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Hepatoma Research: the beginning of a new forum

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INTRODUCTION

Liver cancer, primary hepatocellular carcinoma (HCC) or hepatoma has become the third leading cause of death from cancer worldwide.^[1,2] In 2008, the GLOBOCAN reported 746,300 new cases of HCC diagnosed worldwide with 695,900 HCC-related deaths and a 1.07 incidence to mortality ratio making it the third most fatal cancer world-wide with the vast majority (84%) of cases concentrated in the developing countries in Asia and Africa.^[2,3] HCC is a disparate cancer preferentially afflicting the middle to lower socio-economic segment of the world.^[4] The economic cost of HCC is staggering with global expense estimated at \$895.2 billion a year only followed by cardiac (\$753.2 billion) and cerebrovascular disease (\$298.2 billion).

Hepatoma Research (*Hepatoma Res*, ISSN 2394-5079, <http://www.hrjournal.net/>), this new open access online journal, has been created to improve and promote the international exchange of clinical and academic information about HCC. We invite our peers, clinical and research collaborators alike to contribute to this new journal to improve the international exchange of information in real time to meet this global challenge. Our journal will address all aspect of HCC, including cell biology, pathophysiology, genetics, immunology, pharmacology, medical management as well as radiological and surgical interventions.

ETIOLOGY

Currently, HCC predominately (78%) arises from two chronic liver infections: hepatitis B virus (HBV) and hepatitis C virus (HCV). HBV represents the etiologic factor in 50% of world-wide HCC cases and was recognized in 1994 by the WHO/IARC with a relative risk ranging from 5 to 98.^[5,6] Inactive HBV is also an established risk for HCC with a hazard ratio of 4.6. HBV in Asia, especially in China and Korea, has shown a steady decline through HBV immunization programs. Alternatively, the United States and Japan witnessed a rise in HCV acquired from intravenous drug abuse in the 60's and 80's, which was associated with 80-90% of HCC cases in Japan and 40-60% of cases in Italy and the United States with an odds ratio of 1.3-134.^[7-9] After decades of frustration treating HCV introduction of new protease inhibitors are achieving 80-100% viral eradication, which is associated with a decreased the relative risk for the development of HCC.^[10-12] Unfortunately complete virologic response does not eradicate the risk of HCC in established HCV-related cirrhosis.

Unfortunately, the progress in viral hepatitis has not addressed the looming cloud of obesity, nonalcoholic fibrotic liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) on our horizon. In a world of advancing technology, the standard of living including food stores has dramatically increased and subsequently the mean body mass index and incidence of obesity. NAFLD/NASH as an etiologic factor results in excess fatty acids, and hepatocellular steatosis, which elicits fatty acid oxidation and reactive oxidative stress thought to produce epigenetic changes.^[13,14]

CARCINOGENESIS

No matter what the agent viral hepatitis, fatty liver or diabetes the principle risk factor in HCC is the presence of a pre-neoplastic liver.^[15,16] In HBV-related HCC, the presence of serum HBV

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DNA has been shown to be a predictor of HCC development synergistic with inflammation.^[17] Viral DNA replication and hepatitis B core antigen expression are halted in HBV HCC, while 20% of cells persist production of hepatitis B surface antigen triggering an immune responses and the secretion of cytokines tumor necrosis factor- α , interferon and interleukin-2, which can down regulate the accumulation of HBV RNAs.^[18-20]

Development of HCV-related HCC is a multistep process including the up regulation of inflammatory cytokines and induction of oxidative stress from chronic hepatitis, fibrosis, liver regeneration, and cirrhosis.^[21] The intermediate step is represented by dysplastic nodules with the coexistence of epigenetic and genetic changes that develop into HCC. Multiple pro-inflammatory states appear to be synergistic with HCV including: alcohol, HBV and HIV co-infection, diabetes mellitus, older age, African American race, thrombocytopenia, and smoking.^[21-24]

Nonalcoholic fibrotic liver disease and NASH is an entity previously classified with cryptogenic cirrhosis with a high relative risk for HCC. Pro-inflammatory states from fatty acids release cytokines, pro-oncogenic signals and stimulate epigenetic changes even in the absence of cirrhosis.^[18] Subsequently obese type II diabetics are at twice the risk to develop HCC.^[25-27] Alternatively, African Americans are at a lower relative risk for HCC compared to Caucasians based on fat distribution and metabolism. The estimated yearly incidence of HCC development in NASH-cirrhosis (2.6%) is similar to HCV-cirrhosis (4%).^[28]

Genes involved in hepatocarcinogenesis include *p53*, *PIKCA*, and *β -catenin*. In addition, there are two signaling pathways for cellular differentiation that are frequently disrupted: (1) *Wnt- β -catenin* and (2) *Hedgehog*. WNT signaling appears to be associated with a higher incidence of transformation and pre-neoplastic adenomas.^[29]

SURVEILLANCE

The American Association for the Study of Liver Diseases advocates bi-annual ultrasound surveillance for high-risk patients.^[30] Cost-effectiveness is met with two criteria: (1) annual incidence > 1.5% per year and (2) threshold of \$50,000 per quality-adjusted life year (QALY). Several economic analyses confirm Child-Pugh Class A patients increase life expectancy with cost effectiveness of \$26,000 and \$55,000 per QALY. The best data on surveillance comes from a prospective Chinese trial.^[31-35] Surveillance is recommended for all cirrhotics, HBV carriers if they are Africans older than age 20 years, Asians older than 40 years or have a family history of HCC. However, debate on the utility of AFP continues.

Unfortunately ultrasound is highly operator dependent with a variable sensitivity of 30-70%, and most importantly < 20% of patients compliant with biannual exams.^[36-38]

DIAGNOSIS

Hepatocellular carcinoma is diagnosed by contrast-enhanced computerized tomography (CT) or magnetic resonance imaging (MRI). Early arterial phase enhancement is seen in the tumor followed by venous phase dropout. These characteristics carry result in 90% sensitivity and 95% specificity for lesions greater than one centimeter.^[39] In 2013, the American College of Radiology introduced the Liver Imaging Reporting and Data System to standardize the reporting and data collection of CT and MRI for HCC.^[40] In efforts to improve lower cost technology, contrast-enhanced ultrasound was introduced with a 90% sensitivity, 99% specificity and 89% diagnostic accuracy.^[41] The diagnostic accuracy of MRI has been improved by dual contrast agents like Eovist[®], which is a hepatobiliary excretion and vascularization markers used to diagnosis HCC.^[42] Despite these advances in technology, diagnostics are still encumbered by operator variability and inadequate diagnostic resolution in tumors under 2 cm. The deficiencies in diagnostic screening and sensitivity are seen by the mean tumor size of HCC with the state of Louisiana being 6.5 cm well above Milan Criteria.

STAGING AND PROGNOSIS

Multiple staging systems exist for HCC, but the Barcelona Clinic Liver Cancer (BCLC) staging, and prognostic system appear to be the most widely accepted. BCLC incorporates tumor stage, cirrhosis stage, and functional performance status and links stage with a treatment algorithm.^[43-45] Despite multiple valid staging systems, the most attractive system would be the staging of HCC on genomic finger printing directing therapy and resource allocation such as liver transplants.

Very early stage HCC (Stage 0) are tumors < 2 cm have the best prognosis but are hard to identify on imaging. Early stage HCC (Stage A) is solitary lesions or up to three lesions < 3 cm with preserved liver function (Child-Pugh Class A or B) and reasonable functional status (PS 0-2) with. Their 5-year survivals reach 50-75%. Intermediate stage HCC (Stage B) is multi-nodular with preserved liver function (Child-Pugh Class A or B) and good functional status (PS 0), and no cancer-related symptoms or evidence of vascular invasion. Advanced stage HCC (Stage C) demonstrates vascular invasion or extra-hepatic spread with compromise of functional status (PS 1 or 2) due to HCC. Terminal stage HCC (Stage D)

have tumor marked with vascular invasion and extra-hepatic spread with decompensated cirrhosis (Child-Pugh Class C), poor functional status (PS > 2).

TREATMENT OPTIONS

Surgical resection is an excellent option but has a limited utility due to advanced cirrhosis and is employed in < 5% of patients.^[46] Candidates for resection include: (1) Child-Pugh Class A; (2) hepatic venous pressure gradient < 10 mmHg; (3) platelet count > 100,000; (4) future remnant > 25% (non-cirrhotic); and (5) 50% (cirrhotic) resulting in a 70% 5-year survival.^[47,48] The future remnant can be augmented by pre-operative portal vein embolization. Unfortunately, the majority of patients develop either new HCC or recurrent tumor within 5-year exceeding 70% but if the tumor burden remains within Milan they are candidates for salvage transplant.^[49,50]

Liver transplantation is reserved for unresectable or decompensated cirrhotics with HCC within the Milan criteria: (1) One lesion ≤ 5 cm and (2) three lesions ≤ 3 cm could provide a > 70% 5-year survival.^[51] Current organ allocation in the United States is performed utilizing Model for End-stage Liver Disease with HCC receiving exception point varying from 22 to 34 points while patients that exceeding Milan are required to be downstaged by pre-transplant locoregional to reduce dropout and potentially post-operative recurrence.^[52,53] European centers take an alternative approach whereby laparoscopic resection is liberally employed, and those patients with the highest risk for recurrence are sent for the liver transplant. These factors include lymphovascular invasion and nonencapsulated tumors.

Locoregional therapies include: (1) percutaneous ethanol injection; (2) cryotherapy; (3) radiofrequency ablation; (4) microwave therapy; (5) irreversible electroporation (IEP); and (6) yttrium. Percutaneous ethanol is the least expensive and frequently performed in the office with ultrasound. Thermal ablation is more complex but very effective in smaller tumors (2-3 cm): 70-80% and intermediate tumors (3-5 cm): 50%. Radiofrequency ablation, microwave ablation, and IEP all result in thermal injury, tissue necrosis and apoptosis propagation.^[54,55]

Several drug delivery systems have been introduced including ThermoDox[®] and Delcath[®] a percutaneous intrahepatic, hepatic perfusion device. ThermoDox[®] is a liposomal delivery system for doxorubicin triggered by heat delivered by an ablation device.^[56] The Delcath[®] device delivers high doses of chemotherapy to the liver in an isolated circuit under hyperthermic conditions.

Radioembolization or Y-90 is the radiation delivered through microembolization beads. Two versions of Y-90 exist, smaller

beads for end capillary embolization and the larger for arterial embolization both designed to deliver up to 150 Gy of beta radiation.^[57] Both have relative complications related to their size, embolization stasis methods and radiation intensity. Elevated bilirubin and portal vein thrombosis have become relative contraindications using selective and super-selective approaches. Y-90 has a median survival of 17.2 months in Child-Pugh A cirrhotics and 7.7 months in Child-Pugh B cirrhotics.^[58]

Trans-arterial chemoembolization (TACE) is a widely adopted therapy for HCC embolizing tumor's arterial supply with or without doxorubicin. TACE has a survival advantage at 1 year (82% vs. 63%) and 2 years (63% vs. 27%) compared to controls.^[59,60] Increased bilirubin (> 2.5 mg/dL) and portal vein thrombosis are no longer an absolute contraindications utilizing a selective or super-selective approach to tumors.^[61] Drug-eluting beads have been developed to provide stable and prolonged delivery to decrease doxorubicin toxicity resulting in higher rates of complete response.^[62,63] Chemoembolization results in the tumor ischemia and hypoxia, which stimulate angiogenic growth factors including vascular endothelial growth factor (VEGF), which potentially-induce tumor angiogenesis and tumor recurrence.^[64]

Sorafenib is a tyrosine kinase inhibitor that was shown to have a survival benefit over best supportive care in two pivotal studies: (1) sorafenib in patients with advanced HCC and Asian Pacific trials in patients with Child-Pugh Class A cirrhosis, and (2) advanced HCC compatible with Stage C.^[65,66] Sorafenib is currently the primary chemotherapeutic agent for the treatment of unresectable or recurrent HCC. Multiple adjuvant trials are under way to evaluate the synergistic effects of sorafenib post-resection and ablative therapies. Brivanib is an oral selective dual inhibitor of the fibroblast growth factor and the VEGF pathway, which is being evaluated as a second-line therapy for the management of VEGF stimulation.^[67] Other agents under investigation include: erlotinib, bevacizumab, lapatinib, gefitinib and cetuximab.

CONCLUSION

Hepatocellular carcinoma is the third leading cause of cancer mortality world-wide preferentially afflicting lower socioeconomic patients. Dramatic advances have been made to reduce the incidence of HBV and HCV including HBV immunization strategies and the introduction of new direct acting antiviral drugs for the treatment of HCV. With eradication strategies for HBV and HCC, NAFLD and NASH will become the principle etiology for HCC. HCC will become a disease of the obese.

Obesity itself will complicate HCC management particularly surgical interventions including resection and liver transplantation. Concentrated efforts should be placed on early diagnosis. Early diagnosis not only improves patient survival and is far more cost

effective than any late intervention. Several diagnostic strategies lie in the future with the potential identification of circulating tumor cells or identification of a premalignant signature like epigenetic changes. Despite our best efforts to diagnose HCC in earlier stages, this will not be feasible in most. Therapeutic efforts should be redoubled into the development of new drug delivery systems and platforms to improve chemotherapeutic monotherapy or platform assisted surgery with agents such as nanoparticle and liposomal delivery systems.

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Laparoscopic hepatectomy in cirrhotic patients with hepatocellular carcinoma: technical aspects and potential benefits

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INTRODUCTION

Surgical procedures are considered more challenging in cirrhotic patients with hepatocellular carcinoma (HCC) than in non-cirrhotic patients, because of the former's high morbidity and mortality following surgery.^[1] In open liver surgery, the extremely long incision required for mobilization and resection of the liver can result in significant intraoperative blood loss or postoperative intractable ascites, followed by reduced collateral circulation in the abdominal wall and ligaments around the liver. These complications may progress to postoperative hepatic failure in some patients.^[2]

Innovations in technology and surgical skills for hepatectomy have been applied to minimize postoperative complications. Some of these are as follows: (1) sophisticated instruments for liver transection, such as ultrasonic aspirators and high-energy devices; (2) inflow and outflow vascular control, such as inflow occlusion of the portal triad (Pringle maneuver), selective hepatic vascular occlusion, and total hepatic vascular exclusion;^[3] (3) an anterior approach without liver mobilization in order to prevent liver compression and tumor rupture;^[4] and (4) a liver-hanging maneuver to minimize bleeding in the deeper parenchymal plane and to guide the direction of the parenchymal transaction.^[5]

Since the late 1990s, laparoscopic surgery has gained popularity, resulting in a paradigm shift in liver surgery. Laparoscopic hepatectomy is thought to be a less-invasive procedure than open hepatectomy.^[6] The benefits of laparoscopic hepatectomy may be particularly advantageous for reducing intraoperative blood loss and retaining postoperative ascites. In matched-paired comparative studies and a comprehensive meta-analysis, laparoscopic hepatectomy was found to have several perioperative advantages with no differences in oncological outcomes.^[7-11] Recent technological advances and accumulation of surgical experience have gradually expanded the indications for laparoscopic hepatectomy to include treatment for HCC. Laparoscopic hepatectomy has now been performed even in cirrhotic patients with HCC. There are several tips and techniques for safely performing laparoscopic resection on the cirrhotic liver, as described below.

LAPAROSCOPIC LIVER MOBILIZATION

We strongly recommend laterally dissecting the coronary and triangular ligaments, after identifying the supra-hepatic inferior vena cava (IVC) ("medial-to-lateral approach").^[12] Dissection of the cranial ligamentous attachment using the medial-to-lateral approach helps avoid injuries to the IVC and hepatic veins as well as to potential collateral vessels at the lateral edges of the triangular ligament, which can be difficult to control in some cirrhotic patients. Achieving careful ligation of the short hepatic veins with sealing devices and/or clips under a clear vision is essential for liver mobilization. Surgeons need to utilize gravity and retraction effectively to provide a clear view and avoid blind procedures. Moreover, after the surgeon acquires adequate experience with the technique, laparoscopic liver mobilization provides

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better visualization and a more precise procedure than does open surgery.

Liver mobilization itself may result in postoperative refractory ascites, considering the destruction of collateral blood and lymphatic flow.^[13] In laparoscopic surgery, depending on the location of the tumor, liver rotation by gravity and gentle manipulation often enables liver resection without liver mobilization.

LAPAROSCOPIC LIVER TRANSECTION TECHNIQUE (SUPERFICIAL PRE-COAGULATION, SEALING, AND TRANSECTION METHOD)

Liver transection is the most challenging aspect of hepatectomy in terms of bleeding. Pre-coagulation of the superficial parenchyma using a radiofrequency ablation device is useful for controlling intraoperative bleeding.^[14] We introduced an original laparoscopic liver transection technique, the superficial pre-coagulation, sealing, and transection method.^[15] This method consists of four steps: Superficial pre-coagulation from the liver surface using a needle-type electrode with the VIO 300 D soft-coagulation system (ERBE Elektromedizin, Tübingen, Germany); exposure of vessels and bile ducts with an ultrasonic aspirator; sealing of the vessels and bile ducts with energy devices; and transection of the liver parenchyma. In this method, bleeding can be well controlled even during transection, which enables bloodless transection without inflow and outflow vascular occlusion. In our previous report, this method yielded good results for patients with deteriorated liver function.^[15] The other benefit of this method is its eco-friendly nature. All devices used in this method are reusable by autoclave sterilization. This simple, safe, and eco-friendly transection method has the potential to become the standard method of laparoscopic liver transection.

HARNESS TRACTION TECHNIQUE (HARNESS)

We also developed a novel method for controlling the transection plane, which we refer to as the “Harness Traction Technique (HARNESS)”, for safe and precise dissection in pure laparoscopic hepatectomy, especially anatomical resection. The idea for this technique was originally derived from the liver-hanging maneuver.^[5] The characteristics of HARNESS, which are different from those of the open hanging maneuver, includes the creation of a groove all along the transection line and tying tape along the groove. This technique enables maintenance of a precise transection plane and control of the location and direction of the dissection plane freely in the abdominal cavity, similar to a horse being controlled by traction of the harness, which results in minimized bleeding,

movement of the transection point to the appropriate position, and creation of good tension for parenchymal transection at the transection point. The original hanging maneuver can only be applied in right hemihepatectomy and extended posterior sectionectomy; however, HARNESS can be applied to various kinds of laparoscopic hepatectomies, even those without a natural hook for the tape, such as posterior sectionectomy, anterior sectionectomy, and partial hepatectomy.

The described techniques have resulted in good clinical outcomes, as described in our previous reports.^[12,15] These less-invasive and systematic procedures have the potential to prevent postoperative hepatic failure. Keeping these points in mind ensures that laparoscopic hepatectomy becomes a simple and safe procedure, even for cirrhotic patients with HCC. Furthermore, laparoscopic hepatectomy is associated with fewer postoperative adhesions than conventional open hepatectomy. For the cirrhotic liver, which is a well-known precancerous condition requiring multimodal treatment, this benefit could enable any future surgical treatments to be performed much more easily in case of recurrence. For tumors on the liver surface, the procedure also carries a lower risk of peritoneal dissemination than radiofrequency ablation. With regard to both surgical and oncological aspects, these advantages make laparoscopic hepatectomy ideal as a bridging therapy for curative liver transplantation.^[6,16]

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The evolving role of transarterial chemoembolization in the management of hepatocellular carcinoma

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Globally, hepatocellular carcinoma (HCC) is one of the most frequent causes of cancer-related mortality, being third overall in terms of cancer-related mortality, in addition to being the fifth most common cancer in men and seventh most frequent in women.^[1] It represents a complex disease, as it usually develops in a background of cirrhosis, which signifies that in order to properly manage the patient, both issues must be addressed. Based on this, there is a wide range of treatments, depending on the stage and extent of the HCC, as well as on the hepatic function and patient's overall condition. There are curative treatments, including surgical resection and orthotopic liver transplantation for tumors that fall within certain criteria, with liver transplantation having the advantage of addressing both the HCC and the hepatic disease. There are also a variety of locoregional treatments, such as radiofrequency ablation, microwave ablation, irreversible electroporation and transarterial chemoembolization (TACE), which can potentially have a therapeutic role in small lesions (usually < 2 cm), or more frequently play a role in the management of more advanced HCC or in patients with poor functional status, either related to their cirrhosis or to overall comorbidities. The first experience with hepatic arterial chemoembolization was in the early 1980s.^[2] Over the years, TACE has managed to gain a lot of attention as it represents a type of treatment

with limited stress for the patient, which has been shown to offer significant advantages. That is not to say that there are no side effects, as the patients after a treatment can experience abdominal pain, fever, nausea, emesis, hepatic and gallbladder inflammation and possible infection. However, given its efficacy in different stages of the disease, the challenge remains identifying its proper role and place in the continuum of care for these patients.

Transarterial embolization involves the transcatheter delivery of either solid particles or coils (transarterial embolization) or chemoembolization (TACE) or chemoembolization using drug-eluting beads (DEB-TACE) or radioembolization (for example with Yttrium-90 microspheres). A more recent endeavor is the use of targeted radionuclide therapy, which is an elegant step towards increased targeted therapy.^[3] Overall, it is considered that transarterial embolization by itself is not enough, as with minimal extra effort we could offer these patients much more if the chemotherapy is included. In a review of available literature up to October 2013 by a Canadian group, it was concluded that TACE does offer a survival benefit to these patients, and that DEB-TACE (although it may have a slightly better safety profile compared to TACE) is equivalent to standard TACE regarding increased overall survival.^[4]

Despite these encouraging results, there are several questions that need to be taken into consideration, regarding both the validity of our data, as well as the evolution of our practices. Although TACE can be used in other diseases and organs as well, this editorial will mainly comment on its application in the liver. Specifically, we have to understand that the success of the method depends significantly on the technical characteristics, that is whether we are using chemotherapy or DEB or radiation microspheres, ultimately this may affect the end-result. In

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addition, apart from what is being delivered, it is essential that it is delivered correctly, which means that the interventional radiologist has to aim for lobar or sublobar branches of the hepatic artery and not simply deliver the therapeutic regimen to the main hepatic artery, as this will include the liver as a whole and create more side-effects by affecting an already-fragile remaining healthy liver. Significant progress and improvement in the results have been seen in the last decade or so when microcatheters have been increasingly used, thus providing more accuracy in the delivery. Furthermore, although the preferred treatment in patients with cirrhosis and tumors within the Milan criteria is liver transplantation, TACE has been shown to have either a neoadjuvant role of sorts by providing a downstaging or bridging therapy or an adjuvant treatment role (alone or with sorafenib) in the case of HCC recurrence, which is the main problem after liver transplantation.^[5] There is also a significant ongoing discussion regarding the contraindications in the use of TACE, as these are also evolving with the once absolute contraindication of portal vein thrombosis, now being a relative one with proper patient selection.^[6] Finally, when discussing a treatment, it is important to have ways of assessing its efficacy, other than patient overall survival. In the case of TACE, this has led to the use of the assessment of re-treatment with TACE prognostic system in the case of multiple treatments and to the modified Response Evaluation Criteria in Solid Tumors criteria.^[7]

Although the above may mean that there still several unresolved issues regarding the use of TACE in the treatment of HCC, the key fact remains that the technique continues to evolve and as we

understand more about tumor biology, it will be easier to identify those patients that will benefit the most from this treatment.

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Notch signaling in hepatocellular carcinoma: molecular targeting in an advanced disease

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ABSTRACT

The use of alternative therapeutic approaches in advanced carcinogenesis is a growing investigative base. One such cancer, primary liver cancer, is one of the most commonly occurring cancers worldwide and often presents in late stage disease consequently preventing traditional curative modalities. As a result, hepatocellular carcinoma (HCC), representing the majority of primary liver cancer, is the third most common cause of cancer-related deaths globally. Survival rates are linked to stage of presentation as well as concomitant cirrhosis limiting the 5-year survival in these patients to < 20%. Alternative strategies are in dire need as patients in this cohort have limited palliative options. Currently, sorafenib is the only approved systemic therapy; however, it has a limited survival advantage and low efficacy prompting the empirical need for further evaluation. Understanding of cancer therapy has led to an enhanced focus on the Notch pathway as a potential target for advanced HCC. Notch signaling is a critical component of development and cell fate and has been linked to various modalities including liver regeneration and as a key driver in carcinogenesis. In this review, we will provide a review of the current status of the Notch signaling in liver cancer and of Notch as an alternative potential strategy for advanced HCC.

Key words: Hepatocellular carcinoma; liver regeneration; Notch inhibitors; Notch signaling; sorafenib

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a significant health concern representing the sixth most common cancer globally.^[1] Over the past 20 years, HCC has become one of the most frequent occurring tumors worldwide with the incidence in the United States steadily increasing.^[2-5] In addition, coupled with an increase in the incidence, HCC mortality has also increased substantially. Currently, it is the third most common cause of cancer-related deaths throughout the world.

Approximately one-third of patients are amenable to curative therapy through the use of localized radiofrequency ablation or resection.^[6,7] Moderate stage disease indicative of multifocal intrahepatic carcinogenesis has led to alternative approaches such as trans-arterial chemoembolization (TACE). TACE has provided a relatively efficacious avenue for patients in this category.^[7-10] Patients progressing to or presenting as late stage disease have limited treatment options. Approximately, 70% of patients will initially or eventually present at this late stage. Consequently, this leads to a 5-year survival in patients with HCC of < 20%.^[11] In addition, HCC is characteristically coupled with concomitant cirrhosis, further exacerbating disease morbidity and mortality.^[12] Therefore, there is an urgent and critical need to expand alternative and effective approaches to these patients in advanced, nonresectable disease. This need for additional therapy and the evolving understanding of molecular pathways has led to a concentrated focus on therapeutic molecular targeting in many organ-specific cancers as well as HCC.

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Several signaling pathways are of interest due to their specific oncogenic and/or tumor suppressor characteristics. For instance, the ras/raf pathway provides the only current approved therapeutic approach in advanced HCC through the use of sorafenib.^[13,14] Other traditional pathways such as PI3k/Akt/mammalian target of rapamycin,^[15-17] Wnt/ β -catenin,^[18] as well as Notch signaling^[19-21] have been investigated. Further delineation into the manipulation of these pathways is critical for future alternative strategies for HCC. One of these pathways of interest is Notch signaling. As a functionally conserved pathway, it is involved in the regulation of several cellular properties including differentiation, proliferation, homeostasis and survival. First studied in *Drosophila*, Notch was linked to neural development. Future studies were able to identify the homology between species as well as accurately describe the Notch transmembrane receptor and provide evidence in its role as cellular regulator of differentiation, proliferation, and survival.^[22-28] Continued work in the field of Notch signaling would inevitably showcase its role in a myriad of cellular processes centered on the development.

NOTCH SIGNALING

The Notch signaling pathway consists of Notch receptors, ligands, negative and positive modifiers, and transcription factors. In mammals, these efficient modules have several members and the interplay between these molecules is not yet fully understood, but its role in several processes is being teased out including regulation of metabolism, inflammation, liver regeneration and repair.^[29] Notch signaling is important from other conserved signaling pathways because its role in the mechanism of signal transduction is crucial. Compare to other intercellular signaling pathways such as Wnt, Hedgehog, and transforming growth factor- β , Notch is distinctive in several traits. First, the signaling of Notch is unique. It is comprised of both canonical and noncanonical signaling. The traditional canonical pathway occurs in a juxtacrine process that is unique to Notch. Cell-to-cell interaction is required for subsequent signaling. A transmitter cell releases one of the five major Notch ligands (Jagged 1, 2, and Delta-like 1, 3, 4) and binds to one of the four transmembrane Notch receptors (Notch 1-4) on the associate cell. Signaling is through several cleavage steps. The Notch receptor is cleaved by furin-like convertases in the trans-Golgi network, which results in two subunits of the mature/functional receptor. The extracellular Notch receptor subunit consists of a ligand-binding domain that is composed of epidermal growth factor-like repeats.^[30] In addition, mammalian Notch-1, -2 and -3 receptors contain cytokine response regions and transcriptional activation domains.^[30] Successful binding of the ligand to the representative receptor triggers a

cleavage cascade of the Notch intracellular domain (NICD) via γ -secretase with NICD translocated to the nucleus.^[31] NICD then binds with the RBP-Jk family activating the complex as well as recruiting co-activator MAML1 that initiates transcription of Notch downstream targets including hairy enhancer of split, hairy enhancer of split with YRPW motif families, p21, and Sox-9.^[32-36] Deactivation of the Notch signal is rapidly induced by phosphorylation and degradation. NICD is phosphorylated within the PEST domain by the CDK8 kinase and targeted for proteasomal degradation by E3 ubiquitin ligases that include Sel10/Fbw7.^[37,38] Transcription activation of the ternary complex is disassembled and reset for the next round of signaling. With no second messenger to amplify its signal, deactivation is acute and tightly regulated.

Noncanonical Notch signaling involves a multiple of parallel pathways as cross-talk between these pathways dominates the influence of Notch through paracrine regulation.^[31,39] One particular, well documented, example is the signaling of Notch and the Wnt/ β -catenin pathways. Both pathways can act in synergistic concert through traditional Notch signaling or opposing interactions.^[40] Conversely, antagonistic signaling is through noncanonical effects. A second crucial aspect particular to Notch signaling is the counteracting effects. Notch signals involve either the promotion or suppression of cell proliferation, cell death, and activation of differentiation programs. This happens in cells throughout development of the organism and during the maintenance of self-renewing adult tissues. Therefore, gain or loss of Notch signaling mechanisms has been directly linked to multiple human disorders. Even more conflicting in nature, opposing actions of Notch has been linked to similar disease processes in the liver.^[41,42]

NOTCH SIGNALING IN THE LIVER

The role of Notch signaling in the liver remained relatively unknown until the discovery and investigation of Alagille syndrome (AGS). As an autosomal dominant disease, AGS is characterized by ductopenia and cholestasis. Diminished development of intrahepatic bile ducts is the hallmark of AGS. Through genetic testing, near the turn of the century, it was demonstrated that mutations in the *Jagged 1* gene and to a lesser extent Notch-2 led to AGS.^[43-46] Therefore, it evidenced the role of Notch signaling in hepatogenesis, more specifically hepatic duct morphogenesis. Further research into the role of Notch in liver development focused on liver regeneration following injury. During injury and subsequent liver regeneration (i.e., partial liver resection) hepatocyte and cholangiocyte proliferative properties are often inhibited; therefore, precursor hepatic progenitor cells (HPCs) are activated in response to massive liver injury.^[47-49] HPCs

can differentiate or give rise to hepatocytes as well as cholangiocytes through Notch activation thus further strengthening the role of Notch in hepatogenesis and morphogenesis.^[50-52] During repair and regeneration and at the height of concentrated HPC involvement, several pathways are activated to assist in morphogenesis including the Notch pathway. In addition, activation of the Notch pathway (specifically Notch-1 and -2 isoforms) is in concert with parallel pathways during regeneration.^[40,53,54] These critical and emerging studies linking Notch activation to intrahepatic morphogenesis was a fundamental building block to the transition to reviewing the role of Notch in carcinogenesis.

NOTCH IN CARCINOGENESIS

As the expanding role of Notch signaling in the development of organogenesis continued, the role of Notch in carcinogenesis was ongoing. Notch-1 identification as an oncogene was first discovered through investigation into T-cell acute lymphoblastic leukemia (T-ALL). T-ALL gain of function mutations in Notch-1 led to overexpression and constitutive activation of Notch-1 receptor and thus enhanced proliferation.^[55] In addition, the oncogenic ability of Notch was also exhibited in colorectal cancer.^[56] According to Ambros, most Notch-mediated processes require a transient pulse of activity that in some cases lasts only as long as a fraction of the cell cycle degradation of the NICD.^[57] This is of particular interest as Notch transduction has a 1:1 ratio of input to output without the presence of second messengers. Therefore, constitutive activation will provide constant transduction and thus aberrant proliferation. In addition to mutations in Notch-1 signaling leading to oncogenic enhancement, alterations in Notch-1 signaling also has led to changes in angiogenesis.^[58]

The enhanced discoveries of the Notch signaling pathway lead to further advancement in additional solid tumors. Robinson *et al.*^[59] studied the overexpression of Notch-1 and -2 fusion proteins in benign breast epithelial cells. Subsequent constitutive expression resulted in altered growth characteristics while the inhibition of Notch signaling reduced the growth of the *Notch* gene fusion-expressing breast cancer xenografts. Zender *et al.*^[60] showed that overexpression of the Notch signaling pathway modulates the dysregulation of the oncogene cyclin E, resulting in the development of cholangiocellular carcinoma. In addition, inhibition of Notch activity blocks tumor cell proliferation and induces apoptosis in cholangiocellular carcinoma.^[60] A retrospective analysis in oral squamous cell carcinoma (OSCC) showed that the Notch pathway was defective in 66% of patients and the studies of mechanism

showed that the functional Notch-1 signaling inhibited proliferation of OSCC cell lines.^[61]

Notch-1 dysregulation is not the only isoform involved in carcinogenesis. Studies have demonstrated that up regulation of the Notch-3 isoform was required for induction of p21 expression in senescent cells.^[62] Inactivation of Notch-3 by γ -secretase inhibitor (GSI) or short interference RNA (siRNA) decreased cell proliferation and induced apoptosis in the chemoresistant ovarian cancer cells.^[63] Finally, Lu *et al.*^[64] have showed that the Notch-3 was positively correlated with Jagged-1 at the mRNA and protein levels. Therefore, they concluded that Notch-3 and Jagged-1 may play an important role in the initiation and proliferation of human nonfunctioning adenomas.^[64]

Despite these early indications of Notch as a potential target for cancer therapy, the reality of Notch signaling in carcinogenesis remains opaque. Although there is growing evidence as to Notch acting as an oncogenic process, other cancers exhibit Notch's role as a tumor suppressor in nature. For instance, in neuroendocrine tumors, Notch-1 acts as a tumor suppressor with overexpression leading to a reduction in cellular proliferation and growth.^[26,65-67] Furthermore, despite thorough studies involving either the activation or inhibition of the Notch pathway in the modulation of carcinogenesis, there is limited data surrounding the expression of Notch receptors and their link with cancer. Additionally, noncanonical pathway activation of Notch further confuses and complicates the underlying roles of this pathway during times of aberrant cellular growth.

TARGETING NOTCH IN HEPATOCELLULAR CARCINOMA

Despite promising results of Notch mediation in multiple organ-specific cancers, there is limited and conflicting data on Notch signaling in HCC. In several studies, Notch-1 acts as a tumor suppressor.^[41,68] On the other hand, there is evidence that Notch is oncogenic in nature.^[69-71] As time passes, growing evidence may indicate that although individual HCC signatures may include Notch as a tumor suppressor, the majority of HCC Notch mediation is through overexpression and oncogenic activation. For instance, Villanueva *et al.*^[72] revealed that the conditional expression of NICD1 in a mouse model led to HCC in all test subjects within the 1st year. Biopsied tumors represented varying stages in the mice test group. Moreover, overexpression of Notch-1 was closely linked to insulin-like growth factor 2 and Sox-9 expression levels and interestingly, the NICD1 conditionally active mice genetic signature was evidenced in a subset of human patients with HCC.^[72] Further studies have shifted the tide toward Notch acting as an oncogene in liver carcinogenesis. Sox-9, a downstream target of Notch signaling cascade, is linked to cellular proliferation

and carries a worse prognosis. Overexpression of Sox-9 leads to a transition to HPC-type activity and consequently less differentiated cell types.^[73]

As we advance our understanding of the Notch signaling pathway, initial studies including several early phase I clinical trials are underway in various stepwise components within the pathway. Given the intricacy of the canonical Notch pathway, it comes as no surprise that there are multiple avenues to target Notch signaling [Figure 1]. Targeting either the ligands and/or the receptors, inhibiting cleavage of the active NICD, and preventing transcription of downstream targets are the major targeted aims in Notch mediation. In this review, we will briefly discuss the most common techniques aimed at inhibiting Notch signaling.

The most studied area is inhibition of GSI and the subsequent release of NICD. In fact, GSI examination is not inclusive to HCC. Rather, the extensive research of Notch signaling in neural development has led to GSI application for Alzheimer's disease.^[74] There has been countless preclinical and phase I clinical trials examining the efficacy and effectiveness of GSI mediation in many cancer types.^[75-77] Unfortunately, to date, there is no phase I evidence of the role of GSIs in

HCC. Moreover, given the pan-inhibition nature of GSIs, the toxicity profiles are relatively disconcerting given the off target effects, especially intestinal adverse effects specifically through down regulation of Notch-1 and -2 isoforms.^[29]

As a result of the nonspecific inhibition of GSIs, alternative strategies should be considered. One particular area of increasing interest is the use of monoclonal antibodies and decoys at both the ligand and receptor sites. Antibody and decoy (competitive antagonist) application has a more specific efficacy, thus limiting the dose-escalated toxicities and potentially providing a concentrated result. There are multiple monoclonal antibodies currently being tested in preclinical studies. Notch-1 receptor antibodies have shown promising results.^[78,79] Both Notch-2 and Notch-3 antibodies have transitioned to phase I clinical trials.^[79,80] In addition to Notch receptor blockade, antibodies against Notch signaling ligands have been investigated. Delta-like ligand four antibodies have shown interesting results from multiple avenues.^[58,81,82] Similarly, decoys provide excellent Notch inhibition and act as a competitive antagonist either at the Notch receptor or the ligand binding sites. Notch-1 decoys have been studied as well as Jagged-1 ligand decoys.^[83,84] Finally, prevention of NICD-mediated transcription is a novel process to modulate carcinogenesis. Peptides that block the transcription of NICD provide interesting applications to Notch signal inhibition.^[75] In addition to this review of potential Notch mediation through the alteration of multiple events, Espinoza and Miele^[75] recently compiled a comprehensive table and analysis including a majority of current preclinical and clinical studies using a myriad of Notch inhibitors that further details the current effectiveness of Notch alteration.

Traditional mechanisms of Notch inhibition have and will continue to be thoroughly investigated; however, there is growing interest in targeted gene inhibition, more specifically in the context of Notch signaling. Historically, AGS and the mechanism of hepatogenesis were further delineated through *Notch-2* gene manipulation; therefore, an approach to targeting specific genes within the Notch pathway may provide additional support in lieu of traditional Notch inhibitors. There are several approaches to gene silencing, two of which are frequently used and include using a small hairpin RNA or short hairpin RNA (shRNA) or siRNA. Mao *et al.*^[85] reported that the shRNA mediated knock-down of Notch-1 inhibited the breast cancer cell line MCF-7's proliferation and induced cell apoptosis through multiple mechanistic actions. One in particular, the down regulation of the anti-apoptotic protein nuclear factor-kappa B, proved effective and enhanced the anti-tumorigenic effect when combined with traditional chemotherapeutic agents such

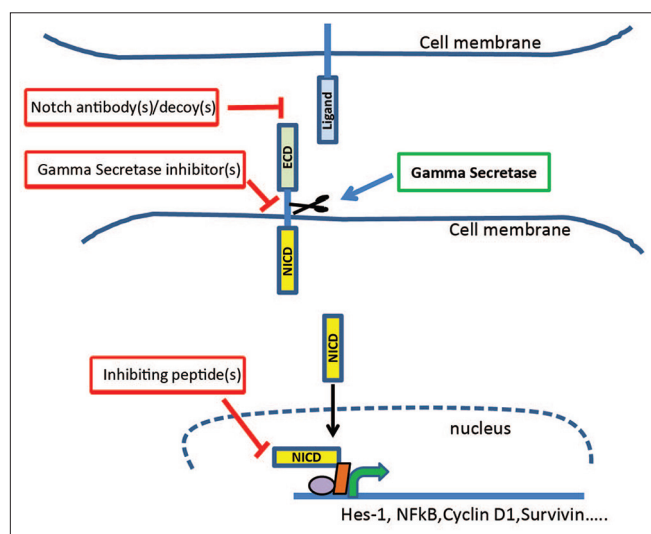


Figure 1: Canonical Notch signaling pathway and potential sites of inhibition. The Notch pathway is primed through cell-to-cell interaction distinguishing it from other regulatory pathways. Following ligand secretion from a transmitting cell, the ligand binds to one of the four Notch receptors on the receiver cell. Ligand-receptor binding facilitates the cleavage of the intracellular component of the transmembrane receptor via γ -secretase. Successful cleavage activates the Notch intracellular component domain (NICD) which translocates to the nucleus where is regulated a host of transcription factors. Given the intricacy of the pathway, there are multiple key regulatory steps poised for targeted therapy. First, monoclonal antibody and decoy administration at both the Notch ligand and receptor is in preliminary, preclinical investigation. Second, γ -secretase inhibitors are the most studied target within the pathway; however, there is limited data within hepatocellular carcinoma. Finally, inactivating transcriptional peptides are a novel trend focused on inhibiting the canonical transcription mediated by NICD

as paclitaxel. Finally, Mao *et al.*^[85] were able to translate this to an *in vivo* study evidencing that genetic knockout of Notch-1 abrogated tumor xenograft growth. Zhao *et al.*^[86] reported that the knockdown of Notch-1 by RNA interference suppressed Akt activation, reduced glioma cell growth rate and induce cell apoptosis.

Notch-1 deletion has also been studied in HCC. Sun and colleagues investigated that knockout of Notch-1 inhibited cell proliferation and significantly suppressed tumor formation of LO2/HBx cells in a BALB/c nude mouse model *in vivo* through activation of apoptotic caspase cascades. In addition, they observed that this blockade arrest the cell cycle in the G0/G1 phase through the down regulation of cyclin D1, CDK4, E2F1 and the up regulation of p21.^[87] Wang *et al.*^[88] suggested that the inhibition of Notch1 by shRNA significantly suppressed the growth of HBx transformed human hepatic cells through G0/G1 cell cycle arrest and apoptosis. The mechanism, they suggested, may be linked to the promoted expression of P16 and decreased expression of Bcl-2.^[88]

Finally, the investigation into microRNAs as a potential strategy is growing in interest. MicroRNAs are small regulators of both post-translational and post-transcriptional markers. They are often at the center of abrogation in many cancer types.^[89,90] Given their stability, they are potential candidates for use in combination studies. For instance, there is increasing data on the use of microRNAs sensitizing HCC to traditional chemotherapy.^[91,92] In addition, genetic profiling of microRNAs in patients with HCC will assist as an alternative and supportive strategy in terms of disease-free progression and overall survival. For example, microRNA-224 expression is associated with a better prognosis and further evaluation into this subtype is currently ongoing.^[93,94]

Despite the plethora of early investigations into Notch inhibition, there are concerns that need to be addressed moving forward. First, there are relatively limited studies advancing in HCC research. As of this publication, there are no clinical trials utilizing Notch inhibition as an alternative strategy for HCC. In addition, in other solid tumor studies, no trial has advanced past phase I. Perhaps we are in the early stages of development, but given the stagnant advancement additional approaches should be addressed.

TRANSITION TO THE FUTURE

The exciting and staggering concept of Notch signaling is that it is still in the infantile stages of development. The majority of evolution in the understanding of this pathway has come within the last 20-30 years. Within that time, there have been

novel and potentially ground-breaking investigations into the role of Notch not only in HCC, but other cancer types. In addition, the study of Notch mediation has radiated toward different fields of medicine with the intent of delineating the roles of isoform-specific NICD. Additionally, the role of cellular homeostasis has interpretive results in a myriad of clinical and basic science indications and perhaps Notch will be at the forefront of these studies. However, the role of Notch in carcinogenesis, albeit, counterintuitive, is both exciting and complex. The early results prove modulation of this pathway could aid in the care of advanced, resistant, and aggressive cancer types. These Notch-based strategies will continue to be evaluated and will also be combined with other pathway mitigation to reduce toxicity profiles, as well as the chemoresistance. Combination with approved and current strategies will further the understanding and commitment to providing alternative and efficacious treatment options to patients with HCC.

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Combined radiofrequency and chemoembolization vs. chemoembolization in management of hepatocellular carcinoma

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ABSTRACT

Aim: Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. If left untreated, liver cancer has a poor prognosis with more than 90% of patients dying of the disease within 5 years of diagnosis. The aim of this study is to assess the value of combined radiofrequency ablation (RFA), followed by trans-arterial chemoembolization (TACE) in the management of HCC. **Methods:** Fifty HCC patients with chronic liver disease were categorized into two groups according to the modality of locoregional treatment: 25 HCC patients treated with RFA followed by TACE within 5 days and 25 HCC patients treated with TACE only. **Results:** Complete response was achieved in 100% and 84% of the HCC patients after 1 month from combined RFA-TACE therapy and TACE only respectively. The rate of objective response after 7 months was 84% and 44% in the RFA-TACE and TACE groups respectively. One year disease free survival rate was 56% and 24% in RFA-TACE and TACE groups respectively, and overall survival rate was 88% in the RFA-TACE group and 80% in the TACE only group. **Conclusion:** Combined RFA-TACE appears to be an effective modality and superior to TACE only for the treatment of HCC.

Key words: Hepatocellular carcinoma; radiofrequency; trans-arterial chemoembolization

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with over 600,000 new diagnoses/year.^[1] In Egypt, the annual incidence of HCC showed a significant rising trend from 4.0% in 1993 to 7.2% in 2002.^[2] Radiofrequency ablation (RFA) is a locoregional modality for tumor ablation which often allows for greater preservation

of unaffected hepatic parenchyma.^[3] The greatest success rate was achieved in non-infiltrating tumors of sizes 3-5 cm.^[4] Trans-arterial chemoembolization (TACE) is an intra-arterial infusion of chemotherapy, which blocks (embolizes) small blood vessels, depriving the tumor of its needed blood supply. However, this procedure is not curative.^[5] The combination of RFA and TACE induces coagulative necrosis in large areas without any possibility of revascularization.^[6]

The aim of this study was to assess the efficacy of combined RFA followed by chemoembolization in the management of HCC.

METHODS

This cross-sectional randomized controlled prospective study was performed at the HCC Clinic at Ain Shams University.

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Ethical consideration

Ethical approval was obtained from the Local Ethical Committee of the Department of Tropical Medicine, Ain Shams University. Written informed consent was obtained from all participants. The right to refuse participation was emphasized.

Patients

The study included 50 cases of diagnosed HCC on top of chronic liver disease divided into two groups according to treatment modality. Group 1: 25 HCC patients underwent TACE only as a control group with 10 (40%) patients with single tumor sizes of 5 cm and 15 (60%) patients with single tumor sizes of 5-7 cm. Group 2: 25 HCC patients underwent RFA followed by TACE (RFA-TACE) within 5 days with 14 (56%) patients with single tumor sizes of 5 cm and 11 (44%) patients with single tumor sizes of 5-7 cm.

All patients fit clinical, biochemical, radiological (ultrasound) criteria of chronic liver disease and had positive hepatitis C virus (HCV) Ab and/or HbsAg. HCC was diagnosed by evidence of high α fetoprotein (AFP) (> 200 ng/mL) and/or presentation of HCC characteristics by triphasic spiral abdominal computed tomography (CT), according to American Association for the Study of Liver Diseases guidelines.^[7]

Inclusion criteria

We included patients with Child-Pugh class A or B presentations, prothrombin concentrations of $> 50\%$, platelet counts of $> 50,000$ per mm^3 , and ultrasound detection of the lesion to be allowed for percutaneous loco-regional ablation therapy.

Exclusion criteria

Patients with Child-Pugh class C presentations tumors in inaccessible sites or close to vital structures, malignant main portal vein involvement and/or extrahepatic metastases. All patients were categorized according to the Barcelona-Clinic liver cancer (BCLC) Staging System of HCC pre- and post-treatment to evaluate the general condition, performance status of liver functionality, Child-Pugh scores and Okuda stage.^[8-11]

Procedures

All of the enrolled patients were subjected to tests of: complete blood count (CBC), alanine transaminase, aspartate transaminase, serum albumin, serum bilirubin, prothrombin time and international normalized ratio (INR), hepatitis markers (HCV Ab, HbsAg and HBcAb), AFP, chest X-ray, abdominal ultrasonography and abdominal triphasic spiral CT.

In post-treatment follow-up studies, all patients were subjected to the following evaluation measures 1-month

afterwards and then every 3 months following the procedure: full clinical examination, CBC, prothrombin concentration, liver function tests, kidney function, AFP and spiral CT of abdomen. Classification of response was conducted according to the European Association for the Study of Liver amendments that take into account the reduction in viable tumor volume due to TACE-induced necrosis as follows. Complete response (CR): complete disappearance of all known disease and no new lesions; partial response (PR): at least 50% reduction in total tumor load of all measurable lesions; progressive disease (PD): at least 25% increase in size of one or more measurable lesions or the appearance of new lesions; stable disease (SD): does not qualify for CR/PR or PD.^[7]

Radiofrequency ablation was performed percutaneously under ultrasound guidance after general anesthesia. RITA (Mountain View, California, USA) and Boston Scientific (Natick, Massachusetts, USA) expandable-type electrodes were used. These electrodes had multiple thin curved monopolar electrodes extending from the central cannula (18-14 gauge). Radiofrequency emanates from each of these hooks, resulting in increased coagulation. Also Valley Lab (Boulder, Colorado, USA) cooled-tip electrodes (17 gauge) were used. These electrodes have two hollow lumens that permit continuous internal cooling of the tip. Using percutaneous endovascular techniques, TACE was performed by selective catheterization of the hepatic segmental arteries nourishing the lesions, using either 5-F catheters (Simmons 1 and Cobra; Mallinckrodt, St. Louis, USA or Hydrophilic Simmons 1 and Cobra; Terumo, Tokyo, Japan) or 3-F coaxial microcatheters (Tracker 18; Vascular Access System, Target, St. José, USA; SP Catheter; Terumo). TACE was used to deliver potent anticancer drugs directly into tumor-feeding arteries. As a result, tumors were exposed to very high drug concentrations, while systemic exposure was minimized. The cytotoxic lipiodol mixture was prepared by mixing 100 mg adriablastin powder with 10 mL of saline, water soluble contrast and 10 mL of oily contrast (Lipiodol Ultra-Fluid; Jubilant HollisterStier General Partnership 16751 Trans-Canada Highway, Kirkland, Quebec, Canada) to ensure a homogenous mixture. Embolization was done by mixing small pieces of gel foam particles and water soluble contrast.

