients complicated with epilepsy during the acute phase

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ABSTRACT

Aim: To compare the difference between viral encephalitis patients complicated with epilepsy group and those without epilepsy. **Methods:** The authors retrospectively study 116 cases of viral encephalitis patients admitted to the General Hospital of Ningxia Medical University and the Cardia-Cerebrovascular Disease Hospital of Ningxia Medical University from January 2011 to December 2016. There were 39 cases with epilepsy and 77 cases without epilepsy. By surveying the Hospital Information System, the authors collected their clinical data including general situations, medical history, physical examination, clinical presentation, laboratory examination, cerebrospinal fluid examination, imaging examination, electroencephalogram examination (EEG), treatment, and discharge conditions. The authors used SPSS for further analysis and statistics. **Results:** Medical history: there were significant differences between the two groups in disturbance of consciousness, cognitive dysfunction, and admission conditions ($P < 0.05$). No significant differences were found in other clinical manifestations. Auxiliary examination: in cerebrospinal fluid (CSF) examination, the patients with epilepsy had higher glucose ($P < 0.05$). Brain imaging examination shows that cortical involvements in patients with epilepsy were higher ($P < 0.05$), and EEG examination showed that patients with epilepsy were more severe ($P < 0.05$). No significant differences were found in other auxiliary

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examination. Treatment and prognosis: all the patients were given antiviral treatment. Twenty-eight cases of the patients with epilepsy (71.79%) are used of antiepileptic drugs. The prognosis of patients with epilepsy was poor ($P < 0.05$). **Conclusion:** Viral encephalitis frequently manifests with seizures in its acute phase, and the incidence of seizures in our study was 33.62%. The main form was generalized seizures (64.10%), which could occur at any age; patients with epilepsy had a higher cortical involvement on imaging and a higher degree of EEG abnormalities than patients without; patients with epilepsy had a higher level of glucose in the CSF; an episode of viral encephalitis complicated with seizures could aggravate disturbances of consciousness and cognitive impairment. The prognosis of patients with epilepsy was poor.

INTRODUCTION

Viral encephalitis refers to an acute inflammatory process of the brain parenchyma due to direct viral infection. The estimated incidence of clinically-diagnosed viral encephalitis is 3.5-7.4/100,000/year.[1] At present, we have found more than 130 viruses that can cause viral encephalitis. The common viruses found in the clinic are the herpes simplex virus Type-I, Type-II (HSV-I, II), varicella-zoster virus, Japanese encephalitis virus, and enterovirus (mainly for ECHO virus and Coxsackie virus). Among the sporadic viral encephalitides, herpes simplex encephalitis (HSE) is perhaps the most frequently associated with epilepsy, which may often be severe. Among the epidemic (usually due to flaviviruses) viral encephalitides, Japanese encephalitis (JE) is the most common and is associated with acute symptomatic seizures, especially in children.[2]

There are many methods for detection of viral encephalitis, but they all have advantages and disadvantages. Even with the best efforts, 30-60% of patients with clinically suspected viral encephalitis still cannot be diagnosed. Virological diagnosis is the "gold standard." But isolation of the virus is seldom accomplished due to the difficulty in obtaining brain tissue through biopsy and a lack of specialized facilities for viral cultures in many places. Moreover, there were greater complications of brain biopsy. Virus antibody testing in serum or cerebrospinal fluid (CSF) are simpler to perform and more widely available than brain biopsy, but it has low sensitivity because many viruses do not express specific antibodies. Virus nucleic acid testing has higher sensitivity and specificity. Polymerase chain reaction (PCR) technology has significantly improved virological diagnosis. But many laboratories fail to meet the technical requirements and, thus, restrict the widespread application in this area of clinical microbiology. Therefore, the diagnosis of viral encephalitis currently depends on cerebrospinal fluid examination, brain magnetic resonance imaging (MRI) or computed tomography (CT), and electroencephalogram (EEG). Cerebrospinal fluid examination manifests with normal or elevated encephalic pressure, normal or mildly elevated protein, and normal sugar and chloride in most cases. The numeration of leukocyte is (50-500) × 10^6/L in the cytological examination and most of them are lymphocytes. Cerebrospinal fluid examination has important significance for distinguishing other types of encephalitis.[3] The appearances on early EEG are mainly abnormal rhythm and increased slow wave in viral encephalitis patients.[4] CT shows foci mainly located in one or both temporal lobes and single or multiple low density foci. CT is currently used only for infant patients and patients who do not cooperate or who move restlessly. MRI is the most important imaging examination. The edge of an abnormal area on a routine MRI scan is often unclear and may include lesions and peripheral edema. It will show a long T1 and long T2 signal and a short T1 and long T2 when it is accompanied by hemorrhage. Lesions also show high signal on diffusion weighted imaging (DWI). An enhanced MRI may have a higher sensitivity for immune compromised patients and mild patients and may include blood brain barrier damage [Figures 1 and 2].[5]

Viral encephalitis frequently manifests with seizures in its acute phase with an incidence of 19.0-62.69%.[6,7] Epileptic seizures may aggravate brain parenchyma damage and induce many severe complications that affect prognosis and quality of life, such as: pulmonary infectious disease; respiration-circulation failure; fractures and tongue biting in patients with general tonic-clonic seizures; and water-electrolyte imbalance in patients with status epilepticus. So far the researches of viral encephalitis complicated with epilepsy are not completely consistent. Herein, we retrospectively studied 116 cases of viral encephalitis and compared the difference between those complicated with epilepsy and those without. As a result, we are able to diagnosis early and take effective treatment to reduce mortality and improve prognosis.

METHODS

Subject investigated

We retrospectively studied 116 cases of viral encephalitis admitted to the General Hospital of Ningxia Medical University and the Cardio-Cerebrovascular Disease Hospital of Ningxia Medical University from January 2011 to December 2016. The inclusion

criteria:[8] (1) acute or subacute onset, some patients had a history of prodromal infections; (2) patients had symptoms of brain parenchymal damage; (3) cerebrospinal fluid examinations were consistent with viral encephalitis; (4) there was brain parenchymal damage or brain edema on imaging and varying degrees of alteration in EEG. The exclusion criteria: (1) patients younger than 14 years were excluded; (2) patients with history of epilepsy or other secondary epilepsy were excluded; (3) we removed other explicit epileptogenic causes such as hypoxia, blood glucose abnormalities, poisoning, uremic damage and so on; (4) the main clinical materials were incomplete.

Content

We collected, through a survey of the Hospital Information System, the clinical data of 116 cases of viral encephalitis including general situation, medical history, physical examination, clinical presentation, laboratory examination, cerebrospinal fluid examination, imaging examination, EEG examination, treatment, and discharge conditions

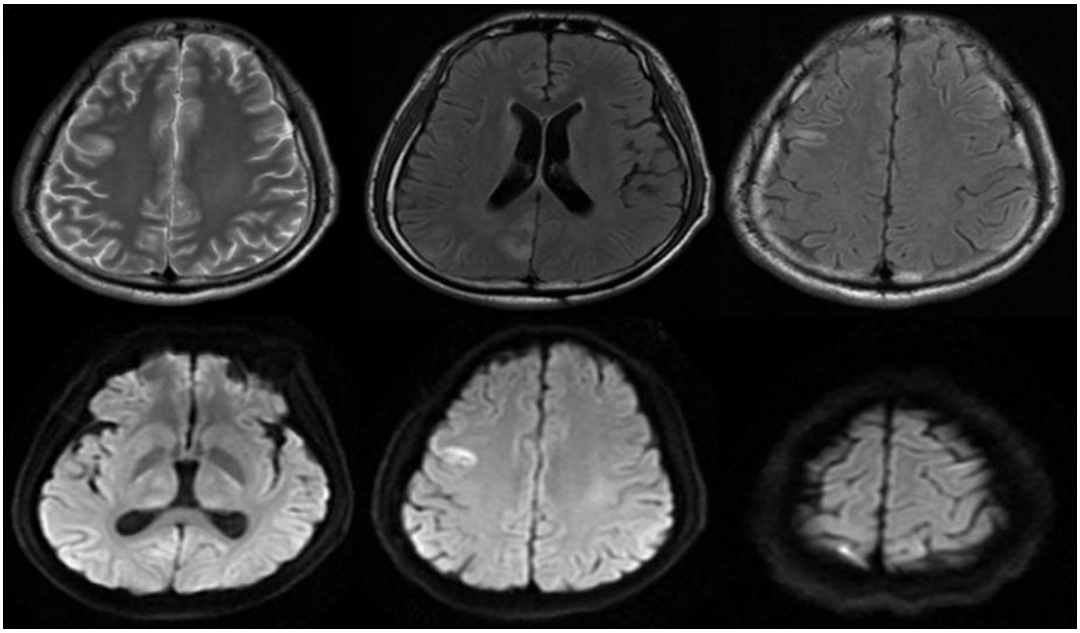

Figure 1: Abnormal signal can be seen in the right frontal lobe, occipital lobe and bilateral dorsal thalamus, caudate nucleus in the brain magnetic resonance imaging, shows high signal on T2WI and a slightly higher signal on Flair, shows high signal diffusion weighted imaging

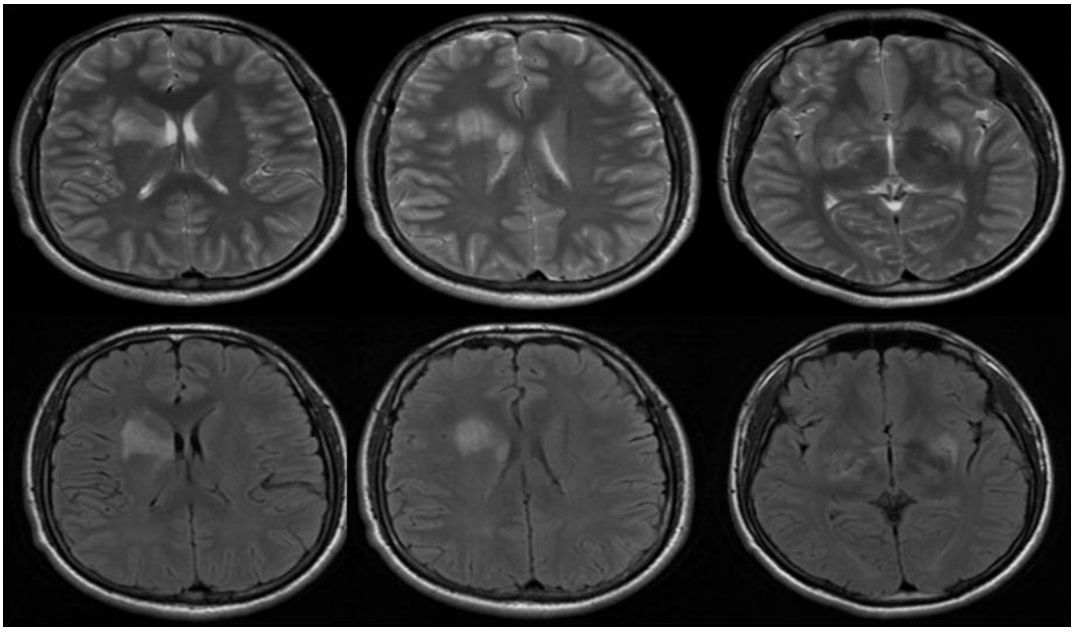

Figure 2: Abnormal signal can be seen in bilateral hippocampus and right putamen in the brain magnetic resonance imaging, shows high signal on T2WI and high signal on Flair

and analyzed the data.

Statistical methods

We used SPSS 17.0 for statistical and further analysis. The measurement data and enumeration data were statistically analyzed with Rank Sum test and χ^2 test respectively.

RESULTS

General situations

In the prior 6 years, the incidence of viral encephalitis had not increased obviously. There were 67 cases of male patients (57.76%) and 49 cases of female patients (42.24%) among all the patients. The mean age of presentation was 41.00 ± 19.06 years in the group with epilepsy and 37.70 ± 15.59 years in the group without epilepsy. The average length of stay was 19.53 ± 16.46 days in the group with epilepsy and 19.84 ± 12.76 days in the group without epilepsy. There were no significant differences between the two groups in gender, nationality, age, average hospitalization days; however, there were significantly differences in the conditions upon entering the hospital (P = 0.020) [Table 1].

Clinical presentation

Among the 116 patients with viral encephalitis, 46 cases had definite causes. Catching cold was the main precipitating factor for 41 cases (89.13%). Two cases (4.34%) were precipitated by psychiatric and psychological factors; 2 cases (4.34%) were precipitated by overwork; and 1 case was precipitated by brain trauma. There were 96 patients (82.76%) with acute onset and 20 patients (17.24%) with subacute onset. Some viral encephalitides have a history of prodromal infections, such as, respiratory infections (especially upper respiratory tract infections), digestive tract infections, and mucocutaneous herpes. Part of the patients were complicated with severe pneumonia, atrial fibrillation, heart failure and other cardiovascular diseases, gastrointestinal bleeding, and other serious medical diseases [Table 2].

There were 7 cases of patients (6.03%) with initial symptoms of epilepsy, which is much lower than that of Misra's study of 30.7% *vs*. 42.6%.[7] Possible reasons are that patients and their families do not recognize partial epilepsy seizure, there are no family members in the presence of seizures, and the patients cannot provide the relevant medical history themselves. There were 3 cases of patients (2.59%) with initial symptom of dizziness, nausea, vomiting and other general symptoms; 1 case of patients (0.86%) with initial symptom of visual disorder; 2 cases of patients (1.72%) with initial symptom of hemiplegia; 2 cases of patients (1.72%) with initial symptom of paresthesia; 1 case of patients (0.86%) with initial symptom of ataxia; and 1 case of patients (0.86%) with initial symptom of conscious disturbance. The main manifestations of viral encephalitis include headache, fever, seizures, limb weakness, consciousness disturbance, mental and behavioral disorders, language disorders, cognitive dysfunction, ataxia, and varying degrees of alteration

Table 1: Comparison of gender, nationality and entering hospital condition between the two groups

Characteristics	With epilepsy group		Without epilepsy group		χ^2	*P*
	Case	Rate (%)	Case	Rate (%)		
Male	23	58.97	44	57.14	0.036	0.850
Female	16	41.02	33	42.85		
Han nationality	28	71.79	57	74.02	0.066	0.798
Non-Han nationality	11	28.20	20	25.97		
Common patients	10	25.64	37	48.05	5.395	0.020
Severe patients	29	74.35	40	51.95		

Table 2: Comparison of clinical manifestations between the two groups

Clinical manifestations	With epilepsy group		Without epilepsy group		χ^2	*P*
	Case	Rate (%)	Case	Rate (%)		
Headache	30	76.92	66	85.71	1.402	0.236
Fever	31	79.49	55	71.43	0.877	0.349
Consciousness disturbance	32	82.05	16	20.78	40.067	0.001
Cognitive dysfunction	24	61.54	28	36.26	6.634	0.01
Sensory disturbance	2	5.13	4	5.19	0.000	1.000*
Involuntary movement	3	7.69	2	2.60	0.628	0.428*
Decreased memory	11	28.21	12	15.58	2.594	0.107
Language disorders	11	28.21	12	15.58	2.594	0.107
Autonomic nervous dysfunction	4	10.26	4	5.19	0.395	0.530*
Central hypoventilation	8	20.51	6	7.79	2.84	0.092*
Meningeal irritation sign	12	30.77	19	24.68	0.491	0.484

*stands for using the chi square test for correction

in sensorium. There were significant indications between the two groups in consciousness disturbance (P = 0.001) and cognitive dysfunction (P = 0.01), both of which were higher in the epilepsy group.

In the 39 cases (33.62%) of viral encephalitis with epilepsy, there were 25 case（64.10%）in which the main form was generalized seizure, which is consistent with current domestic reports.[9] There were 7 cases of status epilepticus, 2 cases of simple partial seizures, 5 cases of complex partial seizures, 7 cases of generalized seizures secondary to partial seizures. In the 32 cases of non-status epilepsy, there were 12 cases whose duration of seizure was within 1 min, 8 with duration of 1 to 5 min, 8 with duration of 5 to 10 min, 1 case with duration of 10 to 20 min, and another 3 cases with unknown duration. There was a range in the number of epileptic attacks: 13 cases were once; 7 cases were 1 to 5 times; 4 cases were 5 to 10 times; and 8 cases were more than 10 times.

Examination

CSF examinations

All patients received a lumbar puncture. There were 53 cases of patients (45.69%) whose intracranial pressure was higher than 180 mmH_2O, including 9 cases of patients whose intracranial pressure was more than 300 mmH_2O. The mean encephalic pressure was 184.10 ± 64.29 mmH_2O in the group with epilepsy and 192.92 ± 70.65 mmH_2O in the group without epilepsy. There are non-significant discrepancies between the two groups in encephalic pressure but the incidence of intracranial hypertension is lower than the study of Gong *et al*.,[10] which is 58.7%. On the one hand, some patients received a lumbar puncture in the early stages of disease, and the intracranial pressure did not increase remarkably; on the other hand, some patients had been using drugs to reduce intracranial pressure before the lumbar puncture.

No clot was found in CSF routine examination. Only 1 case of patients with epilepsy manifests with yellow color and slightly mixed, 4 cases of patients were pale red, 111 cases of patients were colorless and transparent. There was only one case whose protein content in CSF was less than 0.15 g/L, and 38 cases of patients whose protein content was between 0.15 and 0.45 g/L, and 77 cases of patients whose protein content was above 0.45 g/L. There were 12 cases whose glucose content in CSF was less than 2.5 mmol/L, and 93 cases of patients whose glucose content was between 2.5 and 4.5 mmol/L, and 11 cases whose glucose content was above 4.5 mmol/L. There were 30 cases of patients whose chloride in CSF was less than 120 mmol/L, and 80 cases of patients whose chloride was between 120 and 132 mmol/L. There were non-significant differences between the two groups in protein and chloride. However, there was significant difference between the two groups in glucose (P = 0.001), with higher glucose in those with epilepsy. Cytological examination of the CSF showed 95 cases with mainly increased lymphocytes, 16 cases were normal, 4 cases were mainly neutrophil predominant, and 1 case with elevated mononuclear cells. One percent of the phagocytes were found in one case and the same was true for granulocytes and plasma cells. Four cases were neutrophil predominant, 3 of them were from the group with epilepsy and one from the group without epilepsy. A lumbar puncture was performed in the early stages of their disease, and in this stage part of patients were mainly neutrophil predominant. With the treatment and development of disease, lymphocytes and monocytes were gradually increased and prevailed. However, this phenomenon is rarely seen in clinical practice because patients visit the hospital out of this period [Table 3].

Imaging examination

Brain imaging of viral encephalitis patients, using CT or MRI, showed foci mainly located in one or both temporal lobes, followed by frontal lobe, parietal lobe, occipital lobe, insula, hippocampus, and other parts of involvement [Table 4]. There were significant differences between the two groups in cortical involvements (P = 0.001) in which patients with epilepsy were higher [Table 5].

EEG examination

According to the book of Clinical Electroencephalography written by Feng,[11] EEG

Table 3: Comparison of biochemical and cytological examination of cerebrospinal fluid between the two groups

CSF	With epilepsy group M (P_{25}, P_{75})	Without epilepsy group M (P_{25}, P_{75})	Z	*P*
CSF protein (g/L)	0.53 (0.34,0.87)	0.58 (0.43,0.85)	-0.885	0.376
CSF glucose (mmol/L)	3.40 (3.10,4.20)	3.03 (2.76,3.45)	-3.350	0.001
CSF chloride (mmol/L)	122.60 (116.00,125.70)	123.00 (121.15,125.00)	-0.790	0.430
Leukocyte (*10^6/L)	61.00 (25.00,150.00)	37.00 (11.00,92.50)	-2.237	0.025
Lymphocyte (%)	0.84 (0.66,0.90)	0.89 (0.81,0.94)	-1.901	0.057
Monocyte (%)	0.09 (0.06,0.14)	0.08 (0.05,0.15)	-0.763	0.445
Neutrophils (%)	0.04 (0.01,0.18)	0.02 (0.00,0.05)	-1.620	0.105

CSF: cerebrospinal fluid

examination is divided into five grading standards, that is, normality, marginal state, mild abnormality, moderate abnormality, and severe abnormality. The abnormal rate of EEG is 37.07% (43 cases) in my research including 10 cases with epileptiform discharge. There were significant discrepancies between the two groups in abnormality of electroencephalogram (P = 0.001) in which patients with epilepsy are more severe [Table 6].

Treatment

All viral encephalitis patients were given antivirotic treatment. Ninety-nine cases were treated with acyclovir, a broad spectrum antiviral drug, which is a purine nucleoside analog that competitively inhibits the binding of 2'-deoxyuridine to block viral DNA synthesis and interfere with viral replication. The dose is generally 0.5 g Q8 h and treatment for 2 to 3 weeks, critically ill patients can be extended according to their condition. The main adverse reactions are acute renal failure, elevated transaminase, or encephalopathy in patients with normal renal function (seizures, dysarthria, hallucinations, *etc*.).[12] Seventeen cases of patients were treated with ganciclovir, a derivative of acyclovir with the same mechanism of action. The conventional dose is 150 mg Q12 h, and 10-14 days for a course of treatment. The main adverse reactions are for leukopenia and kidney damage.[13] It is reported that interferon can activate natural killer cells and macrophages, enhance B cell and T cell activity, and slow viral replication. However, patients in my research have not used interferon due to its potential to aggravate nausea and vomiting, and, besides, it is expensive. Dehydration is induced in patients with intracranial hypertension to reduce intracranial pressure as well. Glucocorticoids have powerful anti-inflammatory and edema alleviating effects and are used to treat severe patients with white matter demyelination and severe brain edema. Glucocorticoid treatment is accompanied with potassium and calcium supplement and inhibition of gastric acid secretion to protect the digestive tract. Immunoglobulin can neutralize the virus, enhance immune cells, kill cells, neutralize antibodies, and other immune protection and anti-infective function.[14] But immunoglobulin is expensive and has adverse reactions, such as, allergy, transmission of blood diseases, and kidney damage. Therefore, it is currently used for rapidly progressing and severe encephalitis. One case complicated with Guillain-Barre Syndrome developed quickly and was given immune globulin. If necessary, we give electrolyte replacement and rehydration, oxygen treatment, respiratory and circulation support, *etc*. Among the 39 cases of viral encephalitis complicated with epilepsy, and there were 11 cases (28.20%) of patient using antiepileptic drugs (AEDs). There were 18 cases (46.15%) using magnesium valproate to treat epilepsy, and 12 cases (30.77%) using

Table 4: Comparison of imaging data between the two groups

Imaging examination	With epilepsy group		Without epilepsy group	
	Case	Rate (%)	Case	Rate (%)
Frontal lobe	7	16.67	8	14.55
Parietal lobe	5	11.90	3	5.45
Occipital lobe	4	9.52	3	5.45
Temporal lobe	16	38.10	19	34.55
Insular	3	7.14	3	5.45
Brainstem	0	0	2	3.64
Basal ganglia, thalamus, lateral ventricles	1	2.38	11	0.20
Corpus callosum	2	4.76	3	5.45
Ventricle	0	0	1	1.82
Hippocampus	3	7.14	1	1.82
Cerebellum	1	2.38	1	1.82

Table 5: Comparison of cortical involvements between the two groups

Imaging examination	With epilepsy group		Without epilepsy group		χ^2	P
	Case	Rate (%)	Case	Rate (%)		
Cortical involvements	23	58.97	20	25.97	12.085	0.001
Cortical not involved	16	41.03	57	74.03		

Table 6: Comparison of EEG examination between the two groups

EEG examination	With epilepsy group		Without epilepsy group		Z	P
	Case	Rate (%)	Case	Rate (%)		
The normality	16	41.03	57	74.03		
The marginal state	1	2.56	3	3.90		
Mild abnormality	1	2.56	6	7.79	-3.967	0.001
Moderate abnormality	16	41.03	9	11.69		
Severe abnormality	5	12.82	2	2.59		

EEG: electroencephalogram

Table 7: Comparison of prognosis between the two groups

Prognosis	With epilepsy group		Without epilepsy group		Z	*P*
	Case	Rate (%)	Case	Rate (%)		
Cure	0	0	15	19.48		
Improved	35	89.74	56	72.72		
Aggravate	1	2.56	1	1.30	-2.443	0.015
Death	0	0	1	1.30		
Others	3	7.69	4	5.19		

phenobarbital, 4 cases（10.26%）using lamotrigine, 2 cases (5.13%) using levetiracetam, 2 cases (5.13%) using topiramate, and1 case（2.56%）using sodium valproate. There were 7 cases (17.95%) using diazepam to prevent generalized seizure or status epilepticus. Most seizures can be terminated after treatment with one or two kinds of antiepileptic drugs; however, there were still 2 cases with status epilepticus terminated by midazolam or propofol.

Prognosis

According to the standard of prognosis,[15] discharge situation are divided into five conditions, that is, cure, on the mend, aggravated, death, others (due to various reasons such as the interruption of treatment). (1) Cure: all indexes including the symptoms, signs, related auxiliary examination results return to normal; (2) improved: at least one index including symptoms, signs, related auxiliary examination did not return to normal; (3) aggravated: exacerbations; (4) death; (5) others: treatment is interrupted due to the patient's own and other reasons. The discharge situation of the two groups were statistically analyzed with Rank Sum test and the results proved that there were significant discrepancies between the two groups (P = 0.015). The prognosis of patients with epilepsy was poorer [Table 7].

DISCUSSION

Viral encephalitis can occur in any season and at any age. Viral encephalitis frequently manifests with seizures. Its etiology and pathogenesis is not yet entirely clear. The pathogenesis has been found as follows: (1) in acute phase, many pathological changes can be found in viral encephalitis including the release of toxins and viral metabolites accumulation, cerebral edema, cortical arteriovenous thrombosis. All of these pathological changes can reduce the stability of cell membrane and cause epileptic seizure. Electrolyte disorders and excessively dehydration may also increase the incidence of epilepsy; (2) in the later stage, epileptogenic focus is formed in the brain. The manifestations of epileptogenic focus are neuron loss and disorganization because of cerebrocellular necrosis and gliocyte hyperplasia. Metabolic disorders of neurons, owing to ischemia, result in functional disorder ofion pumps across the membrane, resulting in the outflow of potassium ions, inflow of calcium, and sustained depolarization, which can cause seizures;[16] (3) when the hypothalamus is involved, the abnormal secretion of antidiuretic hormone leads to fluctuations of blood sodium, which can further cause brain dysfunction and cause epileptic seizures.[17]

There were non-significant differences between the two groups in encephalic pressure, chloride, protein, and cytologic examination, but the patients with epilepsy had higher levels of glucose. At present, there is no relevant research on this feature. The view that hyperglycemia leads to epileptic seizures has been generally accepted. However, whether glucose in the CSF of epileptic patients is increased or not and what the mechanism might be still needs further research. Brain imaging examination showed that cortical involvements in patients with epilepsy were higher. Therefore, we should be alert to seizures in patients with cortical lesion. EEG examination shows that patients with epilepsy are more severe. We also should be alert to seizures in patients with moderate and severe abnormal EEG. Just as these researches about prognosis of viral encephalitis report, the severer the EEG manifestation, the worse the prognosis.[18] EEG examination plays an important role in the treatment and prognosis of viral encephalitis because EEG changes are usually consistent with changes in patients' conditions. Non-convulsive seizures and non-convulsive status epilepticus can aggravate the severity of dementia in patients with severe encephalitis. Non-convulsive seizures are detectable only by continuous electroencephalographic monitoring, so EEG is particularly important.[19] Conscious disturbance is a common manifestation of brain damage in encephalitis patients during the acute stage. We find that the proportion of patients with conscious disturbance and cognitive impairment in the epilepsy group was higher than that in the non-epileptic group. Therefore, an episode of viral encephalitis complicated with seizures aggravates conscious disturbance and cognitive impairment. Bartolomei *et al*.[20] reviewed the research about the relationship between conscious disturbance and seizures over the past 50 years, and found that patients with seizures complicated with conscious disturbance have poor prognosis. The prognosis of patients with epilepsy is poorer if these izures are more frequent and/or longer duration, or the conscious

disturbance in the acute phase is severer than for other patients.[21] Therefore, this study suggests taking active antiepileptic treatment for patients with frequent seizures. As we lack follow-up investigation of viral encephalitis patients after discharge, we are unable to determine which AEDs have definite advantage over the others. The subject of viral encephalitis complicated with epilepsy remains for further study.

Authors' contributions

Concept: S.Y. Chen, W. Ma, Y.Y. Chen, X.W. Fan
Design: S.Y. Chen, X.W. Fan
Literature search: S.Y. Chen, W. Ma, Y.Y. Chen
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Ethics approval

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Commentary **Open Access**

Hot topics of autoimmune encephalitis

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INTRODUCTION

Within the last few years, a new group of diseases featured with cognitive impairment, seizures and behavior disorders was reported. And these diseases were commonly diagnosed as "viral encephalitis" and "sporadic encephalitis" than autoimmune encephalitis (AE) before AE had confirmed etiological. With the development of autoantibody serological tests, many patients who had been previously considered as patients with "viral encephalitis" can now be definitively diagnosed with AE. Currently, AE has become one of the hotspots in neuroimmunology. A retrospective review suggests that earlier treatment of N-methyl-D-aspartate receptor (NMDAR) antibody encephalitis in children results in better outcomes.[1] Also, there is another study that showed that people who received immunotherapy within 40 days from onset had a better recovery than those who received immunotherapy after 40 days from onset.[2] Therefore, early immunotherapy could improve the prognosis of AE. Also, treatment regimens vary from different types of AE. For example, anti-NMDAR (detected) encephalitis usually requires intensive immunosuppression, whereas encephalitis associated with LGI1 antibodies usually responds well to steroids alone.[3] The lower frequency of neurological relapses is likely due to better recognition of the disorder, earlier treatment, andincreasing use of second-line immunotherapy.[4] Early diagnosis of these disorders is, therefore, required.

DISCUSSIONS ON "A CLINICAL APPROACH TO DIAGNOSIS OF AUTOIMMUNE ENCEPHALITIS"

In a paper published in *The Lancet Neurology*, Graus *et al*.[5] propose new guidelines for the diagnosis of AE. This guideline includes five diseases involving limbic encephalitis (LE), anti-NMDAR encephalitis, Bickerstaff brain stem encephalitis, acute disseminated encephalomyelitis (ADEM) and hashimoto encephalopathy. These diseases show some similar characteristics of onset and clinical features. When AE is suspected, a stepwise diagnosis should be made according to the recommended diagnostic path. This guideline classifies the diagnosis of AE into three levels: possible, probable and definite. The two former levels do not rely on the tests of auto-antibodies, while the definite diagnosis of AE generally need positive antibody result. Each level needs some supportive and exclusive points to diagnosis.

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Diagnosis of possible AE should exclude other probable diseases reasonably. Increased signal on T2-weighted fluid-attenuated inversion recovery (FLAIR) imaging in the medial aspect of the temporal lobes is an important differential diagnosis essential. Cerebrospinal fluid (CSF) analysis and characteristic clinical manifestations also count. However, some diseases are difficult to preclude due to atypical clinical manifestations or due to the lack of relevant detection methods, such as viral encephalitis.[6-8] Whether to continue in accordance with the recommendations of diagnostic path remains to be further verified. Concerned to the imaging features of LE, the guideline emphasizes that bilateral temporal lobes involvement is one of the necessary factors for the definite diagnosis of autoimmune LE. However, it does not imply to deny the existence of LE which is just unilateral temporal lobe involved or with normal imaging.[9,10] When the autoantibody test results are unknown, the diagnostic criteria of autoimmune LE can exclude the disease involving the limbic system as well, such as viral encephalitis, glioma, epilepsy, *etc*., to avoid over-diagnosis.

The guideline lists anti-NMDA receptor encephalitis separately,[11] and for the first time presents detailed probable and definite diagnostic criteria. The diagnosis is based on the specific symptoms, auxiliary examination, autoantibody detection and tumor evidence. Anti-NMDAR encephalitis is singled as an independent diagnosis may be due to the following reasons: (1) anti-NMDAR encephalitis has a characteristic manifestation other than typical LE, such as the involuntary movement of the face, speech disorders, autonomic dysfunction and central hypoventilation; (2) although the exact incidence of various types of AE cannot be calculated, anti-NMDAR is much higher than other types of AE based on current data;[5,12] (3) there was a clear correlation between NMDAR encephalitis and teratoma; (4) the most common magnetic resonance imaging manifestations of anti-NMDAR encephalitis are abnormal non-specific signals in cortical or subcortical, and the positive rate is low. Moreover, Imaging evidence of bilateral limbic system involvement is the necessary condition of the clinical diagnosis of autoimmune LE.[5,12] To sum up, anti-NMDAR encephalitis is currently diagnosed independently of autoimmune limbic encephalitis disease. It is noteworthy that the diagnostic criteria for anti-NMDAR encephalitis do not include near-term memory impairment in the clinical manifestations. Because it is difficult to assess memory impairment in those patients with psychiatric symptoms or in child patients.[5] As the more common symptom of the disease,[4] memory disorders can often be obtained by asking history. So, near-term memory disorder could be included into the diagnostic criteria, but still needs further clinical validation.

AUTOIMMUNE ENCEPHALITIS AND OTHER NERVOUS SYSTEM DISEASE OVERLAP SYNDROME

Demyelinating disease

A recent case study of a large number of patients showed that a group of patients with anti-NMDAR antibody encephalitis have an overlap in clinical/MR manifestations with optic neuromyelitis and myelitis.[13] In this group of patients, MR usually shows multifocal, subscapular or extensive T2/FLAIR phase abnormalities which suggests an overlap with demyelinating disease such as optic neuromyelitis. More than half of these patients suffered demyelinating disease before or after the onset of anti-NMDAR antibody encephalitis. In some patients in this group, anti-NMDAR antibody encephalitis and demyelinating disease occurred at the same time, which greatly challenges the diagnosis. Some patients with early diagnosis of ADEM, neuromyelitis optica or multiple sclerosis could appear psychiatric symptoms, faciobrachial dystonic seizures and/or autonomic dysfunction which strongly suggests a diagnosis of anti-NMDAR encephalitis.

Herpes simplex virus encephalitis (HSE)

It is reported that HSE has a recurrence rate of 25%.[14] Some of the recurrent patients would recover after antiviral treatment. But some recurrent patients presented with new symptoms such as choreoathetosis and so on. As for them, virological examination was negative and antiviral therapy was not effective. This part of patients' serum and CSF anti-NMDAR antibody was positive.[15] Their conditions improved after immunotherapy. Some studies have reported anti-NMDAR antibody encephalitis could occur after HSE.[16,17] Since then, HSE recurrent patients were found other non-specific neuronal surface-mediated antibody positive in serum or CSF, suggesting that HSE infection may be one of the causes of AE.

AE combined with other antibodies

Overlap anti-neuronal antibodies in AE is rare. Anti-NMDAR encephalitis is the most common type of AE. This disease combined with other neuronal antibodies, such as anti-Aquaporin antibody, anti-myelin oligodendrocyte glycoproteinantibody is rarely reported. The proportion of anti-GABABR encephalitis with multiple antibodies appears to be higher. Höftberger *et al*.[18] reported 20 patients with GABABR antibodies, seven of them had

overlap antibodies. Five patients with small-cell lung cancer had additional onconeuronal antibodies (Ri, amphiphysin, or SOX1), and 2 without tumor had GAD65 and NMDAR antibodies. In addition, AE can also overlap with other immune mediated non-nervous system autoantibodies, such as antibodies to thyroid peroxidase or GAD65.[19]

DETECTION METHODS OF AUTOIMMUNE ENCEPHALITIS ANTIBODY

Even though the launched diagnostic criteria of AE do not depend on the detection of autoantibody, it still needs to emphasize the importance of autoantibody detection. Commonly used methods for autoantibody detection include cell-based assay (CBA), immunohistochemical staining of brain tissue, immunocytochemical staining of cultured single hippocampal neurons in rodents (only for animal experiments).[5] CBA method is the more recognized detection method in domestic laboratory. The sensitivity and specificity of the CSF detection of CBA is 98.5% and 100%, respectively. The sensitivity and specificity of serum antibody detection of CBA were 85.5% and 98.2%.[20,21] In clinical practice, there are also many patients that meet the autoantibody-negative AE diagnostic criteria, which may be due to the limited types of antibody that can be detected or the false negative test results can be recorded. The guidelines also recommend that patients who meet the criteria of probable AE, but do not have well characterized autoantibodies, investigation of CSF and serum for new antibodies in reference laboratories is important. And if the immunohistochemical staining was positive, even if not sure what kind of antibody, it still highly suggested of AE.

TREATMENT OF AUTOIMMUNE ENCEPHALITIS

Selecting the appropriate therapeutic time window and the appropriate drugs and drug doses of early standardized treatment of AE are particularly important. Currently, the treatment of AE is divided into first-line immunotherapy, second-line immunotherapy and tumor resection (with tumor patients). First-line immunotherapy including steroids, intravenous immunoglobulin and/or plasma exchange, combined with tumour removal, second-line immunotherapy is mainly immune inhibitors such as rituximab and/or cyclophosphamide. Currently, there is no research to compare which treatment regimen is better, but generally it is accepted to take the first-line treatment of a program or a combination of two options. If the first-line treatment is invalid, then switch to the second-line treatment. For patients with AE associated with tumor, tumor resection should be carried out as soon as possible.[15]

CONCLUSION

With the introduction of new diagnostic criteria for AE, the diagnosis of AE can be standardized. Early diagnosis of AE, starting immunotherapy or resection of the tumor is conducive to improving the prognosis of the disease. However, the sensitivity and specificity of the diagnostic criteria remain to be further clinical validated. For antibody-negative AE, the existence of new antibodies still needs further exploration.

Authors' contributions

Drafting/revising the manuscript, study concept or design: Y. Peng, J.W. Wang

Study supervision, intellectual contribution: J.W. Wang

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Ethics approval

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Topic: Infectious Disease of Central Nervous System **Open Access**

Developments in auxiliary examination of Creutzfeldt-Jakob disease

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ABSTRACT

Creutzfeldt-Jakob disease (CJD), which is caused by prion scrapie protein, is a rare, chronic, transmissible and fatal disease. Clinical manifestations of CJD include rapidly progressive dementia, cerebellar ataxia, visual disturbance, as well as pyramidal and extrapyramidal tract signs. Four subtypes of CJD have been reported, including sporadic, familial or genetic, iatrogenic and variant. Given the infectiousness and high mortality of the disease, it is imperative that earlier and more accurate diagnostic methods are developed. In the past years, 14-3-3 protein testing and periodic sharp wave complexes in electroencephalogram have been widely used in CJD clinical diagnosis; and the abnormal hyper-intensity in diffusion weighted imaging has also been used. Recently, there has been a focus on the diagnostic value of 18F-fluorodeoxyglucose positron emission tomography/computed tomography. New findings of potential biomarkers in cerebrospinal fluid and decreases in diffusion tensor imaging measures have emerged as having an association with CJD. Magnetic resonance spectroscopy has also drawn attention as an emerging method for diagnosis. In this review, the progress in auxiliary examinations of CJD is discussed and the potential, future diagnostic methods are introduced.

INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a rare human transmissible spongiform encephalopathy with a subacute disease course, a long incubation period and a mortality rate of 100%. CJD is associated with central

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nervous system (CNS) degeneration that manifests as rapidly progressive dementia, cerebellar ataxia, visual disturbance, and pyramidal/extrapyramidal tract signs, *etc*. Relatively rare symptoms of CJD include epilepsy, bulbar palsy, amyotrophy and stroke-like episodes. Onset of disease in most patients is at 64-66 years and death typically occurs 1-2 years thereafter. The causative agent in CJD is prion scrapie protein (PrPsc), which results from the misfolding of prion protein and is able to self-replicate.

Four subtypes of CJD have been reported, including sporadic (sCJD), familial or genetic (fCJD or gCJD), iatrogenic (iCJD) and variant (vCJD). sCJD is the most common type and accounts for 85-95% of all cases.[1] It occurs worldwide without geographic or seasonal clustering,[1,2] and may arise because of random mutation or post-translational modification of the PrP gene (PRNP). Based on the genotype and biochemical properties, sCJD is classified into six different molecular strains: MM1, MM2, MV1, MV2, VV1, and VV2.[3,4] MM1/MV1 is the most common and typical type with 60-70% of sCJD and has very high positive rate in magnetic resonance imaging (MRI), periodic sharp wave complexes (PSWCs) and 14-3-3 protein testing. VVI is negative in PSWCs and has a longer duration of illness-about 21 months.[3-6] fCJD accounts for about 10% of prion disease cases.[7] It shows autosomal dominant inheritance of mutations in PRNP with high penetrance，and over 50 different mutations in PRNP have been found.[8] The common form of fCJD results from mutation at codon 200, and the phenotype in patients is similar to that of sCJD.[9] Some other forms of fCJD with the phenotype different from sCJD have been classified as distinct types such as Gerstmann Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI).[5] GSS even could have a much longer disease duration lasting 5 years. The first iCJD case (a person who was infected by corneal transplant from a CJD patient) was reported in 1974.[10] CJD can be transmitted by intracerebral electrodes, corneal transplantation, dura mater grafts, injections of growth hormone extracted from human pituitary glands and contaminated neurosurgical instruments. Blood transfusion or blood products of CJD patients are also infectious.[11] vCJD was first described in 1996, and was associated with ingestion of beef with bovine spongiform encephalopathy (BSE). After the spread of BSE in the UK and the transmission to humans in 1980s-1990s, the secondary spread of the disease appeared in the 2000s from asymptomatic infected individuals to others by routes such as blood transfusion or organ grafting.[12-14] vCJD has different manifestations from sCJD such as younger age at onset (28-29 years, mean), longer disease course (14 months, mean), prominence of psychiatric and sensory symptoms, and negative of PSWCs.[5]

The clinical manifestations of CJD lack specificity, thus it is difficult to distinguish from other dementia diseases such as Alzheimer disease, autoimmune encephalitis, neurologic paraneoplastic syndrome and hepatolenticular degeneration, just depending on clinical symptoms. And the detection of PrP or the genetic diagnosis has not been extensively used clinically. Therefore, auxiliary examinations are important for accurate diagnosis of CJD. 14-3-3 protein testing and electroencephalogram (EEG) are the traditional technologies that are widely used. Diffusion weighted imaging has been intensively studied, which has a high sensitivity and plays a significant role in diagnosis. Recently, scientists propose some emerging auxiliary examination methods, such as the new biomarkers in cerebrospinal fluid (CSF), diffusion tensor imaging, magnetic resonance spectroscopy and 18F-fluorodeoxyglucose positron emission tomography/computed tomography (18F-FDG PET/CT). We make a review on the progress of auxiliary examinations on CJD as follows.

AUXILIARY EXAMINATION

CSF biomarkers

14-3-3 protein is the most widely used CSF biomarker for CJD diagnosis. It has the highest expression in neuron synapses and plays a role in signal transduction, neurotransmission, cell differentiation and apoptosis.[15,16] 14-3-3 protein can be detected in CSF of CJD patients and in the neurofibrillary tangles upon pathologic examination.[17] The mechanism by which 14-3-3 protein induces CJD onset is unknown, and the diagnostic value of 14-3-3 protein is controversial. Some previous studies have reported the high sensitivity (85-97%) and specificity (68-97%) of the immunoblot test for 14-3-3 protein in CJD diagnosis.[17,18] When 14-3-3 protein testing is taken among unselected patients with rapidly progressive dementia (disease duration less than 12 months), the false positive rate is about 12%. But in some other studies about pathology confirmed cases, the negative rate is very high.[19] And the contamination of CSF by 14-3-3 protein containing blood cells may lead to false-positive results. In addition, the expression of 14-3-3 protein is also found in many conditions of neuronal injury such as infectious diseases of the CNS, metabolic encephalopathy and in the acute stage of cerebral infarction, reducing the specificity of 14-3-3 protein testing.[20] One study showed that only 17 of 32 confirmed sCJD patients (biopsy- or autopsy-confirmed) had positive results of

14-3-3 protein with the sensitivity of only 53%.[21] And in another analysis of 420 CJD patients confirmed by biopsy, the specificity was only 28%.[22] The levels of 14-3-3 protein in vCJD and fCJD subtypes are different.[17] Distinct from sCJD, vCJD has about only 50% positive rate of 14-3-3 protein. Furthermore, GSS and FFI are nearly negative for 14-3-3 protein testing.[5]

With the progress of the research, some other biomarkers of CSF are found. Total tau (t-tau) and phosphorylated tau (p-tau) proteins in CSF are considered as useful biomarkers of CJD. Tau is a brain microtubule-associated protein which can assemble into filamentous structures by itself that forming neurofibrillary tangles under pathological conditions. This kind of tau neurofibrillary tangles, referred to as tauopathies, is a common feature in neurodegenerative disease such as Alzheimer's disease. Recent studies show increased T-tau levels and increased T-tau to P-tau ratios in CJD patients,[23] with the diagnostic accuracy about 79.6%.[24] Levels of tau in CSF have also been suggested as a marker for molecular subtype (codon 129 genotype) of sCJD.[25] Other CSF biomarkers, such as neuron specific enolase, brain-specific creatine kinase, S100β protein and desmoplakin, have also been investigated as potential biomarkers, but have yet to be used clinically. Perhaps the combination of more than one CSF marker could contribute to the differential diagnosis of CJD.[26-29]

EEG

EEG is a reliable non-invasive diagnostic method for CJD, especially sCJD, and was extensively used in clinic even prior to 14-3-3 protein testing.[30] The typical feature of sCJD patient in EEG is periodic sharp wave complexes (PSWCs). The duration of PSWCs is usually 100-600 ms and the intervening background among PSWCs is generalized slow waves with low amplitude. PSWCs appear relatively later than the onset of clinical symptoms. In the early stages of disease, EEG usually shows diffuse slow waves. PSWCs become apparent by 8 to 12 weeks after the disease onset in most cases and occur even later in a few cases, but disappear in the end-stages of disease. Thus, the time to take EEG examination is extremely important.[31,32] In the regular EEG examination, the sensitivity of PSWCs is 64-66%,[32,33] and the specificity is about 80%.[34] Twenty-four-hour ambulatory EEGs could increase the sensitivity to about 79.6%.[19] EEG recording can increase the accuracy of the diagnosis from "possible" to "probable" sCJD if generalized PSWCs are demonstrated. Continuous video EEG monitoring may be useful in identifying the connection between clinical symptoms and EEG signal. In fact, PSWCs had some relevance to myoclonus.[35] Different from sCJD, vCJD shows non-specific slow-wave abnormalities or normal waves in EEG, but no typical PSWCs.[5,30] In iatrogenic CJD patients, the region of PSWCs is corresponding to the site of operation. In genetic CJD patients, the positive rate of PSWCs is only about 10%. The source and the pathophysiological mechanism of PSWCs remain unclear, although cortical and subcortical mechanisms have been proposed. The PSWCs of CJD are diffused discharges and may have multiple cortical sources or alternating ways of activation in cortex, involving a subcortical pacemaker. Frontal intermittent delta activity and triphasic wave-like activity are supposed to be forerunners of PSWCs and appear when cortical and subcortical gray matter are involved. Basal ganglia, thalamus and frontal cortex have also been suggested to be involved in generating PSWCs in CJD, but, experimental studies based on animal model are needed to validate this hypothesis.[36] PSWCs are also found in toxic encephalopathy, encephalitis and metabolic encephalopathy, especially hepatic encephalopathy.[33] Sometimes unilateral PSWCs can be observed in acute unilateral cerebral injury like cerebral infarction, encephalopyosis, and brain tumor. There are few breakthroughs in PSWCs for CJD diagnosis in recent years, compared to the in-depth studies on medical imaging of CJD. Further investigations of the pathophysiological mechanism of PSWCs and the relevance to clinical manifestation are warranted.

Diffusion weighted imaging

Diffusion weighted imaging (DWI) is a form of functional MR imaging which can detect the Brownian motion of water molecules in the pathological state by the means of Echo Planar Imaging technology. It is sensitive to the cytotoxic edema and is applied in the diagnosis of cerebral infarction most frequently. DWI is also the most widely used, intensive studied and highly valued MRI sequence for accurate radiological diagnosis and differential diagnosis of CJD at present. The typical performance of DWI on CJD patients is the abnormal ribboning hyperintensities in cerebral cortex and/or the abnormal hyperintensities in basal ganglia region. DWI signal changes reflect the presence of micro-vacuolation of brain tissue causing spongiform degeneration, which is confirmed by autopsy.[37] And it correlates well with the clinical manifestation and PrPsc accumulation.[38] The sensitivity of DWI for diagnosis is about 85-96%, and the specificity is about 93%, which is superior to that of conventional MRI (T1, T2 and FLAIR).[19,39-41] In a study on the diagnostic utility of CSF biomarkers and DWI, MRI-DWI was the best predictor, with a diagnostic accuracy of 97%. T-tau had a diagnostic accuracy of 79.6%,

and that of 14-3-3 protein was 70.4%.[24] The similar DWI signal can also be observed in patients affected by mitochondrial encephalomyopathy and toxic encephalopathy such as mercury poisoning.

The majority of sCJD patients (80-90%) have cortical abnormal hyperintensities on DWI, and the percentage of basal ganglia alterations is about 50-69%.[19,42] Other studies on DWI showed a fewer detection of increased signal in the thalamus of CJD patients. Thalamus involvement is more frequent in vCJD and VV2 subtype of sCJD patients. GSS is rarely abnormal in DWI.[5] A quantitative analysis of apparent diffusion coefficient(ADC) value can display slight changes in the thalamus of sCJD patients although abnormal signal may not be found visually. The DWI scan sequence of MRI shows high sensitivity for the abnormal hyperintensities in cerebral cortex and basal ganglia, but none of the standard MRI sequences reveal abnormal signal in cerebellum or brain stem of CJD patients. DWI appears sensitive to the restricted diffusion of water in cortex and basal ganglia but not in the cerebellum and brain stem. The abnormal regions may be unilateral at the disease onset then bilateral with time. In some cases the signal intensity decreases as the disease progresses.[40,43] Hyperintensities can be observed on DWI in early stage of sCJD when 14-3-3 protein and PSWCs are still negative. Recent study proposed that the patients with abnormal DWI hyperintensities in basal ganglia lesion had shorter disease duration and higher incidence of myoclonus. The lower apparent diffusion coefficient in basal ganglia indicated the faster presence of akinetic mutism and a shorter disease course.[44] However, others have not obtained the same results. Contrary to other rapid dementias, sCJD patients manifest wider range of hyperintensity on DWI than on FLAIR sequence.[41] The area of abnormal DWI hyperintensities are in accordance with clinical manifestation and the area of PSWCs. With the thorough researches on DWI, CJD diagnostic criteria are continuously updated. The abnormal signal of DWI was described as one of the diagnosis standards officially in 2009 (Zerr *et al*.[18]); Vitali *et al*.[41] and Meissner *et al*.[45] described the DWI hyperintensities in detail and proposed the MRI diagnostic criteria of CJD in 2009 and 2011, respectively.

Diffusion tensor imaging

Diffusion tensor imaging (DTI) is a relatively new MRI scan technique that reflects the diffusion anisotropy of water in cerebral white matter and the integrity of white matter fiber tracts.[46] The fractional anisotropy (FA) image of DTI can visually display the structure of white matter fiber. Mean diffusivity (MD) reflects the average diffusion of water molecules from all directions-the greater MD measured, the more water molecules unrestricted in the tissue. When there is fiber loss causing the increase in extracellular space, MD increases. When there is microstructural pathology like myelination defects in the fiber, FA decreases. DTI can reflect dynamic microstructural changing in brain tissue.[47] Previously, DTI was used to study pathology in neurodegenerative diseases such as Alzheimer's disease and dementia with Lewy bodies.[48,49] Recently, advances have been made in DTI for CJD diagnosis. For instance, significant decreases have been observed in MD, but not FA, in the caudate and pulvinar of sCJD patients compared to other rapidly progressive dementia patients and normal controls. Some studies hypothesized that the spongiform changes in CJD could restrict water molecule diffusion and lead to decreased MD and a relative preservation of FA.[50,51] Along with the changing of DWI hyperintensity in the disease course of CJD, MD decreases in the early stage of disease and tends to be normal or increase in the terminal stage. The increasing MD is associated with more significant loss of function. Neuronal loss increases water diffusion and augmentation of MD measurement in the late stages of CJD. In addition, the increased size of micro-vacuolation and their coalescence in end-stage of CJD might cause an increased free water flow.[52] The DTI test is highly sensitive, but not very specific. Therefore, DTI is an important tool for diagnosis, but alone is not sufficient for a CJD diagnosis.

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS) is a noninvasive examination that can quantitatively analyze specific atomic nucleus and their chemical components based on MRI technique and chemical shift. MRS displays the metabolism and biochemistry of pathological tissue in the form of spectrum. Proton magnetic resonance spectroscopy (^{1}H-MRS) is the most widely applied MRS technique, which can detect the resonance peak of more than twelve brain metabolite and neurotransmitter like N-acetyl-aspartate (NAA), creatine (Cr), myo-inositol (mI), and choline. Currently, ^{1}H-MRS is mainly used to study metabolic disorders of the CNS, tumors, and dementia disease. There have been very few studies on CJD although MRS provides information on chemical metabolism.

In one case report of sCJD, MRS detected marked extensive decreased NAA, and displayed increased myo-inositol/creatine ratio in basal ganglia and the insular cortex, along with slightly reduced choline/creatine ratio.[53] Similarly, other case studies revealed decreased NAA in basal ganglia, thalamus

and cortex.[54,55] MRS imaging in brain tissue of CJD patients, especially striatum, display moderate reduction in NAA, suggesting neuronal loss or dysfunction. ^{1}H-MRS can detect the absence of NAA, creatine and choline peaks in late-stage CJD patients, indicating no neuronal activity.

The metabolic changes of MRS can be detected in the initial clinical course of CJD, prior to the abnormal changes detected in DWI. For instance, the reduction in NAA/Cr ratio can be found in the brain tissue of CJD patients when DWI hyperintensity is still negative.[53] In one research on the differential diagnosis of patients with rapidly progressive neurological signs similar to the clinical symptoms of sporadic prion disease, ^{1}H-MRS presented great diagnostic value. The percentage of correctly diagnosed prion cases was 86% for DWI, 86% for thalamic NAA/Cr ratio, 90% for thalamic NAA/mI ratio and 86% for CSF 14-3-3 protein. The prion disease patients had reduced NAA/Cr ratios ≤ 1.21. In this study, researchers proposed that the combination of thalamic MRS and DWI may increase the diagnostic accuracy of the MRI scan,[56] whereas, other studies indicate that these changes in metabolism are sensitive but nonspecific. NAA is a neuronal marker that decreases in many conditions of neuronal injury, including degenerative disease. And myo-inositol is a glial marker, increased in glial proliferation. The increased myo-inositol may also be found in Alzheimer's disease, herpes simplex encephalitis, neuro-cysticercosis and progressive multifocal leukoencephalopathy.[53] Although MRS can quantitatively reveal the changes of chemical components in brain tissue infected by CJD, the studies on the diagnostic value of MRS lack specificity data. Larger sample controlled clinical trials are certainly needed.

18F-FDG PET/CT

18F-FDG PET/CT is a nuclear medicine examination method that can reveal the glucose metabolism of tissue without influence on the internal environment of human body. Clinical use of PET is now well established in early diagnosis of tumor, Parkinson's disease, Alzheimer's disease and in the accurate positioning of epileptogenic focus. PET is also used to study neural receptors, neurotransmitters, and clinical pharmacology. Theoretically, there is hypometabolism in the CJD patients' brain tissue, which is probably related to vacuolation and PrPsc accumulation. PET, as one of the most sensitive techniques to detect glucose metabolism of tissue, may be of great value for early diagnosis and appropriate differential diagnosis of CJD. PET is expected to detect cortical, basal ganglia or thalamic hypometabolism in CJD patients, while the FFI patients only present a slight hypometabolism in the thalamus. Generally, hypometabolism is more frequent and more severe in cerebral lobe cortex than in basal ganglia structures, and rarely in cerebellum and brainstem.[57] The metabolic alterations on PET appear earlier than DWI hyperintensities, partly because some vacuoles in hypometabolism tissue are too small to be detected by DWI. Some abnormal lesions which cannot be seen on DWI can be detected by PET especially in the early stage of CJD duration, suggesting a higher sensitivity of PET.[42] Others proposed PET was more sensitive than DWI for cortex, and DWI was more sensitive than PET for basal ganglia.[57]

PET is highly sensitive and an effective means of identifying early-stage tumors which is important for the differential diagnosis of CJD, particularly in identified from cases of paraneoplastic neurologic syndromes which shares similar performance including rapidly progressing dementia, prominent psychiatric symptoms, seizures and dystaxia.[19] Hypometabolism can also be detected in other rapidly progressive dementia diseases by PET, however, data on the specificity of PET among a large populations are limited. In Alzheimer's disease, the parietal lobe and the temporal lobe are most likely involved in hypometabolism on PET, whereas abnormal signals are less pronounced or occur later in the frontal lobe. In dementia with Lewy bodies, hypometabolism is most frequently detected in the occipital and temporoparietal cortex together with contrary hypermetabolism in putamen and pallidum. Hypometabolism in thalamic and upper brainstem can be observed in progressive supranuclear palsy.[57] Further study should be carried out to explore the value of PET for differential diagnosis between CJD and other rapidly progressive dementia diseases.

In addition, some studies have reported that the abnormal hyperintensities on DWI and hypometabolism areas on PET are correlated with the clinical symptoms.[19,42,58] For instance, abnormal changes in basal ganglia predict extrapyramidal tract signs and the radiographic abnormality appears earlier than clinical symptoms. Additionally, there are consistencies among the regions of abnormalities on DWI, PET and the regions of PSWCs.[59,60]

DEVELOPMENTS OF AUXILIARY EXAMINATION CAUSING A REVOLUTION IN DIAGNOSTIC CRITERIA

Pathology results from autopsy and brain biopsy

are still the gold standards for CJD diagnosis. The classic pathological features of CJD are spongiform degeneration, astrocytes gliosis and nerve cell loss. But the percentage of pathological diagnosis among CJD patients is very low. Some surgical and pathology departments are not active to do autopsy or biopsy because of the infectious and incurable characteristic. And the sterilization methods for contaminated instruments have not been promoted in many countries. Furthermore, many people refuse biopsy partly due to fear of invasive examination or refuse autopsy because of traditional cultural beliefs. Less invasive biopsy of the olfactory mucosa or skeletal muscle and other novel ultrasensitive seeding assays based on the amplified detection of PrP, such as real-time quaking-induced conversion, have been developed, but have not been extensively used in clinic.[61,62] Thus the diagnosis of CJD based on clinical manifestations and non-invasive examinations (14-3-3 protein testing, EEG, DWI, PET, *etc*.) remains significant.

The clinical diagnostic criteria for CJD were first proposed in 1979, using a combination of clinical features and EEG.[63] 14-3-3 protein test was assessed in subsequent years and added to the diagnostic criteria later. The World Health Organization (WHO) formulated detailed diagnostic guidelines in 1998, including clinic symptom, PSWCs of EEG, 14-3-3 protein and exclusion diagnosis. According to the diagnostic guidelines of WHO, 14-3-3 protein can provide a possible-to-probable diagnosis.[31,64] Abnormal findings on MRI were mentioned at that time but without high attention. In the manual for surveillance of human transmissible spongiform encephalopathies 2003, WHO described the diagnostic criteria for the four CJD subtypes (sCJD, gCJD, iCJD and vCJD, separately) in more detail. And the bilateral symmetrical pulvinar high signal on MRI brain scan was incorporated into the diagnostic criteria for vCJD.[30] As research has progressed, MRI has played an increasingly significant role in CJD diagnosis.[40,60,65,66] The clinical criteria for the diagnosis of CJD have been revised to include abnormal hyperintensities on DWI based in part on key studies (Zerr *et al*.,[18] Centers for Disease Control and Prevention[67]). Vitali *et al*.[41] also proposed detailed MRI diagnostic criteria. Although DTI and PET have received much attention, they are not readily used in diagnosis. In fact, PET is more often used for the exclusion diagnosis.

CONCLUSION

CJD is infective, untreatable and fatal. The treatment method which could reverse the disease course does not exist currently. Prior reports indicate that intensive life-sustaining treatments, such as assisted breathing with ventilator and nutritional support, can prolong the patients' lives, but could not prevent death.[68] The causative agent, prions, are not completely inactivated by exposure to high temperature, ultraviolet and ionizing radiation, or chemicals that are effective against common viruses.[69] In order to prevent transmission of this devastating disease and reduce the risk to public health, earlier and accurate clinic diagnosis of CJD are critically important. Auxiliary examination plays a significant role in CJD diagnosis. EEG is most widely used as the traditional examination method in clinic and DWI has the highest sensitivity and specificity. The value of 14-3-3 protein testing remains controversial. The use of MRS for CJD diagnosis in clinic is limited. Detection of tau protein, DTI and PET have considerable potential in the differential diagnosis of different rapidly progressive dementia, especially in the situation of overlapping clinical manifestations. In addition, DWI and DTI may be useful to assess pathological changes of CJD. The abnormal results of auxiliary examination, such as the region of PSWCs, the abnormal hyperintensity area of DWI and the low metabolism area of PET, are consistent with the symptoms and the area of pathologic change. Multiple combined auxiliary examination methods may increase the accuracy of diagnosis and differential diagnosis among CJD patients. Further investigation into potential auxiliary examination methods for earlier and more accurate diagnosis of CJD are certainly needed.

Authors' contributions

Concept and design: J.T. Zhang
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Conflicts of interest

There are no conflicts of interest.

Patient consent

No patients were involved.

Ethics approval

Not applicable.

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Relationship of cerebral microbleeds to inflammatory marker levels

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ABSTRACT

Aim: The purpose of this study is to investigate the incidence, distribution and risk factors of cerebral microbleeds (CMBs) and the relation between CMBs and inflammation in ischemic cerebrovascular disease. **Methods:** Two hundred and one patients without acute infarction or transient ischemic attack were enrolled. The presence and number of CMB were assessed on susceptibility-weighted imaging. The traditional risk factors of CMB were recorded. Levels of high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), and matrix metalloproteinase-9 (MMP-9) were tested. Logistic regression analyses were used for multiple-factor analysis of risk factors of CMB. **Results:** Of the 201 patients, 49 (24.38%) had CMB. Multivariate logistic regression analyses showed that the age, the prevalence of hypertension, silent lacunar infarction, white matter lesion, Montreal Cognitive Assessment Score, the using rate of antithrombotic drugs and levels of hs-CRP, IL-6, MMP-9 were the risk factors for CMB. After adjustments for traditional risk factors, inflammatory marker levels remained to be associated with CMBs. The adjusted odd ratios of hs-CRP, IL-6 and MMP-9 were 1.745 (1.342-2.270), 1.223 (1.018-1.533) and 1.284 (1.082-1.423), respectively. Furthermore, inflammatory marker levels were the risk factor for deep or infratentorial CMBs and lobar CMBs. **Conclusion:** The age, prevalence of hypertension, silent lacunar infarction, white matter lesion, MoCA Score, the using rate of antithrombotic drugs and serum hs-CRP, IL-6, and MMP-9 levels were the independent risk factors for CMBs.

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INTRODUCTION

Cerebral microbleeds (CMBs) are designated as a microvascular disease which has no related symptoms and physical signs.[1] CMBs has a round or ovoid signal of hypointensity (d < 10 mm) in T2* Gradient-Recall Echo (GRE) and susceptibility-weighted imaging (SWI) sequences.[2] Previous studies suggested that damage of cerebral microvasculature resulted from hypertensive vasculopathy, cerebral amyloid angiopathy (CAA) and ischemic brain damage.[3] CMBs are associated with hypertensive vasculopathy tends to occur in basal ganglia, whereas advanced CAA is associated with a lobar distribution.[1] CMBs exists widely in patients of ischemic and hemorrhagic stroke, but also in normal elderly individuals.[1] Previous studies have shown that stroke patients combined with CMBs have a significantly higher risk of recurrence.[3] The presence of CMBs was also independently associated with cognitive functions.[4] Multiple CMBs always indicate serious microvascular lesion which will increase risk of rebleeding.[5] At the same time, patients with CMBs had a higher risk of spontaneous hemorrhage while receiving anticoagulation and thrombolytic therapy.[5] CMBs are related to antiplatelet-related intracerebral hemorrhage.[6] Recently, an individual participant meta-analysis showed that C-reactive protein concentration was associated with the risk of coronary heart disease and ischemic stroke.[7] Inflammatory marker interleukin-6 (IL-6) retained a significant association with coronary heart disease and ischemic stroke.[8] A review revealed that matrix metalloproteinase-9 (MMP-9) levels were significantly correlated with severity of stroke, larger infarct volume and worse functional outcome.[9] For the above reason, we sought to choose high-sensitivity C-reactive protein (hs-CRP), IL-6 and MMP-9 to investigate the relationship between inflammatory marker levels and CMBs so as to prevent CMBs from developing.

METHODS

Population

Between June 2013 and March 2015, we recruited 286 participants with a age was greater than 45 years of age from both outpatient and in-patient services of the Department of Neurology, Peking University Binhai Hospital. In this study, the majority of the patients were without acute or transient neurologic impairment. We excluded 18 patients with a history of intracerebral hemorrhage and 9 patients with a history of traumatic brain injury. In addition, infectious diseases (*n* = 12), collagen disease (*n* = 8), malignant disease (*n* = 4) and patients with severe co-morbid medical conditions (*n* = 10) and those using anti-inflammatory medications (*n* = 13) were also excluded. We further excluded patients whose hs-CRP levels were higher than 8 mg/L (*n* = 11). Finally, 201 patients were included in the analysis. We divided the patients into 2 groups based on SWI, CMBs group (*n* = 49) and no CMBs group (*n* = 152), CMBs group was further divided into deep or infratentorial CMBs group (*n* = 30) and lobar CMBs group (*n* = 19) based on CMBs location.

Risk factors assessment

The data including age, gender, body weight, height and medical history of patients such as smoking, alcohol intake, heart disease，blood pressure, blood glucose, serum lipid and the use of antithrombotic drugs were recorded for analysis. Hypertension was defined as blood pressure ≥ 140/90 mmHg on measurements taken on at least two occasions, or patients with a history of hypertension and using antihypertensive drug. Hyperlipidemia was defined as triglyceride level ≥ 1.7 mmol/L; low-density lipoprotein cholesterol level ≥ 3.4 mmol/L; total cholesterol level ≥ 5.72 mmol/L, or use of cholesterol-lowering therapy. Diabetes was defined as fasting blood-glucose ≥ 7.0 mol/L and/or postprandial blood sugar ≥ 11.1 mol/L, or use of antidiabetic therapy. Body mass index was defined as weight [kg]/height [m]^2. Smoking was defined as ever smoking but giving up and current smoking. Habitual alcohol intake was defined as alcohol drinking more than 20 g/day antiplatelet drugs and anticoagulant drugs are regarded as antithrombotic drugs. Montreal Cognitive Assessment Scores were used to assess cognitive function.

Magnetic resonance imaging parameters and diagnosis of CMB

Brain magnetic resonance imaging (MRI) was performed with a 3.0-tesla MR unit (Achieva; Philips). Susceptibility-weighted imaging (TR = 17 ms, TE = 24 ms, 10-mm-thick slices with 5-mm spacing) was included in the routine protocol. All the images were compared and analyzed on the workstation by trained and experienced radiologist and neurologist who were blinded to clinical information at the same time according to the Microbleed Anatomical Rating Scale MARS.[10] A single CMB was defined as a small, round, or ovoid hypointensity of < 10 mm in diameter, evident on T2* GRE or SWI MRI sequences.[2] Microbleed mimics (e.g. vessels, mineralization, air-bone interfaces, partial volume artifact at the edges of the cerebellum) were excluded.[10] CMBs were divided into lobar microbleeds (cortex, subcortex, and white matter) and deep or infratentorial

microbleeds (basal ganglia, thalamus, brainstem and cerebellum).[11] Further, we made categories for "strictly lobar microbleeds" (persons with ≥ 1 new microbleeds restricted to a lobar location) and "deep or infratentorial microbleeds" (persons with ≥ 1 new microbleeds in a deep or infratentorial location with or without lobar microbleeds).[12] Silent lacunar infarction was defined as an area of low signal intensity on T1-weighted images with corresponding high signal intensity on T2-weighted images, whose diameter was > 3 mm and < 15 mm (e.g. dilated perivascular space).[13] Diagnostic criteria of white matter lesion: periventricular white matter lesions (WMLs) were scored according to the following patterns: no lesions (0 points); pencil-like or cap-like thin lesions (1 point); smooth haloes at lesion site (2 points); and irregular periventricular high signals extending to deep WM (3 points). Deep WMLs were scored according to the following patterns: no lesions (0 points); punctate separate lesions (1 point); fused lesions (2 points); and large fused lesions (3 points).The total score was obtained by adding the periventricular and deep WMLs scales together.[14]

Measurement of inflammatory markers

Blood was drawn with minimally traumatic venipuncture for measurement of serum inflammatory markers. Blood were centrifuged by 3,000 *g* for 15 min at 4 °C, and then aliquots were stored at -70 °C. Serum hs-CRP was measured by latex turbidimetric immunoassay with a sensitivity of 0.01 mg/L. Serum IL-6 and MMP-9 were measured by enzyme-linked immunosorbent assay (High Sensitivity Quantikine kit; R&D System) with a sensitivity of 0.01 pg/mL. Serum MMP-9 was also measured by enzyme-linked immunosorbent assay (High Sensitivity Quantikine kit; R&D System). The detectable limit for Serum IL-6 and MMP-9 were 0.01 pg/mL and 0.01 ng/mL, respectively.

Statistical analysis

Data was analyzed using SPSS 19.0. Measurement data was described as mean ± standard deviation (SD). Enumeration data was described as number (%). *T*-test and one-way analysis of variance was used for comparisons of continuous variables. We used χ^2 test for enumeration data. Skewed distribution data was described as (Media and Q1-Q3). Kruskal-Wallis test followed by the Mann-Whitney *U* test were used for comparisons between groups. Multivariate logistic regression analyses were used for calculation of odds ratio, in which logarithmically transformed values of inflammatory markers were used. The results are shown as the odd ratios (OR) with 95% confidence interval (CI). Probability values were 2-tailed, and values of $P < 0.05$ were considered significant.

RESULTS

CMB distributional characteristics

Of the patients, 49 (24.38%) had CMBs. The spatial distribution of the CMB number and location was as follows: deep or infratentorial, 166 in 30 patients (61.22%); lobar, 88 in 19 patients (38.78%).

Relations between CMBs and traditional risk factors

The baseline characteristics of the patients in this study are shown in Tables 1 and 2. There were significant differences in the traditional risk factors such as age, the prevalence of hypertension, coronary heart disease, silent lacunar infarction (SLI) and WMLs,

Table 1: Comparison of baseline characteristics between CMB group and no CMB group

Characteristics	CMB group (*n* = 49)	No CMB group (*n* = 152)	*P*
Age (years)	68.61 ± 7.76†	61.76 ± 11.06	0.011
Male (*n*, %)	29 (59.18)	79 (51.97)	0.483
BMI (kg/m^2)	26.89 ± 2.96	25.63 ± 2.76	0.068
Smoking (*n*, %)	21 (42.85)	37 (47.37)	0.642
Alcohol intake (*n*, %)	26 (53.06)	74 (48.68)	0.494
Hypertension (*n*, %)	33 (67.34)‡	61 (40.13)	0.001
DM (*n*, %)	20 (40.81)	60 (39.47)	0.868
HLP (*n*, %)	21 (42.85)	57 (37.50)	0.506
CHD (*n*, %)	29 (59.18)†	64 (42.10)	0.048
SLI (*n*, %)	35 (71.42)‡	47 (30.92)	0.001
WML (M, Q1-Q3)	5 (4-6)‡	2 (1-4)	0.000
Antithrombotic drugs (*n*, %)	27 (55.10)†	54 (35.53)	0.019
MoCA Score	24.45 ± 0.29‡	26.62 ± 0.21	0.006
hs-CRP [mg/L (M, Q1-Q3)]	6.83 (5.91-9.73)‡	3.20 (2.10-5.34)	0.000
IL-6 [pg/mL (M, Q1-Q3)]	8.23 (6.68-12.20)‡	5.59 (3.72-7.79)	0.000
MMP-9 [ng/mL (M, Q1-Q3)]	15.98 (13.65-18.46)‡	11.66 (7.78-15.77)	0.001

†$P < 0.05$, ‡$P < 0.01$, compared with the no CMB group. CMB: cerebral microbleeds; BMI: body mass index; DM: diabetes mellitus; HLP: hyperlipemia; CHD: coronary heart disease; SLI: silent lacunar infarction; hs-CRP: high-sensitivity C-reactive protein; IL: interleukin; MMP: matrix metalloproteinase

MoCA Scores or the using rate of antithrombotic drugs between the CMB group and no CMB group ($P < 0.05$, Table 1). Furthermore, we also observed similar associations between traditional risk factors and deep or infratentorial CMBs [Table 2]. Similarly, there were significant differences such as age, or the prevalence of alcohol intake, SLI and WML between the lobar CMB and no CMB group ($P < 0.05$, Table 2). Compared with the lobar CMB group, there was higher prevalence of hypertension and lower alcohol intake in deep or infratentorial CMB group [Table 2]. On logistic regression analysis, the association between traditional risk factors and CMBs are summarized in Table 3. The ORs (per 1SD increase; 95% CI) of age, the prevalence of hypertension, SLI and WML, MoCA Score and the using rate of antithrombotic drugs were 1.666 (1.062-2.632), 2.634 (1.067-4.767), 2.345 (1.056-5.226), 1.717 (1.132-2.603), 1.198 (1.023-2.268) and 1.234 (1.116-2.584), respectively. In addition, the association between traditional risk factors and deep or infratentorial CMBs were also stronger [Table 3]. However, for strictly lobar CMB, the only independent risk factor was age and the prevalence of alcohol intake [Table 3].

Relations between CMBs and inflammatory marker levels

We observed that serum hs-CRP, IL-6 and MMP-9 levels (median: 6.83, 8.23, 15.98, respectively) in the CMB group were higher than in those (median: 3.20, 5.59, 11.66, respectively) without CMB group [Table 1]. Similarly, we observed that hs-CRP, IL-6 and MMP-9 levels were higher in the deep or infratentorial CMB group (median: 7.01, 9.31, 17.63, respectively) or lobar CMB group (median: 6.82, 7.98, 15.84, respectively) than in those without [Table 2]. There was no significant difference between the deep or infratentorial CMB group and lobar CMB group. Connections between CMB and inflammatory markers are summarized in Table 4. Logistic regression analysis showed that each 1SD-increase in each inflammatory marker level was significantly associated with the presence of CMB after adjustment for age and sex (Model 1). After adjustment for age, sex, BMI, smoking, alcohol intake, hypertension, diabetes, hyperlipidemia, coronary heart disease, the useful of antithrombotic drugs and the presence of SLI and WML, inflammatory marker level remained to be associated with CMB (Model 2).

The adjusted ORs of hs-CRP, IL-6 and MMP-9

Table 2: Comparison of baseline characteristics between deep or infratentorial CMB group and lobar CMB group

Characteristics	Deep or infratentorial CMB group ($n = 30$)	Lobar CMB group ($n = 19$)	No CMB group ($n = 152$)
Age (years)	69.77 ± 7.76‡	67.54 ± 7.88‡	61.76 ± 11.06
Male (*n*, %)	19 (63.33)	10 (52.63)	79 (51.97)
BMI (kg/m^2)	26.65 ± 3.35	25.39 ± 2.89	25.63 ± 2.76
Smoking (*n*, %)	11 (36.67)	9 (47.37)	37 (47.37)
Alcohol intake (*n*, %)	12 (40.00)*	14 (73.68)†	74 (48.68)
Hypertension (*n*, %)	24 (80.00)‡**	9 (47.37)	61 (40.13)
DM (*n*, %)	13 (43.33)	7 (36.84)	60 (39.47)
HLP (*n*, %)	14 (46.67)	7 (36.84)	57 (37.50)
CHD (*n*, %)	20 (66.67)†	10 (52.63)	64 (42.10)
SLI (*n*, %)	24 (80.00)‡	11 (57.89)†	47 (30.92)
WML (M, Q1-Q3)	5.5 (4-6)‡	5 (4-6)†	2 (1-4)
Antithrombotic drugs (*n*, %)	18 (60.00) †	9 (47.37)	54 (35.53)
MoCA Score	24.20 ± 1.56‡	24.58 ± 1.07†	26.62 ± 0.21
hs-CRP [mg/L (M, Q1-Q3)]	7.01 (5.23-9.93)‡	6.82 (5.67-9.80)‡	3.20 (2.10-5.34)
IL-6 [pg/mL (M, Q1-Q3)]	9.31 (8.15-13.82)‡	7.98 (5.80-10.81)‡	5.59 (3.72-7.79)
MMP-9 [ng/mL (M, Q1-Q3)]	17.63 (14.02-18.91)‡	15.84 (13.02-16.74)‡	11.66 (7.78-15.77)

†$P < 0.05$, ‡$P < 0.01$, compared with the no CMB group; *$P < 0.05$, **$P < 0.01$, compared with the lobar CMB group. CMB: cerebral microbleeds; BMI: body mass index; DM: diabetes mellitus; HLP: hyperlipemia; CHD: coronary heart disease; SLI: silent lacunar infarction; hs-CRP: high-sensitivity C-reactive protein; IL: interleukin; MMP: matrix metalloproteinase

Table 3: OR (95% CI) for CMB status per 1SD increase in traditional risk factors

Variable	CMB OR (95% CI)	Deep or infratentorial CMB OR (95% CI)	Lobar CMB OR (95% CI)
Age	1.666 (1.062-2.632)†	1.773 (1.172-2.641)†	1.436 (1.134-2.442)†
Hypertension	2.634 (1.067-4.767)†	3.875 (1.795-5.955)‡	
SLI	2.345 (1.056-5.226)†	2.438 (1.226-4.750)‡	
WML	1.717 (1.132-2.603)†	1.850 (1.291-2.643)†	
MoCA Score	1.198 (1.023-2.268)†	1.230 (1.100-2.448)†	
Antithrombotic drugs	1.234 (1.116-2.584)†	1.290 (1.001-2.229)‡	
Alcohol intake			1.023 (1.008-1.058)†

†$P < 0.05$, ‡$P < 0.01$, compared with the no CMB group. SD: standard deviation; CI: confidence interval; OR: odd ratios; CMB: cerebral microbleeds; SLI: silent lacunar infarction; WML: white matter lesion

Table 4: OR (95% CI) for CMB status per 1SD increase in inflammation factors (values were log transformed for analysis)

Variable	CMB		Deep or infratentorial CMB		Lobar CMB	
	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)
hs-CRP	1.852 (1.435-2.391)‡	1.745 (1.342-2.270)‡	2.372 (1.680-3.262)‡	2.302 (1.520-3.482)‡	1.578 (1.043-2.337)‡	1.534 (1.009-2.259)‡
IL-6	1.469 (1.204-1.792)†	1.223 (1.018-1.533)‡	1.480 (1.102-2.492)‡	1.334 (1.008-3.660)†	1.422 (1.001-2.678)‡	1.508 (1.097-3.428)†
MMP-9	1.397 (1.196-1.632)‡	1.284 (1.082-1.423)‡	1.420 (1.241-1.799)‡	1.287 (1.145-1.599)‡	1.293 (1.142-2.423)‡	1.242 (1.178-1.409)‡

†$P < 0.05$, ‡$P < 0.01$, compared with the no CMB group. Model 1: adjusted for age and sex; Model 2: adjusted for age, sex, body mass index, smoking, alcohol intake, hypertension, diabetes, hyperlipidemia, coronary heart disease, the presence of silent lacunar infarction and white matter lesion and antithrombotic drugs. CMB: cerebral microbleeds; SD: standard deviation; CI: confidence interval; OR: odd ratios; hs-CRP: high-sensitivity C-reactive protein; IL: interleukin; MMP: matrix metalloproteinase

were 1.745 (1.342-2.270), 1.223 (1.018-1.533) and 1.284 (1.082-1.423), respectively. Furthermore, the associations between inflammatory marker level and deep or infratentorial CMB or lobar CMB remained significant after adjustment for age and sex (Model 1) and after additional adjustment for the traditional risk factors (Model 2). The adjusted ORs of hs-CRP, IL-6 and MMP-9 for the presence of deep or infratentorial CMB were 2.302 (1.520-3.482), 1.334 (1.008-3.660) and 1.287 (1.145-1.599), respectively. For the presence of lobar CMB, the adjusted ORs of hs-CRP, IL-6 and MMP-9 were 1.534 (1.009-2.259), 1.508 (1.097-3.428) and 1.242 (1.178-1.409), respectively.

DISCUSSION

The prevalence of CMBs varies from different population. A meta-analyze found that incidence of CMBs was 44% in ischemic stroke, 83% in intracerebral hemorrhage, and 5-6% in healthy adults.[15] In this study, the incidence of CMBs was 24.38%, 30 patients (61.22%) had deep or infratentorial CMB, 19 patients (38.78%) had lobar CMB. The prevalence of CMB in our study was higher than that in healthy adults, but, lower than that in ischemic stroke. The first possible reason was the patients who participated without acute infarction or transient ischemic attack. The second possible reason was the use of SWI, it may increase the prevalence and number of CMB detected, compared with T2 GRE.

On assessment, CMB are often categorized according to location, separating strictly lobar CMB from deep or infratentorial CMB. Lobar CMB represents cerebral amyloid angiopathy, but, deep and infratentorial CMB is attributed to hypertensive arteriopathy.[16] Poels *et al*.[17] found an association between atherosclerosis and deep or infratentorial CMB in individuals with uncontrolled hypertension. This may explain the theoretical assumption that deep or infratentorial CMB are attributed to hypertensive vasculopathy, whereas lobar CMB are a result of amyloid angiopathy.[18] Several studies have demonstrated strictly that lobar CMB was associated with the apolipoprotein E4 allele and diastolic blood pressure. On the other hand, systolic blood pressure, pulse pressure, silent lacunar infarction, white matter hyperintensities and smoking were associated with CMB in the deep or infratentorial regions.[19] In our study, hypertension, SLI, WML were associated with higher incidence of CMB, and thus were strong risk factors of CMB. The associations between traditional risk factors and deep or infratentorial CMB remained significant. Our results were consistent with previous studies. Shams *et al*.[16] observed that patients with CMB were significantly aged, had hypertension, and had lower cognitive function. A number of studies have shown that age is an independent risk factor of CMB. Vernooij *et al*.[11] found that the incidence of CMB increased with age from 17.8% in persons aged 60-69 years to 38.3% in those over 80 years. There were significant differences in MoCA Scores and using antithrombotic drugs between the CMB group and no CMB group. Several studies reported an association between the presence of CMB and impaired cognitive function.[20,21] Small vessel disease can be visualized as white matter hyperintensities and SLI but also as CMB on brain MRI.[21] A number of studies found that almost all the CMB patients had various degrees of WML and different numbers of SLI. Gregoire *et al*.[6] found that CMB were more numerous and prevalent in antiplatelet users who developed symptomatic ICH compared with matched antiplatelet-users who did not develop ICH. This data suggested a potential role for CMB as a risk factor for antiplatelet-associated ICH. The relationship between alcohol intake and CMB needed to be further studied.

Our study showed that the levels of hs-CRP, IL-6, MMP-9 in CMB group were significantly higher than those in no CMB group. The regression analysis showed that inflammatory factors such as hs-CRP, IL-6 and MMP-9 were the independent risk factors of CMB. Also, they were also the independent risk factors of deep or infratentorial CMB and lobar CMB. This suggested that

CMB can be seen as the common downstream product of hypertensive vasculopathy and cerebral amyloid angiopathy pathways.[22] In old patients, hemosiderin deposited around the capillaries, the hemosiderin leaking through expansile vascular clearance would accelerate the inflammation and result in small vessel disease and CMB.[23] Previous studies found that levels of hs-CRP, IL-6, and IL-18 are related to both deep and lobar CMB.[22] Hoshi *et al*.[13] found that IL-6 could result in increase of hs-CRP as an important cytokine in inflammation, it can be a predictor of SLI. Koh *et al*.[24] found that the levels of MMP-9 and hs-CRP were significantly higher in patients with CMB than in those without. Hemosiderin of brain microbleed is well known to activate MMP-9, increased MMP-9 activity enhances inflammatory markers related to deterioration of neurological function in ischemic stroke.[24] Pantoni *et al*.[25] found that destruction of Blood Brain Barrie played an important role during the process of CMB. MMP-9 would destroy the blood-brain barrier (BBB) by degrading the extracellular matrix, leading to the increased permeability of BBB.[26] The animal experiment also proved a close relationship between increased MMP-9 and chronic destruction of BBB.[27] CMB resulting from cerebral amyloid angiopathy could be confirmed as the presence of β-amyloid in the vessel wall, which activated microglia and T lymphocytes expressing heme oxygenase-1 activity and complement activation were prominent. The above studies supported that inflammatory reaction participate the dysfunction of vascular endothelium and destruction of BBB, finally leading to the occurrence of CMB.[28]

This study has several limitations. Firstly, the results of this research have limited effectiveness because of the relatively small number of cases, especially deep or infratentorial and lobar CMB group. More patients must be enrolled in a large clinical trial. Secondly, in the cross-sectional study, we did not recruit the general population as a control group. The relationships of CMB and inflammation in the general population remain unclear. Thirdly, we choose hs-CRP, IL-6 and MMP-9 to study the relationship between inflammatory marker and CMB. Other inflammatory factors need to be studied further to confirm the relationship between inflammatory marker and CMB.

In conclusion, our results demonstrated that CMB were closely related with the age, prevalence of hypertension, SLI, WML, MoCA Score, the using rate of antithrombotic drugs, levels of hs-CRP, IL-6, and MMP-9, suggesting a role of inflammatory processes in CMB. Patients with CMB have been consistently found to have an elevated risk of hemorrhagic stroke or an antithrombotic-related hemorrhagic complication.[29] In the near future, we may reduce levels of circulating inflammatory markers to prevent CMB from happening.

DECLARATIONS

Authors' contributions

Substantially contributed to the general idea and design of the study: C. Li, Z.R. Jia
Took part in designing the protocol: Y. Song
Contributed to data collection and drafted the manuscript: Q.L. Lu
Helped with the data analysis: L. Wang
Read and approved the final manuscript: Q.L. Lu, C. Li, Y. Song, L. Wang, Z.R. Jia

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Conflicts of interest

The authors declare that they have no conflict of interest.

Patient consent

Informed consent was obtained from all individual participants included in the study.

Ethics approval

All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Editorial **Open Access**

Neurocysticercosis and hippocampal damage: a causal link favored by epileptogenesis or neuroinflammation?

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Cysticercosis is a frequent parasitic infection of the nervous system that occurs when humans become intermediate hosts of the pork tapeworm *Taenia solium* (*T. solium*), after ingesting its eggs. The disease is usually transmitted person-to-person, from Taenia carriers to healthy individuals, through non-hygienic handling of food or by direct contact with human feces. Ingestion of undercooked pork contaminated with cysticerci as the cause of human cysticercosis is a common misconception, since the role of pigs is to maintain the infection cycle by causing human taeniasis.[1]

While cysticerci may invade the subcutaneous tissue, striated muscles and many other organs, clinically relevant disease is often observed in patients with neurocysticercosis (NCC), defined as the infection of the central nervous system and its coverings by the encysted larval stage of *T. solium*. Most symptomatic NCC patients (up to 80% of cases) develop recurrent seizures (epilepsy), which is most often seen in patients with parasites located in the brain parenchyma.[2] NCC is a major cause of secondary epilepsy in many developing countries and is currently considered the single most important disease explaining the higher number of epilepsy seen in these regions.[3] In addition, travelling and immigration of people from endemic to non-endemic areas has caused increased prevalence

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rates of epilepsy secondary to NCC in developed countries of North America and Western Europe.[4]

For a better understanding of mechanisms involved in the pathogenesis of epilepsy secondary to NCC, a review of the stages of involution of parenchymal brain cysticerci is mandatory. After lodging in the brain parenchyma, cysticerci establish as vesicular (viable) cysts, which provoke little or no inflammatory changes in neighboring tissues. In most cases, vesicular cysts degenerate and are transformed into calcified nodules due to the attack of the host's immune system. Intermediate involutive stages during which cysticerci experience such degeneration have been called "colloidal" (cysticerci showing degenerative signs but still with liquid contents) and "granular" (compact lesions with more advanced degenerative changes) stages, respectively.[5]

Parenchymal brain cysticerci in any of the aforementioned stages may be associated with reactive seizures. Compressive effect on the brain parenchyma is the most likely explanation for seizures occurring in patients with vesicular cysts. Degenerating cysticerci may be associated with seizures due to the inflammatory reaction induced by the host's immune attack. In cases of calcified cysticerci, the gliosis that develops around dead parasites or the exposure of remaining parasitic antigens (trapped in the interior of calcifications) to the brain parenchyma could be the cause of recurrent seizures.[6]

Semiology of seizures related to NCC is varied. Patients with multiple parasites may present with focal seizures and those with a single lesion develop generalized seizures.[7] The lack of anatomic-semiologic correlation between cysticerci location and seizure semiology in a sizable proportion of patients with epilepsy and NCC has long been a cause of debate and concern. This has even led to the hypothesis that both epilepsy and NCC might simply occur by chance in areas where NCC is endemic. While this is theoretically possible, MRI findings of inflammatory changes surrounding calcified cysticerci after a seizure in about 50% of cases, provides strong evidence favoring a cause-and-effect relationship between NCC and seizures.[8] In other cases, particularly in NCC patients with medically refractory epilepsy, chronic seizures have been shown to come from an associated atrophic or sclerotic hippocampus and not from the parasites themselves.[9] In these cases, the damaged hippocampus is for sure not an innocent bystander, but the result of signals or forces -- epileptogenic or inflammatory -- coming from the parasites.

Most patients with hippocampal sclerosis and epilepsy -- particularly mesial temporal lobe epilepsy (MTLE) have history of perinatal trauma, recurrent febrile seizures, status epilepticus, or traumatic brain injury. Such initial precipitating injuries often lead to the development of neuronal loss in CA1 and CA3 hippocampal layers.[10] The increasingly recognized association between granular or calcified lesions NCC lesions located within the hippocampus or in the adjacent cerebral tissue, points to this parasitic disease as the initial precipitating injury causing hippocampal atrophy and sclerosis.[11-13] In addition, pathological reports have shown neuronal loss in the CA1 layer and gliosis, as well as the presence of a severe inflammatory reaction in the brain parenchyma surrounding calcified cysticerci.[14] In this view, it has been postulated that calcified cysticerci could generate the development of both seizures and late hippocampal atrophy that will perpetuates the seizure disorder.

Hippocampal atrophy has also been documented in patients with calcified cysticerci located outside hippocampal areas. Different studies from Brazil and the Indian subcontinent, have revealed a significantly higher than expected prevalence of cysticercotic lesions among patients with MTLE undergoing surgery for medically intractable epilepsy.[11-15] This, together with the finding that patients with NCC and MTLS/hippocampal sclerosis have less often history of other types of initial precipitating injuries (febrile seizures) than those with MTLS/hippocampal sclerosis alone, led to the concept that a causal relationship between NCC and MTLE with hippocampal sclerosis exists. In addition, a population-based study conducted in an Ecuadorian rural population showed a strong association between NCC and HS in community-dwelling adults.[16] In the same population, it was demonstrated that this association is strongly related to age, suggesting that NCC-related hippocampal atrophy takes a long time to develop.[17] Interestingly, many of the studied individuals do not had epilepsy or electroencephalography evidence of paroxysmal abnormalities, showing that the association between NCC and hippocampal atrophy may occur irrespective of seizure activity (unpublished data).

In NCC patients, the relationship between epilepsy and hippocampal atrophy/sclerosis is most likely bidirectional. According to a current hypothesis, parasite-induced inflammation is the trigger for repetitive seizures, which may cause hippocampal atrophy, which is the pathological substrate for the subsequent development of MTLE. Parasites may be located outside the hippocampal region, suggesting

a remote harmful effect of NCC-induced reactive seizures. It is also possible that cysticerci may lead to an inflammation-mediated hippocampal damage, not requiring recurrent seizures as a causative factor, as has been demonstrated in experimental studies.[18,19]

Longitudinal studies are required to demonstrate a causal relationship between NCC and hippocampal atrophy. If this causal relationship truly exists, specific biomarkers could be identified to examine mechanisms of hippocampal epileptogenesis in humans, which have been scarcely investigated.[20] This knowledge might allow the development of interventions directed to prevent chronic epilepsy in NCC and other forms of acquired epilepsy.

DECLARATIONS

Authors' contributions

Study design and manuscript drafting: O.H. Del Brutto
Literature review, significant contribution to intellectual manuscript content: V.J. Del Brutto

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Conflicts of interest

There are no conflicts of interest.

Patient consent

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Ethics approval

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Case Report **Open Access**

Cytology abnormal of cerebrospinal fluid in superficial siderosis of the central nervous system

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ABSTRACT

Superficial siderosis of the central nervous system (SSCNS) is usually caused by chronic subarachnoid hemorrhage which leads to the accumulation of hemosiderin in the subpial layers of the brain and the spinal cord. The exact clinical manifestations and T2-weighted magnetic resonance imaging (MRI) the patient presented here is diagnosed SSCNS mainly due to the cytology of cerebrospinal fluid (CCSF) and the superficial siderosis of T2-weighted MRI. CCSF can be a good complementary to the diagnosis of SSCNS.

INTRODUCTION

Superficial siderosis of the central nervous system (SSCNS) is a rare disorder that is resulted from recurrent and persistent hemorrhage which leads to the accumulation of hemosiderin in the surface of the brain and the spinal cord. These are typically clinical syndromes: sensorineural deafness, cerebellar ataxia, dementia and positive signs of pyramidal tract. Here, we describe one case of a 72-year-old male patient who was diagnosed with SSCNS using magnetic resonance imaging (MRI) and cytology of cerebrospinal.

CASE REPORT

The patient was 72-year-old and presented with a 3-month history of progressive sensorineural hearing loss, cerebellar ataxia, several absence seizures and dizziness and was admitted in our hospital. No relevant trauma or acute symptom onset was reported. He had no particular hobbies and there was no significant family history of neurological illness.

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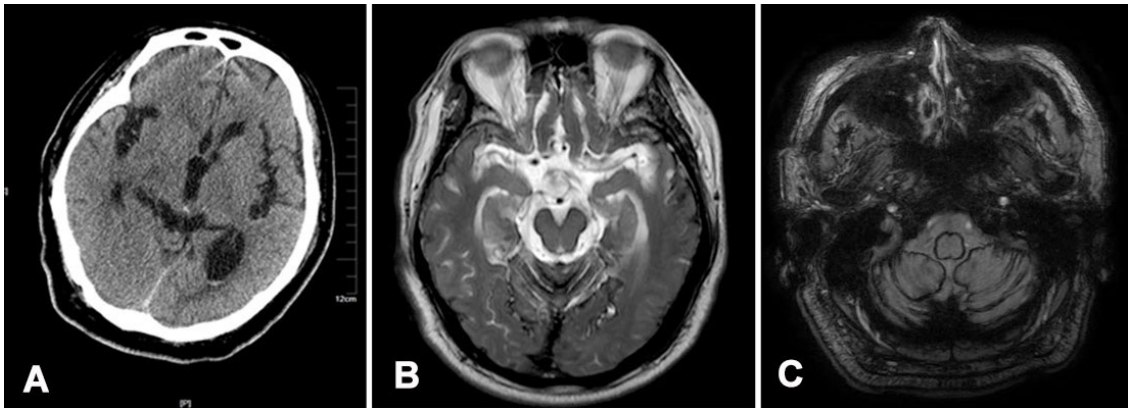

Figure 1: (A) Computed tomography scan showed that there was some blood in the posterior horn of the lateral ventricle; (B) T2-WI showing linear hypointensities along the surfaces of the brainstem and cerebellum; (C) susceptibility weighted imaging showing a dark rim around the cerebellar lobes and midbrain brain, corresponding to hemosiderin deposition

Abnormalities on neurological examination included decreased hearing in both ears, positive Babinski's sign on the both sides, and a wide-based gait. He was unable to maintain stable when asked to keep his eyes closed. Routine laboratory tests were normal. Computed tomography (CT) scan showed high density in the posterior horn of the lateral ventricle, and bilateral subarachnoid gap widened [Figure 1A]. No parenchymal hemorrhage was seen in the CT scan, T2-weighted and susceptibility weighted MR images (SWI) of the brain [Figure 1B and C] showed areas of linear hypointensity along the sylvian fissures, cortical sulci, surfaces of the brainstem and cerebellum. There was also evidence of significant cerebellar atrophy. Low signal could be seen in the posterior horn of the lateral ventricle form SWI [Figure 1C]. Extrallinear low signal on sylvian fissures and cerebral cortical sulci could be observed in SWI images. Lumbar puncture was performed, and it revealed the normal pressure of 140 mmH_2O. The color of the cerebrospinal fluid (CSF) was pale. And CSF analysis showed 46,050 red blood cells (RBCs)/mm^3 and 28 white blood cells (WBCs)/mm^3. Sugar in CSF was normal. Chloride is 111 mmol/L, protein was 1,324 mg/L. Immunoglobulin was normal in CSF. The acid-fast stain and ink smears were negative. We gave our patient an additional CSF cytology (CCSF). CCSF revealed that the white blood cell counted 30/mm^3, the red blood cell number counted 50,000 cells/mm^3. May-Grunwald-Giemsa staining was conducted and no abnormal shaped cells were seen. Lymphocytes were 54%, monocytes were 14%, neutrophile granulocytes were 27%. We could see RBC phagocytes 1%, hemosiderin phagocytes 4% [Figure 2]. Electroencephalography showed the sharp waves in the focal right antero-mid temporal together with normal background. The patient was then diagnosed with SSCNS, subarachnoid hemorrhage and epilepsy. The reason of bleeding from subarachnoid hemorrhage was not detected after detailed examinations including digital subtraction angiography of brain and spinal cord.

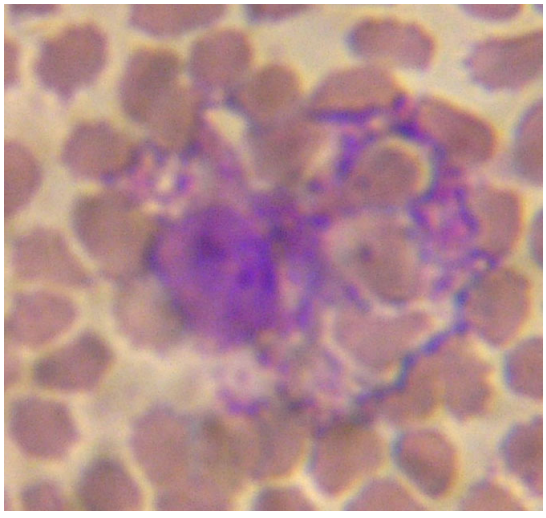

Figure 2: Cerebrospinal fluid cytology. A different number of red blood cells could be seen (MGG staining, ×1,000)

DISCUSSION

SSCNS is an uncommon disorder. Until 2006 less than 300 cases of SSCNS have been reported.[1,2] Hemosiderin deposits in the subpial layers of the spinal cord and the brain. The pathogenesis of SSCNS was not cleanly known by us. So far the general thought is that excessive iron from recurrent subdural bleeding leads to the loss of neurons and myelin, resulting in the development of a neurological deficit.[3] The most likely explanation for SSCNS is recurrent bleeding in the subarachnoid space. It may have lots of causes, including idiopathic (35%), CNS tumor (15%), head trauma (13%), arteriovenous malformation (9%). It is rarely seen in intradural neurosurgical operations, brachial plexus injury, nerve root avulsion, or other causes of subarachnoid hemorrhage (SAH).[4-6] In our case, the patient refused to the whole spinal

MRI. We were unable to identify the exact source of the bleeding, however, bleeding was present in the subarachnoid space.

T2-weighted MR imaging typically reports hypointensity along the surface of cerebellum and brainstem, corresponding to the deposition of hemosiderin. Cerebellar atrophy is also commonly associated with SSCNS.[7] In our case, T2-weighted images showed a rim of hypointensity around the cerebellum, brainstem, and Sylvian fissure, which helped us to diagnose this patient with SSCNS. In recent years, RBCs have been observed in CSF studies with SSCNS. The presence of RBCs in the CSF is the defining feature of SSCNS. A non-traumatic lumbar puncture was performed in our patient, the color was pale red. CSF was full of characteristics following analysis and cytology. It showed a large number of RBCs and obvious neutrophilic response. There were erythrocyte phagocytes and hemosiderin phagocytic cells measure in the CCSF. Seven days later, we were able to see bilirubin phagocytes. All the above types of phagocytic cells suggested that the cerebral hemorrhage had not stopped or might be a sign of rebleeding.[8] This kind of performance was consistent with its pathogenesis and pathophysiology. From what has been discussed above, CCSF can prompt the pathogenesis of the SSCNS, it was identified a beneficial supplement to CSF analysis. We come to a conclusion that CCSF can provide one of the objective bases of clinical diagnosis of SSCNS.

At present, we have no effective treatment for SSCNS.[9] Surgical treatment can be used to remove the potential source of recurrent SAH in order to arrest the clinical deterioration, but a number of patients has been reported to have experienced aggravated symptoms after surgery.[10] Iron chelating drugs were identified ineffective because of the blood-brain barrier (BBB). Recently, Schirinzi *et al*.[11] used soluble deferiprone that could cross BBB. He found that soluble deferiprone can improve ataxia. The CSF-ferritin concentration could be slightly reduced. However, the neuroimaging remained unchanged. After administering our patient with carbamazepine 100 mg three times a day, epilepsy symptoms disappeared. We could not find the cause of bleeding, no surgical intervention was performed. Now (1.5 years from the last discharge), he is still alive and sensorineural hearing loss. His cognitive function has badly declined and reports of always being in bed and needs the assistance of family members. He also reports of incontinence, but no reports of seizures. CCSF findings revealed the etiology and pathology mechanism of SSCNS. T2-weighted MRI and SWI showed that reliable changes of the head imaging. Combined with the patient's clinical manifestations, the diagnosis of SSCNS was obtained. T2-weighted MRI used together with the CCSF is strongly recommended in differential diagnosis of SSCNS.

DECLARATIONS

Authors' contributions

Conception, diagnosis and design: C. Wang
Manuscript preparation: J.X. Diao
Pathology diagnosis: S.M. Li
Manuscript revision: C. Wang
Final approval of manuscript: C. Wang

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patient.

Ethics approval

There is no ethics issue in this paper.

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Topic: Stroke **Open Access**

Role of neuroinflammation in ischemic stroke

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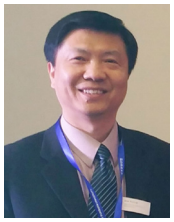

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ABSTRACT

Ischemic stroke causes the depletion of energy and induce excitotoxicity and neuroinflammation in the brain that results from thrombotic blockage. Neuroinflammation occurs initially depending on activated resident microglia that has the same function as the macrophage. Activated microglia participates in the neuroinflammatory process by phagocytosing the injured brain cells and producing the pro- and anti-inflammatory mediators. In this review, the authors present an overview of the role of microglia in mediating neuroinflammation in ischemic stroke.

INTRODUCTION

Stroke is an acute episode of focal dysfunction of the brain, retina or spinal cord lasting longer than 24 h, or for any duration if imaging (computed tomography or magnetic resonance imaging) or autopsy show focal infarction or hemorrhage relevant to the symptoms. Stroke is comprised of ischemic stroke (most common at approximately 85%) causing cerebral, retinal, and spinal infarction and hemorrhagic stroke (15%) that may result from intracerebral hemorrhage and subarachnoid hemorrhage [Figure 1]. Almost 90% of strokes are attributable to risk factors such as hypertension, regular physical inactivity, high apolipoprotein, insufficient diet quality, psychosocial factors, current smoking, cardiac causes, high alcohol

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consumption, and/or diabetes mellitus.[1]

Ischemic stroke is caused by arterial embolism and *in situ* small vessel diseases. Embolism in brain results in oxygen and glucose deprivation, leading to brain damage and neurologic deficit. The cellular and molecular mechanisms underlying ischemic stroke-induced brain damage have been extensively investigated. Excitotoxicity, oxidative stress, and inflammation have been considered as major contributors to ischemic neuronal injury.[2] Cerebral ischemia induces large release of glutamate that causes over-activation of NMDA receptors and large inflow of Ca^{2+}, leading to excitotoxicity-induced cell death.[3-7] The process of ischemia-reperfusion induces the production of superoxide and nitric oxide from damaged neurons and astrocytes and depletes glutathione, a primary antioxidant to protect against reactive oxygen species-mediated DNA damage.[8-10] Inflammation occurs after ischemia-reperfusion injury, which is caused by the dying cells and debris in the absence of microbes.[11,12]

There is an increasing evidence to showing complex role of the immune system in the pathophysiological changes that occur following ischemic stroke.[13] For example, brain injury activates neutrophils and macrophage/microglia,[14] as well as the lectin pathway of complement activation and the toll-like receptors (TLRs) that are the sensors in the innate immune system,[15,16] which leads to amplification of the inflammatory cascades. The immune system is closely involved in all the stages of ischemic stroke-induced brain damage and tissue repair by the parenchymal processes.[17,18] When activated, the adaptive immune system is intervened by lymphocyte populations that include T - B cells and regulatory T cells.[19] Additionally, stroke induces the deleterious antigen-specific autoreactive responses, but it also has beneficial effects.[20] The ischemic brain can act through the autonomic nervous system to have suppressive effect that can induce intercurrent infections and contribute to the morbidity and mortality after stroke.[21-23] Therefore, immune system-mediated inflammation is critically involved in determining the fate of the brain following ischemic stroke.[24-26] Understanding the mechanisms underlying role of neuroinflammation in ischemic stroke would provide important targets for the development of therapy in ischemic stroke.

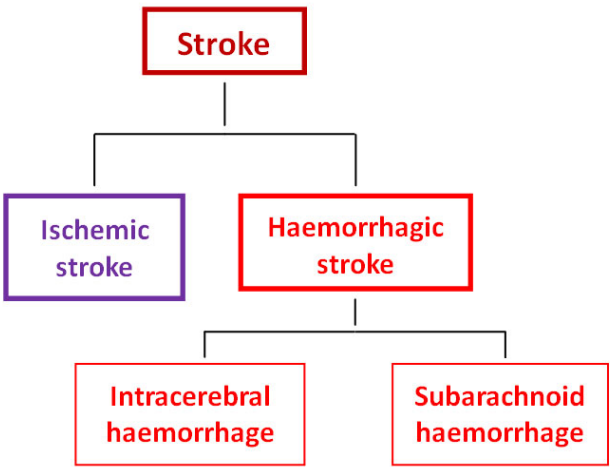

Figure 1: Stroke is comprised of ischemic stroke (85%) and hemorrhagic stroke (15%) (intracerebral hemorrhage and subarachnoid hemorrhage)

The aim of this review is to offer an overview of the current knowledge about the immune system and the neuroinflammatory processes in ischemic stroke. We focus on how the neuroinflammatory processes are triggered by ischemic stroke, and how microglia cells play a role in neuroinflammation after ischemic stroke.