Statistical analysis

The collected data were statistically analyzed using the program SPSS (Statistical Package for Social Sciences, software version 18.0, Echosoftware Corporation, USA). Data were expressed as mean \pm standard deviation for quantitative parametric measures in addition to median percentiles for quantitative non-parametric measures and both number and percentage for categorized data. The following tests were

used: Student's *t*-test, Wilcoxon rank sum test, Chi-square, and Kaplan-Meier.

RESULTS

Both groups were matched with regards to age and sex. Comparison between both groups regarding the different scoring systems shows no statistically significant difference ($P > 0.05$). There were 23 (92%) Child-Pugh class A and 2 (8%) class B patients in the TACE only group vs. 24 (96%) and 1 (4%) in the RFA-TACE group. Performance status grades of 0 were present in 18 (72%) patients and 7 (28%) with grade 1 in TACE vs. 14 (56%) and 11 (44%) in RFA-TACE. BCLC stage B was found in 18 (72%) and 7 (28%) in stage C TACE vs. 14 (56%) and 11 (44%) in RFA-TACE respectively [Table 1]. Table 2 shows the response to the treatment after 1 month, with all patients underwent RFA-TACE achieved CR, but with no statistically significant difference between both groups ($P > 0.05$). After 7 months of treatment, the rate of the objective response (which includes both CR and PR for at least 6 months) was higher in RFA-TACE than that of TACE alone ($P < 0.01$). It was noted that lesions of more than 5 cm were more liable to PR and PD after chemoembolization alone. Performance status was improved in the RFA-TACE groups, shifting from a grade of 1-0 ($P < 0.01$). As summarized in Table 3, 1-year total recurrence rates and local tumor progression rates were higher in patients that underwent chemoembolization alone ($P < 0.01$). In Table 4, 1-year disease free survival rates and overall survival rates were higher after the combined therapy ($P < 0.001$). Figure 1 shows the median survival time for the two studied groups, which was 13 months with no statistically significant difference ($P > 0.05$). Comparison between both groups with regards to survival rates at 7 months and at 1 year is shown in Figure 2. No major complication was reported after combined therapy or after TACE only; only post-embolization syndrome was reported as a minor complication in 68% and 72% of the patients in RFA-TACE and TACE groups, respectively.

DISCUSSION

Hepatocellular carcinoma is the fifth most common cancer worldwide and the third leading cause of cancer-related mortality,^[12] with its incidence increasing worldwide ranging between 3% and 9% annually.^[13] The European Association for the Study of Liver and the American Association for the Study of Liver Diseases recommends RFA as a non-surgical technique for the treatment of early stage HCC (Child-Pugh class A or B, solitary HCCs or up to 3 nodules with each ≤ 3 cm in size).^[7] TACE has become the treatment of choice

Table 1: Scoring systems before treatment

Variable	n = 25 (n (%))		P
	TACE	RFA-TACE	
Child-Pugh class			
A	23 (92.0)	24 (96.0)	> 0.05
B	2 (8.0)	1 (4.0)	
Okuda stage			
I	24 (96.0)	25 (100)	> 0.05
II	1 (4.0)	0 (0)	
PST			
0	18 (72.0)	14 (56.0)	> 0.05
1	7 (28.0)	11 (44.0)	
BCLC			
B	18 (72.0)	14 (56.0)	> 0.05
C	7 (28.0)	11 (44.0)	

RFA: radiofrequency ablation; TACE: trans-arterial chemoembolization; PST: performance status test; BCLC: Barcelona-Clinic liver cancer

Table 2: Treatment response in the studied groups

	n = 25 (n (%))		P
	TACE	RFA-TACE	
After 1 month			
Complete response	21 (84)	25 (100)	> 0.05
Partial response	2 (8)	0 (0)	
Progressive disease	2 (8.0)	0 (0)	
After 7 months			
Objective response	11 (44)	21 (84)	< 0.01

RFA: radiofrequency ablation; TACE: trans-arterial chemoembolization

Table 3: Overall recurrence rates at 13 months following the procedure

	n = 25 (n (%))		P
	TACE	RFA-TACE	
Total recurrence of HCC (same lesion and/or new lesion)	18 (72)	9 (36)	< 0.05
Recurrence of HCC in same lesion only	14 (56)	4 (16)	< 0.01

RFA: radiofrequency ablation; TACE: trans-arterial chemoembolization; HCC: hepatocellular carcinoma

Table 4: The disease free survival rate and the survival rate in the studied groups

	n = 25 (n (%))		P
	TACE	RFA-TACE	
Disease free survival rate at 1 year	6 (24)	14 (56)	< 0.001
Overall survival rate at 1 year	20 (80)	22 (88)	> 0.05

RFA: radiofrequency ablation; TACE: trans-arterial chemoembolization

for multinodular HCC,^[14] and for large HCCs in patients who are not surgical candidates.^[15] Another promising role of RFA is to be combined with TACE for the treatment of intermediate and large tumors,^[16] so as to obtain a large area of coagulation.^[17] The combination of TACE with RFA has two theoretical merits: (1) occlusion of hepatic arterial flow by means of embolization may contribute to the decrease in the heat-sink effects during RFA and increase the ablation volume by RFA; and (2) combined treatment may have the

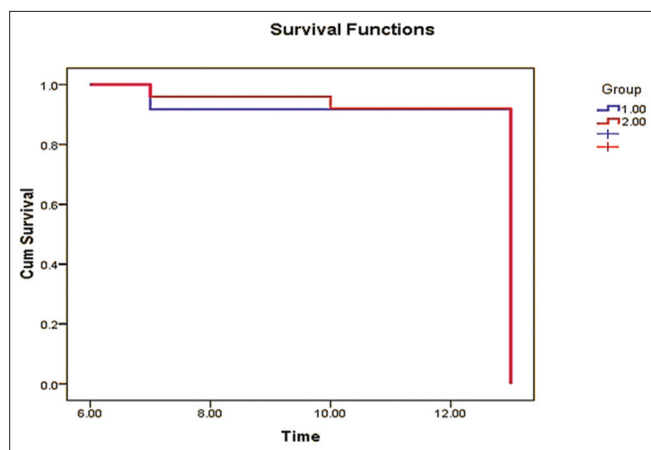


Figure 1: Kaplan-Meier curve showing the median survival time for the two groups. Kaplan-Meier curve showed the median survival time for the two groups, which were 13 months and there was no statistically significant difference

effect of anticancer agents on cancer cells, which is enhanced by the hyperthermia.^[18] In this work we evaluated the efficacy of combined RFA followed by TACE (RFA-TACE) in single HCCs (5-7 cm) in comparison to TACE only. The study results showed CR after 1 month in 100% and 84% of patients in combined RFA-TACE and TACE alone groups respectively. This is in agreement with other groups of researchers who reported that CR was achieved in 79.9% and 92.5% of HCC patients after combined (TACE-RFA) and RFA therapy respectively.^[19,20] In this work, both medium HCCs (5 cm) and large HCCs (> 5 cm) achieved CR in 100% of patients in the RFA-TACE group, whereas Wang *et al.*^[21] showed that CR was achieved in 57.6% of medium sized HCCs and in 6% of large sized HCCs after (TACE-RFA) therapy. Another study documented that CR was achieved in 40% of patients with large sized HCC after (TACE-RFA) therapy.^[22] In our study, the rate of objective responses after 7 months (CR and/or PR for at least 6 months) were 84% and 44% in RFA-TACE and TACE groups respectively, while Cheng *et al.*^[23] reported that the rate of objective response after 6 months was 54% and 35% in TACE-RFA and TACE group respectively. In the current work, there was improvement in the performance status of many patients following RFA-TACE therapy as 44% of patients shifted from a PST score of 1-0 after 1 month of the combined therapy while 4% of patients were shifted from a PST of 0-1 after TACE only. Sacco *et al.*^[24] revealed that there was worsening of clinical status in patients after TACE only. These results ensured that combined therapy had a positive impact on the performance status of HCC patients, also improved their survival and disease free survival rate and shifted them from BCLC stage C, where patients were restricted to certain supportive medical treatments, to stage B, where other different modalities could be implemented for HCC patients and with a good prognosis. In this study, local tumor progression rate at 1 year was 16% after RFA-TACE

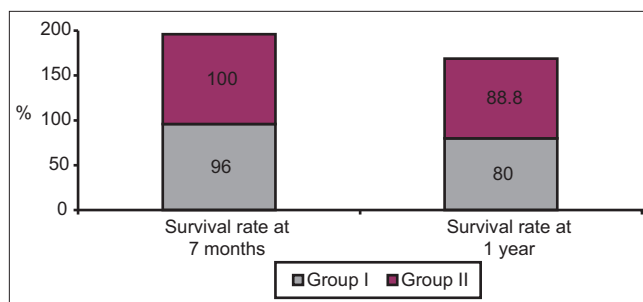


Figure 2: Survival rates at 7 months and 1 year. Survival rate at 7 months was 100% in radiofrequency ablation (RFA) + trans-arterial chemoembolization (TACE) group and 96% in TACE group. One year survival rate was 88.8% in RFA + TACE group and 80% in TACE group with statistically significant difference. RFA: radiofrequency ablation; TACE: trans-arterial chemoembolization

therapy. This result is comparable to Takaki *et al.*^[22] and Kim *et al.*^[25] who reported that local tumor progression rate at 1 year was 15% and 9% after TACE-RFA respectively. In the present work, no major complications were reported after combined therapy or after TACE only. Only post-embolization syndrome was reported as a minor complication in 68% and 72% in RFA-TACE and TACE groups respectively. Another study reported that major complications were observed in 2.2% of patients and minor complications were observed in 2.2% of patients post-combined TACE-RFA therapy.^[19] Takaki *et al.*^[22] stated that a minor complication was observed in 3% of patients post-combined TACE-RFA therapy. In the current study, 1-year survival rate was 88% and 80% in RFA-TACE and TACE only respectively, which is comparable with previous studies showing 1-year survival rates at 93%, 98% and 100% in TACE-RFA treated groups.^[19,23,26] The present study found that, 1-year recurrence-free survival rate was 56% and 24% in RFA-TACE and TACE respectively, which is in concordance with other studies reporting 1-year recurrence free survival rates of 74% and 64.5%.^[22,26] Comparative studies have previously described TACE-RFA, a combined technique in which TACE was performed before RFA, and proved that it is much more better than mono-therapy, especially in medium and large sized HCC lesions. Although it is different from the technique discussed in this study in which TACE was done after RFA (RFA-TACE), both have nearly the same results, with RFA-TACE presenting better responses with regards to CR and objective response than TACE-RFA and mono-therapy, especially in management of medium sized HCC. In TACE-RFA, occlusion of hepatic arterial flow by means of embolization may contribute to the decrease in the heat-sink effects during RFA, increase in the ablation volume, and induce coagulation necrosis in large areas without any possibility of revascularization. During RFA, the high rate of local recurrence may be due to residual cancer cells or adjacent microscopic satellite tumor nodules, so TACE could be used as an adjuvant therapy after RFA to eradicate the peripheral viable tissue and micro-metastasis with more concentrated

local chemotherapeutic agents, also enhancing the effects of anticancer agents on cancer cells by the hyperthermia following RFA. So our conclusion is combined RFA and TACE appear to be effective modality and superior to TACE alone for the treatment of HCC

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Role of diabetes mellitus on the recurrence rate of hepatocellular carcinomas after radiofrequency ablation in chronic hepatitis C patients

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ABSTRACT

Aim: The aim was to assess the impact of hyperglycemia on the recurrence of hepatocellular carcinoma (HCC) as well as evaluate survival after curative ablation by radiofrequency. **Methods:** This study, which was conducted retrospectively on 107 chronic hepatitis C (CHC) patients with 159 HCCs, was presented to the Hepatology Unit of Internal Medicine Department at Tanta University Hospitals. All lesions were curatively treated by radiofrequency ablation (RFA) and the surveillance of HCC recurrence was evaluated radiologically every 3 months for periods between 6 and 36 months. Of 107 subjects, 70 were males and 37 were females, with mean age 50.4 ± 9.4 years. All patients were divided according to their glycemic state into the following three groups: Group I, which included 37 type 2 diabetic patients, with adequate maintenance of blood glucose, has 52 HCCs; Group II, which included 25 type 2 diabetic patients with inadequate maintenance of blood glucose, has 43 HCCs; and Group III, which included 45 euglycemic non-diabetic patients, has 64 HCCs. **Results:** Our results showed that, there was significant increase in recurrence rate in diabetic patients with inadequate maintenance of blood glucose (Group II) compared to those in Group I and Group III ($P < 0.0001$). Interestingly, there was no significant difference concerning HCC recurrence between diabetic patients with adequate maintenance of blood glucose (Group I) and non-diabetic euglycemic patients (Group III). Our results also identified that, inadequate maintenance of blood glucose in diabetic patients was also a significant predictor of poor survival. **Conclusion:** Inadequate maintenance of blood glucose in diabetic patients is a significant risk factor for recurrence of HCC and for poor survival after curative RFA therapy in CHC patients.

Key words: Hepatocellular carcinoma; hyperglycemia; radio frequency ablation; recurrence; survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is considered as one of the most common cancers worldwide and its incidence has been increasing in many countries.^[1] It accounts for 80% of primary liver cancers, complicating liver cirrhosis in most

cases and the third most common cause of cancer-related deaths.^[2]

There is considerable geographical variation in the incidence of HCC, which is thought to be related to differences in the prevalence of underlying risk factors, in particular hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.^[3]

Hepatocellular carcinoma surgical resection, liver transplantation, and local ablation therapy, such as radio frequency ablation (RFA) therapy, have been considered as efficient curative therapies for HCC.^[4] Currently, RFA has gained popularity based on the ease of use, safety, reasonable cost and applicability to minimally invasive techniques.^[5]

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RFA therapy is now widely indicated in patients with small HCC^[5] in which the survival rates were similar to surgical resection.^[6]

Hepatocellular carcinoma has a high rate of recurrence after curative resection or local ablation therapy, reaching approximately 80% within 5 years.^[7-9] The recurrence of HCC and patient survival are associated with the number of HCC nodules and their sizes.^[10,11] Hepatic reserve function at the time of HCC therapy is another factor that is associated with the recurrence of HCC and patient survival.^[12] Antiviral therapy targeting HCV^[13,14] or HBV^[15] has been shown to decrease HCC recurrence, and improve hepatic reserve function and survival.

There is a strong association between diabetes mellitus and increased cancer risk in liver, the key organ involved in the metabolic derangements typical of diabetes.^[16] Metabolic factors, such as obesity and diabetes, are closely linked to the etiology of nonalcoholic steatohepatitis, which is also considered a cause of HCC.^[17] If these metabolic factors are related to the recurrence of HCC, therapeutic intervention targeting these factors may lead to prevention of frequent recurrence of HCC and improved patient survival.

The impact of diabetes on the recurrence of HCC, after treatment, has been discussed, but with conflicting results.^[18-21] In our study, the possible impacts of hyperglycemia on the recurrence of HCC in chronic hepatitis C (CHC) patients after curative RFA were analyzed and we found that inadequate maintenance of blood glucose was related to the high rate of HCC recurrence.

METHODS

This study was conducted retrospectively on 107 patients with 159 hepatic focal lesions. The patients were curatively treated by RFA therapy from January 2010 to June 2013. There were 70 males and 37 females with mean age 50.4 ± 9.4 years.

An informed consent was taken from all participants. All the records were confidential. The results of this research were used only for scientific purpose. Any unexpected risks that appeared during the course of the research were cleared with the participants and the clinical committee on time.

We selected the patients for this study according to the following inclusion criteria: maximum diameter of HCC ≤ 4 cm, number of HCC nodules ≤ 3 , no previous history of treatment of HCC and follow-up observation for at least 6 months and up to 36 months after RFA therapy. The

etiological background of liver disease was HCV infection for all patients as other etiology of liver disease were excluded from this study. Exclusion criteria for our study were as following: maximum diameter of HCC > 4 cm, number of HCC nodules > 3 , previous history of treatment of HCC, follow-up observation for less than 6 months after RFA therapy and cirrhotic patient by other than CHC.

All patients were divided according to glycemic state to the following three groups: Group I included 37 controlled type 2 diabetic patients, with adequate maintenance of blood glucose, with 52 HCC nodules; Group II included 25 uncontrolled type 2 diabetic patients with inadequate maintenance of blood glucose, with 43 HCC nodules; and Group III included 45 euglycemic patients with 64 HCC nodules. The Child-Pugh classification grade was either A ($n = 50$) or B ($n = 57$). The number of HCC nodules was 1 in 61 patients, 2 in 40 patients, and 3 in 6 patients. The maximum diameter of HCC nodules was 3.11 ± 0.53 cm.

Inadequate maintenance of blood glucose was defined as an average value of casual blood glucose ≥ 200 mg/dL. The level of hemoglobin A1c (HbA1c) was not used in the present study because the lifespan of erythrocytes is shortened due to hypersplenism in patients with chronic hepatitis or cirrhosis, leading to lower HbA1c levels relative to the blood glucose level.^[22] Diagnosis of type 2 diabetes was made according to the American Diabetes Association criteria of a fasting blood glucose level ≥ 126 mg/dL and/or HbA1c level ≥ 6.5 .^[23] Obesity was defined as a body mass index > 25 kg/m² according to the definition of the Japan Society for the Study of Obesity.^[24]

After initial treatment of HCC by RFA, the ablated area was confirmed by triphasic computed tomography (CT) or dynamic magnetic resonance imaging (MRI) within 1-week. If the ablated area was not sufficient, then RFA therapy was repeated until the HCC nodule was completely ablated.

Diagnosis of HCC was based on abdominal ultrasonography, triphasic CT, dynamic MRI and alpha-fetoprotein (AFP). Classical HCC was diagnosed for tumors showing vascular enhancement with washout on at least two types of diagnostic imaging. Tumor biopsy was used to diagnose tumors with non-classical imaging findings.

For surveillance of HCC recurrence after curative therapy with RFA, patients were evaluated by abdominal ultrasonography, contrast-enhanced triphasic CT, or contrast-enhanced dynamic MRI every 3 months.

Recurrence of HCC was diagnosed based on reappearance of arterial enhancement in the ablated lesions or new focal

lesions detected by ultrasonography showing vascular enhancement with washout on triphasic CT or dynamic MRI. If the tumor was not hypervascular, a tumor biopsy was performed to confirm the diagnosis.

Statistical analysis

All patients' data were tabulated and processed using SPSS 10.0 (SPSS Inc., Chicago, USA). The data were presented by mean and standard deviation and compared using one way analysis of variance test. In addition, the data were presented by frequency and percent and compared using Chi-square test or Fischer's exact test when appropriate. For analysis of survival and recurrence, the time of initial RFA treatment was defined as day 0. Survival rate was analyzed by the Kaplan-Meier method and log rank test. Multivariate analysis was performed using a Cox proportional hazard model. In all tests *P* value was considered significant if < 0.05 .

RESULTS

The clinical and laboratory characteristics of patients undergoing curative RFA for HCC are summarized in Table 1.

Our results showed that, upon comparison of the three groups, that is, the diabetes with inadequate maintenance of blood glucose group (Group I), the diabetes with adequate maintenance of blood glucose group (Group II), and the euglycemic non-diabetes group (Group III), the recurrence rate was significantly higher in the diabetes with inadequate maintenance of blood glucose group than in the other two groups ($P < 0.0001$) [Figure 1 and Table 2]. On the other hand, there was no significant difference in the HCC recurrence rate between the diabetes patients with adequate

maintenance of blood glucose group and the non-diabetes group ($P = 1.0000$).

With regard to the number of HCC nodules, namely, solitary or multiple, the recurrence rate was significantly higher in patients with multiple HCC nodules in all groups. Within each subgroup of patients with single and multiple HCC nodules, diabetes with inadequate maintenance of blood glucose was significantly associated with recurrence of HCC in comparison to other groups as shown in Figure 1 and Table 2. In terms of the initial level of serum AFP ≥ 200 ng/mL, the recurrence rate was significantly higher in patients with AFP ≥ 200 ng/mL in all groups. Within each subgroup of patients with AFP ≥ 200 ng/mL and < 200 ng/mL, diabetes with inadequate maintenance of blood glucose was associated with a higher rate of recurrence in comparison to other groups as shown in Figure 1 and Table 2. On the other hand, obesity was not significantly associated with HCC recurrence in all groups.

As shown in Figure 2, the survival rate was significantly lower in diabetic patients with inadequate maintenance of blood glucose (solid line) than in diabetic patients with adequate maintenance of blood glucose (blood glucose < 200 mg/dL, broken line) or non-diabetic patients (dotted line) ($P = 0.0060$). There was no significant difference in survival rate between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

DISCUSSION

The effect of metabolic factors, such as hyperglycemia, diabetes, and obesity, on the recurrence of HCC after curative RFA therapy was analyzed retrospectively. Our results identified that inadequate maintenance of blood glucose in diabetic patients was a significant and independent risk factor for early recurrence of HCC, whereas obesity and

Table 1: The clinical and laboratory criteria for all patients undergoing RFA

Variable	Group I (<i>n</i> = 37) (52 HFL)	Group II (<i>n</i> = 25) (43 HFL)	Group III (<i>n</i> = 45) (64 HFL)
Age (years)	50.8 \pm 8.6	53.4 \pm 9.4	50.9 \pm 8.6
Sex (male/female)	27/10	15/10	28/17
ALT (IU/L)	57.7 \pm 15	59.7 \pm 17.9	61 \pm 18.2
AST (IU/L)	50 \pm 7	54.4 \pm 11.4	50.3 \pm 13.4
Child-Pugh grade (A/B)	17/20	13/12	20/25
Casual blood sugar (mg/dL)	159.9 \pm 7.47	262.0 \pm 44.35	121.3 \pm 25.7
Maximum diameter of HCC (cm)	3.17 \pm 0.51	3.11 \pm 0.53	3.16 \pm 0.46
Number of patient with 1/2/3 HCC	24/11/2	9/14/2	28/15/2
AFP (ng/mL)	170.3 \pm 160	169.5 \pm 139.9	182.6 \pm 153.3
AFP ≥ 200 (ng/mL)	21/37	15/25	28/45
BMI (kg/m ²)	27.9 \pm 0.5	27.2 \pm 2.9	26.5 \pm 3.1

RFA: radiofrequency ablation; ALT: alanine aminotransferase; AST: aspartate aminotransferase; HCC: hepatocellular carcinoma; AFP: alpha-fetoprotein; BMI: body mass index; HFL: hepatic focal lesion

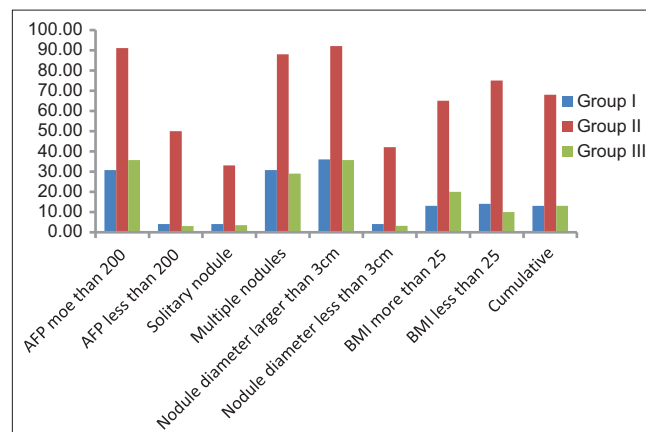


Figure 1: The recurrence of all studied patients as regard all risk factors after radiofrequency ablation (AFP: alpha-fetoprotein; BMI: body mass index)

Table 2: The recurrence of all studied patients as regard all risk factors after RFA

Group		Recurrence of HCC as regard independent risk factors		Recurrent lesions	
		Risk factor	<i>n</i>	<i>n</i> (%)	<i>P</i>
I (<i>n</i> = 37)	AFP ≥ 200	13	4 (30.7)	0.0423	0.0423
	AFP < 200	24	1 (4)		
	Solitary nodule	24	1 (4)		
	Multiple nodules	13	4 (30.7)	0.0207	
	Nodule diameter ≥ 3 cm	11	4 (36.3)		
	Nodule diameter < 3 cm	26	1 (3.8)		
	BMI ≥ 25	23	3 (13)	1.0000	
	BMI < 25	14	2 (14)		
	Cumulative	37	5 (13)		
II (<i>n</i> = 25)	AFP ≥ 200	11	10 (90.9)	0.0421	0.0099
	AFP < 200	14	7 (50)		
	Solitary nodule	9	3 (33.3)		
	Multiple nodules	16	14 (87.5)	0.0112	
	Nodule diameter ≥ 3 cm	13	12 (92)		
	Nodule diameter < 3 cm	12	5 (41.6)		
	BMI ≥ 25	17	11 (64.7)	1.0000	
	BMI < 25	8	6 (75)		
	Cumulative	25	17 (68)		
III (<i>n</i> = 45)	AFP ≥ 200	14	5 (35.7)	0.0080	0.0228
	AFP < 200	31	1 (3)		
	Solitary nodule	28	1 (3.5)		
	Multiple nodules	17	5 (29)	0.0080	
	Nodule diameter ≥ 3 cm	14	5 (35.7)		
	Nodule diameter < 3 cm	31	1 (3.2)		
	BMI ≥ 25	15	3 (20)	0.3843	
	BMI < 25	30	3 (10)		
	Cumulative	45	6 (13)		
<i>P</i>	AFP ≥ 200	<i>P</i> ^a = 0.0045	<i>P</i> ^b = 0.0119	<i>P</i> ^c = 1.0000	
	AFP < 200	<i>P</i> ^a = 0.0017	<i>P</i> ^b = 0.0005	<i>P</i> ^c = 1.0000	
	Solitary nodule	<i>P</i> ^a = 0.05	<i>P</i> ^b = 0.0375	<i>P</i> ^c = 1.0000	
	Multiple nodules	<i>P</i> ^a = 0.0027	<i>P</i> ^b = 0.0013	<i>P</i> ^c = 1.0000	
	Nodule diameter ≥ 3 cm	<i>P</i> ^a = 0.0078	<i>P</i> ^b = 0.0044	<i>P</i> ^c = 1.0000	
	Nodule diameter < 3 cm	<i>P</i> ^a = 0.0078	<i>P</i> ^b = 0.0042	<i>P</i> ^c = 1.0000	
	BMI ≥ 25	<i>P</i> ^a = 0.0019	<i>P</i> ^b = 0.0155	<i>P</i> ^c = 0.6632	
	BMI < 25	<i>P</i> ^a = 0.0083	<i>P</i> ^b = 0.0007	<i>P</i> ^c = 0.6467	
	Cumulative	<i>P</i> ^a < 0.0001	<i>P</i> ^b < 0.0001	<i>P</i> ^c = 1.0000	

^aP: P value between Group I and II; ^bP: P value between Group II and III; ^cP: P value between Group I and III. Group I: inadequate maintenance of blood glucose; Group II: adequate maintenance of blood glucose; Group III: non-diabetic euglycemic. HCC: hepatocellular carcinoma; AFP: alpha-fetoprotein; BMI: body mass index; RFA: radiofrequency ablation

diabetes with adequate maintenance of blood glucose were not. This was based on the results that showed that diabetic patients with inadequate maintenance of blood glucose had a higher rate of HCC recurrence compared with diabetic patients with adequate maintenance of blood glucose and non-diabetic patients. In other words, even in patients with diabetes, if the blood glucose was adequately maintained, the HCC recurrence rate did not differ significantly compared with those in non-diabetic patients. These results indicate the

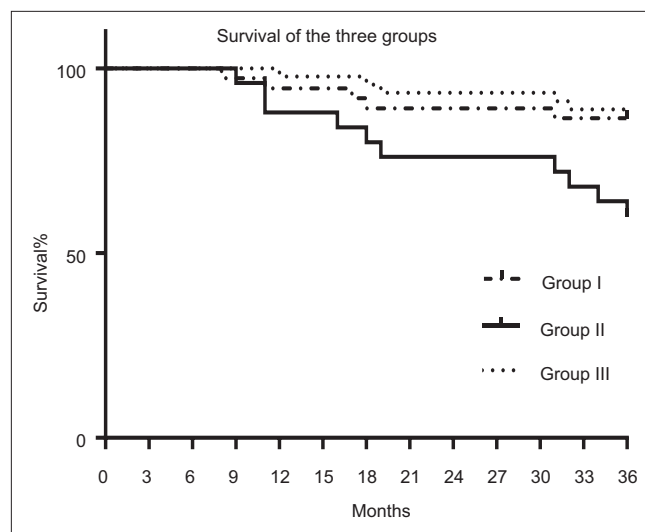


Figure 2: Survival rate in all groups

possibility that adequate management of hyperglycemia may lead to a reduction in the risk of HCC recurrence.

These results have been confirmed by several authors.^[25-29] There may be several mechanisms involved in the relationship between hyperglycemia and HCC recurrence. Hyperglycemia promotes cancer cell proliferation in pancreatic cancer cells and breast cancer cells through accelerated cell cycle progression or through the production of reactive oxygen species, leading to activation of protein kinase C and increased DNA synthesis in cancer cells.^[30-33]

Takahashi *et al.*^[34] proved that post-challenge hyperglycemia is a significant risk factor for the development of HCC in patients with CHC while hyperglycemia at fasting was not. A possible reason for this result may be that patients with post-challenge hyperglycemia may have higher fluctuations in daily glucose levels that lead to oxidative stress.^[34] This is because it was reported that acute fluctuations in blood glucose levels cause greater oxidative stress than sustained chronic hyperglycemia.^[35] Taken together, a possible mechanism for the relationship between higher level of casual blood glucose and development of HCC in the present study may be that daily fluctuations in serum glucose levels caused greater oxidative stress. Alternatively, hyper-insulinemia or increased level of insulin-like growth factor, which are caused by hyperglycemia, may be related to carcinogenesis.^[36]

The results of our study identified that, the survival rate was significantly lower in diabetic patients with inadequate maintenance of blood glucose than in diabetic patients with adequate maintenance of blood glucose or non-diabetic patients. There was no significant difference in survival rate between diabetic patients with adequate maintenance of blood glucose and non-diabetic patients.

These results were confirmed by Hosokawa *et al.*^[25] who suggested that glycemic control in diabetic patients, more so than diabetes itself, plays a role in the survival. The mechanism by which glycemic control and survival are related is unknown, but frequent recurrence of HCC in hyperglycemic patients and the accumulation of damage in liver function because of repeated treatment intervention for HCC may lead to worsening survival. Diabetes accelerates liver fibrosis and inflammation with increased inflammatory markers and cytokines resulting in severe liver failure and poor cancer prognosis.^[25]

In conclusion, inadequate maintenance of blood glucose in diabetic patients is a significant risk factor for recurrence of HCC and for poor survival after curative RFA therapy in CHC patients.

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Repeat liver surgery by laparoscopy for a malignant recurrence after previous open or laparoscopic resection

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ABSTRACT

Aim: This paper reported the experience of one center on repeat laparoscopic liver surgery for metastasis and hepatocellular carcinoma (HCC) with a review of the literature. **Methods:** This retrospective study included 24 patients who underwent laparoscopic re-intervention (hepatic resection and radiofrequency ablation) for recurrent HCC in cirrhosis ($n = 17$) and for recurrent malignant metastases ($n = 7$) after a previous open or laparoscopic procedure. Patients were divided into two groups according to the first surgical approach. Group 1 underwent open resection and laparoscopic procedure (7 patients), and Group 2 underwent laparoscopic resection and laparoscopic procedure (17 patients). **Results:** Mean operative time for re-intervention was significantly longer for Group 1 (220.14 ± 80.06 min) than for Group 2 (150 ± 56.18 min; $P = 0.001$), whereas the mean blood loss and mean hospital stay were comparable in both groups. According to Dindo-Clavien classification, overall morbidity ranged between Grade I and IIIa and was similar in both groups. **Conclusion:** This study suggests that repeat laparoscopic surgery for recurrent hepatic malignant diseases in selected patients is a feasible and safe procedure with good short-term outcomes, but further prospective studies are needed to support these results.

Key words: Recurrence; repeat liver surgery; resection

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INTRODUCTION

Current literature reports significant efficacy of repeat hepatectomies in the treatment of recurrent malignant diseases (both primary and secondary) of the liver.^[1-5] The improved clinical outcomes after multidisciplinary treatment have led surgeons and oncologists to work on a new challenge - the management of recurrence. In hepatic surgery, the laparoscopic approach is becoming a widely accepted alternative to open approach especially for tumors located on

anterior segments of the liver. Nevertheless, at the present, few studies have been done on repeat laparoscopic surgery of the liver because of some technical difficulties of repeated interventions, which is even more challenging if carried out by a minimally invasive approach.

We previously published data on laparoscopic re-interventions for hepatocellular carcinoma (HCC) in cirrhotic liver that described peri-operative outcomes, safety, and feasibility of this procedure.^[6] In this paper, our experience on repeat laparoscopic liver surgery for malignant primary and secondary diseases with a review of the literature is reported.

METHODS

Patients and inclusion criteria

From January 2004 to December 2013, 24 patients underwent a laparoscopic re-intervention (hepatic resection

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and radiofrequency ablation [RFA]) for recurrent HCC in cirrhosis ($n = 17$) and for recurrent metastases from colorectal carcinoma ($n = 7$) after a previous open or laparoscopic procedure. The primary surgical interventions were 7 open and 18 laparoscopic procedures (a laparoscopic segmentectomy was associated with a laparoscopic RFA in 1 patient). Details of hepatic procedures are explained in Table 1. A wedge resection (WR) was performed in association with a laparoscopic left hemicolectomy in 1 case of synchronous metastases; a left lateral sectionectomy with an open left hemicolectomy and a cholecystectomy was performed for a similar case.

The inclusion criteria for the laparoscopic re-intervention were: A well-compensated chronic liver disease (Child-Pugh Class A) without signs of severe portal hypertension in cirrhotic patients, a performance status of Karnofsky ≥ 70 , an American Society of Anesthesiology status ≤ 3 , either a single HCC (≤ 5 cm) or 1 or more metastases when located in the anterior hepatic segments (segments II, III, IVb, V and VI), or a small (3 cm) deep HCC for laparoscopic RFA

in which major hepatectomy is not recommended. No tumor was biopsied pre-operatively.

The patients were divided into two groups according to the first surgical approach [Table 2]. Group 1 underwent open resection (OR) and laparoscopic procedure (7 patients), and Group 2 underwent laparoscopic resection (LR) and laparoscopic procedure (17 patients). Results from the two groups were compared in a retrospective study. Between the two groups, we analyzed and compared operative time for re-intervention, blood loss, hospital stay, post-operative morbidity, and mortality. Data were expressed as mean \pm standard deviation and represented in Table 3. Differences in means between the groups were compared using Student's *t*-test. $P < 0.05$ was considered statistically significant. The hospital review board approved this study.

Surgical technique

The surgical technique for the repeat laparoscopic hepatic resection was described elsewhere.^[6-8] In brief, continuous CO₂ pneumoperitoneum was induced using access technique

Table 1: Features of hepatic procedures

Patients	First approach	First procedure	Recurrence site	Second approach	Redo procedure for recurrence	Operative time for second procedure (min)	Type of lesion (number)	Size (mm)
1	OR	Subsegmentectomy	II	LR	Left lateral sectionectomy	115	HCC (1)	40
2	OR	Segmentectomy	III	LR	Left lateral sectionectomy	100	HCC (1)	38
3	OR	Segmentectomy	IV	LR	Subsegmentectomy (converted to laparotomy)	120	HCC (1)	38
4	OR	Segmentectomy	IV	LR	Subsegmentectomy	130	HCC (1)	40
5	OR	Subsegmentectomy	VIII	L-RFA	L-RFA	120	HCC (1)	28
6	LR	Left lateral sectionectomy	VII-VIII	L-RFA	L-RFA	100	HCC (1)	33
7	LR	Subsegmentectomy	II	LR	Left lateral sectionectomy	60	HCC (1)	48
8	LR	Segmentectomy	IV	LR	Subsegmentectomy	80	HCC (1)	30 exophityc
9	LR	Subsegmentectomy	IV	LR	Subsegmentectomy	60	HCC (1)	48 exophityc
10	LR	Subsegmentectomy	V	LR	Segmentectomy	50	HCC (1)	40
11	LR	Subsegmentectomy	VIII	L-RFA	L-RFA	80	HCC (1)	35
12	LR	Segmentectomy	VI	LR	Segmentectomy	40	HCC (1)	38
13	LR	Segmentectomy	II	LR	Left lateral sectionectomy	80	HCC (1)	45
14	LR	Subsegmentectomy	II-III	LR	Left lateral sectionectomy	65	HCC (1)	38
15	LR	Subsegmentectomy	V	LR	Segmentectomy	60	HCC (1)	45
16	LR	Segmentectomy + L-RFA	VII	L-RFA	L-RFA	50	HCC (1)	28
17	LR	Left lateral sectionectomy	II	LR	Segmentectomy	50	HCC (1)	35
18	LR	WR	IV	LR	WR	60	MTX (1)	28
19	LR	WR	III, VI	LR	WR	100	MTX (3)	10, 23, 25
20	LR	WR	VI, V	LR	WR	80	MTX (3)	17, 20, 27
21	OR	WR	II, V, VI	LR	WR	120	MTX (3)	10, 28, 25
22	OR	Left hemicolectomy + cholecystectomy + left lateral sectionectomy	IV-V	LR	Bisegmentectomy Third procedure: WR	220	MTX (1)	76
23	LR	Left hemicolectomy + WR	II, III, IV	LR	Left lateral sectionectomy + WR Third procedure: WR	240	MTX (4)	28, 35, 30, 38
24	LR	WR	VI	LR	WR	120	MTX (1)	22

OR: open resection; LR: laparoscopic resection; WR: wedge resection; MTX: metastases; HCC: hepatocellular carcinoma; L-RFA: laparoscopic radiofrequency ablation

Table 2: Perioperative results

	Group 1 (%)	Group 2 (%)	P	S/NS
Extensive adhesions (grade 3-4)	5 (71.4)	2 (11.7)	0.01	S
Operative time, min (mean ± SD)	220.14 ± 80.06	150 ± 56.18	0.03	S
Blood loss, mL (mean ± SD)	297 ± 134	272.2 ± 120	1.0	NS
Morbidity	5 (29.4)	2 (28.5)	1.0	NS
Grade I atelectasis	1	1	-	-
Grade I ascites	1	0	-	-
Grade II pneumonia	2	0	-	-
Grade II bleeding	1	0	-	-
Grade IIIa perforation	0	1	-	-
Mortality	Nil	Nil	-	-
Conversion	1	0	-	-

S/NS: significant/nonsignificant; SD: standard deviation

Table 3: Classification of adhesions

Grade	Description of adhesions
0	None
1	Thin film, divided by blunt dissection
2	Thin vascular, easily divided by sharp dissection
3	Extensive thick vascular, requires division by sharp dissection
4	Dense, bowel at risk of injury with division

of open laparoscopy with the Hasson trocar. In some cases, a safe access to the abdominal cavity was carried out by use of a Visiport® (Covidien, Mansfield, MA, USA), opening the abdominal wall layer by layer, after pneumoperitoneum was achieved with a Verres needle.

During the exploratory laparoscopy, parietal and visceral adhesions were dissected. Such adhesions had to be dissected carefully with the use of specific surgical devices without causing any damage to the gastrointestinal tract before obtaining surgical access to the liver. In this phase, the pneumoperitoneum allowed adhesions to become strained to allow more meticulous assessment and lysis of adherences. The Pringle maneuver was prepared for all patients but was performed only in selected cases (8/24).

Anatomical resections (segmentectomy, subsegmentectomy of IVb, bisegmentectomy, and left lateral sectionectomy) were performed for treatment of HCC, and WR was performed for liver metastases.

After an extensive adhesiolysis has been performed, staging abdominal laparoscopy and laparoscopic ultrasonography were carried out to confirm the extension of the lesions and their relationships to the vasculature, to visualize their margins inside the parenchyma, and to exclude a widespread peritoneal carcinosis that might hinder the procedure. Laparoscopic transections were performed with a harmonic

scalpel (Harmonic Ace Shears®; Ethicon, Endo-Surgery, Cincinnati, OH, USA) or with a vessel sealer (Enseal Tissue Sealer®; Ethicon, Endo-Surgery, Cincinnati, OH, USA) or (Ligasure™; Covidien, Mansfield, MA, USA), and was performed with reduced bleeding, due to a reduction of portal inflow of up to 30% because of the pneumoperitoneum. The resection bed surfaces were treated with a biologic fibrin glue (Tissucol; Baxter, Wien, Österreich), or a hemostatic gel (FloSeal; Baxter, Wien, Österreich), or a sealant patch (TachoSil®; Takeda, Linz, Österreich) to minimize risk of biliary leak and to ensure hemostasis.

Bipolar electrocoagulation was used for minor bleeding, and larger structures were secured with ties or either multiple absorbable or nonabsorbable clips.

In order to facilitate the maneuver of left lateral sectionectomy, the left hepatic vein was stapled, and the device was introduced through the trocar located on the right of the patient, and then angled toward the left.

Laparoscopic radiofrequency ablation

A three-trocar configuration was routinely used. A 12-mm port at the umbilicus housed the 30° laparoscope. After an extensive adhesiolysis has been performed, staging abdominal laparoscopy and laparoscopic ultrasonography were carried out to identify the positions of the lesions.

As previously described,^[9] RFA was carried out with multi-electrode 15-gauge radiofrequency probes (RITA Medical Systems, Mountain View, CA, USA). Hook-shaped retractable electrodes were deployed to a maximum diameter of 3 cm. After every electrode had reached a temperature of 100 °C, the ablation was performed in a step-by-step fashion, with a single step lasting approximately 8-10 min. In two patients with a deep HCC, the size of the lesion was slightly larger than that recommended for a standard RFA (35 and 33 mm, respectively). In these two cases, a Pringle maneuver was carried out during laparoscopy causing vascular occlusion to reduce blood flow and to increase the volume of the ablation.

After track ablation, hemostasis of the liver surface was ensured by bipolar electrocoagulation.

RESULTS

Repeat laparoscopic hepatic procedures were performed in 24 patients: 6 were treated by left lateral sectionectomy (1 associated with a WR), 4 by segmentectomy, 4 by subsegmentectomy (1 had conversion to laparotomy), 1 by bysegmentectomy associated with a WR, 4 by laparoscopic

RFA of HCC, and 5 by WR. Two patients were subjected to a third repeat procedure consisting of laparoscopic WR of segment II and VI, respectively, for a second recurrence of liver metastases.

The laparoscopic procedure was successfully completed in 23 cases (95.9%). Adhesions were graded by the staff surgeons using the scale presented in Table 3, similar to that used in a multi-center study on adhesion prevention.^[10] Grades 3 and 4 adhesions were present in 5 patients (71.4%) in Group 1 and 2 patients (11.7%) in the Group 2.

Of the 24 patients, one underwent conversion to laparotomy in Group 1, not because of adhesions but due to inadequate control of the resection margin for a HCC located in segment IV. One patient, receiving a laparoscopic RFA of a HCC of 28 mm in VII segment after primary intervention of segmentectomy associated with laparoscopic RFA, was subjected to intestinal resection associated with ileostomy to treat peritonitis from intestinal perforation that occurred during laparoscopic RFA.

The mean operative time for re-intervention was significantly longer for Group 1 (220.14 ± 80.06 min) than for Group 2 (150 ± 56.18 min; $P = 0.001$), whereas the mean blood loss was comparable in both groups: 297 ± 134 mL in Group 1 and 272.2 ± 120 mL in Group 2 ($P > 0.05$). The mean hospital stay was 6.4 ± 2.5 days in Group 1 and 5.2 ± 3 days in Group 2 ($P > 0.05$). The resection margins were disease-free in all the patients.

The overall post-operative morbidity and mortality rates were 29.1% (7/24) and 0%, respectively. According to Dindo-Clavien classification,^[11] overall morbidity varied between Grades I and IIIa. Morbidity rate was 29.4% in Group 1 and 28.5% in Group 2. In Group 1, 2 patients had atelectasis treated by physical therapy (Clavien's Grade II), 2 had pneumonia treated by antibiotics (Clavien's Grade II) and 1 had bleeding from one trocar site treated by compression (Clavien's Grade II). In Group 2, 1 patient presented post-operatively with moderate ascites, 1 with atelectasis (Clavien's Grade I) and 1 presented with intestinal perforation that occurred during a laparoscopic RFA, requiring a re-intervention (Clavien's Grade IIIa).

Long-term outcomes in terms of hepatic recurrence have not yet been evaluated.

DISCUSSION

Recurrence rate for liver malignancy is estimated at 77-100% for HCC^[12,13] and 60% for metastasis from colorectal carcinoma.^[14] Nevertheless, current data report efficacy of

repeat hepatectomies in the treatment of primary or secondary tumors of the liver.^[1-5] At present, studies on laparoscopic hepatic re-interventions are limited. Technical difficulties of both repeat hepatectomy and laparoscopic approach have slowed the spread of laparoscopic re-interventions on the liver. Few papers are available on this procedure, and investigations are biased due to the retrospective nature of these studies, and to the time differences between the series of open and laparoscopic interventions [Table 4].^[6,15-22]

Tsuchiya *et al.*^[20] reported a cohort of 14 patients affected by HCC, who underwent laparoscopic repeat resection after a primary procedure (laparoscopic hepatectomy, RFA, resection of extrahepatic metastasis, or diagnostic assessment). They demonstrated that 2-year survival in patients with intrahepatic recurrence (100%) is significantly higher than in those with the extrahepatic recurrence (42.9%).

Indeed, the surgical strategy can be changed, and survival can be impaired because of the presence of concomitant peritoneal recurrence or because of extensive peritoneal adhesiolysis. Biopsies of suspicious lesions are mandatory to identify carcinomatous foci in dense adhesions to treat the extrahepatic recurrence if possible, or to abstain from a surgical procedure.

Shafae *et al.*^[18] analyzed the experience of laparoscopic repeat liver resection of three institutions recruiting 76 patients (61 with liver metastasis, 3 with HCC, and 12 with benign lesions) divided into two groups according to the first surgical approach. Peri-operative outcomes (in terms of estimated blood loss and intra-operative transfusions) were better in patients with previous LR than in patient with previous ORs. Furthermore, long-term outcomes in terms of hepatic recurrence and the need for laparoscopic re-interventions were compared with those of open repeat resection in other studies,^[1-5] and similar outcomes were observed.

Table 4: Retrospective studies about laparoscopic repeat surgery of the liver

Year	Author	Number	Tumor
2009	Belli <i>et al.</i> ^[6]	12 cases	HCC
2009	Liang <i>et al.</i> ^[15]	1 case	HCC
2010	Cheung <i>et al.</i> ^[16]	1 case	HCC
2011	Hu <i>et al.</i> ^[17]	6 cases	HCC
2011	Shafae <i>et al.</i> ^[18] (tri-institutional)	76 cases	HCC + metastasis
2011	Nakahira <i>et al.</i> ^[19]	15 cases	HCC + metastasis
2012	Tsuchiya <i>et al.</i> ^[20]	16 cases	HCC
2013	Kanazawa <i>et al.</i> ^[21]	40 cases	HCC
2014	Shelat <i>et al.</i> ^[22]	19 cases	HCC + metastasis
2015	Cioffi <i>et al.</i> (this series)	24 cases	HCC + metastasis

HCC: hepatocellular carcinoma

Kanazawa *et al.*^[21] reported a series of 40 patients who underwent hepatic repeat resection for HCC. Twenty patients were previously operated with the open approach and 20 with the laparoscopic approach. Intra-operative blood loss and the incidence of post-operative complications and consequently, post-operative hospital stay were significantly lower in the laparoscopy group.

Shelat *et al.*^[22] reported a series of 19 patients who underwent repeat operated in whom peri-operative data of laparoscopic primary and repeated hepatic resection were compared (outcomes from minor and major resections were considered separately). Liver metastases were the most common indication for repeat resections. The operative time and blood loss were both significantly greater in laparoscopic repeat resection, whereas length of stay and complications did not differ between the groups.

In previous papers that reported our experience in repeat surgery for HCC in cirrhotic liver, we highlighted that a minimally invasive approach applied during the first hepatectomy determines minimal post-operative adhesions and faster and safer adhesiolysis in terms of blood loss and risk of visceral injuries.^[23] These factors highlight the advantages of the minimally invasive approach in the management of oncological recurrence of selected cirrhotic or metastatic patients.

In our study, patients with HCC on cirrhosis represent the most part of the cases. This is because patients with multiple lesions in recurrent liver metastases are less often selected for a multiple laparoscopic WR. The mean operative time for re-intervention was significantly longer for the group with previous OR, whereas the mean blood loss and the hospital stay were comparable in both groups. The resection margins were disease-free in all the patients.

A good training in laparoscopic adhesiolysis during minimally invasive incisional hernia repair even in cirrhotic patients can accelerate the learning curve in the lysis of hypervascularized adhesions, facilitated by laparoscopic pneumoperitoneum and optical magnification.^[24]

The only case of severe complication in our study was in a patient previously treated with a LR followed by a laparoscopic RFA for a recurrent HCC. At the time of re-operation, he was affected by severe thrombocytopenia. The need to perform a safe hemostasis by electrocoagulation on the liver surface after extraction of the RFA probe from the hepatic parenchyma induced us to perform a RFA with the laparoscopic approach. During laparoscopy, the presence of a few thin adhesions (grade evaluated: 0-1)

induced us to consider the visceral damage not as a specific complication of adhesiolysis *per se*, or of the re-operation, but a generic adverse event of laparoscopy. Subsequently, we have restricted indications for the laparoscopic approach of RFA that seems to increase morbidity of an otherwise safe procedure.

In conclusion, this study suggests that repeat laparoscopic surgery for recurrent hepatic malignant diseases in selected patients is a feasible and safe procedure with good short-term outcomes, but further prospective studies are needed to support these results.

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Salvage transplantation for post-resection recurrence in hepatocellular carcinoma associated with hepatitis C virus etiology: a feasible strategy?