NEUROINFLAMMATION

Neuroinflammation, an inflammatory response in the brain, occurs in a variety of acute brain diseases.[27,28] The non-diseased brain is separated by the blood brain barrier (BBB) from periphery.[29] The BBB prevents immune cells that are in the blood from entering brain tissue.[30] Brain is an independent immune-privileged organ with the innate. Neuroinflammation is regulated by the production of reactive oxygen species (ROS), cytokines and chemokines.[31] Once neuroinflammation happens, it enhances the release of several cytokines in the brain.[32,33] It also involves the reaction of innate immune cells (i.e. the microglia) in the parenchyma, the infiltration of myeloid cells and the adaptive immune cells (i.e. lymphocytes).[34] But the own innate immune system of brain operates mainly dependent on microglia, astrocyte and the expression of TLRs on these glia as well as the release of interleukins.[35,36]

Microglia is an innate immune cell that is well-characterized as the resident macrophage of the brain.[37] Astrocyte is important mediator of homeostasis in the brain.[38] These two cells are key players in the multicellular response to central nervous system (CNS) trauma and disease, including the immune reactions.[39,40] TLRs, the well-defined pattern recognition receptors of the immune system,[41] can initiate an immune response upon exposure to harmful microorganisms[42] and play a key role in macrophage activation. Neuronal TLR's play a central role in connecting the interactions between the immune system and the nervous system.[42] Interleukin's act as

essential innate immune modulators and conduct an array of biological processes.[43]

The neuroinflammation process is decided by the scene, duration and course of the neurological insult.[44] Neuroinflammation can perform function that are either supportive or destructive by which is determined by the immune signals relayed to the CNS. The nature of neuroinflammatory function can depend on the conditions and the intensity and duration of inflammation.[45] The positive role associated with neuroinflammation is only present for a brief, controlled inflammatory situations and responses and this can be considered as performing a protective function to the host organism.[46-48] For example, during low transient inflammation that may occur during infections, the immune cell signals to the brain by increasing the expression of interleukin (IL)-1 cytokine, this then increasing the 'survellience' role of glia cells in the brain if infected.[49,50] The transient inflammation of traumatic CNS injury, following the expression of IL-4, has been shown to promote injury recovery and axonal regrowth.[51,52] On the contrary, the negative aspects of neuroinflammation mainly represent maladaptive inflammatory responses.[53,54] The common characteristics of this aspect is increasing, supraphysiological production of cytokines [IL-1 and tumor necrosis factor (TNF)], ROS, and other inflammatory mediators including inducible nitric oxide synthase.[55] These markers are highly evident in the high traumatic CNS, giving rise to collateral damage.[56] Following the acute phase of CNS trauma, the IL-1 and IL-6 drive a low-level and chronic inflammatory response, leading to cognitive impairments and reduced neuronal plasticity.[57]

MICROGLIA AND NEUROINFLAMMATION

Microglia are the innate immune cells of the CNS, and are key modulators of the immune response in the brain.[37] Microglia is considered as the resident macrophage in the brain and the initial responders to tissue damage.[58] Microglia express receptors that respond to various stimuli that may as a consequence result in there activation.[59] A large number of studies indicate that microglia expresses different proteins and cytokines that display different role to express different function.[60] Activated microglia have two phenotypes: classically activated (M1) and alternatively activated (M2).[61] The M1 microglia are pro-inflammatory and thus secrete cytokines and oxidative metabolites such as IL-1β, TNF, IL-6 and nitric oxide,[62] whereas M2 microglia contributes to recovery after brain injury. M2 microglia expresses anti-inflammatory mediators, such as IL-10, IL-4 and give out various neurotrophic factors, which prevent inflammation and improve injury [Figure 2].[63] M1 microglia tends to induce neuronal cell death. Recent research has demonstrated that the M1 phenotype microglia can be switched to the M2 phenotype.[64] One study has shown that HIV-associated dementia initiates and maintains M1 phenotype microglia in the CD40 ligation by CD40L and TNFα. These microglia may later switch microglia to the M2 phenotype via up-regulation of CD45.[65] In a pathological condition, the corresponding stimuli may active microglia and cause them to change their shape and function and initiate phagocytosis.[66] Microglia works in close association with astrocytes to release cytokines that lead to a cascade of events which can modulate the neuroinflammatory respond. Meanwhile, the microglia cells produce and release excitotoxic metabolites that can damage surrounding tissue. Sometimes a short-term neuroinflammatory response is likely good for recovering the damages or infected tissue.[67] On the contrary, a long period of time neuroinflammatory process may damage the surrounding brain tissue.[68]

ROLE OF MICROGLIA IN NEUROINFLAMMATION AFTER STROKE

Neuroinflammation occurs in different types of brain injuries including ischemic stroke. Ischemic stroke mediated brain injury results in necrosis and apoptosis.[69-71] The damaged cells and debris induces neuroinflammation in areas in and around the ischemic injury in the brain.[72] Ischemia-induced cell debris and increased ROS lead to neuroinflammation by activating resident microglia and astrocytes as well as attracting infiltrating leukocytes from circulating blood.[73] These cells increase major

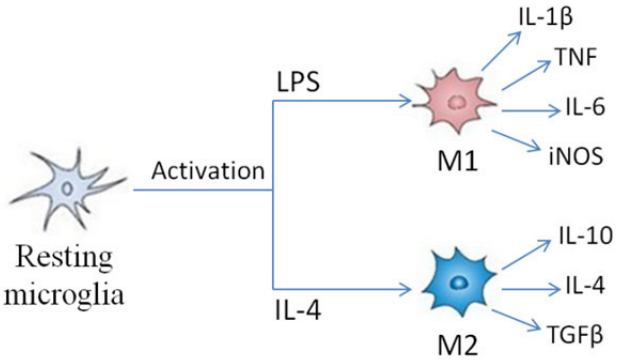

Figure 2: Activated microglia has two activation phenotypes: classically activated (M1) and alternatively activated (M2). M1 microglia is considered as pro-inflammatory, activated by LPS which produces pro-inflammatory cytokines and oxidative metabolites such as IL-1β, TNF, IL-6 and nitric oxide. M2 microglia contributes to recovery after brain injury activated by IL-4 and express anti-inflammatory mediators, such as IL-10, IL-4, TGFβ. LPS: lipopolysaccharide; IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor

histocompatibility complex class II molecules and cytokines.[74-76] Following activation of microglia, the release of pro-inflammatory mediators from these microglia favor the permeability of the BBB. Together with the secretion of chemokines, this promotes the successive entry of systemic leukocytes including neutrophils, macrophages and lymphocytes, which share several functional features with microglia.[77,78]

Microglia is the resident macrophage of the brain and a key modulator of immunologic responses after ischemic stroke. Under normal conditions, microglia is primarily involved in activity-dependent synaptic pruning and repair.[37] When ischemic stroke occurs, the native microglia undergoes morphological transformation from a ramified resting state in preparation for the forthcoming immune response.[79,80] Once reperfusion beginning, microglia come to be activated to an active, characterized by many branching processes in the penumbra, motile amoeboid state.[81] These activated microglia start to engulf endothelial cells via phagocytosis, which allows the entrance of blood serum components.[82] Active microglia phagocytoses foreign organisms as well as injured brain cells.[60,83] In ischemic stroke, activation of microglia is the early stages of the neuroinflammation process even within minutes.[83-85] Several reports have demonstrated that defective microglial activation increased the infarction and apoptosis after ischemic stroke.[86]

Microglial activation following ischemic stroke can promote activated microglia to migrate toward the ischemic hemisphere of the cerebral cortex.[87] It is suggested that active microglia have predominantly harmful effects in the acute stages of ischemic stroke and most beneficial effects appear in delayed stages.[62,88] Microglia morphology is changed either to M1, the typically activated phenotype, or to M2, an alternatively activated phenotype, after stroke.[61,89,90] M1 microglia activated by LPS and the pro-inflammatory cytokine interferon-gamma (IFN-γ) shows harmful effect after stroke.[91] In contrast, M2 phenotype microglia contribute to stroke recovery through anti-inflammatory cytokines such as IL-4.[92] In ischemic stroke, the M2 phenotype is dominant in both local microglia and newly recruited macrophages at earlier stages. The M1 phenotype increases progressively in peri-infarct regions. Thus, ischemic neuron induces changes towards the M2 phenotype in microglia and macrophages.[62] Considering the opposing roles of microglia phenotypes in ischemic stroke, it is critical to develop therapeutic strategy by restraining the morphological transformation and promoting the beneficial of microglia.

ROLES OF CYTOKINES IN CEREBRAL ISCHEMIA

IFN-γ

IFN is a type cytokines that plays a key role in the immune system. The IFN family cytokines are divided into two types. Type I IFNs constitute by a largest IFN class and comprise the IFN-α, -β, -ε, -κ, and -ω, type that share notable sequence homology and are produced by most cell types. IFN-γ is a unique member of the type II IFN.[93,94] IFN-γ is principally secreted by monocytes, macrophages, T cells, natural killer (NK) cells, dendritic cells and B lymphocytes. IFN-γ is a critical regulator of immune function and provides a robust first-line of defense against invading pathogens. Additionally, IFN-γ has plenty of biological functions including regulation of several aspects of the immune responses, stimulation of antigen presentation via upregulating class I and class II major histocompatibility complex (MHC) molecules on the surface of macrophages and T cells. IFN-γ when bound to its cognate receptor can activate a variety of downstream signaling pathways, particularly the Janus kinase (JAK)/signal transducer and activator of transcription (STAT).[95,96] All of these characteristics potentially influence the process of atherogenesis. Numerous lines of evidence have indicated that IFN-γ is highly expressed in atherosclerotic lesions and believed to have a critical role in the atherogenesis.[97] Stroke is the main atherosclerosis disease.[98] Under inflammatory conditions, MHC class II specific CD4+ cells will be activated. Activated CD4+ cells easily infiltrate through BBB into the CNS following cerebral I/R.[99] Therefore, microglia have the opportunity to retain and further stimulate CD4+ cells already primed to differentiate into T helper 1 (TH1) cells producing proinflammatory cytokines (IL-2, IFN-γ, TNF-α) or into T helper 2 (TH2) cells producing cytokines that support antibody-mediated responses (IL-4, IL-5, IL-10, IL-13).[100] IFN-γ is thought to have a key role in the polarization of microglia. TH1 cells produces proinflammatory cytokines IFN-γ that can return to activation microglia into M1 phenotype, shows pro-inflammatory response, and produces pro-inflammatory cytokines and oxidative metabolites.

IL-1β

IL-1β belongs to the family IL-1. IL-1β is a key immunoregulatory and proinflammatory cytokine that affects almost all cell types. IL-1β is produced following the formation of a inflammasome; such as monocytes and macrophage/microglia.[101] After Ischemic stroke, IL-1β can activate nuclear factor (NF)-κB via the activation of TLRs allowing NF-κB to transactivate genes associated with cytokines,

chemokines and other proinflammatory mediators.[102] In a pathological condition, IL-1β also connects with the activation and proliferation of astrocytes and microglia. After Ischemic stroke, the microglia will be activated, the M1 phenotype of microglia can express IL-1β which act as a proinflammatory cytokines to play neurotoxic effect.[62] In addition, IL-1β can prime the endothelium for increased leukocyte adherence and edema formation.[103] At supraphysiological levels IL-1β can be neurotoxic, however, IL-1β can also promote astrocytes to secrete survival promoting factors.[104] IL-1β when bound to its cognate receptor the IL-1 receptor (IL-1R) can also result in IL-1R-dependent increase in NF-κB pathways. However, if the levels of IL-1β are increased above a specific threshold, it can result in the increase of greater amounts of the IL-1 receptor antagonist (IL-1Ra). It is this balance between IL-1β and its antagonist the IL-1Ra that is more important for its global effect and role than just the IL-1β itself.[105] Thus, we predict that balance of IL-1β and IL-1Ra might be good predictor for patient outcome following ischemic stroke. However, few clinical studies have made use of their level as stroke biomarkers. IL-1β levels mostly were associated with poor long-term functional outcome in study,[106] while IL-1Ra levels have shown to be predictive of the development of post-stroke infections.[107]

Transforming growth factor beta

Transforming growth factor beta (TGF-β) proteins are multifunctional cytokines with pleiotropic functions.[108] TGF-β can regulate various biological processes, including hematopoiesis, angiogenesis, cell proliferation, differentiation, migration and apoptosis. TGF-β also plays an important role in the regulation of the immune system. TGF-β is a superfamily, including inhibins, activins, growth differentiation factors (GDFS), bone morphogenetic proteins (BMPs), TGF-β isoforms, and glial cell derived factors.[109] The main research object is TGF-β isoforms. TGF-β exists in at least three isoforms: TGF-β1, TGF-β2, and TGF-β3.[110] In the TGF-β superfamily, only TGF-β1, produced by activated microglia, and TGF-β2, produced by astrocytes and neurons.[111] TGF-β1 and TGF-β2 increased prominently after ischemic stroke. After Ischemic stroke, TGF-β produced by activated M2 phenotype macrophage, plays an anti-inflammatory role and contributes to recovery after brain injury.[63] TGF-β reduces microglial activation and thus reduces the potential harmful effects associated with activated microglia. TGF-β decreases the expression of other poisonous cytokines and suppresses the release of oxygen and nitrogen derived products. TGF-β can also stimulate the release of IL-1Ra and promote angiogenesis.[112] Its protective effects, however, are limited to the peri-infarcted area, as TGF-β can inhibit apoptosis but not necrosis.[113]

IL-4

IL-4, its congener of IL-13, a product of select immune cells that has highly polyfunctional properties. IL-4 is known to regulate a variety of immune and inflammatory responses, including T cell differentiation and IgE class in B cells.[114] IL-4 is primary produced by TH2 cells.[115] During CD4+ cellular activation, cytokines are through T cell receptor mediated signaling and co-stimulation. For instance, IL-4 mediated activation of the signal transducer and activator of transcription 6 plays an important role during TH2 cell differentiation.[116] IL-4 have an unique properties as it polarizes macrophages/microglia toward the M2 phenotype which is anti-inflammatory phenotype.[117] M2 macrophages/microglia expresses anti-inflammatory mediators and give out various neurotrophic factors that aid in the resolution of inflammation via increased trophic input and the augmentation of phagocytosis and proteolysis of dead, diseased cells/proteins, ultimately paving the way for tissue repair.[118] Consequently, IL-4 may have a neuroprotective function to promote tissue repair and may act as a therapeutic factor.

STROKE-ASSOCIATED INFECTION AND NEUROINFLAMMATION

Infection frequently occurs in both and after stroke that can induce immune and neuroinflammatory responses.[119-122] The characteristics of post-stroke infections include immune suppression, elevation of IL-6, decreases in TNF-α levels and inflammation are among the factors. Along with stroke-associated infection, inflammatory responses are the defense mechanism against infection and it can also be a pathogenic mechanism that precipitates stroke and neurological sequelae.[123] It is generally recognized that stroke-associated infection may be a source of inflammation and autoimmunity as infection facilitates the maturation of APCs into potent immunostimulatory cells.[124] Stroke-associated infection is mostly induced by virus.[125-127] Virus enters the CNS through two pathways: (1) hematogenous dissemination through BBB;[125] (2) neuronal retrograde dissemination.[126] It also suggested that virus can replicate in macrophage and CCR5+ T cells in the CNS.[127]

CONCLUSION

The role of neuroinflammation in ischemic stroke has drawn increasing attention. In this review, we summarize the relevance of inflammation in the nervous system and introduce the neuroinflammatory cells

and mediators that occur following ischemic stroke. Microglia is the resident macrophages of the brain. After ischemic stroke, the M1 and M2 phenotype of microglia play different roles at different times. The M1 phenotype tends to induce neuronal cell death, but M2 microglia contributes to the recovery after brain injury. Down-regulation of M1 phenotype and up-regulation of M1 phenotype are considered to be the potential strategy to counteract ischemic brain injury. Recent research has demonstrated that the M1 phenotype can be switched to the M2 phenotype. But the underlying mechanisms remain unclear. Thus, understanding how and why the M1 phenotype is down-regulated and the M2 phenotype up-regulated are important current and next steps to improve our understanding of the differing role of microglia post-stroke. Probing the mechanisms of M1-M2 switch could provide new approach to protect against ischemic neuronal death. Properly controlling the transformation of microglia is an important task in the treatment of ischemic stroke.

DECLARATIONS

Authors' contributions

Concept and design: Q. Wan
Data analysis, manuscript preparation and editing: R. Liu
Literature search: M.X. Pan, J.C. Tang, Y. Zhang, H.B. Liao, Y. Zhuang, D. Zhao

Financial support and sponsorship

None.

Conflicts of interest

There are no conflicts of interest.

Patient consent

No patients were involved.

Ethics approval

Not applicable.

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Advancements in diagnosis and treatment of meningeal carcinomatosis in solid cancer

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ABSTRACT

Meningeal carcinomatosis (MC) is a disease that malignant tumor cells cultivate in the cerebrospinal fluid or meninges. With the development of therapy methods and new techniques, survival time of patients with tumor is prolonged, and the incidence of MC is increasing. Diagnosis is based on the evaluation of clinical manifestations, cerebrospinal fluid and neuroimaging findings. Furthermore, in recent years, the diagnostic value of the tumor-derived cell-free DNA in the cerebrospinal fluid (CSF) is promising and may improve the diagnostic yield of CSF analysis. Traditional treatments of MC include surgery, radiation therapy, systemic therapy, and intrathecal therapy. Recently, molecular targeted therapy and immunotherapy have received more and more attention. The authors review the epidemiology, pathogenesis, clinical manifestation, diagnosis and treatment of MC in solid cancer, and discuss the diagnosis and treatment options currently available as well as those under investigation.

INTRODUCTION

Meningeal carcinomatosis (MC), also called neoplastic meningitis is a disease in which intracranial primary tumors or extracranial malignant tumors diffuse, disseminate or focally invade into the meninges and spinal subarachnoid.[1-4] In 1870, Swiss pathologist Eberth[5] demonstrated the selective infiltration of carcinoma cells in the leptomeninges in an autopsy case with lung cancer, while pathological anatomy revealed no inflammation in meninges. The term MC was proposed first to describe the clinical condition

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by Siefert in 1902. This disease was uncommon at that time, and usually confirmed by autopsies. In the past few decades, 10-30% of people with solid tumors progress to nervous system metastasis, with 4-15% developing into MC.[6,7] The metastases most frequently come from carcinoma of the breast, lung, gastrointestinal tract, and melanoma. Adenocarcinoma is the most common histologic types.[8] This disorder is easy to be misdiagnosed because of the diverse clinical manifestations and lack of specificity.

This article systematically reviewed the epidemiology, pathogenesis, clinical manifestation, auxiliary examination, diagnosis, treatment and prognostic aspects.

EPIDEMIOLOGY

With the development of imaging technique and therapies, the survival time of patients with MC is prolonged and the incidence of MC is growing. MC can occur secondary to tumors have not been discovered and in antitumor therapy, which was most common in older individuals. About 4-7% of patients with solid tumor suffer meningeal metastasis,[9-11] with lung cancer (9-25%), gastrointestinal tumor (4-14%), breast cancer (2-5%) and malignant melanoma (23%) as the most common causes. MC can be also detected clinically in 5-15% of patients with hematological malignancies (lymphomatosis or lymphomatous) and primary brain tumors (gliomatosis).[12] However, there are still cancers with an unknown primary (1-7%). Above all, MC is a relatively late event in carcinoma process.

PATHOPHYSIOLOGY

In many MC cases, the damages can be seen in autopsies as brain tissue edema, enlarged cerebral gyrus, meningeal congestion, cerebrospinal membrane greyish white in color, ventriculomegaly on sectioning. Microscopically, tumor cells can be seen in the cerebrospinal membrane and subarachnoid space diffusely or focally, while nodules are not obtained in cerebral parenchyma.

Cancer cells may leave the primary tumor to meningeal by following routes: malignant neoplasms cells may shift to subarachnoid space or cerebral ventricles by hematological invasion, with later spread to the cerebrospinal fluid (CSF). Both the perineural or perivascular spaces and cranial or radicular nerve pathway carry tumor cells to dura mater, leptomeninges, or the ependyma, leading to tumor deposits. Neoplastic cells may spread to the meninges directly.

DIAGNOSIS

The diagnosis of MC may be confirmed on the basis of the National Comprehensive Cancer Network guidelines.[13] The guidelines indicated that any one of the criteria listed below are sufficient to diagnose MC; positive CSF cytology; neuroimaging findings consistent with MC, supportive clinical signs and symptoms and a nonspecific but abnormal CSF changes (increased white blood cell count, decreased glucose, and high protein concentration) in patients suffering from tumor. Despite substantial false negative rate, CSF cytology remains the gold diagnostic standard. In addition to the cytological/neuroimaging/clinical diagnosis, other CSF parameters such as β-glucuronidase, creatine-kinase BB isoenzyme (CK-BB), *etc*. may be regarded as adjuvant diagnosing for MC and are also used to monitor the treatment response. Furthermore, the diagnostic value of the tumor-derived cell-free DNA in the CSF is promising and may improve the diagnostic yield of CSF analysis.

Clinical characteristics

Multiple interrelated events result in clinical symptoms of MC, such as obstruction of CSF reflux leading to hydrocephalus, nutrient metabolism competition between neoplastic cells and normal cells resulting in neurological function deficit, tumors invasion of Vichow Robin Spaces. Most patients with MC first presented with headache, nausea, vomiting, epilepsy, cervical radicular pain, hemiplegia and unconsciousness. In a cohort of 60 patients with breast cancer leptomeningeal metastases, headache was the most common presenting symptoms (55%), followed by various cranial neuropathies and epilepsy (50% and 12%, respectively). Vertigo presented in 12 patients (20%).[14] Classically, MC presents with various clinical signs and symptoms in three domains of neurologic function: the cerebral hemispheres; the cranial nerves; and the spinal cord and associated roots.[15]

Headache, nausea, vomiting are the most frequent manifestations of cerebral hemisphere dysfunction. Other signs include hemiplegia, aphasia, changes in mental status, seizures and cognitive impairments. However, simple focal ischemic cerebral injury and non-communicating hydrocephalus are uncommon. Diplopia, hearing impairment, hemianopsia, and trigeminal sensory loss are common symptoms of cranial nerve involvement with the VI cranial nerve being the most frequently impaired, followed by cranial nerve III and IV. The most frequent spinal

signs and symptoms include lower motor weakness, dermatomal sensory loss, pain in the neck or back and radiculopathy. Nuchal rigidity is less common which present in less than 15% of cases.[16,17]

CSF examination

Routine CSF examination

Intracranial hypertension (> 200 mmH_2O) is observed in 46% cases with MC. More than 90% of patients with MC have abnormal routine test and biochemistry indicators in CSF with increased leukocytes (> 4/mm^3) in 57%, elevated protein (> 50 mg/dL) in 76%, and decreased glucose (< 60 mg/dL) in 54%.[18] The nonspecific routine CSF examination should not dissuade consideration of this diagnosis.

CSF cytology

Cytological examination of CSF is still the golden criteria. The literatures reported[19] that the sensibility of May-Grunwald Giemsa stain method for diagnosing MC was 75-90%, with the specificity 100%. Prior related foreign researchers[20] suggest that the positive rate of CSF cytology with the first lumbar puncture is 45%, which increased to 80-90% with a second CSF exam. Little benefit is obtained from a third lumbar puncture. Of the 42 patients with MC accepted into the trial reported by He *et al*.,[21] the sensitivity of a first lumbar puncture is 85.7%, while the tumor cells were found in remaining 14.3% of cases from repeat lumbar punctures. There are still some false positive rates of CSF cytology check. Sometimes it is hard to distinguish the normal cells from the lymphoma cells.

Some simple measures can improve the sensitivity for the diagnosis including CSF sample disposal. The CSF specimen should be processed within an hour after collection which will improve the sensitivity of CSF cytology. Large CSF sampling volumes (> 10.5 mL) is also critical to improve the yield of CSF sensitivity.[22] May-Grünwald-Giemsa staining is better than Papanicolou stain for delineation of nuclear morphological characteristics and cytoplasmic limits. Nonetheless, there remains 25-30% of patients with MC diagnosed based on clinical picture, and radiographic findings, and persistently negative CSF cytology.[14,18]

Tumor markers

The evaluation of serial biochemical markers in the CSF may be of value in the adjunctive diagnosis of MC and assessment in therapeutic efficacy. Some biomarkers may be nonspecific, such as β-glucuronidase, CK-BB, lactate dehydrogenase, tissue polypeptide antigen, beta2-microglobulin, carcinoembryonic antigen, vascular endothelial growth factor (VEGF), which can be helpful as indirect indices of MC.[23,24] Other tumor markers such as carbohydrate antigen 15-3, carbohydrate antigen, carbohydrate antigen 125, carbohydrate antigen, neuron specific enolase, alfa-fetoprotein, CYFRA 21-1, and epidermal growth factor receptor (EGFR) can be relatively specific for MC when increased in CSF compared to serum.[25-27] Combined assay of different markers may enhance the sensitivity of MC diagnosis.[26] Occasionally, the biomarkers provide diagnostic support for MC in cases suspected as MC with negative CSF cytology.[28] However, detection of tumor cells in the CSF by CSF cytology remains the golden criteria for diagnosis of MC.

Genetic testing

When tumors diffuse into the central nervous system (CNS), the patients are usually already in an advanced disease stage and is unresponsive to therapy. Mechanisms of cancer dissemination and development within the CNS are unknown due to limited access to tumor tissue. Sasaki *et al*.[29] analyzed the EGFR mutation status of CSF straightly using real-time polymerase chain reaction that was more sensitive than cytology to diagnose MC in seven patients with non-small cell lung carcinoma (NSCLC) harboring an EGFR mutation (sensitivity of 100% *vs*. 28.6%). A separate study used next-generation sequencing by Pentsova *et al*.[30] to reveal somatic alterations in tumor-derived DNA from CSF in patients with CNS metastases of solid tumors and primary brain tumors. These studies demonstrated that identification of genomic mutations in tumor-derived cell-free DNA from CSF using a sufficiently sensitive platform in patients with CNS involvement. These techniques may be useful in complementing the diagnosis of MC, monitoring response to therapy and identifying resistance mutations. Therefore, in recent years, CSF has attracted the greatest attention and may be considered as a “liquid biopsy” for patients with MC. Currently, the technology of high-throughput sequencing of CSF may recognize cancer-related DNA in cases with known or suspected CNS involvement, which will provide significant aid for the diagnosis and treatment response.

Neurological imaging

Computed tomography (CT) is not sensitive in diagnosing MC with an estimated 23-38% of sensitivity of scan reported.[31,32] Magnetic resonance imaging (MRI) is considered the standard for the cancer patients with clinical suggestive of MC.[33] The sensitivity of MRI in the diagnoses of MC varied from 20% to 91%.[11,14,34] Subarachnoid or ventricular enhancing nodules, diffuse or focal leptomeningeal enhancement, ependymal, sulcal, and nerve root enhancement are common MRI findings in MC. Brain parenchymal metastases can be observed in 21-82% of MC.[34-37]

Any stimulation of the pia mater, such as subarachnoid blood, infection and cancer can produce enhancement of MRI. Lumbar puncture itself can induce a meningeal reaction resulting in leptomeningeal enhancement, so it would be better to conduct MRI examination prior the procedure.[38] Nevertheless, negative findings cannot be excluded the diagnosis of MC absolutely.

Researches on radionuclide using either ^{111}Indiumdiethylenetriamine penta-acetic acid or ^{99}Tc macroaggregated albumin are regarded as effective technique of choice to monitor and evaluate CSF flow dynamics.[39,40] CSF flow blocks have been demonstrated in 30-70% of patients with MC, with blocks usually arises in the skull base, within the spine and over the cerebral convexities.[40,41] Patients with CSF flow obstruction confirmed by radionuclide show shorter survival time when compared with those with normal CSF flow.[42,43] Managements of affected areas radiotherapy to the location of CSF flow obstruction resume flow in 30% of patients with spinal affected and in 50% of patients with intracranial involved.[44]

TREATMENT

Treatment of MC focuses on two aspects: therapy toward meningeal involvement and toward the primary cancer. In other words, patients with MC were given meningeal involvement therapy based on the primary cancer. As almost all patients with MC have been in advanced stage at presentation, palliative treatment such as radiotherapy, chemotherapy, biotherapy and molecular targeted therapy, *etc*. are usually the main treatment for primary tumor. Current treatments for meningeal involvement include surgery, radiation therapy (RT), systemic therapy, and intrathecal therapy, molecular targeted therapy and immunotherapy. Treatment should be targeted at alleviating the neurological symptoms, improving the quality of life and prolonging the survival time for the patients with MC. Therapy toward meningeal involvement mainly from the following aspects introduced.

Surgery

The main operative treatment in MC is ventriculo-peritoneal shunting for hydrocephalus due to CSF circulatory disorders and implantation of intraventricular reservoir for administration of cytotoxic chemotherapy drugs. Communicating hydrocephalus often occurs in patients with MC leading to symptoms of intracranial hypertension. Increased intracranial pressure can be relieved by surgery with a ventriculoperitoneal shunt to improve clinical symptoms if hydrocephalus continues. If possible, an on-off valve may be placed to permit the administration of intra-CSF chemotherapy.[45,46] Moreover, lumboperitoneal shunting may also be a therapeutic option in relieving clinical symptoms of intracranial hypertension in MC.[47,48] There are two types of reservoirs that be generally inserted in a region in the right frontal lobe: the Rickham reservoir, which be placed over a burr hole, and the Ommaya reservoir, a domed shape device that could be easily palpated.[49] The objective is to ensure a more uniform distribution of the drug within the subarachnoid space and to improve the curative effect of drug.

Radiotherapy

Radiotherapy is an integral part of MC therapy for patients with a syndrome of cauda equina, coexisting parenchymal brain metastases and CSF flow disturbance, which will alleviate symptoms, reduce bulky tumors volume and rectify CSF flow obstructions. Irradiation range of the whole brain irradiation (WBRT) include the cerebral meninges, basis cranii, basilar cistern, and the spinal canal to the plane of cervical vertebrae 1 and 2. WBRT is usually recommended at a dose of 30-36 Gy in fractions of 3 Gy, 40 Gy in 2 Gy fractions administered to patients with favorable prognosis,[45] for cases with a poor prognosis 5 × 4 Gy is an alternative to shortens the course of treatment.[50] It relieved pain and alleviated nervous system symptom but demonstrated no benefit to improve survival.[34] Craniospinal irradiation is rarely administered in MC because of its significant bone marrow toxicity. Focal radiotherapy can be administered safely in patients with bulky disease and obstructive lesions in short periods using a single dose via stereotactic radiosurgery, which is beneficial for patients with obvious syndrome of radicular pain and can result in reduced use of pain medicine.[45] In general, symptoms usually can be controlled after RT.[51,52]

Chemotherapy

Intrathecal therapy

Intrathecal chemotherapy is generally regarded as a modality to evade the blood-brain barrier (BBB) and blood-CSF barriers in MC. Four chemotherapy agents are received FDA approval for intrathecal injection: methotrexate (MTX), cytosine arabinoside (Ara-C), liposomal Ara-C, and thiotepa, with methotrexate as the broadest used drug in the treatment of MC. As antimetabolites, MTX and Ara-C are the firm rock in medical practice for MC caused by any primary cancer in decades. Liposomal Ara-C has similar curative effect, but its advantage lies in decreased frequency of intrathecal injection.[53] Additionally, trastuzumab and topotecan has recently been used in intrathecal chemotherapy in MC from breast cancer.[54-56] Topotecan, an alkylating agent, showed variable

effects.[57] A retrospective review of 149 patients with breast cancer-related MC showed that there was no significant difference in overall survival between patients treated with intra-CSF liposomal cytarabine and methotrexate, with the median overall survival of 4.2 months.[58]

Other retrospective studies demonstrated similar overall survival (OS) and remission rates with one intrathecally administered agent.[59] In addition, randomized studies showed that there was no difference in response of combined medicines (methotrexate, thiotepa, and cytarabine or methotrexate and cytarabine) and single-agent methotrexate in patients with MC.[60-62] Therapeutic effects in patients treated with intrathecal injection may be superior to those without IT treatment (P = 0.001).[34,35] Bone marrow suppression can occur after administration of intrathecal chemotherapies, which will be relieved after rescue with folinic acid (10 mg every 6 h for 24 h). Intra-CSF chemotherapy usually produces transient (< 5 days) chemical aseptic meningitis that manifest as fever, headache, nausea/vomiting, photophobia, meningismus and insanity, which may be mitigated by oral medications such as febrifuges, antemetics, and steroids in most cases.

Intrathecal administration of chemotherapy can be carried out either via spinal punctures or an intraventricular route. IT treatment can be performed by repeated lumbar puncture. Posture impacts ventricular drug levels after intralumbar administration and patients should remain prostration for at least one hour following treatment. Intraventricular administration of chemotherapeutic agents via an Ommaya or Rickham reservoir provide a couple of advantages compared with intralumbar treatment.[63] The process is indolent for the patients and would help physician be more efficient during clinical practice. In addition, IV administration also shows several advantages in pharmacokinetics which can make the drugs distribute uniformly in the entire subarachnoid ventricular spaces.[64] An improved OS was obtained for intraventricular administration compared with intralumbar chemotherapy in one clinical study of breast cancer patients with MC.[65]

Methotrexate. MTX is an anti-folate agent that inhibits dihydrofolate reductase necessary for the synthesis of folic acid required for DNA synthesis and tumor growth. The half-life of MTX is around 4-8 h. MTX is administered on a twice-weekly schedule for treatment induction and followed by weekly administration for consolidation. The following schedules have been recommended. MTX induction: 10-15 mg twice a week for 4 weeks. Consolidation: 10-15 mg once a week for 1 month and then every 2 weeks for 2 months. Maintenance: 10-15 mg every 4-8 weeks. For patients with intraventricular devices, the dose is cut in half. A retrospective study indicated that use of intensive-dose MTX therapy (15 mg/day, 5/7 days, 1 week on 1 week off) in MC patients with breast cancer had a median survival of 4.5-5 months.[37] Intra-CSF MTX eliminates tumor cells in 20-61% of cases with MC.[66] IT MTX treatment in the 1st month can achieve a cytological response predictive of a longer median survival (6 *vs*. 2 months).[67]

Cytosine arabinoside. Ara-C, a pyrimidine analogue, inhibits the synthesis of DNA. The half-life of ara-C is approximately 3.4 h in the CSF, which is much longer than in serum because the cytidine deaminase is low in CSF. The traditional ara-C will be completely cleared from the CSF within 1-2 days.[68] Similar to MTX, ara-C should be administered twice a week for treatment induction. Ara-C is relatively ineffective for MC secondary to solid tumors, but is a well established treatment for lymphomatous meningitis. Liposomal ara-C, a depot encapsulated form of ara-C (DepoCyt), provides a therapeutic ara-C concentration in the CSF for as many as 10-12 days with a half-life of 140 h. Intra-CSF administration of the liposomal ara-C may be once every 2 weeks. A randomized trial analyzed the survival rate difference in solid tumor-related MC treated with intra-CSF liposomal ara-C and MTX and there was no marked significant difference between the two groups (median survival 105 *vs*. 78 days).[69] The improvement in median time to neurologic progression with intra-CSF liposomal ara-C administration improved neurologic progression free survival (PFS) and reduced times of hospitalization for patients.[70] The schedule of intra-CSF administration as follows: liposomal ara-C induction: 50 mg every 2 weeks in weeks 1 and 3. Consolidation: 50 mg every 2 weeks in weeks 5, 7 and 9, followed by an additional dose at week 13. Maintenance: 50 mg every 4 weeks in weeks 17, 21, 25 and 29. Ara-C is initially administered at a dosage of 25-100 mg twice weekly with a 4-week induction, followed by 25-100 mg once weekly for 4 weeks of consolidation and 25-100 mg once a month for subsequent maintenance. If cytarabine is delivered intraventricularly, a dose reduction of 50% should be considered.

Thiotepa. Thiotepa, an alkylating agent, inhibits the cell cycle nonspecifically and available for routine intra-CSF chemotherapy. It shows the shortest half-life of all drugs used for intra-CSF chemotherapy with approximately 20 min and is cleared completely

in CSF within 4 h.

Intrathecal administration of thiotepa may be used in second-line treatment regimens for breast cancer-related MC patients who show poor response or fail to tolerate intra-CSF MTX. A randomized trial demonstrated that MC patients treated with intra-CSF MTX had significantly longer median survival compared with intra-CSF thiotepa (16 *vs*. 14 weeks).[71] Thiotepa Induction: 10 mg 2 or 3 times weekly for 4 weeks. Consolidation: 10 mg once weekly for 4 weeks. Maintenance: 10 mg once a month.

Innovative intra-CSF chemotherapy regimens. The growing number of patients with tumor who develop MC boost the investigation of new intra-CSF chemotherapeutic agents such as topotecan, alpha interferon, trastuzumab, rituximab.

(1) Topotecan. Topotecan, a topoisomerase I inhibitor, shows anticancer activity against various solid tumors of adult and childhood. A phase I study has shown a response in 3 out of 13 children received IT topotecan with primary brain tumors-related MC.[72] It is not clear if Topotecan, with good tolerability, produces any added benefit compared to other intra-CSF therapies. Therefore, IVent topotecan combined with other IVent agents may be an option due to its good tolerance profile. The treatment program is as follows. Induction: 0.4 mg twice a week for 6 weeks. Consolidation: 0.4 mg twice a week for 6 additional doses. Maintenance: 0.4 mg twice monthly for 4 months and then monthly thereafter; (2) biological modifiers. Intra-CSF administration of interleukin-2 has been evaluated in cases with MC secondary to melanoma. As previously reported with systemic therapy, some cases showed a long-term clinical response but some side-effects of therapy appeared.[73] In addition, interferon-alpha exhibited a moderate activity in a phase II trial of 22 patients with MC from a wide variety of solid tumor (median period of response: 16 weeks), combined with a transient chemical arachnoiditis and cumulative fatigue in most cases;[74] (3) monoclonal antibodies. In clinical studies, intra-CSF administration of monoclonal antibodies which targets the tumor antigens have been performed in patients with MC from solid tumors including breast cancer, ovarian cancer, melanoma and showed a rare long period of response (7-26 months).[75]

Trastuzumab. Approximately 3-5% of HER 2 positive breast cancer patients develop meningeal metastasis unlike the parenchymal brain metastasis (about 30%).[76,77] Primary tumor tissues and CSF neoplastic cell share tumor HER 2 status.[78] Trastuzumab CSF/serum ratios vary from 0.0023 mg/dL to 0.02 mg/dL in patients with MC regardless of WBRT, which result in very limited CSF concentration of trastuzumab.[79,80] The clinical practice of intra-CSF trastuzumab shows clinical and cytological success in patients with MC from HER-2 positive breast cancer.[81,82] A patient with MC received 67 cycles of weekly 25 mg IT trastuzumab with a long survival time (27 months) after MC diagnosis and dramatic clinical improvement.[54] Moreover, intra-CSF trastuzumab combined with intra-CSF MTX and ara-C has been performed in two patients with MC. The survival time of the two patients was 13.5 months and 6 months respectively with a clinical benefit and without substantial toxicity.[55] Intra-CSF administration of trastuzumab remains experimental and additional experience and data are required before consideration as a standard treatment.

Bevacizumab. Bevacizumab, an angiogenic inhibitor, target the VEGF ligand. Several studies showed higher levels of VEGF in CSF in patients with MC, supporting the hypothesis that angiogenesis promotes MC. The correlation coefficient was negative between VEGF and survival in these patients.[83,84] Bevacizumab is clinically approved metastatic colorectal cancer and NSCLC. Retrospective study manifested that bevacizumab was found to be safe in CNS metastases without inducing intracranial hemorrhage.[85] The assessment of intra-CSF injection of bevacizumab is ongoing in MC.[86,87] A pilot study of 15 patients with MC showed that bevacizumab resulted in a dramatically decreased CSF VEGF level and relief of clinical symptoms. Furthermore, a preclinical rabbit model of MC treated with intra-CSF bevacizumab has been evaluated.[88]

Systemic chemotherapy

The advantage of intra-CSF chemotherapy in solid tumors-related MC pales in comparison to hematological malignant tumor because of inborn chemical resistance, limited intra-CSF chemotherapy drugs availability, and the insufficient accessibility of large nodules to intra-CSF chemotherapy. In addition, MC is always accompanied with systemic disease, so it is reasonable to use systemic chemotherapy to simultaneously treat systemic disease and MC.[89,90] Treatment options of intra-CSF and systemic chemotherapy have been evaluated in solid tumors-related MC.[91,92] The overall response rate, the long-term survival rate and the median survival of patients with solid tumors-related MC who underwent intra-CSF chemotherapy combined with systemic chemotherapy and radiotherapy did not change despite increased neurotoxicity. Another prospective

study in patients with MC from NSCLC concluded that adding systemic chemotherapy to combined intra-CSF chemotherapy and radiotherapy did not improve the survival time due to insensitivity of the type of cancer.[93] Data from a retrospective study of 135 patients showed that the management of systemic chemotherapy is closely related to a longer survival time, which is a significant positive prognostic factors in patients with MC.[94] However, the choice of the systemic chemotherapy seems to be based not only on the chemical sensitivity of the primary tumor but also on its ability to pass through the blood-brain-barrier and the effective concentrations of drug in the CSF, which can image the chemical characteristics of the systemic agent. Treatment with high-dose intravenous MTX and cytarabine achieved therapeutic CSF levels in patients with hematological malignancy and MC from breast cancer.[22]

Myelosuppression is the dose-limiting factor of these treatment schedules. Moreover, these agents are toxic and limited by their narrow spectrum of activity in most solid tumors.

Molecular targeted therapy

Recently, the application of molecular targeted drugs in the clinic have achieved breakthrough results in patients with MC who show mutations in the *EGFR* gene or rearrangement of the anaplastic lymphoma kinase (*ALK*) gene in lung tumor, amplification of human epidermal growth factor receptor 2 (*HER2*) gene in breast cancer, and positivity of CD20 in B cell lymphoma.

Mutations in the *EGFR* gene and rearrangement of the *ALK* gene are the two the most frequently studied types of genetic mutations in NSCLC. Identification of the mutation status of the *EGFR* gene is crucial because patients harboring *EGFR* gene mutations are highly sensitive to *EGFR* tyrosine kinase inhibitor (TKI). *EGFR* mutations are independent positive prognostic factors in patients with NSCLC-related MC.[95,96] Liao *et al*.[97] indicated that MC patients receiving *EGFR* TKI therapy with an *EGFR* mutation showed longer overall survival compared with those without the mutation (10.9 months *vs*. 2.3 months, $P < 0.001$). *EGFR* TKI can pass through the BBB at levels of 1-3%.[98] A study demonstrated that high-dose gefitinib (750 or 1,000 mg daily) improves neurologic symptoms and achieve therapeutic levels in CSF in 57% of NSCLC patients with MC who is sensitive to *EGFR* TKI.[99] Erlotinib showed higher concentration in CSF (28.7 *vs*. 3.7 ng/mL, $P = 0.0008$) compared to gefitinib.[98] Moreover, a retrospective study indicated that the cytologic transformation rates in erlotinib treatment group were higher (64.3% *vs*. 9.1%, $P = 0.012$) than gefitinib treatment group in NSCLC with MC.[100] In addition, afatinib is an FDA-approved second-generation *EGFR* TKI for NSCLC with *EGFR* mutations and the effective treatment for CNS metastasis (brain metastasis or MC) in NSCLC who had an inadequate response to erlotinib or gefitinib.[101] However, there are no reports of the curative effect of afatinib in patients with MC who failed high-dose *EGFR* TKI. Osimertinib (AZD9291), a third-generation *EGFR* TKI, showed effectiveness in an *in vivo* MC model with a first-and second-generation *EGFR* TKI resistant.[102]

Rearrangement of the *ALK* gene is observed in around 4-5% of NSCLC patients. Identifying *ALK* rearrangement is important because patients with rearrangement of the *ALK* gene can be effectively treated with *ALK* inhibitors.[103] Crizotinib, a first-generation *ALK* inhibitor, shows poorly BBB permeability with a CSF-to-plasma ratio of 0.026, so the CNS remains a frequent site of recurrence for *ALK*-positive cases treated with crizotinib.[104,105] Several case reports showed a higher brain-to-plasma ratio of the second-generation *ALK* inhibitors (ceritinib or alectinib) compared to first-generation *ALK* inhibitors and better efficacy against MC for *ALK* positive patients with brain metastases.[106-108] Evidence from studies show that second-generation *ALK* inhibitors, especially ceritinib, may be treatment choices in MC patients from *ALK*-positive NSCLC.[109]

Amplification of *HER2* is found in about 15-20% of breast cancer cases.

Trastuzumab is a monoclonal antibody that acts via the *HER2* receptor and is effective for patients with *HER2*-positive breast cancer.[110] However, the effects have been limited due to BBB permeability in MC. Stemmler *et al*.[79] found that the ratio of serum trastuzumab to CSF trastuzumab in patients with brain metastases from breast cancer was 420:1 before radiation, 76:1 after radiotherapy, and 49:1 in cases with accompanied MC after radiotherapy. Trastuzumab is a highly effective intrathecal chemotherapy agent that can be used independently, or in combination with other drugs, for the management of MC from *HER2*-positive breast cancer.[55,111,112] Several studies revealed that intrathecal trastuzumab can be used safely and efficiently for *HER2*-postive breast cancer patients with MC with a wide dose range of 4-150 mg.[113] Lapatinib, as a dual TKI of *HER1* and *HER2* is effective for *HER2*-positive breast cancer patients who have progressed while on trastuzumab.[114,115] Nevertheless, there has been no reported data on lapatinib for

treatment of MC. Therefore, intrathecal trastuzumab is the only targeted therapy for MC in patients with *HER2*-positive breast cancer.

Rituximab, an anti-CD20 monoclonal antibody, shows efficacy in patients with diffuse large-B-cell lymphoma, its effects in MC are limited because of its large molecular size leading clinicians to study intrathecal rituximab.[116-118] A case-series analysis of relapsed CNS lymphoma demonstrated that intraventricular administration of rituximab showed efficacy in six cases. Intraventricular rituximab was administered in dose of 10-40 mg, produced a total elimination rate of malignant cells in CSF for three patients and a disappearance of leptomeningeal lymphoma nodules in one patient.[118] Therefore, these results show the potential of intrathecal rituximab for patients of MC with CD20-positive lymphoma.

Vemurafenib, a *BRAF* inhibitors, possesses a good perspective in late stage of melanoma patients with *BRAF* mutatation. In a case report, vemurafenib showed clinical and imaging responses and improvement of survival time.[119]

Immunotherapy

CpG-28, a Toll-like receptor 9 (TLR-9) agonist, can boost both the innate and the adaptive immune system through stimulation of TLR-9 and have antineoplastic activity in animal models.[120] In a phase I trial, 29 patients with MC received injection of CpG-28 both subcutaneously and intrathecally, which indicated the tolerance and feasibility of intrathecal injection with CpG-ODN for cases with MC.[121] The median PFS was 7 weeks and OS was 15 weeks. This new immunostimulating agent was also used in patients with recurrent glioblastoma, which showed good security and some cases of mild reactions.[122,123] Based on the current study results from each phase of clinical trials, immunotherapy has become a new direction of clinical researches on MC.

CONCLUSION

MC is the third most common CNS metastatic complication of systemic cancer with extremely poor prognosis. We summarized the epidemiology, pathophysiology, clinical manifestation, diagnosis and various therapeutic managements for solid tumor-related MC. The symptomatology is characterized by high intracranial pressure (headache, nausea, vomiting, consciousness disorder), cranial nerve involvement and radicular symptoms. The correct diagnosis depends on the contrast-enhanced magnetic resonance imaging of the spine and brain in combination with the cytological CSF analysis. In addition, the technology of high-throughput sequencing of CSF which recognizes cancer-related DNA will provide significant reference for the diagnosis in clinics. Traditional treatments including surgery, RT, systemic chemotherapy and intrathecal chemotherapy, but the prognosis for MC remains very poor with a median survival of < 3 months. Recently, molecular targeting treatment and immunotherapy have been applied to MC and have shown breakthrough results. The prognosis of MC may be affected by several factors such as age, performance status, primary tumor histology. Age of more than 50, low Karnofsky performance status, lung cancer or malignant melanoma as primary tumor may be the negative prognostic factors in cases with MC. Therefore, precise diagnostic techniques remain to be investigated, and novel therapeutic targets need to be found to improve the life quality and prolong the survival time for the patients with MC.

DECLARATIONS

Authors' contributions

Study concept and design: J.Y. He
Manuscript drafting: J.Z. Cui
Manuscript revising: Q. Li
Data collection: X.Q. Li, R.P. Gao, H. Bu
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Patient consent

There is no patient data involved.

Ethics approval

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Peripheral NK and B regulatory cell frequencies are altered with symptomatic exacerbation in generalized myasthenia gravis patients

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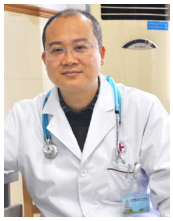

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ABSTRACT

Aim: Myasthenia gravis (MG) is an autoimmune disease, in which immunotherapy can improve symptoms for a period, but the majority of patients still experience symptomatic fluctuation or develop myasthenic crisis. This study aimed to explore the relationship between frequency of peripheral lymphocyte subsets and myasthenia gravis disease stage. **Methods:** The percentages of B regulatory (Breg) cells and natural killer (NK) cells in the peripheral

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blood samples obtained from 54 MG patients and 10 healthy controls were surveyed using flow cytometry. MG patients were subdivided into the ocular MG, generalized MG (GMG) in exacerbation stage and GMG in remission stage. **Results:** The percentage of Breg cells was significantly decreased in both the exacerbation stage (6.93 ± 1.18) and remission stage (6.56 ± 1.32) of GMG patients compared to healthy controls (15.97 ± 2.88). The percentage of NK cells were significantly increased in GMG patients in remission stage (20.69 ± 3.45) compared to healthy controls (11.33 ± 0.95). Frequency of NK cells in the patients in remission stage was significantly increased compared to patients in exacerbation (20.69 ± 3.45 *vs*. 12.32 ± 1.42). **Conclusion:** The Breg cells are involved in the pathogenesis of GMG, and NK cells are closely associated with the fluctuation of MG symptoms. NK cells could be a useful marker for MG activity and for monitoring effectiveness of immunotherapy.

INTRODUCTION

Myasthenia gravis (MG) is one of the most common autoimmune neurological diseases and affects the neuromuscular junction.[1] Although there are many therapeutic methods such as immunosuppressive therapy and steroids which are generally regarded as the effective preventive measures leading to marked symptomatic improvement in the majority of MG patients, there are many cases who still experience fluctuating muscle weakness and fatigability to some degree, or develop myasthenic crisis even after a clinical remission.[2-5] Yet the factors predicting the symptomatic fluctuation have not been well defined.

MG is mainly mediated by acetylcholine receptor antibodies (AChR-Ab), which were demonstrated to be associated with the severity of disease in individuals.[6] However, the heterogeneity of AChR-Ab and no elevated titers of AChR-ab in the minority of MG patients limited the clinical significance of measuring the antibodies.[1,7] In addition, there are some patients with elevated titers of antibodies against muscle-specific kinase (MuSK), Titin, RyR and LRP4,[8-11] signifying the heterogeneity of the autoimmune disease. Identification of specific markers of disease activity and severity is of great interest to clinicians. Changes in peripheral lymphocyte subsets were reported in patients with MG as well as patients with other autoimmune diseases, including systemic lupus erythematosus,[12] rheumatoid arthritis[13] and Sjogren's syndrome,[14,15] suggesting that these abnormal distributions of lymphocyte subsets may be involved in the pathogenesis of autoimmune diseases.[16-19] $CD4^+$ T helper cells have an important role in an experimental animal model of myasthenia gravis (EAMG) and MG patients as they influence autoreactive B cell production of anti-AChR antibodies.[20] Th1, Th2, Th17 and regulatory T (Treg) cells, which were differentiated from Naïve $CD4^+$ T cells, have been shown to be involved in the pathogenesis of MG.[21] B cells, because of the key contribution to humoral immune responses involving the secretion of antibodies, are generally considered to be pathogenic in the majority of autoimmune diseases. Recent evidence indicates that specific B cell subsets can negatively regulate immune responses by constraining Th1 and Th17 responses, indicative of the existence of regulatory B (Breg) cells.[22] Natural killer (NK) cells predominantly participate in the innate immune response to infections. NK cell-derived interferon-gamma activates Th1 helper cells,[23] and NK cells can impact acquired immunity. The subsets of lymphocytes mentioned above have been demonstrated to be involved in the pathogenesis of MG, but the role of lymphocyte subsets in symptomatic exacerbation in MG patients is not fully understood.

NK cells which play an important role during viral infection and tumor diseases, were considered to be involved in the regulation of autoimmunity in animal models and in humans.[24-26] NK cell numbers were found to decline with progression of most autoimmune diseases of humans.[27-29] Interestingly, the number of NK cells was increased significantly after plasmapheresis in patients with MG, indicating that they may become an important marker for monitoring clinical activation in MG patients.[16]

Traditionally, B cells have been thought to contribute to the antigen (Ag)-specific autoantibody production. Nonetheless, the role of B cells extends beyond the production of antibodies in autoimmune diseases. Recently, B cell subsets with regulatory functions were identified and the studies focused on Breg cells, also known as B10 cells, which express IL-10 and $CD1d^+CD5^+CD19^+$ phenotypes.[30-32] Recent studies showed that Breg cells can prevent the development of EAMG,[33] and were involved in patients with autoimmune diseases.[31,34-36] However, whether there are corresponding changes in Breg cells associated with severity of diseases remains elusive.

In this study, we investigated the changes in peripheral NK cells and Bregs in MG patients experiencing deterioration, in attempt to find a reliable marker

for MG activity and for monitoring effectiveness of immunotherapy.

METHODS

Patients

In this study, 54 patients with MG who met the standard clinical criteria[37] and 10 healthy controls (HC), 4 men and 6 women, aged 38.50 ± 16.37. All the participants signed the informed consent prior to the enrollment. The study was performed in accordance with the 1964 Declaration of Helsinki (including amendments) and has been approved by the Ethics Committee of Huazhong University of Science and Technology. Exclusion criteria were severe heart diseases, severe kidney diseases, severe mental illness, acute or chronic hepatitis, tuberculosis or HIV infection. The mean ± standard deviation of the ages of the patients was 40.30 ± 14.81 years. The sex ratio (male:female) was 28:15. The clinical features of the participants were summarized in Table 1. Eleven patients presented with ocular MG (OMG) (age: 35.36 ± 4.57; sex ratio: 5:6), and 43 with generalized MG (GMG). The GMG patients were further divided into two groups according to the clinical status. Group I included 25 patients who were in exacerbation stage (PE), presenting symptomatic exacerbation or myasthenic crisis (age: 44.52 ± 2.58; sex ratio: 8:17). Group II consisted of 18 patients who were in remission (PR), manifesting slight limb weakness or ocular symptoms for at least three months (age: 35.44 ± 3.66; sex ratio: 7:11). Overall, 43 patients were on prednisone and trans-sternal extended thymectomy was performed on 30 patients, including 8 with thymic hyperplasia and 22 with thymoma. The identified factors which provoked the deterioration of MG symptoms were upper respiratory tract infection (14 cases), inappropriate drug use (4 cases), surgery (1), exhaustion (1), and stress (1) [Table 2].