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ABSTRACT

Aim: The aim was to analyze the feasibility of salvage liver transplant after liver resection in hepatocellular carcinoma (HCC) with hepatitis C virus (HCV) etiology. **Methods:** All the patients diagnosed with HCC with HCV etiology who underwent living donor liver transplant from July 2002 to November 2012 were studied. Their recurrence rate, mortality, and prognostic factors were analyzed and compared between primary transplant and salvage transplant for up to 5 years post-transplant. **Results:** One hundred and nine patients underwent a liver transplant for HCC associated with HCV etiology within the University of California, San Francisco criteria. Eighteen were post-hepatectomy salvage transplants and 91 were primary transplants. Median follow-up time was 31 months. One, 3 and 5 years overall survival rates were 76%, 76% and 65% in the salvage group, and 92%, 85% and 85% in primary transplant group respectively. The difference in overall survival rates was statistically significant ($P = 0.031$). However, recurrence-free survivals for 1, 3 and 5 years were 72%, 72% and 46% for salvage group, and 91%, 73% and 46% for primary transplant group; which were not statistically significant ($P = 0.328$). **Conclusion:** Salvage transplantation for post-hepatectomy recurrence for patients with HCC associated with HCV-related chronic liver disease seems to offer inferior overall survival rates than primary transplantation.

Key words: Hepatitis C virus; hepatocellular carcinoma; salvage transplantation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver.^[1] There is an ongoing debate about what is the best strategy to treat HCC, particularly in Child A cirrhosis, when the primary option is resection and transplantation. Some reports have suggested that transplantation is a better choice,^[2] but the opponents of this approach suggest that resection is better because it can

also serve as a bridge to liver transplantation.^[3] Many authors have suggested that results of salvage transplantation are comparable to primary transplantation.^[4,5] However, there also is controversy over management of HCC associated with hepatitis C virus (HCV) related cirrhosis. Chirica *et al.*^[6] suggested that overall and disease-free survival after liver resection for HCV-related HCC is poor and so primary liver transplantation (LT) should be offered to these patients. In this study, we evaluated feasibility of salvage transplantation in HCC patients with HCV-related liver disease.

The aim of this study was to compare the survival rates and recurrence rates of primary as well as salvage transplantation and also to evaluate prognostic factors affecting survival and recurrence in primary as well as salvage transplantation.

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METHODS

Patients selection

All the patients transplanted for hepatitis C related liver disease with HCC were analyzed retrospectively. All the data collected was at the time of liver transplant. Barcelona clinic liver cancer staging criteria were followed to decide treatment options. University of California, San Francisco (UCSF) criteria were used as transplantation indication.^[7] If the tumor was outside UCSF criteria, loco-regional therapies such as transarterial chemoembolization, radiofrequency ablation were used. These patients were scheduled for transplantation when they fulfilled UCSF criteria. Results with down staging are published before.^[8] Patients who underwent liver resections before and subsequently transplanted for intrahepatic recurrence were included in salvage transplant group, and other patients who were transplanted without prior resection were included in the primary transplant group. The review board of Chang Gung Memorial Hospital approved this study.

Follow-up

Patients were followed-up every 3 months for the first year, then every 6 months and yearly after that. Follow-up included liver function tests, alpha-fetoprotein (AFP) and triple-phase computed tomography scan, or magnetic resonance imaging.

Statistical analysis

Chi-square test and Fisher's *t*-test whenever appropriate were used for categorical variables and Mann-Whitney *U*-test for continues variables. Kaplan-Meier survival curves were prepared for recurrences and mortality with the Log-rank test. Multivariate analysis was performed using multivariate Cox regression analysis. SPSS version 21 (IBM, Armonk, NY, USA) was used for statistical analysis. Two-tailed significances were taken into consideration. *P* < 0.05 was considered as statistically significant.

RESULTS

One hundred and nine patients underwent living donor LT for HCV-related HCC between July 2002 and November 2012. Median follow-up time was 31 months. Eighteen patients underwent salvage transplantation for intrahepatic recurrence post-hepatectomy; while 91 patients underwent primary transplants. Patients' characteristics are described in Table 1.

As described in Table 1, age of patients in both groups, mean tumor numbers and size were comparable in both the groups. The primary transplant group had statistically significant higher mean model for end-stage liver disease (MELD) score and mean Child-Turcotte-Pugh (CTP) score.

Table 1: Characteristics of 109 patients with hepatitis C virus liver disease and hepatocellular carcinoma undergoing liver transplantation as salvage or primary treatment

	Salvage transplant (n = 18)	Primary transplant (n = 91)	P
Age (years, mean ± SD)	56 ± 5	56 ± 6	0.874
Sex (male, %)	72	63	0.471
MELD (mean ± SD)	9 ± 4.6	11 ± 4.2	0.026
CTP (mean ± SD)	6 ± 1	8 ± 2	0.045
Associated HBV infection (%)	28	15	0.307
Pre-operative interferon and ribavirin treatment (%)	22	37	0.283
Overall recurrence (%)	11	13	0.836
Pre-operative RFA (%)	33	34	0.971
Percutaneous ethanol injection (%)	22	15	0.433
Overall mortality (%)	28	12	0.090
Pre-transplant within Milan (%)	33	55	0.114
Pre-transplant TACE (%)	67	53	0.299
Microvascular invasion on explant histology (%)	11	25	0.178
Moderate to poor differentiation on explant histology (%)	50	25	0.052
Pre-operative viral load = 0 (%)	22	19	0.744
Recurrence months (mean ± SD)	37 ± 32	37 ± 26	0.544
AFP pre-operative (mean ± SD)	40 ± 509	123 ± 86	0.764
Waiting months for transplant (mean ± SD)	9 ± 13	9 ± 12	0.673
Number of tumor (mean ± SD)	1 ± 2	1 ± 1	0.588
Size of largest tumor (cm, mean ± SD)	2.1 ± 1.6	3 ± 1.8	0.116
Pre-operative viral load (mean ± SD)	44,153 ± 146,079	162,459 ± 549,720	0.752

Data are shown as % or mean ± SD. MELD: model for end-stage liver disease; CTP: Child-Turcotte-Pugh; HBV: hepatitis B virus; RFA: radiofrequency ablation; TACE: transarterial chemoembolization; AFP: alpha-fetoprotein; SD: standard deviation

Survival and recurrence rates comparisons in both the groups

The two recurrences occurred in the salvage transplant group that exhibited extrahepatic metastasis. In the primary transplant group, total of 12 recurrences were noted. In two cases the recurrence was intrahepatic, and 10 were extrahepatic metastasis. One, 3 and 5 years recurrence-free survival rates were 72%, 72% and 46% in the salvage transplant group, and 91%, 73% and 46% in the primary transplant group respectively. The difference was not significant statistically ($P = 0.328$ on Log-rank analysis). One-year recurrence-free survival was low in salvage transplant group, but it did not achieve statistically significant level ($P = 0.08$ for 1 year). Kaplan-Meier survival curves were shown in Figure 1. One, 3 and 5 years survival rates were 76%, 76% and 65% in salvage transplant group, and 92%, 85% and 85% in primary transplant group. Kaplan-Meier survival curves were prepared and Log-rank analysis was done [Figure 2]. One, 3 and 5 years survival rates were significantly lower in salvage transplant group ($P = 0.031$).

Analysis of prognostic factors

Prognostic factors were evaluated in all 109 patients. On the log-rank analysis, on univariate analysis high MELD score ($P = 0.01$), no pre-transplant interferon therapy ($P = 0.002$), salvage transplant, no prior transarterial chemoembolization (TACE) ($P = 0.03$) were associated with worse survival rates. On multivariate Cox regression analysis salvage transplantation ($P = 0.04$) and no pre-transplant TACE ($P = 0.02$) were independently associated with worse survival rates. Higher AFP levels were associated with worse recurrence-free survival ($P = 0.005$).

DISCUSSION

Poon *et al.*^[9] suggested that 80% of the intrahepatic recurrences after resection are transplantable. Based on

this result, many authors such as Belghiti *et al.*^[10] suggested that liver resection should be the first line of treatment, followed by salvage transplantation for recurrence. They also showed that 3- and 5-year survival rates were not different between primary transplantation and salvage transplantation. However, Bozorgzadeh *et al.*^[11] showed that survival outcome for transplantation for HCC associated with HCV were significantly lower than for other etiology. Chirica *et al.*^[6] suggested that overall and disease-free survival after liver resection for HCV-related HCC is poor and so primary LT should be offered to these patients. However Cucchetti *et al.*^[12] suggested good outcomes after liver resection for HCV patients.

The aim of our study was to analyze feasibility of salvage transplantation for HCC associated with HCV etiology. In this study both the salvage transplant and the primary transplant groups were comparable however the primary transplant group had significantly higher pre-operative MELD scores as well as CTP scores. MELD scores and CTP scores were not significantly associated with survival or recurrence at any step of the analysis. There were no differences with regard to pre-operative viral load and pre-operative treatment taken between two groups.

In our study, there was not statistical significant difference in recurrence-free survival between salvage transplant and primary transplant group. However, 1-, 3- and 5-year overall survival rates were significantly lower in the salvage transplant group. These results indicate that primary transplant may be a better treatment strategy for transplantable HCC in case of associated HCV etiology. Adam *et al.*^[13] also showed inferior overall and recurrence-free survival in the salvage transplant group. However, they did not study HCV etiology separately. Belghiti and Durand^[14] in their editorial mentioned that in the study by Chirica

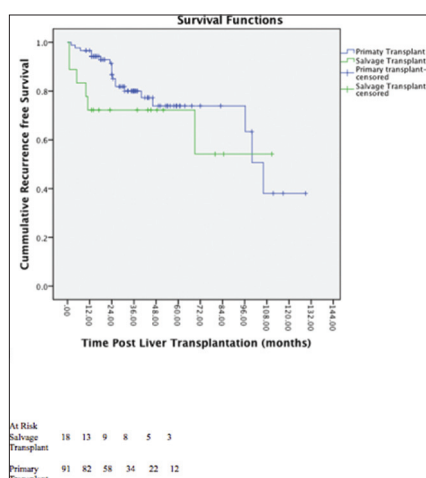


Figure 1: Recurrence free survival salvage transplant vs. primary transplant (Log rank test $P = 0.328$)

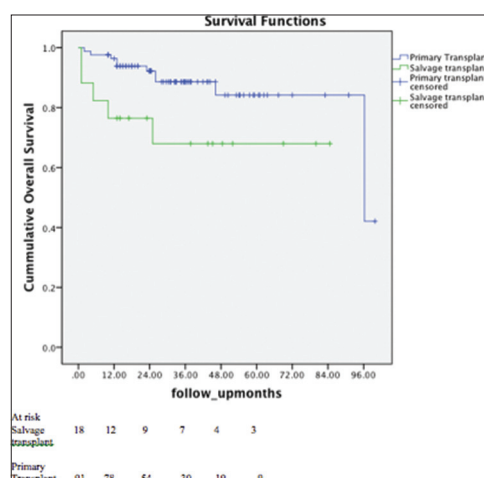


Figure 2: Overall survival in salvage transplant vs. primary transplant (Log rank test $P = 0.03$)

et al.^[6] tumor recurrences were outside Milan criteria after resection for HCV etiology so they may not be candidates for subsequent transplantation so primary transplant can be a good option for HCV-related HCC.

We also analyzed prognostic factors affecting survival and recurrence in both primary transplant and salvage transplant groups. High pre-operative AFP levels were associated with high recurrence rates. Vibert *et al.*^[15] also found pre-operative AFP as a significant prognostic factor for poor survival and recurrence. High MELD score, no pre-transplant interferon therapy, salvage transplant and no prior transarterial chemoembolization was associated with worse survival rates. In multivariate Cox regression, analysis salvage transplant and no prior transcatheter arterial chemoembolization were independent predictors of worse outcome. Shimoda *et al.*^[16] suggested that advanced tumor stage and particularly vascular invasion are poor prognostic indicators for tumor recurrence. The authors showed that early pathological tumor-node-metastasis stage, adjuvant chemotherapy, and pre-operative chemoembolization were associated with better outcomes for LT for concomitant HCV and HCC. In our studies, however, factors like vascular invasion, tumor number, tumor size, and presence of vascular invasion, tumor differentiation or histological grade did not achieve statistical significance. HCV viral load did not achieve statistical significance in predicting neither recurrence nor survival ($P = 0.8$ and 0.9 respectively). Pre-operative MELD and CTP scores were significantly higher in the primary transplant group but still these patients achieved better survival rates than the salvage transplant group. Even in advanced underlying disease cases, primary transplant achieved best results. Patients in the salvage transplants had mean CTP of 6, indicating that the majority of them had a compensated, or Child A cirrhosis and their survival was lower than the primary transplant group. There was no statistical difference between recurrence-free survival between primary and salvage transplant group, however 1 year recurrence free survival was 72% in case of salvage transplant and 91% in primary transplant with $P < 0.1$ (though not < 0.05), and most of the death were due to HCV and HCC recurrences. One death was due to post-operative bleeding as salvage transplant is technically more difficult than primary transplant. Pre-operative sustained virological response and pre-operative viral load was less in salvage transplant group though non-significant.

In the era of new and more efficacious anti-HCV drugs, this survival difference will be probably overcome. Thus, patients with HCC and HCV can receive salvage LT but probably they should receive pre-emptive antiviral therapy.

There are certain limitations of this study as this are a retrospective analysis. We also recognize that the numbers of patients in the salvage transplantation group were relatively low and with just two recurrences in salvage transplant group, statistical significance of the recurrence rate is weak. Another limitation is that we did not have complete pathological details of prior liver resection specimens in salvage transplant group as some of them were referred to us after resection; in addition, some patients underwent resection before 2002, and complete pathological analysis was not available.

In conclusion transplantation for post-hepatectomy recurrence for patients with HCC associated with HCV-related chronic liver disease seems to offer inferior overall survival rates than primary transplantation. However, results in the era of new anti-HCV drugs need to be evaluated further.

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Combined sarcomatoid hepatocellular and cholangiocarcinoma: a case report and literature review

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ABSTRACT

Hepatic sarcomatoid carcinomas are very rare. The majority of cases contain sarcomatoid features with either hepatocellular carcinoma (HCC) or cholangiocarcinoma (CC) elements alone. These are aggressive tumors and carry an unfavorable prognosis. We describe an extremely rare tumor sub-type of combined sarcomatoid HCC and CC in a hepatitis B virus carrier presenting with abdominal pain. Pre-operative imaging suggested a segment VI hepatocellular cancer with no metastatic spread. *En bloc* surgical resection with the right adrenal gland, Gerota's fascia and right hemidiaphragm was performed. The patient suffered early peritoneal tumor recurrence and lymph node metastasis. Pre-operative diagnosis of such sarcomatoid tumors is difficult. Current evidence for adjuvant treatment is also limited. Prognosis of these patients remains extremely poor, and surgery appears to be the only curative option in cases of early disease. It is essential that clinicians carry a high index of suspicion and awareness of this rare pathological entity to improve patient outcome.

Key words: Cholangiocarcinoma; hepatocellular carcinoma; sarcomatoid; spindle cell sarcoma

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INTRODUCTION

Hepatic sarcomatoid carcinomas are very rare. The reported incidence is < 10% with most cases only identified at autopsy. Diagnosis after surgical resection has been found in 1.8% of cases.^[1] Most patients are reported as individual case reports or small series only. There is a confusing and varied pathological terminology including carcinosarcoma, sarcomatoid carcinoma and spindle cell carcinoma,^[1-3] all likely to describe the same pathology. The majority of cases contain sarcomatoid features together with either hepatocellular carcinoma (HCC) or cholangiocarcinoma (CC) elements alone.

A sarcomatous tumor with both HCC and CC components is extremely rare. These tumors have an aggressive behavior and are associated with poor prognosis. Management options are currently unclear and limited. We report a case of combined sarcomatoid HCC and CC with a literature review of reported cases based on their characteristics and treatment options.

CASE REPORT

A 69-year-old Asian man presented with right-sided abdominal pain. He was a known hepatitis B virus (HBV) carrier but otherwise had no significant past medical history. He had no regular follow-up and was not on anti-viral therapy. Physical examination was unremarkable. Laboratory investigations were consistent with Child's A liver cirrhosis. Liver function tests were normal apart from a raised alkaline phosphatase level at 168 µmol/L. Alpha-feto protein (AFP) level was raised to 21 ng/mL. Antibody to hepatitis e antigen (HBeAg) was positive whereas HBeAg and anti-hepatitis C virus were negative. HBV DNA level was 178 IU/mL. Computed tomography (CT) identified a

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cirrhotic liver with a 5.9 cm × 5.7 cm irregularly shaped mass with heterogeneous contrast enhancement and contrast washout in portovenous phase arising from segment VI with suspected invasion into the postero-lateral chest wall [Figures 1 and 2]. Incidental bilateral renal cysts were also identified. Dual tracer positron emission tomography (PET) CT scan confirmed a segment VI lesion with predominantly 18F-fluorodeoxyglucose uptake (SUVmax 14.2), suggestive of a moderate to poorly differentiated HCC [Figure 3]. There was another discrete hypermetabolic ¹¹C-acetate avid only lesion (SUVmax 9.8) in segment IV/VIII consistent with multifocal HCC. There was no evidence of metastatic spread. Left lobe liver volumetry was measured at 34.5% of estimated standard liver volume.

We proceeded to a laparotomy with a plan for curative resection. A large tumor was seen in the right subphrenic space with invasion into at least 50% of the right hemidiaphragm and segment VI of the liver. Intra-operatively it was difficult to determine whether the tumor was hepatic in origin [Figure 4]. Dense adhesions were also identified around the right adrenal gland and Gerota's fascia. The liver was severely cirrhotic with numerous regeneration nodules. Intra-operative ultrasound

could not identify any lesions at segment IV/VIII. A segment VI resection was performed together with *en bloc* resection of the right adrenal gland, upper Gerota's fascia and the invaded area of the right hemidiaphragm. The diaphragmatic defect was closed primarily with nylon sutures and reinforced with polytetrafluoroethylene mesh. Diaphragmatic satellite nodule frozen section suggested probable high-grade malignant tumor, but the origin could not be determined. Radiofrequency ablation was applied to the resection margins. Post-operative recovery was uneventful, and the patient was discharged on day 6. On follow-up CT scan 1-month after the operation, peritoneal nodularities up to 1.5 cm were identified. PET/CT scan confirmed metastatic deposits at the diaphragmatic mesh (2.7 cm, SUVmax 14) in addition to nodal deposits in the paracaval (2.8 cm, SUVmax 12.6) and pre-aortic regions (1.8 cm, SUVmax 9.6). The patient declined palliative chemotherapy and is currently receiving symptomatic care.

Pathological examination of the resected specimen revealed a 10 cm × 9 cm × 7 cm firm tan-colored tumor with a pushing margin, invading into diaphragmatic skeletal muscle [Figure 5a and b]. The background liver showed features consistent with cirrhosis. Hepatitis B surface antigen was positive. Majority of the tumor was composed of

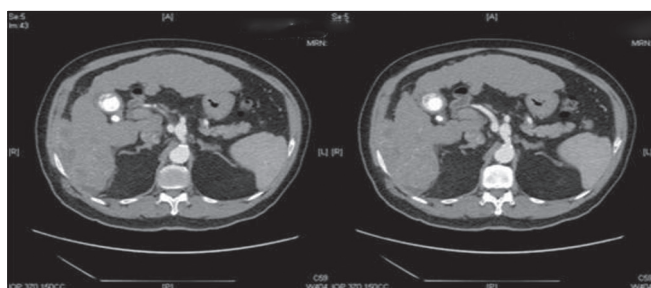


Figure 1: Computed tomography scan in arterial phase showing a segment VI heterogeneously enhancing mass with invasion to the postero-lateral chest wall

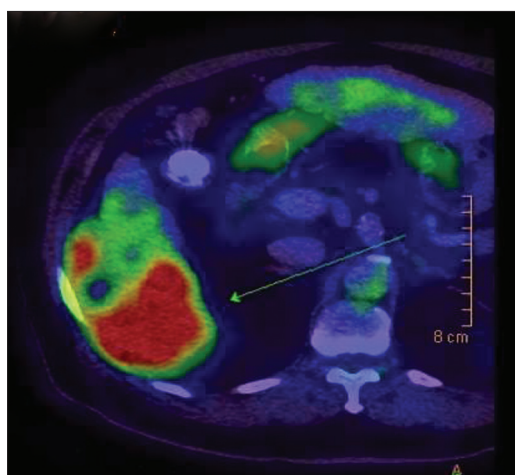


Figure 3: Positron emission tomography/computed tomography showing a predominantly 18F-fluorodeoxyglucose-avid liver mass suggestive of moderately to poorly differentiated hepatocellular carcinoma (SUVmax 14.2)

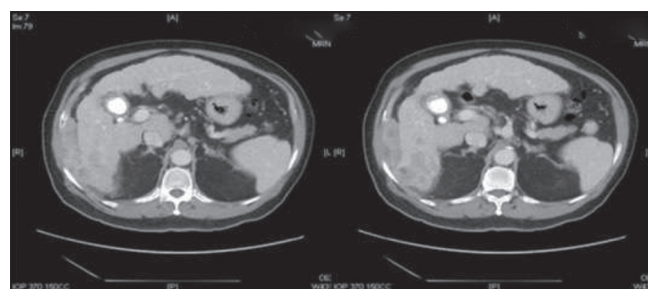


Figure 2: Computed tomography scan in portovenous phase showing a segment VI mass with contrast washout



Figure 4: Intra-operative photo showing a cirrhotic liver with a segment VI mass invading into the right hemi-diaphragm and chest wall

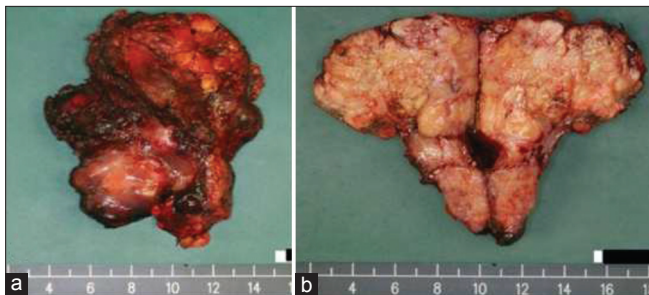


Figure 5: (a) Macroscopic appearance of the tumor and (b) gut section showing a tan-colored tumor with a pushing border

epithelioid to spindle-shaped cells with moderate cellularity. Other parts of the tumor composed of closely packed oval cells with high nuclear-cytoplasmic ratio and occasional acinar formation [Figure 6a]. Some parts of the tumor also composed of pleomorphic polygonal and multinucleated cells. The cells displayed moderate to severe nuclear pleomorphism and large patches of necrosis. Immunohistochemical staining showed diffusely strong membranous staining for pan-cytokeratin (CK) MNF116 (monoclonal antibody for carcinoma), low molecular weight CK CAM5.2 (monoclonal antibody for carcinoma), focal strong membranous staining for CK19 (CK19, monoclonal antibody for CC detection) and weak to moderate positivity for hepatocyte paraffin-1 (HEP-PAR-1, monoclonal antibody for hepatocyte detection for HCC). In areas with acinar formation, dot-like staining of CK7 (CK7, monoclonal antibody for bile duct differentiation) and CK19 was noted in apical parts of the cells toward the lumen. The overall features were consistent with a sarcomatoid carcinoma consisting of both hepatocellular (HEP-PAR-1 positivity) and CC (CK7 and CK19 positivity) differentiation [Figure 6b and c]. Final pathological staging was pT4 (American Joint Committee on Cancer, 7th edition).

DISCUSSION

Liver sarcomatoid carcinoma is a rare pathological entity. This highly malignant tumor usually contains an epithelial (hepatocellular or CC) element together with sarcomatous mesenchymal cells. Less than 100 such sarcomatoid cases have been reported in the literature based on either a hepatocellular or CC element. The case reported here of combined sarcomatoid HCC, and CC is extremely rare and only a few such cases have been reported [Table 1].

Although the published nomenclature is inconsistent, they share common pathological features. As found in the present patient's tumor, large area of central necrosis is a characteristic feature of hepatic sarcomatoid carcinoma. The rapidly dividing sarcomatoid cells outgrow the neovasculature of the tumor, resulting in necrosis.^[1] Microscopically,

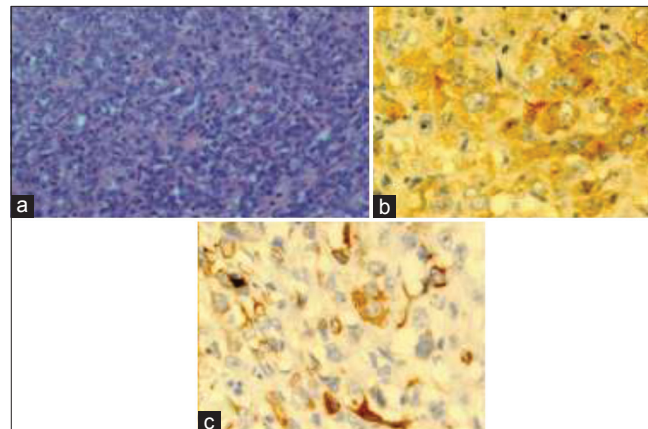


Figure 6: (a) Hematoxylin and Eosin slide of the tumor (x400), and (b) tumor slide showing hepatocellular differentiation, hepatocyte paraffin-1 positive (x400), and (c) bile duct differentiation, cytokeratin 19 positive (x400)

malignant epithelioid cells of the liver are found together with pleomorphic spindle-cells diagnostic of sarcomatous change. The pathogenesis is unclear but most believe that these tumors are as a result of either a differentiation of totipotent stem cells into mesenchymal cells and epithelial cells or the transformation of HCC or CC cells undergoing metaplasia into sarcomatous cells.^[2] A transitional phenomenon has been observed in previous reports. Kakizoe *et al.*^[10] identified positive immunohistochemical staining of AFP and CK in sarcomatous cells suggesting the presence of a cell type transformation. In addition, the sarcomatous component could further differentiate into specialized cell types including rhabdomyoblastic,^[11] chondroid^[12] and hepatoblastoma-like.^[13]

No definite identifiable risk factor for hepatic sarcomatoid carcinoma exists so far. Previous series reported an approximately 50% HBV infection rate in these tumors. There is however, no evidence to suggest HBV infection is associated with an increased risk of their development. It has been suggested that previous cancer treatment including systemic target therapy and trans-arterial chemoembolization (TACE) may increase the risk of developing these tumors.^[14] Kojiro *et al.*^[15] reported that previous anti-cancer therapy such as TACE might pre-dispose HCC cells to undergo a metaplastic change into sarcomatoid cells. They observed a higher incidence of sarcomatoid carcinomas in those treated with TACE.

Clinically, these tumors pose a diagnostic challenge pre-operatively as they resemble HCCs in presentation. Their behavior however is much more aggressive than ordinary HCC. The diagnosis of a sarcomatoid element prior to pathological specimen examination has proven difficult. As in the present patient, most would initially be considered

Table 1: Case reports of combined sarcomatoid hepatocellular carcinoma and cholangiocarcinoma

Study	Age, gender	Symptoms	Hepatitis serology	Treatment	Follow-up and prognosis
Nakajima <i>et al.</i> ^[4]	74 years, male	Right upper quadrant pain	HBsAb	TACE	Lung metastasis; death at 17 months; diagnosis at autopsy
Papotti <i>et al.</i> ^[5]	59 years, male	Lumbar pain	Negative	Hepatectomy	Biliary fistula; death at 4 months
Jeong <i>et al.</i> ^[6]	60 years, female	Right upper quadrant pain/mass	NA	Hepatectomy	Tumor recurrence; death at 12 months
Kim <i>et al.</i> ^[7]	67 years, male	Synchronous colon tumor	NA	NA	NA
Boonsakan <i>et al.</i> ^[8]	28 years, male	Fever	HBsAg	Palliative resection	Lost to follow-up
Pua <i>et al.</i> ^[9]	71 years, male	Fever, weight loss, anorexia	Negative	Hepatectomy	Lung and pleural metastasis; death at 6 weeks

NA: not available; HBsAb: hepatitis B surface antibody; HBsAg: hepatitis B surface antigen; TACE: trans-arterial chemoembolization

ordinary HCC with positive hepatitis serology, cirrhosis and raised AFP levels. Liver function parameters may or may not be unstable depending on the size of the tumor and degree of underlying cirrhosis. The liver was grossly cirrhotic [Figure 4] in our patient; however, his child's score was only Class A. This might be attributed to his relatively large liver volume reserve compensating for cirrhosis. Some authors have reported high fever and abdominal pain as frequent symptoms of sarcomatoid HCC.^[13,16] Although these were not present in this patient, the suspicion of liver abscess based on these symptoms may delay and make the diagnosis more difficult. In a patient described by Inoue *et al.*,^[13] abscess drainage followed by biopsy were performed before the diagnosis was made. At 3 weeks after admission, there was already pleural metastasis. Despite a trial of chemotherapy, the patient died of multiorgan failure on day 27. Radiologically, our patient's tumor was also suggestive of HCC with arterial enhancement and portovenous contrast washout. Honda *et al.*^[17] described delayed or prolonged contrast enhancement as a feature of sarcomatoid HCC. This finding may be attributed to the presence of active cancerous tissue with fibrous stroma. The aggressiveness of this type of tumor is highlighted by the high incidence of local invasion and metastatic spread either at the time of surgery or early recurrence. Indeed, there was invasion into the right hemidiaphragm of > 50% in our patient. Both intra and extrahepatic metastasis as well as lymphadenopathy is common.^[16] Metastasis to multiple organs including the lungs, hepatic hilar lymph nodes, greater omentum, stomach and diaphragm as seen in our patient have been reported.^[4]

Prognosis is inevitably poor with such aggressive behavior of these tumors with high risk of metastasis. In the cases reported in the literature, most patients have developed metastatic lesions within 3-6 months of diagnosis and survival rarely exceeded 12 months. This is exemplified in the present patient, where metastatic lymph nodes and peritoneal nodules were already identified 1-month post-surgical resection. In a study reported by Aishima *et al.*,^[18] 7 (17.5%) out of 40 patients had a sarcomatous component in combined HCC and CC. The rest of the patients

were divided according to the percentage of CC. Sarcomatous patients were found to have significantly higher chance of vascular invasion, lymph node metastasis as well as high CC percentage group, and were associated with a much shorter survival compared to those without a sarcomatous and lower CC component ($P < 0.0048$). Such aggressive lesions make the diagnosis and treatment difficult. Patients are either diagnosed only at post-mortem or at an advanced unresectable stage. There is currently no recommended treatment strategy for sarcomatoid carcinomas, and surgical resection appears to be the only curative option, albeit only at the early stage of tumor formation. There is yet no report of successful adjuvant treatment with chemotherapy or radiotherapy described in the literature to prolong survival in patients with this aggressive tumor.

In conclusion, a case of hepatic combined sarcomatoid HCC and CC is reported here. This is an extremely rare tumor with an aggressive behavior. The prognosis is extremely poor, and survival has been limited to within a few months in most cases. Pre-operative diagnosis and distinction from ordinary HCC is difficult. Reports in the literature suggest surgery as the treatment of choice at present. Due to the rarity of the disease, adjuvant chemotherapy or radiotherapy has not shown any promise. Early diagnosis with a high index of suspicion appears to give the best chance of cure.

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Successful living donor liver transplantation in a cystic fibrosis patient with combined hepatocellular carcinoma and cholangiocarcinoma

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ABSTRACT

Combined hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) is a rare tumor entity. In this report, we describe a case of a young patient who developed a liver tumor in a cirrhotic liver caused by cystic fibrosis. All diagnostic findings suggested that this tumor was an HCC. We performed living donor liver transplantation. Histological examination of the tumor revealed combined HCC and CC as an incidental finding. Two years after the transplantation, the patient is in good clinical condition and is disease-free.

Key words: Cholangiocarcinoma; cystic fibrosis; hepatocellular carcinoma; liver transplantation; liver tumor; living donation

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INTRODUCTION

Liver cirrhosis is one of the major risk factors for the development of hepatocellular carcinoma (HCC), which is the one of the leading causes of cancer-related deaths worldwide.^[1] Cystic fibrosis (CF) might be associated with liver diseases including liver cirrhosis requiring liver transplantation or combined lung-liver transplantation. These hepatobiliary diseases are the third-leading cause of death in CF patients.^[2] Combined HCC and cholangiocarcinoma (CC) is a rare tumor entity, which is defined as a tumor in which both HCC and CC components co-exist in either the same tumor or the same liver.^[3]

As yet, there is no report of a combined HCC and CC in cirrhotic liver caused by CF. We present a case of a young

patient who was transplanted at our center with an incidental finding of a combined HCC and CC in a cirrhotic liver due to CF.

CASE REPORT

A 29-year-old patient was admitted to our transplantation unit due to a recently diagnosed liver tumor with underlying liver cirrhosis. The cirrhosis was known and treated for years since the patient suffered from CF since his childhood. The single liver tumor was localized in segment V with a diameter of 6.5 cm. The computed tomography scan showed a hyper-vascularized tumor in the arterial phase with the aforementioned extent [Figure 1]. Tumor marker analysis showed a marked elevation of α -fetoprotein (808.7 ng/mL, normal range: < 6.6 ng/mL) and a slight increase of carbohydrate antigen (CA) 19-9 (48.9 U/mL, normal range: < 37 U/mL). All other tumor markers were normal. Taken together, we diagnosed an HCC in a cirrhotic liver caused by CF. To rule out any extra-hepatic tumor manifestation, a positron emission tomography scan (18-fludeoxyglucose, activity: 222 MBq) was performed showing a moderately increased glucose metabolism within the liver tumor (standard uptake volume in tumor: 6.5; standard uptake volume in liver: 2.4), but no signs of any extra-hepatic tumor spread [Figure 2].

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Figure 1: Computed tomography scan of the tumor (white arrows) in the arterial phase

This young patient with a relatively large suspected HCC seemed to be a good candidate for liver transplantation. Despite the CF, the lung function of the patient was good (forced expiratory volume in one second 69%, forced vital capacity 88%), and, therefore, a combined lung-liver transplantation was not necessary.

Because the tumor exceeded the Milan criteria, listing the patient for liver transplantation with a Model for End-stage Liver Disease (MELD) exception was not possible. As a “bridging-to-transplant” therapy, we performed a transarterial chemoembolization [Figure 3]. The patient’s liver function was still good, reflecting a MELD score of 8 (bilirubin 16 $\mu\text{mol/L}$; creatinine 76 $\mu\text{mol/L}$; international normalized ratio 1.2); therefore the chance of receiving an organ offer within a short time period *via* a MELD-based allocation was low.

We decided to perform living donor liver transplantation (LDLT). The brother of the patient offered to be the donor. To rule out CF in the donor, we supplemented our standard donor evaluation procedure with consultation by CF experts. Three months after diagnosis of the tumor, we performed successful LDLT using the right liver lobe as graft.

Surprisingly, the tumor not only showed signs of a HCC (positive for cytokeratin 8, TTF-1 and hepatocyte paraffin 1), but also signs of a CC (positive for cytokeratin 7 and CA 19-9) in the histopathologic examination. Therefore, a combined HCC and CC (Allen and Lisa type C) was diagnosed.

Two years after LDLT, the patient remains disease-free and is in good clinical health. Tumor markers are all in the normal range (α -fetoprotein: 3.9 ng/mL; CA 19-9: 20.1 U/mL).

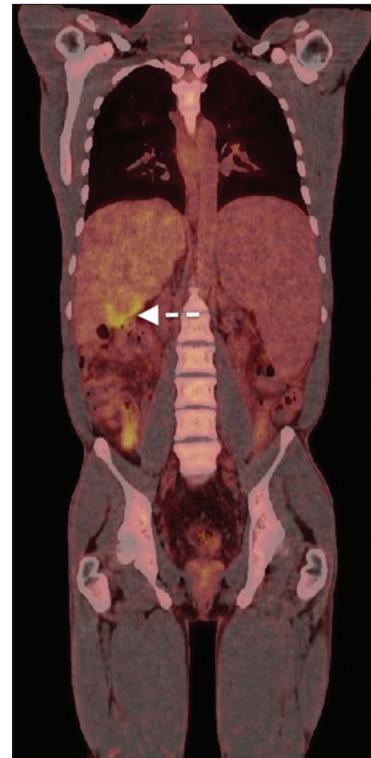


Figure 2: Positron emission tomography scan of the tumor (white arrows) indicating a moderately increased glucose metabolism

DISCUSSION

To our knowledge, this is the first case of a combined HCC and CC in a patient suffering from CF as the underlying disease.

Liver resection provides similar mid-term survival between patients with HCC and patients with combined HCC and CC. However, liver transplantation provides significantly greater 3 years survival for patients with HCC (78%) compared with patients with combined HCC and CC (48%).^[4] Park *et al.*^[5] described a 5-year survival rate of 60% after liver transplantation for combined HCC and CC. Most of the patients in this cohort experienced tumor recurrence within the 1st year after transplantation. In a small patient cohort, two of three patients died due to a metastatic tumor recurrence in the 1st year after transplantation.^[6] Thus, liver transplantation is discussed controversially for patients with combined HCC and CC. In our case, the final diagnosis of the combined HCC and CC was an incidental finding. We did not perform a biopsy of the tumor since the α -fetoprotein elevation, and the typical signs in the CT scan suggested an HCC diagnosis. It is a theoretical question whether we would have performed liver resection instead of transplantation if we had known the definitive diagnosis of combined HCC and CC prior to the transplantation. As mentioned before, most publications showed better survival outcome in such patients following liver resection.^[7,8]

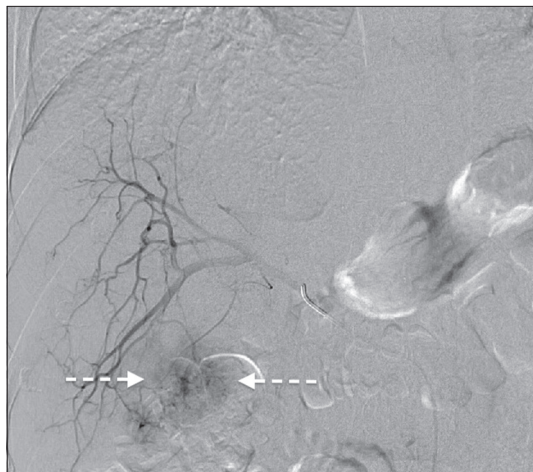


Figure 3: Tumor (white arrows) during the transarterial chemoembolization

The occurrence of a malignant liver disease in patients with CF is rare. There are a few case reports of HCC in young CF patients.^[9-11] Beyond this, there is only one case report describing a CC in a CF patient, who died soon after the diagnosis.^[12]

In this report, we present the first case describing a combined HCC and CC in cirrhotic liver caused by CF. After successful LDLT, the patient remains disease-free.

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The evolving role of laparoscopic ablation

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In the last two decades, the etiology of hepatocellular cancer has shifted from hepatitis B to hepatitis C with the incidence of nonalcoholic steatohepatitis (NASH) rising dramatically. This sudden rise in NASH and newly diagnosed cases of hepatocellular cancer may represent the tip of an epidemic. Current recommendations for patients with chronic hepatitis B or hepatitis C infections should be screened with routine use of ultrasound. Despite this Surveillance, Epidemiology, and End Results, data would suggest that the average size of newly diagnosed hepatocellular cancer is beyond the Milan criteria at the time of diagnosis. In the setting of fatty liver disease and NASH, an early serologic diagnosis is not evident.

Significant limitations already exist in the availability of liver allografts for liver transplantation. This constraint in organ availability necessitates improved loco-regional therapies. Open and laparoscopic liver resection have been traditionally limited to Child's A and early B cirrhotics. In recent years, more sophisticated evaluations have been proposed including transjugular pressure gradients and vital dye excretion. With the unfortunate limitations in transplantation and resection, ablation has become the mainstay of therapy. Chemoembolization and later radioembolization with yttrium have been most frequently employed a technique for ablation.

Surgical ablation initially was hampered by technologic limitations. This included liver fracture and profuse bleeding associated with cryotherapy, and prolonged treatment times and patient hyperthermia with radiofrequency ablation. Radiofrequency ablation with its slow thermal evolution was susceptible to heat sink and inadequate ablations. The most recent evolution in ablation was the development of microwave ablation. Microwave catheters were designed to decrease treatment times and overcome thermal sinks.

Despite the improved treatment capacity, microwave catheters create more oblong ablation cavities. The treatment areas are not exact with potential marginal recurrence from inadequate ablations. Next generation microwave catheters are being designed to generate uniform ablation defects. Alternative treatment options include advanced imaging registration and catheter placement. In this current issue of *Hepatoma Research*, the role of laparoscopic ablation is discussed and evaluated. Additional articles discuss the mechanism and role of sorafenib in the adjuvant and primary treatment of unresectable hepatocellular cancer.

In the next decade, the number and incidence of unresectable and untransplantable liver cancers will increase dramatically. This necessitates improvements in loco-regional control including ablation technologies and adjuvant systemic therapies.

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Preneoplastic foci in mice fed diethyl 1, 4-dihydro, 1, 4, 6-trimethyl 3, 5-pyridine decarboxylate are resistant to protoporphyrin accumulation

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It has been shown that preneoplastic liver cell foci and hepatic nodules generated by thioacetamide (TAA) in drug-primed mice, which were first fed diethyl 1, 4-dihydro, 1, 4, 6-trimethyl 3, 5-pyridine decarboxylate (DDC) or griseofulvin (GF) for 5 months were resistant to protoporphyrin accumulation.^[1] DDC or GF are potent porphyrinogenic drugs and accumulate protoporphyrin in the mouse liver. Although DDC or GF are withdrawn for 1 month, when treated with TAA, the nodules formed on the 5th or 7th day of treatment were devoid of protoporphyrin deposits. Feeding DDC or GF for 8 months induced liver tumors which were devoid of protoporphyrin deposits. In contrast, the surrounding liver tissue contained numerous protoporphyrin deposits. This could be attributed to the decreased activity of delta-aminolevulinate synthase, the first rate-limiting enzyme in protoporphyrin synthesis. Furthermore, there could be an increased activity of ferrochelatase, which catalyzes the incorporation of iron into protoporphyrin, and increased activity of heme oxygenase, the last enzyme in porphyrin degradation. Protoporphyrin deposits were found in the normal liver parenchyma, but not in the hyperplastic nodules (HNs) or hepatocellular carcinomas formed in mice fed GF or DDC for 5-12 months.^[2-4] C3H male mice, aged 4 weeks (Harlan, Sprague-Dawley, San Diego, CA, USA), were fed a protein-rich 25% semisynthetic

complete standard Tekland test diet *ad libitum* (Tekland, Madison, WI, USA) with 0.1% DDC or 2.5% GF for 1 year and then sacrificed. Control mice were fed the basal diet without DDC. As soon as the mice were killed, their liver was removed. Portions of the liver were fixed in formalin and embedded in paraffin for light microscopic analysis by hematoxylin-eosin and gamma glutamyl transpeptidase (GGT) was accessed by histochemistry. Figure 1 illustrates an HN from a mouse fed DDC for 1 year. The tumor is devoid of protoporphyrin and is surrounded by normal liver parenchyma filled with protoporphyrin deposits. Figure 2 shows hyperplastic foci stained red for GGT, a marker for HN, showing that the precursor lesion is devoid of protoporphyrin deposits. HN or foci are generated during the process of liver cancer development in response to chemical carcinogens or dietary

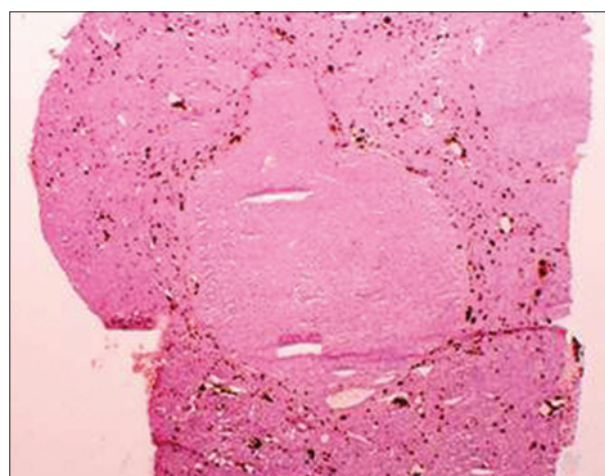


Figure 1: Liver from a mouse fed diethyl 1, 4-dihydro, 1, 4, 6-trimethyl 3, 5-pyridine decarboxylate for 1 year (x57). Note the hyperplastic nodule is devoid of brown protoporphyrin pigment, whereas the surrounding normal liver parenchyma is filled with protoporphyrin deposits

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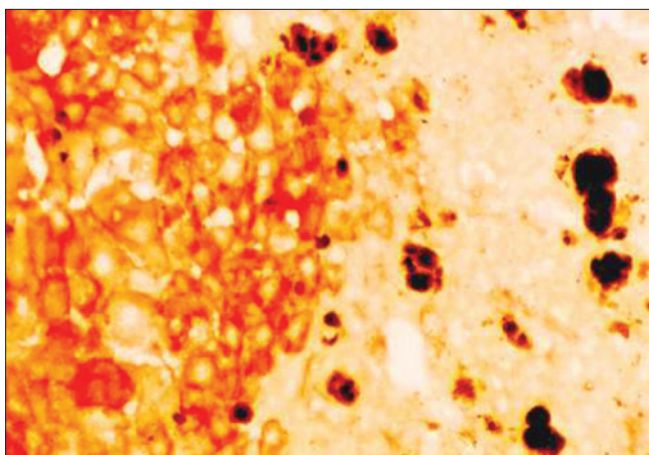


Figure 2: Liver from a mouse fed diethyl 1, 4-dihydro, 1, 4, 6-trimethyl 3, 5-pyridine decarboxylate (DDC) for 5 months, then withdrawn from DDC for 1 month, then refed DDC for 3 days (x567). Note that the hyperplastic nodule stained red for gamma-glutamyl transferase is devoid of protoporphyrin pigment whereas the normal liver parenchyma has numerous protoporphyrin deposits as shown

manipulations. HN is a population of cells from which hepatocellular carcinoma can develop. The HN studied here resembles resistant phenotypes which grow in an otherwise toxic environment of potent porphyrinogenic drugs DDC and GF. The HN is resistant to protoporphyrin accumulation whereas the surrounding normal liver is filled with protoporphyrin. This observation is further supported by the fact that HNs generated by diethylnitrosamine initiation and various promoting regimens are resistant to their own promoting agents. For example, the nodules produced by resistance to a cytotoxicity model using 2-acetyl aminofluorene (2-AAF) are resistant to 2-AAF metabolism.^[5] The nodules produced by choline methionine deficient diet are resistant to fat accumulation.^[6] The HN nodules generated in orotic acid models are resistant to imbalances of nucleotide pools created by orotic acid;^[7] nodules generated

in lasiocarpine model are resistant to megalocytic effect of *Senecio* alkaloid.^[8] These selective resistances to cytotoxicity models appear to be precursor lesions for hepatocellular carcinoma development.

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Radiofrequency, microwave, and laser ablation of liver tumors: time to move toward a tailored ablation technique?

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INTRODUCTION

Primary and secondary hepatic tumors are quite common and constitute a significant source of mortality. Primary liver cancer is the fifth most common cancer worldwide and the third most common cause of cancer mortality, and secondary involvement of the liver, particularly from colorectal carcinoma, is even more common.^[1,2]

Although surgical resection remains the gold standard for eligible patients with hepatocellular carcinoma (HCC) or liver metastases (LM), and liver transplantation is considered the standard therapy for selected patients with HCC, in the last years the role of ablation therapies in the treatment of primary and secondary liver tumors continued to increase, as they have widely been proven to be effective and safe.^[3-10] They play a key role in the treatment of patients who are not eligible, poor candidates for surgery, or who refuse surgery and are increasingly used as a bridge to liver transplantation in patients with HCC. In addition, some recent studies suggested that radiofrequency ablation (RFA) is as effective as surgical resection in the treatment of very early HCC.^[11,12] Depending on tumor size and number, thermal ablation can be chosen as the only treatment, combined with systemic therapies, surgery, or other regional treatments, in order to utilize a multimodality approach to the patient aimed at the best treatment result.

Thermal ablation techniques include either heating ablation [RFA, microwave ablation (MWA), laser thermal ablation (LTA), and high-intensity focused ultrasound] or freezing ablation (cryoablation). This paper will deal with the “hot” ablation techniques, focusing on RFA, MWA, and LTA. RFA is the most used technique worldwide, and its efficacy has been largely proven over the last 20 years. However, a lot of clinical studies suggest that MWA and LTA are as effective as RFA, and the choice of the thermal ablation modality is usually determined by the experience and preference of the interventional oncologists and radiologists, as well as by the availability of the different devices in the single centers.^[13-16] RFA, MWA, and LTA share some main technical aspects. In brief, all of them rely on controlled thermal energy delivery aimed at raising the tissue temperature between 60 °C and 100 °C to determine coagulative necrosis of tumor lesions,^[17,18] as well as the placement of a needle (RFA electrode, MWA antenna, or LTA fiber through a fine needle) into the target lesion. On the other hand, each thermal technique shows peculiar advantages and limitations that could make each of them more suitable than the other ones to treat patients and tumors with different characteristics.

Therefore, the opening question of this paper is the following: is it time to move toward a tailored approach to thermal ablation?

RADIOFREQUENCY ABLATION

RFA is based on alternating current of RF waves. The alternating current transmitted via an insulated electrode tip inserted into the tissue generates ionic agitation and frictional heats that extend into adjacent tissue by conduction. When temperature > 60 °C is achieved, the tissue heating results in coagulative necrosis of the tumor.

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RFA is the most used ablative modality worldwide, and its effectiveness and safety have been fully proven.^[3] It is an established therapeutic choice for non-surgical patients with early stage HCC, and some recent papers suggested that RFA can be as effective as surgical resection in terms of overall survival and recurrence-free survival rates in patients with small, centrally located HCC.^[11,12] RFA has also been reported to be an effective treatment of LM \leq 3 cm, in particular from colorectal cancer,^[8,9,19] and it may be indicated in resectable lesions as an adjunct to resection, in inoperable lesions that demonstrate complete or partial response after chemotherapy, or in recurrent and progressive lesions.^[18,20]

The efficacy of RFA is influenced by tumor location and size. RFA of HCC $<$ 3 cm diameter achieves complete response in over 90% of cases, whereas 50-70% of tumors 3-5 cm in size are completely ablated. RFA of HCC $<$ 5 cm can achieve equal survival benefit compared with surgical resection whereas results for ablation of HCC larger than 5 cm are poor.^[21-23] Reported rate of mortality of percutaneous RFA is $<$ 1%, and major complication rate ranges from 0.6% to 8.9%.^[24,25]

Advantages of RFA

RFA is the best established and well experienced thermal technique among all the thermal modalities available. Its efficacy, feasibility, and safety have been largely proved.