Flow cytometric analysis

The peripheral blood samples from all the participants were obtained by venipuncture and stored in tubes containing ethylene diamine tetraacetic acid (5.4 mg/tube). Each sample was divided into 2 tubes, each one containing 100 μL fresh whole blood which was further incubated with 5 μL of mAbs (Becton Dickinson) for 20 min in dark at room temperature. Breg cells were stained with FITC conjugated anti-human CD19, PE conjugated anti-human CD1d, APC conjugated anti-human CD5, and NK cells were analyzed after staining with PE conjugated anti-human CD16, PerCP conjugated anti-human CD3, APC conjugated anti-human CD56, respectively. Isotype-matched immunoglobulin served as control for analysis of $CD1d^{+}CD5^{+}CD19^{+}$ B cells and $CD3^{-}CD16^{+}CD56^{+}$ NK cells. After incubation, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). The remaining cells were resuspended in PBS then at least 30,000 lymphocyte events were acquired from each tube to determine their proportions using the FACS Caliber flow cytometer (Becton Dickinson).

Statistical analysis

All statistical analyses were performed using the SPSS software application. The data were expressed as the mean ± standard error. For a comparison of the different subgroups of the MG patients and the healthy controls, the data were analyzed using two-tailed Student's *t*-test. *P*-values lower than 0.05 were considered to be statistically significant.

Table 1: Clinical characteristics of the MG patients

Characteristic	Patients in exacerbation (number)	Patients in remission (number)	Ocular MG (number)
Gender			
Female	17	11	6
Male	8	7	5
Age (year, mean ± SD)	44.52 ± 2.58	35.44 ± 3.66	35.36 ± 4.57
Osserman's classification			
Class I			11
Class IIa	1	6	
Class IIb	21	12	
Class III	1		
Class IV	2		
Thymoma	9	11	2
With thymectomy	6	8	1
Thymic hyperplasia	1	5	2
With thymectomy		3	1
Use of prednisone	24	17	2
Immunosuppressant			
Tacrolimus	1		

MG: myasthenia gravis; SD: standard deviation

RESULTS

Frequencies of Breg cells decreased in the peripheral blood of GMG patients

We have analyzed the frequencies of peripheral Breg cells in the MG patients. $CD19^+CD1d^+CD5^+$ B cells are routinely regarded as Bregs. To characterize the Breg cell subpopulation, we have determined their surface expression of CD1d and CD5 with CD19 gating, the 2 markers that are believed to be co-expressed by "regulatory" B cells.[38] The data revealed that the percentage of $CD1d^+CD5^+$ cells among the $CD19^+$ were significantly decreased in both the exacerbation stage (6.93 ± 1.18) and remission stage (6.56 ± 1.32) of GMG patients compared to healthy controls (15.97 ± 2.88; P = 0.001 and P = 0.002, respectively) and topatients with OMG (16.08 ± 2.88; P = 0.001 and P = 0.002, respectively). Bregs in the peripheral blood of OMG patients did not show any decrease, instead a slight increase. But no significant difference was found between those in the ocular MG patients and the controls (P = 0.978). The imbalance of Bregs in GMG was more obvious. Usually the patients with GMG display different levels of severity, and a lot of patients may experience symptomatic fluctuation or develop myasthenic crisis. In order to clarify whether Breg cells play a key role during symptomatic exacerbations, we further compared the frequencies of these cells at different stages of GMG patients, and found that the frequencies of Breg cells in GMG patients were not significantly different between stages (6.93 ± 1.18; 6.56 ± 1.32; P = 0.834) [Table 3; Figure 1].

Table 2: Factors leading to exacerbation

Factor	Number	Ratio (%)
Infection	14	56
Inappropriate drug use	4	16
Unknown cause	4	16
Surgery	1	4
Exhaustion	1	4
Stress	1	4

Frequencies of NK cells correlate with clinical stages of GMG patients

NK cells ($CD3^-CD19^+CD56^+$) are an important player in the regulation of acquired immunity and the levels in the peripheral blood of MG patients have been analyzed. The percentage of $CD3^-CD19^+CD56^+$ NK cells was increased to different degrees, especially in GMG patients in remission stage (20.69 ± 3.45) compared to healthy controls (11.33 ± 0.95; P = 0.017). In the patients with OMG, who presented with mild symptoms, NK cells tended to increase compared with those in the healthy controls (18.98 ± 3.81). Yet the increase was less than that in GMG patients in remission stage and there was no statistical difference. The percentage of NK cells in the GMG patients at exacerbation stage also tended to increase, but the difference was not significant when compared with that in the healthy controls (12.32 ± 1.42). In order to determine whether NK cell frequencies were associated with symptomatic exacerbation, the percentages of NK cells were compared between the two subgroups of GMG patients. The frequency of NK cells in the patients in remission were significantly increased compared to patients in exacerbation (20.69 ± 3.45 *vs*. 12.32 ± 1.42, P = 0.017, Table 4, Figure 2). The data suggest that NK cells are involved in the pathogenesis of MG and mayplay a protective role during development of the disease.

Frequency of lymphocyte subsets in patients with exacerbation and infection

It's well known that various factors such as infections, inappropriate drug use, or neuro-endocrine dysfunction

Table 3: Frequencies of Breg cells in the peripheral blood of MG patients (%)

Group	Number	$CD19^+CD1d^+CD5^+$ B cells	P value[a]	P value[b]	P value[c]
Patients in exacerbation	25	6.93 ± 1.18	0.834	0.001	0.001
Patients in remission	18	6.56 ± 1.32		0.002	0.002
Ocular MG	11	16.08 ± 2.88			0.978
Healthy controls	10	15.97 ± 2.88			

$CD1d^+CD5^+CD19^+$ represent the Breg cells; the results represent the mean ± standard error; P value[a] as compared between the patients in exacerbation and patients in remission; P value[b] as compared with ocular MG; P value[c] as compared with healthy controls. MG: myasthenia gravis

Table 4: Frequencies of NK cells in the peripheral blood of MG patients (%)

Group	Number	$CD3^-CD19^+$ $CD56^+$ NK cells	P value[a]	P value[b]	P value[c]
Patients in exacerbation	25	12.32 ± 1.42	0.017	0.126	0.569
Patients in remission	18	20.69 ± 3.45		0.751	0.017
Ocular MG	11	18.98 ± 3.81			0.077
Healthy controls	10	11.33 ± 0.95			

$CD3^-CD19^+$ $CD56^+$ cells represent the NK cells. The results represent the mean ± standard error; P value[a] as compared between the patients in exacerbation and ones in remission; P value[b] as compared with ocular MG patients; P value[c] as compared with the healthy controls. NK: natural killer; MG: myasthenia gravis

will lead to of muscle weakness in MG patients. Among the predisposing factors, infections were the most frequently identified for symptomatic exacerbation in our study [Table 5]. So, we have further analyzed peripheral blood lymphocyte subsets in the patients with infections at the exacerbation stage. Our data showed that the patients with infections had significantly lower percentage of NK cells those without infections during exacerbation (10.97 ± 1.74; 14.03 ± 2.36, Table 5, Figure 3), suggesting that the NK cells may be consumed by infections or by a similar mechanism during deterioration of muscle weakness. Furthermore, the difference in NK cells percentages between the patients in exacerbation with infections and the cases in remission was significant ($P = 0.028$), but the difference between the patients in exacerbation without infections and the cases in remission was not statistically significant [Table 5; Figure 3]. The

Table 5: Frequencies of NK and Breg cells in PE with infection and without infection (%)

Group	Breg cells				NK cells			
	Frequencies	*P* value[a]	*P* value[c]	*P* value[d]	Frequencies	*P* value[a]	*P* value[c]	*P* value[d]
PE with infection (14)	6.84 ± 1.59	0.891	0.007	0.931	10.97 ± 1.74	0.028	0.871	0.295
PE without infection (11)	7.05 ± 1.85	0.825	0.016		14.03 ± 2.36	0.177	0.307	
Patients in remission (18)	6.56 ± 1.32		0.002		20.69 ± 3.45		0.017	
Healthy controls (10)	15.97 ± 2.88				11.33 ± 0.95			

The results represent the mean ± standard error; *P* value[a] as compared between the patients in exacerbation and the ones in remission; *P* value[c] as compared with the healthy controls. *P* value[d] as compared between PE patients with infection and without infection. NK: natural killer; PE: patients in exacerbation

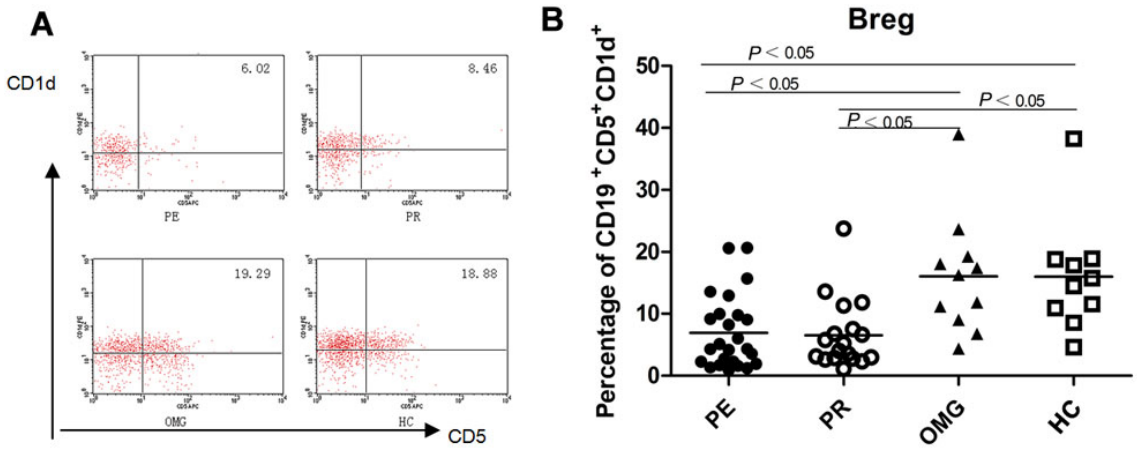

Figure 1: (A) Breg cells flow cytometry scatter plot in the PE, PR, OMG patients and the HC; (B) the percentage of Breg cells in GMG significantly lower than those in the OMG patients and in the controls, the difference was statistically significant ($P < 0.05$), but there was no obvious difference between the two subgroups of GMG patients. PE: patients in exacerbation; PR: patients in remission; OMG: ocular myasthenia gravis; HC: healthy controls; GMG: generalized myasthenia gravis

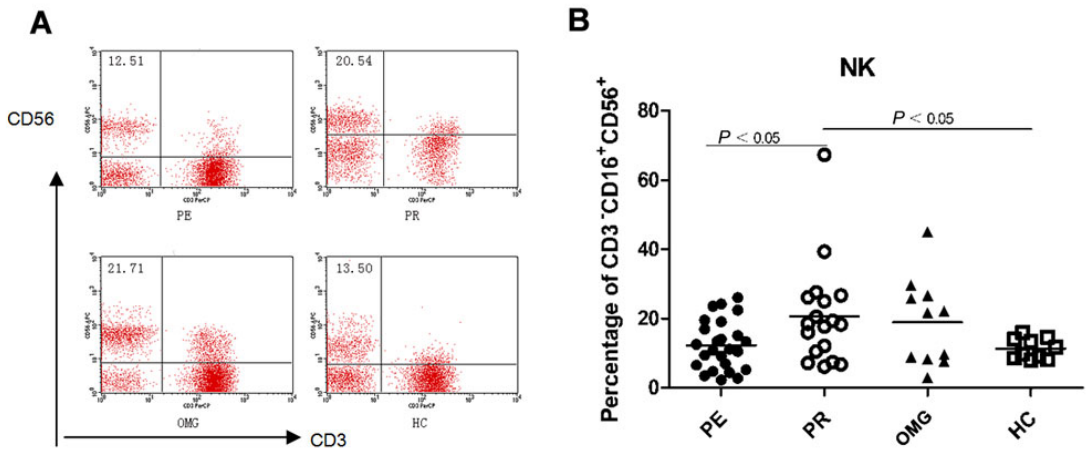

Figure 2: (A) NK cells flow cytometry scatter plot in the PE, PR, OMG patients and the HC; (B) the percentage of NK cells in the patients remission significantly higher than that in the patients in exacerbation and the healthy controls ($P < 0.05$). NK: natural killer; PE: patients in exacerbation; PR: patients in remission; OMG: ocular myasthenia gravis; HC: healthy controls

percentage of Bregs did not differ between the patients in exacerbation with infections (6.84 ± 1.59) and those without infections (7.05 ± 1.85).

DISCUSSION

Disorders of neuromuscular junction can be of immunological, toxic, or genetic origin; and among these rare disorders, MG is the most common. The clinical hallmark of MG is a fluctuating weakness and fatigability of the affected voluntary muscles. With the advent of immunotherapy, the long-term outcome has been improved significantly,[39] but the symptomatic deterioration after symptomatic remission takes places in majority of MG patients including both the OMG and GMG cases.[40-42] Thus, reliable markers that reflect the activity of the disease to guide the clinical therapy are critical.

MG patients present with heterogeneous clinical patterns in terms of onset-age, initial symptoms, mode of development, thymic abnormalities, immunological profiles, and responsiveness to treatment. MG is currently considered to consist of a heterogeneous group of autoimmune diseases, which share common aspects, such as the impairment of neuromuscular transmission induced by autoimmunity, manifested by muscle weakness and fatigability and the response to both pyridostigmine and immunosuppressants.[43]

In MG, the presence of multiple autoantibodies against numerous targeted molecules (e.g. AChR-ab). These antibodies suggest altered immunity, but any single antibody determination is hardly reflective of the progression or activity of the disease. The production of the above-mentioned antibodies are likely the result of the dysfunctioned lymphocytes, thus measuring the peripheral lymphocytes subsets in MG patients may be a promising way to monitor the progression of the disease.

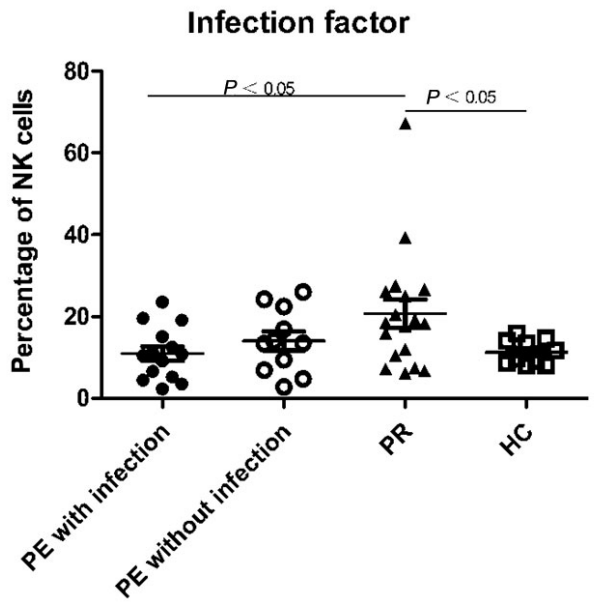

Figure 3: The percentage of NK cells in PE with infections was statistically lower than PR. The difference between PR and PE without infections was not statistically significant. NK: natural killer; PE: patients in exacerbation; PR: patients in remission; HC: healthy controls

B cell abnormalities contribute to the development and progress of autoimmune diseases. Traditionally, the predominant function of B cells was thought to be limited to production of autoantibodies. However, B cells have both positive and negative regulatory roles during immune responses. During murine development the absence of B cells results in significant quantitative and qualitative abnormalities within the immune system, including a remarkable decrease in thymocytes numbers,[44] defects within spleen dendritic cells and T cells compartments.[45,46] Through production of immunomodulatory cytokines, B cells can also negatively regulate cellular immune responses. A variety of regulatory B cell subsets have been described. Whether Breg cells can serve as a marker for disease activity in MG remains to be determined. Our observation showed that the percentage of Breg cells was significantly decreased in the peripheral blood of GMG patients, indicating that Breg cells are affected during the development of the disease. But when we further focused on the changes of Breg cells between the two subgroups of GMG, no significant difference was found between them. This interesting finding suggests that the peripheral Breg cells dysfunction may contribute to the development of MG, but are likely not a main factor in the acute exacerbation of disease. Thus, more detailed studies on the subsets of Breg cells may provide valuable insights into the role of Breg cells in MG.

NK cells are large granular cells that constitute 5-10% of circulating lymphocytes in humans, and are important effectors in innate immunity.[29] Increasing studies report that NK cells can also act as regulators in adaptive immunity by producing cytokines which modulate the downstream immune factors.[47-49] In addition, NK cells were found to play a protective role in several autoimmune disease models.[50-52] In EAMG, Liu *et al*.[53] reported that NK cells proliferate in the early stages of the disease; the percentage of NK cells then decrease with disease progression. Based on the observations, NK cells were suggested to activate $CD4^+$ T lymphocytes. A previous report showed that the activity of NK cells in the blood of MG patients was lower than that of the controls.[54] Further, Suzuki *et al*.[55] showed that the frequencies of NK cell

subclasses were similar among the control thymuses, hyperplasic thymuses and thymomas; suggesting that circulating NK cells may have migrated to sites of inflammation. In our study, we discovered that the percentage of NK cells was increased significantly in the patients in remission stage, but nearly unchanged in exacerbation stage and in PE with infections. The data suggest that NK cells may protect against autoimmune response, especially in patients with infections; yet, the mechanism for the increase in NK cells is unknown. These findings are consistent with Suzuki *et al*.,[55] which suggested that NK cells in the blood of patients at exacerbation stage may transfer to the neuromuscular junction, where immune cells obviously accumulate. At the stage of remission, NK cells might then transfer back to peripheral blood. It will be very interesting to compare the NK cell density in the neuromuscular junction among the patients in different stages. However, how the NK cells migrate at different stages and how NK cells are involved in the pathogenesis of MG remains to be fully elucidated. Based on our initial study, we also suggest that developing approaches that restore and boost the activity of NK cells may improve the MG symptoms and have therapeutic value.

Studies on the immune mechanism of OMG are scarce. However, in our study, both the Breg cells and NK cells were not obviously changed in the OMG patients. Actually, childhood onset MG (CMG), predominantly manifested isolated ocular symptoms, shows many different aspects from the adult onset MG, such as the rare transformation from the ocular type to generalized types, the scarcity of thymomas, and the lacking benefit of thymectomy in CMG.[41] We are convinced that the CMG with ocular type represents a different clinical entity in which the immunologic pathogenesis is far from being understood.

In conclusion, lymphocyte subsets were obviously disordered in GMG patients. The decrease of the peripheral Breg cells is linked with development of GMG, while the increase of peripheral NK cells is associated with reduced MG symptoms. Our research reveals that the frequency of the NK cells could be a reliable marker for MG activity in GMG patients. Restoring and boosting the activity of NK cells could be of therapeutic value. Therefore, a prospective, randomized clinical trial is required to further delineate the significance of NK cells in MG patients.

DECLARATIONS

Authors' contributions

Concept, design, and manuscript review: B.T. Bu

Concept, statistical analysis, and manuscript editing: L.X. Li

Literature search, experimental studies, manuscript preparation, and manuscript editing: X.Y. Lai

Clinical studies, and data acquisition: M.C. Gui, J. Lin, Y. Li, X. Luo, X.Y. Lai

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Conflicts of interest

There are no conflicts of interest.

Patient consent

All the participants signed the informed consent prior to the enrollment.

Ethics approval

The study has been approved by the Ethics Committee of Huazhong University of Science and Technology.

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Letter to Editor **Open Access**

Acute demyelinating lesions with restricted diffusion in multiple sclerosis: a new variant?

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Typical acute demyelinating lesions in relapsing-remitting multiple sclerosis (RRMS) exhibit vasogenic edema with increased diffusion, as demonstrated by the appearance of a bright signal on apparent diffusion coefficient (ADC) maps using diffusion weighted magnetic resonance imaging (MRI),[1] while acute ischemic stroke lesions demonstrate restricted diffusion and low signal on ADC maps.[2]

In order to identify multiple sclerosis (MS) patients with acute demyelinating lesions with restricted diffusion (ADLRD), a retrospective review of the medical records and MRI scans of 582 patients was performed. For inclusion in this study, patients must have been diagnosed with RRMS and present acute symptoms and neurological semiology. The following pulse sequences were required to have been performed within 19 days from the onset of symptoms: T2-weighted imaging (T2WI), fluid-attenuated inversion recovery, pre- and post-contrast T1-weighted imaging (T1WI), ADC and diffusion weighted imaging; ADLRD were considered as present if they were demonstrated on the MRI and exhibit locations corresponding to patients' acute symptoms and neurological semiology.

Five MS patients qualified for the study (0.85%), 3 females and 2 males, with ages ranging from 27 years to 42 years. Based on available medical records, 2 of the patients had clinically definite RRMS, for 9 and 12 years accordingly, while in the other 3 patients,

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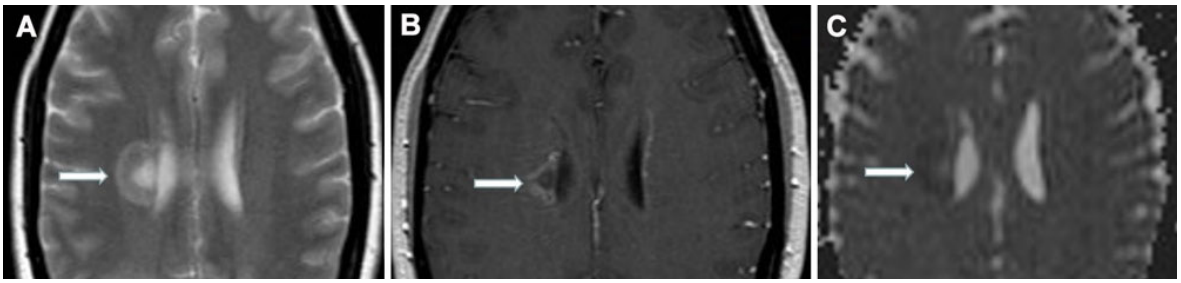

Figure 1: (A) Axial T2-weighted image reveals a high signal periventricular lesion; (B) axial T1-weighted image shows peripheral open ring enhancement after contrast administration; (C) apparent diffusion coefficient map shows restricted diffusion at the periphery of the lesion

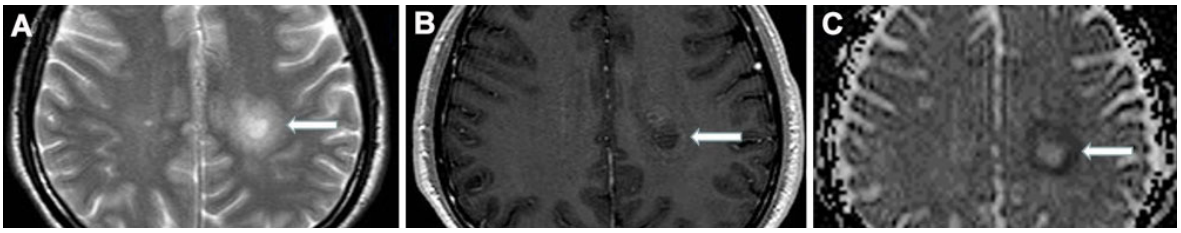

Figure 2: (A) Axial T2-weighted image reveals a high signal lesion in the centrum semiovale; (B) axial T1-weighted image shows mild enhancement after contrast administration; (C) apparent diffusion coefficient map shows restricted diffusion at the periphery of the lesion

the RRMS diagnosis followed by the performance of an MRI and the symptoms which corresponded to ADLRD, were the first indications of MS symptoms. The patients with the established diagnosis of MS received intravenous corticosteroids prior to MRI performance.

An MRI was performed within one week from the onset of symptoms onset and five acute demyelinating lesions, one in each patient, were demonstrated in the centrum cemiovale and in the periventricular region. The diameter of the lesions was 12-25 mm. The lesions exhibited restricted diffusion at their periphery [(1.2-1.6) × 10^{-3} mm^2/s], with reduced signal on ADC maps [Figures 1 and 2]. Four of the lesions showed peripheral enhancement on T1WI sequences after contrast administration [Figures 1 and 2].

Selected clinical MS cases with ADLRD are reported in the literature,[3,4] with the restriction of the diffusion involving either the entire lesion or part of the lesion.[1] In our case series the restriction was confined to the periphery of the lesions, sparing the central area, which were detected one week after the onset of symptoms. It is uncertain whether the ADLRD, that do not enhance, represent a phase of ADLRD development before or after the potential contrast enhancement or whether this lesion never enhanced.

ADLRD is a new diagnostic challenge in young patients. In acute stroke cases the ADC maps show restricted diffusion the first 2 days and pseudo-normalization between 7-10 days, without enhancement after gadolinium administration.[2] On ADLRD the restricted diffusion remains for at least 13 days, as reported by Balashov *et al*.[1]

It is suggested that ADLRD may represent a new variant of MS and possible mechanisms of inflammatory cascades in MS should be investigated, such as early leukocyte migration, cytokines effects on oligodendrocytes, astrocytes or microglia within the periventricular white matter of a developing lesion.[1,4,5]

Prospective studies with a large number of patients are required to better characterize these lesions and monitor the clinical course of MS patients with ADLRD.

DECLARATIONS

Authors' contributions

Writing the paper: S. Markoula, A. Zikou
Reviewing patients' data: S. Markoula
Reviewing MRI imaging data: A. Zikou, P. Margariti
Editing the paper: M. Argyropoulou, A.P. Kyritsis

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Topic: Stroke **Open Access**

The roles of endoglin gene in cerebrovascular diseases

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ABSTRACT

Endoglin (ENG, also known as CD105) is a transforming growth factor β (TGFβ) associated receptor and is required for both vasculogenesis and angiogenesis. Angiogenesis is important in the development of cerebral vasculature and in the pathogenesis of cerebral vascular diseases. ENG is an essential component of the endothelial nitric oxide synthase activation complex. Animal studies showed that ENG deficiency impairs stroke recovery. ENG deficiency also impairs the regulation of vascular tone, which contributes to the pathogenesis of brain arteriovenous malformation (bAVM) and vasospasm. In human, functional haploinsufficiency of *ENG* gene causes type I hereditary hemorrhagic telangiectasia (HHT1), an autosomal dominant disorder. Compared to normal population, HHT1 patients have a higher prevalence of AVM in multiple organs including the brain. Vessels in bAVM are fragile and tend to rupture, causing hemorrhagic stroke. High prevalence of pulmonary AVM in HHT1 patients are associated with a higher incidence of paradoxical embolism in the cerebral circulation causing ischemic brain injury. Therefore, HHT1 patients are at risk for both hemorrhagic and ischemic stroke. This review summarizes the possible mechanism of ENG in the pathogenesis of cerebrovascular diseases in experimental animal models and in patients.

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INTRODUCTION

In human, endoglin gene (*ENG*, or CD105) is located on chromosome 9q34.11. It is a type III transforming growth factor β (TGFβ) receptor interacting with TGFβRI (TGFβ receptor, type I) and/or TGFβRII (TGFβ receptor, type II)[1]. In the endothelium, ENG interacts with the activin receptor-like kinase 1 (ALK1 or ACVRL1), a type 1 TGFβR. ENG binds with TGFβ1 and TGFβ3 with high affinity in the presence of other TGFRs but not with TGFβ2[1-4]. ENG also binds to activin-A, bone morphogenetic protein 2 (BMP2) and BMP7[1]. Protein studies suggested that ENG plays an important role in modulating the TGFβ signaling pathway[4].

ENG gene expresses in many cell types, including endothelial cells[5,6], activated monocytes and macrophages[7], mesenchymal cells, fibroblasts[8], and vascular smooth muscle cells [Table 1][9,10]. Animals studies have revealed that ENG may be dispensable during vasculogenesis, a process from which primary capillary plexus is formed; but ENG is required in angiogenesis, a process that remodels the primary endothelial network into a mature circulatory system[11,12]. Immunohistochemical analysis showed that in normal human brain, ENG is expressed in the endothelial cells of brain vessels, as well as the endothelial and adventitial layers of leptomeningeal arteries [Table 1][13]. ENG expression is upregulated in endothelial cells during wound healing and tumor vascularization, and in inflammatory tissues and developing embryos[1,14,15], indicating that ENG is an endothelial proliferation marker[16,17].

Ischemic stroke is caused by occlusion of a cerebral artery. After ischemic stroke, blood supply to the affected brain tissue is reduced, which leads to oxygen deprivation to brain cells. Ischemia induces a significant increase in microvascular density, a sign of angiogenesis, in the penumbra of the cerebral infarct[18]. The degree of increased vessel-density in the ischemic penumbra is positively correlated with the survival rate of stroke patients[19]. In addition, increased angiogenesis was associated improvement of functional outcome in both animal models and stroke patients[20-23].

Table 1: Summary of ENG expression patterns in tissue and cell lines

Tissue	Tissue samples	Cell lines
Brain	Humanendothelium[13] Human adventitia[13]	
Non-brain	Human placenta[6] Human spleen[7] Murine ovary and uterus[8] Murine heart[8] Murine muscle[8] Murine placenta[8] Murine spleen[8]	HUVEC[5] HOON[6] U-937[6,7] HL-60[7] Cultured monocytes[7] NCTC-2071[8] VSMC[9] HASMC[10]

Mutations in the *ENG* gene are associated with type 1 hereditary hemorrhagic telangiectasia (HHT)[24], also known as Osler-Rendu-Weber Syndrome. HHT is an autosomal dominant disease. The clinical features of HHT patients are telangiectases in mucocutaneous membrane and arteriovenous malformation (AVM) in multiple organs, including the skin, liver, lung, intestine and brain. AVMs are abnormal vessels that shunt blood directly from arteries to veins[25]. Brain AVM (bAVM) tends to rupture, which can cause life-threatening intracranial hemorrhage and hemorrhagic stroke[25]. Hemorrhage from bAVM can also cause long-term disability. Elevated levels of angiogenic factors including vascular endothelial growth factor (VEGF) were found in sporadic bAVM patients[26,27]. High levels of VEGF are also associated with increase of blood-brain barrier (BBB) permeability and bAVM hemorrhage[27-29]. Similarly, HHT patients that have a higher incidence of AVMs in multiple organs also have an increased level of plasma VEGF[30]. All of these evidence suggest that angiogenesis is involved in the pathogenesis of bAVM.

Since ENG plays an important role in the angiogenesis, in this review, we summarize the influences of ENG on endothelial function and the angiogenesis, as well as how ENG-deficiency contributes to the pathogenesis of cerebrovascular diseases, including ischemic stroke and intra-cranial hemorrhage, as well as cerebrovascular malformation, stenosis and occlusion.

THE FUNCTION OF *ENG* GENE IN ANGIOGENESIS

To study the functional role of ENG in development, *Eng* gene knockout mice were generated[11,31]. Homozygous deletion of *Eng* gene in mice causes embryonic death by E10.5-11.5[11,31]. The endothelial cells derived from *ENG* deficient human embryonic stem cells failed to organize effectively into tubular structures *in vitro*[12]. VEGF induced vascular network was also reducedin the metatarsal bone of *Eng* heterozygous knockout (*Eng*$^{-/-}$) mouse embryo[12]. Consistently, depletion or inhibition of *ENG* gene in human endothelial cells mitigated VEGF-induced angiogenesis[12]. These findings suggest that ENG is required for the differentiation and sprouting of endothelial tubes, which are important processes of angiogenesis.

ENG also mediates endothelial-mesenchymal communication during angiogenesis[11,32,33]. The recruitment of vascular smooth muscle cells and pericytes to newly formed vascular network is impaired in *Eng* deficient mouse embryos[11].

ENG DEFICIENCY IS AN IMPORTANT RISK FACTOR FOR BOTH HEMORRHAGIC AND ISCHEMIC STROKES

As mentioned in previous sections, ENG deficiency is associated with HHT1, a familial disease that has bAVM as one of its major phenotypes. Brain AVM contains abnormal vessels, that are prone to rupture, causing intracranial hemorrhage and hemorrhagic stroke. In addition, patients with ENG deficiency (HHT1) have a higher incidence of pulmonary AVM (PAVM), which is associated with a high incidence of paradoxical embolism in the cerebral circulation and ischemic brain injury[34]. To understand bAVM pathogenesis and to develop therapeutic strategies, many *Eng* deficient mouse models were generated. Using these animal models, we are able to elucidate bAVM pathogenesis and test new therapies.

Since homozygous deletion of *Eng* gene in mouse causes embryonic lethality[11,31], mice with heterozygous deletion of *Eng* ($Eng^{+/-}$)[31] are used to study the pathogenesis of HHT patients. $Eng^{+/-}$ mice exhibit many phenotypes that resemble those of HHT1 patients, including mucocutaneous telangiectases, external bleeding, and AVMs in the liver, lung, brain and gastrointestine[35]. Enlarged cerebrovascular structure was found in some $Eng^{+/-}$ mice with evidence of hemorrhage[35]. However, penetrance of bAVM in $Eng^{+/-}$ mice is very low, only 7%[35], suggesting that heterozygous *Eng* deletion alone is not sufficient to cause bAVM formation. In addition, the differences of the penetration of HHT phenotypes in 129/Ola and C57BL/6 $Eng^{+/-}$ mice suggests that modifier genes are contributing to the severity and heterogeneity of AVMs in HHT patients[35].

Based on clinical studies, we and others found that VEGF levels are increased in the plasma of HHT patients and in surgically resected sporadic human bAVM specimens[26,27,30]. The intensity of VEGF staining is also correlated with microvessel density in nasal mucosa from HHT patients[36]. Together, abnormally high level of VEGF appears to be a fundamental part of AVM pathophysiology[25,30,37-39]. Based on these studies, we overexpressed VEGF in the mouse brain in conjunction with *Eng* deletion to generate bAVM models. In adult $Eng^{+/-}$ mice, intra-brain injection of an adeno-associated viral vector expressing VEGF (AAV-VEGF) significantly increased the penetrance of cerebrovascular abnormality[40]. Almost all-adult $Eng^{+/-}$ mice that received intra-brain injection of AAV-VEGF showed cerebrovascular abnormality[40]. However, unlike HHT1 patients, the vascular abnormality in $Eng^{+/-}$ mice is at the capillary level.

Bone marrow-derived cells can infiltrate into the brain angiogenic region. We found that macrophages are the major bone marrow-derived cells recruited to the brain angiogenic foci[41]. Since the accumulation of bone marrow-derived macrophage in VEGF-induced brain angiogenic regions peaks earlier than the increase of vessel density, macrophages likely play a role in angiogenesis.

Using $Eng^{+/-}$ mice, the influence of bone marrow derived cells in the development of bAVM has been studied. Transplantation of $Eng^{+/-}$ bone marrow to wild type mice induced vascular dysplasia in the brain angiogenic regions, while transplantation of wild type bone marrow to $Eng^{+/-}$ mice reduced the severity of vascular dysplasia in the brain angiogenic foci of $Eng^{+/-}$ mice[42]. These data suggested that *Eng* gene mutation in bone marrow cells cause vascular dysplasia. Importantly, these data suggested that transplantation of normal bone marrow cells to bAVM patients could be a therapeutic option.

Although we were able to induce vascular dysplasia in the brain of $Eng^{+/-}$ mice by overexpression of VEGF, arteriovenous shunts were not detected in these mice. Studies have shown that a combination of homozygous *Eng* inactivation and additional stimulations are needed for robust bAVM formation. Genetic studies also indicated that mutations of *Eng* modifier genes contribute to AVM formation[43,44].

To avoid embryonic death caused by homozygous *Eng* deletion, Allinson *et al.*[45] generated an *Eng*-floxed ($Eng^{2f/2f}$) mouse line that have the *Eng* gene exons 5-6 flanked by loxP sites. When Cre recombinase is present, the DNA sequence between the loxPsites will be deleted. To test whether homozygous *Eng* gene deletion plus angiogenic stimulation can initiate bAVM formation, an adeno virus expressing Cre recombinase (Ad-Cre) and AAV-VEGF were co-injected into the brain of $Eng^{2f/2f}$ mice[45,46] to induce brain focal *Eng* gene deletion and angiogenesis. $Eng^{2f/2f}$ mice with focal *Eng* gene deletion and angiogenic stimulation developed vascular dysplasia beyond the capillary level around the AAV-VEGF injection site eight weeks after the vector injection[46]. Robust bAVM have also developed

in the AAV-VEGF induced brain angiogenic region in mice subjected to global *Eng* deletion at the age of 8 weeks old[47]. The bAVM phenotype in these mice highly resembled the phenotype of human bAVM[47]. Furthermore, *Eng*-null endothelial cells were found in the dysplastic vessels in the bAVM lesion[47]. Our studies are consistent with the studies on skin AVM development, and support the notion that an injury (angiogenic stimulation) is needed to induce bAVM.

Eng-deficient bAVM mouse models have been used to analyze the function of macrophages during bAVM pathogenesis. Although *Eng* deficiency has been shown to impair monocyte migration into injured tissue[48-50], an increased number of bone barrow-derived macrophages and activated residential microglia was found in the bAVM lesion in mouse and human. Compared with normal macrophages, *Eng*-deficient macrophages show slower but more persistent infiltration into the brain angiogenic regions[51]. Delayed clearance of macrophages and persistent inflammation could exaggerate abnormal vascular phenotypes in bAVM[51].

In addition to conditional knockout of *Eng* gene in adult mice, several cell-specific cre transgenic mouse lines have been used to induction of *Eng* deletion in specific cell-types. For example, the promoter of SM22α (smooth muscle actin) is used express cre in smooth muscle specifically. Although SM22α is predominantly expressed in smooth muscle cells in normal mice, Cre expression driven by the SM22α promoter in this transgenic mouse line was also found in other cell types, including endothelial cells[52,53]. SM22α Cre; $Eng^{2f/2f}$ mice have *Eng* gene deleted in the SM22α expressing cells during the embryonic developmental stage. We found 90% of SM22α Cre; $Eng^{2f/2f}$ mice have spontaneously developed bAVM by 5 weeks of age and 50% of them died by 6 weeks of age[47]. BAVM lesions varied in size and location in these mice[47]. In addition to bAVMs, some of SM22α Cre; $Eng^{2f/2f}$ mice also developed spinal and intestinal AVMs[47]. Because AVM develops in this mouse line spontaneously without exogenous VEGF stimulation, this model is an ideal model for testing new therapeutic strategies.

As mentioned above, *Eng* gene not only expresses in endothelial cells[5,6], but also expresses in activated monocytes/macrophages[7], mesenchymal cells, fibroblasts[8], and smooth muscle cells[9,10]. Using transgenes that express cre specific cell-types, the *Eng* gene was conditionally deleted in different cell types in adult mice to determine which cell type is most crucial for AVM development[54,55]. In SclCreER; $Eng^{2f/2f}$ mice, which have *Eng* deleted in endothelial cells only, AVM formed in the skin around the ear wound and back wound[54,55]. We found that bAVM develops in the brain angiogenic region in Pdgfb-iCreER; $Eng^{2f/2f}$ mice that have *Eng* gene deletion specifically in endothelial cells. Myh11CreER-mediated *Eng* deletion in smooth muscle cells in adult mice did not cause AVM formation in the wound area of the skin[54]. Furthermore, LysMCre; $Eng^{2f/2f}$ mice, which have *Eng* deleted in macrophages, did not develop AVM in any organ and in the brain angiogenic regions[47]. These studies indicate that *Eng* deletion in endothelial cells is essential for AVM formation in the brain and other organs[47,54].

Eng-deficient bAVM mouse models were valuable resources to test new therapies for the treatment of bAVM. Current treatments for bAVM are mostly invasive and associated with high morbidities and mortalities[56]. Since high VEGF level is involved in the pathogenesis of bAVM, we have tested the feasibility of use soluble FMS-like tyrosine kinase 1 (sFLT1) gene therapy to treat bAVM. Soluble FLT1 is an alternative transcript of FLT1 (or VEGFR1) containing only the extracellular domains of the receptor. Soluble FLT1 binds VEGF with high affinity in tissue, reduces VEGF signaling through its membrane-bound receptors, and thus inhibits VEGF-induced angiogenesis[14]. Systemic delivery of AAV9-sFLT1 into a bAVM mouse model that has *Eng* gene deleted globally reduced abnormal vessels in the bAVM region[57]. Intravenous delivery of AAV9-sFLT1 to SM22α Cre; $Eng^{2f/2f}$[57] mice that have spontaneously developed bAVMs reduced mortality and bAVM penetrance[57]. This study demonstrated that mouse models are important tools to test new therapies.

HYPOXIA INDUCES ENG EXPRESSION

Hypoxia induces the expression of ENG in human and mouse brain microvascular endothelial cells[16,22,58], which ameliorates endothelial cells apoptosis regardless of the presence or absence of TGFβ[59]. During hypoxia stress, TGFβ induces apoptosis of endothelial cells[60,61], but reduces the death of neurons[62] and vascular smooth muscle cells[61]. Therefore, ENG is likely to antagonize the inhibitory effects of TGFβ1 on human vascular endothelial cells[17,63] and protect endothelial cells against apoptosis via TGFβ signaling or other independent pathways[59].

Under hypoxia conditions, ENG expression increases in many cell-types, such as, human microvascular endothelial cells-1 (HMEC-1) and monocytic U-937 cell. It is likely that hypoxia regulates ENG expression through crosstalk of several signaling pathways[58].

The transcriptional regulation of ENG expression under hypoxia condition was studied by a reporter assay using HeLa cells, and by the electrophoretic mobility shift assay (EMSA) using human umbilical cord vein endothelial cells (HUVECs). These assays confirmed the presence of a hypoxia response element (HRE) in the enhancer region of *ENG* gene[64]. Therefore, ENG expression can be induced by hypoxia through hypoxia-inducible factor-1 (HIF-1). A subsequent study suggested that hypoxic induction of Eng expression in bEnd.3 (a mouse brain endothelial cell line) cells was activated through ERK-p38 MAPK and JNK pathway[16], instead of HIF-1[58]. In addition, Smad3 was reported to interact with HIF proteins to induce the overexpression of ENG[64]. Although these studies implicated links among multiple factors, further studies are required to better elucidate the exact transcriptional regulation of ENG expression under hypoxia conditions.

ENG EXPRESSION IS UPREGULATED AFTER STROKE INJURY

Previous studies revealed that ENG was highly expressed in the penumbra region of human stroke lesion, where an increase of angiogenesis was found[22]. However, it was not clear at that time whether the angiogenesis was beneficial. In acute ischemic stroke patients, there is a robust mobilization of immature hematopoietic cells, colony-forming cells and long-term culture initiating cells[65]. It has been suggested that the degree of immature hematopoietic cell mobilization is directly correlated with the recovery of neurological function[66,67]. An increase of ENG positive micro-particles including exosomes and shedding vesicles, which are small vesicles released by specific cells (endothelial or MSC)[68], were detected in patients' sera collected 3 days after stroke compared to that of healthy people[69]. Certain types of ENG positive micro-particles increased further in stroke patients with severe disability. The ENG positive micro-particles decreased gradually after the initial increases[69]. The number of these circulating ENG positive micro-particles was positively correlated with the stroke severity, even after adjusting for other demographic and clinical variables, such as hypotension and other stroke comorbidities[69]. Similarly, ENG positive circulating micro-particles released from endothelial cells were also increased in patients with acute ischemic stroke. The increase of ENG positive cells was positively associated with the severity of neurological function at hospital admission, larger brain lesion volume and unfavorable functional outcome at hospital discharge[70]. The increased level of circulating ENG positive micro-particles after acute stroke may have been caused by either increased circulating cells as a self-repair response to stroke or a sign of increased apoptosis of circulating cells in response to hypoxic conditions[69].

The role of ENG in stroke injury is complex, and is influenced by the local microenvironment. Constitutive expression of ENG enhances the TGFβ signaling and promotes new vessel wall remodeling[11]. ENG overexpression also protects against TGFβ-induced apoptosis of endothelial cells[17,59]. Reduction of vascular cell-apoptosis after hypoxia improves blood supply to ischemic tissue[58,71]. Increase of ENG expression in endothelial cells could also be hazardous, because BBB permeability was increased in some of the capillaries that express high level of ENG, which was accompanied with mononuclear cell infiltration in the surrounding brain tissues[72]. These findings suggests that pronounced ENG overexpression might impair vessel wall integrity. Alternatively, lack of ENG expression may indicate severe vessel damage[72]. ENG and TGFβ are involved in the pathogenesis of post-ischemic brain injury in human. Abnormal ENG and TGFβfunction might lead to long-term neurological deterioration or cognitive disturbance after acute ischemic stroke[72,73]. Homeostasis of ENG expression is crucial for maintaining normal angiogenesis, vascular remodeling and reduction of stoke injury.

THE EFFECT OF ENG DEFICIENCY IN ISCHEMIC STROKE INJURY

The survival of neurons in peri-infarcted regions is associated with the extent of patient recovery after stroke[74]. Nutrient supply supporting neuron survival is carried through blood. Higher microvessel density in the peri-infarct region is associated with lower morbidity and mortality[22]. Hypoxia-induced angiogenesis increases blood flow and oxygen delivery to ischemic tissues, which contributes to the recovery after stroke[28].

Angiogenesis occurs in human brain after stroke. Through examining human postmortem brain samples with ischemic infarcts caused by occlusive vascular diseases, capillary networks with regular connection and micro-vessels were found in the brain samples of patients who died within one week after stroke, and the neo-vasculature was in filled with blood in the brain samples collected from patients that died 2-3 weeks after stroke[75]. The micro-vessel density remains higher in the infarct area compared with the corresponding contralateral side three months after stroke[22]. Increased vessel density restores cerebral blood flow, salvages ischemic tissue, enhances neuronal survival and improves functional recovery of

stroke survivors[76].

ENG is expressed in proliferating vascular endothelial cells[77] and is elevated in inflammatory tissue and healing wound[78]. In patients, somepro-angiogenic genes, including Tie-2, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), hepatocyte growth factor-α (HGF-α) and monocyte chemoattractant protein-1 (MCP-1), were upregulated in ENG expressing micro-vessels in stroke affected tissue. These key angiogenic elements play important roles in endothelial cell migration, differentiation and tube-formation, as well as vessel stabilization and stem cell homing into the region of angiogenesis and revascularization[79].

In *Eng* deficient mice (*Eng*$^{+/-}$ mice), the functional performance after stroke was poorer than wild type animal both in the acute phase and the sub-chronic stage (one month after stroke), suggesting that there is an association between delayed functional recovery and *Eng* deficiency[50]. The infarct volume and atrophic volume are larger in *Eng*$^{+/-}$ mice[50]. The density of micro-vessels within the infarct and peri-infarct region are lower in *Eng*$^{+/-}$ mice than wild type mice[50,80]. *In vitro* study showed that *Eng*$^{+/-}$ endothelial cells express a lower level of VEGF[81] compared to that of wild type endothelial cells. *Eng*$^{+/-}$ macrophages express lower levels of VEGF receptor 1 (VEGFR1) and 2 (VEGFR2) at the baseline and lower level of VEGFR2 after VEGF stimulation than wild type macrophages[42]. Although *Eng*$^{+/-}$ macrophages and wild type macrophages express similar levels of MMP9 at the baseline, unlike in wild type macrophages, the expression of MMP9 did not increase in *Eng*$^{+/-}$ macrophages after VEGF treatment[42]. In the brain of *Eng*$^{+/-}$ mice, VEGF-induced upregulation of VEGFR2 expression was also impaired[82]. Together, these data suggest a reduced angiogenic response in the absence of normal *Eng* function may be responsible for the impairment of tissue repair in *Eng* deficient mice after experimental stroke.

In addition, our study suggested that *Eng* deficiency is associated with impairment of macrophage recruitment and clearance in the peri-infarct area during stroke recovery[50]. Eng expression was upregulated during the transition from monocyte to macrophage[7]. *Eng* deficiency in endothelial cell reduced adhesion and transmigration of leukocytes in response to ischemic injury[83]. Recruitment of monocytes to the infarcted tissue and subsequent vessel formation was severely impaired in HHT1 patients (who have *ENG* haploinsufficiency)[80] suggesting that *ENG* deficiency impairs monocyte adhesion and migration. In the acute phase (3 days) of stroke, *Eng* deficient mice had fewer macrophages in the peri-infarct region[50]. However, at 60 days after stroke, a time that is considered as recovery stage, there was an increase number of macrophage in the peri-infarct region of *Eng* deficient animals[50], suggesting a delayed homing and clearance of over-activated macrophage. However, the roles of post-ischemic inflammation might be bidirectional[84]. The inflammatory response after ischemic stroke could contribute to a secondary brain injury, because the influx of inflammatory cells amplifies brain cell death. On the other hand, inflammation also facilitated the clearance of damaged tissues and promoted tissue repair[85]. Therefore, the consequences of impaired macrophage in homing and clearance in the stroke tissue require further studied.

Interestingly, *Eng*$^{+/-}$ mice had severer brain injury than wild type mice since the first day of experimental stroke[50], which could not be explained by impaired tissue repair. As discussed in above, hypoxia induce endothelial *Eng* expression, which prevents hypoxia-induced apoptosis of endothelial cells. Therefore, vascular damage in *Eng*$^{+/-}$ mice could be more severe than in wild type mice after ischemic injury. In addition, *Eng* haploinsufficiency has been shown to be associated with reduced production of nitric oxide and increased production of superoxide under eNOS induction[86]. Nitric oxide produced by endothelial cell induces vascular relaxation[87]. Bioavailability of nitric oxide is lower in *Eng*$^{+/-}$ mice than in wild type mice[88]. Enhancing superoxide production in *Eng* deficient mice reduces vascular relaxation, and increases vessel damage and oxidative stress, all of which increases brain injury during the acute stage of ischemic stroke.

Since ENG plays an important role in angiogenesis and lack of ENG dampens angiogenesis, therapeutic stimulation of ENG could promote angiogenesis, vascular remodeling and improve stroke recovery, as well as reduce morbidity and mortality of stroke patients.

CIRCULATING SOLUBLE ENG MODULATES CEREBRAL VASCULAR REMODELING AND PLAYS ROLES IN VASOSPASM AFTER SUBARACHNOID HEMORRHAGE

Soluble ENG (sENG) is an alternative transcript of *ENG* gene, which contains only the extracellular domain of the full-length ENG. Soluble ENG enters the circulation in various conditions that related to

the endothelial injury, activation, inflammation and senescence[89]. Our group showed that sENG level is increased in the surgical resected human bAVMs[90]. We have also shown that co-injection of an adenoviral vector expressing sENG with AAV-VEGF into mouse brains caused capillary dysplasia. It is still unclear how overexpression of sENG causes cerebrovascular malformation. One of the possibilities is that circulating sENG acts as a decoy inhibiting the effect of ENG on the endothelium, leading to vascular malformation during angiogenesis.

Nitric oxide (NO) is a potent vascular smooth muscle relaxant, which is synthesized by the vascular endothelium. *Eng*$^{+/-}$ mice have a lower level of NO metabolites (nitrites) in the plasma and in the urine than that of wild type mice[91], suggesting that the NO level is lower in *Eng* deficient animals. The hypotensive and vasodilatory response induced by endothelium-dependent vasodilators was less intensive in *Eng*$^{+/-}$ mice than wild type mice. However, the difference of this vasodilation effect between *Eng*$^{+/-}$ mice and wild type mice disappeared after NO synthesis was inhibited[91]. These findings suggested that the NO level or the subsequent vessel response to NO is reduced in *Eng*$^{+/-}$ mice. However, after eliminating the endogenous NO, the vasodilatory effect induced by exogenous NO donor (nitroprusside) was similar in *Eng*$^{+/-}$ and wild type mice[91]. The peripheral progenitor cells of HHT patients expresses lower level of eNOS (endothelial nitric oxide synthase) mRNA[92]. Endothelial NOS produces NO in response to humoral and mechanical stimuli. However, resistance arteries in *Eng*$^{+/-}$ mice displayed an eNOS-dependent impairment in the myogenic response (normal resistance arteries contract in response to increases of perfusion pressure) despite of a reduced eNOS level. *Eng* deficient endothelial cells had uncoupled eNOS, which produce less NO but more superoxide[86]. Taken together, these studies indicate a role of *Eng* in the regulation of vascular tone.

Cerebral vasospasm is one of the most common complications of subarachnoid hemorrhage (SAH) and is associated with high morbidity and mortality. NO is found to be an important mediator of vasospasm[93]. The potential role of ENG on the production of NO suggests that ENG might be associated with vasospasm after SAH. In patients with SAH, the level of sENG increased in the cerebrospinal fluid (CSF) and decreased in the serum[94,95]. In the subgroup with cerebral infarction due to post-SAH vasospasm, the level of sENG was higher in the CSF and lower in the serum than the patients who did not have post-SAH cerebral infarction[94,95]. The level of sENG during the first two weeks of SAH might be a predictive factor for the long-term outcome, such as, 6 months after SAH[95]. Similar to sENG, the ENG positive endothelial micro-particles were increased in SAH patients with vasospasm[96].

Soluble ENG are present in both healthy people and patients with pathological conditions (such as preeclampsia and SAH)[89]. Several studies suggest that sENG is a naturally occurring antagonist of TGFβ[97]. In contrast to the lower level of sENG, the level of TGFβ1 in the serum was higher in patients with vasospasm after SAH than those without vasospasm[95]. Moreover, sENG interferes the binding between TGFβ1 and its receptors[89]. TGFβ1 has been suggested to be involved in eNOS activation[89]. Therefore, the reduced sENG levels in patients with post-SAH vasospasm might reflect an impaired production of vasorelaxant factors, such as NO. However, there is no direct evidence supporting the cause-and-effect relationship between vasospasm and sENG. Further studies of post-SAH vasospasm in *Eng*-deficient mice might be helpful in exploring the association of sENG and vasorelaxation.