Limits of RFA

Large lesions can require multiple overlapping ablations to create an adequate safety margin. Although it has recently been reported that the treatment of sub-capsular or high-risk located nodules does not increase the rate of treatment-related complications,^[26] the sub-capsular or high-risk position of the nodules can represent a relative contraindication to RFA. Tumors strictly close to large vessels can be incompletely treated due to the heat-sink effect, in which thermal energy produced by ablation is partially shunted away from the tumor by the cooler blood.^[27,28]

MICROWAVE ABLATION

MWA utilizes MW frequency (typically at 900-2,500 MHz) to cause oscillation of polar molecules in tissue (primarily water), increasing their kinetic energy and the temperature of the tissue. Although the final effect of MWA consists in coagulative necrosis of the lesion like RFA, the mechanism of heating differs substantially, since MW energy radiates into the tissue through an interstitial antenna that determines direct heating of the lesion. Whereas RF heating requires an electrically conductive path, MWs can propagate even through tissues with low electrical conductivity, high impedance, or low thermal conductivity, like charred or desiccated

tissues.^[29,30] MW can generate very high temperatures inside the lesion in a very short time, potentially leading to improve treatment efficacy and to obtain larger ablation volumes.

However, the use of MWA was limited for a long time because of technical limitations of some currently available MW systems. Major limitations included low power, shaft heating, large diameter probes (13-14 gauge), small ablation areas requiring multiple insertions, and non-spherical ablation volumes, which have discouraged clinical application of MWA in many western countries. Adding a cooling jacket around the antenna was demonstrated to decrease cable heating, thus increasing the amount of power that can be safely delivered.^[31] The introduction of a choke coil into the distal portion of the antenna was also proposed to decrease back heating effects, but this remedy caused remarkable thickening of the antenna, making the devices unsuitable for the percutaneous application. Recently, a miniaturized device has been developed (the so-called Mini Choke[®]), which minimizes the back-heating effects using slender MW antennas (14-16 gauge), and allowing for the percutaneous application.^[32]

Several prior studies demonstrated that early-generation MWA had equal effectiveness, safety, and survival when compared with RFA, with shorter ablation time.^[13,33-35] The recent technical advances (in particular, the Mini Choke[®]) have been reported to achieve coagulation of areas larger than RFA.^[36-38] Such a capability could result useful in the treatment of tumors \geq 3 cm. A randomized prospective comparison of MWA and RFA in the treatment of HCC did not demonstrate any difference in the rates of residual or untreated disease,^[39] and a local control rate up to 95% over a median follow-up of 33 months was reported in the intraoperative treatment of colorectal LM.^[40] Mortality and major complication rates using the most recent MWA devices are similar to RFA.^[32]

Advantages of MWA

MWA offers some advantages compared with RFA, including greater intratumoral temperature, deeper penetration of energy, propagation across the poorly conductive tissue, less sensitivity to the heat-sink effect, and larger ablation volume. Such peculiarities enable to treat larger tumors with adequate safety margin, and nodules closed to large vessels. In addition, MWA does not need the use of grounding pads.

Limits of MWA

Microwave energy is more difficult to distribute than RF energy. MW energy is carried in wavelengths, which are more cumbersome than the small wires used to feed energy to RF electrodes, and are prone to heating when carrying large amount of power. Consequently, MWA appears less

feasible than RFA in the treatment of high-risk located and subcapsular nodules. In addition, MWA is more expensive than RFA and LTA.

LASER ABLATION

LTA utilizes laser devices that convert electrical into light energy, which determines tissue heating and cellular death by coagulative necrosis. Neodymium: Yttrium aluminum garnet (wavelength of 1,064 nm) and diode (wavelength of 800-980 nm) lasers are most commonly used, as penetration of light is optimal in the near infrared spectrum. Light is delivered via flexible bare tip fibers with a diameter from 300 to 600 μm . A bare-tip fiber provides an almost spherical thermal lesion of 12-15 mm in diameter, and a beam-splitting device or a multi-source device allows the use of up to four fibers, simultaneously delivering the light into each single fiber.^[15-17] The optical and thermal characteristics of the tissue, as well as the proximity of blood vessels, determine the thermal diffusion of the light energy and define the ablation area. Bare tip fibers are inserted through 21-gauge needles into the lesions. Usually, one to two fibers are used to treat nodules up to 1.5 cm in diameter, three fibers to treat nodules from 1.5 to 2.5 cm, and four fibers with tips arranged in a square configuration to treat nodules > 2.5 cm. In addition, the pullback technique can be used to treat larger nodules.

LTA has been investigated less vigorously than the other ablation techniques, but it seems to show the same efficacy and safety profile than RFA, with a shorter treatment time per session.^[16,17,41-43] Most of the studies on LTA are focused on the treatment of HCC. Using from one to four fibers on the basis of tumor size, the reported complete response rates range from 82% to 97%.^[44-46] Mortality rate is < 1%, and major complication rate ranges from 0.1% to 3.5%.^[47]

Advantages of LTA

The main advantage of LTA is its feasibility, as LTA utilizes very fine needles to insert the fibers into the lesion. Such a characteristic makes LTA particularly safe for the treatment of nodules with difficult location. Furthermore, the possibility to use from one to four fibers allows to achieve ablation areas different in size, enabling to treat lesions different in size or multiple small lesions in the same session, sparing the normal parenchyma as far as possible. In our experience, LTA is the cheapest ablation technique when up to three fibers are used, and it is cheaper than MWA when four fibers are used.

Limits of LTA

The correct placement of the fibers can result technically

difficult, particularly if more than two fibers are needed and should be performed by very skilled operators. The efficacy of LTA can be limited by the heat-sink effect.

FINAL CONSIDERATIONS

Surgical resection is the treatment of choice for LM, and liver transplantation, whenever possible, is the best curative option for HCC. However, in recent years thermal ablation therapies have become more and more central in the treatment of liver lesions, as the majority of patients are not eligible for surgery. Moreover, some recent studies suggested that RFA is as effective as surgical resection, or even preferable in selected patients, in the treatment of small HCC lesions.^[11,12] Good local tumor control and survival comparable with surgical resection were also reported in subgroups of patients with LM from colorectal and breast cancer.^[18,19] Thermal ablation of HCC in patient waiting for liver transplantation can be performed as a bridge to the transplantation. Likewise, thermal ablation combined with resection and/or systemic chemotherapy has been demonstrated to improve the survival in patients with LM.

Thermal therapies are minimally invasive, well tolerated, and demonstrate a very low rate of major complications. Although RFA represents the “historical” and more experienced thermal ablation technique, both MWA and LTA have been demonstrated to be as effective and safe as RFA when performed by skilled operators.^[13-16] Therefore, to date in most centers of interventional oncology or interventional radiology the choice of the ablation technique usually depends on the physicians’ preference and experience. However, when all the three “hot” ablation techniques are available in a single center, which thermal treatment should be preferred?

Each thermal modality presents peculiar technical characteristics, advantages, and limitations. Likewise, patients can have some contraindications to some ablation technique (for instance, the presence of a pacemaker is a relative contraindication to RFA), and tumors can differ in number, size, and location. It follows that, in our opinion, the choice should be based on the characteristics of the patient, tumor, and ablation techniques. For this purpose, we suggest the algorithm adopted in our Section of Interventional Ultrasound, aimed at tailoring the thermal treatment on the single patient to achieve the best outcome [Figures 1 and 2].

In short, a single nodule 2 cm or smaller in size can efficaciously be ablated using all the thermal modalities. Both RFA and LTA are cheaper than MWA and should be preferred unless the

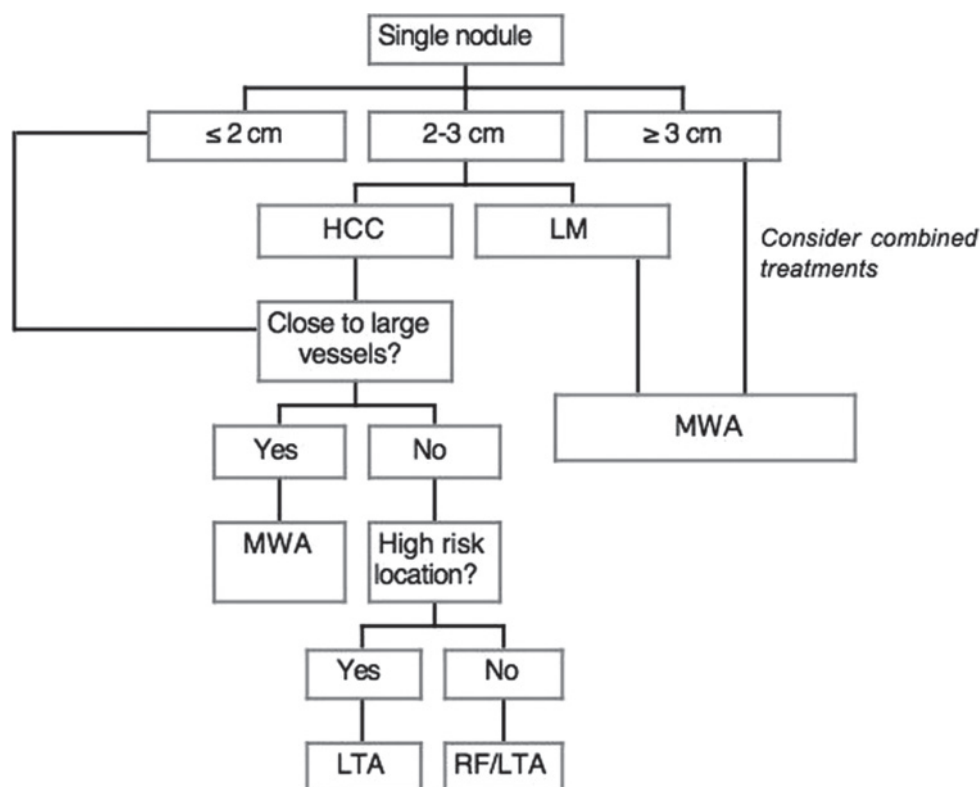


Figure 1: Proposed algorithm for thermal ablation of single nodule. HCC: hepatocellular carcinoma; LM: liver metastases; MWA: microwave ablation; LTA: laser thermal ablation; RF: radiofrequency

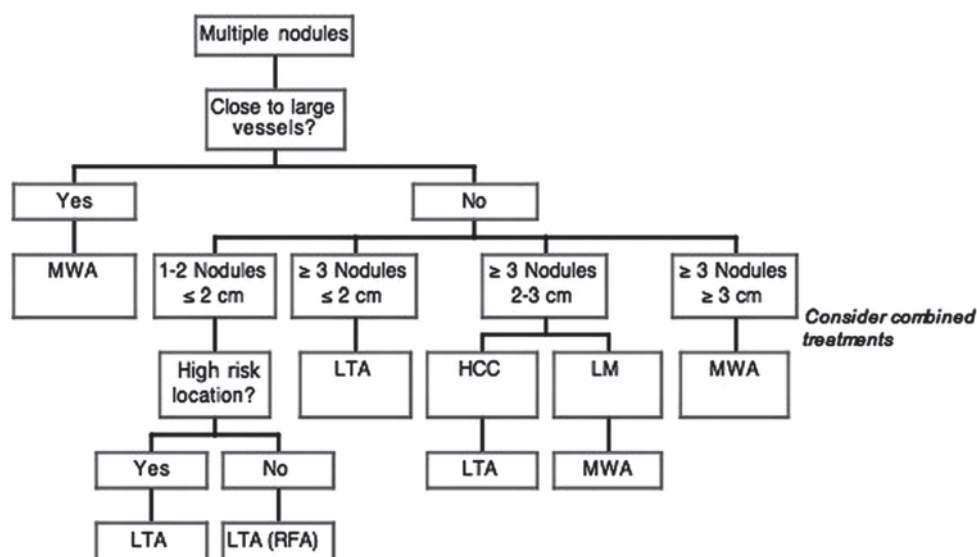


Figure 2: Proposed algorithm for thermal ablation of multiple nodules. HCC: hepatocellular carcinoma; LM: liver metastases; MWA: microwave ablation; LTA: laser thermal ablation; RFA: radiofrequency ablation

tumor is close to large vessels, as MWA is not affected by the heat-sink effect. In addition, LTA could be more feasible and safe when the nodule has a difficult or risky location, as the needles used to insert the fibers are considerably finer than RFA electrodes. Single HCC 2-3 cm in size could be ablated following the same indications, even though multiple overlapping insertions can be needed for RFA, and at least

three fibers should be used for LTA. Conversely, LM 2-3 cm in size can take advantage of MWA because the safety margin has to be greater than for HCC. Likewise, both single HCC and LM ≥ 3 cm should be treated with MWA.

Multiple small lesions (maximum 2.5-3 cm in diameter), especially if they vary in size, should preferably be treated

with LTA, as the ablation area can be diversified according to the lesion size using from one to four fibers, thus sparing the normal tissue as far as possible. Finally, MWA should always be preferred when the tumors are close to large vessels or are ≥ 3 cm in diameter, independently of the number of the nodules.

CONCLUSION

We have to answer the opening question of this paper. In our own personal opinion, the answer is: yes, it's time to move toward a tailored approach to thermal ablation.

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Hepatocellular carcinoma in elderly patients: a concise review on systemic therapy with sorafenib

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ABSTRACT

The treatment of hepatocellular carcinoma (HCC) in elderly patients is unclear. In particular, the efficacy and safety of sorafenib as a systemic treatment in these patients is still under debate. We performed a concise review of sorafenib therapy in this population. However, it is important to make any decisions on treatment for elderly patients with HCC through a multidisciplinary team that includes experts in the liver disease. Patients with good clinical conditions should be treated with sorafenib.

Key words: Hepatocellular carcinoma; sorafenib; treatment; chemotherapy; elderly patients

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid organ malignancy worldwide and the second cause of cancer-related mortality,^[1] with the highest incidence rates in areas where hepatitis B virus infection is endemic such as South-East Asia and sub-Saharan Africa.^[2] Similar to other common cancers, the incidence of HCC increases in relation to age. In Western countries, it tends to peak at the age of 75 years and in the United States it rarely occurs before the age of 40 years. In Chinese and in black African populations it generally occurs in younger patients.^[2]

Well-known risk factors typically characterize the development of HCC. The most frequent conditions include chronic viral hepatitis (types B and C),^[3] alcohol intake, and aflatoxin

exposure.^[4] Cirrhosis is another important risk factor, which may be triggered by chronic viral hepatitis, alcohol or inherited metabolic diseases. Therefore, in up to 90% of cases, HCC becomes progressively worse on account of underlying liver diseases so that in most patients, prognosis and management are influenced by the presence of two separate entities: Chronic hepatitis with or without cirrhosis and HCC.^[3,5,6] Consequently, the choice of the appropriate HCC therapy should consider the limitations presented by underlying liver diseases.

In the most recent HCC guidelines (EASL/EORTC, AASLD, AISE, AIOM), disease staging includes tumor characteristics, underlying liver cirrhosis, and performance status. Treatment allocation is then based on these parameters. Patient age is not taken into account though there is an increasing focus on elderly patients. Due to the aging of the population, this group represents the fastest growing segment of populations with cancer.

The aim of this investigation was to review all the gathered experience of using sorafenib, a targeted multikinase inhibitor, in the treatment of HCC, with a focus on the evaluation of safety and the efficacy of this agent in the elderly as compared to younger patients.

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DEFINITION OF “ELDERLY PATIENT”

Due to the fact that aging involves a progressive shortening of life expectancy and reduction in the functionality of organ systems, the definition of an “elderly patient” is somewhat hazy and still a matter of debate.^[7] What is the upper limit of age in which a patient is considered as old? Should the cut-off of 70 years used in clinical studies and in current clinical practice to be considered an acceptable and reasonable boundary? Is age the only parameter to take into account for defining an elderly patient or should the so-called stage of aging be evaluated more extensively? It has been demonstrated that the standardized geriatric evaluation systems, assessing additional parameters such as comorbidities, cognitive, and health status, may better correlate with therapy toxicity and patient outcomes,^[8] and could subsequently supply additional information to the standard performance status scale, such as KPS or ECOG PS, normally used in oncology. However, there has yet to be a general consensus on what the best geriatric evaluation system to use is.

Due to their fragile conditions, elderly patients are generally perceived to be more susceptible to the toxic effects of cancer therapy and, as a result, gain less clinical benefits from treatments due to frequent dose interruptions or permanent discontinuations of the drug. This perception could be related to the fact that this population is underrepresented in clinical trials, which are often conducted excluding patients exceeding a certain age limit or those bearing comorbidities.

However, a growing body of evidence suggests that older patients with adequate organ function and a reasonable life expectancy should receive the same treatment as younger patients. A retrospective analysis is evaluating 13 different molecularly targeted cancer therapies found similar frequencies of drug-related adverse events among the elderly as in younger patients.^[9] To limit this gap and identify the most appropriate treatment of elderly patients, it is advisable to assess how available treatment options behave in the framework of both well-conducted clinical trials and in everyday clinical practice.

TREATMENT WITH SORAFENIB

Therapeutic options for HCC range from surgeries (resection or liver transplantation) to loco-regional therapies (percutaneous ethanol injection, radiofrequency ablation or trans-arterial chemo-embolization) and systemic treatment, depending either on the stage of tumor disease or on the underlying liver disease. Currently, treatment with sorafenib has been recognized as the only standard systemic therapy for HCC. It is indicated for patients with well-preserved liver function (Child-Pugh A class) and with advanced tumors

or for those with tumor progressing upon loco-regional therapies.^[10]

The efficacy of sorafenib in HCC was established in SHARP^[11] and Asian-Pacific,^[12] two randomized, phase III multicenter, double-blind, placebo-controlled trials that led to the approval of drug by the International Health Authorities. In both studies, sorafenib administered at the dose of 400 mg twice daily demonstrated a statistically significant improvement of overall survival and time to progression than compared to the placebo in patients with well-preserved liver function (Child-Pugh A). Sorafenib proved to be well-tolerated, with skin toxicity (hand-foot skin reaction), diarrhea, and asthenia representing the most common adverse effects. Temporary treatment interruptions and/or dose reductions along with immediate specific treatment of adverse events proved effective in managing adverse drug effects.

In the SHARP trial, the median age of patients treated with sorafenib was 64.9 ± 11.2 years, whereas the Asia-Pacific trial was 51 years (range 23-86). In both studies, the inclusion criteria did not set an upper age limit. The Asia-Pacific study included younger patients on account of the earlier onset of HCC in those regions. The preplanned subgroup analysis according to the patients' age grouping in the Asia-Pacific study showed that sorafenib provided similar clinical benefits in both younger (< 65 years) and older (≥ 65 years) patients. Following the two registrative trials, the use of sorafenib has also been evaluated in large patient populations treated according to everyday clinical practice.

Global Investigation of therapeutic DEcisions in HCC and Of its treatment with sorafeNIB, is an international, post-approval, prospective, non-interventional study undertaken to evaluate the safety and the efficacy of sorafenib in patients with unresectable HCC, in which the inclusion criteria of patients closely corresponded to that of real-life practices.^[13] This study carried out in 39 countries worldwide, enrolled 3,371 patients; of these, 3,202 were available for the evaluation of safety. Two interim analyses (after the accrual of 500 and 1,500 patients, respectively) and the final analysis confirmed the safety profile of sorafenib previously recorded in the phase III pivotal trials, without detecting any new unexpected adverse events. A breakdown of the safety of sorafenib according to age groups was available only for the second interim analysis (1,571 patients).^[13] The comparison of sorafenib safety profiles in younger (< 65 years, $n = 883$) and older patients (≥ 65 years, $n = 688$) showed that the incidence of adverse events, drug-related adverse events, and serious adverse events was independent of age, similar in both older and younger patients. It is interesting to

note that even the adverse events resulting in permanent discontinuation of the drug were similar in both groups.^[13]

The impact of age on the effects of sorafenib in clinical practice was also examined in different single and multicenter experiences^[14-20] and discussed in a few reviews.^[21,22] A table summarizing the efficacy and the safety of older and younger patients treated with sorafenib reported in most papers so far published are reported [Table 1].

The first non-Asian study investigating the use of sorafenib in a large cohort of elderly patients was published in 2013. Di Costanzo *et al.*^[14] analyzed a cohort of consecutive patients not eligible for surgery or loco-regional treatment, with Child-Pugh score ≤ 7 , treated with sorafenib. Clinical outcomes and treatment-related adverse events were compared between younger (< 70 years) and older (≥ 70 years) patients. Overall, 150 patients (90 in the younger and 60 in the older group) were evaluated. The study

Table 1: Synoptic table outlining the results in younger and older patients with HCC treated with sorafenib

Authors	Study design	Efficacy	Safety
Cheng <i>et al.</i> ^[12]	Open, randomized, preplanned subgroup exploratory analysis	Similar efficacy between < 65 years and > 65 years (OS = 6.5 months)	-
Lencioni <i>et al.</i> ^[13]	Open	-	Safety of sorafenib according to age groups only for the second interim analysis (1,571 patients). Comparison of sorafenib safety profile between younger (< 65 years, $n = 883$) and older patients (≥ 65 years, $n = 688$) showed that the incidence of adverse events, drug-related adverse events, and serious adverse events was similar in both older and younger patients independently of age Grades 3-4 AEs: < 70 years (15.7%), ≥ 70 years (9.2%)
Di Costanzo <i>et al.</i> ^[14]	Open	150 patients < 70 years ($n = 90$): treatment duration = 4 months, TTP = 8 months, OS = 12 months ≥ 70 years ($n = 60$): treatment duration = 4 months, TTP = 12 months, OS = 16 months	
Edeline <i>et al.</i> ^[15]	Retrospective	129 patients < 70 years ($n = 78$): PFS = 5.6 months, OS = 9.6 months ≥ 70 years ($n = 51$): PFS = 5.6 months, OS = 12.6 months	Similar between the two groups: occurrence of severe toxicities (41.0% vs. 51.0%) and hospitalization due to toxicity (9.0% vs. 13.7%). Asthenia and bleeding more frequent in the elderly
Jo <i>et al.</i> ^[16]	Retrospective	185 patients < 80 years ($n = 161$): OS = 10.5 months ≥ 80 years ($n = 24$): OS = 11.7 months No difference in response rate	No difference as for frequency and severity of AEs
Montella <i>et al.</i> ^[17]	Retrospective	60 patients > 60 years Disease control rate = 80%, stable disease = 76.6%, TTP = 7 months, OS = 10 months	Thrombosis correlated to TTP. Full doses in 11 out of 60 patients (18.3%)
Francini and Bianco ^[18]	Retrospective	31 patients, aged between 70 and 83 years	AEs were reported in all patients, mostly during the 1st month and of grade 1 or 2. Grade 3 side effects: fatigue (22.6%), hand-foot skin reaction (19.3%), thrombocytopenia (12.9%), hyperbilirubinemia (9.7%), abdominal pain (9.7%), and only in one case, diarrhea (3.2%). No grade 4 toxicity Sorafenib has a positive impact on self-sufficiency and quality of life
Morimoto <i>et al.</i> ^[19]	Retrospective	76 patients < 75 years ($n = 52$), ≥ 75 years ($n = 24$) Average OS and the median TTP were comparable between two dose regimens 400 bid and 400 qb (5.3 months vs. 5.0 months, $P = 0.839$)	The median treatment duration and overall incidence of ADRs were not statistically different with increasing age Subgroup analysis revealed that treatment discontinuation because of ADRs was more frequent among the ≥ 75 years (41.7%) than among the < 75 years (15.0%) AEs with a standard dosage of sorafenib: 41.7% in patients ≥ 75 years and 15.0% in patients < 75 years. This difference is statistically significant. With half-dose regimen, no difference between the age groups was observed
Wong <i>et al.</i> ^[20]	Retrospective	172 patients ≥ 70 years ($n = 35$): PFS = 2.99 months, OS = 5.32 months < 70 years ($n = 137$): PFS = 3.09 months, OS = 5.16 months	Grades 3-4 AEs: ≥ 70 years (68.6%), < 70 years (62.7%)

HCC: hepatocellular carcinoma; AEs: adverse events; OS: overall survival; TTP: time to progression; PFS: progress free survival; ADRs: adverse drug reactions

showed that in elderly HCC patients with cirrhosis, sorafenib is as safe and effective as in younger patients. No unexpected adverse events related to advanced age were observed. Temporary and permanent sorafenib discontinuations were more frequent in older than in younger patients. However, this difference did not turn out to be statistically significant.

A recently published retrospective study on 129 patients compared the efficacy and safety of sorafenib in HCC patients with different ages (≥ 70 and < 70 years).^[16] The efficacy and the overall safety were found to be similar between the two groups. Asthenia and bleeding were more frequent in older patients as a result of a higher use of platelet aggregation inhibitors in this population.

The efficacy and safety of sorafenib in patients ≥ 80 years old were examined in a multicenter Japanese retrospective study.^[17] One hundred and eighty-five patients were reviewed, 24 of them being ≥ 80 years old and 161 being < 80 years old. Median overall survival was greater in older patients (11.7 months) than compared to those < 80 years old (10.5 months), with a good tolerability in both groups.

Many elderly patients are frail. Frailty implies a reduced organ function, the presence of comorbidities and impairment of physical function.^[7] Concomitant assumption of different drugs could moreover interfere with sorafenib absorption. However, studies on the use of sorafenib in the elderly suggest overcoming the predisposition to consider older patients associated with poor prognosis and poor tolerance to drugs. In these first experiences, in fact, efficacy and safety of sorafenib do not seem influenced by the age. All elderly patients undergoing sorafenib treatment should be strictly monitored to evaluate physical (blood pressure, vital signs) and laboratory parameters to prevent and promptly manage adverse events. Dose adjustments, in order to alleviate adverse events, may be a successful strategy to avoid permanent discontinuation and maximize the benefit of the drug.

A comprehensive geriatric assessment (CGA) should be performed by geriatricians to evaluate the functional and global health status of these elderly patients because CGA results are closely related to the prognosis of elderly patients in general.

CONCLUSION

Given the challenges of managing the complexity of HCC often associated with underlying liver disease and the complex health conditions of elderly patients, it is extremely

important to make any decisions on treatment through a multidisciplinary team that includes experts in liver disease and in clinical oncology to perform a personal non-protocol approach for the oncological care and management of elderly patients with HCC. However, elderly patients with good clinical conditions should be treated with sorafenib.

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Hepatoma and trematode infestation: a short review

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ABSTRACT

Hepatoma is a common cancer that can be seen around the world and clinical correlation between infection and hepatoma is evident. Hepatitis virus infection is proved for its relationship with hepatoma. However, the knowledge of other infections is still limited. In this short review, the relationship between hepatoma and some trematode infestation including echinococcosis fascioliasis, opisthorchiasis, clonorchiasis and schistosomiasis are described and discussed. Opisthorchiasis and clonorchiasis are confirmed for cholangiocarcinoma carcinogenesis but still lack evidence for hepatoma carcinogenesis. Schistosomiasis can increase the severity of hepatoma.

Key words: Hepatoma; infestation; trematode

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INTRODUCTION

Hepatoma is a common malignancy seen around the world. The clinical correlation between infection and hepatoma is very interesting. Relationship between hepatitis virus infection and hepatoma is confirmed. Chronic hepatitis B and chronic hepatitis C infects can result in hepatoma carcinogenesis. However, the knowledge on other infections is still limited. Many tropical infections have been continuously studies for the relationship with hepatoma. Here, the authors summarize and present the information on hepatoma and trematode infestation.

HEPATOMA AND ECHINOCOCCOSIS

Echinococcosis is a parasitic infestation primarily observed in

liver (in humans), which is associated with liver cystic disorder called hydatid cyst. Previous reports indicate a positive correlation between liver hydatid cyst and hepatoma.^[1-6] Although it is rare, hydatid disease needs to be included in differential diagnosis of hepatoma.^[7,8] Hoffmann *et al.*^[7] noted that the final diagnosis could be derived only if the histopathological examination is done. At present, it is accepted that the co-incidence between hydatid disease and hepatoma is possible, however, there is still no conclusion on the carcinogenesis process due to hydatid disease. Kübeck *et al.*^[6] recently noted that some authors considered echinococcosis as a trigger for hepatoma and suggested for further study on such relationship. In fact, hydatid disease has a role in causing liver fibrosis and cirrhosis,^[9] which is a precancerous liver lesion. However, the lesion due to hepatic cyst and fibrosis is usually severe. The patient should not have a long survival to have a fully developed hepatoma as a consequence.

HEPATOMA AND FASCIOLIASIS

Fascioliasis is another common human parasitic infestation.^[10] The relationship between fascioliasis and biliary cirrhosis is speculated in some publications. Vítovec^[11] mentioned for the role of biliary cirrhosis of fasciolar origin

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on the hepatoma carcinogenesis is cattle. However, this is not the finding in human beings. Kabaalioglu *et al.*^[12] studied on human fascioliasis and concluded that “long-term complications are rare in fascioliasis, and malignancy or cirrhosis related to the disease has not been observed.”

HEPATOMA AND OPISTHORCHIASIS

Liver fluke infestation or opisthorchiasis is another important parasitic infestation. It is common in Indochina and has been proved for its relationship in the occurrence of cholangiocarcinoma.^[13] However, the role of opisthorchiasis on hepatoma carcinogenesis is still not conclusive at present. There are some reports on co-incidences between opisthorchiasis and hepatoma.^[14,15] Nevertheless, the epidemiological investigation still reveals no clear evidence that opisthorchiasis can induce hepatoma carcinogenesis. Suksumek *et al.*^[16] recently studied *Opisthorchis viverrini* DNA in patients with hepatocellular carcinoma (HCC) and found that the presence of parasite had no relationship to any cancer. In fact, the finding by Suksumek *et al.*^[16] is not surprising since the main mechanism of cholangiocarcinoma carcinogenesis is due to chronic biliary tract irritation by parasitic infestation. Since the *O. viverrini* does not infest in hepatic parenchyma, the induction of the HCC should not occur.

HEPATOMA AND CLONORCHIASIS

Similar to opisthorchiasis, clonorchiasis is known for its relationship with cholangiocarcinoma carcinogenesis.^[17] The mechanism is the same as that described in opisthorchiasis model. For hepatoma, there are some reports on the co-incidence between clonorchiasis and hepatoma.^[18] However, the role of clonorchiasis in hepatoma carcinogenesis is still controversial. A recent report by Tan *et al.*^[19] concluded that clonorchiasis could be an important risk factor for hepatoma. When the course of clonorchiasis is prolonged, the risk of hepatoma could increase.^[19] Hepatitis B virus (HBV) infection, alcohol consumption, and clonorchiasis might have synergistic actions in the development of hepatoma.^[19] Chen *et al.*^[20] found that excretory/secretory products of the parasite might plays an important role in hepatoma carcinogenesis. However, the study reported by Chen *et al.*^[20] is only an *in vitro* study. Chen *et al.*^[20] noted that “Csseverin”, an important excretory/secretory products, might exacerbate hepatoma carcinogenesis, however, this is only a speculation at this point.

HEPATOMA AND SCHISTOSOMIASIS

The role of schistosomiasis on hepatoma carcinogenesis is widely discussed. In animals, liver cirrhosis due to infestation

can induce hepatoma carcinogenesis.^[21,22] In humans, there are some reports on positive correlation between schistosomiasis and hepatoma.^[23-32] Since the induction of liver fibrosis in human due to schistosomiasis is observed, it is proposed that this pathology can be the underlying cause of hepatoma which may occur in future.^[25-27] In a case-control study by Khella *et al.*,^[26] it was found that the history of schistosomiasis is significantly different between case and control. A similar observation was also reported by el-Zayadi *et al.*^[31] Badawi and Michael^[30] found that schistosomiasis increased the severity of HBV infection and elevated the risk of HCC associated with the HBV infection. However, Nakashima *et al.*^[24] studied necropsies with hepatoma coincident with schistosomiasis, and concluded that chronic schistosomiasis, on its own, is unlikely to be the cause of primary liver cell carcinoma. Nakashima *et al.*^[32] also reported that HCC related to viral hepatitis B and/or C also increased in cases with underlying schistosomiasis. Yosry^[33] concluded that there is inadequate evidence for the carcinogenicity of *Schistosoma mansoni* in humans. *S. mansoni* may still be linked to HCC through potentiating effects of HBV and hepatitis C virus (HCV) on the liver. El-Tonsy *et al.*^[34] concluded that schistosomiasis accelerates hepatic dysplastic changes in the presence of other risk factors making cancer appear early and with a more aggressive nature, compared to the same risk in absence of schistosomiasis. Therefore, it can be summarized that superimposing the effects of HBV and HCV on the liver can be expected in the cases with combined schistosomiasis and hepatitis virus infection.^[33]

Conclusively, schistosomiasis can induce liver fibrosis, which is a precancerous lesion. In addition, chronic schistosomiasis is common. The increased argyrophilic nucleolar organizer regions proteins which related to increased dysplasia can be observed in the chronic schistosomiasis.^[35] In addition, new observation on the deteriorating immunological status in chronic schistosomiasis is also reported.^[36] Dysregulation of cellular immune responses, impaired T-lymphocytes and natural killer cells, can be seen in the patients and can ease the occurrence of cancer.^[36] The poor immunity is said to be partially due to the poor nutritional status,^[37] which is a common complication in chronic schistosomiasis.^[38] Hence, it is no doubt that schistosomiasis can be the cause of hepatoma carcinogenesis. Focusing on the case with combined chronic schistosomiasis and chronic hepatitis B or hepatitis C infection, a more severe liver pathology can be expected. The precancerous liver pathological change due to hepatitis virus can be superimposed by liver fibrosis induced by schistosomiasis and this should be the explanation for finding that schistosomiasis increase the severity of hepatitis related hepatoma.

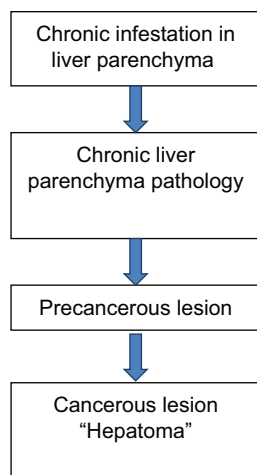


Figure 1: Common mechanism that trematode can induce hepatoma carcinogenesis

CONCLUSION

There are many reports on hepatoma and trematode infestation. Opisthorchiasis and clonorchiasis are confirmed for cholangiocarcinoma carcinogenesis, but there is still lack for the evidence on hepatoma carcinogenesis. For schistosomiasis, it is found that schistosomiasis increase the severity of hepatitis related hepatoma. Focusing on the mechanism, the trematode that has a possible role in hepatoma carcinogenesis usually has a chronic form of infestation. The chronic liver parenchyma pathology, especially for liver fibrosis and cirrhosis, is the main precancerous lesion that can further develop into hepatoma [Figure 1].

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Anti-proliferative and apoptotic efficacy of diallyl disulfide on Ehrlich ascites carcinoma

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ABSTRACT

Aim: This study was conducted to assess the *in vivo* and *in vitro* anti-tumor effects of diallyl disulfide (DADS) against Ehrlich ascites carcinoma (EAC) and to suggest its probable mechanism of action. **Methods:** EAC was induced in female mice by intraperitoneal injection of EAC-cells from stock mice. EAC-bearing mice were orally treated with 100 mg/kg body weight for 2 weeks beginning from the 1st day of EAC intraperitoneal transplantation. Cytotoxicity effects of DADS against EAC-cells *in vitro* were investigated at different concentrations (0, 6.25, 12.5, 25, 50, and 100 µg/mL) of DADS using trypan blue exclusion assay. **Results:** Data from this study exhibited a significant decrease in EAC-aliquot volume as well as total and alive EAC-cell number and a marked increase in dead EAC-cell number and percent in EAC-bearing mice treated with DADS as compared with EAC-bearing control. These changes were consistent with increased number of cells which exhibited phenotypic apoptotic signs marked by a decrease in the expression of anti-apoptotic protein Bcl-2, an increase of pro-apoptotic and cell cycle arrest mediator p53 and an elevation of DNA fragmenting indicator terminal deoxynucleotidyl transferase in EAC-bearing mice treated with DADS. In addition, the tumor marker sialic acid level was markedly decreased in plasma and Ehrlich ascites in EAC-bearing mice treated with DADS. *In vitro*, DADS also produced anti-proliferative and anti-tumor cytotoxic potentials against EAC. **Conclusion:** DADS may have anti-cancer effects which may be mediated via modulation of apoptosis and cell cycle arrest.

Key words: Anti-proliferative effects; apoptosis; diallyl disulfide; Ehrlich ascites carcinoma; imaging; semi-quantitative analysis

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INTRODUCTION

Cancer still has a high mortality rate. Recently, considerable attention has been focused on identifying naturally occurring chemopreventive and chemotherapeutic substances capable of inhibiting, retarding or reversing the multistage carcinogenesis.^[1] Thus, a preventive and/or therapeutic use

of phytochemicals could open new avenues in the search for strategies against proliferation of tumor cells. It was found by many authors that numerous biologically active phytochemicals kill cancer cells by targeting epigenetic alterations that occur during carcinogenesis and by affecting the cell cycle which is tightly regulated by a series of cell cycle regulators.^[2,3]

Organosulfur compounds of garlic including diallyl disulfide (DADS) exhibit a wide range of biological activities, including anti-mutagenic, anti-oxidant, anti-proliferative effects which could protect against critical events that are involved in the cancer process.^[4-8] In addition, available epidemiologic evidence is consistent in showing a correlation between consumption of the high amount of garlic and a reduced risk of cancer, particularly colon cancer, at

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most sites.^[9] Moreover, many publications attributed the anti-carcinogenic properties of garlic principally to allyl sulfides, among them DADS, which was shown to suppress growth and facilitate the death of different tumor cells.^[2,6-10]

This study was designed to assess the anti-proliferative and anti-tumor cytotoxic efficacies against Ehrlich ascites carcinoma (EAC) (which corresponds to mammary adenocarcinoma in female mice) *in vivo* and *in vitro*. In addition, the apoptogenic effect of DADS was assessed via following the changes in the anti-apoptotic protein, Bcl-2 and apoptotic markers, p53 and terminal deoxynucleotidyl transferase (TdT) by immunohistochemical methods, imaging and semi-quantitative analysis. The experimental study was approved by review board of Beni-Suef University.

METHODS

Tested agent (DADS)

DADS (C₆H₁₀S₂) was obtained from Fluka Chemie GmbH, Buchs, Switzerland (lot and filling code 427432/1 55004115). DADS was dissolved in dimethyl sulfoxide (DMSO) for *in vivo* and *in vitro* studies and was administered orally by gastric gavage at dose level of 100 mg/kg body weight (b.w.)/day^[11] for 2 weeks. Its structure is CH₂=CH-CH₂-S-S-CH₂-CH=CH₂ as indicated in the previous publication.^[12]

Animals and EAC-bearing model

Normal albino mice were obtained from Animal House, Institute of Ophthalmology, Giza, Egypt. EAC-bearing stock mice were obtained from Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. To induce EAC in mice for the experimental study, 0.2 mL EAC aliquot aspirated from stock mice was added to 9.8 mL saline (dilution is 1:50) and 0.2 mL of this diluted EAC was intraperitoneally administered by syringe into each mice.

Animal grouping

The EAC-injected mice were divided into two groups each containing 12 animals. Mice in group 1 (control group) were administered 10% DMSO as a vehicle in a volume equivalent to that given to treated animals. Group 2 was treated with DADS, dissolved in 10% DMSO, at a dose of 100 mg/kg b.w. The treatments were orally applied by gastric intubation between 10 and 12 AM daily for 2 weeks beginning from the 1st day of EAC-intraperitoneal inoculation.

Animal survival

The number of animals survived in each group was determined at the end of the experiment and the survival percent in each group was calculated as follows: survival percent = (number of survived animals/total number of animals) × 100.

Sampling and detection of EAC-volume and cell number

At the end of the experimental period, animals were sacrificed under diethyl ether anesthesia and 0.2 mL saline was intraperitoneally injected. One minute later, EAC-aliquot was aspirated by a sterile syringe into a test tube. The volume of EAC-fluid for each mouse was measured. The number of alive and dead EAC-cells were determined using trypan blue exclusion assay.^[13] Alive and dead EAC-cells were counted by Neubauer hemocytometer. Briefly, 40 µL of EAC-aliquot was added to 4 mL 2% trypan blue (dissolved in 0.9% saline) and the mixture was left for 5 min. One drop from the mixture was taken on Neubauer hemocytometer and the number of stained cells (dead cells) and nonstained cells (viable or alive cells) were counted. Total number of EAC-cells and percent of dead EAC-cells were calculated for each EAC-bearing mouse. This procedure was adopted from the methods of Freitas *et al.*^[14] and Chandru *et al.*^[15]

Blood samples were obtained from carotid artery of each mouse into ethylenediaminetetraacetic acid (15%)-containing tubes under mild diethyl ether anesthesia and were centrifuged at 3,000 g for 15 min. The plasma was aspirated into Eppendorf tubes and kept in deep freezer at -30 °C until used for plasma sialic acid determination. One milliliter of EAC fluid from each mouse was homogenized in 2 mL saline (0.9% NaCl) and centrifuged at 3,000 g for 15 min. The supernatant was aspirated into Eppendorf tubes and kept in deep freezer at -30 °C until used for ascites sialic acid determination.

Part of EAC-aliquot from each tumor-bearing mouse was centrifuged at 3,000 g for 15 min and the precipitated EAC-cells were fixed in neutral buffered formalin for histological and immunohistochemical studies.

Determination of plasma and ascites sialic acid concentration

Plasma and ascites sialic acid level was determined according to the method of Warren.^[16] In this method, sialic acid is oxidized into formylpyruvic acid which reacts with thiobarbituric acid to form a pink color product. The color intensity measured at 549 nm is proportional to the concentration of sialic acid in the sample.

Histological and immunohistochemical investigations

The fixed samples were transferred to the Department of Pathology, National Cancer Institute for processing, blocking, sectioning and staining with hematoxylin and eosin or mounting on positive slides for immunohistochemical investigations. Sections mounted onto positive-charged slides (Fisher Scientific, Pittsburgh, PA, USA) were used to detect the Bcl-2 and p53 reactivity or apoptotic cells using

the TdT-mediated dUTP nick end labeling (TUNEL) assay.^[17] The TUNEL assay was performed using a kit (*in situ* cell death detection kit, Roche Molecular Biochemicals, Mannheim, Germany) according to the protocol provided by the manufacturer, while Bcl-2 and p53 reactivity were determined following method of Gao and Zhou.^[18] Briefly, before the incubation with antibodies, endogenous peroxidase activity was quenched, slides washed and then incubated in a blocking solution of hydrogen peroxide 1% in methanol, in darkness for 15 min. Antigen retrieval occurred in citrate buffer 10 mmol/L, pH = 6.0. After cooling, sections were rinsed in tap water and then phosphate buffer saline (PBS). Primary antibodies for either Bcl-2 (DakoCytomation, USA) or p53 (Lab Vision Corporation, 47777 Warm Springs Blvd, Fremont, CA, USA), diluted 1:150 and 1:100, respectively in PBS, were applied for 1 h at 37 °C. Secondary biotinylated antibody diluted 1:100 and 1:200 in PBS was applied for a period of 30 min at 37 °C. Streptavidin-biotin or avidin-biotin complex with horseradish peroxidase (ABC/HRP) was applied for 10 min at room temperature. Bound antibody complex was visualized by the reaction of 3, 3'-diaminobenzidine (DAB) substrate and counterstained with hematoxylin. Secondary biotinylated antibody, ABC/HRP and DAB were obtained from Zymed Laboratories (Invitrogen Immunoprotection, 561 Eccles Avenue, South San Francisco, CA, USA). Hematoxylin was obtained from Sigma Chemical Company, USA.

Imaging and semi-quantitative analysis of Bcl-2, p53 and terminal deoxynucleotidyl transferase

The yellowish brown colored stained area were analyzed in pixels; percent area and intensity were detected by ImageJ software, US National Institutes of Health, Bethesda, Maryland, USA (<http://imagej.nih.gov/ij/>).

Anti-tumor cytotoxicity against EAC-cells *in vitro*

The viability of cells as a result of 6 different concentrations (0, 6.25, 12.5, 25, 50, and 100 µg/mL) of DADS was tested by trypan blue exclusion assay according to the method of Ahmed and Ahmed.^[19] Briefly, EAC-cells at concentration 2.5×10^5 cells/mL suspended in PBS were incubated at 37 °C for 2 h in the presence of different concentrations of DADS dissolved in dimethyl sulfoxide. At the end of incubation period, equal volume of trypan blue solution was added to sample cells, then the stained cells (dead cells) and unstained cells (alive cells) were counted using Neubauer hemocytometer. The percent of dead cells for each test was calculated.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by a least significance difference (LSD) test to compare the groups with each other.^[20] Data were expressed

as mean \pm standard error. Values with $P > 0.05$ are not significantly different while values with $P < 0.05$ and $P < 0.01$ are significantly and highly significantly different respectively. F-probability expresses the general effect between groups.

RESULTS

Effects on animal survival and mortality

After 2 weeks of EAC-intraperitoneal transplantation, 8 of 12 mice administered 10% DMSO as a vehicle survived; the survival percentage was 66.66%. The treatment of EAC-bearing mice with DADS markedly increased the survival percentage to reach 83.33% at the end of the experiment [Table 1].

Effects on EAC-fluid volume and cell number *in vivo*

The inhibitory effect of DADS on EAC-cells *in vivo* was tested in terms of EAC-fluid volume, number of total and alive EAC-cells, and number and percent of EAC-dead cells. The daily treatment of EAC-bearing mice with DADS for 2 weeks after EAC-cells intraperitoneal transplantation induced a significant decrease (LSD; $P < 0.05$) of EAC-fluid volume, number of total and alive EAC-cells. On the other hand, the number and percent of dead EAC-cells exhibited a potential increase (LSD; $P < 0.01$) as compared to EAC-bearing control counterparts. The ratio of EAC-fluid volume to total EAC-number was also highly significantly (LSD; $P < 0.01$) increased in EAC-bearing mice treated with DADS as compared with control mice [Table 1].

With regards to one-way ANOVA, it was found that the effect between groups on number of total and alive EAC-cells and percent of dead cells was very highly significant (F-probability; $P < 0.001$), while the effect on EAC-volume and number of dead cells was only highly significant ($P < 0.01$) [Table 1].

Effect on plasma and ascites sialic acid level *in vivo*

Plasma sialic acid level in EAC-bearing mice was highly significantly ($P < 0.01$; -35.05%) decreased as a result of treatment with DADS. The ascites sialic acid concentration was also highly significantly ($P < 0.01$; -28.49%) decreased in EAC-bearing mice treated with DADS as compared with EAC-bearing control [Table 2].

Histological and immunohistochemical effects on EAC-cells

As indicated by low and high magnification, EAC cells of EAC-bearing control female mice [Figure 1a and c] were characterized with abundant basophilic and dark stained cytoplasm as well as moderate sized nuclei. As a result of treatment with DADS [Figure 1b and d], EAC-cells decreased in number and size and appeared with a narrow rim of

Table 1: Effect of DADS administration on animal survival percent EAC-aliquot volume, EAC-cells number and percent of dead cells in EAC-bearing mice

Groups	Animal survival percent (%)	EAC-aliquot volume (mL)	Total EAC-cell number ($\times 10^7$)	EAC-aliquot volume/total EAC-number (%)	Alive EAC-cell number ($\times 10^7$)	Dead EAC-cell number ($\times 10^7$)	Percent of dead EAC-cells
EAC-bearing mice control ($n = 8$)	66.66	6.56 ± 0.49	121.31 ± 14.13	5.41 ± 0.63	118.33 ± 13.64	2.71 ± 0.23	2.38 ± 0.19
EAC-bearing mice treated with DADS ($n = 10$)	83.33	$4.90 \pm 0.19^{**}$	$51.81 \pm 4.24^{**}$	$9.46 \pm 0.61^{**}$	$48.09 \pm 4.27^{**}$	$3.72 \pm 0.19^{**}$	$7.61 \pm 0.98^{**}$
F-probability	-	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.001$
LSD at the 5% level	-	1.027	28.45	2.51	27.62	0.63	2.38
LSD at the 1% level	-	1.416	39.20	3.64	38.05	0.86	3.28

Data are expressed as mean \pm SE. $^{**}P < 0.01$: effect is highly significant; difference between two means of the same parameter is higher than the value of LSD at the 1% level. EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide; LSD: least significance difference; SE: standard error

Table 2: Effect of DADS administration on plasma and ascites sialic acid concentration in EAC-bearing mice

Groups	Plasma sialic acid concentration (mg/100 mL)	Percent change	Ascites sialic acid concentration (mg/g protein)	Percent change
EAC-bearing mice control ($n = 8$)	91.72 ± 1.26		241.59 ± 11.77	
EAC-bearing mice treated with DADS ($n = 10$)	$59.57 \pm 4.99^{**}$	-35.05	$172.74 \pm 4.72^{**}$	-28.49
F-probability	$P < 0.001$		$P < 0.001$	
LSD at the 5% level	12.13		24.84	
LSD at the 1% level	16.71		34.23	

Data are expressed as mean \pm SE. $^{**}P < 0.01$: difference is highly significant; difference between two means is higher than value of LSD at the 5% level. EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide; LSD: least significance difference; SE: standard error

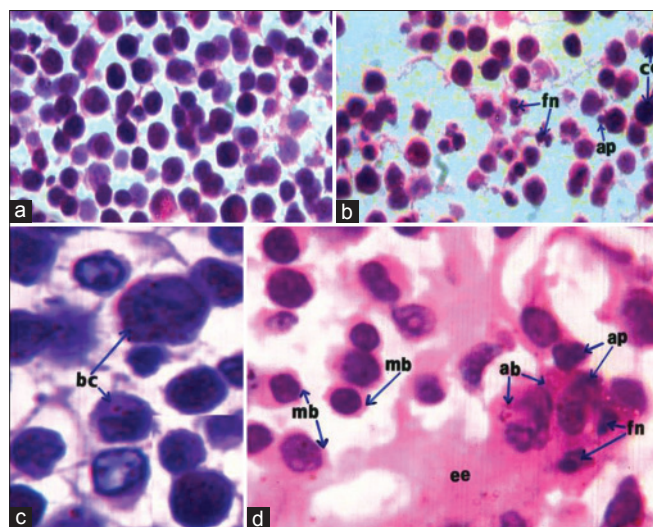


Figure 1: Photomicrographs of HE stained EAC-cells sections showing decreased number of cells, plasma membrane blebbing (mb), fragmenting nuclei (fn), chromatin compaction or condensation (cc), apoptotic bodies (ap) and extracellular exudates (ee) (b, $\times 100$; d, $\times 1000$), as a result of treatment of EAC-bearing mice with DADS as compared to EAC-bearing control mice (a, $\times 100$; c, $\times 1000$) which have EAC-cells with bigger size, abundant basophilic cytoplasm (bc), and moderate sized-nuclei. EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide

light stained eosinophilic cytoplasm with azurophilic lytic bodies. Many EAC-cells, after treatment with DADS, exhibited apoptotic signs including shrinkage, blebbing plasma membrane, apoptotic bodies, nuclear chromatin compaction, and fragmenting nuclei. Between EAC-cells, there was a large amount of eosinophilic material or exudates.

After noticing that DADS induces EAC-apoptosis, the changes in anti-apoptotic protein Bcl-2 and pro-apoptotic mediator p53 as well as DNA fragmenting marker TdT were followed to determine the mechanism of EAC killing.

As indicated in Figure 2, the treatment of EAC-bearing mice with DADS induced a potential decrease of Bcl-2 expression (yellowish brown color) in the cytoplasm of EAC-cells [Figure 2b] as compared to the control [Figure 2a]. In contrast, p53 protein concentration was noticeably increased in the cytoplasm and nuclei of EAC-cells of DADS-treated mice [Figure 2d] as compared to the control [Figure 2c]. Similarly, TdT expression was remarkably increased in the nuclei of EAC-cells in DADS-treated mice [Figure 2f] as compared with the control counterpart [Figure 2e].

Data of imaging and semi-quantitative analysis

Imaging and semi-quantitative analysis results are represented in Figures 3 and 4.

Photomicrographs obtained from imaging analysis depicted that the amount of expressed Bcl-2 has clearly decreased in EAC-bearing mice treated with DADS [Figure 3b] as compared to EAC-bearing control mice [Figure 3a]. On the other hand, the expressed p53 [Figure 3d] and TdT [Figure 3f] are much higher in EAC-bearing mice treated with DADS than in those of the corresponding EAC-bearing controls [Figure 3c and e].

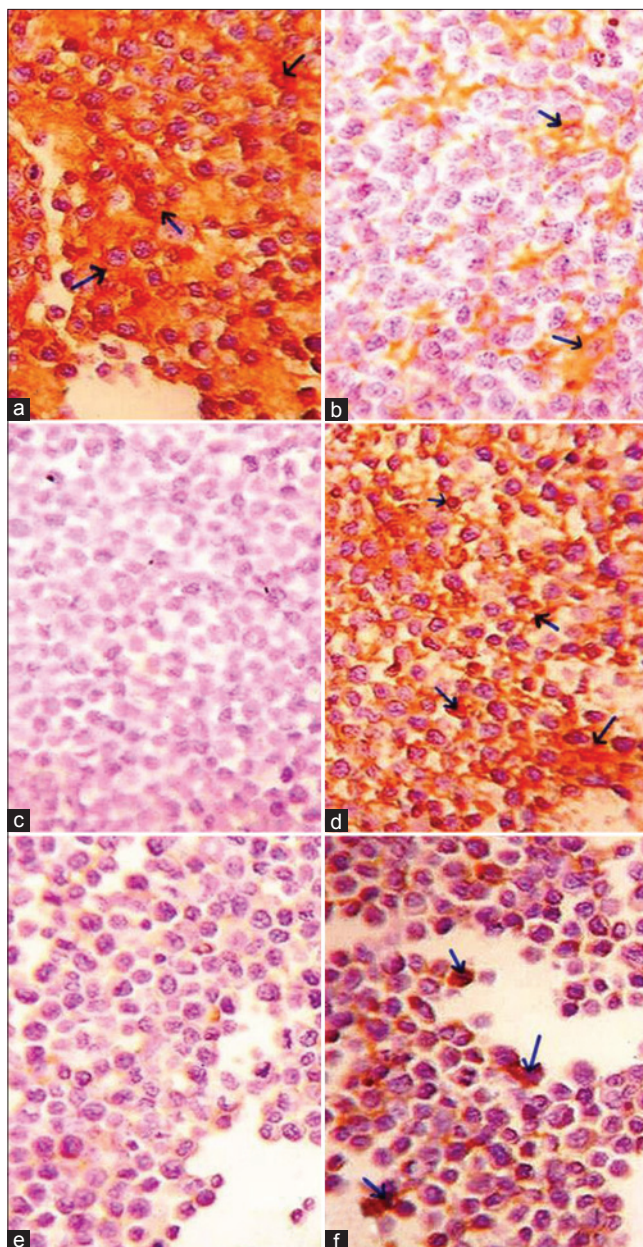


Figure 2: Photomicrographs of immunohistochemically stained EAC-sections. Images respectively show the greater amount of Bcl-2 (arrow; yellowish brown color) in the cytoplasm of EAC-cells in control mice (a, $\times 100$) as compared with mice treated with DADS (b, $\times 100$), the higher amount of p53 (arrow; yellowish brown color) in the cytoplasm and nuclei of EAC-cells in mice treated with DADS (d, $\times 100$) than in EAC-bearing control mice (c, $\times 100$), and the higher level of TdT (arrow; yellowish brown color) in the nuclei of EAC-cells in mice treated with DADS (f, $\times 100$) than in EAC-bearing control mice (e, $\times 100$). EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide; TdT: terminal deoxynucleotidyl transferase

As indicated in Figure 4, the area in pixels, percent area and intensity of yellowish brown color of immunoreactive anti-apoptotic marker, Bcl-2, were remarkably decreased in EAC-bearing mice treated with DADS as compared to those of EAC-bearing control mice. In contrast, the area in pixels, percent area and intensity of yellowish brown color of immunoreactive apoptotic markers, p53 and TdT were

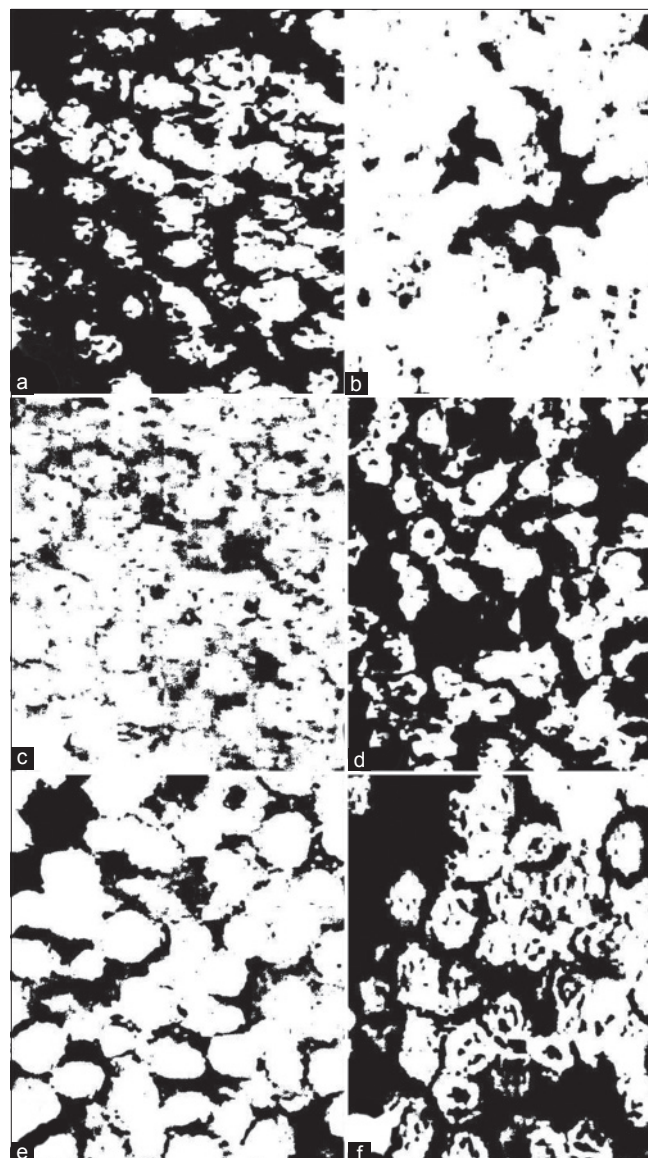


Figure 3: Photomicrographs of image analysis of immunohistochemically stained EAC-sections for Bcl-2 (b), p53 (d) and TdT (f) of EAC-bearing mice treated with DADS as compared to their respective EAC-bearing mice controls (a, c and e). The black colored areas reflect the yellowish brown stained areas. The figures depict a decreased expression of Bcl-2 and an increased expression of p53 and TdT as a result of treatment with DADS. EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide; TdT: terminal deoxynucleotidyl transferase

potentially elevated in EAC-bearing mice treated with DADS as compared with those of EAC-bearing control mice.

Effects on EAC cells *in vitro*

Incubation of EAC-cells ($2.5 \times 10^5/\text{mL}$ suspended in PBS) with 0, 6.25, 12.5, 25, 50, and 100 $\mu\text{g/mL}$ of DADS (dissolved in DMSO) for 2 h produced 0, 16.5%, 35%, 70%, 90%, and 100% inhibition of cell viability, respectively. Thus, the effect seemed to be dose dependent and IC_{50} was 19.500 $\mu\text{g/mL}$ [Table 3].

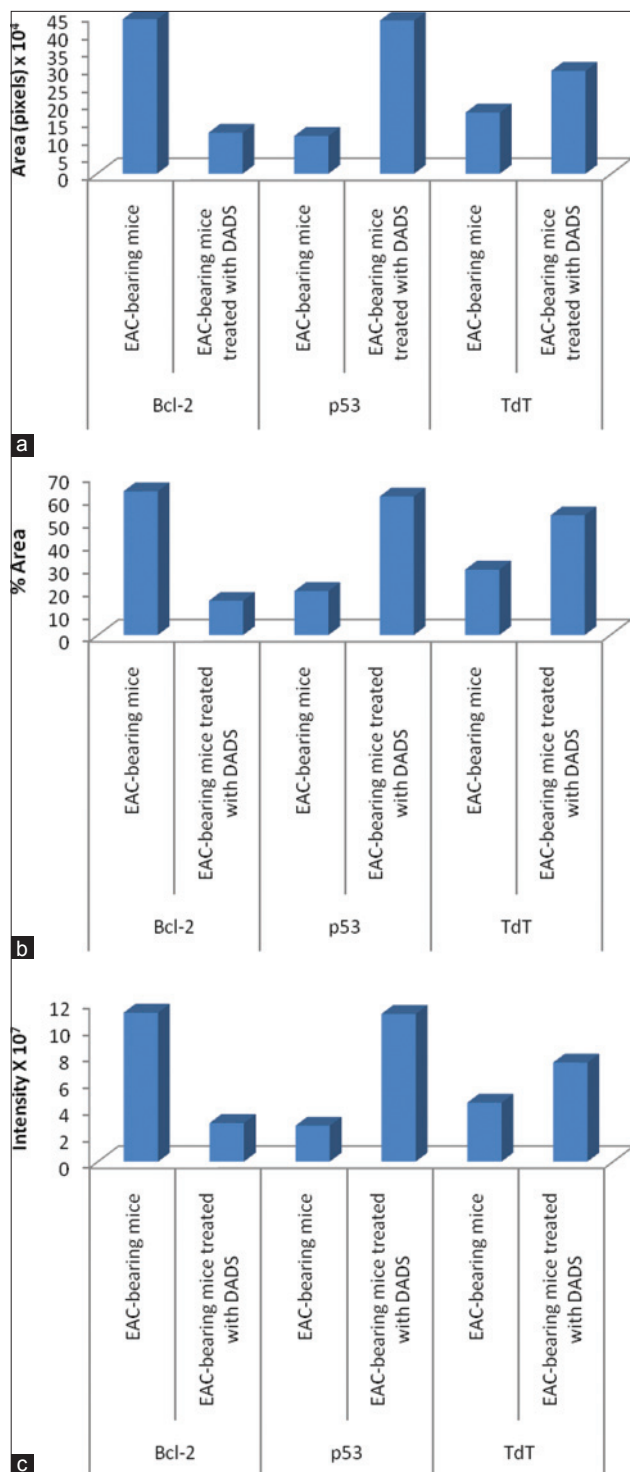


Figure 4: Data of image analysis of immunohistochemically stained EAC-sections showing (a) area in pixels, (b) percent area, and (c) the intensity of yellowish brown color of immunoreactive Bcl-2, p53 and TdT. EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide; TdT: terminal deoxynucleotidyl transferase

DISCUSSION

The goal of cancer chemoprevention is to slow, to block or to reverse the process of carcinogenesis. With regard to this principle, the study is a trial to evaluate the anti-tumor

Table 3: Effect of DADS, at various concentrations, on percent inhibition of EAC-cell viability *in vitro*

Compound	Percentage of inhibition of cell viability (µg/mL)					
	0	6.25	12.5	25	50	100
DADS	0	16.5	35	70	90	100

IC50: 19.500 µg/mL. EAC: Ehrlich ascites carcinoma; DADS: diallyl disulfide

cytotoxicity effects of DADS against EAC *in vivo* and *in vitro*. In addition, the apoptogenic effects of this agent were assessed by investigating the histological changes and measuring some apoptotic and anti-apoptotic mediators by imaging and semi-quantitative analysis of immunohistochemically stained sections of EAC-cells.

The current *in vivo* study revealed that DADS has a potential antiproliferative and cytotoxicity effect against EAC (a transplantable neoplasia from a malignant epithelium that corresponds to mammary adenocarcinoma) in EAC-bearing female mice.^[14,21] This activity was confirmed by a significant decrease in EAC-aliquot volume and total and alive EAC-cell number, and a tremendous increase in dead EAC-cell count and percent as a result of treatment with DADS in comparison to a vehicle. The anti-tumor cytotoxicity of DADS was associated with an improved survival and increased the life span of EAC-bearing mice. The plasma and Ehrlich ascites tumor marker, sialic acid, was significantly decreased in EAC-bearing mice treated with DADS as compared to EAC-bearing control mice. These *in vivo* results were supported by *in vitro* study which indicated that DADS induced unexpected anti-proliferative and anti-tumor cytotoxicity effects against EAC-cells.

In concurrence with the present results, the sialic acid, the component of glycoproteins and glycolipids that constitute the cell surface, was reported to be shed or secreted by tumor cells leading to its increased level in blood of humans with cancer and in animal cancer models.^[22] The sialic acid level in serum and plasma was reportedly decreased in C57BL/6 mice lung metastasis of B16F-10 melanoma cells^[23] and in alloxan diabetic rats,^[24] respectively. The anti-proliferative and apoptotic effects of DADS derived from *Allium sativum* in different carcinomas both *in vivo* and *in vitro* were also elucidated by various authors.^[25-27]

The histological findings of this study indicated that many cells seemed apoptotic, after treatment with DADS, as they were shrunk and had blebbing plasma membrane, apoptotic bodies and fragmenting nuclei which are considered as phenotypical or morphological signs of apoptosis.^[28] In addition to these histological results, immunohistochemical results depicted that the expression of Bcl-2 working primarily by blocking apoptotic pathway,^[29] was noticeably

reduced while the pro-apoptotic and anti-tumor suppressor protein p53 causing cell cycle arrest of EAC-cells,^[30-32] was increased as a result of DADS treatment. The stimulating effect of DADS on the expression of p53 may result from inhibition of cyclin-dependent kinases.^[33] It was reported that p53 stimulation produced a dual effect on EAC-cells. It may upregulate the pro-apoptotic protein Bax on one hand and/or mediate growth arrest involving p21 as a major effector on the other.^[28,34] Similar to the effect on p53, the DNA fragmentation marker, TdT^[28] also increased as a result of treatment with the tested compound. From these findings, it can be suggested that the induced inhibition of EAC-cell growth and proliferation by DADS are due to induction of apoptosis and cell cycle arrest. These results and suggestion are in accordance with other several publications.^[35-37]

It was reported that in EAC-bearing mice, extensive formation of new capillary blood vessels (neovascularization or angiogenesis) provides more nutrients and oxygen supply to the highly divided EAC-cells leading to the induction of growth and proliferation.^[38-40] Thus, it should not be excluded that DADS may have anti-angiogenic effects which in turn may have a crucial role in anti-proliferative effects.^[15]

As suggested by Freitas *et al.*^[14] and Senger *et al.*,^[41] the production of ascitic extracellular fluid in Ehrlich carcinoma is said to occur due to increased capillary permeability present in the peritoneal cavity. This vascular change occurs due to increased receptor expression for autocrine motility factor (AMF).^[14] AMF link to its receptor induces angiogenesis and changes in endothelial cell morphology causing a subsequent increase in vascular permeability with increased amount of ascitic fluid^[42] in addition to the increasing number of proliferated cells. In the present study, the volume of EAC-aliquot relative to total cell count was increased as a result of DADS treatment. In our opinion, this means that the extra-cellular fluid volume was increased at the expense of EAC-cells which exhibited a greater rate of apoptosis and death after treatment with DADS. This assumption was supported by the present histological results which depicted increased extracellular exudates in EAC-bearing mice treated with DADS.

The present *in vitro* study indicated that DADS succeeded to produce amazing potential anti-tumor cytotoxicity and antiproliferative effects against EAC-cells. These results are in accordance with those obtained by many previous authors who found that DADS cause marked inhibition of HepG2 cell proliferation^[6,43,44] and HCT116 cell growth.^[2,10] The present results are also in accordance with *in vivo* studies of Ahmed *et al.*^[45] and Abdel-Aleem *et al.*^[46] who found that DADS had anti-tumor effects in liver and

kidney of carbon tetrachloride-intoxicated rats. Different hypotheses have evolved to explain the mechanisms by which DADS produced its anti-carcinogenic effects. García *et al.*^[47] reported that DADS exerted its anti-carcinogenic effect by inhibition of cytochromes CYP2E, CYP2A6 and CYP1A1 and activation of UDP-glucuronyl-transferase. Knowles and Milner,^[35] Robert *et al.*^[36] and Oommen *et al.*^[37] stated that the anti-tumor effect of garlic may be due to the induction of apoptosis of tumor cells. Furthermore, Iciek *et al.*^[43] attributed the anti-cancer effects of DADS to both suppression of cell division (stimulation of cell cycle arrest) and induction of apoptosis in tumor cells. This latter postulation is concomitant with the present study, which revealed a decreased expression of anti-apoptotic protein Bcl-2 in cytoplasm and increased levels of pro-apoptotic and cell cycle arrest protein in cytoplasm and nuclei as well as increased concentration of DNA fragmentation marker TdT in nuclei of tumors cells after treatment with DADS as compared with vehicle. This attribution was supported by imaging and semi-quantitative analysis which revealed that the area in pixels, the percent area and the intensity of yellowish brown color of immunoreactive pro-apoptotic protein, Bcl-2, decreased while those of the yellowish brown colored immunoreactive apoptotic markers, p53 and TdT, increased.

In conclusion, DADS has effective anti-tumor potentials against EAC-cells in both *in vivo* and *in vitro* studies. These anti-tumor effects may be mediated via modulation of tumor cell cycle as well as the balance between pro-apoptotic and anti-apoptotic factors.

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Aqueous extract of *Corchorus olitorius* decreases cytotoxicity of aflatoxin B₁ and fumonisin B₁ in H4IIE-*luc* cells

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ABSTRACT

Aim: Aflatoxin B₁ (AFB₁) and fumonisin B₁ (FB₁) are important food-borne mycotoxins. Co-contamination of foodstuffs with these two mycotoxins is well-known and has been implicated in a possible development of hepatocellular carcinoma in humans living in regions of the world where exposures to these mycotoxins in grain are greatest. The aim of the current study was to evaluate the potential protective effects of an aqueous extract of *Corchorus olitorius* (*C. olitorius*, moroheiya) against cytotoxicity of AFB₁ and/or FB₁ in H4IIE-*luc* rat hepatoma cells, using assays to measure cell viability and disruption of DNA integrity. Although this transactivation assay was originally developed to specifically respond to aryl hydrocarbon agonists, this cell line was used because of its hepatic origin. **Methods:** H4IIE-*luc* cells were incubated with different concentrations of AFB₁ and/or FB₁ for 24 and 48 h with or without aqueous extract of *C. olitorius*. **Results:** Both mycotoxins decreased cell viability and increased DNA damage. Cytotoxicity was more pronounced when cells were exposed simultaneously to AFB₁ and FB₁. **Conclusion:** Aqueous extract of *C. olitorius* protected cells against cytotoxicity of mycotoxins. *C. olitorius* contains a water-soluble, natural chemo-preventative agent for cancer that should be isolated and identified.

Key words: Anticancer; cytotoxicity; DNA; liver; moroheiya; mycotoxins

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INTRODUCTION

Co-occurrence of various mycotoxins in foodstuffs and animal feed is common because each toxigenic fungus can produce more than one mycotoxin and foodstuff can be colonized by several fungi either while growing in the field or during storage or transport.^[1] Processed products are often composed of various raw materials which might be

contaminated with mycotoxins.^[2] Poor harvest practices and inadequate conditions during drying, handling, packaging, storing and transporting can contribute to the growth of fungi and an increased risk of production of mycotoxins.^[3] The importance of co-occurrence of mycotoxins lies in the changes that might occur in the combined toxicity of mycotoxins.^[4] In addition, the existence of relationships in the occurrences of mycotoxins allows predictions of the presence of individual mycotoxins from the presence of others.^[1]

Among these mycotoxins, aflatoxin B₁ (AFB₁) is the predominant contaminant in cereals and oilseed and presents a significant risk,^[5] due to being hepatotoxic and carcinogenic to humans and animals.^[4,6-8] AFB₁ is classified by the International Agency of Research on Cancer (IARC) as a Group 1 carcinogen.^[9] This mycotoxin is also mutagenic, teratogenic, and immunosuppressive in farm, and laboratory animals,^[10-12] and primarily affects cell-mediated immunity.^[13] AFB₁ is also able to induce reactive oxygen species (ROS),^[8,14-16] possibly requiring activation of cytochrome P450.

Fumonisin, mainly produced by *Fusarium verticillioides* and *F. proliferatum*, are mycotoxins commonly found on corn. The most toxic and abundant of these is fumonisin B₁ (FB₁), which causes esophageal and hepatic cancer in humans and liver and kidney cancer in rodents.^[17-19] IARC evaluated FB₁ and classified it as probably carcinogenic to humans (Group 2B).^[20] Moreover, FB₁ modulates immunity in animals and decreases viability of lymphocytes in poultry.^[21]

Humans and animals are constantly exposed to small concentrations of these mycotoxins, either individually or in combination.^[22] Mycotoxicoses occur seasonally in areas that have not implemented effective prophylactic measures.^[23] While interactions between mycotoxins had been discussed,^[24] few studies have been conducted with these combinations.

Cochorus olitorius (*C. olitorius*, Tiliaceae family) is indigenous to the Middle East, including Egypt and South Africa. Young leaves of *C. olitorius* are regarded to be a healthy vegetable in East Asia and Japan, typically known as moroheiya.^[25,26] Its health benefits have been reported to include antitumor activity by inhibiting tumorigenesis,^[27] antioxidant properties,^[28] and antibacterial activity.^[29] Young leaves of *C. olitorius* are rich in calcium, potassium, phosphate, iron, ascorbic acid, carotene and other nutrients, and contain a large amount of mucilaginous polysaccharides.^[28,30] It has also been reported that compounds such as carotenoids, flavonoids, and vitamin C, isolated from leaves of *C. olitorius*, exhibit significant antioxidant characteristics.^[30] In addition, leaves of *C. olitorius* have been reported to have ethno-medicinal

importance as a demulcent and febrifuge^[31] and also possess anti-inflammatory, analgesic, and antimicrobial activities.^[32,33] The aim of the current research was to assess possible protective effects of *C. olitorius* extracts against cytotoxic effects and disruption of DNA integrity induced by FB₁ and AFB₁ in the rat hepatoma cell line (H4IIE-*luc*).

METHODS

Chemicals

Aflatoxin B₁ and FB₁ (98% purity) were purchased from Sigma Chemicals (St. Louis, MO, USA). The DNA extraction kit (DNeasy Blood and Tissue Kit) was obtained from Qiagen (Hilden, Germany). A DNA ladder, polymerase chain reaction (PCR) master mix containing 100 base pairs and RNase free water were obtained from Fermentas Inc., (Glen Burnie, MD, USA). Supertherm *Taq* polymerase was purchased from JMR Holdings (London, UK). Forty primers were obtained from Operon Technologies (Alameda, CA, USA). All solvents used were analytical grade from Burdick and Jackson (Muskegon, MI, USA).

Plant materials

Stems and leaves of *C. olitorius* were collected from a residential garden in the city of Potchefstroom, North West Province, South Africa. The plant material was freeze-dried, pulverized, and 1 g was infused with 10 mL water for 24 h at room temperature. After centrifugation, the supernatant was freeze-dried and stored at 4 °C until used.

Cytotoxicity

Rat hepatoma cells (H4IIE-*luc*) were used as the mammalian model. This cell line had been stably transfected with a firefly luciferase reporter gene under control of the dioxin response element and the aryl hydrocarbon receptor mechanism.^[34-37] These cells were originally developed as a reporter gene assay to determine the presence of, and to semi-quantify the concentrations of certain groups of persistent organic pollutants^[38] including mixtures.^[39]

H4IIE-*luc* cells were seeded at a density of 10,000 cells/mL media (Dulbecco's Modified Eagle's Medium, Sigma: D2902; St. Louis, MO, USA) in the inner 60 wells of a 96-well microplate. A volume of 250 µL of culture medium, supplemented with 0.044 mol/L NaHCO₃ and 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), was added to each well. To avoid edge effects and to create a homogenous microclimate across all wells containing cells, outer cells received 250 µL Dulbecco's phosphate buffered saline (PBS) (Tewksbury, MA, USA). Two sets of plates were incubated, at 37 °C in humidified air with 5% CO₂, with one set for 24 h and the other set for 48 h. After incubation,

the medium was removed and replaced with medium containing *C. olitorius* extract at either of two concentrations (20 or 40 µg/mL) and incubated for another 24 h. The medium was replaced with medium containing varying concentrations of AFB₁ (50, 25, 2.5, 0.25, 0.025 µmol/L) dissolved in methanol, or of FB₁ (200, 100, 10, 1, 0.1 µmol/L) dissolved in methanol. A combination of the already mentioned concentrations of AFB₁ and FB₁ were also tested: 50 µmol/L AFB₁ + 200 µmol/L FB₁; 25 µmol/L AFB₁ + 100 µmol/L FB₁, and so on. Exposure to mycotoxins was carried out in triplicates. Cells in six wells in each plate were exposed only to the aqueous extract of *C. olitorius* and cells in 11 wells were not exposed to anything except the growth medium.

To determine the viability, based on metabolic activity of cells, a colorimetric assay was performed using the yellow dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Montigny-le-Bretonneux, France). In this assay, MTT is converted to formazan (blue) by mitochondrial reductase enzymes in living cells.^[40] A final concentration of 500 µg/mL MTT was added to each well and incubated for 30 min. Blue formazan crystals that were formed by reduced MTT were dissolved with dimethylsulfoxide and absorbance by the formazan was measured spectrophotometrically at 560 nm. The amount of blue formazan produced is proportional to the amount of viable cells, and the percentage of viable to dead cells was calculated by comparison with a control (untreated and solvent control). Viability among various *C. olitorius* treatments described above were compared to the viability of cells treated only with mycotoxins by applying the same protocol described before, but omitting aqueous extract of *C. olitorius*.

Extraction of DNA

Harvested cells were washed with PBS to remove the nonadherent dead cells. The adherent cells were removed by trypsinizing (0.25% trypsin, 0.1% versene EDTA; purchased from Thermo Scientific, Rockford, IL, USA) and activity was stopped by addition of media. The cell suspension was centrifuged at 3,000 g for 5 min at room temperature. Genomic DNA was extracted from cells according to the Qiagen instruction manual and concentrations determined spectrophotometrically by use of the NanoDrop ND-1,000 Spectrophotometer. Purity of DNA was assessed by examining the 260/280 nm ratio.^[41]

Random amplification of polymorphic DNA-polymerase chain reaction analysis

Amplification of DNA fragments was carried out using an ICycler (Bio-Rad, Herts, UK) thermal cycler using 20 primers from the Operon Biotechnologies (BioCampus Colonge Nattermannalle, Germany). PCR amplification was conducted in 25 µL reaction volumes containing 10 ng genomic DNA,

12.5 pmol/L master mix (×2) (Thermo Fisher Scientific, Carlsbad, CA, USA), 1.0 units of Supertherm *Taq* polymerase and 50 pmol/L primer. The PCR reactions were carried out in a thermocycler (Bio-Rad C1000, Bio-Rad, Hercules, CA, USA), programed with a first denaturation for 5 min at 95 °C, followed by 40 cycles for 30 s denaturation at 95 °C, 30 s annealing at 37 °C and 1 min extension at 72 °C. Final extension at 72 °C for 5 min was allowed before holding at 4 °C for 5 min. Reaction products were stored at -80 °C prior to electrophoresis.

Gel electrophoresis

Amplified products together with marker (100 bp DNA) were resolved by gel electrophoresis (60 V/cm for 135 min) on 2% agarose gel in tris-acetate-EDTA buffer containing 0.001 mg/mL ethidium bromide purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gels were photographed by Gel Documentation System (Gensnap) software (Synegen, UK).

Band analysis

The gels for control and exposed DNA were run for each of the 20 primers [Table 1]. A DNA ladder of 100 bp was also run in each gel. The bands for PCR products were analyzed by TotalLab Quant (V11.5: TL100-LX59-7YF4-EX). The fluorimetric profiles of each amplification reaction were studied both qualitatively and quantitatively by comparing profiles from control and DNA exposed to the extracts. Each change observed in random amplification of polymorphic DNA (RAPD) profiles of treated groups (disappearances and appearance of bands in comparison to the control RAPD profiles) was given the arbitrary score of +1. The mean was then calculated for each experimental group exposed to the mycotoxins for varying time periods. Template genomic stability (%) was calculated as “100 - (100a/n)” where “a” is the average number of changes in DNA profiles and “n” is the number of bands selected in control DNA profiles.^[42]

Statistical analysis

All data were statistically analyzed with the Graphpad Prism 4.02 Inc. (La Jolla, CA, USA). The significance of the

Table 1: Sequences of the primers used to amplify DNA of H4IIE-*luc* rat hepatoma cells

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
D01	ACCGCGAAGG	D11	AGCGCCATTG
D02	GGACCCAACC	D12	CACCGTATCC
D03	GTCGCCGTCA	D13	GGGGTGACGA
D04	TCTGGTGAGG	D14	CTTCCCCAAG
D05	TGAGCGGACA	D15	CATCCGTGCT
D06	ACCTGAACGG	D16	AGGGCGTAAG
D07	TTGGCACGGG	D17	TTTCCCACGG
D08	GTGTGCCCCA	D18	GAGAGCCAAC
D09	CTCTGGAGAC	D19	CTGGGGACTT
D10	GGTCTACACC	D20	ACCCGGTCAC

differences among treatment groups was determined with two-way analysis of variance. The assumptions of parametric statistics were confirmed. Normality was confirmed by the Kolmogorov-Smirnov test and homogeneity of variance was confirmed by use of Levine's test. All statements of significance were based on a probability of $P \leq 0.05$.

RESULTS

The results of cell viability assay revealed that H4IIE-*luc* cells that were treated with both concentrations (20 and 40 $\mu\text{g/mL}$) of *C. olitorius* extract were statistically significantly more viable after 24 h exposure than the ones that were not treated with the plant extract. However, there was no significant protection by the plant extract against FB_1 after 48 h of exposure. Furthermore, *C. olitorius* extract could not protect the cells from the AFB_1 concentration series or the combination exposure ($\text{AFB}_1 + \text{FB}_1$) irrespective of the exposure period (24 or 48 h) [Table 2]. A dose-dependent decrease of cell viability after exposure to increasing amounts of AFB_1 was observed only after 48 h [Figure 1a]. After 24 h exposure, the response was not linear (hormetic effect). Except for the lowest concentration, cytotoxicity was more pronounced after 48 h. However, protective effects of the *C. olitorius* extract were observed after both 24 h and 48 h of exposure to AFB_1 . After 48 h, viability, expressed as a percentage of H4IIE-*luc* cells affected by FB_1 , was approximately 40% less than that of cells exposed to FB_1 alone. After 48 h, there was also no dose-dependence, but cytotoxicity was less pronounced. Protective effects of 20 or 40 $\mu\text{g/mL}$ *C. olitorius* extract were observed. After both 24 and 48 h of exposure, production of MTT formazan was greater in the presence of both concentrations of *C. olitorius* extract at all tested doses of FB_1 compared to those in the absence of *C. olitorius* extract [Figure 1b]. No significant differences were found between 20 and 40 $\mu\text{g/mL}$ of *C. olitorius* extract after 24 h of exposure.

Incubation of H4IIE-*luc* cells with $\text{AFB}_1 + \text{FB}_1$ for 24 h resulted in greater cytotoxicity to cells as measured by the MTT assay, with significant toxicity at the sum of the two mycotoxin concentrations 12.5 and 125 $\mu\text{mol/L}$ [Figure 1c]. The cells were least viable when they were exposed to the mixture of 250 $\mu\text{mol/L}$ mycotoxin. Addition of *C. olitorius* extract to cells resulted in slightly greater viability. At lesser concentrations of AFB_1 (1.25 $\mu\text{mol/L}$) + FB_1 (12.5 $\mu\text{mol/L}$), protective effects of aqueous extracts of *C. olitorius* on viability of cells was greater relative to the cells that did not receive plant extract [Figure 2].

The EC_{50} values for AFB_1 were 6.9 and 1.8 after 24 and 48 h of exposure, respectively. When *C. olitorius* extract was added, the EC_{50} values were 4.3 and 2.49 after 24 or 48 h of

exposure, respectively [Table 3]. At the lesser concentration, FB_1 did not cause measurable cytotoxicity. However, the MTT assay revealed cytotoxicity at the greater concentration (200 $\mu\text{mol/L}$) although all doses studied were less than those required to obtain an EC_{50} .

Only 5 of 10 oligonucleotide primers, primers D07, D09, D13, D15, and D16, used to measure responses of molecular-genetic parameters of cells among various treatments, gave detectable bands [Figure 3]. A total of 75 DNA sequences, ranging from 144 to 2,000 bp, were observed. All of the bands were "polymorphic" given 100% polymorphism for control cells and the other treatments for the 2 time periods using all primers. Quantitative analysis of these bands, expressed as a percentage of band loss, showed a time-dependent relationship [Figure 3 and Table 4]. Similarly, in the case of losses of bands after the shorter period of exposure (24 h), 12 of 75 bands (16%) had disappeared [Figure 3a]. At the longer duration of exposure (48 h), 21 of 75 bands (28%) had disappeared [Figure 3b]. Protective effects of *C. olitorius* extract were observed after 24 h, when 25 of 75 bands (33.3%)

Table 2: Summary of Wilcoxon matched pair tests to compare the viability of rat hepatoma H4IIE-*luc* cell line treated with *C. olitorius* extract

Mycotoxins	Exposure time	<i>C. olitorius</i> extract concentrations	
		20 $\mu\text{g/mL}$	40 $\mu\text{g/mL}$
FB_1	24 h	0.04*	0.04*
	48 h	0.69	0.89
AFB_1	24 h	0.9	0.5
	48 h	0.35	0.89
$\text{FB}_1 + \text{AFB}_1$	24 h	0.69	0.5
	48 h	0.22	0.08

* $P \leq 0.05$. AFB_1 : aflatoxin B₁; FB_1 : fumonisin B₁; *C. olitorius*: *Cochorus olitorius*

Table 3: EC_{50} values of AFB_1 , FB_1 , and $\text{AFB}_1 + \text{FB}_1$ alone or in combination with the *C. olitorius* extract after 24 and 48 h and exposure measured by the MTT bioassay using H4IIE-*luc* rat hepatoma cells

Mycotoxin and/or plant extract treatments	Time exposure (h)	Cytotoxicity (EC_{50}) H4IIE- <i>luc</i>
FB_1	24	ND
	48	ND
AFB_1	24	6.90
	48	1.95
$\text{FB}_1 + \text{AFB}_1$	24	14.5
	48	6.8
$\text{FB}_1 + \text{C. olitorius}$ (20 $\mu\text{g/mL}$)	24	542.8
$\text{FB}_1 + \text{C. olitorius}$ (40 $\mu\text{g/mL}$)	24	26646
$\text{AFB}_1 + \text{C. olitorius}$ (20 $\mu\text{g/mL}$)	24	4.32
$\text{AFB}_1 + \text{C. olitorius}$ (40 $\mu\text{g/mL}$)	24	2.42
$\text{FB}_1 + \text{AFB}_1 + \text{C. olitorius}$ (20 $\mu\text{g/mL}$)	24	18.5
$\text{FB}_1 + \text{AFB}_1 + \text{C. olitorius}$ (40 $\mu\text{g/mL}$)	24	21.77

AFB_1 : aflatoxin B₁; FB_1 : fumonisin B₁; *C. olitorius*: *Cochorus olitorius*; ND: not detectable; MTT: methylthiazole tetrazolium

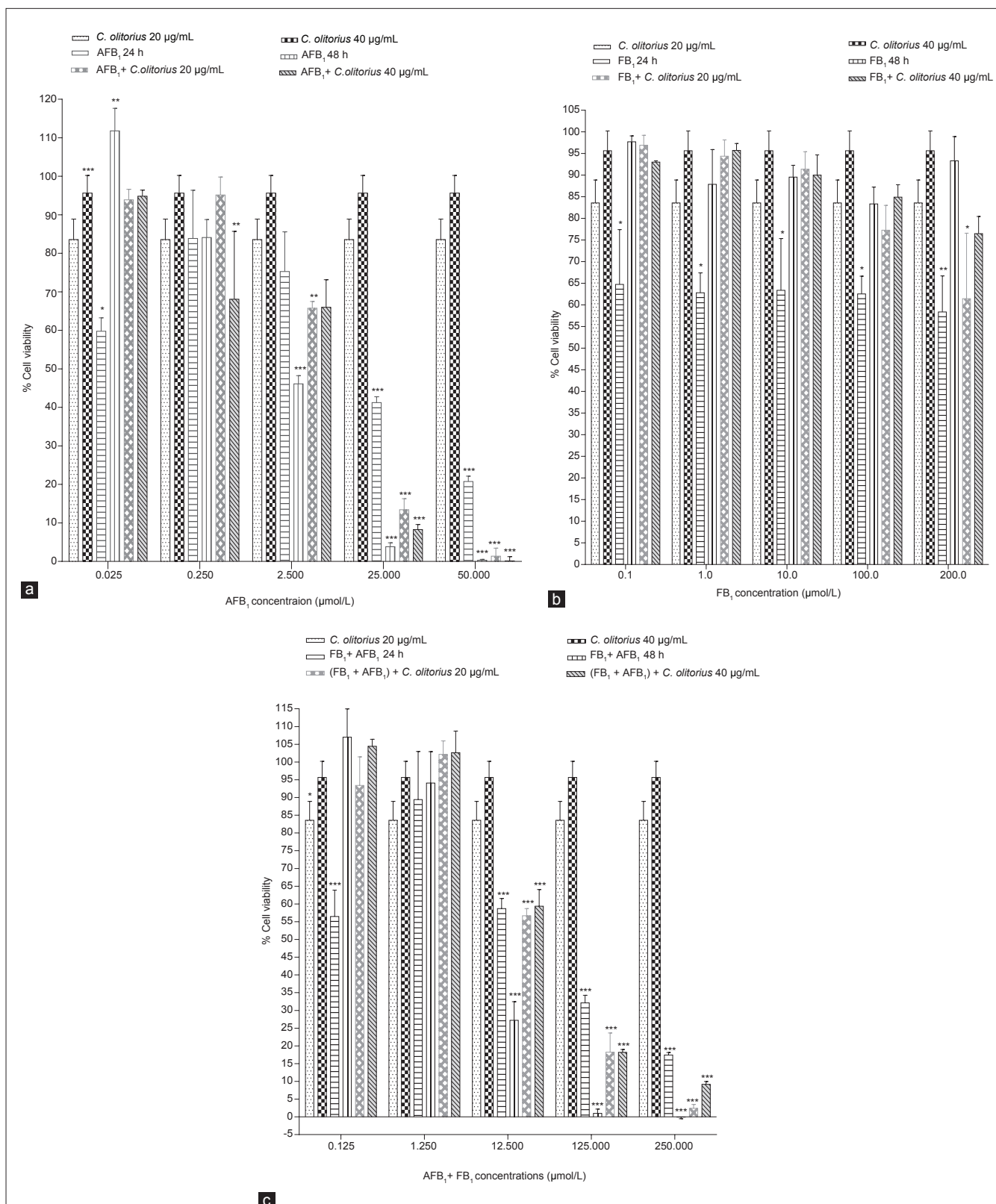


Figure 1: Cytotoxicity of (a) AFB_1 at concentrations of 0.25-50 $\mu\text{mol/L}$ without and with *C. olitorius* extract, (b) FB_1 at concentrations of 1-200 $\mu\text{mol/L}$ without and with *C. olitorius* extract, and (c) AFB_1 at concentrations of 0.25-50 $\mu\text{mol/L}$ together with concentrations of 1-200 $\mu\text{mol/L}$ FB_1 , on proliferation of H4IIE-*luc* cell line determined by MTT bioassay. Data represent mean \pm SEM of triplicates (significance of the differences among treatment groups: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). AFB_1 : aflatoxin B_1 ; FB_1 : fumonisin B_1 ; *C. olitorius*: *Cochorus olitorius*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM: standard error mean

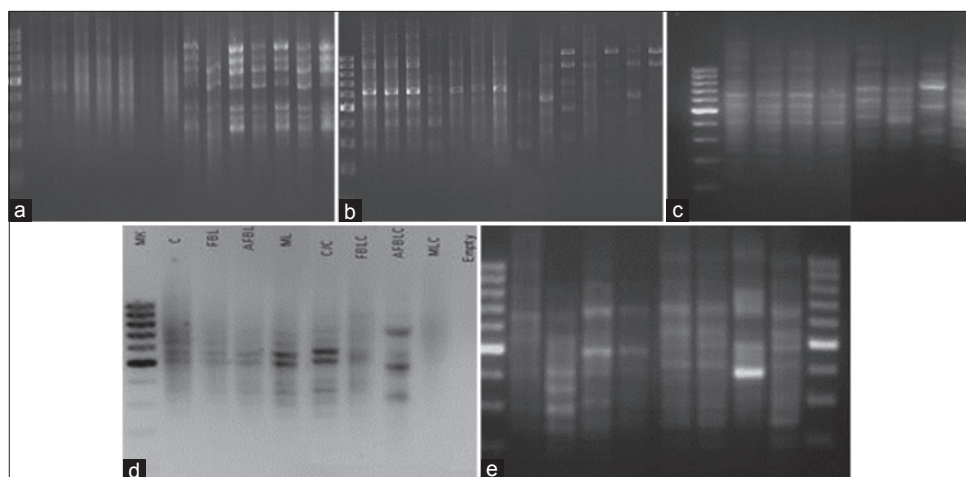


Figure 2: RAPD profiles of genomic DNA from cell line of rat, hepatoma (H4IIE-luc) cells, following exposure to FB₁ and/or AFB₁ for various time periods. (a) PCR products with primer OPD 07. (b) PCR products with primer OPD 09. Lane 1: the DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB₁ (1 µmol/L); lane 4: cells plus FB₁ (200 µmol/L); lane 5: cells plus AFB₁ (0.25 µmol/L); lane 6: cells plus AFB₁ (50 µmol/L); lane 7: cells plus mixture (1 µmol/L FB₁ + 0.25 µmol/L AFB₁); lane 8: cells plus mixture (200 µmol/L FB₁ + 50 µmol/L AFB₁); lane 9: cells plus *C. olitorius* (40 µg/mL); lane 10: *C. olitorius* (40 µg/mL) plus FB₁ (1 µmol/L); lane 11: *C. olitorius* (40 µg/mL) plus FB₁ (200 µmol/L); lane 12: *C. olitorius* (40 µg/mL) plus AFB₁ (0.25 µmol/L); lane 13: *C. olitorius* (40 µg/mL) plus AFB₁ (50 µmol/L); lane 14: *C. olitorius* (40 µg/mL) plus (1 µmol/L FB₁ + 0.25 µmol/L AFB₁); and lane 15: *C. olitorius* (40 µg/mL) plus (200 µmol/L FB₁ + 50 µmol/L AFB₁). (c) PCR products with primer OPD 13. (d) PCR products with primer OPD 16. Lane 1: DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB₁ (1 µmol/L); lane 4: cells plus AFB₁ (0.25 µmol/L); lane 5: cells plus mixture (1 µmol/L FB₁ and 0.25 µmol/L AFB₁); lane 6: cells plus *C. olitorius* (40 µg/mL); lane 7: *C. olitorius* (40 µg/mL) plus FB₁ (1 µmol/L); lane 8: *C. olitorius* (40 µg/mL) plus AFB₁ (0.25 µmol/L); and lane 9: *C. olitorius* (40 µg/mL) plus mixture (1 µmol/L FB₁ and 0.25 µmol/L AFB₁). (e) PCR products with primer OPD 16. Lane 1 and 10: DNA marker (100 pb); lane 2: cells only; lane 3: cells plus FB₁ (200 µmol/L); lane 4: cells plus AFB₁ (50 µmol/L); lane 5: cells plus mixture (200 µmol/L FB₁ and 50 µmol/L AFB₁); lane 6: cells plus *C. olitorius* (40 µg/mL); lane 7: *C. olitorius* (40 µg/mL) plus FB₁ (200 µmol/L); lane 8: *C. olitorius* (40 µg/mL) plus AFB₁ (50 µmol/L); and lane 9: *C. olitorius* (40 µg/mL) plus mixture (200 µmol/L FB₁ and 50 µmol/L AFB₁). AFB₁: aflatoxin B₁; FB₁: fumonisin B₁; *C. olitorius*: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction. (a) OPD 07 for lesser (24 h) exposure; (b) OPD 09 for greater (48 h) exposure; (c) OPD 13 for greater (48 h) exposure; (d) OPD 16 for lesser (24 h) exposure; (e) OPD 16 for greater (48 h) exposure

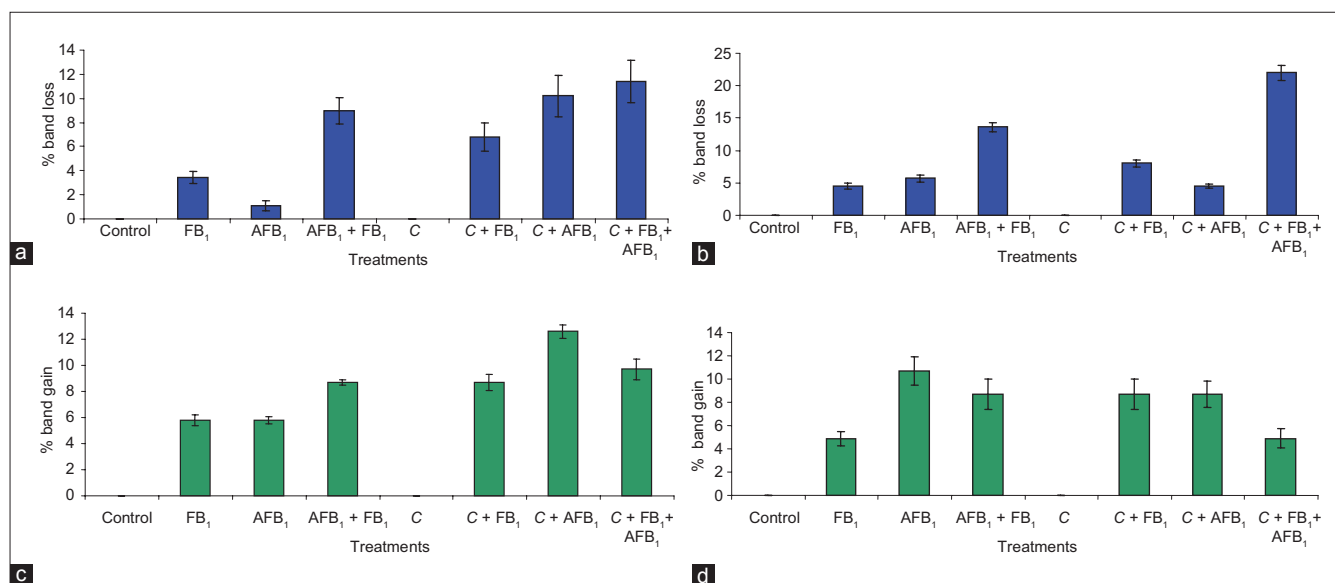


Figure 3: Genomic damage: the percentage of altered bands in each treatment at high concentration detected by RAPD-PCR. (a) Average band loss at lesser concentrations for 24 h; (b) average band loss at greater concentrations for 48 h; (c) average band gains at lesser concentrations for 24 h; and (d) average band gain at greater concentrations for 48 h. AFB₁: aflatoxin B₁; FB₁: fumonisin B₁; C: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

had disappeared, while for the 48 h and exposure, 30 of 75 bands (40%) had disappeared.

In cases where bands were gained after exposure to *C. olitorius* extract at the shorter duration of exposure,

21 new bands out of 75 (28%) were amplified. A similar trend was observed during the longer exposure, where 25 of 75 bands (33.3%) appeared [Figure 3c]. Protective effects of *C. olitorius* extract were observed as new bands appeared during the 24 h, since 32 of 75 bands (42.7%) appeared;

during the longer exposure, 23 of 75 bands (30.7%) disappeared [Figure 3d].

When OPD 9 primer was used, a maximum of 10 RAPD-PCR disappeared when cells were exposed to the mixture of FB₁ and AFB₁ + aqueous extract of *C. olitorius* for 48 h [Table 4]. However, when with OPD 15 was used as the primer, the maximum appearance of new bands showed the same number of bands lost (10) that was observed in cells exposed to AFB₁ + aqueous extract of *C. olitorius* after 24 h.