Interestingly, the changes of sENG in the cerebrospinal fluid (CSF) and the serum of patients with SAH and vasospasm are opposite[94,95]. In patients with Doppler sonographic vasospasm, the serum level of sENG was similar to those without vasospasm[95]. However, the serum level of sENG was reduced in patients with cerebral infarction due to severe vasospasm and hydrocephalus[95], suggesting that the sENG level in the serum might beserved as a biomarker for cerebral ischemia subsequent to vasospasm. Cerebral hypo-perfusion or hypoxia could induce increases of focal expression of ENG and might contribute to the increase of sENG in the CSF of patients with vasospasm. Both extravasation of sENG from blood or intrathecal production of sENG could cause the increase of sENG in the CSF and decrease of sENG in the plasma. Further studies are needed to ravel the origin of sENG during post-SAH vasospasm.

Although it is not clear how ENG-positive micro-particles and sENG increased in patients with post-SAH vasospasm, the results of these studies indicated that, the circulating sENG is a promising biomarker for cerebral vasospasm after SAH.

ALTERNATIONS OF ENG EXPRESSION IN ATHEROSCLEROTIC PLAQUES AND STENOTIC CEREBRAL VESSELS

Carotid atherosclerotic stenosis is a major cause of

ischemic stroke. As mentioned in earlier sections, ENG is expressed mainly in endothelial cells, smooth muscle cells and macrophages, which are the three major cells involved in the pathogenesis of atherosclerosis[10]. The expression of ENG is very low in normal human arteries and is restricted to the endothelial cells of adventitial microvessels[10]. In contrast, higher ENG expression is present in the advanced atherosclerotic plaque of human patients[10]. The site of ENG expression are slightly difference between atherosclerotic plaques in carotid arteries and aorta. In aortic atherosclerotic plaque, ENG is predominantly expressed in smooth muscle cells. However, in carotid plaque, ENG is expressed in endothelial cells of neo-vessels within the lipid core and plaque shoulders[98]. ENG expression is higher in carotid plaque containing higher levels of collagen and lessintra-plaque thrombi, which are characteristics of stable plaques[99]. These evidence indicate that ENG may promote the formation of intra-plaque neo-vessels and collagen and reduce the vessel leakage and hemorrhage. However, ENG expression in the neo-vessels of carotid atherosclerosis has also been found to be positively correlated with the advanced grade of plaques[100]. The distinct ENG expression patterns in different types of plaques suggest that ENG might play different roles in the course of atherogenesis progression.

Atorvastatin is a drug to treat carotid atherosclerotic plague. In a mouse model of atherosclerosis, atorvastatin treatment decreased the level of Eng in the serum and increased Eng expression in the plaque[101]. Therefore, Eng may serve as a biomarker for evaluating the therapeutic effect of drugs in treating atherosclerosis. More studies are needed to elucidate the role of ENG in the pathogenesis of atherosclerosis.

Moyamoya disease (MMD) is a rare, progressive cerebrovascular disorder caused by blocked arteries at the base of the brain in an area called the basal ganglia. MMD is one of the major causes of stroke in children and adults characterized by progressive stenosis or occlusion of terminal portion of internal carotid arteries and development of fragile collateral vessels[102]. Middle cerebral artery (MCA) of MMD patients had thicker intimal walls than control vessels collected from aneurysm patients[103], indicating intimal hyperplasia in MMD. The expression of ENG and HIF-1 are increased in the intima of MMD patients[103]. In addition, TGFβ3 expression was also detected, which was predominantly in the endothelium and was co-localized with HIF-1 and ENG[103]. Although the study did not find an association between cerebral blood perfusion and ENG expression[103], the low spatial resolution method used to evaluate the cerebral blood flow of the entire MCA territory might not be accurate enough to detect the real perfusion through the MCA branches that were used to measure the ENG expression. The increased expression of ENG and HIF-1 in MMD is consistent with the increased expression of ENG under hypoxia condition. Therefore, ENG may play roles in the pathogenesis of cerebrovascular stenosis or occlusion.

THE PROSPECTIVE OF MODULATING ENG EXPRESSION FOR THE TREATMENT OF CEREBROVASCULAR DISEASES

Since ENG has been implicated in the pathogenesis of various cerebrovascular diseases [Table 2], modulation of ENG expression might be a potential treatment for these conditions. Although currently there is no treatment available for patients with human cerebrovascular diseases through targeting ENG, several agents that affect ENG expression specifically or non-specifically, are clinical available for treating patients or are used in clinical trials. TRC105 is a chimeric IgG1monoclonal antibody specifically against ENG that inhibits angiogenesis, induces antibody-dependent cellular cytotoxicity (ADCC) and apoptosis of proliferating endothelium. The safety and activity of TRC105 have been tested in a Phase I and a preliminary Phase II clinical trials in cancer patients[104]. Resveratrol is a natural component of a number of fruits, including grapes, blueberries and raspberries. The skin of red grapes is used to extract resveratrol. *In vitro*, resveratrol reduces sENG secretion and pro-inflammatory factors of cultured endothelial cells[105]. Therefore, it might be a promising non-specific inhibitor of sENG. A proper level of ENG expression might be crucial for maintaining normal angiogenesis and vascular remodeling in the brain. However, there is no report of direct regulation of ENG for treating cerebrovascular disease to date. *In vitro* study showed that statins could increase sENG secretion from endothelial cells[106]; and *in vivo* administration of stain increased Eng expression in the carotid plaque of a mouse model[101]. Statins are a group of medications that has been used to treat patients with carotid artery atherosclerosis and other ischemic cerebrovascular diseases. More studies are needed to test whether ENG can be used as a target for developing new therapies for the treatment of cerebrovascular diseases.

CONCLUSION

In summary, ENG plays a critical role in angiogenesis,

Table 2: Cerebrovascular diseases and ENG level

Cerebrovascular diseases	Species	Specimen	ENG level	Clinical or biological observation	Author and year
Stroke	Human	Brain	Increased	-	Krupinski *et al.*,[22] 1994
	Human	Serum	Increased	Positive correlation with stroke severity	Kim *et al.*,[69] 2012; Simak *et al.*,[70] 2006
	Eng$^{+/-}$ mouse	Brain	Decreased	Poorer functional performance, larger infarction, less angiogenesis, impaired macrophage recruitment and clearance	Shen *et al.*,[50] 2014
Brain AVM	*Eng*$^{+/-}$ mouse	Brain	Decreased	Cerebrovascular dysplasia after VEGF stimulation	Hao *et al.*,[37,40] 2010
	Eng$^{2f/2f}$ mouse	Brain	Decreased after gene KO	Brain AVM after VEGF stimulation and *Eng* KO	Choi *et al.*,[46] 2012; Choi *et al.*,[47] 2014
	HHT1 patient	Somatic cell	Decreased	Higher incidence of AVM in brain and pulmonary	McAllister *et al.*,[24] 1995
Vasospasm SAH	Human patient	Serum	Decreased	patients with cerebral infarction,	Dietmann *et al.*,[95] 2012
	Human patient	CSF	Increased	patients with cerebral infarction	Testai *et al.*,[94] 2011
Carotid stenosis	Human patient	Carotid plaque	Increased	Positive correlation with stage of plaque	Conley *et al.*,[10] 2000; Bot *et al.*,[99] 2009; Luque *et al.*,[100] 2009
	Mouse	Carotid plaque	Increased	Atrovastatin increase *Eng* expression	Rathouska *et al.*,[101] 2011
Moyamoya disease	Human patient	Intima of MCA	Increased	-	Takagi *et al.*,[103] 2007

AVM: arteriovenous malformation; CSF: cerebrospinal fluid; HHT: hereditary hemorrhagic telangiectasia; MCA: middle cerebral artery; KO: knock-out; SAH: subarachnoid hemorrhage; VEGF: vascular endothelial growth factor

vascular development and regulation of vascular tone. ENG deficiency is associated with the development of AVM in HHT patients, exacerbates stoke injury and impairs stroke recovery. ENG might be a potential biomarker for vasospasm after SAH and cerebrovascular stenosis. Therefore, experimental or therapeutic modulating of ENG expression are useful ingeneration of disease models in animals to study disease pathogenesis and indevelopment of novel therapies to treat cerebrovascular diseases. The exact function of ENG in cerebrovascular diseases remains to be revealing.

DECLARATIONS

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There are no conflicts of interest.

Patient consent
There is no patient data involved.

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Not applicable.

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Case Report **Open Access**

A case of atypical progressive multifocal encephalopathy mimicking acute ischemic stroke: case report and review of literature

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ABSTRACT

Progressive multifocal encephalopathy (PML) is a rare but often fatal infectious brain disease caused by the reactivation of John Cunningham polyomavirus. Reactivation occurs in immunocompromised individuals with AIDS and leukemia, on chemotherapy or being treated with immunosuppressant drugs (e.g. monoclonal antibodies). Cases of PML have been described in patients treated with natalizumab, efalizumab and rituximab used, respectively, for the treatment of (1) multiple sclerosis, (2) psoriasis and (3) haematological malignancies or systemic autoimmune diseases (rheumatoid arthritis and systemic lupus erythematosus). The authors describe an unusual case of acute brainstem and cerebellar PML following chemotherapy for chronic lymphatic leukemia diagnosed 4 years before the onset of PML in a 75-year-old man. The patient was treated with high dose chemotherapy and rituximab with complete response. The onset of symptoms of PML was very rapid and occurred after more than two years from last rituximab infusion; patient had a sudden neurological deterioration, with rapid progression to death in about a month from the onset of symptoms. Lesions were localized in the cerebellum, brainstem and such pattern has been reported in very few cases in the literature.

Quick Response Code:

INTRODUCTION

Progressive multifocal encephalopathy (PML) is a demyelinating infectious disease of the central nervous system caused by reactivation of John Cunningham polyomavirus (JCV) and often leads to death resulting from progressive oligodendrocytes infection and lysis[1]. Prior to human immunodeficiency virus era, this infection was seen in severely immunosuppressed patients, including individuals with hematological malignancies, organ transplantation or chronic inflammatory conditions, such autoimmune disorders, with an incidence of 4 cases/100,000[2-4].

Clinical presentation is heterogeneous: PML usually begins as a subacute illness that typically evolves from focal or multifocal neurological deficits progressing over days to weeks, leading to severe disability and, ultimately, to death. In some cases focal neurological syndromes may present acutely and can be mistaken for stroke. The ratio of cerebral to brainstem involvement is estimated approximately to be 10:1. For reasons that are unclear, brainstem involvement is more common in acquired immunodeficiency syndrome patients, with a ratio of approximately 4:1[1].

Diagnosis of PML is clinical and radiological, principally magnetic resonance imaging (MRI) based; diagnosis is then confirmed by demonstration of JCV DNA in the cerebrospinal fluid by polymerase chain reaction (PCR) test[4,5].

Key MRI diagnostic features are: (1) cortical and deep cerebellar nuclei (in infratentorial involvement) sparing; (2) absence of mass effect on subarachnoid or ventricular spaces and on adjacent areas; and (3) lack of contrast enhancement (CE). Diffusion-weighted images (DWI) sequences are also able to assess the extension of white matter lesions; regions with increased DWI intensity represent white matter areas characterized by reduced water diffusion due to cytotoxic edema[2-6].

Demyelination is usually multifocal, involving hemispheric white matter (parietal, frontal and occipital lobes) and/or cerebellar peduncles. In literature, rare cases of posterior fossa localization are described[7].

To date, there is no established therapy for PML and the treatment is mostly supportive[8].

CASE REPORT

The patient was a 75-year-old male with a previously (2012) diagnosed chronic lymphocytic leukemia. He was treated with chlorambucil in 2012, followed by 6 cycles of fludarabine, cyclophosphamide and lenalidomide in 2013, with complete response; he was then treated with rituximab and steroids for hemolytic anemia (for 4 weeks in 2014) with complete regression.

In March 2016, he was admitted to the emergency department for acute dizziness and ataxia followed, after one day, by dysarthria and left limbs ataxia. A cerebral unenhanced computed tomography (CT) scan showed diffuse ipodensity of the left cerebellum and the middle cerebellar peduncle without mass effect [Figures 1 and 2].

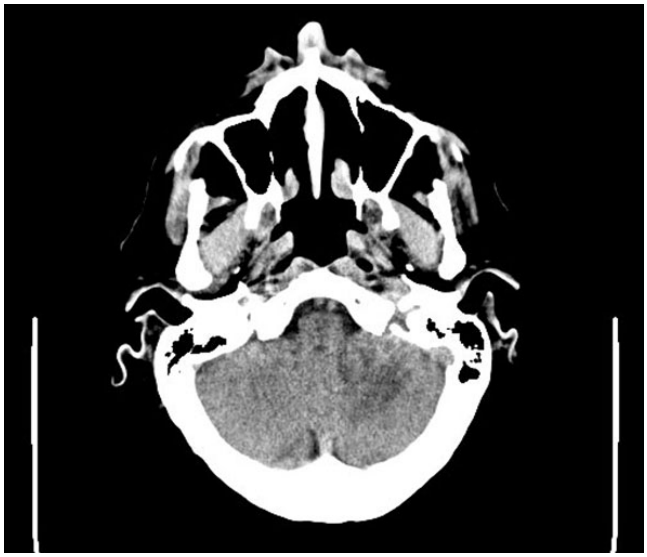

Figure 1: Brain computer tomography scan without contrast showing ipodensity al left cerebellar hemisphere without mass effect

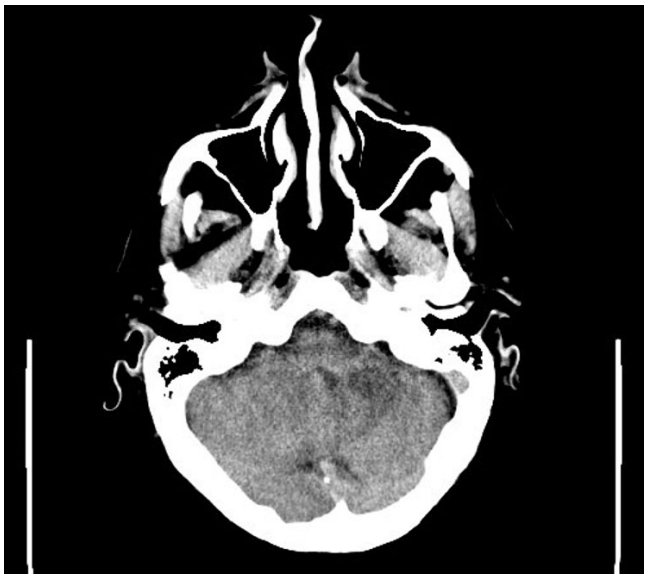

Figure 2: Brain computer tomography scan without contrast showing ipodensity al left cerebellar hemisphere spreading to middle cerebellar peduncle

Neurological examination showed that the patient had dysarthria and left limbs dysmetria, left-beating nystagmus and balance difficulties. Acute ischemic stroke was suspected and antiplatelets therapy with aspirin was started, with transitory symptoms improvement.

Seven days later he presented with worsening of dizziness, nausea and lack of appetite; a second brain scan CT (unchanged) and gastroenterological investigations (negative) were performed.

The patient was discharged, but 10 days later, he had acute onset of involuntary movements in left arm that were interpreted as partial epileptic seizures. Antiepileptic therapy with levetiracetam was started with good response. Two days later he had a rapid worsening of symptoms, with alteration of consciousness; he was responsive only to pain stimuli, left limbs hyposthenia and left gaze deviation also appeared. A CT scan showed an extension of the brainstem lesion with middle cerebellar peduncle involvement. Such a finding, together with clinical deterioration, suggested the presence of a partial basilar thrombosis/embolism. A CT angiography was then performed, showing patency of basilar and vertebral arteries. CT scan findings were interpreted as caused by a mass effect of the pre-existing lesion instead of a true extension of the lesion itself, due to the intrinsic low contrast resolution of the exam. Due to the presence of fever and rigor nucalis, infective encephalitis was suspected, and a lumbar puncture was performed, showing only mirror pattern oligoclonal bands on cerebrospinal fluid (CSF) and serum, PCR for neurotropic viruses (HSV, VZV, CMV, EBV, Adenovirus and Enterovirus) and cultural CSF examination were negative. A broad-spectrum antiviral and antibacterial therapy was started without improvement. Blood test exams were normal.

A brain MRI revealed an asymmetric T2/FLAIR hyperintensity in bilateral cerebellar, middle cerebellar peduncles, upper pons and mesenchephalum white matter without oedema, without CE; DWI showed signal increase without detectable apparent diffusion coefficient changes [Figures 3-5].

Another lumbar puncture was performed and PCR for JCV virus on CSF tested positive with 11,300 copies/mL.

Patient quickly deteriorated and died 10 days after the diagnosis of PML; due to the severity of clinical condition, the rapid progression of symptoms, and the lack of evidence of efficacy of specific therapies, he was treated only with supportive therapy.

DISCUSSION

Treatment with monoclonal antibodies is a newly identified predisposing factor for PML development. Among monoclonal antibodies those that increase the risk for PML development are natalizumab, efalizumab and rituximab. At present, more than 70 cases of PML have been associated with the use of rituximab, predominantly in patients treated for

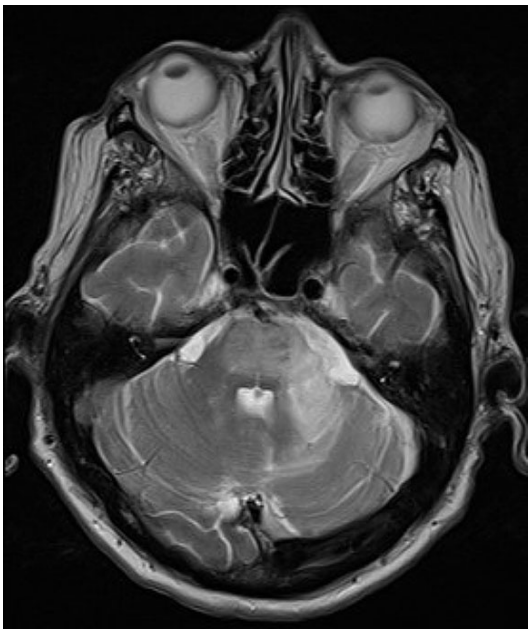

Figure 3: Axial brain magnetic resonance imaging, T2 sequences, revealing asymmetric T2 hyperintensity of the white matter of middle cerebellar peduncles and upper pons without oedema

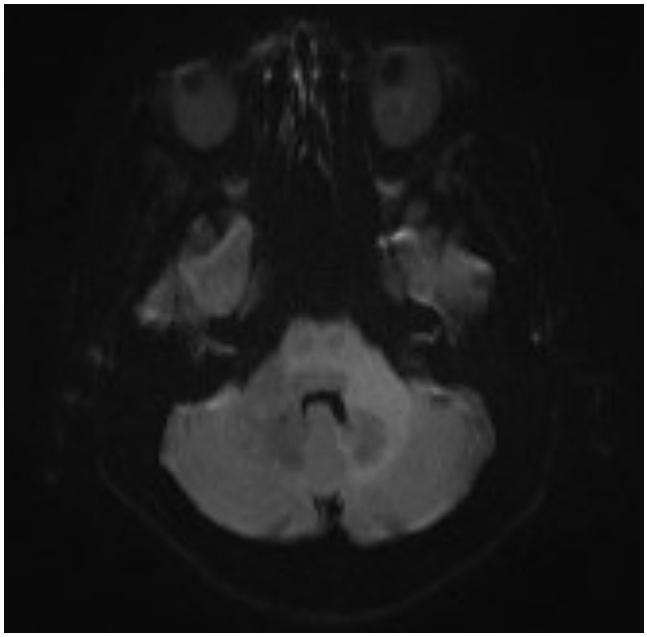

Figure 4: Brain magnetic resonance imaging, diffusion weighted images sequences: increase of signal in the same regions without detectable apparent diffusion coefficient changes

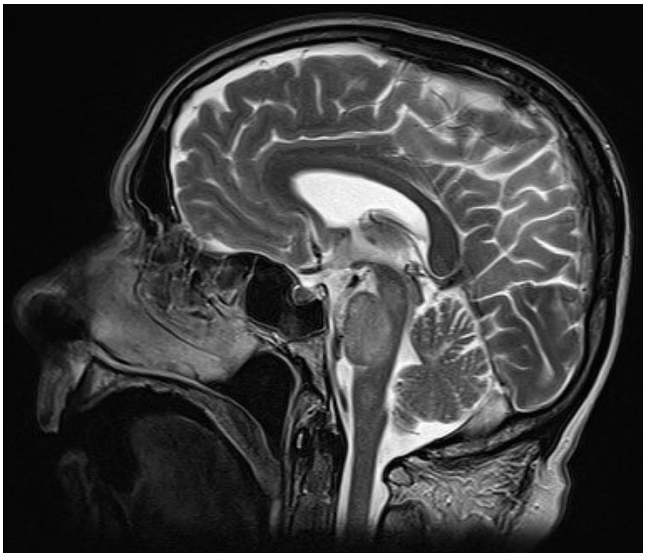

Figure 5: Sagittal brain magnetic resonance imaging, T2 sequences: presence of an asymmetric T2 hyperintensity of white matter in bilateral cerebellar, middle cerebellar peduncles, upper pons and mesenchephalum without oedema

lymphoproliferative disorders. Rituximab is a chimeric human/mouse IgG1 monoclonal antibody that targets CD20 antigen expressed on the surface of both normal and malignant B lymphocytes; rituximab was approved for the treatment of CD20 positive hematological malignancies, and for non-malignant autoimmune disorders, as rheumatoid arthritis and systemic lupus erythematosus; it can also be used with an "off-label" indication in multiple sclerosis and neuromyelitis optica[7-9].

In general population, the risk of PML is 1 in 200,000 people[7]. However, even if the use of rituximab may increase the risk of developing PML, the absolute risk of PML is probably low and does not overcome the benefits in term of mortality in patients with hematological malignancies. The disease in this group of patients appears to set early (following the start of rituximab therapy, with median time of onset from the last dose being about 6 months), with rapid progression and fatal course (median time to death following diagnosis about 2 months). Predisposing factors for rapid progression of the disease include CD4 count < 500 cells and PML diagnosis within three months following therapy initiation[7-10].

The pathophysiology of the rituximab-PML association is unclear and, as reported in the literature, does not seem to be solely due to a B cells depletion[11,12].

In this case, the association of many atypical characteristics: (1) posterior fossa presentation (as opposed to the classical hemispherical lesions); (2) absence of recent immunosuppression (last rituximab dose administered two years before PML onset) without signs of hematological abnormalities; and (3) rapid neurological deterioration (day-by-day worsening with "sudden" onset of the symptoms) made PML diagnosis particularly tricky. Despite single atypical characteristics have already been reported in the literature, association of such findings have never been reported so far.

As shown by MRI and CT scan, absence of a distinctive vascular territory lesion distribution, despite the acute clinical course and the posterior fossa localization could have been an early clue to a correct diagnosis.

In this case, diagnosis of PML has been delayed because of the clinical presentation (acute onset of cerebellar symptoms), initial lack of anamnestic data (i.e. previous chemotherapies that have been told later) and the presence of hypodense lesion in the first cerebral CT scan, which had been interpreted as acute ischemic lesion, reason why cerebral MRI was not performed in the acute phase.

DECLARATIONS

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Authors' contributions
Drafted the manuscript: M.T. Infante, G. Novi, L. Barletta
Contributed to the analysis and interpretation of data: L. Malfatto, R. Gentile, L. Castellan, C. Serrati, M.T. Infante, G. Novi, L. Barletta
Revised the manuscript, gave final approval and agreed to be fully accountable for ensuring the integrity and accuracy of the work: M.T. Infante, G. Novi, R. Gentile, L. Malfatto, L. Castellan, C. Serrati, L. Barletta

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Conflicts of interest
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Patient consent
The patient and his family gave the informed consent.

Ethics approval
All the study procedures of this case report were conducted according to the Declaration of Helsinki.

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Case Report **Open Access**

Dramatic recovery from stroke following intravenous thrombolysis in a patient on prasugrel for recent percutaneous coronary angioplasty

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ABSTRACT

The authors report the first case of thrombolysis in a patient already receiving both aspirin and prasugrel following a recent ischemic coronary event. A 55-year-old gentleman was treated for inferior wall myocardial infarction with aspirin, prasugrel and percutaneous angioplasty of right coronary artery. Three days following the procedure he developed acute ischemic stroke due to a left middle cerebral artery infarction with a National Institute of Health Stroke Scale (NIHSS) of 24 and was treated with alteplase. Therapy was interrupted after completion of 29 mg (for a body weight of 65 kg) dose due to oral bleeding. Fifteen minutes post thrombolysis NIHSS was 5 and dropped to zero after 12 h. This report highlights the benefits of alteplase in the context of several relative contraindications like the setting of acute myocardial infarction treated with percutaneous intervention and high NIHSS.

Quick Response Code:

INTRODUCTION

Intravenous thrombolysis is the standard of care in acute ischemic stroke, and is associated with significant improvement in outcome measures[1]. Intracerebral hemorrhage is an absolute contraindication for thrombolysis. While due diligence must be exercised, strict interpretations of relative contraindications might prove a barrier to potentially life changing thrombolysis in stroke[2,3]. Hence decisions regarding thrombolysis are made on a case-by-case basis[4]. We report a patient who, whilst on prasugrel and aspirin for post-myocardial infarction (MI), made a dramatic recovery from acute ischemic stroke after thrombolysis.

CASE REPORT

A 55-year-old gentleman was referred to our hospital two days following the development of inferior wall myocardial infarction (IWMI) diagnosed as ST-elevation myocardial infarction (STEMI) that had been treated with antiplatelets and statin. The last ECG at the time of discharge after the IWMI revealed evolved infarction. Three days after the onset of IWMI he underwent percutaneous angioplasty of the right coronary artery. Three days following this procedure, he was discharged on the following medications: aspirin 150 mg/day, prasugrel 10 mg/day, and atorvastatin 40 mg/day. While being discharged he developed weakness of right sided limbs, inability to comprehend or talk, and became drowsy. There was no history of headache, vomiting, or convulsions. At the emergency room, his vital parameters were: BP 110/60 mmHg, pulse 64/min. He had global aphasia, right gaze palsy, right hemiplegia, hemianopia, hemihypoaesthesia, and sensory inattention. National Institute of Health Stroke Scale (NIHSS) was 28. Blood sugar was 160 mg%. CT scan of the brain was unremarkable. MRI of the brain, done 30 min after the onset of stroke, revealed a moderate sized infarct involving the left middle cerebral artery territory [Figure 1A and B]. MR angiogram revealed diffuse pruning of M1 and significant reduction of signals in M2 and M3 segments [Figure 1C]. Echocardiogram revealed no intracardiac clot.

The risks and benefits of intravenous thrombolysis were discussed with the family. After obtaining informed consent alteplase was administered. Stroke-onset to needle-time was 55 min. Following the bolus of 5.5 mg, a mild improvement in motor power was noted when he could minimally move the right lower limb. There was a gradual improvement of limb power during the infusion of alteplase. He developed an oral bleed after 29 mg of the drug had been infused. Further infusion was discontinued. Limb power improved to grade 3/5 over the upper and lower limbs, and verbal comprehension was normal. However, he continued to have Broca's aphasia. CT scan of the brain, done immediately, did not reveal intracerebral hemorrhage. No further administration of alteplase was done. Fifteen minutes post thrombolysis Broca's aphasia also significantly improved and he could speak several words fluently. NIHSS was 5. Twelve hours post thrombolysis, NIHSS was zero. Repeat CT scan did not reveal any hemorrhagic complications. Aspirin, 150 mg/day, was started after 24 h and clopidogrel, 75 mg/day, after 48 h. He maintained improvement and was discharged 4 days after the onset of stroke.

DISCUSSION

Alteplase is the only approved intravenous thrombolytic therapy for stroke and is recommended in the first 4.5 h following the onset of acute ischemic stroke[1]. Contraindications to its use were derived from the exclusion criteria utilized in major stroke trials, and violation of protocols have been shown

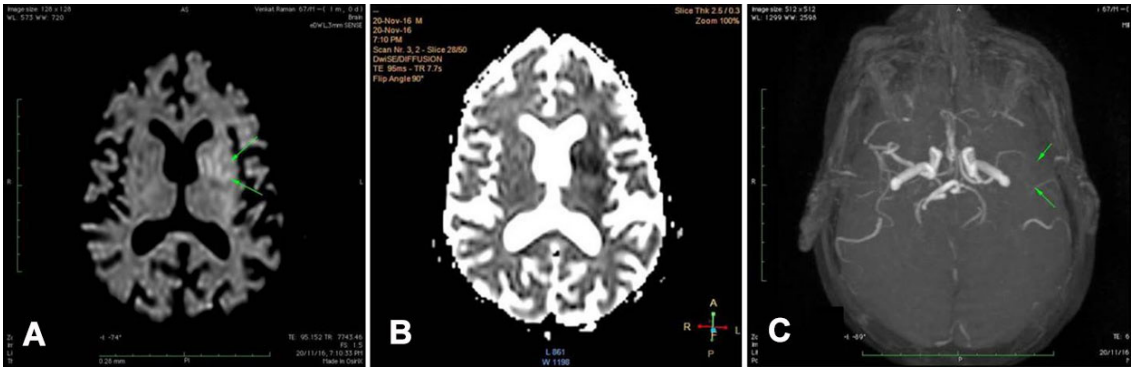

Figure 1: (A) is a diffusion weighted image showing diffusion restriction involving left gangliocapsular and perisylvian regions (shown by arrows) corresponding to left middle cerebral artery territory; (B) is the corresponding apparent diffusion coefficient map showing low signals in the left gangliocapsular region; (C) shows diffuse pruning of M1 and significant reduction of signals in M2 and M3 segments of the left middle cerebral artery (shown by arrows)

to be associated with complications, importantly intracerebral hemorrhage[5]. However, many of the contraindications have proven to be unnecessarily restrictive in real-world clinical practice, and patients have been thrombolysed off-label with consistent benefits[4,6]. Our patient had a recent myocardial infarction, traditionally considered as a contraindication for intravenous thrombolysis. Since the preferred option of mechanical thrombectomy is available only in a few select centers with such expertise, we opted for intravenous thrombolysis which has the inherent risks of myocardial rupture, pericardial hemorrhage and cardiac tamponade. Fortunately, these complications are less likely to occur with inferior wall myocardial infarction in contrast to anterior, and patients have undergone thrombolysis in this situation with benefits[7]. Also, we did not consider the percutaneous intervention, done 3 days prior, as a specific contraindication for therapy. Traditionally, an NIHSS of more than 20 or 25 has been considered as an exclusionary criterion for administration of alteplase. However mounting evidence has shown the benefits of intravenous thrombolysis, despite high NIHSS[1,8]. The occurrence of stroke while in the hospital allowed us to institute treatment within one hour, a factor that could have contributed to the dramatic recovery in our patient[9,10]. Prior antiplatelet therapy is not considered a contraindication for stroke thrombolysis. While thrombolysis has been reported in patients on aspirin or clopidogrel or both, this is the first report of thrombolysis in a patient on prasugrel.

In conclusion, stroke thrombolysis has to be considered on a case-to-case basis after careful consideration of the risks-to-benefits ratio and must be pursued where benefits outweigh the risks.

DECLARATIONS

Authors' contributions

All the authors were part of the treating team. The manuscript was prepared by the corresponding author, and later read and acknowledged by all the authors.

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Patient consent was obtained for publishing the case report.

Ethics approval

Ethics committee approval was obtained for publishing the case report.

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Original Article **Open Access**

LPS-induced TLR4 neuroinflammatory signaling in CHME-5 microglial cells

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Escherichia coli lipopolysaccharide, nuclear factor-kappa B p65, cluster of differentiation 68, toll-like receptor 4, neuroinflammation

ABSTRACT

Aim: In the field of neuroinflammation, identifying specific effects of pharmacological agents and other factors is problematic given the relative difficulty and expense in obtaining and culturing primary microglia. Immortalized microglial cell lines are very useful, but only a limited number have been characterized for inflammatory signaling. Therefore, characterization of lipopolysaccharide (LPS)-induced toll-like receptor 4 (TLR4) signaling in CHME-5, a microglial cell line, is expected to be of value as an experimental model of inflammatory signaling in the central nervous system (CNS). **Methods:** It was recently suggested that CHME-5 cells are of rat origin, not human, hence, verification of this claim using short tandem repeat genotype sequencing, along with immunoblotting, reverse transcription-polymerase chain reaction, and immunocytochemistry techniques to validate that CHME-5 retain morphological, phenotypical, and functional characteristics of primary microglia were undertaken. **Results:** LPS induced inhibitor kappa B-alpha and nuclear factor-kappa B (NF-κB) p65 activation, NF-κB p65 binding activity, and *tumor necrosis factor alpha* gene expression. Additionally, results also confirmed the maintenance of microglial phenotype as seen with increased cluster of differentiation 68 gene and protein expression, immunofluorescence, and the absence of glial fibrillary acidic protein-immunoreactivity.

Quick Response Code:

TLR4 gene expression and immunofluorescence were significantly increased after LPS treatment. **Conclusion:** These data demonstrate that CHME-5 cells are not human, but are indeed a beneficial tool for studying microglial inflammatory signaling.

INTRODUCTION

The innate immune response is instrumental in combatting infection and in response to stress and physical injury. One family of receptors expressed on innate immune cells is toll-like receptors (TLRs). TLRs can recognize a host of patterns produced by bacteria, viruses, and fungi, known as pathogen-associated molecular patterns, or self-products released from apoptotic cells, called damaged-associated molecular patterns[1]. To date, 10 TLRs have been identified in humans, 12 in rodents[2,3].

TLR4 was discovered as the receptor responsible for detection of Gram-negative bacterial lipopolysaccharide (LPS), which leads to production of pro-inflammatory cytokines and up-regulation of co-stimulatory molecules necessary for initiation of the adaptive immune response[4-6]. TLR4 recognizes LPS with the aid of several accessory proteins, including LPS-binding protein (LBP), cluster of differentiation 14, and myeloid differentiation 2. Activation of TLR4 leads to initiation of 2 distinct signaling pathways, MyD88-dependent and TRIF-dependent pathways[3,7].

Until the past decade the central nervous system (CNS) has been generally considered as an "immune privileged" site because of the extensive defense and regulatory mechanisms available to protect this organ from foreign cells and pathogens[8]. But we now know there are cells expressed in the CNS that detect infection or injury and respond accordingly.

Microglia, the primary immune cells, are also known as "macrophages of the CNS". Microglia interact with other glia, namely astrocytes, to support neuronal function, survey/patrol for and clear foreign or harmful particles, and regulate neuroinflammation through pro-inflammatory mediators[9,10]. Microglial activation is characterized by morphological changes, proliferation, and up-regulation of receptors including scavenger, complement, cytokine/chemokine and pattern recognition receptors. Recognition of pathogens or insult leads to activation and release of pro-inflammatory and neurotoxic factors including tumor necrosis factor-α (TNFα), interleukin-1β, interferon gamma inducible protein-10 (CXCL10), nitric oxide (NO) and reactive oxygen species (ROS)[9,11,12]. Microglial activation has been implicated in numerous neurodegenerative diseases including multiple sclerosis, Alzheimer's disease, and Parkinson's disease[13-16].

TLR4 is the most widely studied TLR, and its expression is more abundant in microglia than in any other resident cell in the CNS[17,18]. TLR4 signaling is well defined in peripheral immune cells, but less clear in the CNS. Here, we characterized LPS-induced TLR4 inflammatory signal transduction in the CNS using a fetal-derived microglial cell line, CHME-5. To our knowledge, this is the first report to validate the finding that CHME-5 is not a human cell line, and show that microglial responses in CHME-5 cells are comparative to human primary microglia. Therefore, we demonstrate that CHME-5 can be used as an experimental tool for microglial research and neuroinflammatory signaling.

METHODS

Cell culture

CHME-5 cells were originally developed by Janabi *et al.*[19] and were gifted to our lab by Dr. Pierre Talbot, Quebec, Canada. CHME-5 growth media consisted of Dulbecco's modified eagle medium (DMEM) with 4.5 g/L glucose and sodium pyruvate without L-glutamine, 10% fetal bovine serum, 200 mmol/L L-glutamine, penicillin-streptomycin (100 U/mL potassium penicillin, 100 µg/mL streptomycin sulfate), and 250 µg/mL amphotericin B. CHME-5 cells were maintained in growth media at 37 °C, with 5% CO_2. For experimental assays, growth medium was aspirated from cells and replaced with serum-free media (SFM) for no less than 16 h at 37 °C.

LPS treatment

Lipopolysaccharide from *Escherichia coli* O55:B5 (#L2880, Sigma-Aldrich, St. Louis, MO, USA) was purified by phenol extraction. The lyophilized powder was reconstituted in HyPure cell culture grade water and sterile filtered to a stock concentration of 1.2 mg/mL. Cells were stimulated with 1.0 µg/mL LPS unless otherwise noted. For dose-response studies, 0.001-10 µg/mL was used for stimulation.

STR genotyping

PowerPlex 21 System (Promega-#DC8902) was used to validate Short tandem repeats (STR) regions in CHME-5 cells. Reactions were set up using PowerPlex 21 5× Master Mix [5.0 µL/reaction (rxn)], PowerPlex 21 5× Primer Pair Mix (5.0 µL/rxn), DNA template (0.5 ng), control DNA (0.5 ng), and water (up to 25 µL). Thermocycler settings were as follows: 96 °C-1 min, (94 °C-10 s, 59 °C-1 min, 72 °C-1 min for 30 cycles), 60 °C-10 min. Results were analyzed using GeneMapper-ID Software (Applied Biosystems).

Protein extraction

Depending on the experiment, either whole cell lysate or cytoplasmic/nuclear extractions were isolated for subsequent assessment of protein expression. For whole cell lysates, cells were washed with ice-cold phosphate-buffered saline (PBS) and then 400 µL of cell Lysis Buffer (CBL, Cell Signaling) was added to each 100-mm dish. Following a 5-min incubation at 4 °C, lysates were collected and centrifuged at 14,000 × *g* (4 °C) for 10 min. The supernatant was collected and stored at -20 °C. For cytoplasmic and nuclear extracts, cells were washed with and collected in 1 mL ice-cold PBS. Cells were centrifuged at 129 × *g* (4 °C) for 5 min. The supernatant was aspirated and combined with 400 µL Lysis buffer 1 [10 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) (pH 7.9), 10 mmol/L KCl, 0.10 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.10 mmol/L ethylene glycol-bis (β-aminoethyl ether)-tetraacetic acid (EGTA), 1 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin, and 10 µg/mL aprotinin] and vortexed vigorously for 10 s, followed by incubation on ice for 15 min. Next, 100 µL 5.4% igepal was added, samples vortexed for 10 s, and then centrifuged at 14,000 × *g* (4 °C) for 5 min. The supernatant was collected and stored at -20 °C; then 100 µL Lysis buffer 2 (20 mmol/L HEPES, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 1 mmol/L leupeptin, 10 µg/mL aprotinin) was added to the remaining pellet. The samples were then vortexed for 15 min at 4 °C and then centrifuged at 14,000 × *g* (4 °C) for 22 min. The supernatant was collected and stored at -20 °C.

Immunoblot analysis

Protein extracts were subjected to 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. Briefly, protein (50 µg) in loading dye (0.25 mmol/L Tris, pH 6.8, 10 mmol/L DTT, 30% glycerol, 10% SDS, 0.05% bromophenol blue, and 50 µL/mL β-mercaptoethanol) was boiled for 10 min and then loaded into gels. Electrophoresis was performed in running buffer (250 mmol/L glycine, 25 mmol/L Tris, 0.1% SDS) at 100 V for 15 min, and then at 125 V for 145 min for an overall running time of 2 h. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes in transfer buffer (195 mmol/L glycine, 25 mmol/L Tris, pH 8.0, 20% methanol) at 100 V for 90 min. After transfer, PVDF membranes were rinsed and blocked in bovine serum albumin (BSA) in 1× Tris-buffered saline-Tween (TBST) (150 mmol/L NaCl, 25 mmol/L Tris, pH 8.0, 2 mmol/L KCl, 0.1% Tween-20-TBST) for 2 h at 25 °C with rocking. For cluster of differentiation 68 (CD68), 2% BSA was used and 5% BSA was used for all other antibodies. Primary antibodies diluted in blocking buffer (1:500-1:2,000), were added to membranes and rocked overnight at 4 °C. Membranes were then washed 3 times with TBST for 5 min with each wash. Alkaline phosphatase-linked secondary antibodies diluted in blocking buffer (1:1,000-1:5,000) were added to membranes and rocked for 2 h at 25 °C and then washed 3 times with TBST for 20 min. Enhanced Chemifluorescence (ECF) substrate (#45-000-947, GE Healthcare Amersham) was used to image blots using the Typhoon Scanner 9410. Image J software (National Institutes of Health) was used to obtain the mean grey intensity for the bands of interest.

MTT assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed to determine cell viability after treatment. Fresh SFM (1 mL) was added to each well followed by addition of 111 µL MTT. Cultures were then incubated at 37 °C for 45 min. Media was aspirated from each well and 1.5 mL dimethyl sulfoxide added followed by rocking at 25 °C for 30 min. Absorbance was read at 492 nm with the Synergy 2 plate reader (Biotek Instruments).

NF-κB p65 binding assay

Nuclear factor-kappa B (NF-κB) activation was measured using the NF-κB p65 transcription factor kit (#89859, Thermo Scientific) per manufacturer's instructions. Briefly, binding buffer (50 µL) was added to each well, which was pre-coated with the NF-κB binding consensus sequence. Then, 10 µL of nuclear extract was added to each well, in duplicate. Following incubation for 1 h at 25 °C with mild agitation, wells were washed 3 times with 200 µL of wash buffer, then 100 µL of primary antibody (1:1,000) was added to each well, followed by a 1-h incubation at 25 °C, without agitation. Wells were washed as described above and 100 µL of secondary antibody (1:10,000) was added to each well, followed by 1-h incubation at 25 °C, without agitation. Finally, wells were washed 4 times with 200 µL of wash buffer, and 100 µL of chemiluminescent substrate was added to each well. Chemiluminescence was measured immediately with a Nikon plate reader.

RNA extraction

Following cell stimulation, cells were washed 3 times with ice-cold PBS, incubated in Trizol (1 mL/100 mm dish) at 25 °C for 5 min, and then lysates were collected in nuclease-free tubes. Chloroform (0.2 mL) was added to lysates followed by manual shaking of tubes for 15 s. Lysates were then incubated at 25 °C for 3 min, centrifuged at 12,000 × *g* for 15 min (4 °C)

and then the upper aqueous phase was collected in fresh microcentrifuge tubes. Isopropanol (0.5 mL) was added to the aqueous phase, followed by incubation at 25 °C for 10 min, and then centrifuged at 12,000 × *g* for 10 min (4 °C). Next, the supernatant was removed and RNA pellets washed with 70% ethanol, and centrifuged at 7,500 × *g* for 2 min (4 °C). This ethanol wash step was repeated twice. RNA pellets were dried at 25 °C for 15 min, followed by addition of 35 µL nuclease-free water. RNA samples were incubated in a 65 °C water bath for 10 min and then stored at -80 °C. To obtain RNA concentrations, samples were thawed on ice and ng/mL was determined with a Nanodrop Spectrophotometer 1000 (Thermo Scientific), and validated with the 260/280 ratios between 1.8 and 2.0.

RNA integrity

RNA gels were prepared with NorthernMax-Gly Reagents (Ambion) to determine RNA integrity. A 1% agarose gel was prepared with NorthernMax-Gly buffer. To prepare samples, 3 µL RNA, 3 µL nuclease-free water and 6 µL glyoxal sample loading dye were incubated at 50 °C for 30 min. Samples were loaded and electrophoresed at 100 V for 45 min. RNA gels were stained with SYBR Safe (3 µL/50 mL in NorthernMax-Gly buffer) and rocked on a Multimixer (Lab-Line Instruments, LLC-Melrose Park, IL) for 30 min. To visualize the 28S and 18S bands, RNA gels were imaged on Typhoon Scanner 9410 (GE Healthcare Life Sciences) at 450 V.

Real-time-polymerase chain reaction

RNA was reverse transcribed using the SuperScript IV VILO Master Mix (Invitrogen, #11766050) and cDNA (100 ng) was used to perform real-time polymerase chain reaction (RT-PCR) for genes of interests. The primer for CD68 was designed using rat CD68 messenger RNA (mRNA) from the NIH National Center for Biotechnology Information website and obtained from IDT Technologies. The rat primers for TLR4, TNFα, and β-actin were obtained from IDT Technologies. RT-PCR mix included 2× PowerUp SybGreen, 0.5 µmol/L forward primer, 0.5 µmol/L reverse primer, and nuclease-free water up to 15 µL. Thermocycler settings were as follows: 50 °C-2 min, 95 °C-2 min, (95 °C-15 s, 60 °C-1 min for 40 cycles), 95 °C-15 s, 60 °C-1 min, 95 °C-15 s. Primer sequences used were as follows: TLR4-forward: 5'-GAA GCT ATA GCT TCA CCA ATT TCT CAC AA-3', 60.2 °C; reverse: 5'-GAT AGG GTT TCC TGT CAG TAC CAA GGT TG-3', 60.1 °C; CD68-forward: 5'-CTC AGC AGC TCT ACC ATG AGG TTC-3', 59 °C; reverse: 5'-CTT CCG GTG GTT GTA GGT GTC TC-3', 59.2 °C; TNFα-forward: 5'-CAG ATC ATC TTC TCA AAA CTC GAG TGA CA-3', 60.3 °C; reverse: 5'-GTT GGT TGT CTT TGA GAT CCA TGC CAT TG'-3', 60.1 °C; and β-actin-forward: 5'-GAA GGA TTC CTA TGT GGG CGA CGA-3', 60.5 °C; reverse: 5'-GAG CCA CAC GCA GCT CAT TGT AG-3', 60.3 °C.

Immunocytochemistry

Cells were exposed to SFM with or without LPS (1 µg/mL), for 10 min at 37 °C. After stimulation, cells were gently washed 3 times with ice-cold PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at 25 °C. Cells were washed 3 times with ice-cold PBS and permeabilized with 0.01% Triton-X in PBS for 10 min at 25 °C. Next, cells were washed as described above and primary antibodies mouse-TLR4 (1:1,000) or rabbit-CD68 (1:1,000) in PBS added to respective wells and incubated overnight at 4 °C. The next day, cells were washed 3 times with TBST and incubated with fluorescently labeled secondary antibodies, anti-rabbit-AlexaFluor-647 (1:1,000) or anti-mouse-AlexaFluor-555 (1:1,000) while rocking for 2 h at 25 °C. Negative control cells were unstimulated cells only incubated with secondary antibodies. Cells were washed 3 times with TBST and labeled with [4'-6-diamidino-2-phenylindole (DAPI)-PBS)] for 10 min, rocking at 25 °C. Finally, cells were washed 3 times with TBST, cover slips removed from wells, and mounted on slides using anti-fade gold mounting media (Invitrogen). Slides were dried overnight and then imaged.

Epifluorescence microscopy

TLR4 and CD68 immunofluorescence was imaged with an epifluorescence microscope (Olympus BX51) using Spot 5.1 software. Images were captured with the 20× objective using fluorescent channels for DAPI (350 nm), TLR4 (TRITC-555 nm), and CD68 (Cy5-647 nm). Images were processed with CellProfiler cell image analysis software[20], and quantified in GraphPad Prism 7.0.

CellProfiler

All images of nuclei were processed in Image J, with global contrast settings to clean-up and define nuclear boundaries for CellProfiler's automatic nuclear recognition algorithm. A CellProfiler pipeline was created for each fluorescent channel image set to define global pixel intensity thresholds. All image sets were then processed for high-throughput quantification in CellProfiler to measure area and mean grey intensity. High-throughput quantification can process hundreds of images while standardizing the recognition and measurements of each cell in every image. This process increases sample size while removing inherent subjectivity of hand tracing.

Confocal microscopy

The Leica SPE Scanhead RYBV confocal microscope was used to capture images for brightfield, and immunofluorescence and all images were processed in Leica Application Suite Advanced Fluorescence. For brightfield imaging, the 488 laser (55%) was used to capture images with the 100× oil objective in unstimulated and LPS-stimulated cells, which were fixed with 4% PFA for 10 min at 25 °C. For fluorescent imaging, cells were prepared as described in the immunocytochemistry section and images captured with the 100× oil objective using fluorescent channels for DAPI (350 nm), TLR4 (Rhodamine-555 nm), and CD68 (Texas Red-647 nm). TLR4 channel (red) gain and laser was set to 925 and 33%, CD68 channel (green) gain and laser was set to 975 and 33%, and DAPI channel (blue) gain and laser was set to 839 and 15%. These settings were applied to all confocal images. One random field-of-view (FOV) was obtained for each group at a resolution of 1024 × 1024 pixels. Eighty images were then taken through the depth (Z) of 6.63 μm for each FOV. This value of 6.63 μm was used to provide equal X, Y, and Z dimensions, providing standardization of collecting depth image sets.

Three dimensional reconstruction

Confocal image sets for each fluorescent channel were converted to 8-bit and processed in image J using Isosurface within the Bone J plugin (http://bonej.org/). TLR4 three dimensional (3D) mesh was produced with a resample rate of 2 and a threshold of 20. CD68 3D mesh was generated with a resample rate of 2 and threshold of 23. DAPI 3D mesh was constructed with a resample rate of 2 and a threshold of 30. 3D meshes for all fluorescent channels were imported into Blender (blender.org) and scaled uniformly. Artifacts due to apparent non-specific and non-cellular labeling were deleted in some channels for better imaging. Lighting, cameras and mesh-materials were created to image each scene. The respective brightfield image for each FOV was then placed at the base of each 3D reconstruction and scaled to fit. Images were rendered using Blender Cycles Render at 3840 × 3840 pixel resolution.

Statistical analysis

Image J Software was used to obtain the mean grey intensity of all immunoblots and the mean was taken for each time point. GraphPad Prism 7.0 was used for transformation, quantification, and graphing of all data. For statistical analysis of mRNA and protein expression, binding activity, cell viability, and immunofluorescence, one-way analysis of variance (ANOVA) with Dunnett's or Tukey's multiple comparison tests were used, unless otherwise stated in the results section. Significance was determined at $P < 0.05$.

RESULTS

CHME-5 cells are not of human origin (STR genotyping of CHME-5 cells)

STR Genotyping was performed to validate the claim that CHME-5 cells are not of human origin. STR genotyping confirmed that CHME-5 cells are devoid of any human genomic DNA [Supplementary Figure 1].

LPS-induced activation of NF-κB p65 in CHME-5 cells

Activation of NF-κB p65 is a crucial step for nuclear translocation and transcription of pro-inflammatory mediators. Phosphorylation of NF-κB p65 was used as a measure of NF-κB activation. Immunoblot analysis indicated that LPS increased nuclear levels of phospho-NF-κB p65 [Figure 1A]. Further analysis with a Student's *t*-test revealed that the increase was statistically significant, $P < 0.02$ [Figure 1B].

LPS is not toxic

The MTT assay was used to determine the extent to which LPS stimulation affects cell viability. ANOVA revealed that LPS (0.001-10 μg/mL) at 30 min did not significantly ($P = 1.00$) affect viability of CHME-5 cells [Figure 1C].

LPS-induced NF-κB p65 binding activity

For transcription of pro-inflammatory mediators to occur, the NF-κB p65 subunit must bind to nuclear consensus sequences. NF-κB p65 activation was also determined by assessing NF-κB p65 binding activity in nuclear extracts. Tubulin and histone 3 expression were measured as internal controls to confirm cellular compartmentalization in cytoplasmic and nuclear fractions [Supplementary Figure 2]. ANOVA and uncorrected Fisher's LSD tests indicated that NF-κB p65 binding activity was significantly increased at 10 ($P < 0.04$) and 90 min ($P < 0.01$) [Figure 1D]. Binding activity at 30 and 180 min remained at basal levels.