There was a significant difference in stability of the DNA template between control and each of the treated groups [Figure 4]. However, no significant difference was observed in stability of the DNA template between control and cells exposed to the aqueous extract of *C. olitorius* alone. The protective effect of the aqueous extract of *C. olitorius* on DNA was observed in the cells exposed to FB₁ and AFB₁.

DISCUSSION

Aflatoxin B₁ and FB₁ are the most frequently observed mycotoxins in food and animal feed. In African and European countries, both mycotoxins are found in maize.^[43] Toxicity and carcinogenicity of AFB₁, which has been classified as Group 1 carcinogen are thought to be directly linked to its bioactivation, resulting in a reactive form of AFB₁, the 8, 9-epoxide. Bioactivation of AFB₁ occurs primarily by a microsomal cytochrome P450-dependent epoxidation of the terminal furan ring of AFB₁, which is responsible for binding to cellular macromolecules such as DNA, RNA and other protein constituents.^[44-47] The MTT assay is more sensitive and reproducible than testing intact animals and is valuable in determining the modes of action of toxins. In the current study, H4IIE-*luc* cells responded to FB₁ and AFB₁ as well as a mixture of the two mycotoxins. Cytotoxic effects of FB₁ have been previously observed for murine microglial cells and primary astrocytes,^[48] rat glioblastoma cells,^[49,50] human keratinocytes and esophageal epithelial cells,^[51] primary

Table 4: Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from H4IIE-*luc* rat hepatoma cell line following exposure to FB₁ and/or AFB₁ alone and in combination with the *C. olitorius* extract for 24 and 48 h

Primer	Change in the RAPD profile	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
OPD 7 (24 h)	Appeared	0	3	5	4	4	0	6	0	0	1	1	1	3	0
	Disappeared	0	0	0	0	0	0	0	0	2	0	0	0	0	0
OPD 9 (48 h)	Appeared	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Disappeared	0	0	0	1	3	3	2	0	0	1	5	0	4	10
OPD 13 (48 h)	Appeared	0	0	0	0	3	3	0	0	0	0	0	0	0	0
	Disappeared	0	0	3	0	0	0	2	0	4	6	4	4	6	5
OPD 15 (24 h)	Appeared	0	0	0	1	4	6	3	0	5	2	10	3	0	0
	Disappeared	0	3	1	0	0	0	0	0	0	0	0	0	0	4
OPD 16 (48 h)	Appeared	0	2	0	1	0	0	0	0	4	6	3	6	7	5
	Disappeared	0	0	0	0	2	5	8	0	0	0	0	0	0	0

T1: control; T2: FB₁ (1 μmol/L); T3: FB₁ (200 μmol/L); T4: AFB₁ (0.25 μmol/L); T5: AFB₁ (50 μmol/L); T6: 1 μmol/L FB₁ + 0.25 μmol/L AFB₁; T7: 200 μmol/L FB₁ + 50 μmol/L AFB₁; T8: *C. olitorius* 40 μg/mL; T9: *C. olitorius* 40 μg/mL + 1 μmol/L FB₁; T10: *C. olitorius* 40 μg/mL + 200 μmol/L FB₁; T11: *C. olitorius* 40 μg/mL + 0.25 μmol/L AFB₁; T12: *C. olitorius* 40 μg/mL + 50 μmol/L AFB₁; T13: *C. olitorius* 40 μg/mL + (1 μmol/L FB₁ + 0.25 μmol/L AFB₁); T14: *C. olitorius* 40 μg/mL + (200 μmol/L FB₁ + 50 μmol/L AFB₁). AFB₁: aflatoxin B₁; FB₁: fumonisin B₁; *C. olitorius*: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA

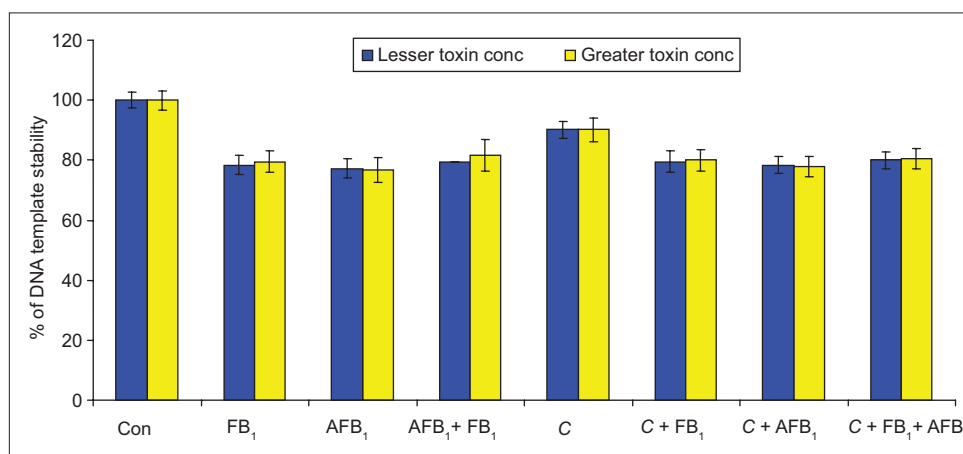


Figure 4: Stability (%) of DNA templates, as determined RAPD-PCR in rat hepatoma cells (H4IIE-*luc*) following exposure to FB₁ and/or AFB₁ for 24 or 48 h. Con: control; AFB₁: aflatoxin B₁; FB₁: fumonisin B₁; C: *Cochorus olitorius*; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

rat hepatocytes and rat liver *in vivo*.^[52] In human and rat glioblastoma cells and mouse hypothalamic cells, production of ROS was increased after exposure to 10-100 $\mu\text{mol/L}$ of FB_1 for 48-144 h.^[53] Exposure to 10-100 $\mu\text{mol/L}$ of FB_1 for 72 h had no effect on production of ROS in human fibroblasts, or in primary cultures of rat astrocytes exposed to the same concentrations of FB_1 for as long as 6 days.^[54,55] Exposure to concentrations as high as 20 $\mu\text{mol/L}$ of FB_1 did not significantly reduce the viability of IPEC-J2 cells.^[56]

In the current study, EC_{50} could not be calculated for FB_1 because viability of cells exposed to 200 $\mu\text{mol/L}$ was reduced only 41.6%, which is consistent with previously published results.^[44] In yet another study, FB_1 was only weakly cytotoxicity.^[57] The EC_{50} for AFB_1 was 1.87 $\mu\text{mol/L}$, which is similar to that observed previously by others,^[58-60] who reported EC_{50} values ranging from 0.065 $\mu\text{mol/L}$ for B-CMV1A2 cells to 14 $\mu\text{mol/L}$ in BE12-6 cells. Exposure of H4IIE-*luc* cells to greater concentrations of AFB_1 and FB_1 resulted in lethality that was a concentration- and time-dependent. This effect was greater in cells treated with AFB_1 or $\text{AFB}_1 + \text{FB}_1$. The interaction of FB_1 and AFB_1 in the induction of DNA damage and its correlation with biomarkers of cellular oxidative status has previously been reported to occur *in vivo*.^[4,8,22,61] These reports suggested that genotoxicity and carcinogenicity of AFB_1 were enhanced by exposure to FB_1 .^[8] The *in vivo* results indicated that these effects were due to the production of ROS, which resulted in lipid peroxidation.^[4,61]

AFB_1 is a well-known genotoxicant. When the mechanism by which the aqueous extract of *C. olitorius* protected H4IIE-*luc* rat hepatoma cells against genetic damage caused by AFB_1 and/or FB_1 was investigated by use of RAPD analysis, there were statistically significant differences in the profiles of expression of the investigated genes, between the control and the treated cell lines at all concentrations tested.^[33] Differences in the profile between the control and the treated samples were due to point mutations and/or base modifications of the genome caused by AFB_1 and/or FB_1 .^[62] Changes were observed for all genes for which primers were used. In our study, both qualitative and quantitative analyses showed that both mycotoxins increased instability of DNA templates of cells, in time- and concentration-dependent manners. This result supports the conclusion that both mycotoxins are direct-acting, genotoxicants that have the potential to attack hotspots present in DNA. The number of stable bands increased as a function of time and dose. Inconsistency in profiles of bands in RAPD analyses might have been observed because the two mycotoxins are acting directly as genotoxicants. However, they might

act as genotoxicants through generation of free radicals during metabolism of the toxins through reactions of either electrophiles or nucleophiles with DNA. This interaction creates changes in their sequences that ultimately results in the formation of new priming sites and/or disappearances of existing priming sites for the RAPD primers. Thus, it gives different RAPD profiles for cells exposed to toxins.^[63]

Random amplification of polymorphic DNA-PCR suffers from inherent limitations such as a lack of reproducibility and occurrence of pseudo-bands which prevent its routine application.^[64] However, if conditions of the assays are properly optimized, these limitations can be resolved.^[65,66] By optimizing conditions of the analysis, cloning the PCR products and further sequencing the products, RAPD can be useful in analyzing the nature and mode of action of the genotoxicants.^[65,66] While in the present study RAPD could detect toxin-induced DNA damage, further studies would be needed before it could be used regularly as a tool in the detection of alterations in DNA sequence due to the genotoxicants.

Previous studies have demonstrated that certain compounds in the diet can offer protection against toxicity of mycotoxins.^[67] Natural vitamins, carotenoids, polyphenol and trace elements are potentially beneficial in protection against mycotoxicosis.^[68] Green leafy vegetables are known to be dietary sources of minerals, trace elements and phytochemicals that contribute to health.^[69] Molecular evidence has suggested that trace elements and antioxidant molecules in green, leafy vegetables lessen risks of cancer and cardiovascular diseases through mechanisms that modulate free radical attack on nucleic acids, proteins, and polyunsaturated fatty acids.^[70] *C. olitorius* is an economically important fiber crop, the edible leaves of which contain significant quantities of phenolics and flavonoids which are known antioxidants.^[33,71-74] Although in the current study the active compound(s) in the aqueous extract of *C. olitorius* were not isolated or identified, flavonoids are possible candidates among the active compound(s) in *C. olitorius*. *C. olitorius* contains abundant amounts of a number of flavonoids that could act as antioxidants, including: 5-caffeoylquinic acid, 3,5-dicaffeoylquinic acid, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside), quercetin 3-(malonylgalactoside), ascorbic acid, α -tocopherol, and chlorophyll.^[29] Furthermore, *C. olitorius* contains relatively high levels of quercetin glycosides. Several novel flavonol glycosides named corchorusides A and B, in addition to a major component, capsugenin-25, 30-O- β -diglucopyranoside have been isolated from *C. olitorius*.^[26] Recently, several flavonoids, such as rutin, and quercetin and phenolic compounds, including gallic acid, chlorogenic acid, p-cumaric acid, ferulic

acid, and ellagic acid have been isolated from extracts of *C. olitorius*.^[75] Consequently, protective effects of the aqueous extract of *C. olitorius* against cytotoxicities of AFB₁ and FB₁ in H4IIE-*luc* might be due to the antioxidant capacity and the abundant occurrence of the flavonoid compounds. Another candidate for the active compound(s) is chlorophyll. Numerous *in vitro* studies have indicated that derivatives of chlorophyll, including chlorophyllide A and B and pheophorbide A and B can attenuate chemical genotoxicity by forming a molecular complex with pro-mutagens,^[74-76] which might involve strong chlorophyll-AFB₁ and/or FB₁ interaction via their planar unsaturated cyclic rings.^[75] Derivatives of pheophorbide A and B provided additional protection not only by direct trapping, but also by increasing glutathione S-transferase activity against hepatic AFB₁ metabolites.^[76]

In conclusion, both AFB₁ and FB₁ induced oxidative stress, which resulted in cytotoxicity and fragmentation of DNA of H4IIE-*luc* rat hepatoma cells after various durations of exposure to these toxins singly or in combination. Exposure to these mycotoxins resulted in appearance of new bands in the RAPD analysis, in addition to DNA damage. Treatment with an aqueous extract of *C. olitorius* resulted in a significant improvement in viability of cells and reduced damage to DNA in H4IIE-*luc* cells exposed to mycotoxins. Due to these effects, *C. olitorius* is suggested to be a traditional edible plant containing potential chemo-preventive agents for human cancers. However, additional studies on the uptake, metabolism, and disposition of the active ingredients in *C. olitorius* need to be further studied. Currently, the active ingredient (s) are unknown, and it is also not known whether these constituents that are effective *in vitro* can have similar effects *in vivo*.

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Laparoscopic radiofrequency ablation for hepatocellular carcinoma

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ABSTRACT

Aim: The optimal treatment for hepatocellular carcinoma (HCC) is either surgical resection or liver transplantation, but only one-third of the patients are suitable candidates for surgery. Laparoscopic radiofrequency ablation (RFA) in selected patients is a safe, feasible technique, which has proved to be superior to the percutaneous approach in patients with severe liver disease or in lesions in which the percutaneous approach is impossible. The aim of this study is to present our experience with laparoscopic RFA and demonstrate its safety as an alternative therapeutic procedure in selected patients with HCC.

Methods: This is a retrospective study of patients with HCC who underwent laparoscopic RFA between March 2009 and December 2014. **Results:** Thirty-two patients with 37 tumors underwent laparoscopic RFA. Median tumor size was 2.24 cm (0.7-4.45 cm). Major complications occurred in 8 patients. Initial complete ablation was achieved in 94.6% (35/37) lesions and sustained complete ablation rate was 62.85% (22/35). Overall survival rates at 1-, 2-, and 3-year were 89%, 67.5%, and 40%, respectively. **Conclusion:** Laparoscopic RFA of HCC is safe and the long-term outcomes are similar to those achieved with liver resection. Further trials combining chemoembolization and RFA are needed to improve long-term outcomes and to limit local tumor progression.

Key words: Hepatocellular carcinoma; radiofrequency ablation; laparoscopic approach

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths and the fifth most common form of cancer worldwide.^[1] Due to the nature of HCC, tumor stage, liver function, and performance status are the main prognostic variables and treatment allocation is

based on the Barcelona-Clinic Liver Cancer (BCLC) staging system^[1-3] [Figure 1]. Liver transplantation and surgical resection are considered the optimal curative strategy,^[1-4] but only one-third of patients with HCC are suitable candidates for surgery.^[4] Radiofrequency ablation (RFA) is considered the most effective local ablative therapy for patients who cannot undergo surgery due to the number and distribution of the nodules and/or the liver impairment,^[1,2,5] and can be performed percutaneously, by laparotomy or by laparoscopy.^[6,7] Laparoscopic RFA is an interesting alternative when percutaneous RFA (perRFA) cannot be performed due to the tumor location (e.g., in the case of subcapsular lesions, nodules adjacent to diaphragm without a therapeutic window, proximity to adjacent structures, and lesions in deep locations)^[7-10] or because of the impossibility of visualizing the tumor by percutaneous ultrasound.^[9] It has been reported

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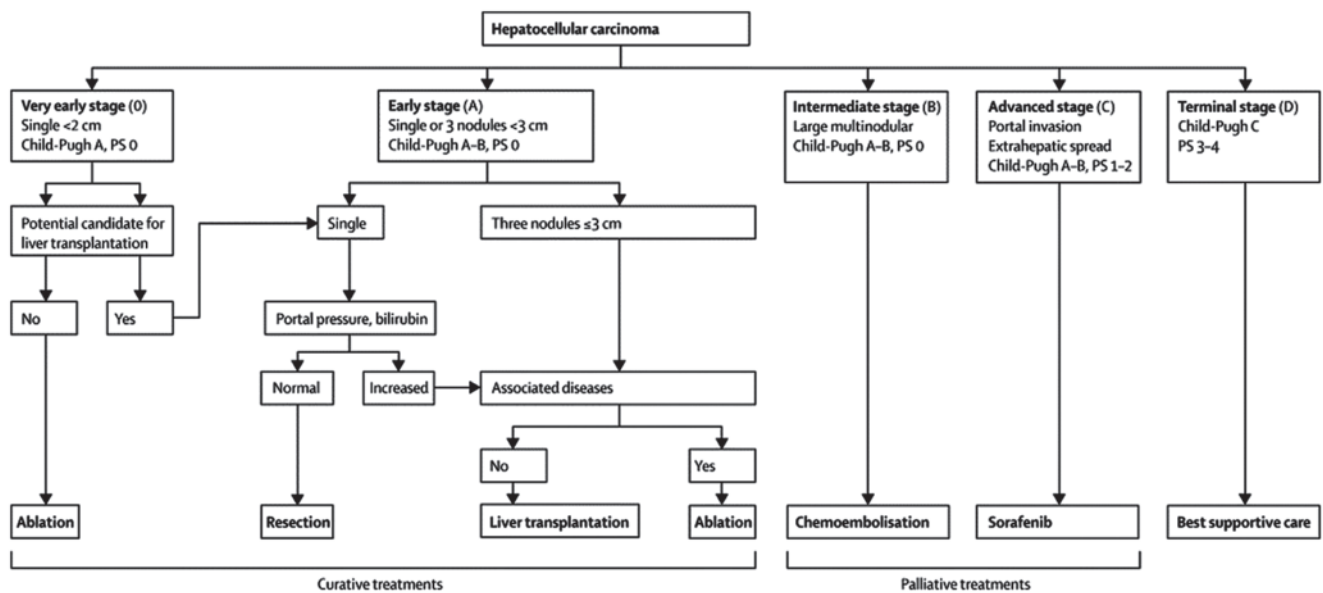


Figure 1: The Barcelona-Clinic Liver Cancer staging system for hepatocellular carcinoma (from Bruix *et al.*^[2]). PS: performance status; M: metastasis classification; N: node classification; Bb: bilirubin; LT: liver transplantation; RFA: radiofrequency ablation; TACE: transarterial chemoembolization

that intra-operative laparoscopic ultrasound (IOLUS) permits detection of 25% of new HCC nodules^[11] and allows much more accurate staging.^[8]

In this paper, we present our experience in laparoscopic RFA, analyzing the outcomes to show the safety and utility of this technique as a valid therapeutic alternative in the selected patients with HCC.

METHODS

Patients and methods

Between March 2009 and December 2014, all patients with HCC attended at the Hospital Son Llàtzer in Palma de Mallorca were entered in a prospective database. A complete medical report was obtained in each patient. Age, gender, etiology, patient characteristics such as comorbidities and liver function, size and location of the tumors, main and associated procedures, post-operative complications, treatment effectiveness and long-term results were recorded. In all, 149 patients were included in the database, with a mean of 24.83 new cases/year. Thirty-seven percent of the patients were suitable for curative treatments such as liver transplantation (which is not performed in our center), liver resection, alcoholization, and radiofrequency. The study was approved by review board of Hospital Son Llàtzer.

A retrospective study was carried out of patients undergoing laparoscopic RFA for HCC. The procedure was performed in: patients with a single lesion or a maximum of three lesions smaller than 5 cm who, due to medical problems or their age, were not candidates for liver transplantation; patients not

suitable for liver resection in whom perRFA was contraindicated for the following reasons: (1) lesions in proximity to the viscera; (2) subcapsular lesions with a high risk of tumoral seeding; (3) lesions not visible by perRFA; and selected patients as a bridging therapy in order to meet the Milan criteria.

The follow-up period of each patient was recorded as the time from the surgical procedure until last clinical evaluation, loss to follow-up or death.

Post-operative complications were recorded and classified by the modified Clavien-Dindo Classification System.^[12]

Surgical procedures

Procedures were performed with the patient under general anesthesia and in the supine position. The pneumoperitoneum was performed with insufflation of CO₂ through a Veress needle inserted through a small 2-3 cm incision above the umbilicus. Patients with nodules in segments VI and VII were positioned in left decubitus position and an 11-mm port was placed in the anterior axillary line. In all cases, the abdominal pressure was maintained under 12 mmHg. In our procedures we used a 30° camera (Karl Storz GrubH and Co KG, Tuttlingen, Germany) and a first complete inspection of the intraperitoneal organs were performed to rule out any extrahepatic disease. A second 5-mm trocar was placed in the epigastric area on the left side of the falciform ligament to introduce the ultrasound device. When lesions were located in the upper part of segment VIII, a hole was made through the falciform ligament in order to provide better access for the ultrasound transducer. Usually an auxiliary 5-mm trocar

was placed on the right side. More trocars were used if it was necessary to mobilize the liver, perform adhesiolysis between the liver and other adjacent organs or release an associated procedure like cholecystectomy.

Intra-operative laparoscopic ultrasound of the entire liver parenchyma was performed to confirm location of the tumor to be treated and to rule out the presence of new nodules. In cases of lesions that were not visible by ultrasound, a piece of a 1-cm (22G) needle was inserted preoperatively into the tumor guided by computed tomography (CT) or ultrasound with a signal enhancer. We did not perform an intra-operative biopsy of the tumor prior to the RFA. Interventional radiologists came to the operating room to perform the RFA procedure and the IOLUS.

The RFA was carried out using the Cool-Tip RFA system (Covidien, Boulder, CO, USA), which uses internally cooled electrodes (ICEs) for ablation. This system circulates chilled saline to the tip of the needle electrode, thus lowering the temperature of the tissue immediately adjacent to it, minimizing tissue charring, and improving the delivery of energy to surrounding tissues.^[13]

The RFA electrode was inserted through a separate percutaneous puncture and the needle was placed as parallel as possible to the plane of the ultrasound so that its entire path could be seen on the ultrasound image as it traversed the liver parenchyma. A single needle 20-cm long and with a 2-cm tip exposure was used for tumors < 3 cm, and for tumors \geq 3 cm a cluster was used to achieve an adequate tissue margin. A 12-min RFA cycle was performed as per the manufacturer's recommendations [Figure 2].

The ICEs have sensors in their tips which measure tissue temperature and impedance at the end of the ablation procedure.^[13] The ablation was considered satisfactory if the end-tissue temperature after 12 min of RFA was $\geq 60^{\circ}\text{C}$, which is enough to cause instantaneous cellular necrosis.^[13] If the end-tissue temperature was $< 60^{\circ}\text{C}$, another RFA cycle lasting 6-10 min was performed.

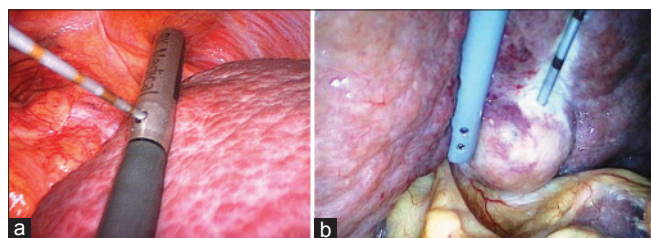


Figure 2: Surgical images of radiofrequency ablation of (a) a lesion next to the diaphragm and (b) a lesion close to duodenum and colon

After finishing the nodule ablation, the intrahepatic needle track was treated with thermocoagulation to avoid track seeding.

Follow-up and definition of clinical outcome

Treatment outcome was evaluated with an enhanced CT scan 2 months after laparoscopic RF.^[14] The inflammatory reaction makes the proper assessment of the treated nodule difficult during the 1st month. Thereafter, patients were followed with CT or magnetic resonance imaging and with the α -fetoprotein every 3 months during the 1st year and every 6 months in the 2nd year.

Initial incomplete ablation was determined as the presence of enhanced areas within the treated nodule in the first follow-up imaging. Sustained complete ablation was defined as the absence of enhanced areas within the treated area at the end of the follow-up period.

Local tumor recurrence was defined as the presence of a growing tumor in the ablation zone after complete ablation had been determined in the first follow-up CT. Distant recurrence or new tumor progression was determined as a growing nodule occurring away from the ablation zone.

Statistical analysis

Data for all patients with HCC were recorded prospectively and introduced into a Microsoft ACCESS database. Data from patients undergoing laparoscopic RFA were analyzed retrospectively using the statistical software SPSS version 18 (SPSS Inc., Chicago, IL, USA). Continuous data were described as means and analyzed with Student's *t*-test if the distribution was normal or with Mann-Whitney *U*-test otherwise. Discontinuous data were presented as percentages and were analyzed by the Chi-square of Fisher's exact test. Overall survival curves and cumulative recurrence curves were analyzed by the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

RESULTS

Patient and tumor characteristics

Between March 2009 and December 2014, 149 new cases of HCC were recorded. Eight perRFA and 40 surgical RFA were performed during this period. Only 3 lesions with radiological features of HCC from the 40 surgical RFA were treated by an open approach, while 37 lesions from 32 different patients were treated with laparoscopic RFA. We recorded the age of all patients, gender, liver function (Child-Pugh Classification), etiology [hepatitis C virus (HCV⁺) or non-HCV⁺], and previous treatment. For tumors we recorded the number of nodules (uninodular or binodular), size, and location (subcapsular, intrahepatic or adjacent to viscera).

All patient and tumor characteristics of the laparoscopic RFA procedures are shown in Table 1. The main reasons for preferring laparoscopy to the percutaneous approach were: subcapsular location in 26 cases, intrahepatic location in eight cases which were difficult to define by perRFA, and location very close to adjacent viscera in three cases.

Although according to BCLC guidelines patients with liver function Child C are not initially candidates for RFA, one patient with Child C finally underwent RFA as palliative treatment after discussion with the Hepatobiliary Committee.

Two patients had a simultaneous surgical procedure associated with the laparoscopic RFA. One of them had a cholecystectomy due to the proximity of one of the tumors to the gallbladder, which was performed prior to the RFA. The specimen was removed by using an endobag and no tumoral seeding was reported. The other simultaneous procedure was a hysteroscopy for a uterine biopsy.

Radiofrequency ablation results

An initial complete ablation was achieved in 35 of the 37 lesions that underwent laparoscopic RFA (94.6%). In one patient without initial complete ablation the lesion was subsequently treated by laparoscopic alcoholization and in the other transarterial chemoembolization (TACE) was performed.

Morbidity

No complications were reported during any of the laparoscopic RFA procedures nor in relation to other simultaneous techniques. Conversion to open surgery was not needed in any patient. The mean post-operative hospital stay was 4.9 days (1-30). All complications are detailed in Table 2 following the modified Clavien-Dindo Classification System.^[12] Eight patients presented some kind of complication: only one of them required emergency reintervention, following a hemoperitoneum (Clavien IIIb). One patient presented liver decompensation with mild ascites and was successfully treated with diuretics (Clavien I).

Recurrence

The median follow-up period was 28.3 ± 2.3 months. After achieving initial complete ablation (35 procedures), local recurrence was seen in 13 cases, after a median delay of 8.38 months (range 3-30). Six recurrent lesions were treated by TACE, 5 by new laparoscopic RFA, 1 by perRFA, 1 by sorafenib, and 1 underwent symptomatic treatment only.

Cumulative recurrence rates at 6, 12, and 36 months were 22.85%, 34.28%, and 37.18%, respectively. A higher

Table 1: Patient and tumor characteristics

Variable	Result
Patient characteristics (n = 32)	
Age	63.56 (38-83) years
Gender (male/female)	21/11
Etiology	
HCV	23
Non-HCV	9
Child	
Child A	23
Child B	8
Child C	1
Cirrhosis	25
Non-cirrhosis	7
Previous treatment	
TACE	2
perRFA	1
Tumor characteristics (n = 37)	
Uninodular	27 (84%)
Size	2.24 (0.7-4.5) cm
≤ 2 cm	19
2.1-3 cm	14
> 3 cm	4
Location	
Subcapsular	26
Intrahepatic	8
Adjacent to viscera	3

perRFA: percutaneous radiofrequency ablation; TACE: transarterial chemoembolization; HCV: hepatitis C virus

Table 2: Post-operative complications

Variable	Result
Clavien I	3
Clavien II	3
Clavien IIIa	0
Clavien IIIb	1
Clavien IVa	0
Clavien IVb	0
Clavien V	1

local recurrence rate was observed in cirrhotic patients, HCV⁺, with subcapsular tumors, although the differences were not statistically significant. Twenty-two lesions of the 35, which achieved initial complete response did not present local recurrence at the end of the follow-up period, representing a rate of sustained complete ablation of 62.85%. Mean follow-up was 18.72 months (range 3-44). Distant recurrence was observed in 15 patients after a mean delay of 16.66 months of follow-up (range 4-39).

Mortality and survival

One patient was lost to follow-up and 16 had died by the end of the follow-up period. No mortality related to the main procedure was reported. One death was reported 30 days after the surgical procedure in a patient with Child-Pugh C cirrhosis who, after RFA, also had complications related to

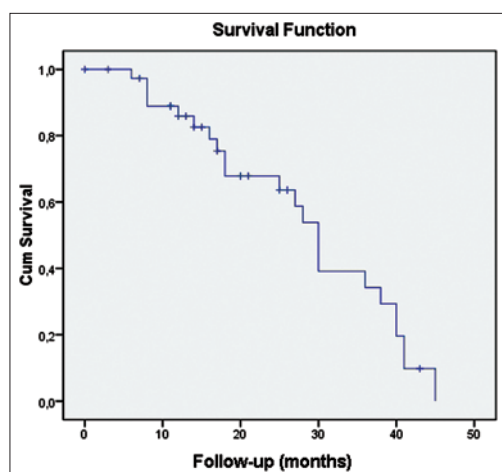


Figure 3: Overall survival of patients with hepatocellular carcinoma treated by laparoscopic radiofrequency ablation

acute vascular ulcers following severe sepsis and multi-organ failure. Only 3 deaths were related to HCC progression.

Overall survival rates at 1, 2, and 3 years were 89%, 67.5%, and 40%, respectively [Figure 3]. There was no association between local or distant recurrence and survival.

DISCUSSION

Patients with HCC have very poor prognosis. Most have poor liver function or major comorbidities at the time of diagnosis that rule out major resections; in fact, liver resection can only be performed in one third of the patients with the disease.^[4] Modern techniques such as RFA are not only potentially curative, with results similar to those achieved with liver resection, but are also minimally invasive.^[15] Laparoscopic RFA was introduced by Jung *et al.*^[16] in 2002 as an alternative technique in cases in which the percutaneous approach was not feasible. Another indication for the procedure is as a bridging therapy to liver transplantation for patients with HCC and terminal liver disease awaiting liver transplantation.

Compared to perRFA, the laparoscopic approach allows a complete vision of the entire intervention, especially when associated with ultrasound, which has demonstrated its utility in reporting new malignant lesions intra-operatively,^[4,10,11] and offers the possibility of treating other tumors simultaneously. Some groups have shown better oncological outcomes^[6,16] and less tumoral spread^[9] with laparoscopic RFA than with the percutaneous procedure. Moreover, simultaneous procedures can be performed together in laparoscopic RFA such as cholecystectomy or liver resection if needed. However, de la Serna *et al.*^[9] reported that laparoscopic RFA for HCC adjacent to the gallbladder seems to be associated with a decreased ablation efficacy in terms of both initial and long-term

complete tumor response. The complications associated with perRFA reported in the literature include intraperitoneal hemorrhage, hepatic infarction, hepatic abscess formation, intestinal perforation, bile peritonitis, and carcinoma seeding. Laparoscopic RFA of HCC is associated with a low rate of major complications, most of them related to bleeding from hepatic puncture sites or trocar accesses and to iatrogenic malignant seeding. We reported only one case of major bleeding requiring reintervention, and no tumor spread was observed. There were no treatment-related deaths in our series.

In the literature, few reviews of minimally invasive RFA are available, since most surgical RFA procedures are still performed by laparotomy.^[6] Today the advantages that the laparoscopic approach can offer, in terms of creating fewer adhesions^[4] and achieving earlier recovery, are well known. The procedure also appears to minimize the surgical insult, with less post-operative morbidity in cirrhotic patients,^[4,9,11,16] so laparoscopic RFA seems preferable to open RFA in these patients as well.^[6] The laparoscopic approach has also shown lower morbidity, lesser hepatic decompensation, and blood loss, and fewer pulmonary complications.^[6,9,17] Moreover, the increased intraperitoneal pressure necessary to perform laparoscopy reduces the portal venous flow, thus improving thermal conduction, enhancing ablation efficacy, and enlarging the ablation zone.^[18]

The rates of initial complete ablation, sustained complete ablation, local recurrence, and survival in the present report are similar to those in previously published reviews,^[4,9,10,13] despite the limitation of our study in terms of its retrospective nature and its small sample size. de la Serna *et al.*^[9] reported an initial complete ablation rate of 94% in a study including 51 treated lesions, with a sustained complete ablation rate of 70%, slightly higher than our rate of 62.85%. Our 1-year cumulative recurrence of 34.28% is an improvement on the rates of 47.4% published by Lee *et al.*^[10] and of 39% published by de la Serna *et al.*^[9]

Some authors have reported that pre-treatment α -fetoprotein and poorly differentiated HCC were independent predictors of local tumor recurrence.^[19,20] This suggests that performing an intra-operatively biopsy prior to laparoscopic RFA, as some groups do systematically, may help to predict long-term results, although it has also been reported to contribute to malignant seeding.^[5,21]

Patients with HCC have a dismal prognosis, with a moderate rate of local recurrence and low long-term survival. Moreover, most of the patients who are treated with laparoscopic RFA are elderly, have severe comorbidities or have impaired liver

function which may make prognosis even worse. Further prospective randomized controlled trials with larger sample sizes should focus on combining RFA with therapies like TACE in order to decrease local tumor progression.

As a conclusion, laparoscopic RFA of HCC is a safe and effective curative strategy in selected patients with unresectable disease, especially when the percutaneous approach is very difficult. In combination with ultrasound, this technique offers clear advantages over other approaches. The incidence of major complications within 30 days after the procedure is very low and the long-term outcomes are similar to those achieved with liver resection. Further trials are needed to assess long-term results in terms of local recurrence and survival.

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Long-term immune-modulatory side effects of radiofrequency ablation in patients with liver metastases and hepatocellular carcinoma

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ABSTRACT

Aim: Used as a palliative therapy for unresectable liver cancer, radiofrequency ablation (RFA) is associated with the induction of immunological responses. Here, we show strong evidence of tumor-specific peripheral blood mononuclear cells (PBMCs) 12 months after RFA. **Methods:** Three patients with colorectal cancer (CRC) metastases to the liver and two patients with primary hepatocellular carcinoma (HCC) were enrolled in this study. PBMC, isolated 12 months after RFA, were stimulated with normal and tumor tissue lysate. Interferon gamma secretion was evaluated by flow cytometry and indirectly, by luciferase assay for adenylate kinase activity in PBMC-stimulated lysates of target cells. Baseline data were detected before RFA and 4 weeks after treatment. **Results:** Two CRC patients and one HCC patient had recurrence-free survival. One patient with CRC developed secondary metastases; one patient with HCC developed a local recurrence. Recurrence-free patients showed a significantly higher cytolytic activity of PBMC against matched tumor cells 12 months after RFA treatment. Interestingly, patients with malignant recurrence showed a decreased cytolytic activity. **Conclusion:** RFA seems to overcome immune-tolerance toward tumor antigens and/or presents new tumor antigens. Patients seem to benefit from a prolonged increase in cytolytic activity. The immune-modulatory effects of RFA need further investigations in multimodality anticancer therapies.

Key words: Liver cancer; radiofrequency ablation; immune response; interferon gamma; peripheral blood mononuclear cells

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INTRODUCTION

Surgical resection is still the gold standard for the treatment of hepatocellular carcinoma (HCC) and liver metastases of colorectal cancer (CRC). However, more than 75% of these patients are elected as unresectable due to the volume and localization of the tumor. Radiofrequency ablation (RFA) is a common therapy option for unresectable liver tumors.^[1-4] It was shown that RFA and laser-induced thermotherapy (LiTT)

can achieve, in selected patients, a survival prolongation time comparable to surgical resection.

An earlier study suggested that RFA has adjunctive immune-modulatory side effects.^[5] By using the VX 2 hepatoma model in rabbits, we showed that RFA can induce a strong mononuclear infiltration around the implanted tumor. More recently, we demonstrated a marked tumor-specific peripheral T cell response in RFA-treated vs. untreated rabbits with the VX 2 hepatoma.^[6,7] We further investigated whether this strong immune response to RFA can be observed in humans. We observed that significantly elevated levels of CD8⁺ T cells appeared 4 weeks after RFA, and this effect lasted at least up to 8 weeks after RFA. There was also significant cytolytic activity of isolated peripheral blood mononuclear cells (PBMCs) 8 weeks after RFA.^[8]

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The aim of this study was to investigate the immunological features of HCC and CRC metastases patients 12 months after RFA.

METHODS

The study was approved by the Ethical Committee of the University of Erlangen-Nuremberg (Ethikkomitee der Universität Erlangen Nürnberg) and performed according to the declaration of Helsinki. All treated HCC and CRC metastases were confirmed histologically prior to therapy.

Selection and description of participants

All enrolled patients took part in a prior trial in which baseline data before RFA treatment were recorded according to the present protocol.

Patients with up to 3 tumor nodules within the liver, with a maximum diameter of 6 cm per lesion, were enrolled in the study. Prior local ablative therapy (LiTT, RFA, ethanol injection) or prior chemo-embolization of the malignant liver tumor was an exclusion criterion. The possibility of curative treatment by resection had to be ruled out. Therefore, all cases were discussed in our tumor conference, including gastroenterologists, oncologists, surgeons, and radiologists.

Patients with at least one of the following findings were also excluded: Karnofsky index < 60, thrombocytes < 50,000/ μ L, prothrombin activity < 50%, partial thromboplastin time > 80 s. No transfusion of platelets or fresh frozen plasma was performed. Informed consent was obtained from every patient no later than 24 h before treatment.

The size and number of tumor nodules were determined by ultrasonography and by computed tomography (CT) (dynamic spiral CT with intravenous application of contrast medium) prior to RFA.

Five consecutive male patients with 8 tumor nodules in total (CRC patients with 2 nodules each and HCC patients with 1 nodule) who met the inclusion criteria were enrolled. Mean patient age was 64 years (range: 59-74 years). Three patients suffered from CRC metastases to the liver while 2 suffering from HCC.

Radiofrequency ablation technique

The whole procedure was performed under ultrasound guidance (Elegra Advanced®, Siemens, Erlangen, Germany) under sterile conditions. The proposed puncture site was infiltrated with a local anesthetic (2% mepivacaine hydrochloride) and the perfused radio-frequency (HF)

needle (Integra, Rättingen, Germany) advanced into the tumor. Midazolam (0.5-5 mg) and/or pethidine (25-100 mg) were administered intravenously as necessary. Patients were monitored by pulse oximetry during the whole procedure.

Two mm (14 G) diameter RFA needle applicators and 15 mm active electrode with microbores were used. During HF application (40 W power output) the RFA needle was continuously perfused with isotonic saline via the bore holes. RF energy was delivered by a computer-assisted radiofrequency generator (Elektrotom 106 HF®, Integra, Rättingen, Germany) and continuous perfusion of the RFA needle was secured by a syringe pump (Pilot C, Fresenius Medical Care, Alzenau, Germany) linked to the RF generator. Perfusion was adjusted according to impedance by means of an electronic interface between generator and perfusor, automatically increasing in response to a rise in impedance (> 400 Ohm). The RF energy was applied for 10-15 min at each needle position, leading to a coagulation zone of 30-35 mm in diameter. Tumors larger than 20 mm were targeted using different applicator positions to create overlapping coagulation zones, in order to treat the entire lesion with a safety margin > 5 mm. Larger tumors were treated with up to 3 simultaneous needle insertions arranged in a triangle (2-4 cm) or square (for larger tumors).

Interferon gamma secretion assay and lymphocyte staining

Heparinized blood (LI-Heparin 10 mL) was collected 12 months after RFA. The samples were stored at 4 °C and tests performed within 12 h after sampling. A liver biopsy of normal and tumor tissue was collected from every patient directly before RFA and stored at -20 °C.

Tissue-lysates were freshly prepared in cold phosphate buffer (50 mmol/L, pH 7.2) using a glass homogenizer as described.^[6] The suspension was filtered with a filter tip (pore size 1.2 mm) to adjust the fragment size to < 1.2 mm. The protein concentration was measured spectrophotometrically using the Bradford assay, and adjusted to 1 mg/mL.

Autologous test antigens (normal and tumor lysate, 12.5 mg) were added to 250 μ L heparinized blood and cultured in a 15 mL conical polypropylene tube for 16 h at 37 °C under 5% CO₂. A negative control without the addition of antigen lysate was included, while staphylococcal enterotoxin B served as positive control antigen. Thereafter, the samples were put on ice and washed with ice cold washing solution [phosphate buffer saline containing 0.5% bovine serum albumin and 2 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4] and the cell suspension centrifuged at 300 g for 10 min at 4 °C. The cell pellet was resuspended with 80 μ L ice cold culture medium (RPMI1640 containing

10% human AB serum). “Catch” reagent (20 μ L) containing a bivalent CD45 capture and interferon gamma (IFN γ) binding antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added; the suspension was kept for 5 min on ice and 5 mL medium was added before incubation at 37 °C in closed roller tubes for 45 min. Thereafter, 20 μ L phycoerythrin-conjugated antibody against IFN γ (1:5 dilution ratio) as well as 10 μ L fluoresceine-thiocyanate conjugated anti-CD8 antibody (1:400 dilution ratio) (both from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added to the cooled and washed cell suspension and incubated for 10 min on ice. Erythrocyte lysis buffer (5 mL), containing 0.155 mol/L NH $_4$ Cl, 10 mmol/L KHCO $_3$ and 0.1 mmol/L EDTA diluted 1:10, was added for 10 min and the cell suspension centrifuged at 300 g for 10 min. The cell pellet was resuspended in 500 μ L ice cold washing buffer and immediately analyzed by flow cytometry (FACS Calibur, BD Biosciences, Heidelberg, Germany) after addition of 0.25 mg propidium iodide in 5 μ L distilled water. FACS data were analyzed using software WinMDI Version 2.8 (Scripps Research Institute, La Jolla, CA, USA).

Cytotoxicity assay

Cytolytic activity of T cells was measured by adenylate kinase (AK) release assay. Cells (10 4) were incubated with 1,000 effector cells in a final volume of 200 μ L growth medium with fetal calf serum in round-bottom 96-well microtiter plates. After incubation for 4 h at 37 °C, 100 μ L of supernatant was harvested and stored at -20 °C for further analysis.

The human HCC cell line HepG2 and the human CRC cell line CaCO $_2$ served as target cells for T cells isolated from patients with HCC and CRC metastases, respectively. All cell lines were human leukocyte antigen matched (ABO-system) and tested previously. HepG2 (ACC-180) and CaCO $_2$ (ACC169) cells were purchased from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig Germany) and were cultured with RPMI1640 and DMEM supplemented with 10% fetal bovine serum, penicillin (107 U/L) and streptomycin (10 mg/L) (Biochrom AG, Berlin, Germany) as previously described.^[9,10]

Maximum AK release was obtained by incubating target and effector cells with Total-Lysis Reagent™ (Lonza, Cologne, Germany), and baseline AK release was obtained by incubating cells with medium alone. Baseline release from T cells and tumor cells were < 10% of maximum release in all experiments, and baseline value was subtracted from each value.

The activity of AK was determined by detection of auto-luminescence using a luciferase assay (ToxiLight Kit,

Lonza, Cologne, Germany). Supernatant (20 μ L) was incubated with AK-detection reagent (Lonza, Cologne, Germany) for 5 min at room temperature. Bioluminescence was measured by a luminometer (BD Monolight 3096 Microplate Luminometer, BD Biosciences, Heidelberg, Germany) and expressed as relative luminescence units (RLU).

Statistical analysis

The stimulation index (SI) of CD8 $^+$ T cells was determined by the ratio of IFN γ^+ CD8 $^+$ vs. IFN γ^- CD8 $^+$ T cells stimulated with tumor tissue lysate vs. stimulation with normal liver tissue, respectively, and calculated with Excel® 2003 software (Microsoft, Seattle, USA). Results were analyzed statistically by the SPSS® software package (Version 14, SPSS GmbH Software, Munich, Germany). SI values were divided into two groups according to the histological origin of the tumors. SI values were calculated numerically and are presented as columns. RLU were expressed numerically and are shown as columns. The significance of the enhanced SI after RFA treatment and augmented cytotoxic activity was tested with Fisher’s test for dependent samples. $P < 0.05$ were considered significant.

RESULTS

Of 3 patients suffering from CRC, 2 had a recurrence-free survival and 1 developed secondary metastases. One patient with HCC developed a local recurrence, and the other one had a disease-free survival after 12 months [Table 1].

All patients had a significant activation of tumor-specific T cells (SI baseline = 2.02, SD \pm 0.2; SI CRC at 12 months = 12.3, SD \pm 0.14; SI HCC at 12 months = 11.8, SD \pm 0.23) ($P < 0.05$) [Figure 1].

Disease-free patients showed a significantly risen cytolytic activity of PBMC against matched tumor cells even 12 months after treatment ($P < 0.05$). The cytolytic activity after 12 months was comparable to baseline data (RLU before RFA = 10.4, SD \pm 1.3; RLU 4 weeks = 354.7, SD \pm 42.1; RLU CRC 12 months = 298.4, SD \pm 23.1; RLU HCC 12 months = 317.4). In contrast, patients with recurrence of malignancy showed a significantly decreased cytolytic activity (RLU for CRC at 12 months = 76.2; RLU for HCC at 12 months = 102.5) ($P < 0.05$) [Figure 2].

DISCUSSION

In the last few years, local ablative therapies such as laser induced or radiofrequency induced thermal ablation have become more attractive as therapeutic options for patients with unresectable solid tumors. The results of local ablative

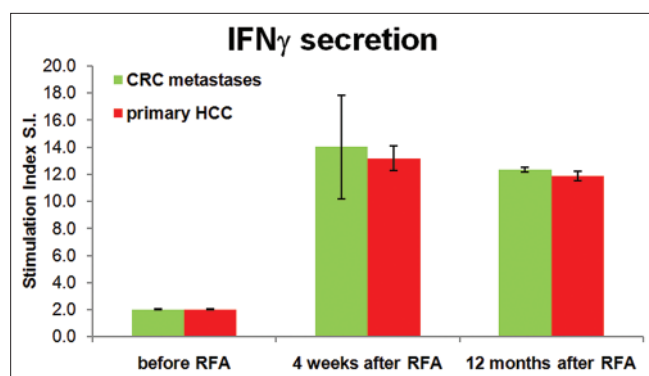


Figure 1: Stimulation index of CD8⁺ T cells was determined by the ratio of IFN γ -CD8⁺ vs. IFN γ -CD8⁻ T cells after stimulation with autologous tumor antigens. Shown are mean \pm SD. IFN γ : interferon gamma; CRC: colorectal cancer; HCC: hepatocellular carcinoma; RFA: radiofrequency ablation

Table 1: Summary of general data with entity of cirrhosis and entity of primary in case of metastases

Patient	Entity	Stage of disease 12 months after RFA
Primary		
CRC1	Colon descending	Disease free
CRC2	Rectum	Metastases to lung
CRC3	Rectum	Disease free
Cirrhosis		
HCC1	Alcohol	Disease free
HCC2	Alcohol	Local recurrence

CRC: colorectal cancer; HCC: hepatocellular carcinoma; RFA: radiofrequency ablation

therapies have shown encouraging survival rates similar to R0 resection of tumors.^[1,2,11] In addition, T cell vaccination or vaccination with dendritic cells is regarded as a promising strategy for the treatment of various tumor types such as malignant melanomas,^[12-20] and high expectations have been placed on cytokine-modulated immunotherapy for liver tumors.

It is well-known that RFA can induce an unspecific immune stimulation. Thus, thermal coagulation causes an inflammatory reaction with lymph-plasma-cellular infiltration that can be visualized as a hypervascular rim in contrast CT and contrast-enhanced ultrasound. This hypervascular rim can be so intense as to impede proper assessment of treatment success.^[5] We recently demonstrated a specific T cell response, after RFA application, toward the orthotopic VX 2-tumor implantation in rabbit livers.^[6] In the clinical setting, it was also shown that a tumor-specific immune response could be detected in patients after RFA. Our preliminary study showed a significant appearance of tumor-specific CD4⁺ and CD8⁺ T cells in peripheral blood up to 8 weeks after RFA. The cytolytic activity of isolated PBMC was also significantly elevated.^[8]

In this study, we investigated the presence of tumor-specific T cells and the cytolytic activity 12 months after RFA

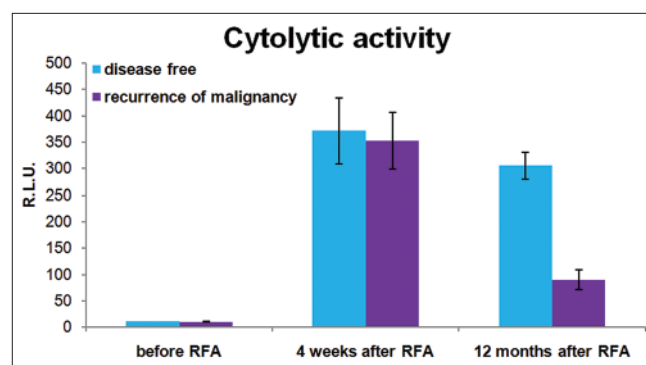


Figure 2: Cytolytic activity of T cells measured by adenylate kinase (AK) release assay. AK release was quantified by luminescence and expressed as RLU. A significant difference between patients with recurrence of malignancy and patients with disease-free survival is shown after 12 months ($P < 0.05$) only. Shown are mean \pm SD. RFA: radiofrequency ablation; RLU: relative luminescence units

treatment. As these tissue samples were obtained by fine-needle biopsy prior to RFA, we decided to concentrate on the measurement of CD8⁺ cells. These cells are reported to be the most promising cell population for anti-tumor therapies. All patients still had significantly increased levels of CD8⁺ cells containing cytotoxic T cells, NK cell and NK T cells as well. Whether these observed cells are resting T cells or any other activated form of T cell remains unclear.

We observed no differences between patients with recurrence of malignancy or with disease-free survival. Interestingly, cytolytic activity was significantly lower in the two patients with tumor recurrence compared. Perhaps, recurrence of malignancy is caused by an ineffective cytolytic/cytotoxic activity of CD8⁺ cells but needs to be confirmed with further investigations.

Prior data showed an enhanced tumor growth if RFA or resection of liver metastases of CRC were not properly performed with remaining micrometastases due to hypoxia and growth factors induced by the process of wound healing.^[21,22] This might indicate that the immune response is not strong enough to control tumor growth or that the immune response is not tumor specific or not effective by the lack of tumor-specific cytolytic cells.