LPS-mediated IκBα phosphorylation in CHME-5 cells

Inhibitor kappa b alpha (IκBα) is the negative regulator for NF-κB and thus must be activated for release of NF-κB into the nucleus. Phosphorylation of IκBα in cytoplasmic lysates was used as a measure of IκBα activation [Figure 2A]. ANOVA and Dunnett's multiple comparison tests revealed LPS significantly increased

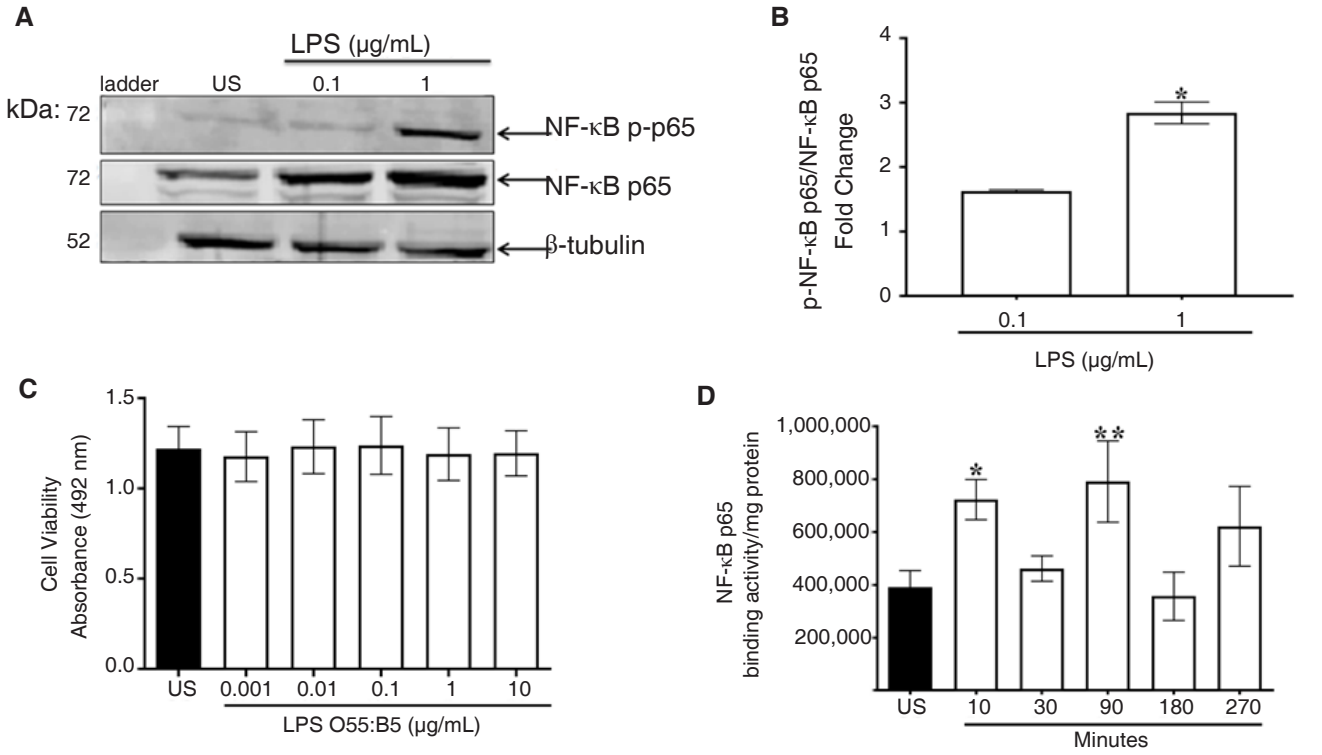

Figure 1: LPS-induced NF-κB p65 activation. CHME-5 cells were exposed to LPS (0.1-1 μg/mL) or media alone at 37 °C for 30 min. A: nuclear lysates were subjected to SDS-PAGE electrophoresis and immunoblotted with NF-κB p-p65 (1:1,000), NF-κB p65 (1:1,000), and β-tubulin (1:1,000) antibodies; B: data is normalized to US and expressed as fold change, integrated density, (*$P < 0.02$) *vs.* 0.1 μg/mL, images are representative of 3 independent experiments ($n = 3$) for each treatment group; C: CHME-5 cells were exposed to LPS (0.001-10 μg/mL) or media alone at 37 °C for 30 min, cell viability absorbance was read at 492 nm, 3 independent experiments were performed in duplicate ($n = 3$) for each treatment group; D: CHME-5 cells were stimulated with LPS (1 μg/mL) at 37 °C for 10-270 min, nuclear extracts were analyzed for NF-κB p65 binding activity, (*$P < 0.04$, **$P < 0.01$) *vs.* US. Image is representative of 5 independent experiments ($n = 5$) for each treatment group. Bars for all groups are presented as mean ± SEM. LPS: lipopolysaccharide; NF-κB: nuclear factor-kappa B; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; US: unstimulated

IκBα activation at 10 min following stimulation, ($P < 0.02$) [Figure 2B]. At 30-270 min, stimulation levels of p-IκBα were similar to control cells. At early time points, IκBα did not undergo degradation in CHME-5 cells [Supplementary Figure 3].

LPS-induced *TNFα* gene expression

Once NF-κB p65 is phosphorylated and translocated into the nucleus, it binds to pro-inflammatory consensus sequences for initiation of gene transcription mediators, such as TNFα. Compared to unstimulated cells, stimulation with LPS (1 μg/mL) significantly increased *TNFα* gene expression at 3 ($P < 0.002$), 6 ($P < 0.02$), and 18 ($P < 0.003$) h, as indicated by Kruskal-Wallis and Dunn's multiple comparison tests [Figure 3A]. As an internal control, RNA integrity was verified by visualizing the 28S and 18S ribosomal RNA (rRNA) bands [Supplementary Figure 4].

LPS is not toxic at later time points

The MTT assay revealed that LPS was not cytotoxic, rather LPS treatment resulted in a minimal increase in cell viability at 6 h, as indicated by ANOVA and Dunnett's multiple comparison tests ($P < 0.01$) [Figure 3B].

CD68 gene expression in CHME-5 cells

CD68 is an established microglial marker, which is up-regulated during activation. Quantification of CD68 mRNA revealed a significant up-regulation of *CD68* gene expression at 3 ($P < 0.0001$) and 6 ($P < 0.01$) h compared to unstimulated cells, as indicated by ANOVA and Dunnett's multiple comparison tests, while expression returned to basal levels by 18 h [Figure 4A].

CD68 protein expression in CHME-5 cells

CD68 protein expression was analyzed after LPS stimulation in whole cell lysates [Figure 4B]. CD68 expression was significantly greater in LPS-stimulated cells at 10 min compared to unstimulated cells, as indicated by Student's *t*-test, ($P < 0.0002$) [Figure 4C]. Multiple CD68 protein bands were detected in LPS-stimulated cells, most prominently at 68, 90, and 110 kilodaltons, which reflect the glycosylated form of CD68 protein[21].

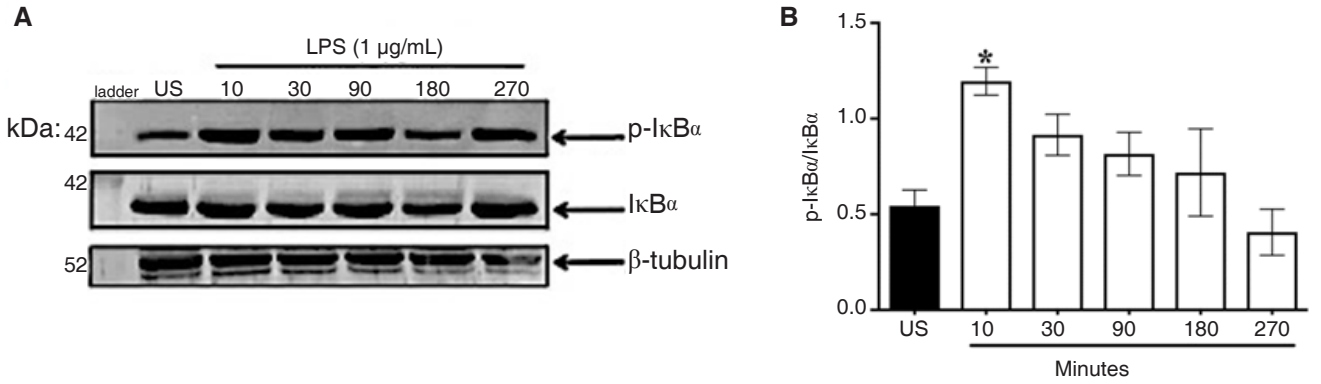

Figure 2: LPS-induced IκBα activation. CHME-5 cells were stimulated with LPS (1 μg/mL) at 37 °C for 10-270 min. A: cytoplasmic lysates were subjected to SDS-PAGE electrophoresis and immunoblotted with p-IκBα (1:1,000), IκBα (1:1,000), and β-tubulin (1:1,000) antibodies; B: integrated density, **P* < 0.02 *vs.* US. Image is representative of 4 independent experiments (*n* = 4) for each treatment group. Bars for all groups are presented as mean ± SEM. LPS: lipopolysaccharide; IκBα: inhibitor kappa b alpha; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; US: unstimulated

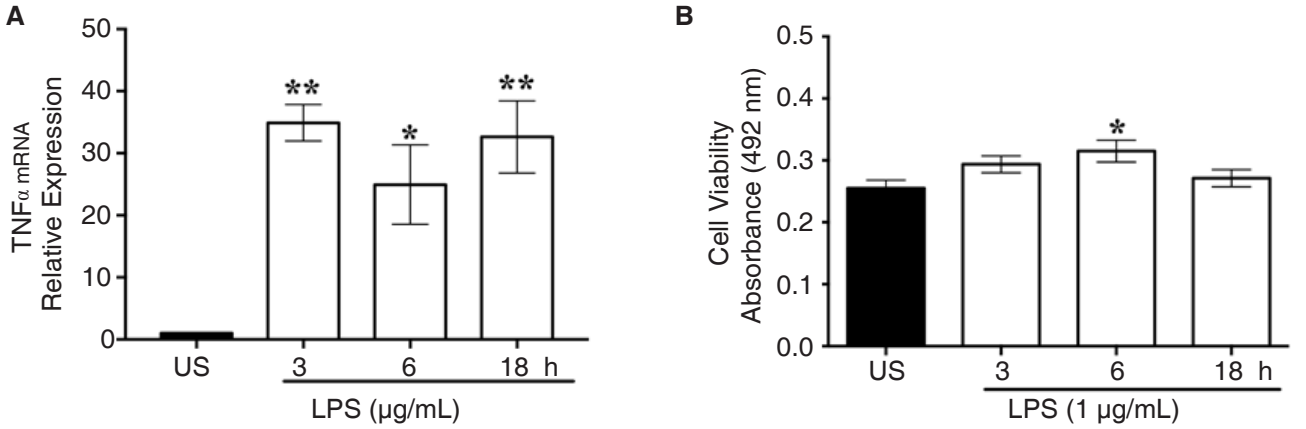

Figure 3: LPS-induced TNFα gene expression. A: CHME-5 cells were treated with LPS at 37 °C for 3, 6, and 18 h. TNFα mRNA expression was analyzed by RT-PCR analysis with β-actin as housekeeping gene, 3 h (***P* < 0.002), 6 h (**P* < 0.02), and 18 h (***P* < 0.003) *vs.* US. Image is representative of 3 independent experiments (*n* = 3) for each treatment group; B: cell viability absorbance was read at 492 nm, **P* < 0.01 *vs.* US. Experiments were carried out in duplicate (*n* = 3) for each treatment group. Bars for all groups are presented as mean ± SEM. LPS: lipopolysaccharide; TNFα: tumor necrosis factor-α; RT-PCR: real-time polymerase chain reaction; US: unstimulated

CHME-5 cells are GFAP negative

To further confirm CHME-5 cells are microglia and not astrocytes, glial fibrillary astrocytic protein (GFAP) was measured at 10 min using whole cell lysates from normal human astrocytes (NHA) as a positive control [Figure 4D]. ANOVA and Tukey's multiple comparison tests revealed that GFAP expression in unstimulated and LPS-stimulated NHA was significantly greater than in CHME-5 cells (US-*P* < 0.0002, LPS-stimulated-*P* < 0.0001); indeed GFAP-immunoreactivity in CHME-5 cells was negligible [Figure 4E].

TLR4 gene and protein expression in CHME-5 cells

LPS induces the innate immune response through binding TLR4 and initiating intracellular signal transduction, which occurs rapidly, especially if proteins are not made de novo, and therefore, protein expression was analyzed as early as 10 min. On the other hand, RNA transcription is a lengthy process and therefore we chose to look at later time points (3, 6, 18 h). ANOVA and Dunnett's multiple comparison tests revealed that TLR4 mRNA expression was significantly elevated 3 h (*P* < 0.0001) and 6 h (*P* < 0.006) after LPS treatment; by 18 h, expression was similar to unstimulated control [Figure 5A]. We also determined the extent to which LPS modulates TLR4 protein expression in CHME-5 cells. ANOVA and Dunnett's *post hoc* analysis of immunoblots revealed significant increases in TLR4 protein expression at 90 (*P* < 0.005) and 270 (*P* < 0.01) min after LPS treatment [Figure 5B and C].

Quantitative analysis of CD68 and TLR4 immunocytochemistry

Epifluorescence microscopy was employed

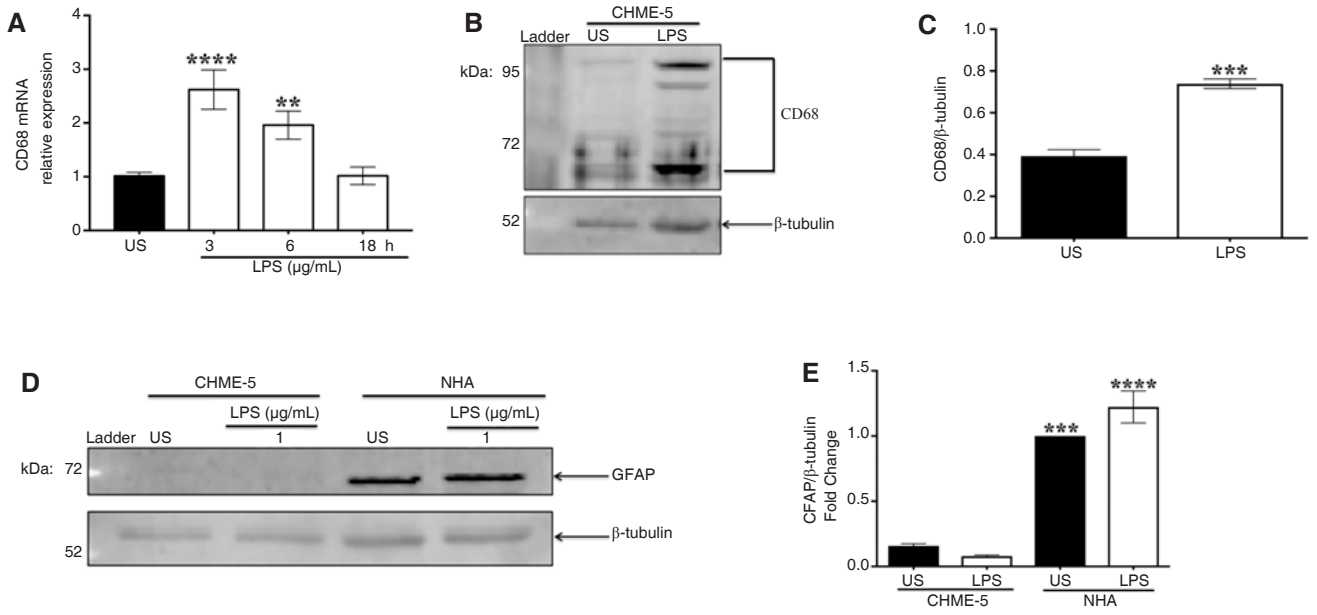

Figure 4: CHME-5 cells are CD68-positive and GFAP-negative. CHME-5 cells were stimulated with LPS (1 μg/mL) at 37 °C for 3, 6, and 18 h. A: CD68 mRNA expression was analyzed using RT-PCR analysis with β-actin as housekeeping gene, 3 h (****$P < 0.0001$) and 6 h (**$P < 0.01$) *vs.* US. Image is representative of 3 independent experiments ($n = 3$) for each treatment group; B: CHME-5 cells were stimulated with LPS (1 μg/mL) at 37 °C for 10 min, whole cell lysates were subjected to SDS-PAGE electrophoresis and immunoblotted with CD68 (1:500) and β-tubulin (1:1,000) antibodies; C: integrated density, ***$P < 0.002$ *vs.* US. Image is representative of 4 independent experiments ($n = 4$) for each group; D: CHME-5 and NHA cells were stimulated with LPS (1 μg/mL) at 37 °C for 10 min, whole cell lysates were subjected to SDS-PAGE electrophoresis and immunoblotted with GFAP (1:2,000) and β-tubulin (1:1,000) antibodies; E: integrated density, (***$P < 0.002$, ****$P < 0.0001$) *vs.* CHME-5. Image is representative of 3 independent experiments ($n = 3$) for each treatment group. Bars are presented as mean ± SEM. CD68: cluster of differentiation 68; GFAP: glial fibrillary astrocytic protein; LPS: lipopolysaccharide; RT-PCR: real-time polymerase chain reaction; US: unstimulated; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

to visualize and quantify CD68- and TLR4-immunofluorescence in unstimulated and LPS-stimulated CHME-5 cells [Figure 6A and B]. Epifluorescence microscopy revealed that CD68 protein expression is constitutively expressed in unstimulated CHME-5 cells. Analysis with CellProfiler revealed a significant increase in CD68-immunofluorescence, in each cell, compared to unstimulated and negative control cells, as assessed with Kruskal-Wallis and Dunn's multiple comparison tests, ($P < 0.0001$) [Figure 6C]. Kruskal-Wallis and Dunn's multiple comparison tests also revealed a significant increase in TLR4-immunofluorescence, in each cell, in LPS-stimulated cells compared to unstimulated cells and negative controls, ($P < 0.0001$) [Figure 6D].

Qualitative analysis of CD68 and TLR4 immunocytochemistry/3D reconstruction

Next, we captured images of unstimulated and LPS-stimulated cells with brightfield and fluorescent imaging, using confocal microscopy, to observe cellular morphology and protein expression, respectively. Overall, unstimulated cells displayed elongated cellular bodies with longer processes, while LPS-stimulated cells exhibited rounded or swollen bodies with shorter processes [Figure 7A]. TLR4 immunofluorescence was observed in both unstimulated and LPS-stimulated cells [Figure 7B]. Similarly, CD68 immunofluorescence was also expressed in unstimulated and LPS-stimulated cells [Figure 7C]. Both CD68 and TLR4 immunofluorescence was merged with DAPI [Figure 7D]. A chosen FOV superimposed onto brightfield images showed TLR4 and CD68 in 3D reconstruction [Figure 7E]. 3D reconstruction images of a close-up side view displayed CD68 and TLR4 immunofluorescence in punctate form, in both experimental treatment groups [Figure 7F].

DISCUSSION

Microglia are considered the macrophages of the CNS and are central to inflammation in the brain[22]. Microglia are of monocytic lineage and take residence in the CNS during the first and second trimesters of embryonic development[22,23]. Much of what is known about microglial cells is derived from *in vitro* studies using primary or transformed cell lines of mouse or rat origin. The establishment of a microglial cell line, CHME-5, was an important advancement for investigating microglia. CHME-5 cells were previously immortalized and validated to have similar morphological and functional properties of primary microglia[19,24].

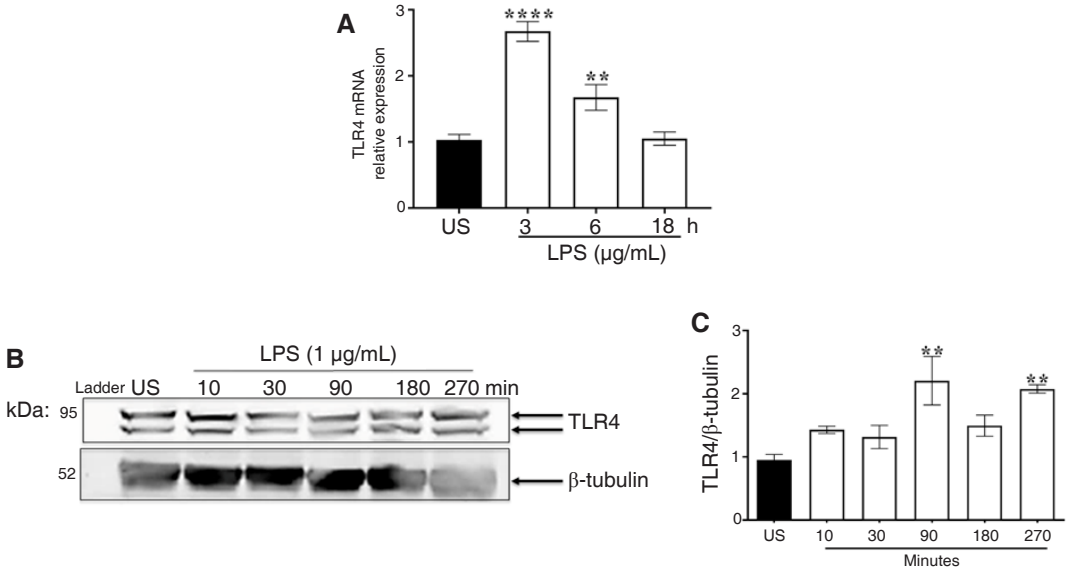

Figure 5: LPS-induced TLR4 expression. CHME-5 cells were stimulated with LPS (1 µg/mL) at 37 °C for 3, 6, and 18 h. A: TLR4 mRNA expression was analyzed using RT-PCR analysis with β-actin as housekeeping gene, 3 h (****$P < 0.0001$) and 6 h (**$P < 0.006$) *vs.* US. Image is representative of 3 independent experiments ($n = 3$) for each treatment group; B: CHME-5 cells were stimulated with LPS (1 µg/mL) at 37 °C for 10-270 min, whole cell lysates were subjected to SDS-PAGE electrophoresis and immunoblotted with TLR4 (1:1,000) and β-tubulin (1:1,000) antibodies; C: integrated density, 90 min (**$P < 0.005$) and 270 min (**$P < 0.01$) *vs.* US. Images are representative of 5 independent experiments ($n = 5$) for each treatment group. Bars are presented as mean ± SEM. LPS: lipopolysaccharide; TLR4: toll-like receptor 4; RT-PCR: real-time polymerase chain reaction; US: unstimulated; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

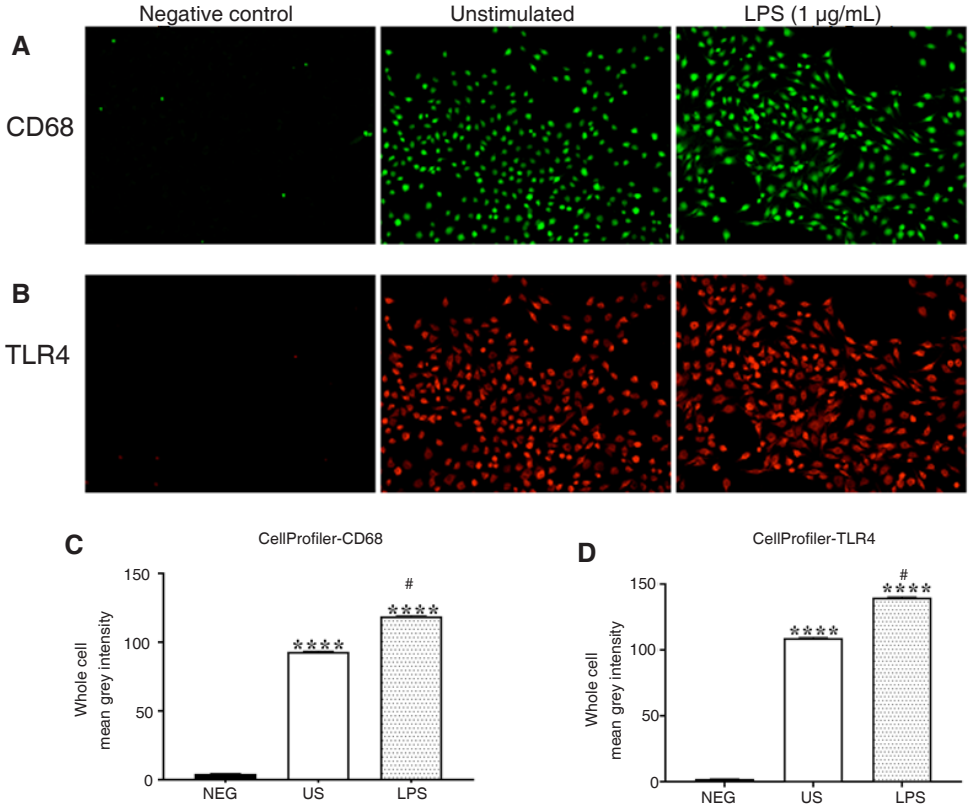

Figure 6: Quantitative analysis of CD68 and TLR4 immunofluorescence. CHME-5 cells were stimulated with LPS (1 µg/mL) at 37 °C for 10 min. Cells were fixed and stained. A: CD68 (1:1,000) and anti-rabbit-Alexa Fluor 647 (1:1,000); B: TLR4 (1:1,000) and anti-mouse-Alexa Fluor 555 (1:1,000) antibodies; A-B: epifluorescence microscopy-40× objective; C-D: CellProfiler analysis for mean grey intensity, (****$P < 0.0001$) *vs.* NEG and (#$P < 0.0001$) *vs.* US. Bars are presented as mean ± SEM. CD68: cluster of differentiation 68; TLR4: toll-like receptor 4; LPS: lipopolysaccharide; NEG: negative control (only incubated with secondary antibody not primary), US: unstimulated

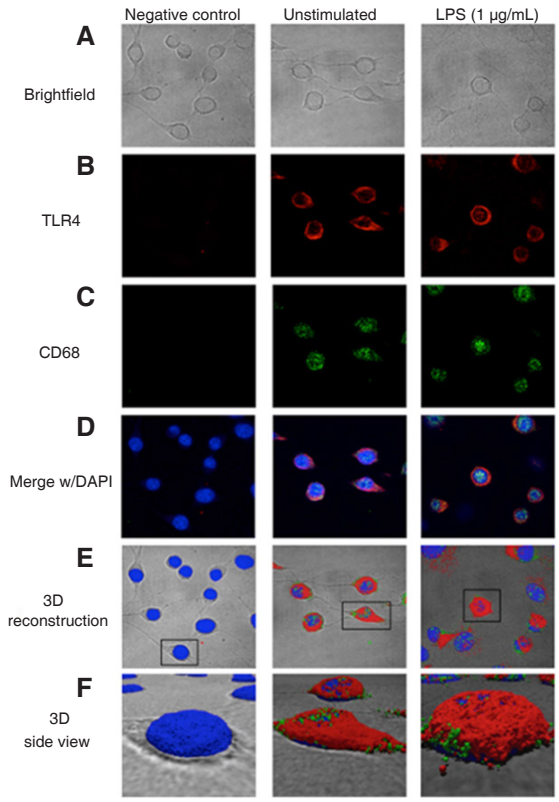

Figure 7: Visualization and 3D reconstruction of CD68 and TLR4 immunofluorescence. CHME-5 cells were stimulated with LPS (1 µg/mL) at 37 °C for 10 min. A: brightfield images were captured with 100× oil objective, cells were fixed and labeled; B: TLR4 (1:1,000) and anti-mouse-Alexa Fluor 555 (1:1,000); C: CD68 (1:1,000) and anti-rabbit-Alexa Fluor 647 (1:1,000) antibodies and DAPI (blue); B-D: confocal microscopy-100× oil objective; D: images merged with DAPI; E: 3D reconstruction with overlay on brightfield images; F: 3D reconstruction of close-up side view. CD68: cluster of differentiation 68; TLR4: toll-like receptor 4; LPS: lipopolysaccharide; DAPI: 4'-6-diamidino-2-phenylindole

Interestingly, a recent publication[25] suggested that CHME-5 cells are of rat origin, not human. In the study, they performed genotyping by macrosatellite analysis, and investigated the CYCT1 gene expression in CHME-5 cells using both human and rat primers, and showed rat CYCT1 gene expression, but not the human counterpart in these cells[25]. The CHME-5 cell line currently being used in numerous labs is apparently of non-human origin, but to our knowledge this has yet to be independently verified. In the present study, human STR genotyping was performed on CHME-5 cells, which confirmed that these cells are not of human origin.

Importantly, we have used CHME-5 cells to advance our understanding of TLR4-mediated signaling in microglial cells. Conversely, during our review of the literature we identified an article characterizing primary human microglia in which data revealed large inconsistencies in microglial and inflammatory genes between primary human microglia and human microglial cell lines, including CHME-5 cells[26]. Knowing what we do now about the non-human origin of CHME-5 cells from Garcia-Mesa *et al.*[25] 2017, the inability to detect gene expression might have been due to the use of human primers. Taking this into consideration, we characterized TLR4 neuroinflammatory signaling in CHME-5 cells, as a rat cell line, validated that these cells are not of human origin, and demonstrated that CHME-5 cells remain a viable tool to study microglial-like inflammatory responses.

Brightfield imaging revealed morphological characteristics of "resting" *vs.* "activated" microglia. Unstimulated and LPS-treated cells displayed morphological signatures of microglia, as seen in previous studies[22,27-29], which included smaller bodies and elongated processes, or amoeboid-shaped and rounder cellular bodies, respectively. This demonstrates that CHME-5 cells retain characteristics that define their role as microglial cells.

Microglia up-regulate several activation markers in response to damage, disease, or loss of homeostatic conditions, such as CD68, which is a microglial activation marker expressed in endolysosomes and in the plasma membrane[30-32]. CD68 gene and protein expression were increased after LPS treatment during early time points, consistent with the rapid responsiveness of microglia as first responders in the CNS[33,34], and, aligns with their role of surveying and returning their environment to homeostasis[9,35]. The expression of CD68, along with morphological attributes observed with immunocytochemistry, indicates that these cells retain phenotypic properties of microglia. To ensure that our culture was not contaminated with astrocytes, GFAP-immunoreactivity was assessed in CHME-5 cells, and as expected, cells did not express GFAP. CHME-5 cells, unlike NHA, showed no GFAP-immunoreactivity, which confirmed CHME-5 cells are not astrocytes. These cells do indeed express CD68, and importantly are GFAP-negative, which eliminates our concern that CHME-5 may be astrocytes or that the culture is contaminated with astrocytes.

We demonstrated that LPS induced inflammatory signaling without inducing cytotoxicity. This finding is consistent with other studies[36], and indicates that results obtained throughout these experiments were not due to LPS toxicity, but rather to specific LPS-induced inflammatory responses.

To investigate LPS-induced TLR4 neuroinflammatory signaling, we used *Escherichia coli* LPS O55:B5 to

model an inflammatory state in the CNS and studied several signaling proteins that are constitutively expressed and activated in the TLR4 pathway. Analysis of 2 crucial intracellular TLR4 signaling proteins, IκBα and NF-κB, were chosen to investigate neuroinflammatory signaling in CHME-5 cells. LPS-induced NF-κB p65 activation was consistent with other microglial cell lines[28,37-39]. Given the fact that many studies investigating microglial responses used the same dose of LPS (1 μg/mL), we are confident that CHME-5 cells were adequately treated to observe inflammatory responses[36,40,41]; unlike the use of a lower dose of LPS (1 ng/mL), which failed to activate and release nitric oxide in CHME-5 cells[42]. Additionally, other studies using human primary microglia and CHME-5 cells have used similar LPS dose range (0.1-1 μg/mL) and time points (6 h for RT-PCR) to investigate inflammatory responses [26,36,37,40,41].

The ability of p65 to be activated, translocate into the nucleus, and bind to NF-κB consensus sequences, is crucial in mediating inflammatory responses in a timely manner. Here, we demonstrated a second, more functional mean of NF-κB activation. LPS-induced NF-κB binding activity was exhibited in 2 waves, at 10 and 90 min. This LPS-induced biphasic activity, has been previously shown in macrophages and is attributed to several possibilities: (1) platelet activating factor (PAF), which is up-regulated during inflammatory stimuli, is involved in NF-κB nuclear translocation, which in turn produces pro-inflammatory cytokines, ultimately up-regulating PAF again in a feedback loop or (2) the release of p65 is not only attributed to IκBα, but IκBβ as well[43,44]. NF-κB is first released from IκBα and then from IκBβ, causing a biphasic response[43]. In fact, we also observed that the NF-κB target gene, TNFα, displayed similar biphasic-like expression in CHME-5 cells following LPS treatment, which was also seen in HeLa cells[45].

LPS-induced IκBα activation as demonstrated by phosphorylation is an early (within 10 min), transient event in CHME-5 cells. Phosphorylation was assessed in cytoplasmic fractions; thus it is presumable that after 10 min IκBα translocated into the nucleus or underwent proteasomal degradation[46]. Additionally, even though the data shows a trend towards IκBα degradation, the analysis revealed that this was not the case during early events in CHME-5 cells. It may be that in CHME-5 cells, evaluation in whole cell lysates is needed to get a detailed assessment of IκBα degradation, as research indicates that IκBα is also present in the nucleus[46,47]. IκBα signal-dependent degradation only occurs in response to ubiquitination of lysine residues[47,48], therefore further analysis of ubiquitination in CHME-5 cells is warranted to elucidate these events.

TLR4, a transmembrane glycoprotein, is part of the innate immune response, and is expressed in the CNS, primarily in microglia[17,18]. Consequently, in addition to assessing LPS-induced signaling, we were interested in the effects of LPS on TLR4 expression. Increased TLR4 gene expression following LPS treatment was consistent with reports showing increases in TLR4 expression in whole blood cells and monocytes as early as 2 and 3 h, respectively[49,50]. The increase observed in TLR4 gene expression in CHME-5 cells following LPS treatment has been reported to occur through binding of the master regulator PU.1 to TLR4 promoter regions, in response to endotoxin[51]. The expression of TLR4 as early as 3 h may be attributed to TLR4 being an early or middle phase gene that peaks at 1 h and 3 h[45]. LPS-induced TLR4 protein expression increased compared to unstimulated cells as seen in both immunoblot analysis and immunocytochemistry. Activation at 270 min may also be due to late-phase NF-κB activation, which is attributed to TRIF-dependent signaling[3]. On the other hand, the lack of TLR4 protein expression at 180 min may be due to negative regulation. There are several negative regulators that control TLR4 inflammatory signaling at different stages in the signaling pathway, such as selective androgen receptor modulator, RP105, and ST2L, which can be induced as early as 10 min, as in the case of IRAK-M[52,53]. Further investigation is warranted to determine mechanisms for regulation of LPS-induced TLR4 signaling in CHME-5 cells.

We provide novel images of CHME-5 cells, showing TLR4 and CD68 immunofluorescence. Epifluorescence imaging revealed that CD68 and TLR4 are constitutively expressed and are robustly up-regulated following LPS stimulation. Understandably, up-regulation of CD68 is expected due to its state of activation in response to LPS. Moreover, confocal imaging provided novel visualization of the expression of TLR4 and CD68 in unstimulated and LPS-stimulated cells. Furthermore, we provided a 3D representation of these proteins in CHME-5 cells, in response to LPS. The ability to reconstruct cells and observe protein expression in a 3D setting provides spatial awareness based on fluorescent intensity. Together, these imaging studies provide new, qualitative information about CD68/TLR4 expression in CHME-5 cells.

In summary, understanding microglial inflammatory responses is very important given the instrumental role of these cells in the innate CNS immune response

and mounting data makes clear that microglia have diverse roles in the CNS. It is generally difficult and expensive to obtain human primary microglia and experiments are often challenging due to limited cell numbers. Cell lines are therefore essential to advance the field of neuroinflammation, in particular, inflammation exacerbating neurodegenerative diseases. Relative to other cell types, availability of microglial cell lines is limited, thus, it is important to maximize our understanding of those tools that are available. Here, we provide novel insights into CHME-5 cells by characterizing TLR4 neuroinflammatory signaling, which aligned with responses seen in other microglial cell lines, such as BV2, HAPI, and human primary microglia. We have also validated very recent findings suggesting that subsets of CHME-5 cells, currently in use, are of a rat, not human origin. With this present research, it is our expectation that CHME-5 will remain a useful tool in the study of microglial cells, particularly as related to neuroinflammation.

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Authors’ contributions

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Design, data analysis, manuscript editing: S. Das

Experimental design, manuscript editing, manuscript review: C.W. Stevens

Concept, definition of intellectual content, manuscript editing and review: R.L. Davis

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Case Report **Open Access**

A rare case of quadrigeminal plate lipoma presenting with the sixth cranial nerve palsy

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ABSTRACT

Intracranial lipomas are rare benign tumour that is slow growing, generally asymptomatic, most frequently located in the midline areas and are usually an incidental finding on imaging and therefore cases are not frequently reported. This study reports a case of a patient with quadrigeminal plate lipoma presenting with obstructive hydrocephalous and the 6th cranial nerve palsy that was successfully treated with ventriculo-peritoneal shunting without addressing the lesion.

INTRODUCTION

Intracranial lipomas are very uncommon slow growing benign tumor with an incidence of less than 0.1% of all intracranial tumors usually found incidentally in autopsy or whenever computed tomography (CT) or magnetic resonance imaging (MRI) is advised for an alternative reason. Intracranial lipomas are generally congenital, benign malformations and most frequently located in the midline areas[1]. CT and MRI are usually sufficient for diagnosis of lipomas because of very low attenuation value on CT and short T1 and T2 on MRI and therefore histopathological confirmation is practically not required. We report a case of 19-year-old male who came to us with the 6th nerve palsy and diagnosed as quadrigeminal plate lipoma. We describe CT and MRI findings with brief review of literature.

CASE REPORT

A 19-year-old male, a student of grade XII was referred to our department with the complaint of headache for 2 years, multiple episodes of vomiting, visual disturbances

and diplopia for 2 months. He also complained of generalized weakness since childhood. There was no history of seizure, loss of consciousness or behavioural changes. On examination, it revealed normal general condition having all systemic examinations within normal except nervous system examination where we found diplopia, the 6th cranial nerve palsy on right side [Figure 1] and bilateral papilloedema on fundoscopic examination.

All laboratory finding including that for fitness for being under general anaesthesia were normal. CT of head revealed a rounded well defined fat density [density-101 Hounsfield units (HU)] area measuring about 17.6 mm × 20.6 mm in the region of quadrigeminal cistern [Figure 2].

MRI findings suggested a rounded well defined hyperintensity lesion measuring about 2.1 cm × 1.9 cm in the region of quadrigeminal cistern in T1-W, T2-W [Figure 3A and B] and fluid attenuated inversion recovery images (FLAIR) sequences [Figure 4] and the lesion didn't take contrast enhancement [Figure 3C] and diffusion weighted image (DWI) sequence suggested no restriction of diffusion [Figure 3D]. The lesion caused compression over cerebral aqueduct resulting in dilation of the 3rd and lateral ventricles. Septum pellucidum was found to be absent [Figure 4]. We diagnosised that this patient had a quadrigeminal plate cistern lipoma with obstructive hydrocephalous due to aqueductal stenosis. Thus, we performed a ventriculo-peritoneal shunting using right Kocher's point and the cerebrospinal fluid (CSF) pressure was found to be elevated. Postoperative state was uneventful and following ventriculo-peritoneal shunting, the 6th cranial nerve palsy was resolved and the headache got subsided gradually.

DISCUSSION

Intracranial lipomas constitute approximately 0.1% of all intracranial tumours. These benign lesions are thought to arise from differentiation of the meninx primitiva, a mesenchymal derivative of neural crest, to lipoma tissue. The vast majority of these types of lesions occur near the midline[1]. More than 50% have been reported to be associated with congenital brain malformations such as agenesis or hypoplasia of the corpus callosum[2]. Others include the absence of the septum pellucidum, cranium bifidum, spina bifida, myelomeningocele, hypoplasia of the vermis and malformation of the cortex[1]. In this case, we found that the septum pallucidum was absent [Figure 4].

The frequency of intracranial lipomas according to location are as following: corpus callosum (64%), quadrigeminal-ambient cistern (13%), infundibular-chiasmatic region (13%), cerebellopontine angle (0.06%) and Sylvian fissure (0.03%)[3]. Eighty percent of cerebellopontine angle lesions, 50% of callosal, 50% of Sylvian fissure and 20% of quadrigeminal-ambient cistern lipomas become symptomatic[2]. Intracranial lipomas are generally asymptomatic lesions and are usually an incidental finding on imaging. Symptomatic lesions are very rare and the symptoms differ per the lipoma's location. Persistent headaches, convulsions[4,5], psychomotor retardation and cranial nerve defects may occur[6].

Symptoms are presented in 20% of cases of lipoma of quadrigeminal plate[7]. The common neurologic findings are features of raised intracranial pressure and hydrocephalous which can be managed easily with ventriculoperitoneal shunting or similar procedures used to treat excess CSF[8]. Most cases of intracranial lipoma involve children and young adults as we had a young teen male presented with intracranial lipoma[9].

Although most of lipoma found incidentally and causes no symptoms and require no intervention[8], our case had triventriculomegaly with features of raised

Figure 1: Showing the case with the 6th cranial nerve palsy on right side (the photograph was taken with the informed written consent of the patient)

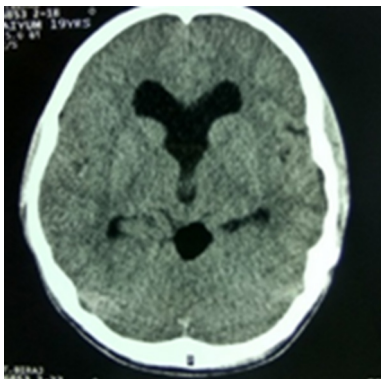

Figure 2: Computed tomography scan of head showing a rounded well defined fat density (density 101) lesion in the region of quadrigeminal cistern

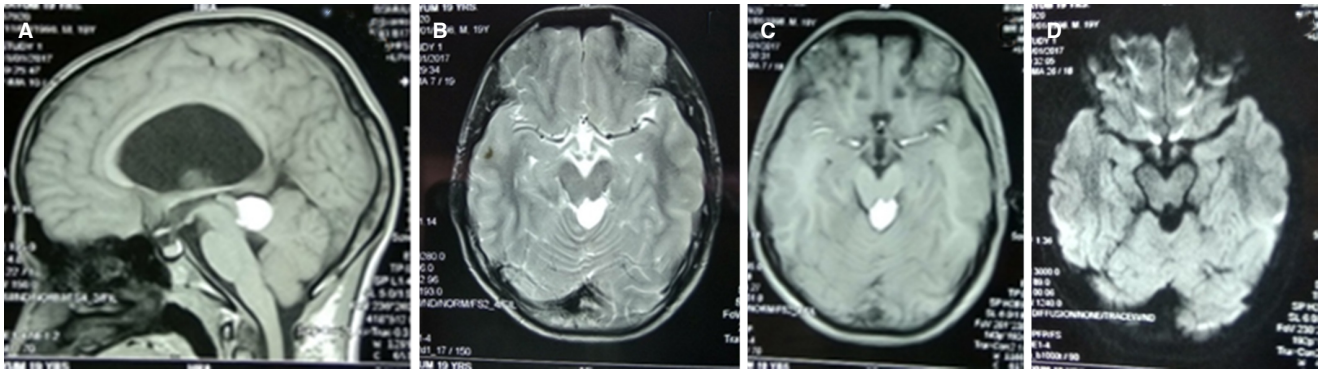

Figure 3: Magnetic resonance imaging of the brain: (A) sagittal T1-weighted (T1-W) sequence showing hyperintense mass over quadrigeminal plate; (B) axial T2-W sequence with hyperintense mass; (C) axial T1-W sequence with contrast showing no contrast enhancement of the lesion; (D) axial DWI sequence showing no restriction of diffusion by the mass. DWI: diffusion weighted image

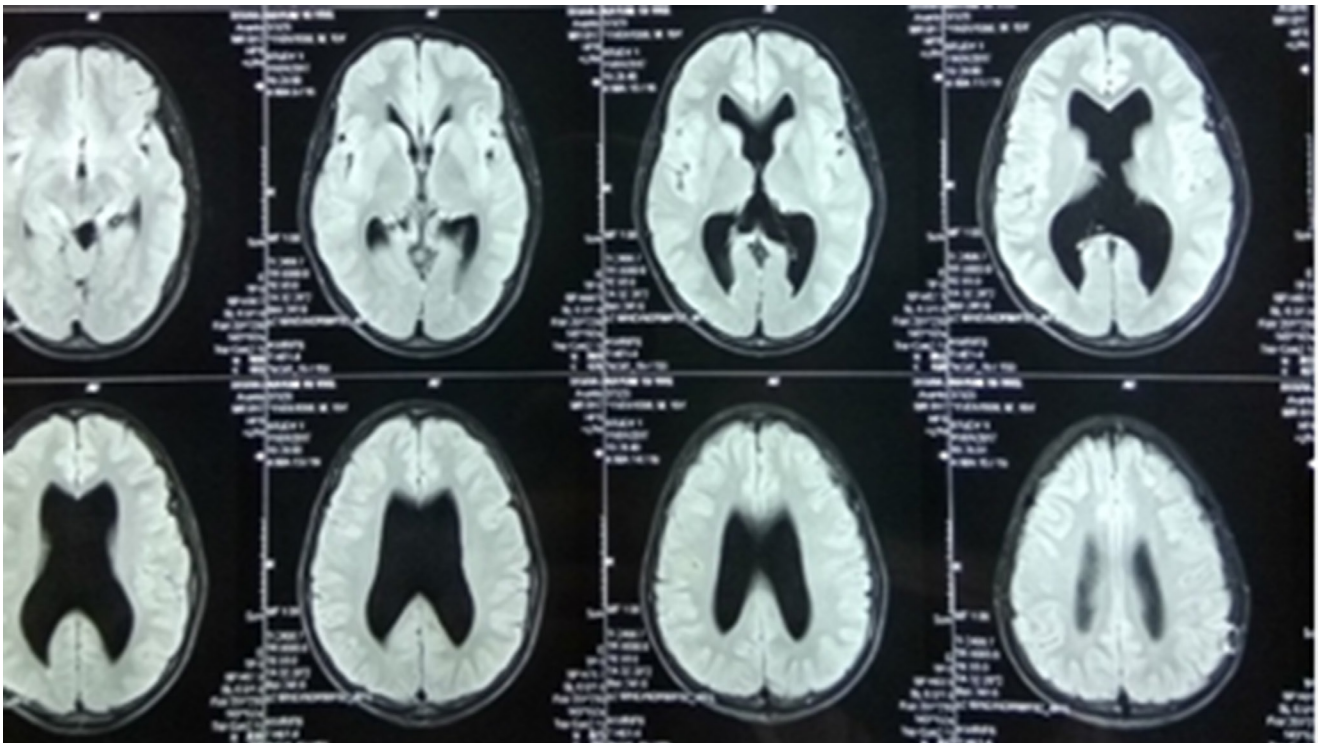

Figure 4: Magnetic resonance imaging of brain with FLAIR sequence showing hyperintense lesion over quadrigeminal cistern with triventriculomegaly and absent septum pellucidum. FLAIR: fluid attenuated inversion recovery images

intracranial pressure and the 6th nerve palsy [Figure 1]. The signs of raised intracranial pressure were thought to be secondary to hydrocephalus which was evident in the case reported on MRI as enlargement of the 3rd ventricle and temporal horns of the lateral ventricles [Figure 4]. On CT, lipomas demarcate areas of marked hypodensity that did not show enhancement after administration of intravenous contrast. They usually have a CT density of between -50 and -100 HU. Calcification is often present in interhemispheric lipomas - usually within the fibrous capsule surrounding the lesion[10]. MRI showed a homogeneous, hyperintense mass on T1-weighted (T1-W) images which where hypointense with fat suppression, hyperintense on T2-W images and hypointense on T2*-W images (due to a magnetic susceptibility and chemical shift effects), no enhancement. Vascular imaging (three-dimensional time-of-flight or MR angiography) may show arterial abnormalities. Dermoids, teratoma, lipomatous transformation of neoplasm or subacute hemorrhage should all be considered in a differential diagnosis[10].

The imaging characteristics of lipomas are very similar to those of dermoid tumours. On CT, both these lesions appear hypodense and neither enhances with contrast. The density of lipomas ranges from 50 to 100 HU,

whereas the density of dermoid tumours ranges from 20 to 40 HU. On MRI, both lesions exhibit high signal intensity on T1-W images and low signal intensity on T2-W images. However, due to the presence of skin appendages and hair, dermoid tumours may be non-homogeneous on MRI[11,12].

In our case, the CT of head revealed a rounded well defined fat density (density-101 HU) lesion in the region of quadrigeminal cistern [Figure 2]. MRI findings suggested a rounded well defined hyperintensity lesion in the region of quadrigeminal cistern in T1-W, T2-W [Figure 3A and B] and FLAIR sequences [Figure 4] without contrast enhancement [Figure 3C] and no restriction of diffusion in DWI sequences [Figure 3D]. The lesion caused compression over cerebral aqueduct resulting in dilation of the 3rd and lateral ventricles. Septum pellucidum was found to be absent [Figure 4]. Treatment of intracranial lipoma depends on the size and location of tumour and can be managed conservatively and surgically if the tumour is causing an mass effect[13]. Primary objectives in the management of quadrigeminal region lipomas include obtaining definitive histology of the lesion, normalizing CSF dynamics, achieving maximum lesion excision and relieving local pressure effects[2]. Radical surgical extirpation is usually contraindicated for 2 reasons. First, the lipoma's generally dense vasculature tends to adhere to the surrounding neural tissue in general and the cranial nerves in particular - making resection technically difficult and thus hazardous. Second, lipomas do not usually involve a mass effect on brain tissue; surgery is unnecessary for stable or asymptomatic cases, since the risks far outweigh the potential benefits[6].

As is true for all intracranial lipomas, conservative management for a lipoma located in the quadrigeminal cistern is reasonable unless the patient becomes symptomatic. Our case was having features of raised intracranial pressure with the 6th cranial nerve palsy and obstructive hydrocephalus due to aqueductal stenosis, we performed ventriculo-peritoneal shunting and the patient was improving gradually. A quadrigeminal plate lipoma occasionally present with hydrocephalous due to aqueductal stenosis which can be managed with CSF diversion procedures. We did ventriculo-peritoneal shunting and our result was satisfactory. To my best knowledge this is the first case of quadrigeminal plate lipoma in South Asian Region presented to us having with features of raised intracranial pressure, the 6th nerve palsy, obstructive hydrocephalus and absent septum pellucidum.

DECLARATIONS

Authors' contributions

Conception, diagnosis and design: B.K. Chaurasia
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Manuscript editing: S. Alam, K.K. Barua
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There are no conflicts of interest.

Patient consent

Patient consent was obtained from the patient.

Ethics approval

There is no ethics issue in this paper.

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Letter to Editor **Open Access**

Peripheral plasmablasts in anti-MuSK myasthenia gravis

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As Hong and Sung[1] previously did, we read with great interest the study published by Guptill *et al.*[2] They reported that anti-muscle-specific kinase (MuSK) myasthenia gravis patients and healthy controls had similar percentages of peripheral plasmablasts. This result is derived from a comparison between 13 female patients (out of the 18 originally included in the study) and 6 controls[2]. Taking into account the effect of rituximab on peripheral plasmablasts[3], those patients treated with it (n = 3) were not included in the previous comparison. Excluding these patients, 6 (40%) were on chronic prednisone treatment, whose dose was between 2.5 mg every other day and 20 mg daily. However, it is already known that prednisone therapy effectively decreases peripheral plasmablasts[4], so this could contribute to the lack of difference between patients and controls. They also reported that immunosuppressed (n = 7) and non-immunosuppressed (n = 6) patients had similar percentages of peripheral plasmablasts. A comparison between non-immunosuppressed patients and controls would have also been quite informative, since it would eliminate the prednisone factor and facilitate the demonstration of a difference between a seemingly normal population of peripheral plasmablasts (controls) and an abnormal one (patients). Finally, vaccination history in controls is also an important point to consider, since it has been documented that peripheral plasmablasts increase after vaccination[5].

Increased peripheral plasmablasts have been found in various immune-based diseases. IgG4-related disease is one of these conditions[6]. In connection with the above, Raibagkar *et al.*[7] recently reported the case of a 54-year-old woman with anti-MuSK myasthenia gravis who also developed retroperitoneal lymphadenopathy histopathologically consistent with IgG4-related disease. They did not report the count of peripheral plasmablasts in this patient. We conducted a search in PubMed and found an additional case of a 72-year-old man with myasthenia gravis who also developed an inflammatory aortic aneurysm. The latter was compatible with a possible diagnosis of IgG4-related disease. Nevertheless, the antibody involved in the diagnosis of myasthenia gravis in this patient is not reported[8].

In the title of their short communication, Raibagkar *et al.*[7] wonder if there is any relationship between the two diseases. It is interesting to note that in anti-MuSK

myasthenia gravis antibodies are directly pathogenic[9], whereas in IgG4-related disease they are probably not[7,10]. We recently stressed that the sphere of IgG4-mediated neurological autoimmune disorders is an expanding one[10]. We believe that the study of the ties between IgG4-mediated neurological autoimmune disorders and IgG4-related disease could represent a very fruitful field in the near future[10], so further studies are needed in this specific area. However, one of the limitations that these studies might face is the low prevalence (or underdiagnosis) of these conditions[11,12]. Multicentric studies through international, collaborative efforts could mitigate this limitation. Regarding the technical aspects, it is also necessary to unify the characterization (gating) of peripheral plasmablasts in order to facilitate dialogue and exchange between different research communities, which may come from different domains of specialization (neurology, rheumatology, immunology, among others).

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Authors' contributions
Literature review, significant contribution to intellectual manuscript content, and manuscript drafting: G. Delgado-García, T. Corona-Vázquez.

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Case Report **Open Access**

Sparganosis of the brain: a case report and brief review

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ABSTRACT

Human sparganosis is a rare disease often affecting muscle, subcutaneous tissue and other locations, but sparganosis invading the brain is rather rare. Cerebral sparganosis has no specific symptoms which makes the diagnosis quite difficult and is usually neglected in the clinic. Here the authors reported a case of a 29-year-old female who was diagnosed with cerebral sparganosis and underwent surgery in their department and a brief review of the literature was conducted as well.

INTRODUCTION

Human sparganosis is a rare but increasing emerging food borne zoonosis caused by the plerocercoid larvae. The genus spirometra constitutes several species including S mansonoides, one of the most commonly reported human sparganosis infections in Asia[1]. The lifecycle of spirometra starts with adult worms living in the intestines of dogs and cats and eggs shed in faeces in the environment, while frogs and snakes usually serve as second intermediate hosts[2]. Humans are mainly infected through drinking untreated water containing spirometra larvae, consumption of undercooked frog or snake meat, or using raw flesh on open wounds as traditional poultices[3-7]. Sporadic human sparganosis has been documented worldwide, but the prevalence is higher in Asia countries, especially in China, Thailand, Japan and South Korea[3,5,8,9]. The most common target places in humans for spirometra are subcutaneous tissue or muscle[10], cerebral sparganosis is relatively rare but represents the most severe type[11], characterized by symptoms like seizures and the wandering sign in magnetic resonance imaging (MRI) scanning[12].

Management of cerebral sparganosis includes surgical removal of the worm as well as postoperative anti-parasite medication[13]. However, cerebral sparganosis cases can be cured with medication alone[14]. Cerebral sparganosis has a good prognosis after treatment[13]. Here, we report a case of a cerebral S mansonoides infection and provide a brief review of the literature.

CASE REPORT

History and examination

A 29-year-old female patient was admitted to the department of Neurosurgery due to intermittent right upper limb epilepsy and a left parietal lobe lesion. The patient experienced her first seizure attack half a year ago consisting of involuntary movements in the right upper limb. She did not take it seriously and did not take any anti-epileptic drugs (AED) medications. Two months before admission to the hospital, seizures became more severe and she found a migratory nodule under her skin on her right thigh. She had lived in Fujian Province her whole life and enjoyed eating frogs in local restaurants. The people in Fujian Province have a habit of eating frogs, crabs, snake, chicken and pig meat, which are all host animals of the sparganosis. Computed tomography (CT) showed a left parietal lesion with large adjacent edema and the MRI showed an irregular hypointense lesion in T1 weighted imaging (T1WI) and hyperintense signal in T2 weighted imaging (T2WI). The lesion was homogenously enhanced in T1WI and with largeperi-lesion edema. MR perfusion showed increased blood flow in the lesion area while spectroscopy showed an increased peak of choline (Cho) and decreased peak of *N*-acetyl-aspartate (NAA) [Figure 1]. A cerebral parasitic infection was suspected since the patient was from the endemic area with a previous history of eating undercooked frogs. Blood and cerebrospinal fluid (CSF) samples were sent to the China Institute of Parasite Research for detection, the antibody for S mansonoides was positive in blood sample but negative in CSF sample. Another lumbar puncture was conducted and this time the antibody for S mansonoide was positive in CSF sample. A craniotomy was performed to resect the lesion.

Operation

A right parietal under-navigation craniotomy was performed. A white long living parasite was seen and extracted. Postoperative pathology confirmed the diagnosis of S mansonoides. The patient received anti-parasite medication after the surgery, postoperative MRI showed complete resection of the lesion. The patient had no seizures after 6-month follow-up [Figure 2].

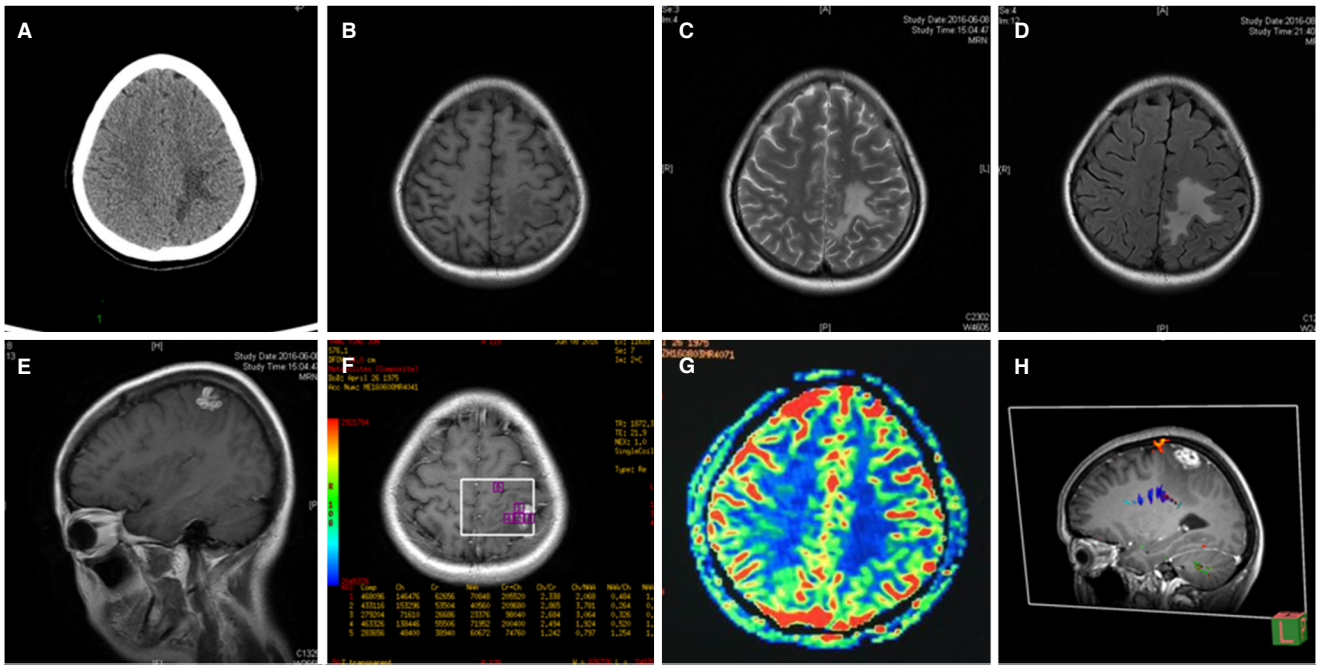

Figure 1: Preoperative radiological features of cerebral sparganosis. A: axial view of CT scanning showed irregular edema of the left parietal lobe; B: axial view of T1WI MRI showed a slight edema of the left parietal lobe; C: axial view of T2WI MRI; D: axial view of FLAIR MRI; E: sagittal view of enhanced T1WI MRI showing an irregular enhanced lesion of the let parietal lobe; F: axial view of enhanced T1WI MRS showed an increase of the Cho/NAA ratio; G: axial view of MR perfuison showed the lesion was hypometabolic; H: sagittal view of preoperative DTI. CT: computed tomography; MRI: magnetic resonance imaging; NAA: *N*-acetyl-aspartate; T1WI: T1 weighted imaging; DTI: diffusion tensor imaging

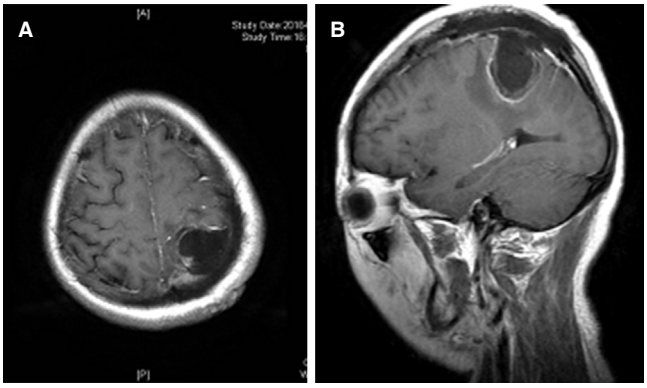

Figure 2: Postoperative enhanced T1WI MRI showed that the lesion was totally resected with a slight edmea around the operative area, A: axial view; B: sagittal view. MRI: magnetic resonance imaging

DISCUSSION

To investigate the epidemic and clinical characteristics of cerebral sparganosis. We searched Pubmed using the keywords “cerebral sparganosis” and “cerebral sparganosis mansonoide”. English literatures with at least abstract available were reviewed.

Epidemiology

Human sparganosis usually occurs when patients consume undercooked frog or snake meat, drink unboiled water contaminated by procercoidlarve. In some rural areas, people use fresh snake or frog meat directly on open wounds as a cure method. Sparganosis can be transmitted through this way, too[5]. Cases of human sparganosis are mostly reported in east Asia, especially in China[5], South Korea[15], Thailand[3] and Japan[16]. Till now, only three cerebral sparganosis cases were reported in Europe[9,17]. Sparganosis can migrate to various tissues and organs, such as subcutaneous connective tissue, muscle, breast, lungs and the central nervous system[2]. Reported cerebral sparganosis cases are quite small compared to other site sparganosis, but according to Hong *et al.*[18] the prevalence of cerebral sparganosis was underestimated, many cases were not reported. In addition, a lot of Asian patients were reported with local language rather than English. The incidence of cerebral sparganosis of children and young people are higher than adults, and people in rural counties who are in underprivileged financial status have a great opportunity to get a sparganosis infection due to hygiene problems[13].

Clinical characteristics

Cerebral sparganosis can cause various symptoms including headache, seizure, parenthesis, confusion, memory loss, *etc*. The symptoms are related to the location of the lesion[14], the most common symptoms were various types of seizures. In some cases, it can even present as an intracranial hemorrhage[19]. Fever is not a common symptom. Lesions are mainly located at frontal-partial lobes but invasion to cerebellums are also reported in very few cases[18]. Diagnosis of cerebral sparganosis is relatively hard since it has no specific manifestations. A history of traveling or living in endemic areas may indicate a possibility for the diagnosis. In China, the majority infected people had a history of consuming undercooked frog or snake meat. Besides histories of traveling or living in endemic areas, an active infection of other organs is also a useful clue for the diagnosis of cerebral sparganosis. In our case, the patient had a migratory nodule on the right upper limb, which makes us highly suspect a diagnosis of cerebral sparganosis.

Laboratory test and neuroimaging

Blood can show an increase of eosinophilia[13]. The disease can also be diagnosed with antigen specified IgG antibodies from blood and CSF samples, although cross-reactivity with other infestations or clonorchiasis limititsspecificity[13,18]. In our case, the first CSF sample was negative for immune-reactivity but positive for blood sample, however, the second blood and CSF sample were both positive.

CT scans usually show a mixed density lesion with peri-lesion edema, punctate calcification is shown in approximately 50% of patients[15,20]. The MRI of cerebral sparganosis is difficult to identify from low grade glioma or other tumors. Typically, ring-like or string-knot enhancement in T1WI images shows the sign of sparganosis movement in the brain parenchyma. Other characteristic features include a tunnel-shape configuration due to immigration of sparganosis through the brain parenchyma. Moreover, serial imaging may demonstrate the tunnel sign from a small nodular lesion and reflect the sparganosis activity in the brain[21-23].

MR spectroscopy of cerebral sparganosis (Bo and Xuejian[24] and Chiu *et al.*[25]) showed increased Cho and decreased NAA peaks in their cases, as in our case. Chiu *et al.*[25] also revealed peaks at 1.3 ppm and a peak between 14 and 1.8 ppm in his 46-year-old female patient, however, our case did not show a similar result. MR perfusion did not show higher cerebral perfusion in our case that was seen in the case of Chiu *et al.*[25].

Surgery

Although some studies report that using anti-sparganosis drugs can successfully cure the disease, the most efficient way to cure the disease still is

surgical removal of the sparganosis from the infested site of the brain. Both stereotactic aspiration and an opening craniotomy were used by neurosurgeons. The goal of surgery is to completely remove the granuloma along with the larval. Deng *et al*.[26] reported a series of 11 cases who underwent stereotactic aspiration surgeries, complete removal as achieved in 10 patients while incomplete removal in 1 patient. Due to the small wounds, stereotactic aspiration is recommended by most neurosurgeons. In the 26 cerebral sparganosis cases of Hong *et al*.[18], 16 of them were treated with craniotomy, 7 of them were treated with stereotactic aspiration and another 3 were treated with praziquantel only. None of them experienced a relapse of the disease. Yu *et al*.[13] reported in 8 of 9 cases of cerebral sparganosis patients underwent a craniotomy due to lack of stereotactic equipment, with 1 patient dying due to unspecified reason. Both investigators reported that symptom duration more than one year indicated worse prognosisafter surgery[13,18]. In our case, we used a navigation guided craniotomy to resect the granuloma as well as the peri-lesion glia proliferation zone. The symptoms resolved immediately after surgery, the patient was administrated AED medication and 5 mg/day of praziquantel for 3 months. After 6-month follow-up, the patient was well without further seizures.

Cerebral sparganosis is a neglected food borne zoonosis and since it has no specific clinical characteristic, it is usually misdiagnosed until postoperative pathological finding. Patients who had a history of consuming undercooked meat or from endemic areas should be highly suspected. Immunosorbent assay for sparganosis antibody using blood and CSF samples as well as MRI images can provide evidence for preoperative diagnosis. Surgery should be performed and postoperative anti sparganosis drugs should be administrated. Usually, cerebral sparganosis had a satisfying outcome based on surgery and drugs.

DECLARATIONS

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Authors' contributions
Conception and design: H.X. Li, S.H. Luan
Acquisition of data: H.X. Li, W. Guo, L.Y. Hua, H.D. Zhu, H.L. Tang
Analysis and interpretation of data: L.Y. Hua, H.D. Zhu, J.J. Deng
Drafting the article: H.X. Li
Critically revising the article: Q. Xie
Reviewed submitted version of manuscript: X.C. Chen, Y. Gong
Approved the final version of the manuscript on behalf of all authors: Q. Xie, Y. Gong
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Administrative/technical/material support: X.H. Chen
Study supervision: Q. Xie, Y. Gong

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Informed consent was obtained from the patient included in the study (KY-2012-019).

Ethics approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of Huashan Hospital, Fudan University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Neuroprotection by minocycline in murine traumatic spinal cord injury: analyses of matrix metalloproteinases

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ABSTRACT

Aim: Minocycline has neuroprotective activities in several models of neurological disorders including spinal cord injury (SCI) where it prevents axonal loss and improves functional recovery. There are still gaps of knowledge on minocycline in SCI including whether it ameliorates neuronal loss at the focal site of trauma, and whether minocycline reduces the activity of matrix metalloproteinases (MMPs), a family of enzymes implicated in the pathophysiology of SCI. This study addressed these gaps. **Methods**: Mice were treated with either minocycline or vehicle control after a spinal cord contusion. MMPs were compared between the two groups using real time polymerase chain reaction and zymography. Immunohistochemistry was used to examine microglial activation and neuronal cell death. **Results**: While several MMP members were elevated in the spinal cord following injury, treatment with minocycline did not affect their expression. Importantly, minocycline reduced the loss of neurons in the epicenter of damage to the spinal cord and in segments caudal and rostral to the injury. **Conclusion**: Despite the inability of minocycline to alter MMPs, the results of neuroprotection at the lesion site support the continued testing of minocycline as a neuroprotective medication in experimental and clinical SCI.

INTRODUCTION

Minocycline, a synthetic derivative of tetracycline, has been used for the treatment of acne for several decades.It has been proposed as a promising neuroprotective agent in multiple central nervous system (CNS) pathologies including stroke, Huntington's disease, amyotrophic lateral sclerosis

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and multiple sclerosis[1-3]. Minocycline has also been demonstrated to have beneficial effects in the treatment of acute spinal cord injury (SCI), a condition for which therapeutic treatments are urgently needed. Indeed, a phase 2 clinical trial administering minocycline in acute human spinal cord injury was completed in Canada with promising early results[4] and a phase 3 clinical trial is ongoing (ClinicalTrials.gov Identifier: NCT01828203). Encouragingly, the efficacy of minocycline in reducing tissue damage and in ameliorating functional impairment in SCI has been demonstrated despite variations in the models used, the species tested and the treatment paradigm[5-7]. The mechanism of action of minocycline in neurological diseases appears not to be related to its antimicrobial activity, but rather to immunomodulation, blockade of excitotoxicity and inhibition of cell death pathways[1,2,5-7], all of which have been implicated in the pathophysiology of SCI.

Minocycline has also been reported to inhibit the production and activity[8-10] of a family of proteolytic enzymes known as the matrix metalloproteinases (MMPs). A large number of MMPs have been identified and, collectively, the MMPs are capable of degrading all components of the extracellular matrix (ECM). As such, MMPs have roles in several developmental processes that involve ECM turnover as well as in wound healing throughout life. However, the aberrant expression of these molecules is detrimental to several diseases of the CNS, including SCI[11]. We have previously shown that several MMPs were upregulated after SCI in mice; in particular, we provided evidence that MMP-12 expression after SCI was linked to increased blood spinal barrier permeability, microglial activation, macrophage infiltration, and worsened functional outcome following injury[12]. In addition, Hsu *et al.*[13] and Noble *et al.*[14] demonstrated that MMP-9 limited functional recovery in the short-term following SCI in rats, although MMP-2 had an opposite outcome 7-14 days after injury.

Given that minocycline is currently in a phase 3 trial in traumatic SCI (ClinicalTrials.gov Identifier: NCT01828203), it is important to have a comprehensive picture of its spectrum of activity. Thus, in this study, we determined whether or not the efficacy of minocycline in SCI was due in part to its ability to inhibit the expression or activity of MMPs. In addition, we examined the response of microglia/macrophages following SCI and the impact of minocycline treatment. Finally, we addressed whether minocycline could protect against the death of neurons at the site of traumatic impact itself, since previous work has examined axons of passage and higher order neurons that project through the spinal cord[5]. These studies are relevant to the potential application of minocycline as a neuroprotective agent after SCI.

METHODS

Surgery and minocycline treatment

CD1 outbred mice were anesthetized, the spinal cord exposed, and extradural mechanical compression of the cord with a modified aneurysm clip was achieved as previously described[5]. In brief, surgery consisted of anesthetizing mice with a mixture of ketamine/xylazine (200 and 10 mg/kg, respectively) intraperitoneally. Animals were subsequently immobilized in a stereotactic frame. An incision was made in the skin and the muscle and tissue overlying the vertebral column were blunt dissected away. Using the spiny process of T2 as a landmark, a laminectomy was performed at the level of T3/T4 and the spinal cord was exposed. A rigid hook was used to clear a path underneath the cord so that a modified aneurysm clip with a closing force of 8 g could be applied. Extradural compression of the cord was achieved by allowing the clip to slam shut on the cord producing mechanical trauma. The clip was maintained in position for one minute producing damage that also had ischemic components. Following injury the clip was removed and the wound was closed using nylon suture. Mice recovered in a room maintained at 27 ºC where they were kept thereafter for the determined survival time. Manual expression of the bladder was required twice daily and the food and water were placed directly in the cage to allow ready access.

One hour after surgery, mice were randomized and injected intraperitoneally with either a solution of minocycline at a dose of 50 mg/kg or saline vehicle control. Subsequent injections were given at 24-h intervals until sacrifice. Following the second injection of 50 mg/kg, the dose of minocycline was reduced to 25 mg/kg for all remaining treatments. This dose regimen is identical to that used in our previous study[5] that demonstrated behavioral recovery by 3 days post-injury in the minocycline compared to vehicle group.

Analyses of MMPs

TaqMan real time PCR was used to profile MMP mRNA levels as detailed[15]. Minocycline and vehicle treated mice were sacrificed 1, 2 and 5 days after injury (4/group) and 4 mice served as uninjured controls. Spinal cords were removed and a 1-cm segment including the injury site was homogenized in Trizol reagent and total RNA extracted. One microgram of RNA was reverse transcribed to make cDNA. Each PCR reaction contained the equivalent

of 5 ng of reverse transcribed RNA. The 18S rRNA gene was used as an endogenous internal control to account for differences in the extraction of original tissue and reverse transcription of total RNA. Primer sequences for the TaqMan assays were previously described elsewhere[15], and statistical validity for the multiple TaqMan targets has been corroborated.

Gelatin zymography of one cm segments of spinal cord was described previously[16] as was the method of *in situ* zymography[17]. Note that while gelatin zymography is a technique reliant on the activity of MMP-2 and -9 to degrade gelatin in-gel, it is ultimately proportional to, and thus a reflection of, the amount of MMP-2 and -9 in the test samples.

While the above in-gel gelatin zymography is a manifestation of protein content, the net gelatinolytic activity (i.e. overall enzyme activity in a milieu that also contains enzyme inhibitors) in an intact non-fixed tissue specimen can be evaluated using *in situ* zymography. For this method spinal cord injury was induced in 14 mice; 7 animals were injected with 50 mg/kg of minocycline 1 h and 24 h after, while the other 7 received saline vehicle at the same time points. One hour after the second injection, mice were given an overdose of ketamine/xylazine intraperitoneally and the spinal cord was carefully removed and directly frozen in isopentane at -70 ºC. Twenty µm thick longitudinal sections were cut on a cryostat, mounted on glass slides and stored at -80 ºC. Sections were thawed and incubated in a humid chamber in 100 mL (50 µL/s) of reaction buffer containing 100 mg/mL of FITC-labeled dye-quenched-gelatin (EnzCheck collagenase kit, Molecular probes, Eugene, OR) for 3 h at 37 ºC[16,17]. At the end of the incubation period slides were rinsed in PBS, fixed and then mounted. Prepared slides were observed using fluorescence microscopy. Sections incubated without dye-quenched-gelatin did not exhibit fluorescence and served as negative controls. Samples from SCI animals were analyzed in a blinded manner using a qualitative rating scale from 0 to 4, with zero representing no fluorescence and a score of 4 depicting maximal *in situ* zymography signal.

Immunohistochemistry

Spinal cord compression was performed in 36 mice as described above. Mice were randomly treated with either minocycline or vehicle and survived for 2 or 5 days (n = 9/group). Animals were given an overdose of ketamine/xylazine and the spinal cords were excised and post-fixed in 10% neutral buffered formalin and subsequently embedded in paraffin wax. Six micrometer thick sections were cut on a microtome and collected at a frequency of 1:10. Three series were obtained for immunohistochemical analysis of microglia, neurons and apoptotic cells using the antibodies Iba1, NeuN (Chemicon) and the Apoptag® Fluorescein In Situ Apoptosis Detection Kit (Tunel, Chemicon). Iba1 and NeuN immunolabeling to detect microglia and neurons, respectively, was performed as previously described[12]. For NeuN labeling, sections had to undergo antigen retrieval by boiling in 10 mmol/L sodium citrate buffer (pH 6.5) for 10 min. Slides were incubated with mouse anti-NeuN (1:50, Chemicon) overnight at 4 ºC. Biotinylated anti-rabbit IgG was used for the secondary antibody and staining was visualized with ABC using DAB as the substrate.

For blinded Iba1 analyses, the degree of microglial/macrophage activation was determined by examining the morphology and density of the Iba1 labeled cells. Considerations were made for the size, shape and relative density of Iba1 labeled cells. Briefly, Iba1-stained sections were scored for microglial/macrophage activation using a scale from 0-4 where 0 was normal cord and 4 was the presence of highly activated microglia/macrophages. For NeuN labeled tissue, longitudinal sections containing central canal were identified as well as sections about 180 µm away on either side of the center section (total of 3 sections/mouse). The number of positively stained cells was manually counted from 5 regions of each section corresponding to lesion area, +1 mm rostral, +2 mm rostral, +1 mm caudal and +2 mm caudal. Unbiased stereology was not used in the blinded counts. Data were analyzed using univariate analysis of variance with scheffe *post-hoc* comparisons. Similarly, for Tunel labeled tissue, the total number of labeled cells in a 1-cm segment of cord, from a section containing central canal and including the lesion epicenter, was counted. In this case, data was analyzed using unpaired *t*-tests at 2 and 5 days.

RESULTS

Expression profile of MMP transcripts in minocycline and vehicle treated SCI animals

The expression profile of 20 MMPs was examined by TaqMan PCR in mouse spinal cord following injury. Figure 1 shows that transcripts encoding MMP-3, -7, -10, -11, -12, -13, -19, -20 and -21 were elevated after spinal cord injury compared to uninjured controls, while those of MMP-23 and -24 were reduced. Compared to vehicle treated SCI samples, minocycline administration had no significant effect on altering the MMP transcripts at 1, 2 and 5 days after injury.

We also measured transcripts encoding physiological antagonists of MMPs, the 4 tissue inhibitors of

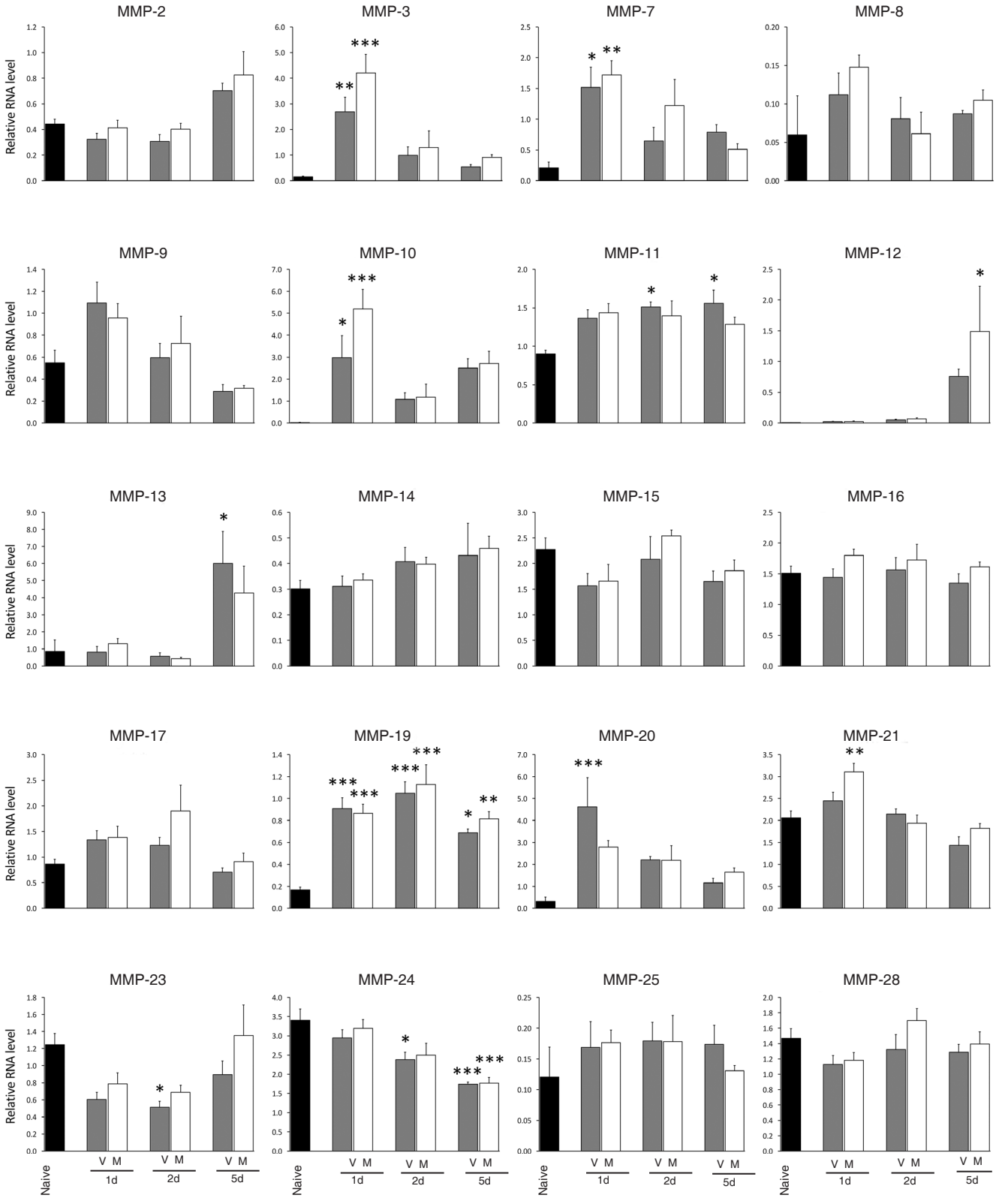

Figure 1: No impact of minocycline treatment on the profile of MMPs after SCI. While the expression of several MMPs was altered after SCI, none of these MMPs were significantly different between the vehicle and minocycline groups. Values are mean ± SEM of 4 samples from vehicle (V) or minocycline (M) group, at 1, 2 or 5 days after injury. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to naïve controls. All analyses were by one-way ANOVA with Tukey's multiple comparisons. MMPs: matrix metalloproteinases; SCI: spinal cord injury

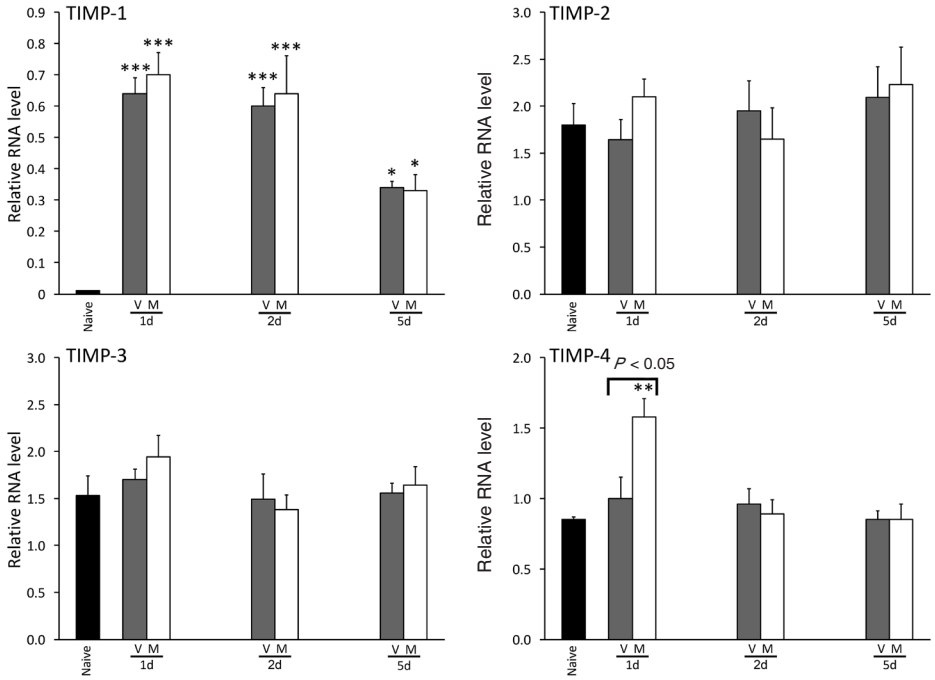

Figure 2: TIMPs remain largely unaltered by minocycline treatment after SCI. TIMP-1 was the only inhibitor that was altered (upregulated) by injury compared to naïve controls, and there was no effect of minocycline treatment with the exception of an increase of TIMP-4 at 1 day after SCI. Values are mean ± SEM of 4 samples from vehicle (V) or minocycline (M) group, at 1, 2 or 5 days after injury. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to naïve controls (one-way ANOVA with Tukey's multiple comparisons). TIMPs: tissue inhibitors of metalloproteinases; SCI: spinal cord injury

metalloproteinases (TIMPs). Figure 2 shows that the expression of TIMP-1, but not TIMP-2, -3 and -4, was upregulated by injury. The levels of all four TIMPs in the minocycline treated cords were not altered from those of the vehicle treatment groups, with the exception of TIMP-4 that was raised by minocycline at 1 day of injury.

Gelatin zymography shows that minocycline does not alter MMP-2 and -9 protein content

Measurement of transcripts encoding MMPs provides a broad overview of the changes occurring to all known MMP members, since several individual MMPs continue to be difficult to measure using Western blot or activity assays. A reproducible and commonly used method for protein levels involves the determination of MMP-2 and MMP-9 by the method of gelatin zymography. Although we did not detect the elevation of their transcripts after SCI [Figure 1], MMP-9 protein can be made outside of the CNS, including in neutrophils, and then deposited into the lesion site. For these reasons, we examined the levels of MMP-2 and -9 protein using gelatin zymography. The pro-MMP-9 protein was minimally expressed in control uninjured spinal cord tissue [Figure 3A]. Injury resulted in an upregulation of both the pro- and active forms of MMP-9 one day after injury. Minocycline treatment had no obvious effect on the injury-induced MMP-9. The pro-MMP-2 species did not elevate profoundly after injury or in the presence of minocycline. The quantification of the gelatin zymograms is displayed in Figure 3B and confirms the lack of effect of minocycline on MMP-2 and -9 protein expression after SCI.

In situ zymography reveals no alterations of net proteolytic activity by minocycline

Although gelatin zymography [Figure 3A] is based on the degradation of gelatin in-gel by MMP-2 and -9, it is a reflection of the content of these gelatinases (MMP-2 or -9) rather than their net enzymatic activity, given that all enzyme co-factors are supplied in optimal amounts for manifestation of catalysis in-gel. In addition, while the mRNA for MMPs are elevated in SCI [Figure 1], so are the transcripts for the TIMP inhibitors [Figure 2], thus making it relevant to address the balance of proteolytic activity in specimens. To determine the net proteolytic activity existent in the injured cord, *in situ* zymography was used, whereby the gelatin-FITC substrate was overlaid onto longitudinal unfixed sections encompassing the injured area[17], and where if there is relative abundance of enzyme over inhibitors, then there would be net proteolysis of the gelatin-FITC. In normal uninjured cord, no fluorescence signal was evident. Twenty-four hours following SCI, varying grades of signals were observed [Figure 3C]. Blinded analyses across 14 injured specimens concluded that there was no

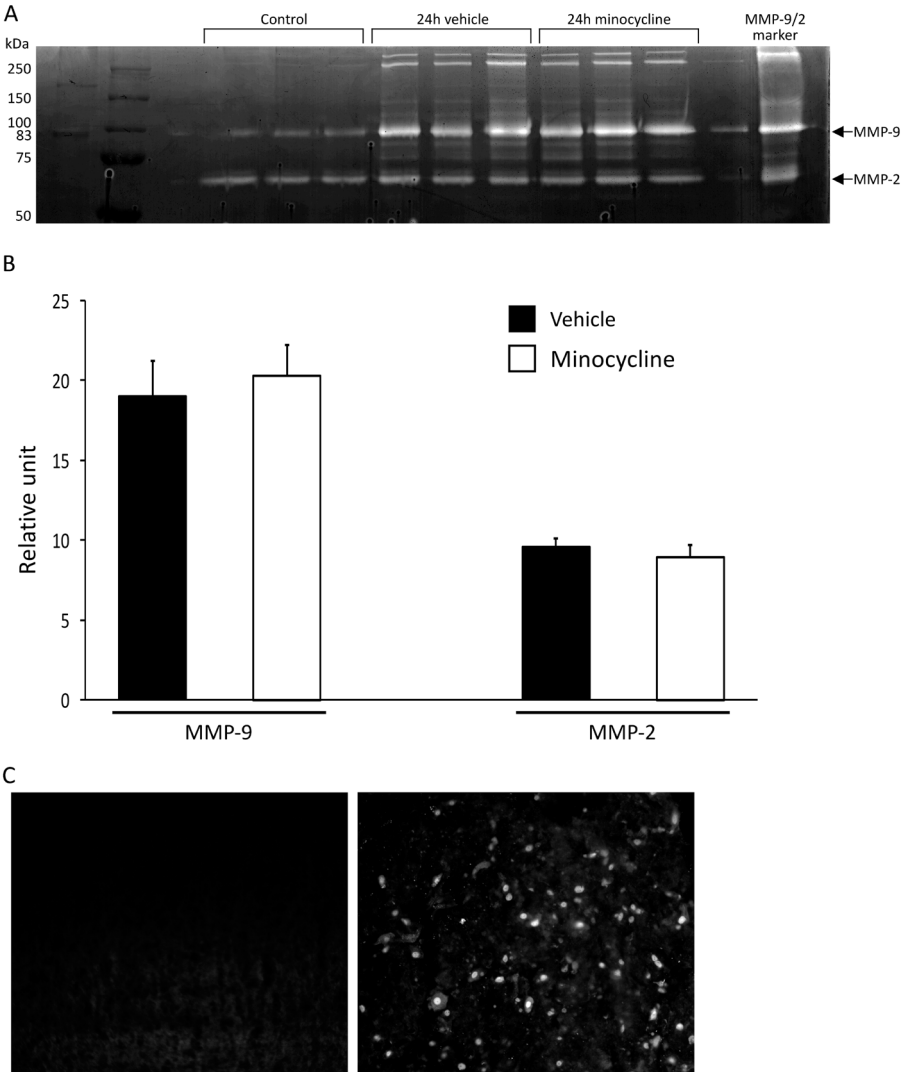

Figure 3: Increased protein levels of MMP-9 after SCI are unaltered by treatment with minocycline. A: Gelatin zymogram displaying pro-MMP-2 and -9 protein species (designated in figure by MMP-2 and MMP-9, respectively) in spinal cords from uninjured control mice; after injury, additional bands running between pro-MMP-2 and pro-MMP-9 represent the activated forms of MMP-9. Minocycline treatment did not markedly change the expression pattern of MMP-2 or -9 levels after SCI (A) and this was corroborated by quantification of band densities through densitometry (n = 6 each) from the zymogram (B); C: *in situ* zymography of a longitudinal section from non-injured (left, slide has been over-exposed to confirm the lack of signal) or vehicle-treated mouse (right, 2 days after injury). *In situ* zymography signal at the lesion site (one longitudinal section containing the central canal per mouse, n = 7 per group) is not different between vehicle- and minocycline-treated mice in blinded analyses (see text). MMPs: matrix metalloproteinases; SCI: spinal cord injury

difference in net proteolysis around the lesion site between vehicle and minocycline samples (1.6 ± 0.3 and 2.0 ± 0.5 graded scores, respectively) (mean ± SEM, P > 0.05, Mann-Whitney non-parametric test).

Minocycline reduces microglial reactivity at the site of injury

Iba1 immunoreactivity was performed on spinal cord sections to assess the degree of microglial/macrophage reactivity. Figure 4 displays Iba1 labeling in the spinal cord of normal uninjured mice (A, B), or in vehicle (C, D) or minocycline treated (E, F) mice after SCI. The Iba1 antibody does not discriminate between microglia and macrophages, so positive cells are collectively referred to as microglia/macrophages. The density of Iba1 positive cells adjacent to the epicenter of injury was prominently increased after SCI (C, D) compared to normal uninjured conditions, and this appeared qualitatively to be reduced in the minocycline treated samples (E, F). Furthermore, the morphology of microglia/macrophages in vehicle treated mice (D) was indicative of highly activated cells with amoeboid morphology and thick stubby processes, contrasting the highly ramified morphology of microglia in the normal cord [Figure 4B]. In contrast, minocycline treated mice had microglia/macrophage morphology that was intermediate between these extremes (F). As the morphological transformation

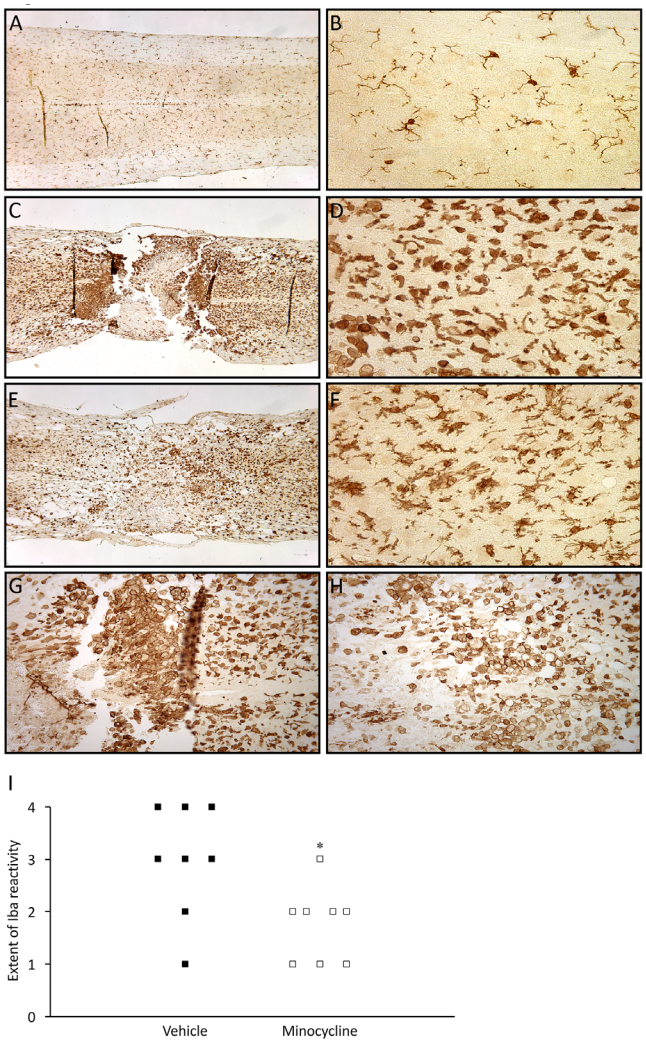

Figure 4: Microglial/macrophage activation and infiltration is reduced in minocycline treated mice. Microglia/macrophages were visualized using Iba1 immunostaining 5 days after injury. A comparison of representative sections from normal uninjured mice (A, B) and SCI mice treated with vehicle (C, D) reveals that there was increased density of cells in the SCI tissue, as well as a more amoeboid morphology characteristic of microglial/macrophage activation after SCI (D). In contrast, in minocycline treated mice (E, F), the density of microglia/macrophages appeared to be qualitatively reduced compared to vehicle controls. In minocycline treated mice (F), the microglia, while demonstrating morphological changes indicative of activation (i.e. shortened and thickened processes), did not progress as much as in the vehicle animals (D). Finally, within the epicenter of the injury in vehicle and minocycline treated animals (G and H respectively), there was an increased number of Iba1 labeled cells displaying an amoeboid morphology, with a greater density of these cells in the vehicle treated mice compared to the minocycline group. Iba1 stained sections were scored blinded for microglial/macrophage activation using a scale from 0-4, where 0 was normal cord and 4 indicated the presence of highly activated microglia/macrophages. There was a significant difference (*$P < 0.05$, Mann Whitney U test) in the morphology and density of Iba1 labeled cells in minocycline treated mice after SCI compared to vehicle (I, where each point represents one longitudinal section containing the central canal per mouse). SCI: spinal cord injury

of microglia from ramified to amoeboid forms is indicative of their increasing state of activation, these results suggest that minocycline reduced microglia/macrophage activation after injury.

We also determined the representation of microglia/macrophages at the epicenter of injury. We found that the density of Iba1 labeled cells was qualitatively lower in minocycline treated mice (H) than in vehicle controls (G), even though the morphology of cells, with the majority being amoeboid, did not differ between the 2 groups. To quantitate the extent of microglial/macrophage reactivity encompassing the lesion and remote areas, Iba1 immunoreactivity was scored by three independent observers blinded to treatment according to previously published methods[12]. Agreement between observers was good in large part and the identical result of 2 reviewers was noted as the score for a particular section. The blinded assessments [Figure 4I] indicated that there was a significant difference in Iba1 immunoreactivity between minocycline and vehicle treated mice 5 days after injury ($P < 0.05$, Mann Whitney U test).

Minocycline decreases apoptotic cell death as revealed by Tunel labeling

The number of Tunel positive cells was counted in sections of spinal cords taken from mice at 2 and 5 days after injury. The data shows cell death occurring at 2 and 5 days,and that minocycline treatment reduced the number of Tunel positive cells at the latter time point [Figure 5]. We did not address whether the Tunel positive cells were oligodendrocytes and/or neurons, and whether there is preferential rescue of one cell type versus another by minocycline.

Minocycline spares neurons at the lesion site after spinal cord injury

Figure 6 displays NeuN labeling in the normal uninjured spinal cord (A, B), in vehicle treated cord at 5 days after injury (C, D), and in minocycline treated mice at comparable points (E, F). While neurons were lost after SCI, there appears to be more neurons preserved in the minocycline versus the vehicle group. To confirm this, blinded counts were taken from the lesion epicenter and areas both rostral and caudal to the injury site. Univariate analysis of variance revealed that there was a significant area effect at 2 and 5 days ($P < 0.001$ for both), where SCI resulted in reduced neuron numbers, with the greatest loss occurring at the lesion site and with more spared neurons as the distance from the epicenter increased rostrally and caudally (lesion site significantly different from areas 1 and 2 mm rostral and caudal from the lesion epicenter, $P < 0.001$) [Figure 6]. Furthermore, while the same pattern of loss was present in minocycline treated animals, there was greater preservation of neurons after SCI when compared to vehicle controls ($P < 0.001$

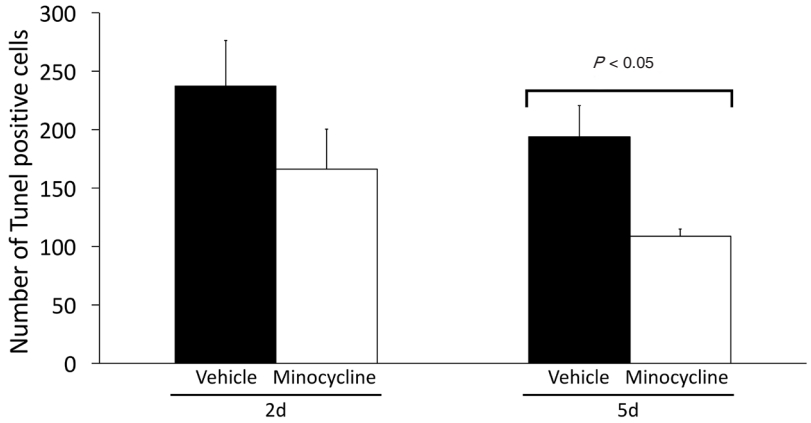

Figure 5: Minocycline treatment reduces the number of cells undergoing apoptotic cell death. Tunel positive cells were counted in sections from vehicle and minocycline treated mice harvested at 2 and 5 days post SCI. Cell death was evident 2 days after injury and was still ongoing at 5 days. The number of Tunel positive cells counted from the minocycline group was significantly lower than the vehicle group at 5 days post SCI (Student's *t*-test, $*P < 0.05$, $n = 9$/group, one longitudinal section containing the central canal per mouse). SCI: spinal cord injury

for both 2 and 5 days) [Figure 6].

DISCUSSION

MMPs are implicated in neural cell death. The application of MMP-1 and -9 to neurons in culture results in their demise[17]. In animal models of SCI, MMP-12 transcripts were upregulated 189 fold over basal levels, and functional recovery was significantly improved in MMP-12 null mice compared to wild-type controls[12]. Similarly, a role for MMP-9 in SCI was demonstrated in a study by Noble *et al.*[14] who showed that MMP-9 was upregulated following a contusion injury and that this was correlated with a reduction in blood spinal barrier integrity and the recruitment of inflammatory neutrophils. These authors also showed that functional recovery from SCI was significantly improved in MMP-9 null mice compared to wild-type controls. In correspondence with these results, inhibitors of MMPs applied acutely following SCI improved histological and functional outcomes[14]. Nonetheless, it must be borne in mind that MMPs also have beneficial functions in the CNS, and that the prolonged usage of MMP inhibitors can impair recovery[18,19].

Minocycline has documented MMP inhibitory activities[8-10]. In a model of ischemia in mice, minocycline reduced injury in wild-type, but not MMP-9 null mice, implicating MMP-9 as a target of minocycline activity in that condition[20]. Thus, it was reasonable to address whether or not the application of minocycline to mice subjected to SCI would result in reduced expression or activity of MMPs at lesion sites. Our overall results, however, do not support a mode of action of minocycline in acute SCI involving MMPs. We did not find reduced expression of transcripts encoding most MMPs in the spinal cord of mice afflicted with SCI and given minocycline. Also, MMP-2 and -9 protein levels were unaltered in the spinal cord of mice administered minocycline. Furthermore, we used *in situ* zymography to determine net proteolytic activity in spinal cord sections and we found no alterations in minocycline versus vehicle treated controls. It is probable that the acute administration of minocycline did not result in concentrations that were high enough to alter MMP activity and levels.

Despite the lack of effect of minocycline on MMPs, the medication did produce neuroprotective effects exemplified by the NeuN data. Previously we have demonstrated that minocycline produces robust neuroprotection in this model of spinal cord compression in the mouse, significantly decreasing lesion size and improving recovery in hindlimb function as assessed by the BBB scale in open field testing and the inclined plane task[5]. Furthermore, in that study, there were indications of white matter sparing determined by Bielchowsky silver stain and retrograde labeling of brain stem neurons by the fluorogold tracer[5]. The current data confirms that there is preservation of the gray matter at the level of the impact injury as well. While several groups have reported improved outcomes from spinal cord injury when minocycline is administered[5-7], these studies have not comprehensively evaluated the survival of neurons throughout the gray matter at the level of injury. Taken together, the existing body of research indicates that minocycline has a global neuroprotective effect.

One reason that minocycline is such an attractive pharmacological agent for neurological disorders is that it has multiple mechanisms of action targeting separate pathological processes simultaneously. Besides

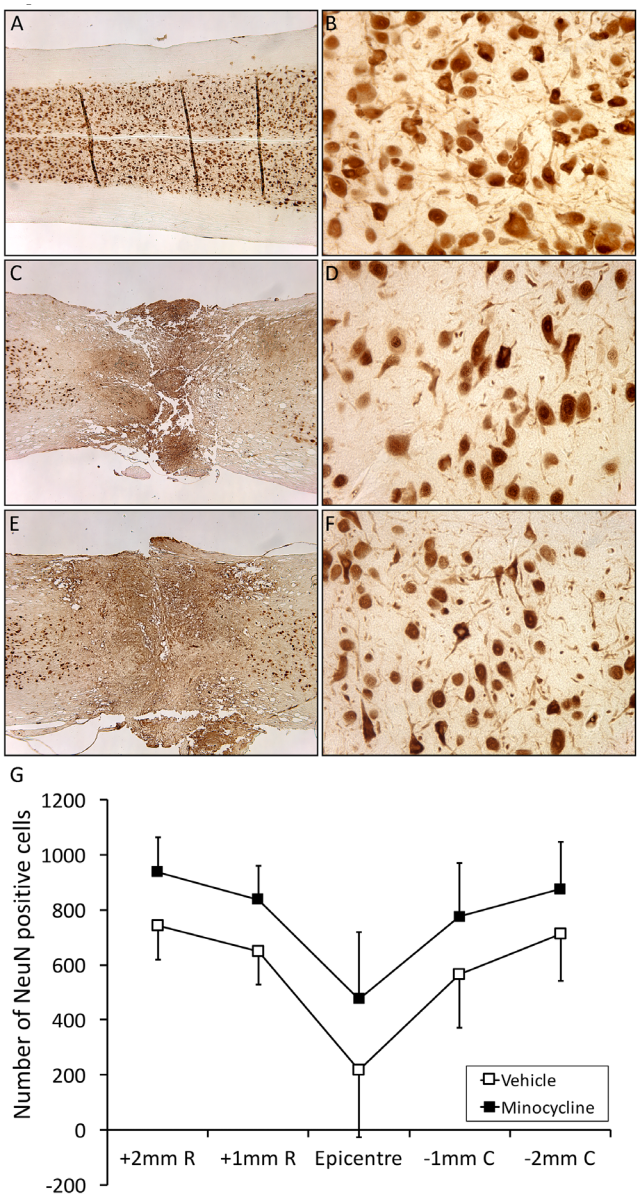

Figure 6: Minocycline treatment increases neuronal survival at the site of SCI. Panels A and B are representative pictures taken from normal uninjured cord immunostained with the neuronal marker, NeuN. Spinal cord compression produces injury at the lesion site that is characterized by a qualitative decrease in the number of NeuN positive cells (C and D, day 5). Minocycline treatment appears qualitatively to preserve some of the neurons within the vicinity of the lesion site (E and F), which was verified by blinded counts of NeuN-positive cells at the lesion epicenter as well as in areas 1 and 2 mm rostral (R) and caudal (C) to the lesion (G, mean ± SD). Panel G shows the data from day 2, and a similar pattern was found at day 5 after injury (data not shown); there were 9 mice per group, where one longitudinal section containing the central canal per mouse was examined. Univariate analysis of variance with scheffe *post-hoc* comparisons revealed that the difference associated with area (-2, -1, 0, +1 or +2) was significant ($P < 0.001$), with the number of cells remaining in the lesion epicenter being reduced compared to adjacent regions. Furthermore, there was a significant group effect with minocycline treated animals having more remaining neurons than vehicle treated controls ($P < 0.001$). SCI: spinal cord injury

reducing inflammatory activity of microglia and T cells[1], the over-activation of which contributes to neural cell death, minocycline reduces toxicity to oligodendrocytes and neurons through potentially direct mechanisms. In this regard, minocycline inhibits the activity of caspases[21,22] and the release of cytochrome c from mitochondria[23], which are both apoptosis-inducing events. Minocycline reduces signaling of the p38 mitogen activated kinase pathway[6,24], and it prevents the activation of poly (ADP-ribose) polymerase[25,26], actions that contribute to the alleviation of neural cell death. This drug has also been shown to decrease apoptosis of oligodendrocytes through a mechanism involving the inhibition of proNGF production by microglia[27]. Minocycline has been reported to reduce glutamate excitotoxicity[28,29], to detoxify free radicals that contribute to neurotoxicity[30,31] and to inhibit lipid peroxidation[32].

A limitation of the present study is that we did not perform neurobehavioral studies to accompany the histological and MMP results. However, the dose regimen employed is identical to that used in our previous study[5] that demonstrated behavioral recovery by 3 days post-injury in the minocycline compared to vehicle group. Another limitation is that for the majority of MMPs examined in the current study, only gene expression and not protein amount or activity was measured. However, protein levels of MMPs-2 and 9 were measured using gel zymography, and net proteolytic activity for these two molecules were examined with *in situ* zymography. Despite this limitation, the transcript expression pattern of MMPs provides valuable information in understanding the mechanisms by which minocycline may exert its effects after SCI.

In conclusion, the novel findings are that minocycline confers protection to neurons at the site of SCI, and that this does not involve the alteration of most MMPs. Given the neurotoxicity that can be inflicted by MMPs acutely after SCI, and the apparent lack of minocycline effect on most MMPs and TIMPs in this study, our results suggest that the combined treatment of minocycline and a specific MMP inhibitor may result in greater recovery than either treatment alone. Nonetheless, even without MMP inhibitory activity acutely after SCI, the myriad of mechanisms attributed to minocycline as aforementioned would position minocycline for further study as a neuroprotective medication after SCI in humans.

DECLARATIONS

Authors' contributions

Conducted experiments, and provided results: T. Rice,

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Co-wrote the first draft: T. Rice, J.E.A. Larsen
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Co-supervised aspects of the project: S. Casha, J. Hurlbert, D.R. Edwards
Provided overall supervision of the project and edited and finalized the manuscript: V.W. Yong

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The authors have no conflicts of interest to declare.

Patient consent
Not applicable.

Ethics approval
All studies involving animals were reviewed and approved by ethics committee at the University of Calgary, Canada.

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Editorial **Open Access**

Neuron specific enolase is a potential target for regulating neuronal cell survival and death: implications in neurodegeneration and regeneration

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Enolase is a multifunctional enzyme primarily involved in catalyzing the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis and the reverse reaction during gluconeogenesis[1-4]. Though typically expressed in the cytosol, enolase has been shown to migrate to the cell surface upon inflammatory signal[3]. It then enhances antigen presentation for the invasion of host cells via plasminogen binding and subsequent plasmin activation, leading to degradation of the extracellular matrix. Cell surface expression of enolase, possibly due to an association with the urokinase-type plasminogen activator (uPA)/uPA receptor complex, additionally induces the production of reactive oxygen species, nitric oxide, and pro-inflammatory cytokines [tumor necrotic factor (TNF)-α, interleukin (IL)-1β, interferon-γ, and transforming growth factor-β] and chemokines [monocyte chemotactic protein 1 and macrophage inflammatory protein (MIP)-1α] to augment neurodegenerative response[3,5]. Lysosomal proteases, especially cathepsins (e.g. Cathepsin X or Cat X), are instrumental in processing several neuronal proteins that generate either harmful or neuroprotective functions. Cathepsins and a neutral protease, calpain, also have regulatory functions in antigen processing and presentation, thereby inducing immune responses that can have either detrimental or beneficial effects on neuronal cells. This editorial discusses the relationships between neuron specific enolase (NSE) and Cat X activity in neuronal cells with a special focus on their implications for neurodegeneration and neuroprotection.