Furthermore, the potential role of a tumor-specific immune response as a target for supportive adjuvant therapy after RFA or resection of metastases has not yet been addressed. Therefore, larger groups with a follow-up for more than 6-12 months are needed to clarify whether lower cytolytic activity levels are correlated with recurrence of malignancy after local ablative therapies. This would suggest the need for an effective combination of drugs with RFA to achieve a long lasting strong cytolytic activity of the Th2 immune response.

Further studies are needed to investigate the potential of the cytotoxic activity of CD8⁺ cells as a prognostic factor. The cytotoxic activity may also play an important role in tumor recurrence. In addition, the possible correlation between an active immune response and disease free survival needs to be clarified. The combination of toll-like receptor agonists with RFA has the potential to improve the outcome of patients with solid tumors in the liver.^[23]

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Sorafenib suppresses hepatitis B virus gene expression via inhibiting JNK pathway

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ABSTRACT

Aim: Hepatitis B virus (HBV) infection is a major cause of chronic liver diseases. Sorafenib is a multikinase inhibitor and an approved anti-liver cancer drug. Here we demonstrated the antiviral effect of sorafenib on HBV gene expression.

Methods: To investigate the effect of sorafenib on HBV gene expression, a luciferase assay was performed with ×1.3 Cp-luciferase HBV construct and reverse transcriptase polymerase chain reaction (PCR), real-time PCR, and Western blotting analyses were performed using HepG2 cells derived from hepatocellular carcinoma and Chang liver cells derived from a normal liver tissue. **Results:** Sorafenib suppressed HBV gene expression via inhibiting the JNK pathway. In this process, the farnesoid X receptor (FXR), a transcription factor that has been reported to increase HBV replication and gene expression, was under control of the JNK pathway. Notably, JNK activation increased FXR protein levels, not mRNA levels.

Conclusion: Sorafenib suppressed HBV gene expression via inhibiting the JNK pathway, which regulates FXR activity.

Key words: Sorafenib; hepatitis B virus; JNK; farnesoid X receptor; antiviral effect

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious public health problem with approximately 350 million people with chronic HBV infection in the world. Among HBV-infected patients, 15-40% develop cirrhosis, liver failure, and hepatocellular carcinoma (HCC).^[1] Persistent infection with HBV is a leading cause of human chronic liver disease. It is well known that for cancer patients with chronic HBV infection who are undergoing cytotoxic chemotherapy, hepatic dysfunction occurs more frequently than that of non-HBV carriers, and this has been attributed mainly to the development of

HBV reactivation. HBV reactivation can be transient and resolve spontaneously but often leads to clinically apparent acute hepatitis. Its occurrence constantly results in delays in chemotherapy schedules and disruption of cytotoxic treatment regimens and in the most severe cases, leads to acute liver failure and death.^[2] Although HBV reactivation can be prevented by antiviral prophylaxis, the mechanism by which HBV contributes to events leading to liver injury in chronic HBV carriers who are receiving cancer chemotherapy remains to be fully understood.

Sorafenib (BAY43-9000, Nexavar) is the first and only medication approved by the USA Food and Drug Administration for the treatment of advanced HCC. Sorafenib is a multikinase inhibitor which has been shown to block tumor cell proliferation and angiogenesis by inhibiting serine/threonine kinases (c-RAF and b-RAF) as well as several receptor tyrosine kinases.^[3] Although several drugs with high efficacy against chronic hepatitis B (CHB) are currently approved in the USA for the treatment of CHB, the drugs have adverse effects

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including: drug resistance, nephrotoxicity, and myopathy.^[4] Therefore, new drug strategies with other mechanisms are extensively required for antiviral therapy.

This study focused on the anti-viral effect of sorafenib based on the function of inhibiting the molecular signaling pathway. We investigated the effect of sorafenib on HBV replication using a human hepatoma cell line and a normal liver cell line and confirmed that sorafenib suppressed HBV replication via inhibiting the JNK pathway, which regulated the activity of the transcription factor, farnesoid X receptor (FXR). These results suggest that HBV replication is associated with the JNK pathway and may be regulated through inhibition of the JNK pathway by sorafenib.

METHODS

Cell culture

HepG2 and Chang liver cells (all obtained from the American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (Gibco BRL, USA) and 1% (v/v) penicillin-streptomycin (Gibco BRL, USA) at 37 °C in a humid atmosphere containing 5% CO₂.

Plasmid constructs and reagents

The ×1.3 Cp-luciferase HBV was generously provided by Y. Shaul (Weizmann Institute of Science, Rehovot, Israel). The 1.2 mer HBV including N-terminal ×3 flagged HBx were kindly provided by W. S. Ryu (Yonsei University, Seoul, South Korea). To construct HBV-Xp-luc and HBV-preS1p-luc, the promoter fragments in HBV genome construct were amplified by polymerase chain reaction (PCR) using cloning primers containing restriction enzyme site, HindIII, and KpnI. After digestion, the fragments were cloned into pGL4 vectors. The sequences were confirmed by automated DNA sequencing. Sorafenib was purchased from Selleckchem (Houston, TX, USA). Chenodeoxycholic acid (CDCA) and Z-guggulsterone (Z-GGS) were purchased from Sigma (St. Louis, MO, USA). SP600125 was purchased from Calbiochem (Billerica, MA, USA). The transfection reagents PolyFect and jetPEI were purchased from Qiagen (Hilden, Germany).

Drug and inhibitor treatment

Cells were treated with indicated chemicals or vehicle controls and incubated for 24 h. Control vehicle treatment (dimethylsulfoxide) was equivalent to the dose range experiments for each tested drug.

Luciferase assay

Cells were transfected with both the reporter vector and the β-galactosidase expression plasmid along with

each indicated expression plasmid using PolyFect. After transfection, the cells were lysed in cell culture lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were performed at least in triplicate.

Cell viability assay

Cell viability was determined by PrestoBlue cell viability reagent (Invitrogen, Carlsbad, CA, USA). Cells were treated with sorafenib at the indicated concentration for 24 h. The medium was removed and replaced with complete cell culture medium containing PrestoBlue (×10) for 1 h. After incubation with PrestoBlue, the medium was placed into 96-well plate for analysis. Absorbance values were determined at 570 nm.

Reverse transcriptase-PCR and real-time PCR

Total RNAs from cells were prepared using Trizol (Invitrogen) according to the manufacturer's recommendation. The cDNA was synthesized from 0.5 μg of total RNA with M-MLV reverse transcriptase (Promega) using oligo-dT at 37 °C for 1 h. The one-twentieth aliquot of the cDNA was subjected to PCR amplification using gene-specific primers [Table 1]. The cDNAs were amplified by PCR and the PCR products were examined by electrophoresis on 1.2% agarose gel. Real-time PCR was performed with TOPreal qPCR ×2 PreMIX with SYBR green (Enzynomics, Daejeon, South Korea) and each of the primers using StepOne™ Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA). The comparative threshold cycle method ($\Delta\Delta C_T$ method) was used to calculate the relative gene expression levels with human β-actin as an endogenous control gene.

SDS-PAGE and Western blotting

Cells were lysed with lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, 1% Nonidet P-40, 10% glycerol and protease

Table 1: Primers used for PCR amplification

Speices	Gene	Type	Sequence (5'-3')
HBV	HBx	Sense	ATGGCTGCTAGGCTGTGCTGC
		Anti-sense	ACGGTGGTCTCCATGCGACG
	HBV core	Sense	ATGCAACTTTTTCACCTCTGC
		Anti-sense	CTGAAGGAAAGAAGTCAGAAG
Human	FXR	Sense	GCCTGTAACAAAGAAGCCCC
		Anti-sense	CAGTTACAAGCATTGAGCAAC
	β-actin	Sense	GACTACCTCATGAAGATC
		Anti-sense	GATCCACATCTGCTGGAA

PCR: polymerase chain reaction; HBV: hepatitis B virus; FXR: farnesoid X receptor

inhibitor and 1 mmol/L PMSF. The protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred on to a PVDF membrane (Millipore, Billerica, MA, USA). For Western blotting, the membranes were incubated with anti-actin (Sigma, Steinheim, Germany), anti-HBx (Chemicon, Danvers, MA, USA), anti-flag (Cell Signaling, Beverly, MA, USA), anti-SAPK/JNK (Cell Signaling), anti-p-SAPK/JNK (Cell Signaling), anti-c-jun (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p-c-jun (Santa Cruz Biotechnology) or anti-FXR (Santa Cruz Biotechnology) antibodies in tris-buffered saline with Tween 20 (TBST) containing 1% Tween 20 supplemented with 3% nonfat dried milk. After washing with TBST, the blotted membranes were incubated with the peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). After washing TBST, the proteins were visualized by the ECL development reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Statistical analyses were carried out by unpaired or paired *t*-test as appropriate. All data are reported as mean \pm standard deviation. $P < 0.05$ were considered significant.

RESULTS

Sorafenib suppresses HBV gene expression

To investigate the anti-viral effect of sorafenib, we obtained promoters of genes contained in the HBV genome. As shown in Figure 1a, the HBV genome contains 4 promoters and 2 enhancers that regulate HBV replication. Of these, the most important one during HBV replication is the precore/core promoter, which has an effect on transcription of pregenomic RNA from cccDNA.^[5] The $\times 1.3$ HBV-Cp-luc construct contains the core promoter [Figure 1a]. The promoter activities of the HBV core, X, and preS1 genes were decreased by sorafenib in a dose-dependent manner [Figure 1b-d]. To investigate if the decrease of promoter activities by sorafenib was induced by cell death, a cell viability assay was performed. The results

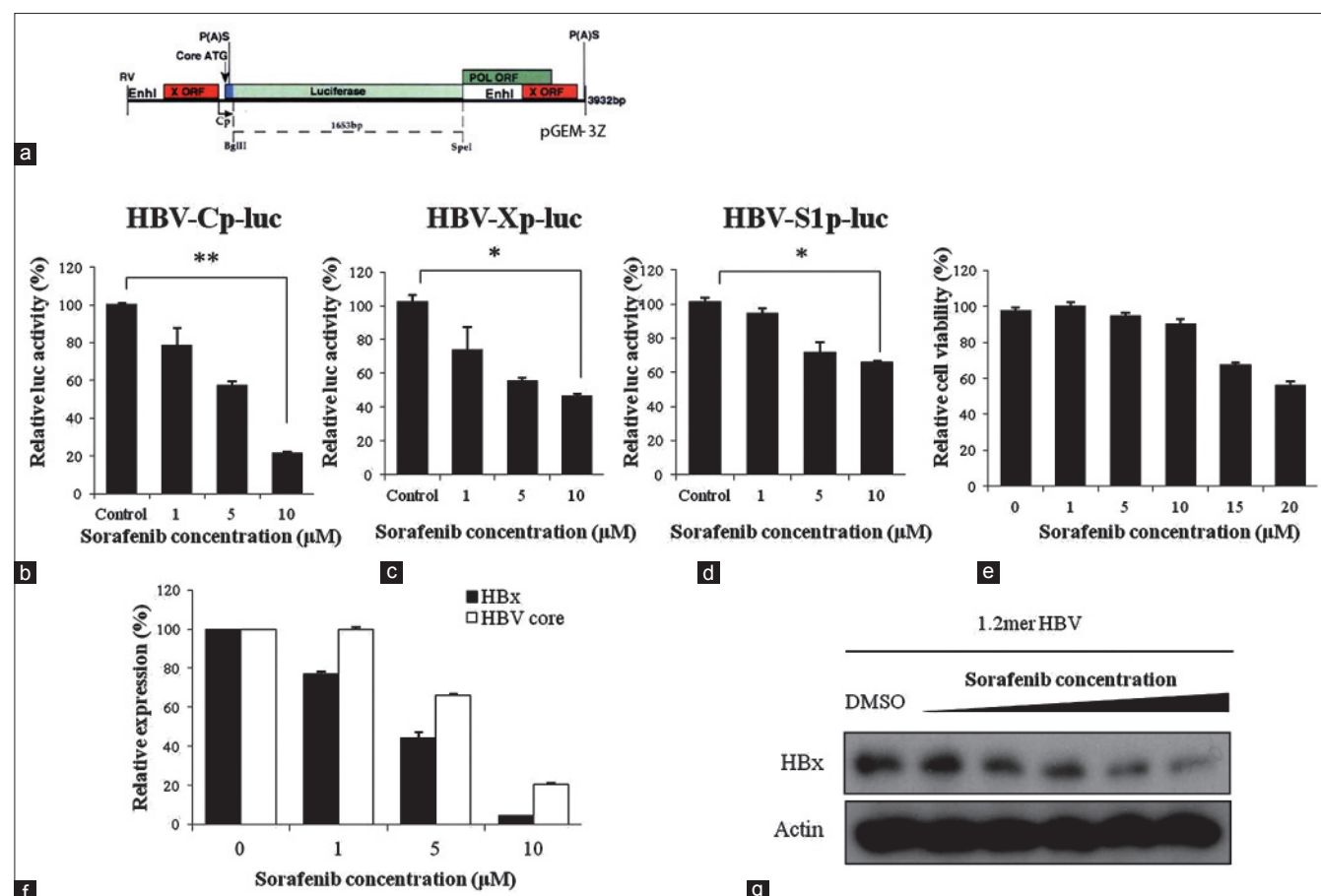


Figure 1: Sorafenib suppresses hepatitis B virus (HBV) gene expression. (a) Structure of the $\times 1.3$ HBV-luc plasmid; (b-d) the effects of sorafenib on HBV promoter activity. HepG2 or Chang cells were transfected with $\times 1.3$ HBV-luc, pGL4-X or pGL4-preS1 constructs for 24 h and treated with sorafenib. The cell lysates were analyzed for luciferase activity. * $P < 0.05$, ** $P < 0.01$, compared with the indicated cells; (e) cell viability assay; (f and g) the effect of sorafenib on HBV RNA or protein levels. HepG2 cells were transfected with 1.2 mer HBV construct for 24 h and treated with sorafenib. The indicated mRNA levels were detected by real-time polymerase chain reaction. Total proteins were prepared from the cells, and then HBx protein levels were detected by Western blotting

showed that there was no cytotoxicity at $< 10 \mu\text{mol/L}$ of sorafenib [Figure 1e]. In addition, mRNA and protein levels of HBx or core, HBV gene products, were decreased by sorafenib in a dose-dependent manner [Figure 1f and g]. These results suggest that sorafenib may suppress HBV gene expression regardless of cell death.

Sorafenib suppresses HBV gene expression through inhibition of the JNK pathway

Sorafenib is a multikinase inhibitor and it blocks several kinase pathways. Of these, the JNK pathway has been reported to be inhibited by sorafenib^[6] and have a possibility to regulate HBV pathogenesis.^[7] Therefore, we investigated if the JNK pathway is blocked by sorafenib and HBV gene expression is suppressed by JNK inhibition. As expected, phosphorylation of JNK and HBV protein levels were decreased by sorafenib [Figure 2a]. This result showed an effect of sorafenib as a JNK pathway inhibitor. In addition, HBV core promoter activity was decreased with inhibition of the JNK pathway [Figure 2b]. HBV mRNA and protein levels were also decreased by inhibition of the JNK pathway [Figure 2c]. The protein levels of c-jun and phosphorylated c-jun were

used as a target of the JNK pathway. Inversely, HBx protein levels were increased by JNK overexpression [Figure 2d] and HBV promoter activity and HBx expression induced by JNK1 were attenuated by sorafenib [Figure 2e and f]. These results suggest that sorafenib may suppress HBV gene expression through JNK pathway inhibition.

Sorafenib suppresses FXR-induced HBV gene expression

To identify the potential transcription factors increasing HBV gene expression and targeted by sorafenib, several hepatocyte-enriched transcription factors were assessed [Figure 3a]. Of these, FXR increased HBV promoter activity and FXR-induced HBV promoter activity was attenuated by sorafenib. FXR enhances synthesis of pregenomic RNA, FXR, and bile acids, the natural ligand of FXR related to the JNK pathway.^[8] Therefore, FXR was considered as a strong candidate targeted by sorafenib. FXR with CDCA, an endogenous FXR ligand, increased HBV core promoter activity [Figure 3b], HBV gene expression in mRNA, and protein levels without a change in FXR gene expression levels [Figure 3c]. To further investigate the effect of FXR on HBV gene expression,

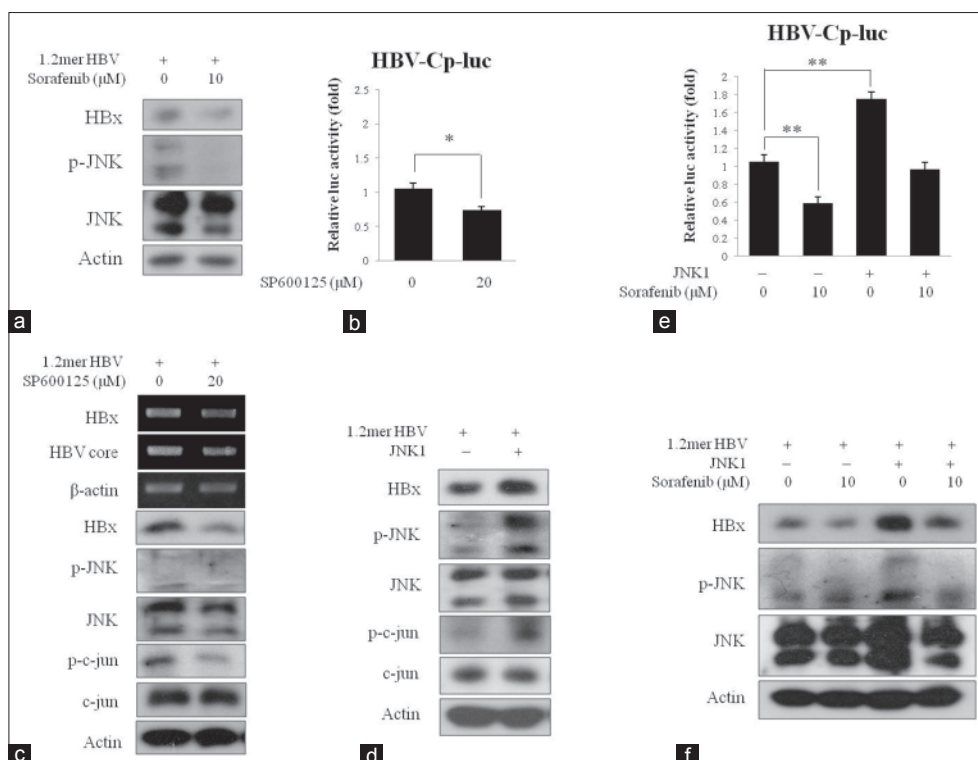


Figure 2: Sorafenib suppresses hepatitis B virus (HBV) gene expression through inhibition of the JNK pathway. (a) The effect of sorafenib on JNK phosphorylation and HBV protein levels. Chang cells were transfected with 1.2 mer HBV construct and treated with sorafenib. The indicated protein levels were detected by Western blotting; (b) the effect of JNK inhibitor on HBV core promoter activity. Chang cells were transfected with $\times 1.3$ HBV-luc construct and then maintained either control conditions or in the presence of SP600125. The cell lysates were analyzed for luciferase activity. $*P < 0.05$ compared with the indicated cells; (c) the effect of JNK inhibitor on HBV gene expression. Chang cells were transfected with 1.2 mer HBV construct and treated with SP600125. The cells were analyzed by reverse transcriptase-polymerase chain reaction and Western blotting; (d) the effect of JNK on HBV gene expression. Chang cells were cotransfected with the indicated constructs. The indicated protein levels were detected by Western blotting; (e) the effect of sorafenib on JNK-induced HBV core promoter activity. Chang cells were cotransfected with the indicated constructs, and the cells were treated with sorafenib. The cell lysates were analyzed for luciferase activity. $**P < 0.01$ compared with the indicated cells; (f) the effect of sorafenib on JNK-induced HBV protein expression. Chang cells were cotransfected with the indicated constructs, and the cells were treated with sorafenib. The indicated protein levels were detected by Western blotting

an antagonist of FXR, Z-GGS was used to inactivate FXR. HBx core promoter activity and HBV gene expression were decreased by FXR inactivation [Figure 3d and e]. To address the mechanism by which sorafenib decreases FXR-induced HBV core promoter activity, we investigated FXR protein levels after sorafenib treatment. As a result, FXR protein levels were decreased by sorafenib [Figure 3f] and FXR-induced HBx protein expression was also decreased by sorafenib [Figure 3g]. These results suggest that sorafenib may suppress HBV gene expression induced by FXR.

Sorafenib suppresses FXR-induced HBV gene expression through inhibition of the JNK pathway

As shown above, sorafenib suppressed HBV gene expression,

FXR, and the JNK pathway. We tried to address the relationship of these molecules involved in suppressing HBV gene expression. First, FXR protein levels were decreased by JNK inhibition and increased by JNK overexpression, but mRNA levels of FXR were not affected [Figure 4a and b]. These results suggest that the JNK pathway affects FXR protein stability and activity, but not FXR gene expression. The promoter activities of HBV genes induced by FXR or activated FXR were attenuated by sorafenib [Figure 4c-e]. In addition, the expression levels of HBx and core genes were increased by FXR and FXR activated by CDCA, but the expression levels were attenuated by sorafenib [Figure 4f and g]. These results suggest that sorafenib may suppress FXR-induced HBV gene expression through JNK pathway inhibition.

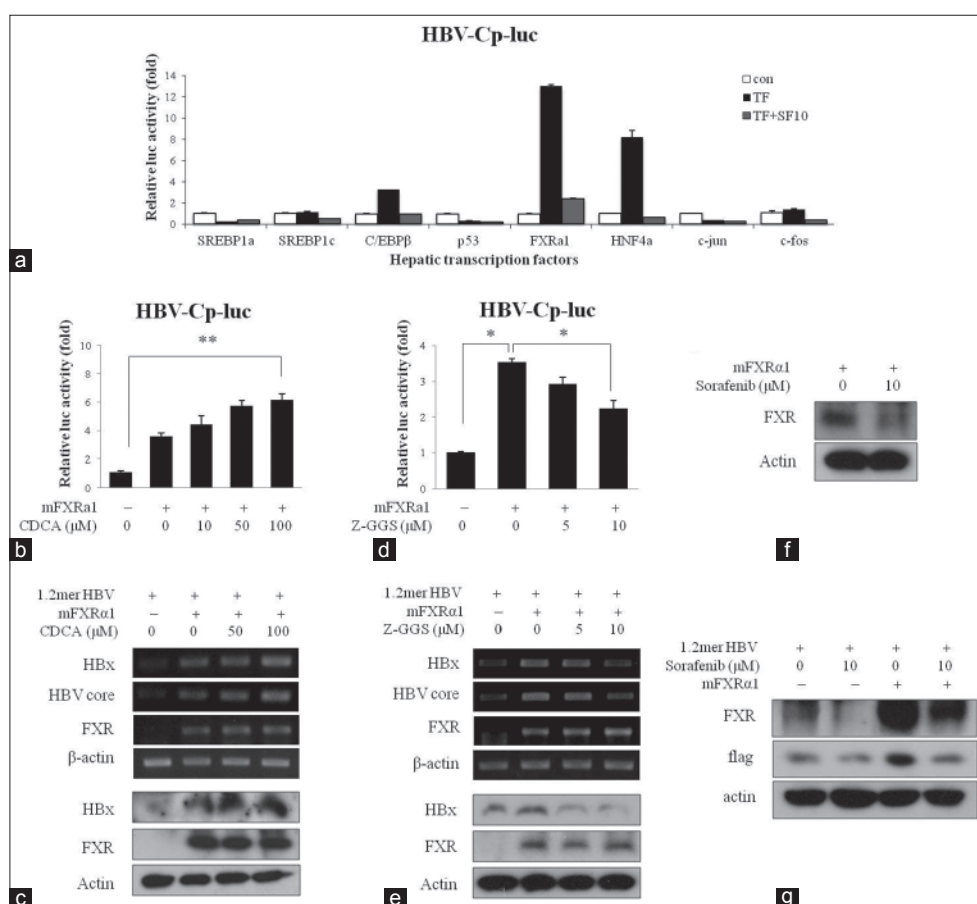


Figure 3: Sorafenib suppresses farnesoid X receptor (FXR)-induced hepatitis B virus (HBV) gene expression. (a) The effect of several hepatic transcription factors and sorafenib on HBV promoter activities. Chang cells were cotransfected with $\times 1.3$ HBV-luc construct and the indicated transcription factors, and incubated in sorafenib. The cell lysates were analyzed for luciferase activity; (b) the effect of FXR $\alpha 1$ on HBV core promoter activity. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of chenodeoxycholic acid (CDCA). The cell lysates were analyzed for luciferase activity. $**P < 0.01$ compared with the indicated cells; (c) the effect of FXR on HBV mRNA and protein levels. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of CDCA. The indicated mRNA or protein levels were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) or Western blotting; (d) the effect of FXR on HBV core promoter activity by the antagonist. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of Z-guggulsterone (Z-GGS). The cell lysates were analyzed for luciferase activity. $*P < 0.05$ compared with the indicated cells; (e) the effect of FXR on HBV mRNA and protein levels by the antagonist. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of Z-GGS. The indicated mRNA or protein levels were detected by RT-PCR or Western blotting; (f) the effect of sorafenib on FXR. Chang cells were transfected with pCMV-mFXR $\alpha 1$ construct, and then maintained either under control conditions or in the presence of sorafenib; (g) the effect of sorafenib on FXR and HBV protein levels. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of sorafenib. The transfected cells were analyzed by Western blotting

DISCUSSION

The antiviral effect of sorafenib on HBV gene expression indicates meaningful approaches to anti-HBV drugs. There are several available agents for the treatment of CHB. These drugs, including immunomodulatory agents and nucleotide/nucleoside analogs, have high efficacy against CHB, but there are several limitations including side effects, tolerance, and drug resistance.^[9] These limitations have been considered as problems to overcome for at least 10 years.^[10] Currently, combined therapies of these drugs show improved efficacy and lower drug resistance in CHB.^[11] However, other drugs with new strategies for HBV replication have not been approved yet. The development of more effective drugs for the management of CHB has proven to be challenging. Here, sorafenib suppressed HBV gene expression by inhibiting the JNK signaling pathway. This new mechanism suggests another possible approach to inhibit HBV replication. Furthermore,

sorafenib could be combined with other established anti-HBV agents to treat CHB because the combined therapy with other mechanisms is expected to show improved efficacy.

Sorafenib is a medicine for HCC and HCC is mainly caused by HBV infection.^[12] Therefore, we expect that sorafenib will help block the progress of hepatitis B and prevent the development of HCC. This might be a unique advantage as an antiviral drug because other currently available anti-HBV agents don't have oncomodulatory effects. In addition, targeting the host molecular signaling pathway is expected to have less drug-resistance compared to nucleotide/nucleoside analogs, which targets viral polymerase with high genetic variation.^[9] The mechanism of the effect of sorafenib on HBV gene expression should be elucidated with a variety of studies *in vitro* and *in vivo*. Especially, the effect of sorafenib on anti-HBV drug-resistant strains should be investigated through combined therapy.

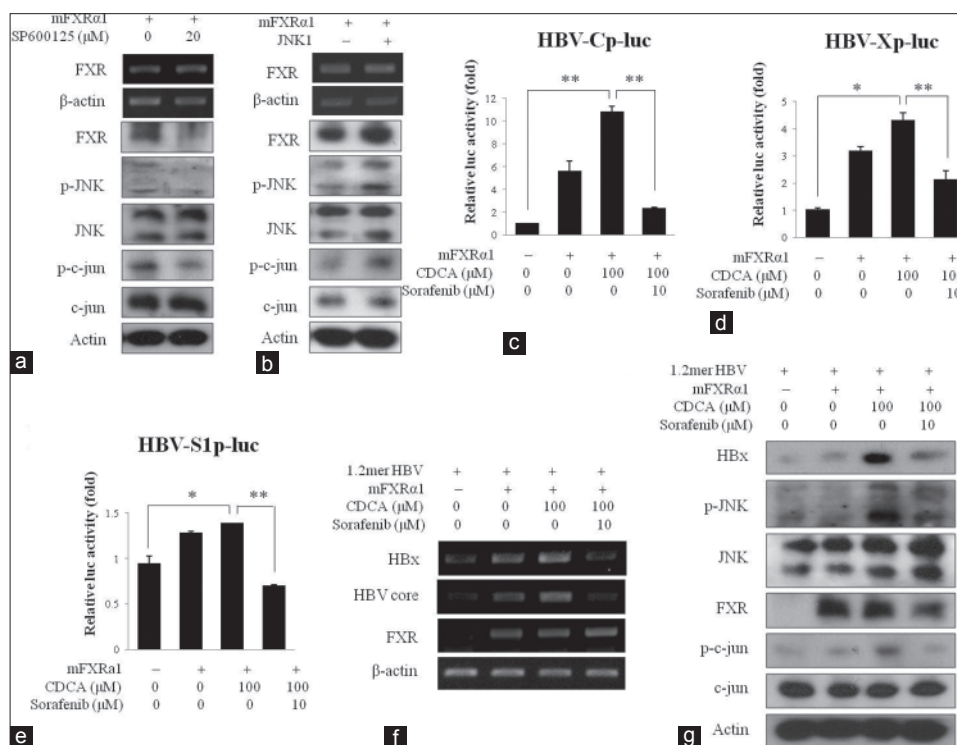


Figure 4: Sorafenib suppresses farnesoid X receptor (FXR)-induced hepatitis B virus (HBV) gene expression through inhibition of JNK pathway. (a) The effect of JNK inhibitor on FXR gene expression levels. Chang cells were transfected with pCMV-mFXR α 1 construct and treated with SP600125. The indicated mRNA or protein levels were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) or Western blotting; (b) the effect of JNK on FXR gene expression levels. Chang cells were transfected with pCMV-mFXR α 1 construct and treated with SP600125. The indicated mRNA or protein levels were detected by RT-PCR or Western blotting; (c) the effect of sorafenib on FXR-induced HBV core promoter activity. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of chenodeoxycholic acid (CDCA) and sorafenib. The cell lysates were analyzed for luciferase activity. ** P < 0.01 compared with the indicated cells; (d) the effect of sorafenib on FXR-induced HBV X promoter activity. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of CDCA and sorafenib. The cell lysates were analyzed for luciferase activity. * P < 0.05, ** P < 0.01 compared with the indicated cells; (e) the effect of sorafenib on FXR-induced HBV preS1 promoter activity. Chang cells were transfected with the indicated constructs, and then maintained either control conditions or in the presence of CDCA and sorafenib. The cell lysates were analyzed for luciferase activity. * P < 0.05, ** P < 0.01 compared with the indicated cells; (f) the effect of sorafenib on FXR-induced HBV mRNA levels. Chang cells were transfected with the indicated constructs as described on each lane, and the cells were treated with CDCA and sorafenib. The indicated mRNA levels were detected by RT-PCR; (g) the effect of sorafenib on FXR-induced HBV protein levels. Chang cells were transfected with the indicated constructs as described on each lane, and the cells were treated with CDCA and sorafenib. The indicated protein levels were detected by Western blotting

Bile acid activates the JNK pathway and FXR, which suppresses cholesterol-7 α -hydroxylase (CYP7A1), independently.^[8] Bile acids are known to inhibit CYP7A1 gene transcription via direct activation of the JNK pathway, and FXR activates the JNK pathway to suppress CYP7A1 in hepatocytes via inducing intestinal fibroblast growth factor 15/19. In this study, JNK activation affected FXR transcriptional activity and FXR protein levels but not FXR mRNA levels, suggesting that the JNK pathway may regulate FXR transcriptional activity and protein stability.

Sorafenib is a well-known Raf kinase inhibitor, but it also inhibits other kinases.^[13] The JNK pathway is one of the signaling pathways inhibited by sorafenib. However, sorafenib has been reported to activate the JNK pathway to induce apoptosis.^[14] It seems to be in conflict with each other, but the effect of sorafenib in a nontoxic concentration was confirmed in this study. Therefore, it may be important to use a proper dose of sorafenib to treat CHB. In this study, the proper dose of sorafenib was < 10 μ mol/L. In the other case, the proper dose of sorafenib that inhibits human cytomegalovirus replication and hepatitis C virus was below 2.5 and 15 μ mol/L *in vitro*, respectively.^[15,16]

In conclusion, these findings suggest that sorafenib, the multi-kinase inhibitor, has an anti-viral effect on HBV gene expression. Further research about the efficacy of targeting the molecular signaling pathway of HBV replication should be evaluated for the treatment of CHB patients.

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Resection of hepatocellular carcinoma after combined treatment with transarterial chemoembolization and sorafenib: a case report and literature review

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ABSTRACT

Hepatocellular carcinoma (HCC) with inferior vena cava (IVC) invasion and metastatic lymph node metastases has a poor prognosis, and surgical resection is seldom indicated. We report how an initially unresectable HCC in a 36-year-old Chinese male with distant lymph node metastases and tumor thrombosis in the IVC was successfully downstaged and ultimately resected together with the IVC. After the disease had been downstaged, curative resection of the tumor and IVC was conducted with immediate reconstruction of the IVC. The patient has survived for more than 2 years after the surgery. In conclusion, tumor and IVC resection can cure metastatic HCC after downstaging treatment combining sorafenib and transarterial chemoembolization.

Key words: Downstaging; hepatocellular carcinoma; lymph node metastases; sorafenib; vascular resection

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INTRODUCTION

There have been a few studies documenting the survival benefits of treating unresectable hepatocellular carcinoma (HCC) with sorafenib,^[1,2] but randomized trial documenting the efficacy of sorafenib as a neoadjuvant therapy is lacking. Sorafenib impedes angiogenesis and induces apoptosis with efficacy,^[3,4] whereas transarterial chemoembolization (TACE) is a potent stimulator for angiogenesis.^[5] Theoretically, the combination of sorafenib and TACE should have a synergistic effect.

CASE REPORT

A 36-year-old Chinese male had painful hepatomegaly for 2 weeks. He was tested positive for hepatitis B surface antigens

and his alpha-fetoprotein (AFP) level was markedly high at 8,195 ng/mL. Computed tomography (CT) conducted in July 2011 showed a 9.7-cm HCC in his right liver lobe, with direct invasion of the retrohepatic inferior vena cava (IVC). There was also an evidence of lymphadenopathy at the porta hepatis and in the para-aortic region. In view of the extrahepatic involvement, HCC was considered unresectable. The patient was put on sorafenib (400 mg, twice a day) and entecavir. He developed tolerable Grade 2 adverse drug reaction (diarrhea and hand-foot syndrome).

One dose of TACE was given in August 2011. Reassessment CT in October 2011 showed interval decrease in the size of the tumor to 5.9 cm, but there were still lymph node metastases and tumor thrombus in the IVC. His AFP level had dramatically lowered to 50 ng/mL. The tumor further reduced in size on subsequent CT in February 2012; however, the AFP level slowly rebounded to 430 ng/mL. Two more sessions of TACE were given in April and July 2012 [Table 1]. Whole-body dual-tracer (¹¹C-acetate and ¹⁸F-fluorodeoxyglucose) positron emission tomography and CT performed in September 2012 confirmed resolution of metastatic lymphadenopathy, but IVC tumor thrombus and the adrenal invasion were still hypermetabolic [Figure 1].

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In order to achieve complete resolution of the disease, surgery was offered. Pre-operative assessment showed that the liver function was satisfactory for a major hepatectomy (with a 6.6% indocyanine green retention rate at 15 min), and there would be an adequate left-liver remnant. Laparotomy was conducted in October 2012. The tumor was found to have infiltrated to the right adrenal gland and the whole length of the retrohepatic IVC. The left liver lobe had an adequate volume and was free of tumor. The right lobe, the caudate lobe of the liver, the right adrenal gland, and the IVC were resected *en bloc* [Figure 2]. Immediate reconstruction of the IVC was performed using a cadaveric vein graft (5.5 cm long, 2.5 cm wide) [Figure 3]. The entire operation lasted 10 h and the total IVC cross-clamping time was 1 h. There was 1.2 L of blood loss.

After the operation, the patient was put on everolimus (1 mg daily). He was discharged 12 days after the operation. Histopathological examination of the resected specimen showed necrotic tumor in the right liver lobe and viable metastatic HCC with the involvement of the right adrenal gland. No viable tumor was seen in the IVC thrombus. The patient was followed-up every 2 months after the operation and had remained free of disease (radiologically and biochemically) for more than 27 months at the time of writing this manuscript.

DISCUSSION

At the beginning, the treatment was meant to be either palliative or neoadjuvant, depending on the radiological and biochemical responses. Ultimately, mixed responses were observed. Disease at the metastatic lymph nodes regressed and the AFP level rebounded substantially over a period of time after treatment. Aggressive surgical resection was offered, and major vascular resection was needed for a potentially curative resection. Sorafenib was continued as an adjuvant therapy. It is hoped that the efficacy and safety of sorafenib in adjuvant treatment of HCC after potentially curative treatment will be demonstrated when the results of the STORM (sorafenib as adjuvant treatment in the prevention of recurrence of HCC) trial are released. A recent German multi-center Phase II trial showed that the combination of TACE and sorafenib could achieve a 74.4% disease control rate according to the criteria set by the European Association for the Study of the Liver. Among the 43 patients, 7% had a complete response, 41.8% had a partial response, and 25.6% had stable disease.^[6] Downstaging by sorafenib alone was reported in two case reports and both eventually underwent curative resection. The histopathological examination of the resected liver showed 35% and 60% of tumor necrosis, and the rightportal branch thrombi were totally necrotic.^[7]

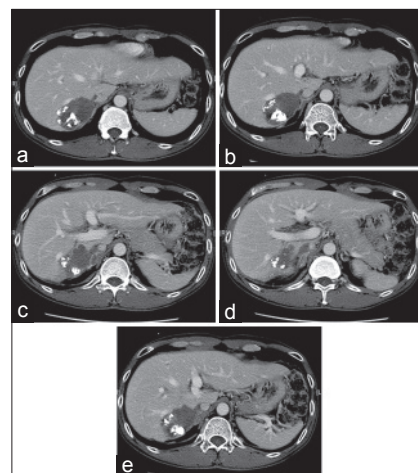


Figure 1: Preoperative computed tomographic scans showing tumor invasion of (a, b) the right hepatic vein, (c) the right adrenal gland, (d) the right adrenal vein, and (e) the inferior vena cava

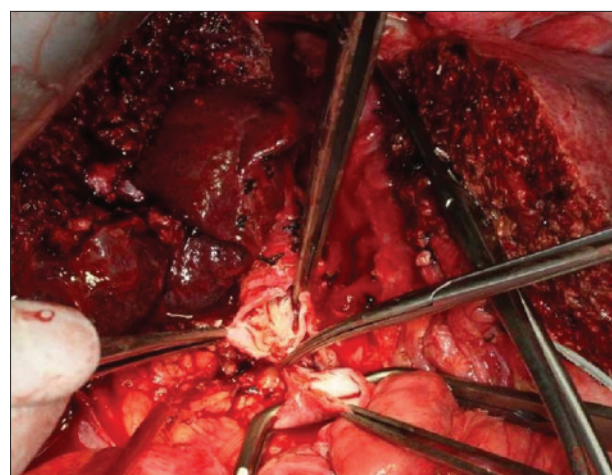


Figure 2: *En bloc* resection of the inferior vena cava, right liver lobe, and right adrenal gland

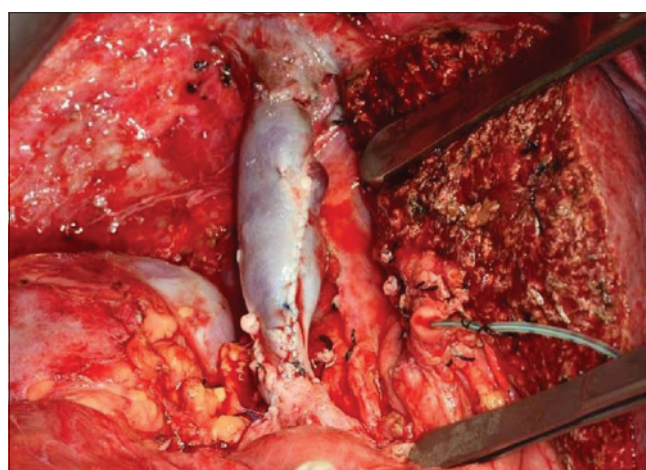


Figure 3: Reconstruction of the inferior vena cava with a cadaveric vein graft

Although successful downstaging is an uncommon occurrence, a multidisciplinary approach in the management of HCC is essential in the modern era. When a tumor is downstaged to a potentially resectable one, early input from

Table 1: Tumor size, imaging and treatment responses in chronological order

	July 2011	October 2011	February 2012	September 2012
Tumor size	9.7 cm (CT scan)	5.9 cm (CT scan)	5 cm (CT scan)	6 cm (PET scan)
AFP (ng/mL)	8,195	50	430	5,653
Treatments	Once dose of TACE + sorafenib	Sorafenib	Two more doses of TACE + sorafenib	Resection of HCC + IVC

AFP: alpha-fetoprotein; CT: computed tomography; TACE: transarterial chemoembolization; PET: positron emission tomography; HCC: hepatocellular carcinoma; IVC: inferior vena cava

surgeons will definitely lead to earlier surgical treatment if it is possible.

A high pre-operative AFP level has been found to be associated with a higher rate of HCC recurrence,^[8] and thus this patient has been put under intensive surveillance. By far, the operation was the only chance of cure for him.

For the time being, clinicians cannot predict which patients will benefit from neoadjuvant treatment, and potential immunohistochemical markers should be explored to identify potential good responders for neoadjuvant treatment with sorafenib.

In conclusion, combined treatment can downstage initially unresectable HCCs to resectable ones in selected patients. Patients with the best chance of good outcomes should be given vigilant reassessment with fine-tuning of treatment options throughout the whole clinical course. Center experience in liver transplantation can help in complicated procedures such as major vascular resection and immediate reconstruction.

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Natural products and hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is a major health problem, with more than 500,000 cases diagnosed annually. It is also an important cause of human mortality in the world. The incidence of HCC is rising due to the widespread of hepatitis and alcoholism, which may be caused by infection, injury, exposure to drugs or toxic compounds, autoimmunity, or genetic defect that leads to the deposition of harmful substances.^[1]

In the broadest sense, natural products (NPs) are chemical compounds or substances produced by a living organism found in nature.^[2,3] Consequently, NPs can be extracted from animals, plants, microbes, and marine organisms.^[4,5] NPs can be considered as a coin with two sides, which have to be considered in the application in modern or alternative medicine. Some NPs have beneficial effects while some others have toxic effects. For example, mycotoxins and some other microbial toxins are carcinogenic, and the International Agency for Research on Cancer classified several mycotoxins as hepatocarcinogenic.^[6] Exposure to some mycotoxins resulted in liver cancer,^[7-10] especially aflatoxin B₁, which is a mutagenic natural compound that contaminates many food sources in some parts of Africa and Asia and is recognized as hepatocarcinogens in humans and many animal species.^[7,11]

The use of herbal medicines can be traced back several thousand years ago in ancient China, ancient Egypt and ancient Roma. Recent research pointed out an increasing

interest concerning the health benefits of a diet rich in NPs.^[12] NPs can act as chemoprotective agents against common liver diseases, such as hepatitis, cirrhosis, liver cancer, fatty liver diseases, and gallstones.^[1]

In general, NPs play a key role in drug discovery and are also a prolific source of novel lead compounds or pharmacophores for medicinal chemistry. Although naturally active substances are usually good lead compounds, most of them can hardly satisfy the demands for druggability. Hence, these structural phenotypes have to be modified and optimized to overcome existing deficiencies and shortcomings.^[13]

Although HCC is always hard to treat, this special issue aims to gather updated progress in this important area and shed the light on the possibility to introduce a new drug based on the benefit of NPs.

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Can sorafenib be discontinued in hepatocellular carcinoma patients with a complete response to treatment?

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INTRODUCTION

Sorafenib is a multi-kinase inhibitor that inhibits angiogenesis by targeting the vascular endothelial growth factor receptor 2 and platelet-derived growth factor receptor pathways while blocking cell proliferation by targeting the Ras/mitogen-activated protein kinase signaling pathway. Two global phase III trials [Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP)^[1] and Asia-Pacific trial^[2]] showed that sorafenib prolonged the survival of patients with advanced hepatocellular carcinoma (HCC). The results of these studies were rapidly disseminated worldwide and were enthusiastically accepted by physicians specializing in liver cancer treatment. Based on the positive results of the SHARP trial,^[1] the EU and USA approved sorafenib for advanced HCC in October and November 2007, respectively. Sorafenib was also approved for patients with unresectable and metastatic HCC in July 2008 in China, but patients have to pay the cost by themselves.

Thereafter, clinicians studied whether enhanced benefits could be derived from combining sorafenib with other therapeutic means. Several studies^[3,4] assessed optimal combinations of sorafenib with transarterial chemoembolization (TACE) or radiofrequency ablation, as well as the sequence of treatment modalities in order to maximize patients' outcomes. Since then, an increasing number of complete remission (CR) cases

were reported.^[5] For patients who have achieved CR, whether sorafenib can be discontinued remains unknown.

PRESENTATION OF THE HYPOTHESIS

Most cases in which sorafenib was discontinued during the course of treatment for HCC resulted from severe adverse events.^[1,2] For patients who have achieved CR, we proposed the concept that sorafenib may be discontinued in CR cases, taking into consideration the high cost for such patients in poor societies, particularly those in developing countries with restrictive coverage for certain pharmaceuticals from national health insurance systems.

SUPPORTIVE OBSERVATION FOR THIS HYPOTHESIS AND FUTURE DIRECTIONS

Recently, a 39-year-old male patient who was diagnosed with HCC (2 cm in diameter) on May 6, 2010 was admitted. He could not receive radical therapy (hepatic resection or radiofrequency ablation) because the tumor was located very close to the right branch of the portal vein. Therefore, he received TACE plus sorafenib therapy. One week later, he had severe drug-related diarrhea (4-8 times bowel movement daily). Five weeks after treatment, the tumor was not detected on both computed tomography scan and

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Table 1: A brief summary of previous reports of CR cases treated with sorafenib as a monotherapy for HCC

References	CR cases	Duration of treatment cessation (months)	Relapse
So <i>et al.</i> ^[7]	1	6	No
Kudo and Ueshima ^[5]	15	NA	No
Wang <i>et al.</i> ^[8]	1	16	No
Sacco <i>et al.</i> ^[9]	1	≥ 6	No
Inuzuka <i>et al.</i> ^[10]	1	8	No

CR: complete remission; HCC: hepatocellular carcinoma; NA: not available

magnetic resonance imaging. Thereafter, he discontinued sorafenib therapy because he could not afford the treatment cost. Up to April 30, 2015, he had CR of tumor status for 58 months.

As we know, most patients treated with tyrosine kinase inhibitors (TKIs) would suffer from drug-related adverse effects. Discontinuation of treatment with TKIs could improve quality-of-life and reduce treatment costs for patients in which a CR is achieved.^[6] To our knowledge, there are several case reports concerning this issue [Table 1], and it seems that relapse of tumor hardly happens in patients who have achieved CR after sorafenib monotherapy, irrespective of drug discontinuation or not. For patients who received sorafenib in combination with other therapeutic means, the CR status may be partly due to the combination treatment, like TACE. Therefore, we propose that sorafenib should be discontinued to reduce the drug-related adverse effects. However, discontinuation of sorafenib in patients with CR carries the risk of progression with new metastases and potential complications. Further investigation in a larger cohort of cases is warranted before such an approach can be regarded as safe. However, this methodology has limitations associated with the small sample of CR cases and interference of other therapeutic means, and hence it may just be a hypothesis.

CONCLUSION

Sorafenib has demonstrated clinical efficacy in HCC patients, and more and more CR cases were reported. For patients who have achieved CR, we propose that sorafenib may be discontinued, irrespective of whether it was used as monotherapy or combination therapy with other therapeutic means. Evaluation of the current hypothesis and investigation with a larger cohort of cases may provide information that

such an approach can be regarded as safe, and thereby improves quality-of-life and reduces treatment costs for patients in which a CR is achieved.

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Nil.

Conflict of interest

There is no conflict of interest.

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Effects of natural compounds in treatment and prevention of hepatotoxicity and hepatocellular carcinoma

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ABSTRACT

Liver diseases are most common disorders in the world and characterized by rapid changes from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). Natural products that attained great attention is to be used in the prevention and treatment of multiple diseases in humans. Several researches have been reported numerous natural and phytochemical compounds that may counteract or prevent the hepatic injury and primary liver cancer. The conservative treatment of liver toxicity and HCC may face awkward challenges in chemotherapy such as therapeutic failure or drug resistance. Accordingly, there is an actual need for safe and effective therapeutic and preventive modalities for liver disorders. The present review aims to focus on the potential protective and therapeutic effects of natural compounds in the prevention and treatment of hepatotoxicity and HCC. It also demonstrates the mechanism of the natural products in enzymatic regulation of antioxidants and its role in apoptosis and proliferation of cancerous lesions of hepatocytes. Accordingly, it highlights the promising role of natural bioactive compounds and provides the rational for further transitional researches, and emphasize on the scientific validation of natural compounds for therapeutic portfolio for clinical use in liver diseases.