Distinct dimeric isoforms of enolase are composed of non-covalently linked α, β, or γ subunits and are tissue-specific[3]. During development, injury, or disease, α-enolase (enolase 1, non-neuronal enolase, ENO1), which is primarily found in adult tissue, can be converted to γ-enolase (enolase 2, NSE) in neurons and neuroendocrine cells. Similarly, α-enolase is

converted into β-enolase (enolase 3, muscle specific enolase, ENO3) in muscle tissue. NSE exists as both αγ and γγ isoforms in neurons, γγ in microglia and oligodendrocytes, and αγ in astrocytes[3,4]. Notably, enolase levels have been shown to be significantly increased in astrocytes and microglia following spinal cord injury (SCI), an observation indicating a possible role for NSE in different pathologies associated with the neuroinflammatory, apoptotic, and neuroprotective activity of these interacting glial cells[6,7]. Due to its specific location in neurons and neuroendocrine cells and its upregulated secretion following axonal damage, NSE has been implicated as a biomarker of functional damage to neurons in SCI and several other pathophysiological conditions: traumatic brain injury, stroke, ischemia-reperfusion injury, cardiac arrest, neuroblastoma, small-cell lung cancer, and Alzheimer's disease (AD)[2-5].

SCI is a debilitating neurological disorder that occurs in two main phases: primary and secondary injury[3,8]. The initial primary injury, resulting from laceration, contusion, compression, and/or contraction of the neural tissue, is the immediate, irreversible damage to the spinal cord and associated axons, cells, and blood vessels. However, a diverse array of secondary injury mechanisms, including hypoxia, ischemia, excitotoxicity, inflammation, and apoptosis, expands the injury site and impairs pro-survival activity following primary injury[9]. These secondary processes are reversible and as such have been a principle target of SCI treatment research[8]. Substantial reduction in blood supply from primary injury triggers ischemia, oxidative stress, and microglial activation that drive the release of pro-inflammatory cytokines and chemokines at the injury site. Under the hypoxic-ischemic conditions immediately following SCI, an influx of Ca^{2+} results in activation of calpain, caspase, and phospholipases[10,11]. Calpain then degrades cytoskeletal proteins and leads to apoptosis. Additional neuronal death can be attributed to glutamate excitotoxicity following the injury[9]. Though these effects are primarily neurodegenerative, macrophages may function as pro- or anti-inflammatory agents in SCI, depending on the M1/M2 macrophage cell ratio in the injured microenvironment.

Our group has shown that SCI treatment in a male Sprague-Dawley rat model using ENOblock, a small molecule inhibitor of enolase, corresponded to a reduction of NSE expression in SCI tissues and a significant decrease in serum NSE, matrix metallopeptidase (MMP)-9 protein expression in injured tissue, serum pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (MIP-1α and IP-10), and glial activation[2]. Elevated MMP-9 expression can promote the activation of microglia and astrocytes, leading to the release of inflammatory cytokines and chemokines that promote cell death. Expression of these pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) is known to induce hyperalgesia, allodynia, and apoptosis of neuronal and glial cells in association with the secondary damages of SCI[2,12-15]. Additionally, MIP-1α has been shown to mediate microglia accumulation and neuroinflammation in brain injury[16]. IP-10, expressed by astrocytes in response to N-methyl-D-aspartate-dependent excitotoxicity, activates the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway in mouse cortical neurons, indicating a role for this chemokine in mediating cell proliferation and apoptosis under neurodegenerative conditions[17,18]. The reduction of MMP-9 and the aforementioned pro-inflammatory cytokines and chemokines by ENOblock indicates the potential for this treatment in attenuating neural damages associated with inflammatory response during secondary injury mechanisms of SCI.

While previous research on the role of NSE in secondary injury mechanisms of SCI has focused on neurodegenerative effects, namely the association between the overexpression of NSE and an inflammatory cascade leading to neuronal cell death, future studies should additionally investigate the role of NSE in pro-survival and regeneration activity via cellular signaling pathways in acute and chronic SCI. NSE has been shown to exhibit neurotrophic activity in controlling neuronal survival, differentiation, and neurite regeneration of human neuroblastoma SH-SY5Y cells via activation of the phosphoinositide 3-kinase (PI3K)/Akt and MAPK/ERK signaling pathways, which regulate cytoskeleton reorganization and transcriptional factor activation in promotion of cell survival and neurite outgrowth[1]. A neuroprotective effect of NSE expression and activity was also observed in a mouse model of AD against amyloid-β-related neurodegeneration[5]. Additionally, the neuritogenic activity of NSE is associated with RhoA kinase inactivation within the PI3K/Akt pathway and affects neurite outgrowth through rapid actin polymerization[1]. NSE likely exhibits similar neuroprotective activity for cell survival, differentiation, and migration following SCI and other neurodegenerative conditions via the PI3K/Akt and MAPK/ERK pathways. Further research evaluating the interaction between NSE and these pathways in SCI secondary injury mechanisms for more clarification would be interesting.

Investigation into the neurotrophic activity of NSE in a mouse model of the neurodegenerative condition AD has shown that NSE can be regulated by Cat X, a lysosomal cysteine protease that cleaves the C-terminal end of the NSE enzyme under acidic conditions[5]. The C-terminal peptide of NSE, which is not involved in plasminogen binding (due to the absence of lysine) or glycolytic function, contains a PDZ-binding domain for the scaffold protein γ-1 syntrophin that enables NSE to relocate to the plasma membrane via actin filament, as evidenced by their co-localization[4]. This C-terminal peptide has been shown to have a pro-survival effect on PC12 cells[19]. The cleavage of NSE at this site by Cat X severely affects its ability to function in neuronal cell differentiation for pro-survival activity or cell death. Because of the known involvement of PI3K/Akt and MAPK/ERK signaling pathways in the activation of cathepsin B, a similar cysteine protease, in glioma, Cat X activity is likely associated with these same pathways[20].

An additional cysteine protease, calpain, is involved in the neuroinflammatory response to SCI[21]. Calpain is found in the cytosol and is active under neutral pH conditions upon Ca^{2+} activation. The role of calpain in apoptosis has been clearly demonstrated, and its activation in SCI conditions has been shown to lead to cytoskeletal and myelin protein cleavage. Calpeptin, a calpain inhibitor, can exhibit neuroprotective effects against excitotoxic apoptosis, reducing neuronal cell death[22]. While inhibition of enolase by ENOblock alters cellular growth, cytokines/chemokines, and inflammatory markers[2,23], it is unknown if ENOblock acts on Cat X and regulates its function. Our group has found increased calpain activity and cell-specific overexpression in astrocytes, microglia, macrophages and T cells in inflammatory demyelinating diseases[24-26]. However, the effects of calpain inhibition (calpeptin) on NSE and Cat X functions remain to be investigated. Calpeptin, which is a cysteine protease inhibitor, could possibly target Cat X, leading to inhibition of NSE-mediated inflammatory events and promotion of neuronal cell survival. Since NSE is a substrate of Cat X, evaluating both ENOblock and calpeptin as potential mediators of NSE expression and activity in neuronal cells following SCI and other neurological disorders.

At certain levels, NSE can support regeneration of neuronal cells[1,5]. NSE-mediated activation of the PI3K/Akt and MAPK/ERK pathways likely supports cell survival and regeneration. On the other hand, these pathways also likely activate Cat X, an enzyme that cleaves NSE. Cat X activity would likely result in a reduction of NSE-mediated activation of PI3K/Akt and MAPK/ERK pathways and result in cell death. It would be interesting to investigate the role of ENOblock in regulating PI3K/Akt and MAPK/ERK pathways and Cat X activity. Future studies to elucidate the role of PI3K/Akt and MAPK/ERK pathways in defining cell fate are warranted. Mediation of the Cat X activity in association with these pathways could result in partial as opposed to total degradation of NSE, thus reducing NSE and Cat X mediated cell death and providing a promising future therapeutic target for reversal of secondary injury mechanisms in acute and chronic SCI as well as other neurological conditions.

In conclusion, several future avenues for research on the mechanisms of NSE expression and activity in neurons and glia and the process of neurodegeneration and regeneration following neurological impairment have been discussed. NSE, once migrated to the plasma membrane, takes part in cellular activation, production of inflammatory cytokines and chemokines, and induction of neuronal cell death (neurodegeneration)[1,3]. The regulated expression of NSE may promote neuronal survival (neuroprotection or regeneration) via cell survival pathways. Previous research has focused on the harmful effects of NSE overexpression following neuronal damage. However, future studies should address the conditions leading to preferential differentiation into pro-survival activity or neuronal cell death and specific methods for regulating NSE and Cat X activity to mediate the secondary damages associated with SCI. The role of Cat X in secondary injury remains unknown, as does the influence of SCI on Cat X expression and activity. Additionally, the direct and indirect targets of ENOblock treatment have yet to be determined. Calpeptin, which acts on calpain to reduce neuronal cell death, may similarly act on Cat X to regulate NSE activity. The effects of these inhibitors on neurodegeneration and/or neuroprotection and their potential interaction with the PI3K/Akt and MAPK/ERK pathways remain to be determined. This editorial has highlighted several potential intermediary effectors associated with neurodegeneration and neuroprotection in SCI and other neuropathological conditions. Significant research is needed to further evaluate these possible mechanisms and their potential for translation into future preclinical and clinical treatments.

DECLARATIONS

Authors' contributions

Overall design and completion of the manuscript: A. Haque

Performed original research on enolase and SCI, and analyzed data published in Neurochemical

Research-2017: M. Capone, A. Hossain, D. Matzelle
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Edited the manuscript: D. Matzelle, N.L. Banik
Reviewed and approved the final version of the manuscript: All authors

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Conflicts of interest

There are no conflicts of interest.

Patient consent

Not applicable.

Ethics approval

Not applicable.

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Letter to Editor **Open Access**

Transcranial magnetic stimulation for schizophrenia: potential and risks

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Recently, transcranial magnetic stimulation (TMS) has increasingly been used to investigate the neurobiology of schizophrenia. In those studies, researchers applied TMS in combination with motor evoked potentials (TMS/MEPs) and high density electroencephalograms (TMS/hd-EEG)[1]. The studies revealed significant impairments in cortical excitability, inhibition, and oscillatory activity, which are more prominent in the frontal brain areas, in patients suffering from schizophrenia compared to healthy controls[1]. Future TMS studies may help explain the underlying neurobiology of schizophrenia, and TMS may help monitor and perhaps further optimize the effectiveness of treatment interventions in patients with this disease. However, despite the potential of using TMS in the investigation of the underlying neurobiology of schizophrenia, three critical notes are essential.

First, so far, TMS studies on schizophrenia have been neither robust, consistent, nor standardized enough and have had high risks of publication bias, as was recently concluded in a large systematic review of forty-one trials by Dougall and colleagues[2]. Studies differ in numerous variables, for example, the stimulation intensity or length, the areas of the brain that are stimulated, and the design of the same TMS condition. Those variables could have great impact on the effects of TMS in the studied population. Therefore, better, well-developed TMS studies that test specific underlying neurobiological working mechanisms in schizophrenia are warranted. Moreover, schizophrenia is not a static, but rather a dynamic, disorder; therefore, TMS studies should focus more on patients with different stages of schizophrenia, i.e. patients experiencing their first episode of schizophrenia versus patients with chronic schizophrenia.

Second, within the studies, insufficient attention has been paid to the safety of using TMS on patients with schizophrenia. This is remarkable considering that a vivid discussion of the risks of using TMS to treat psychiatric patients is ongoing[3]. From the literature, for

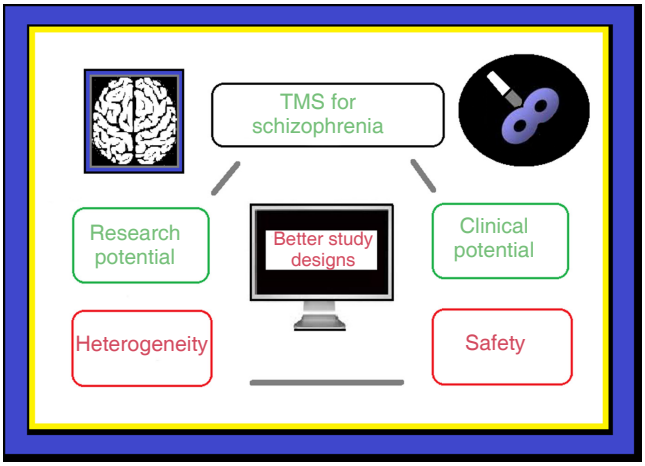

Figure 1: Summarizing the main issues regarding TMS for schizophrenia. TMS: transcranial magnetic stimulation

instance, human subjects, after having been treated with TMS, are known to suffer from minor complaints, such as headaches, pain due to burns caused by the scalp electrodes, local pain, *etc.*[4,5], and major complaints, such as mood changes, seizures, induction of hyper- or hypomania, *etc.*[4,5]. Obviously, in an extremely vulnerable population, such as patients suffering from schizophrenia, those side-effects might be more prominent and may lead to even more adverse events and difficulties in the patient's life.

Third, as to the underlying neurobiology and possible treatment interventions of TMS in patients with schizophrenia, the clinical diagnostic issue of the heterogeneity of the disorder, which is a typical characteristic of schizophrenia, is not taken into account[6]. Although the symptoms may be common, schizophrenia may have many different causes that are influenced by genetic and environmental factors, resulting in a very heterogenic population of patients[7]. The answers to the questions of how informative the TMS findings that have been collected on schizophrenia so far[1] with respect to those subtypes of patient groups with schizophrenia are and what those findings mean at the individual patient level (e.g. with respect to gender differences, interactions with individual pharmacological treatments, *etc.*) remain unclear.

To conclude, the critical issues mentioned above are shown in Figure 1. If progress is to be made in understanding the underlying neurobiology of schizophrenia and in particular, if safe TMS-based treatments are to be developed for patients suffering from this disease, these issues need to be addressed in future, well-designed TMS studies. The results of such studies should more clearly expose the full potential of using TMS in the treatment of patients with schizophrenia.

DECLARATIONS

Authors' contributions

Drafted the manuscript text, developed the intellectual ideas, managed the vivid discussions with the other members of the research group, made the suggested revisions, and approved the final version to be published: P. Bosch, M. van den Noort

Made both intellectual and textual suggestions for improvement, contributed to the vivid discussions with the other members of the research group and approved the final version to be published: S. Yeo, H. Staudte, P. Barisch

Made intellectual suggestions for improvement, contributed to the vivid discussions with the other members of the research group and approved the final version to be published: S. Lim

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Topic: Stroke **Open Access**

Inflammation and genetic factors in stroke pathogenesis

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Stroke is the leading cause of serious, long-term disability in the United States. Each year, approximately 795,000 people suffer a stroke. About 600,000 of these are first attacks, and 185,000 are recurrent attacks. Nearly three-quarters of all strokes occur in people over the age of 65 (www.strokecenter.org/patients/about-stroke/stroke-statistics/). According to the American Stroke Association, stroke is the 5th leading cause of death in the United States, killing 130,000 every year. The prevalence of stroke and its cost will undoubtedly rise as the aging population increases. In addition, stroke incidence and mortality are increasing in less developed countries in which the lifestyles and population restructuring are rapidly changing.

After ischemic stroke, severe disturbance of the blood supply to brain tissue deprives oxygen supply to brain tissue causing neuronal death. Pathophysiological events occur after ischemic stroke include ionic imbalance, neuroinflammation, excitotoxicity, activity of microglia. All of these contribute to neuronal death. Ischemia induces a significant increase in microvascular density, a sign of angiogenesis, in the penumbra of the cerebral infarct. The degree of increased vessel-density in the ischemic penumbra is positively correlated with the survival rate of stroke patients. In addition, increased angiogenesis was associated with an improved functional outcome after ischemic stroke in both animal models and in stroke patients. However, despite considerable research efforts, the exact mechanisms for stroke injury have not been fully understood. None of neuroprotection and angiogenesis strategies has shown therapeutic benefit in clinical setting. Administration of intravenous recombinant tissue plasminogen activator (rtPA) is still the only effective treatment. The therapeutic window of rtPA is limited within 4.5 h after the onset of ischemic stroke[1,2]. Endovascular treatment has been shown to improve functional outcome in 5 randomized clinical trials on selected patients with acute ischemic stroke. Thrombectomy with stent retrievers is now recommended as the standard of care for acute ischemic strokes with a proximal large vessel occlusion in the anterior circulation. However, the therapeutic window for this treatment is also limited[1].

Cerebral ischemia induces a cascade of inflammatory and immune reactions that encompass genomic as well as molecular and cellular events. Immune response has been shown to play a major role in ischemic stroke progression. The extent of neuronal damage seems to

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correlate with the degree of innate immune activity. For example, bone fracture induced systemic inflammation exacerbates ischemic stroke injury in mice[3,4] and reduction of inflammation through activation of a-7 nicotinic acetylcholine receptor reduces ischemic brain injury[5]. However, in recently years, many studies showed that neuroinflammation appear to be double-edged swords in the battle for neurological recovery. For example, microglia/macrophage activation fosters brain recovery by clearing cell debris, which leads to resolving local inflammation. Activated microglia/macrophage also produce a plethora of trophic factors that promote tissue repair[6]. Recent studies show that blocking CCR2 macrophage impairs functional recovery of stroke victims[7]. However, the kinetics of macrophage/microglia polarization switch is different among different models, such as reperfusion *vs*. permanent occlusion[7,8]. Therefore, the contradictory functions of microglia/macrophage might reflect their acquisition of distinct phenotypes in response to different microenvironmental cues[6].

This issue includes a review and a mini review what discussed the role of inflammation in brain injury. Marcet *et al*.[9] discussed the impacts of inflammation in ischemic stroke and traumatic brain injury in their review. They indicated that although the brain damage induced by the initial trauma is most likely unsalvageable, the secondary immunologic deterioration of neural tissue gives many opportunities for therapeutic strategists. This review highlighted the cell death mechanisms associated with injury or center nervous system (CNS) with special emphasis on inflammation. They discussed the sources of inflammation, and introduced the role of the spleen in the systemic response to inflammation after CNS injury. In the mini-review, Liu *et al*.[10] overviewed the role of microglia in neuroinflammatory after ischemic stroke. In addition, a clinical study presented by Lu *et al*.[11] in this issue demonstrated a correlation of cerebral microbleed and the level of inflammatory markers in blood.

In addition to inflammatory, other such as genetic variations influence stroke occurrence and recovery. A review in this issue by Zhu *et al*.[12] discussed the roles of *endoglin* gene in cerebral vascular diseases. Endoglin (ENG) is a transforming growth factor beta associated receptor and is required for both vasculogenesis and angiogenesis. In human, ENG haploinsufficiency is associated with type 1 hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Rendu-Weber Syndrome, which is an autosomal dominant disease. HHT patients have a higher prevalence of telangiectases in mucocutaneous membrane and arteriovenous malformation (AVM) in multiple organs. AVMs are abnormal vessels shunting blood directly from arteries to veins[13]. AVM has abnormal vessel wall structure, which is likely to rupture. Rupture of brain AVM can result in life-threatening intra-cranial hemorrhage and hemorrhagic stroke[13]. Alternatively, pulmonary AVMs in HHT type 1 patients are associated with a higher incidence of paradoxical embolism in the cerebral circulation causing ischemic brain injury than general population.

ENG is required for the differentiation and sprouting of endothelial tubes, which are important processes of angiogenesis. ENG is also an important mediator of endothelial-mesenchymal communication during angiogenesis. *Eng* deficient mouse embryos show impaired recruitment of vascular smooth muscle cells and pericytes to newly form vascular network. Zhu *et al*.[12] discussed, in this issue, the roles of ENG in ischemic stroke and indicated that ENG expression might be a potential biomarker for vasospasm after subarachnoid hemorrhage and cerebrovascular stenosis. Experimental or therapeutic modulating of ENG expression could be useful in generation of animal models for study disease pathogenesis and for development of novel treatments for multiple cerebrovascular diseases.

In summary, we have tremendously expanded our working knowledge of how vascular remodeling in the brain occurs and identified many the key cellular and molecular events underlying this process in recently. In this issue, we have assembled a collection of articles from renowned experts in the field of brain injury and neuroinflammation, and attempted to lift some of the veil on the pathophysiology of stroke.

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Authors' contributions
H. Su contributed solely to the paper.

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Topic: Alzheimer's Disease **Open Access**

Altered filamin A enables amyloid beta-induced tau hyperphosphorylation and neuroinflammation in Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease with proteopathy characterized by abnormalities in amyloid beta (Aβ) and tau proteins. Defective amyloid and tau propagate and aggregate, leading to eventual amyloid plaques and neurofibrillary tangles. New data show that a third proteopathy, an altered conformation of the scaffolding protein filamin A (FLNA), is critically linked to the amyloid and tau pathologies in AD. Altered FLNA is pervasive in AD brain and without apparent aggregation. In a striking interdependence, altered FLNA is both induced by Aβ and required for two prominent pathogenic signaling pathways of Aβ. Aβ monomers or small oligomers signal via the α7 nicotinic acetylcholine receptor (α7nAChR) to activate kinases that hyperphosphorylate tau to cause neurofibrillary lesions and formation of neurofibrillary tangles. Altered FLNA also enables a persistent activation of toll-like-receptor 4 (TLR4) by Aβ, leading to excessive inflammatory cytokine release and neuroinflammation. The novel AD therapeutic candidate PTI-125 binds and reverses the altered FLNA conformation to prevent Aβ's signaling via α7nAChR and aberrant activation of TLR4, thus reducing multiple AD-related neuropathologies. As a regulator of Aβ's signaling via α7nAChR and TLR4, altered FLNA represents a novel AD therapeutic target.

INTRODUCTION

Alzheimer's disease (AD) is a complex neurodegenerative disease characterized by a variety of synaptic and receptor dysfunctions, neuroinflammation, insulin resistance, degeneration and atrophy. Although the pathogenesis of AD is debated, the disease itself can be considered a proteopathy, a disease of abnormal proteins, due to the misfolding and aggregation of amyloid beta (Aβ) and hyperphosphorylated tau in brain areas critical to cognition and memory. These abnormal proteins

ultimately form the histopathological hallmarks of AD brain: amyloid plaques and tau-containing tangles. Misfolded dysfunctional proteins and their aggregation occurs in many other neurodegenerative diseases including Parkinson's disease, dementia with Lewy bodies, multiple systems atrophy, frontotemporal dementia, amyotrophic lateral sclerosis and Huntington's disease[1-4]. Typically, misfolded proteins self-aggregate, creating intracellular inclusions that become extracellular deposits following cell death. In many cases, misfolded proteins propagate in a cell-to-cell "prion-like" manner[1-3,5].

The proteopathy of Aβ in AD is an amyloidosis, meaning the protein converts from an α-helix-rich state to a β-sheet conformation. To enter an amyloid or β-sheet state, proteins must expose the backbone amide N-H and C=O groups to allow hydrogen bonding[4]. Cleavage of amyloid precursor protein into $Aβ_{42}$ by secretases can expose these amide N-H and C=O groups and promote a β-sheet conformation[4]. Elevated concentrations from overproduction or insufficient clearance/processing may also contribute. The hydrogen bonding of the pleated, β-sheet conformation between parallel or anti-parallel β-sheets is much stronger than that in native α-helices, making reversal unlikely. Additionally, the β-sheet conformation allows hydrogen bonding between separate, stacked molecules, promoting oligomerization and eventual plaque formation[4]. Aβ is proposed to form a toxic, small oligomer "seed" requiring 3 or 4 molecules used as a template to "infect" native molecules and propagate in a prion-like manner[6].

The proteopathy of tau, or tauopathy, in AD is primarily caused by hyperphosphorylation. Hyperphosphorylated tau loses its function of stabilizing microtubules and dissociates from them[7]. The increased pool of free tau after dissociation from microtubules is likely an important first step to aggregation in AD[8]. Untethered from microtubules, hyperphosphorylated tau twists together to form the paired helical filaments (PHFs) found in neurofibrillary tangles. In a toxic gain of function, hyperphosphorylated tau also actively disrupts microtubules and inhibits their assembly[7,9] and even sequesters functional tau and other microtubule associated proteins[9]. Hyperphosphorylation also changes tau's localization from axon-predominant to include dendrites, neuronal cell bodies and presynaptic areas, leading to synaptic dsyfunction[10-12].

This mini-review focuses on a third, interconnected proteopathy in AD and its critical role in 2 toxic signaling pathways of soluble Aβ. The newly described proteopathy is an altered conformation of the ubiquitous scaffolding protein filamin A (FLNA), induced by $Aβ_{42}$ and without apparent aggregation[13]. The 1st toxic cascade of Aβ enabled by altered FLNA is Aβ's signaling via α7 nicotinic acetylcholine receptor (α7nAChR) to hyperphosphorylate tau. The 2nd signaling pathway is Aβ's aberrant activation of toll-like-receptor 4 (TLR4), by binding CD14[14], to induce neuroinflammation. In both cases, altered FLNA associates with these receptors to allow their aberrant signaling by Aβ[13,15]. Native FLNA in control brains does not associate with either receptor. Whether the altered conformation precedes or is a consequence of these receptor linkages is discussed. The interdependence of altered FLNA with Aβ signaling to hyperphosphorylate tau and promote neuroinflammation has been elucidated via the reversal of the FLNA proteopathy by a small molecule therapeutic candidate, PTI-125.

Aβ SIGNALING VIA α7NACHR TO HYPERPHOSPHORYLATE TAU

The most toxic form of Aβ is considered soluble Aβ oligomers rather than plaque deposits[16,17]. Evidence that soluble Aβ induces tau pathology has grown, with hyperphosphorylation as the primary pathological modification[18]. Extensive research has elucidated the role of the α7nAChR in the toxicity of soluble $Aβ_{42}$ and the consequent hyperphosphorylation of tau[19-24]. Soluble $Aβ_{42}$ in monomeric or oligomeric form binds and signals via α7nAChR[25-28], essentially hijacking this receptor to abnormally activate various kinases[27,29-31] to heighten tau phosphorylation. Supportive data include co-localization of $Aβ_{42}$ and α7nAChR in AD pyramidal neurons and a complete blockade of $Aβ_{42}$-induced tau hyperphosphorylation *in vitro* by α7nAChR antisense oligonucleotides[32]. $Aβ_{42}$ dose-dependently activates tau kinases to persistently phosphorylate tau at the three proline-directed sites, resulting in elevated hyperphosphorylated tau in neurofibrillary tangles. This Aβ-driven tau hyperphosphorylation can also be blocked by the α7nAChR antagonist α-bungarotoxin or other α7nAChR ligands if administered prophylactically[32-35]. The hyperphosphorylation of tau renders it dysfunctional, alters its cellular distribution and disrupts axonal/dendritic transport, leading to neurofibrillary lesions, dendritic breakdown, and ultimately, neurofibrillary tangles[27]. Importantly, soluble $Aβ_{42}$ binds α7nAChR with an extraordinarily high (high femtomolar) affinity, rendering the $Aβ_{42}$-α7nAChR interaction nearly irreversible[26,36].

Though other targets have been demonstrated for

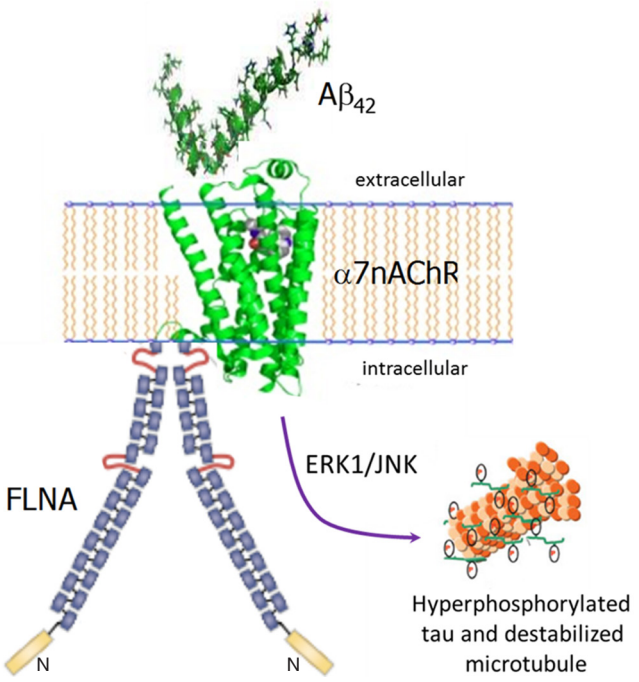

Figure 1: Altered FLNA linkage to α7nAChR enables $Aβ_{42}$'s toxic signaling via α7nAChR to hyperphosphorylate tau. Monomers or small oligomers of $Aβ_{42}$ bind α7nAChR, which recruits FLNA to link to α7nAChR. This recruitment likely alters FLNA's conformation, which in turn increases the affinity of the $Aβ_{42}$-α7nAChR interaction to a femtomolar affinity and enables the signaling. ERK1 and JNK kinases are activated to hyperphosphorylate tau. Hyperphosphorylated tau loses its function of stabilizing microtubules and dissociates from them, eventually creating PHFs and neurofibrillary tangles. FLNA: filamin A; Aβ: amyloid beta; α7nAChR: α7 nicotinic acetylcholine receptor; PHF: paired helical filament

soluble $Aβ_{42}$, their nanomolar or lower affinities suggest high off-rates and limited target engagement, in contrast to $Aβ_{42}$'s nearly irreversible sub-picomolar interaction with α7nAChR. Other targets of soluble Aβ include PrP^C, a prion receptor, which Aβ binds at 50-100 nmol/L to suppress LTP in slice cultures[37]. Acting as a co-receptor for the Aβ-PrP^c complex, mGluR5 also plays a role in the impaired LTP[38]. Soluble Aβ has also been shown to bind neuroligin-1, a post-synaptic cell adhesion protein, in the nanomolar range, and this interaction has been proposed to promote Aβ oligomer formation[39].

Evidence that phosphorylation can alter tau's conformation comes from a study showing that a particular AD-specific phosphorylated tau species is only formed when specific phosphoepitopes in a proline-rich region are sequentially phosphorylated by GSK-3β (at Thr212) and then by PKA (at Ser214)[40]. Whether tau's hyperphosporylation contributes to protein misfolding prior to formation of PHFs is speculative, particularly as its pathological structure has not been elucidated. The tau proteopathy in AD, therefore, involves hyperphosphorylation, but may or may not include misfolding. The formation of PHFs requires hyperphosphorylated tau, and tau protein in neurofibrillary tangles is hyperphosphorylated, most notably at Ser^{202}, Thr^{231} and Thr^{181}[41]. Interestingly, the alpha-synuclein that forms fibrils and is abundant in Lewy bodies in Parkinson's disease is also hyperphosphorylated, at a single serine site[42].

ALTERED FLNA LINKS Aβ AND TAU PROTEOPATHIES

We recently described a third, atypical proteopathy in AD that is critically interconnected with the toxicities of both $Aβ_{42}$ and tau. This third proteopathy is an altered conformation of the scaffolding protein FLNA. It is induced by $Aβ_{42}$, and in reciprocal action, enables $Aβ_{42}$'s toxic signaling via α7nAChR to activate kinases that hyperphosphorylate tau[13]. Altered FLNA enables $Aβ_{42}$'s signaling via α7nAChR by associating with this receptor[13,15]. Although FLNA constitutively associates with other receptors including the mu opioid receptor and insulin receptors[43], FLNA does not normally link to α7nAChR. We hypothesize that upon $Aβ_{42}$ binding to α7nAChR, FLNA is recruited to this receptor and its conformation altered to enable $Aβ_{42}$'s aberrant signaling [Figure 1]. FLNA in control brains can be induced to link to α7nAChR by incubation with $Aβ_{42}$ *in vitro* or by ICV $Aβ_{42}$ infusion *in vivo*[13,15]. The altered conformation of FLNA is also illustrated by the 100-fold difference in binding affinities of the novel drug candidate PTI-125 to FLNA immunopurified from human postmortem AD *vs*. control brain[13]. One distinct difference between altered FLNA and other proteopathies is that the altered conformation of FLNA does not appear to promote self-aggregation.

Best known for cross-linking actin to enable cell structure, flexibility and motility, FLNA is a prominent regulator of the actin cytoskeletal assembly and dynamics[44-46]. The actin cytoskeleton, a vital component in synapses and the dendritic network, is impaired in AD[47]. Hence, the FLNA proteopathy might also disrupt synaptic and dendritic function in AD by disrupting actin dynamics. FLNA exists as an intracellular homodimer and dimerizesin a membrane-bound, C-terminal domain. It is a large (280-kDa) protein with 24 immunoglobulin repeats that are natively β-sheet pleated, forming two rod-like domains separated by two hinge regions. The nature of the conformational change of FLNA in AD is not yet known, thoughit is interesting that native FLNA is predominantly β-sheeted. Demonstrating induction by

Aβ, the altered FLNA conformation exists not only in postmortem human AD brain and in triple transgenic (3xTg) AD mice, but also in ICV $Aβ_{42}$-infused wildtype mice and in $Aβ_{42}$-treated postmortem human control brain[13].

This altered form of FLNA was evidenced by a shift in isoelectric focusing point (pI) from 5.9 in the native state to 5.3 in postmortem human AD brain or brains of mouse models[13]. An altered pI can indicate an altered conformation, reflecting changes in hydrogen bonding, charge-charge interactions or accessibility of ionizable residues within the molecule[48-50]. In this case, the shifted pI is resistant to complete dephosphorylation by alkaline phosphatase. Hence, unlike the proteopathies of tau and alpha-synuclein, the altered conformation of FLNA is not due to changes in phosphorylation state. Further studies are needed to reveal the details of FLNA's conformational change and whether altered FLNA is unique to AD.

The induction of the altered conformation by $Aβ_{42}$ may suggest a direct interaction or some sort of cross-protein templating by $Aβ_{42}$. However, $Aβ_{42}$ and FLNA do not directly interact because neither protein can be co-immunoprecipitated with the other (our unpublished observations). Although it is tempting to presume that FLNA must be in its altered conformation to associate with α7nAChR, we hypothesize that it is FLNA's transmembrane recruitment to this receptor, induced by $Aβ_{42}$'s extracellular binding, that changes FLNA's conformation. $Aβ_{42}$ binds α7nAChR with high femtomolar affinity, the highest known binding affinity of $Aβ_{42}$. Interestingly, prevention of the FLNA linkage to α7nAChR by FLNA-binding compound PTI-125 decreases $Aβ_{42}$'s affinity for this receptor 1,000-10,000-fold, illustrating that FLNA enables not only $Aβ_{42}$'s toxic signaling but also its high-affinity binding for α7nAChR[15]. Although this observation appears to suggest that altered FLNA is responsible for $Aβ_{42}$'s binding, we propose a dynamic, sequential process: (1) $Aβ_{42}$ binds α7nAChR to induce FLNA recruitment; (2) recruitment alters FLNA's conformation; and (3) FLNA's altered form secures (locks in) an ultra-high affinity $Aβ_{42}$-α7nAChR interaction. The reasoning behind this hypothesis is that $Aβ_{42}$ induces both the aberrant FLNA conformation and its recruitment to α7nAChR (either by ICV $Aβ_{42}$ infusion to wildtype mice or by *in vitro* $Aβ_{42}$ incubation of postmortem control brain)[13,15] and that $Aβ_{42}$ and FLNA do not themselves interact.

Enabled by FLNA, $Aβ_{42}$, in monomeric or small oligomeric form[51], signals via α7nAChR to activate kinases to hyperphosphorylate tau protein[30,32,33,52]. We know that the linkage of altered FLNA is required for this Aβ signaling pathway because the novel drug candidate PTI-125 prevents the FLNA-α7nAChR linkage and greatly reduces tau hyperphosphorylation[13,15]. Hyperphosphorylated tau loses its ability to stabilize microtubules and dissociates from them, thereby increasing the pool of free phosphorylated tau that eventually appears in neurofibrillary tangles. We suggest that the signaling of $Aβ_{42}$ via α7nAChR contributes prominently to a variety of AD-related neuropathologies in addition to, or perhaps elicited by, tau hyperphosphorylation because these additional neuropathologies are also reduced by PTI-125's disruption of $Aβ_{42}$'s signaling via α7nAChR[13,15]. Alternatively, they may be related to the neuroinflammatory signaling that is also disrupted by PTI-125 as discussed below. An obvious consequence of the toxic signaling through α7nAChR is that the normal function of α7nAChR is impaired. Because α7nAChR is an upstream regulator of the N-methyl-D-aspartate receptor (NMDAR)[53,54], the impaired NMDAR signaling in AD brain and 3xTg AD mice is very likely related to the impaired signaling via α7nAChR. This assertion is supported by the improvement in signaling function of both receptors by PTI-125[13,15]. $Aβ_{42}$'s signaling to hyperphosphorylate tau also contributes to eventual formation of Aβ deposits and tau-containing neurofibrillary lesions[55,56], because disrupting this signaling via PTI-125 markedly reduces both Aβ deposits and neurofibrillary lesions in 3xTg AD mice or ICV $Aβ_{42}$-infused wildtype mice[13,15]. Finally, reiterating the improved receptor function by PTI-125 and implicating this toxic Aβ signaling pathway in cognitive impairment, PTI-125 also improved working and spatial memory and nesting behavior abnormalities in 3xTg AD mice[13].

ALTERED FLNA ENABLES Aβ-INDUCED NEUROINFLAMMATION

The proteopathy of FLNA enables a second toxic signaling pathway of Aβ: its activation of the innate immune receptor TLR4[13,15]. $Aβ_{42}$ binds the CD14 co-receptor[14], complexed with TLR4, to induce an aberrant FLNA linkage to TLR4[13,15], which appears to reciprocally enable a sustained Aβ-mediated TLR4 activation [Figure 2]. Hence, the FLNA-TLR4 linkage, allowing Aβ activation of TLR4, promotes persistent inflammatory cytokine release and elicits neuroinflammation characteristic of AD. As with α7nAChR, native FLNA in control brains does not associate with TLR4, unless treated with $Aβ_{42}$ by ICV infusion to wildtype mice or by *in vitro* incubation of

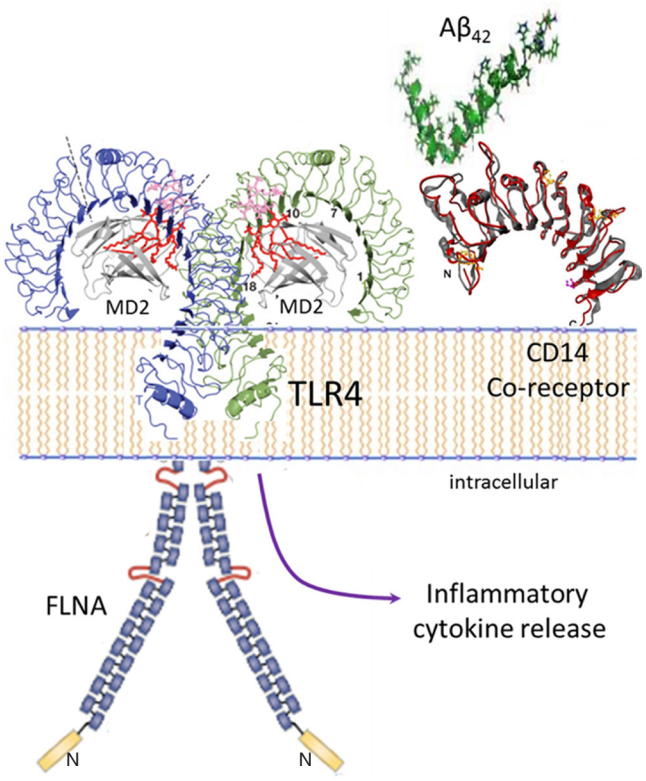

Figure 2: Altered FLNA linkage to TLR4 enables persistent $A\beta_{42}$-induced TLR4 activation and neuroinflammation. $A\beta_{42}$ binds the CD14 co-receptor to induce FLNA recruitment to TLR4. As with α7nAChR, the FLNA linkage likely alters the FLNA conformation. With $A\beta_{42}$ binding CD14, altered FLNA linkage to TLR4 enables a sustained TLR4 activation, leading to substantial inflammatory cytokine release and the neuroinflammation characteristic of AD. FLNA: filamin A; Aβ: amyloid beta; α7nAChR: α7 nicotinic acetylcholine receptor; TLR4: toll-like-receptor 4

control postmortem human brain[13,15]. Like the FLNA recruited to α7nAChR, FLNA that links to TLR4 in the presence of $A\beta_{42}$, or in AD postmortem brain or 3xTg AD mice, has an altered conformation[13]. The question arises again whether $A\beta_{42}$ binding to CD14 induces the FLNA linkage to TLR4 and the altered FLNA conformation in the process, or whether this linkage happens only after FLNA's conformation is altered. With two receptors seemingly controlling FLNA's conformation by recruitment, it is tempting to speculate that one linkage or the other induces the altered conformation, unleashing further aberrant and persistent receptor associations with FLNA. Notably, even in its non-diseased state, FLNA interacts with more than 90 different protein partners including a few receptors and many signaling molecules[44]. With multiple interactions, it is possible that one or more specific protein interactions - induced by Aβ - could alter FLNA's conformation and subsequent behavior. If so, it is then possible that the alteration in FLNA could also affects its interaction with other protein partners and their functions, disrupting the integrity of circuitries to propagate dysfunction in AD brains.

REVERSING FLNA'S ALTERED CONFORMATION

The primary evidence for the role of FLNA's altered conformation in $A\beta_{42}$'s toxic signaling via α7nAChR and TLR4 comes from the small molecule therapeutic candidate PTI-125. The altered conformation of FLNA was first inferred from the efficacy (as well as safety) at relatively low doses of PTI-125 in mouse models and *in vitro*, despite a ubiquitous target. We subsequently determined that PTI-125 binds altered FLNA (in AD postmortem brain, in 3xTg AD mice or in ICV $A\beta_{42}$-infused mice) much more tightly than native FLNA (in control postmortem brain or in control mice): a femtomolar affinity was measured for altered FLNA *vs.* a substantially lower, picomolar affinity for native FLNA[13]. Using the same isoelectric focusing point assessment that demonstrated FLNA's altered conformation, we further showed that PTI-125 binding, by *in vivo* treatment of mice or by *in vitro* incubation of postmortem AD brain, restores the native conformation of FLNA[13]. By reversing the FLNA proteopathy, PTI-125 dramatically reduces FLNA's aberrant linkages to both α7nAChR and TLR4, consequently reducing tau hyperphosphorylation and neuroinflammation[13,15]. By attenuating Aβ's pathogenic drive, PTI-125 treatment improved function of three key receptors: α7nAChR, NMDAR and insulin receptors[13,15]. The improved insulin receptor signaling may also reflect reduced neuroinflammation by PTI-125, as neuroinflammation has been linked to impaired insulin receptor function[57]. Disrupting the FLNA-TLR4 linkage, PTI-125 potently and efficaciously reduced inflammatory cytokines by at least 80% in 3xTg AD mice and in ICV $A\beta_{42}$-infused mice[13,15]. Correlating with the improved NMDAR function, synaptic plasticity was also improved by PTI-125 treatment of either 3xTg AD mice or $A\beta_{42}$-treated postmortem human control brain, evidenced by improved activity-dependent expression of the master synaptic plasticity regulator, activity-regulated, cytoskeleton-associated protein (Arc)[13]. These beneficial effects of PTI-125 in AD mouse models and postmortem AD brain elucidate a critical role of the FLNA proteopathy in multiple toxicities of Aβ and tau, including neuroinflammation [Figure 3]. We also speculate that altered FLNA may alter actin dynamics, given the primary role of FLNA in the actin cytoskeleton.

LIMITATIONS OF INTERPRETATION

There are limitations to our interpretations of our data.

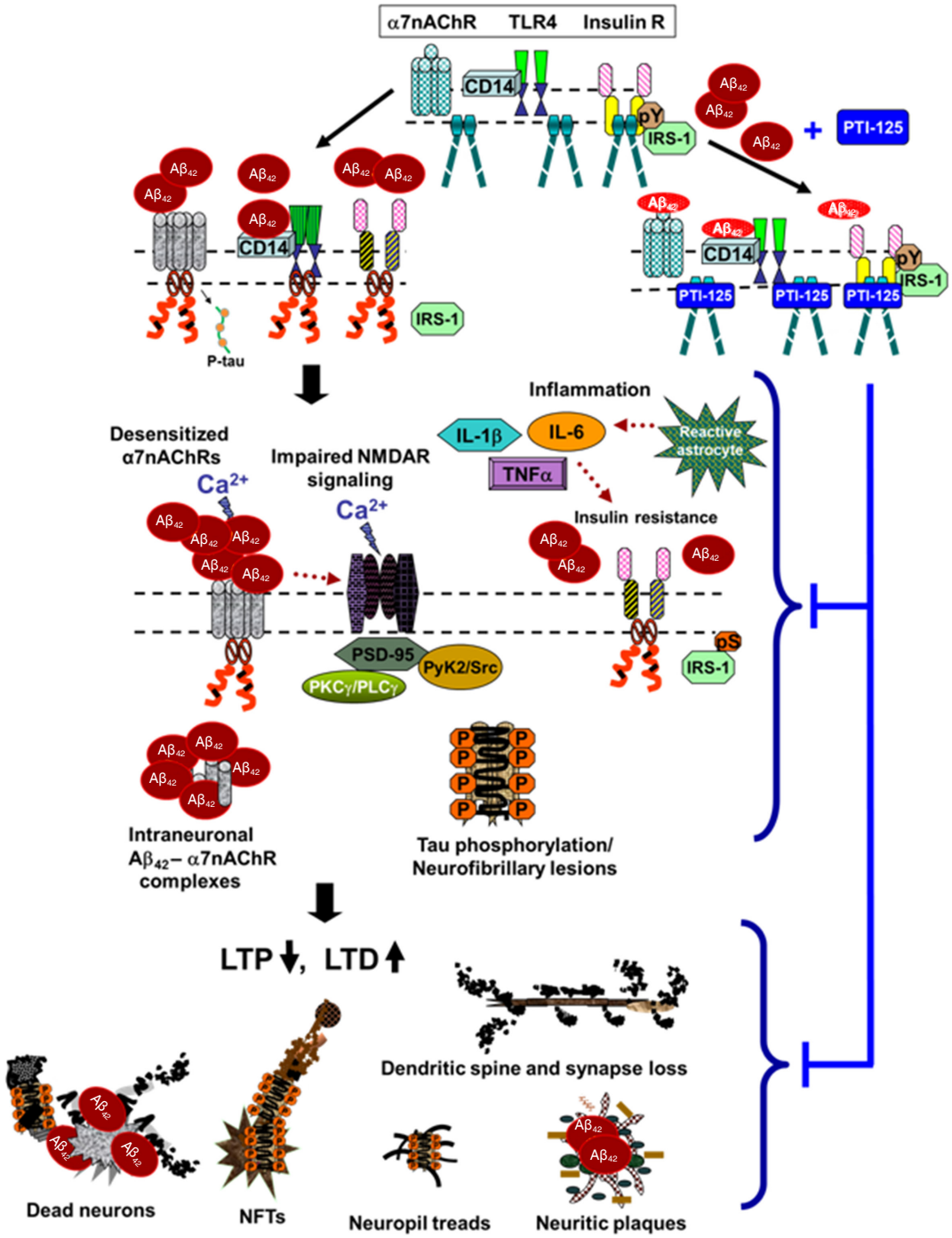

Figure 3: Proposed model of pathological consequences of altered FLNA-enabled $A\beta_{42}$ signaling via α7nAChR and TLR4. Soluble $A\beta_{42}$ monomers or small oligomers bind α7nAChR or CD14, complexed with TLR4, inducing recruitment of FLNA to these receptors. Dimers of native FLNA, coupled to insulin receptors but not to α7nAChR or TLR4, are depicted as straight rods; red curly FLNA depicts the altered form, which is recruited to α7nAChR and TLR4 (and possibly also insulin receptors). Enabled by altered FLNA's new linkages, $A\beta_{42}$ activates α7nAChR to hyperphosphorylate tau and persistently activates TLR4 to induce inflammatory cytokine release (TNFα, IL-1β and IL-6) by reactive astrocytes. This neuroinflammation likely contributes to insulin receptor desensitization[57]. Although the insulin receptor is constitutively associated with native FLNA, it is possible that altered FLNA also contributes to the insulin receptor dysfunction in AD. $A\beta_{42}$'s aberrant signaling through α7nAChR impairs function of α7nAChR and of NMDARs, restricting calcium influx through both receptors. Increasing $A\beta_{42}$ piling onto α7nAChR leads to intraneuronal $A\beta_{42}$-α7nAChR complexes. The hyperphosphorylated tau dissociates from microtubules, disrupting microtubule stability, axonal transport and neuronal function. Along with dysfunctional tau, impaired NMDARs reduce LTP and heighten LTD. Dendritic spines and synapses are lost. Neuritic plaques, neuropil treads and neurofibrillary tangles are formed, and neurons degenerate. FLNA: filamin A; Aβ: amyloid beta; α7nAChR: α7 nicotinic acetylcholine receptor; TLR4: toll-like-receptor 4; TNFα: tumor necrosis factor-α; IL: interleukin; AD: Alzheimer's disease; NMDAR: N-methyl-D-aspartate receptor; LTP: long-term potentiation; LTD: long-term depression

First, the alteration in FLNA has not been directly demonstrated. It is implied by a shift in pI for FLNA in AD *vs.* control brain and supported by the differential binding affinities of PTI-125 to FLNA in control *vs.* AD brain. Representing an aggregate pKa for all amino acids in a protein, a shifted pI is assumed to reflect a change in structure. Second, the possibility exists that PTI-125 has additional significant targets besides FLNA's altered conformation that have confounded our interpretation of its effects. We believe this is a remote possibility because: (1) PTI-125 showed no interactions in a lead profiling screen of 68 receptors, channels and transporters; (2) the binding affinity of PTI-125 in AD brain was virtually identical to its binding affinity for FLNA immunopurified from AD brain[13], indicating a lack of other CNS targets; and (3) PTI-125 shows good CNS penetration and more than sufficient brain levels for a femtomolar target.

Both Aβ and tau as AD therapeutic targets have been questioned following multiple clinical trial failures, and this skepticism may extend to altered FLNA as a viable therapeutic target for AD. Furthermore, several partial agonists or positive allosteric modulators to α7nAChR have also failed, challenging the significance of this Aβ signaling pathway. As commonly suggested, potential reasons for these failures include treating too late in disease progression, because neuropathology precedes symptoms by 10-25 years[58,59], and the possible protective effect of amyloid[60]. Additionally, agents that target aggregation of Aβ or tau may be downstream of significant dysfunction, as suggested by the pathways discussed here. Perhaps most importantly, the femtomolar interaction of $A\beta_{42}$ with α7nAChR imposes substantial competition for antibodies or small molecules seeking to compete directly with this interaction.

An additional caution to interpretation of our data may be our use of synthetic, monomeric $A\beta_{42}$ rather than oligomeric Aβ, thought to be the most toxic Aβ species. Though transgenic mice generate both monomeric and oligomeric Aβ, $A\beta_{42}$ *in vitro* forms a mixture of monomers and small oligomers as well, and both signal via α7nAChR[51].

Finally, despite the broad spectrum of beneficial effects demonstrated preclinically by PTI-125's reversal of FLNA's altered conformation, $A\beta_{42}$ signaling via α7nAChR and TLR4 are only two of many pathogenic cascades in AD. A variety of other approaches have demonstrated similar results in mouse models, and the difficulty of clinical translation remains. We believe our strongest data validating FLNA's altered conformation as a novel target for AD drug development are those using postmortem human AD or $A\beta_{42}$-treated control brainwith postmortem interval < 10 h. PTI-125 demonstrated efficacy in postmortem human brain at concentrations as low as 1 pmol/L[13].

CONCLUSION

Abnormal FLNA is intertwined with the Aβ and tau proteopathies in AD. $A\beta_{42}$ induces the altered conformation of FLNA, and altered FLNA enables sustained $A\beta_{42}$ signaling via α7nAChR and TLR4, possibly by securing high affinity binding of Aβ[13,15]. $A\beta_{42}$'s toxic signaling via α7nAChR activates kinases to hyperphosphorylate tau[25-31], leading to its dysfunction and aggregation, and the FLNA-enabled $A\beta_{42}$ activation of TLR4 enhances neuroinflammation[13,15]. Moreover, the altered conformation of FLNA is an important catalyst of Aβ toxicities and tau dysfunction, including neuroinflammation and synaptic dysfunction. The novel therapeutic candidate PTI-125 offers the possibility of dampening these toxic cascades by restoring the native conformation of FLNA. In addition to its novel target, preferentially binding and reversing an altered protein conformation is a novel mechanism of action for any drug candidate. An advantage of its mechanism is that PTI-125 dramatically attenuates Aβ's aberrant activation of both α7nAChR and TLR4 without directly affecting these surface receptors. PTI-125 preserves tau's neuronal function, preserving axonal transport, and reduces Aβ-induced neuroinflammation and synaptic/dendritic damage while maintaining health of the receptors. AD is a disease of multiple dysfunctions. Interconnected with several toxicities of Aβ and tau and implicated in many AD-related neuropathologies, the proteopathy of FLNA represents an entirely new target for AD drug development.

DECLARATIONS

Authors' contributions

Wrote the manuscript: L.H. Burns
Performed all experimental studies on PTI-125 and edited the manuscript: H.Y. Wang

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Conflicts of interest

LHB is an employee of and HYW is a consultant to Pain Therapeutics Inc., who owns all rights to PTI-125

and related technology.

Patient consent
Not applicable.

Ethics approval
Not applicable.

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AUTHOR INSTRUCTIONS

1. Submission Overview

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Manuscript Type	Definition	Abstract	Keywords	Main Text Structure

Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
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Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

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