Key words: Antioxidant; hepatocellular carcinoma; hepatotoxicity; liver; natural compounds

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INTRODUCTION

Chronic liver diseases are common worldwide disorders characterized by bad sequels started with steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).^[1,2] Indeed, HCC is the fifth most commonly leading cancer, the major cause of death in patients with liver cirrhosis, and the second common cause of cancer-related death in the world.^[3]

The major target strategy in the treatment of liver diseases is to terminate the serial consequences at the pre-fibrotic stage of the liver.^[4] To date, modern medicines have little to offer for alleviation of hepatic diseases. However, natural-based preparations are successfully employed for the treatment of liver disorders.^[5] Accordingly, there is an increased attention in natural products that may counteract

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the detrimental effects of environmental or chemical toxic compounds and prevent multiple hepatic disorders in humans.^[6]

POLYPHENOLS

Polyphenols, commonly presented in vegetables, herbs, seeds, fruits, and other natural sources, represent more than 8,000 different compounds, classified in different classes based on their chemical structure, they are composed of at least one aromatic ring with one or more hydroxyl groups attached.^[7] Polyphenols may be a promising candidate for preventing ethanol-induced liver injury through regulating alcohol metabolic enzymes in a cyclic AMP-dependent manner, polyphenols play a crucial role in the protection of liver against hepatitis due to its potential activity in the reduction of early proinflammatory cytokines [tumor necrosis factor- α and interleukin (IL)-1 β], activation of anti-inflammatory IL-10, and inhibition of lipopolysaccharide-induced activation of nuclear factor kappa B (NF- κ B) in hepatocytes.^[8] Polyphenols are composed of two formulas; phenolic acids and flavonoids and account for 60% and 30%, respectively, of dietary polyphenols.^[9]

Phenolic compounds (PhCs), which are ubiquitously found in plants, have a potent antioxidant activity mainly due to their ability in redox reactions, so they act as reducing agents, singlet oxygen quenchers, hydrogen donors, and chelating agents of metal ions.^[10] Moreover, previous studies revealed that PhCs played an important role in the prevention of hepatotoxicity through increase in the level of reduced glutathione (GSH).^[11]

Flavonoids are a group of polyphenolic compounds, different in chemical structure and characteristics, naturally founded in plants. More than 9,000 different flavonoid compounds were described in plants till now and they play major biological roles through affecting several developmental and important processes.

Flavonoids showed versatile health benefits such as anti-inflammatory, antioxidant, anti-proliferative and anticancer activity, free radical scavenging activity, and antihypertensive effects.^[12] It has been reported that one of the flavonoid compounds luteolin showed a hepatoprotective effect and antioxidant properties against methanol hepatotoxicity.^[13]

HERBAL AGENTS

Milk thistle (*Silybum marianum*) is one of the most famous herbal agents used to treat liver diseases since the 16th century.

Major constituents of milk thistle are the flavonoids, such as silibinin, silidianin, silichristin, and isosilibinin.^[14] Silymarin showed antioxidant properties and hepatoprotective activity, through inhibition of lipid peroxidation, depletion of liver GSH, inhibition of genotoxicity, and enhancement of hepatogenesis.^[15]

Glycyrrhizin, the active constituent obtained from aqueous extraction of the liquorice root (*Glycyrrhiza glabra*), has been used in traditional medicine to alleviate bronchitis, gastritis, and jaundice. The major constituents of licorice are glycyrrhetic acid, flavonoids, hydroxycoumarins, and beta-sitosterol. The latter is likely possessing glucocorticoid and mineralocorticoid properties.^[16] Licorice and their products have been reported to be useful in the treatment of human hepatitis, animal inducible hepatocarcinogenesis, and attenuating titanium dioxide nanoparticles-induced hepatotoxicity.^[17,18]

Ginseng (*Panax ginseng*), a valued Chinese and Korean traditional medicinal herb, has been clinically used in China for thousands of years. Red ginseng elicits a protection against aflatoxin B₁ and fumonisins-induced hepatic pre-cancerous lesions in rats and synergistic action with honey against CCl₄-induced hepatonephrotoxicity.^[19,20]

Ginkgo biloba extract has been shown antioxidant property due to its ability to scavenge free radicals and inhibition of lipid peroxidation.^[21] The most recent discovered *G. biloba* components are polyphenols from which flavonoids and terpene lactones were derived and widely used for treating cardiovascular, non-alcoholic fatty liver, and cerebrovascular diseases.^[22-24]

Dandelion (*Taraxacum officinale*), dandelion water extract (DWE), a herbal medication, may have an effect on the activity of messenger RNA expression of hepatic antioxidant enzymes due to its components that includes sesquiterpene lactones, phenylpropanoids, triterpenoid saponins, and modify lipid profile in streptozotocin-induced diabetes in rats.^[25-27] It has been reported that DWE has anti-fibrotic effect through inactivation of hepatic stellate cells (HSCs) and the enhancement of hepatic regenerative capabilities.^[28]

Garlic (*Allium sativum*) has been widely used as a foodstuff and a traditional medicine for many centuries throughout the world. Garlic is available in different forms such as powder or garlic oil. Garlic has a beneficial value such as anti-atherosclerotic, antihypertensive, antimicrobial, anticancer, immunomodulatory, antioxidant, and radioprotector effects.^[29] On the other hand, allicin (diallyl thiosulfonate), which is the main biologically active

component of freshly crushed garlic cloves, has been produced by the interaction of the non-protein amino acid alliin with the enzyme alliinase (alliin lyase). It has anti-hepatocarcinogenic effect through the *p53* gene modulating apoptosis and autophagy.^[30]

Turmeric (*Curcuma longa*) has been found in the Far East and tropical regions. It had been used to treat menstrual disorders, colic, inflammation, bruising, dyspepsia, hematuria, and flatulence. It also has anticancer and antioxidant actions due to the presence of three chemical components, for example, curcumin I, II, and III.^[31] It suppresses the activation of NF- κ B, so it may be useful in preventing liver disease such as hepatonephrotoxicity.^[32]

Colchicine (*Colchicum autumnale*) is the major alkaloid obtained from *C. autumnale*. Pharmacological properties of colchicine included antimitotic effects and can be used for the treatment of gout.^[33] Moreover, colchicine has been acting as an anti-tumor agent.^[34] Colchicine has been reported to be a safe anti-fibrotic agent when used for long-term treatment of liver disease.^[35]

Thyme (*Thymus vulgaris*) is cultivated in Central and Southern Europe, Africa, and Asia. It is rich with essential oils and anti-oxidative phenolic substances.^[36] It is widely used in folk medicine for the treatment of a variety of diseases including gastroenteric and bronchopulmonary disorders. It is also effective as anthelmintic, antispasmodic, carminative, sedative, diaphoretic, antimicrobial, antioxidant, and antifungal agents.^[37] *T. vulgaris* also

showed hepatorenoprotective effects against aflatoxicosis in rats.^[38]

Marigold (*Calendula officinalis*) is an annual herb native to the Mediterranean region. In Europe and America, it is cultivated for ornamental and medicinal purposes. *C. officinalis* as the marigold or maravilla has been widely used in folk therapy. *Calendula* flower decoction or tincture showed more than 35 properties and its preparations have been used as valuable remedies for burns. *C. officinalis* is mainly used for cutaneous and internal inflammatory diseases of several origins.^[39] Its extract has a protective effect against ultraviolet-induced oxidative stress.^[40] It has been well documented that *Calendula* extract showed anti-genotoxicity and ameliorative effect against hepatotoxicity induced by aflatoxin due to high percentage of total PhCs.^[6,41] The effect of herbal agents has been summarized in Table 1.

MICRONUTRIENTS (VITAMINS AND MINERALS)

Vitamin B₁₂ (cyanocobalamin) molecule contains a cobalt complex, it is known as cobalamin. Molecular weight of vitamin B₁₂ is the highest among all vitamins; therefore, it is known to accumulate at high levels in the liver. Vitamin B₁₂ is a complex organometallic cofactor associated with three subfamilies of enzymes: The adenosylcobalamin-dependent isomerases, the methyl cobalamin-dependent methyltransferases, and the dehalogenases.^[42] In chronic feeding regimen without a methyl-donor, vitamin B₁₂ may lead to the development of HCC.^[43] Previous studies reported that vitamin B₁₂ showed

Table 1: Effect of herbal agents against hepatic disorders

Name	Family	Constituents	Mechanism of action	Major effect
<i>Silybum marianum</i> (Silymarin)	Asteraceae	Silibinin	Inhibit GSH depletion and genotoxicity Inhibited telomerase activity in HCC	Antioxidant
<i>Glycyrrhiza glabra</i> (Liquorice)	Fabaceae	Glycyrrhetic acid	Enhance GSH formation Induce apoptosis in hepatic cancer	Anti-hepatocarcinogenesis
<i>Panax ginseng</i>	Araliaceae	Ginsenosides	Improve GSH synthesis Enhance apoptosis in HCC	Hepatorenoprotective effect
<i>Ginkgo biloba</i>	Ginkgoaceae	Polyphenols	Free radical scavenger Prevention of tumor initiation	Antioxidant
<i>Taraxacum officinale</i> (Dandelion)	Asteraceae	Taraxacin	Enhance mRNA expression of hepatic antioxidant enzymes Prevention of tumor initiation	Anti-fibrotic effect
<i>Allium sativum</i> (Garlic)	Amaryllidaceae	Allicin	Modulation of p53 gene Delay or arrest of the tumor development	Anticancer
<i>Curcuma longa</i> (Turmeric)	Zingiberaceae	Curcumin I, II, and III	Suppresses the activation of nuclear factor kappa B Prevention of tumor initiation	Anticancer and antioxidant
<i>Colchicum autumnale</i>	Colchicaceae	Colchicine	Inhibition of cellular mitosis Delay or arrest of the tumor development	Anti-tumor anti-fibrotic agent
<i>Thymus vulgaris</i>	Lamiaceae	Thymol	Increase GSH synthesis Prevention of tumor initiation	Antioxidant, antimicrobial
<i>Calendula officinalis</i>	Asteraceae	Triterpenoids	Enhance antioxidant enzymes Prevention of tumor initiation	Antioxidant, anti-inflammatory

GSH: glutathione; HCC: hepatocellular carcinoma

hepatoprotective effect against dimethyl nitrosamine in intoxicated rats. Moreover, vitamin B₁₂ suppresses genetic expression of α -smooth muscle actin and heat-shock protein 47, which are markers of liver fibrosis.^[44]

Vitamin C (ascorbic acid) is one of the most required nutrients for a variety of biological functions. The health-promoting effects of vitamin C can be attributed to its biological functions as a cofactor for a number of enzymes, most notably hydroxylases involved in collagen synthesis and as a water-soluble antioxidant.^[45] However, it can exert its antioxidant properties in both aqueous and non-aqueous environments.^[46] Vitamin C is able to decrease hepatic apoptosis and necrosis against cholestatic liver injury in experimental animals.^[47]

Vitamin E (α -tocopherol) is a potent lipid-soluble and chain-breaking antioxidant required nutrient for humans because it is necessary for the prevention of several symptoms, including peripheral neuropathy and hemolytic anemia.^[48] It plays a significant role in preventing or minimizing peroxidation damage in biological systems.^[49] Supplementation with vitamin E inhibits DNA damage due to free radical scavenging activity and its exerting anti-cytotoxicity and anti-genotoxicity.^[50,51] Moreover, α -tocopherol showed hepatoprotective activity against cisplatin-induced oxidative stress, which may be attributed to down-regulations of NADPH oxidase gene expression.^[52]

Zinc (Zn) is an essential trace element with various biological effects, depending on its catalytic and structural role in an enormous number of enzymes and “Zn-finger” proteins.^[53] Zn ions (Zn²⁺) control cell proliferation, differentiation, and have a role in both apoptotic and necrotic cell death.^[54] Zn also has anti-oxidative and anti-inflammatory properties and it postulates hepatonephroprotective effect due to its antioxidant, anti-apoptotic, and anti-inflammatory properties against cadmium-induced hepatotoxicity and reduction of metal accumulation in the organism, which may lead to nephrotoxicity.^[55,56]

The naturally occurring element selenium (Se) plays a major role in a wide variety of biological processes in mammals. Se acts as one of the major components due to its low molecular weight as well as its presence within at least 25 proteins, named selenoproteins, in the form of the amino acid selenocysteine, which is incorporated during translation and is directly involved in redox catalysis.^[57] Although the function of most selenoproteins is still unknown, thioredoxin reductase, GSH peroxidases, and thyroid hormone deiodinases are well described as selenoproteins, which is involved in maintaining the cell reduction-oxidation balance and thyroid hormone

metabolism.^[58] Se administration increases the antioxidant capacity of several intracellular systems. In addition, Se showed hepatoprotective effect against malathion-induced liver injury and diabetic rats.^[59,60] Table 2 demonstrated the effect of micronutrients on hepatic lesions.

DIETARY SUPPLEMENTS

N-acetyl cysteine (NAC) is a derivative of the sulfur-containing amino acid cysteine and an intermediary (along with glutamic acid and glycine) in the conversion of cysteine to GSH. Oral NAC administration leads to an increase in intracellular cysteine and GSH levels.^[61] NAC is the primary antidote for acetaminophen-induced hepatotoxicity.^[62] NAC is able to inhibit genotoxicity due to reactive oxygen species (ROS), protect DNA and nuclear enzymes, and prevent the formation of carcinogen-DNA adducts.^[63] NAC succeeded in the treatment of severe hepatic injury induced by a dietary fitness supplement.^[64]

Alpha lipoic acid (ALA) influences oxidative status by scavenging ROS, regenerating endogenous antioxidants, repairing oxidative damage, and chelating metal ions.^[65] ALA has been proven to be a natural, yet very powerful free radical scavenger and antioxidant. ALA has a protective effect against CCl₄-induced hepatotoxicity and prevents against liver fibrosis due to inhibition of transforming growth factor (TGF)/platelet-derived growth factor-stimulated HSCs activation and ROS generation.^[66-68]

L-carnitine (CAR) is a conditionally essential nutrient, synthesized endogenously from lysine and methionine in the liver, kidney, and brain and it induces its effects on both fat and glucose metabolism.^[69] CAR binds to fatty acyl-coenzyme A and regulates their transport into mitochondrial matrix for β -oxidation. L-CAR is a superoxide scavenger, antioxidant, and DNA cleavage protector.^[70] L-CAR has shown a protective effect against radiation-induced

Table 2: Effect of micronutrients against hepatic injury

Name	Mechanism of action	Major effect
Vitamin B ₁₂	Suppresses genetic expression of α -smooth muscle actin and heat-shock protein 47 Inhibit hepatic fibrosis	Hepatoprotective effect
Vitamin C	Free radical scavenger Prevention of tumor initiation	Antioxidant, anti-apoptosis
Vitamin E	↓Genetic expression NADPH, DNA damage Prevention of tumor initiation	Anti-cytotoxicity and anti-genotoxicity
Zinc	Free radical scavenger, control cell proliferation Prevention of tumor initiation	Anti-inflammatory, anti-apoptosis
Selenium	Catalysis of redox reaction Prevention of tumor initiation	Antioxidant

organotoxicity via induction of endogenous antioxidants.^[71] Reduction of concentration of CAR in blood and tissues is accompanied with hyperlipidemic condition.^[72] It has been well reported that hepatoprotective effect of L-CAR against CCl₄-induced hepatotoxicity is due to significant increase of GSH level.^[73]

Lycopene is the red pigment of tomatoes. Lycopene concentration in human serum tends to be higher than those of all other carotenoid pigments.^[74] Lycopene showed potent anti-inflammatory effects through its action as an antioxidant and free radical scavenger, which may reduce cellular damage.^[75] It plays a crucial role in the protection of cell membranes from lipid peroxidation by neutralizing hydroxyl radicals and may bind to DNA, promoting further protection beyond antioxidant activity.^[76] Lycopene demonstrated potential beneficial effects against oxidative stress. These beneficial functions are due to enhancement of cellular gap junction communication, induction of phase II enzymes through activation of the antioxidant response element of transcription system, and suppression of insulin-like growth factor-1-stimulated cell proliferation. Its effects also include anti-angiogenesis, inhibition of cell proliferation, and induction of apoptosis.^[77] Lycopene showed potent protective effect against hepatic steatosis in knockout mice.^[78]

S-adenosyl-L-methionine (SAME) is an endogenous agent that is a critical precursor for transmethylation and transsulfuration reactions. SAME plays an important role such as a cofactor for many transmethylation reactions of amino acids, proteins, nucleotides, and neurotransmitters and a vital precursor for the transsulfuration pathway that ultimately generates GSH.^[79] SAME has potent activity against acetaminophen-induced hepatotoxicity as compared to NAC.^[80] SAME reduced the cytotoxicity of other hepatotoxicants such as carbon tetrachloride, which may lead to liver fibrosis and alcohol-mediated damage.^[81] SAME was reported to protect liver against hepatic injury and fibrosis through the inhibition of oxidative stress and HSCs formation due to activation of Smad7 (an inhibitor of TGF-beta signaling; regulator of hepatic fibrosis) messenger RNA expression.^[82]

Whey protein concentrates (WPCs) are heterogeneous compounds obtained from milk after casein precipitation at pH 4.6.^[83] WPCs play an important biological role since they act as antioxidants, antihypertensive and anti-tumor, hypolipidemic and antiviral, antibacterial, and chelating agents. WPCs counteract oxidative stress and DNA damage in rats that fed an aflatoxin-contaminated diet.^[84-89] The effect of various supplements has been depicted in Table 3.

Table 3: Effect of dietary supplement against hepatotoxicity and hepatic cancer

Name	Mechanism of action	Use
N-acetyl cysteine	Increase intracellular cysteine and GSH levels Prevention of tumor initiation	Antidote for acetaminophen-induced hepatotoxicity
Alpha lipoic acid	Inhibition of TGF/PDGF-(HSC) Prevention of tumor initiation and hepatic fibrosis	Antioxidant
L-carnitine	Superoxide scavenger, and DNA cleavage protector Prevention of tumor initiation	Antioxidant
Lycopene	Suppression of insulin-like growth factor-1-stimulated cell proliferation Prevention of tumor initiation and hepatic fibrosis	Antioxidant
S-adenosyl-L-methionine	Cofactor for amino acids, inhibition of HSC	Antioxidant
Whey protein concentrates	Free radical scavenger Prevention of tumor initiation	Antioxidants, hypolipidemic agent

GSH: glutathione; TGF: transforming growth factor; PDGF: platelet-derived growth factor; HSC: hepatic stellate cell

It can be concluded that natural bioactive compounds are promising candidate in the treatment and prevention of hepatic injury as well as HCC. The effects may be due to their anti-oxidative properties, modulatory effects in several cytokines, and anti-genotoxic efficacy. The current article highlights on the potential mechanism of the action of natural compounds against hepatotoxicity and suggests further studies for developing novel therapeutic tools in the treatment of hepatic lesions.

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Conflict of interest

There is no conflict of interest.

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Natural products and hepatocellular carcinoma: a review

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ABSTRACT

Hepatocellular carcinoma (HCC) is the fifth commonest cause of malignancy and the third cause of cancer mortality. There are different treatment options for HCC ranging from loco-regional therapy to surgical treatment. Different regimen of systemic chemotherapy has been tried with a poor response. Several studies aimed at discovering more molecules for the management of HCC. Those studies aimed at recognizing and targeting several signaling and molecular pathways that lead to cellular proliferation and tumor formation. In this review, we discussed the role of several agents found in natural and dietary products such as curcumin, resveratrol, flavonoids, *Rubus aleaefolius* Poir total alkaloids, *Livistona chinensis* seed, and crocetin. We had used the names of the above-mentioned products as key words in addition to "HCC" on PubMed to find studies that discussed their roles in HCC. Articles were downloaded for reviewing and discussing natural products that had adequate studies in treating HCC.

Key words: Hepatocellular carcinoma; molecular signaling; natural products

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth commonest cause of malignancy and the third cause of cancer mortality.^[1,2] Most patients with HCC were diagnosed in advanced stage that carried poor outcome with an overall 5-year survival rate of < 9%.^[3,4] An estimation of 21,000 deaths related to HCC was reported in the US in 2012.^[5] HCC has been frequently reported in Sub-Saharan Africa, Europe, North America, and Asia.^[6,7] Most HCC occurs in the patients with liver cirrhosis mainly related to hepatitis B and C infections, hemochromatosis, non-alcoholic steatohepatitis, alcohol consumption, nitrosamines, and aflatoxins.^[8-11]

Hepatic carcinogens, viral hepatitis, and liver cirrhosis induce inflammation and oxidative stress.^[12] Production of free radicals (such as oxygen and nitrogen species) as well as cytokines and chemokines lead to cellular injury.^[12] Following that, cellular proliferation happens, leading to malignant transformation.^[13,14] Moreover, signaling processes at the cellular and molecular levels are involved throughout the development of HCC.^[15-17]

Management of HCC is rather complex compared to other malignancy; it happens mostly in the setting of liver cirrhosis, and the treatment option largely depends on the stage of liver disease, the patient's functional status, number, size and location of tumor, and the presence or

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absence of vascular invasion.^[18] Several staging systems have been developed for HCC, but the best and most widely used is Barcelona Clinic Liver Cancer classification; added to the staging, it has prognostic and treatment implication.^[19]

There are different treatment options for HCC ranging from loco-regional therapy (radiofrequency ablation, arterial chemoembolization, intra-tumor ethanol injection, yttrium-90 intra-arterial delivery as microspheres, and microwave coagulation) to surgical treatment (surgical resection and liver transplantation) and sorafenib.^[9,20,21] Distinctive regimen of chemotherapy has been tried with poor response.

Surgical treatment is the best present-day management options for HCC, but not all patients are eligible for it.^[12] Surgical resection cannot be performed if the tumor is present in multiple sites, in advanced liver disease (Child's B and C) and in the presence of vascular invasion,^[21] and only about 20% of patients are candidates for surgical resection.^[22] Early on, HCC was a contraindication for liver transplantation due to poor result. Mazzaferro *et al.*^[23] published Milan criteria, in which patients with early disease have a good outcome. A significant number of HCC patients do not meet Milan criteria on presentation or drop out due to disease progression while waiting for liver transplantation due to the shortage of donors.^[23] Sorafenib, a tyrosine kinase inhibitor and vascular endothelial growth factor, is presently used in managing unresected HCC.^[24] It increases the average survival time by 3 months in patients with late-stage HCC.^[18] However, sorafenib can be used in Child's A and selected Child's B patients, in addition to the side effect and high cost.^[25,26]

Clearly, the available treatment option is far from optimal, either due to limited efficacy or contraindication due to advanced liver disease (resection and loco-regional therapy for Child's C), this reiterates the need for new treatment option.

Several studies aimed at discovering more molecules for the management of HCC. Those studies aimed at recognizing and targeting several signaling and molecular pathways that lead to cellular proliferation and tumor formation.^[12,27-29]

In this review, we discussed the role of several agents found in natural and dietary products such as curcumin, resveratrol, flavonoids, *Rubus aleaefolius* Poir total alkaloids, *Livistona chinensis* seed, and crocetin. We had used the names of the above-mentioned products as key words in addition

to "HCC" on PubMed to find studies that discussed their roles in HCC. Articles were downloaded for reviewing and discussing natural products that had adequate studies in treating HCC.

CURCUMIN

Curcumin is a polyphenol, a diferuloylmethane and it is among the three main curcuminoids present in turmeric.^[18] Curcumin is a potent anti-inflammatory agent.^[30] Curcumin has been proven to be effective in treating a variety of conditions such as allergy, psoriasis, diabetes, rheumatoid arthritis, asthma, and neurodegenerative diseases.^[18] Moreover, it is cardioprotective, hepatoprotective, carcinoprotective, and neuroprotective.^[18] As mentioned earlier, free radicals generation is an important step in tumor formation, and curcuminoids are known to inhibit oxidation owing to their methoxy group, 1,3 β -diketone moiety, and phenolic hydroxyl group.^[31] Curcumin was found to inhibit nuclear factor-kB (NF-kB), which activated inflammatory cytokines and chemokines, leading to several inflammatory conditions.^[32-34] NF-kB activation promotes cellular proliferation, angiogenesis, and invasion and inhibits apoptosis.^[35,36] In addition, curcumin also inhibits interleukin-1 (IL-1), IL-1B, IL-6, IL-8, tumor necrosis alpha, and cyclo-oxygenase pathways.^[35,37-39] Several studies have supported curcumin's anti-oxidant and anti-inflammatory, particularly in HCC. Dai *et al.*^[27] studied the anti-tumor effects of curcumin *in vitro* and *in vivo*. Curcumin inhibited HepG2's proliferation in a dose and time dependent fashion, with the most potent inhibition at a concentration of 8 $\mu\text{mol/L}$ for 48 h, it leads to HepG2 induced cells apoptosis at high doses, the apoptosis rate increased up to 20% at a curcumin concentration of 16 $\mu\text{mol/L}$. In addition, high doses of curcumin have been shown to elevate casepase-3, an essential protein for apoptosis.^[27] Curcumin has restricted liver tumor growth in HepG2 xenograft mice models *in vivo*; the greatest reduction in tumor volume was around 3740 mm^3 at a high curcumin dose of 60 mg/kg .^[27] Curcumin also mediated apoptosis in HL60, SGC7901, and Bel7402 cells by inhibiting telomerase activity.^[40]

In another study by Lin *et al.*,^[41] curcumin caused a decline migration and invasion of SK-Hep-1 cells as well as matrix metalloproteinase-9 (MMP-9) levels. Also, curcumin has an inhibitory effect of vasculogenic mimicry in SK-Heo-1 cells, a process in which hepatocytes act as endothelial cells and form blood vessels, by inhibiting the STAT3 and Akt pathways.^[42] Moreover, curcumin decreases caveolin-1 levels and epidermal growth factor signaling, and therefore may prevent vascular invasion and metastasis.^[40] Unfortunately, curcumin has poor pharmacokinetics as it undergoes poor

absorption and has low bioavailability.^[43] Curcumin gets directly conjugated once it is absorbed, and only a small amount remains as free curcumin.^[43] It has been suggested that its metabolite, curcumin glucuronide, is responsible for most of its therapeutically assumed action,^[44] however, a recent study showed that curcumin glucuronide has a less potent effect than curcumin itself on HepG2 cells, as the expression of *GSTT1*, *CAT*, *IL-8*, *AREG*, and *ACOX1* genes was greatly downregulated by curcumin than by curcumin glucuronide.^[43] In addition, curcumin is more rapidly absorbed than curcumin glucuronide.^[43] Curcumin is the most studied natural product for HCC; it is clearly effective in HCC at different molecular mechanisms for inflammation, proliferation, and apoptosis, there is a lack of clinical data in humans to confirm the above.

RESVERATROL

Resveratrol (3, 4', 5-trihydroxy-trans-stilbene) is found in red wine, berries, grapes, and peanuts.^[45] Resveratrol has been found to be anti-inflammatory in viral infections, neurodegenerative diseases, cardiovascular diseases, ischemia, and cancer. Resveratrol has anti-cancer effects by suppressing initiation, promotion, and progression of tumor formation.^[46-49] Moreover, it has significant anti-cancer activity by inhibiting inflammation and free radicals generation.^[50,51] Resveratrol has been found to hold rat hepatoma Fao cells and HepG2 cells in S and G2/M phase and prevent them from engaging in mitotic division.^[52] Another study showed that cells exposed to resveratrol were held in G1 phase and had an upregulation in *Bax* and *p21* genes.^[53] It also decreased the invasion of cancer cells by downregulating hepatic growth factor.^[54] It inhibits vascular endothelial growth factor gene expression by inducing hypoxia in HepG2 cells.^[55] In another study, HepG2 cells exposed to high concentrations of resveratrol reaching between 50 and 100 $\mu\text{mol/L}$ for more than 48 h were more prone to apoptosis, in a dose-dependent fashion.^[56] In a study that exposed H22 cells to resveratrol with 5-fluorouracil (FU) vs. 5-FU alone; resveratrol and 5-FU had a greater anti-cancer activity compared to 5-FU alone.^[57] Notas *et al.*^[58] concluded that resveratrol has an anti-proliferative effect against HepG2 cells as well as inducing the production of nitric oxide. It has also been shown to downregulate NF- κB , caveolin-1, and MMP-9.^[59,60] Several *in vivo* studies also support the anti-tumor activity of resveratrol in HCC. Resveratrol was administered to mice that had HCC tumor cells, hepatic tumor growth reduced and cell cycle proteins p34cdc2 and cyclin B1's expression was suppressed.^[61] Resveratrol has shown to have both *in vivo* and *in vitro* effect against HCC though different pathways and appear to have a promising potential, yet there is no clinical data in humans.

FLAVONOIDS

Flavonoids are polyphenols found in vegetables, fruits, flowers, tea, wine, stems, and roots.^[62] There are seven types of flavonoids: Anthocyanidins, flavanones, flavonols, flavones, flavanols, flavononol, and isoflavones.^[63] They have been shown to be cardio-protective and hepato-protective and possess anti-viral and anti-cancer activity.^[64-66] Flavonoids have found to induce apoptosis in HepG2 cells via activation of the mitochondrial pathway, along with the translocation of cytochrome c, activation of caspases such as 9, 8, and 3, abnormal changes in mitochondrial membrane potential, generation of reactive oxygen species, elevation in intracellular calcium, and upregulated transcription of endonuclease G and apoptosis inducing factor-related genes.^[67] Flavonoids have also been found to inhibit HepG2 cells growth by inhibiting the NF- κB pathway via blocking tumor necrosis factor- α .^[68] Administering epigallocatechin-3-gallate (EGCG), which is found in green tea, to HepG2 cells induces their apoptosis by suppressing epidermal growth factor receptor/c-Met signaling; therefore, suppress tumor cell proliferation and invasion.^[69] Quercetin, found in flavonol, has shown to restrain the expression of heat shock proteins 27 and 40, which lead to resistance to chemotherapy, hence potentiating the effect of the chemotherapeutic agent.^[70] Moreover, flavonoids have been found to be anti-hepatitis B virus (HBV) and hepatitis B core. EGCG inhibits HBV replication by altering its DNA synthesis.^[71] Furthermore, hepatitis C virus is inhibited by catechin that interferes with NF- κB and COX-2 pathways.^[72]

Like curcumin and resveratrol, flavonoids appear to have activity against HCC with different mechanisms, through different pathways but need to be tested in clinical trial.

TOTAL ALKALOIDS OF *RUBUS ALEAEFOLIUS* POIR

R. aleaeifolius is a plant used for the management of hepatitis in China.^[73] Hong *et al.*^[74] have reported that components of *R. aleaeifolius*, such as butanol and ethylacetate, are hepatoprotective in mice with acute liver injury after exposure to carbon tetrachloride. Reports from the literature have discussed the protective and therapeutic role of *R. aleaeifolius* Poir in carcinogenesis.^[75,76] Zhao *et al.*^[73] examined the therapeutic effects of total alkaloids in *R. aleaeifolius* Poir (TARAP) on HCC both *in vitro* and *in vivo*. TARAP has been shown to affect HCC growth and induce apoptosis in HepG2 cells via mitochondrion-mediated apoptosis by causing the loss of mitochondrion potential and activation of caspases 9 and 3, apoptosis was dose dependent.^[73] Bax and Bcl-2 are important proteins involved in the process of apoptosis.^[77] Bcl-2 is known to be anti-apoptotic,^[73] and BAX

is pro-apoptotic.^[78] If the ratio of Bcl-2 to BAX is great, then apoptosis does not occur.^[73] TARAP has downregulated the expression of Bcl-2 and upregulated the expression of BAX, decreasing the Bcl-2-BAX ratio, hence inducing apoptosis.^[73] TARAP has been used in China for hepatitis and its use in HCC needs further studies.

LIVISTONA CHINENSIS SEED

L. chinensis seed has been used in China for cancer treatment.^[79] Lin *et al.*^[28] have evaluated the therapeutic role of ethanol extract of the *L. chinensis* seed (EELC) against HCC both *in vitro* and *in vivo*. EELC has inhibited tumor growth in HCC xenograft mice and decreased the tumor weight by 43%, moreover, EELC tumor inhibition was assessed *in vitro* on HepG2 cells, and the maximum reduction in cell viability was around 60% in a maximum time of 24 h, it also induced cell apoptosis in HCC xenograft mice and HepG2 cells.^[28] Moreover, EELC has induced the loss of the mitochondrion membrane potential in HepG2 cells, leading to apoptosis and stimulates the release of caspases 9 and 3 in HepG2 cells and causes a rise in the BAX-Bcl-2 ratio, as what TARAP does.^[28]

CROCIN

Derived from *Crocus sativus*, saffron exhibits a therapeutic effect against depression, cancer, and asthma.^[80] It also acts like oxytocin and as a stimulant.^[80] Three compounds are found in saffron: Picrocrocin, crocin, and safranal.^[81] Crocins give saffron its color.^[80] Several studies discussed the anti-cancer effect of saffron in different types of cancer such as pancreatic, gastric, bladder, and hepatic cancer.^[82-85] Nouredini and Wink^[84] studied the anti-proliferative effects of crocin in HepG2 cells. HepG2 cells exposed to 3 mg/mL of crocin had almost a 59% decrease in telomerase activity. In addition, crocin and safranil have shown to increase the cleavage of caspase-3, arrest the cell cycle, and cause DNA damage in HepG2 cells.^[85] Moreover, Tseng *et al.*^[86] reported that crocetin, a major constituent in saffron, was an anti-oxidant and hepatoprotective by decreasing the synthesis of malondialdehyde in hepatocytes, a marker for fatty acid oxidation and oxidative stress. As concluded by other researchers, crocetin protects against cancer by inducing apoptosis and arresting the cell cycle.^[86]

CONCLUSION

The potential chemo-preventive and therapeutic role of above discussed natural products in HCC is due to their potent anti-oxidant and anti-inflammatory properties as well as their ability to modulate different signaling mechanisms that are implicated in the process of carcinogenesis. They

hold considerable promise as therapeutic agents for HCC. Most of these studies are preclinical with very limited clinical data; therefore, the clinical efficacy of these products is still far from being tested. There is a need to develop a dosing from using the available technology to overcome the low bioavailability and to have a standard dosage for future clinical trials. Once that is achieved, the safety of these products in high doses needs to be ascertained, although they have been in use for hundreds of years.

Lack of good clinical trials testing these products compared to sorafenib and other pharmacological therapy may be due to lack of financial support to conduct such trails.

There is a pressing need for governmental funding and collaboration between centers to conduct multicenter randomized open label studies using the standard of care, with or without these products either individualized or in combination.

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Conflict of interest

There is no conflict of interest.

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Preventive role of chamomile flowers and fennel seeds extracts against liver injury and oxidative stress induced by an immunosuppressant drug in rats

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ABSTRACT

Aim: The present study was conducted to investigate the protective effect of chamomile flowers methanolic extract (CFME) and fennel seeds methanolic extract (FSME) on azathioprine (AZA), an immunosuppressant drug, which induced a liver injury and oxidative stress in rats. **Methods:** Rats were divided into 6 groups (8 rats each) and treated orally for 28 consecutive days as follows. Group 1: rats were given normal saline and used as controls; group 2: rats treated with CFME (200 mg/kg); group 3: rats treated with FSME (200 mg/kg); group 4: rats treated with AZA (25 mg/kg); and groups 5 and 6: rats treated with CFME (200 mg/kg) or FSME (200 mg/kg) 15 min prior to AZA (25 mg/kg) treatment. At the end of experimental period, blood and liver samples were collected from all groups for biochemical analysis and histological examination. **Results:** The obtained data revealed that AZA-induced hepatic injury in the rats as evidenced by the significant increase in serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, cholesterol, and direct bilirubin as well as hepatic malondialdehyde level accompanied with significant decrease in reduced glutathione content and total antioxidant capacity in the liver. Moreover, body weight gain showed the significant decrease and relative liver weight showed the significant increase on AZA treatment. The sequential significant changes in biochemical parameters were accompanied by severe histological changes in the liver tissue, including hepatocytes disorganization with pyknotic nuclei, fatty degeneration, congestion, fibrosis, and bile duct necrosis around the portal tract. The areas of hemorrhages in blood vessels and in between hepatocytes were also seen. However, the results showed the potential hepatoprotective effects of CFME and FSME against AZA-induced liver injury and oxidative stress. They succeeded in restoring the biochemical parameters and improving the histological picture of the liver. This improvement was more pronounced in the rats pretreated with FSME. **Conclusion:** It could be concluded that CFME and FSME have hepatoprotective potentials against AZA probably due to their antioxidant properties and radical scavenging activity.

Key words: Azathioprine; chamomile flowers; fennel seeds; liver; oxidative stress

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INTRODUCTION

Hepatotoxicants, including viruses, fungal products, bacterial metabolites, minerals, environmental pollutants, and chemotherapeutic agents, can induce various disorders of the organ.^[1] Azathioprine (AZA) is a common immunosuppressant drug used in medicine to treat different diseases.^[2] It is now widely used in oncology, dermatology, gastroenterology, and rheumatology for its anti-leukemic and immunosuppressive properties.^[3] AZA (6-(1-methyl-4-nitroimidazol-5-yl) thiopurine) is also used for the prevention of rejection in organ transplants and the treatment of auto-immune diseases.^[4] It is indicated as an adjunct for the prevention of rejection in the renal transplantations. AZA is also used in the prevention of rejection in cardiac, hepatic, and pancreatic transplantations.^[5,6]

The therapeutic use of AZA is associated with many complications. It induces a range of toxic effects that may ultimately result in the discontinuation of treatment. These toxic effects include gastrointestinal disturbances, pancreatitis, reversible alopecia, rashes, fever, tachycardia, pneumonitis, hypotension, and renal dysfunction.^[7] Many studies have frequently reported the hepatotoxicity of AZA *in vitro* and *in vivo*. Many of these studies related the mechanism of hepatotoxicity and liver injury to the oxidative stress. AZA toxicity to rat hepatocytes *in vitro* and the mechanism of AZA toxicity to hepatocytes and decreasing its viability involves the depletion of glutathione (GSH) leading to mitochondrial injury with profound depletion of adenosine triphosphate (ATP) and cell death by necrosis were reported.^[8,9]

Plants rich in natural polyphenolic compounds were intensely studied in recent years due to their potent anticarcinogenic, antioxidant, and immunomodulatory properties. Chamomile (*Matricaria recutita* L.) is one of the most widely used medicinal plants in the world. Aqueous chamomile extract is used as herbal medicine, in the form of tea, demonstrated to possess anti-inflammatory and antioxidant properties.^[10] Chamomile preparations are commonly used for many human ailments such as hay fever, muscle spasms, menstrual disorders, insomnia, ulcers, wounds, gastrointestinal disorders, rheumatic pain, and hemorrhoids,^[11] and also used to treat anxiety, hysteria, nightmares, insomnia, and other sleep problems.^[12] The useful effects of chamomile are related to the presence of several flavonoid constituents (the most abundant phenolic compounds in herbs), and the core structure consists of either flavone (apigenin and luteolin) or flavonol derivatives (quercetin and patuletin). These occur in various forms such as aglyco- mono- and di-glycosides and/or

acyl-derivatives. Other principal components are essential oils such as terpenoids, α -bisabolol and its oxides, and azulenes including chalmuzene and acetylene derivatives.^[13]

Fennel (*Foeniculum vulgare* Mill.), a plant belonging to the family *Apiaceae*, has a long history of herbal uses. Fennel seeds are used as analgesic, carminative, anti-inflammatory, diuretic, and antispasmodic agents.^[14] The antioxidant potential and antimicrobial activity of fennel seed extracts and essential oil have been reported.^[14,15] Fennel seeds methanolic extract contains (FSMEs) high amount of polyphenols, including flavonoids as a major component of polyphenols, gallic acid, caffeic acid, ellagic acid, quercetin, and kaempferol.^[16] Trans-anethole, fenchone, estragole, 4-terpineol, sabinene, alpha-terpinene and monoterpene hydrocarbons (limonene) as the major compounds, were identified in the essential oil.^[17]

The present study was undertaken to elucidate the ability of oral chamomile flowers methanolic extract (CFME) and FSME to alleviate the adverse effects of AZA on the liver through biochemical and histological examinations in albino rats.

METHODS

Experimental animals

Male albino rats (Sprague-Dawley strains) weighing 150-200 g were used in this study. They were obtained from the animal house of National Research Centre, Giza, Egypt and acclimatized for 1 week prior to the experiment. Animals were housed in stainless steel cages at room temperature (20-25 °C) and a photoperiod of 12 h light-dark cycle. Animals were allowed free standard laboratory diet and drinking tap water *ad libitum*. This experimental study was approved by review board of National Research Centre.

Chemicals

AZA B.P uncoated tablets 50 mg manufactured by RPG Life Sciences Ltd., Ceat Mahal, 463, Dr. A B Road, Worli, Mumbai: 400 025, India. Methyl alcohol (98%) was obtained from El-Nasr Pharmaceutical Chemicals Co., "ADWIC" (Egypt). Perchloric acid and trichloroacetic acid (extra pure 99%) were manufactured by SISCO Research Laboratories PVT LTD (Mumbai, India). Thiobarbituric acid was purchased from MERCK (Darmstadt, Germany). Other solvents and chemicals used were either analar or of analytical grade unless otherwise specified. Plant materials: Dried chamomile (*Matricaria chamomilla*) flowers and dried fennel (*F. vulgare*) seeds were purchased from Abd El-Rahman Harraz (Bab El-Khalk Zone, Cairo, Egypt).

Preparation and extraction of the plant materials

Three samples of dried chamomile flowers and fennel seeds were ground. Eighty grams of each ground sample were

transported into 1 L Erlenmeyer flasks, and then 800 mL of 80% methanol (80:20, methanol:water, v/v) were added to the samples. Extraction was carried out using an orbital shaker at a room temperature for 8 h, they were filtrated through filter paper (Whatman No. 1), the residue was re-extracted twice for complete extraction, and then, the combined extracts of every sample were evaporated at 45 °C, using a rotary vacuum evaporator (Rotavapor R-114 BÜCHI, Switzerland) and stored at -4 °C until use.^[15]

Animal grouping

Sixty rats were divided into six groups (10 rats each) based on their body weight and treated daily for 28 consecutive days as follows. Group 1: rats were administered with normal saline by gastric intubation and served as control group; group 2: rats received CFME at a dose of 200 mg/kg (dissolved in normal saline) by gastric intubation;^[18] group 3: rats received FSME at a dose of 200 mg/kg (dissolved in normal saline) by gastric intubation;^[19] group 4: rats were orally administrated with AZA at a dose of 25 mg/kg (dissolved in normal saline) by gastric intubation;^[20] group 5: rats were orally administrated with CFME at a dose of 200 mg/kg followed by AZA, after 15 min, at a dose of 25 mg/kg by gastric intubation; and group 6: rats were orally administrated with FSME at a dose of 200 mg/kg followed by AZA, after 15 min, at a dose of 25 mg/kg.

During the experiment, the animals were weighed twice every week. Body weight gain (BWG) of each control and respective treated rats was calculated with reference to the initial body weight recorded at the beginning of the experiment and the final body weight at the end of the experiment.

Collection of blood samples

At the end of the experimental period, animals were fasted overnight. They were slightly anesthetized with diethyl ether. Blood samples were withdrawn from the retro-orbital venous plexus into serum tubes and left to clot and then centrifuged at 3000 g for 15 min at 4 °C where the clear sera were separated for the determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, and total cholesterol, triglycerides, and total- and direct-bilirubin levels.

Tissue sampling

At the end of blood collection, each animal was rapidly sacrificed, and the liver was dissected out and weighed then apart from its left lobe was immediately kept in 10% buffered formalin-saline solution for a later histopathological examination. Another part from the same lobe of the liver was washed with saline, dried, weighed, and homogenized in 50 mmol/L phosphate buffer (ice-cold) solution (pH 7.4)

to give 20% homogenate (w/v).^[21] The homogenate was centrifuged at 3000 g for 20 min. The supernatant was separated and stored at -70 °C until the determination of the levels of malondialdehyde (MDA), total antioxidant capacity (TAC), and reduced GSH content. Furthermore, the relative liver weight of each animal was then calculated as follows: Relative organ weight = (absolute organ weight [g] × 100)/(body weight of rat on sacrifice day [g]).

Analytical determinations

Colorimetric determinations of serum AST and ALT activities were carried out using UV-160 1PC UV-visible spectrophotometer (Shimadzu, Japan) for reading the absorbance. The assay was performed according to the instruction manual of RANDOX reagent kits manufactured by RANDOX Laboratories Ltd. (Admore, Diamond road, Crumlin, Co., Antrim, UK BT29 4QY). Colorimetric determination of serum ALP activity was performed according to the instruction manual of Reactivos GPL kits manufactured by Reactivos GPL (Barcelona, Espana, Spain). Serum total- and direct- bilirubin levels, triglycerides, and total cholesterol levels were determined colorimetrically using UV-160 1PC UV-visible spectrophotometer for reading the absorbance. The assays were performed according to the instruction manual of Reactivos GPL kits manufactured by Reactivos GPL (Barcelona, Espana, Spain). MDA as an indirect index for lipid peroxidation was determined in liver, based on its reaction with thiobarbituric acid which forms a pink complex that can be measured photometrically.^[22] Colorimetric determination of hepatic GSH content and TAC were carried out using UV-160 1PC UV-visible spectrophotometer for reading the absorbance using Kits produced by Biodiagnostic Co., Egypt.

Histopathological examination

Pieces of the livers from rats of control and treated groups were fixed in 10% formalin saline for 24 h. More washing in tap water overnight was followed by dehydration in graded alcohol, clearing in xylene for 20 min, and embedding in paraffin wax. Transverse serial sections were then cut at 5 µm thickness and mounted on albuminized slide.^[23] Sections were stained with hematoxylin and eosin and investigated by light microscopy.

Statistical analysis

The obtained data were subjected to one-way analysis of variance. The analysis was performed using Statistical Analysis System (SAS) program software; copyright (c) 1998 by SAS Institute Inc., Cary, NC, USA. Tukey test was used to evaluate the significance between the individual groups at $P < 0.05$.^[24] The values in this study were expressed as a mean ± standard error.

RESULTS

AZA treatment resulted in a significant increase in hepatic MDA level concomitant with a significant decline in hepatic GSH content and TAC as compared to control rats [Figure 1]. However, the administration of either CFME or FSME alone revealed insignificant changes in the mentioned parameters when compared to control rats except in rats that received FSME where a significant increase in hepatic GSH was observed. Pre-administration with CFME or FSME significantly reversed the elevation in hepatic MDA level and also reversed the decrease in hepatic GSH content and TAC-induced by AZA treatment toward the normal values of the controls.

The selected and specialized serum markers of liver functions among the different groups are shown in Figures 2 and 3. It is clearly indicated that CFME or FSME had no effect on AST, ALT, and ALP activities as well as total- and direct-bilirubin levels when compared with the control group. The treatment of rats with AZA alone resulted in significant increases in AST, ALT, and ALP activities, direct-bilirubin levels and a non-significant increase in total-bilirubin level. However, the administration of CFME or FSME to rats succeeded significantly in preventing the AZA-induced changes in the above mentioned parameters. In addition, the more

prominent preventive effect was observed in the group pre-treated with FSME.

In the present study, serum triglyceride level showed insignificant change among the different studied groups, but serum cholesterol level increased significantly in AZA-treated rats as compared with the control group. The animals those were administrated with CFME or FSME alone showed an insignificant change in serum cholesterol level as compared with the control group. The administration of CFME in combination with AZA offered little protection against AZA-induced changes in cholesterol level, whereas the administration of FSME to rats succeeded in ameliorating significantly the AZA-induced changes in the mentioned parameter [Figure 4].

AZA treatment resulted in a significant decrease in BWG (%), when compared to control rats [Figure 5a]. The administrated with CFME produced a non-significant decrease in BWG, but FSME administration caused a significant decrease in BWG when compared to control rats. Pre-treatment with CFME before AZA treatment did not ameliorate the decrease in BWG induced by AZA treatment, while as pre-administration with FSME before AZA treatment induced a significant increase in BWG when compared to the AZA-treated group.

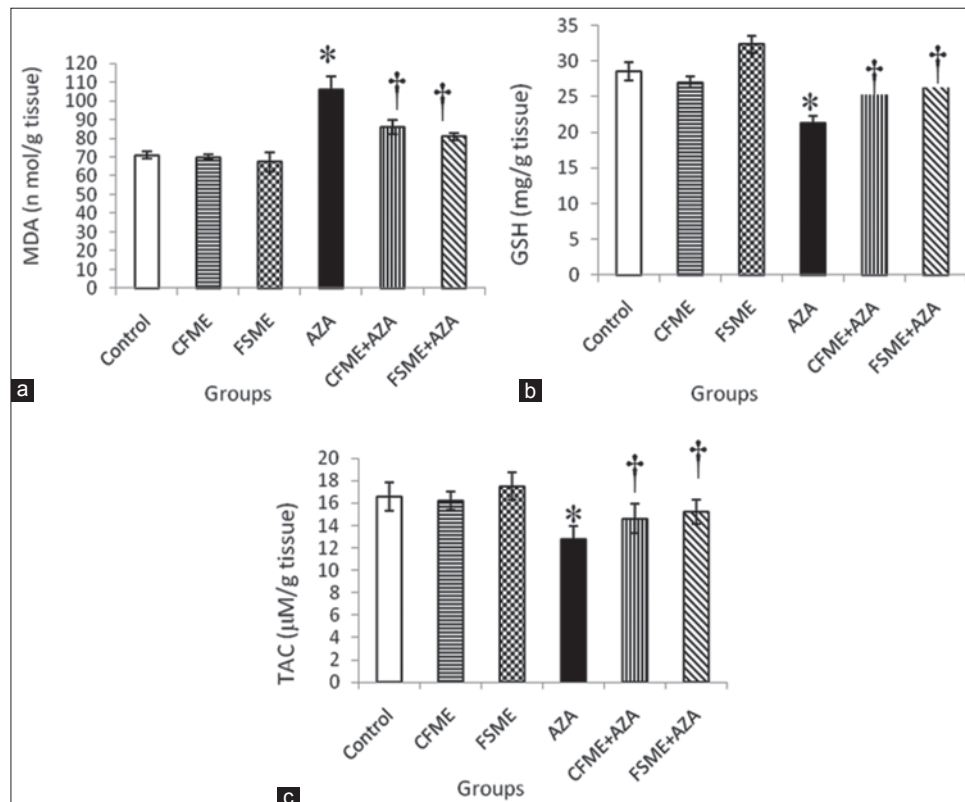


Figure 1: (a) Hepatic malondialdehyde (MDA) level; (b) hepatic reduced glutathione (GSH) level; and (c) total antioxidant capacity (TAC) in rats treated daily for 28 consecutive days with chamomile flowers methanolic extract (CFME), fennel seeds methanolic extract (FSME), azathioprine (AZA), CFME + AZA, and FSME + AZA. Data represent the mean \pm standard error ($n = 8$). * $P \leq 0.05$ compared to normal control rats; † $P \leq 0.05$ compared to AZA-treated rats

Data in Figure 5b show that AZA treatment induced a significant increase in mean relative liver weight (RLW) when compared to normal control rats. However, the oral administration of CFME or FSME did not significantly affect the mean RLW. The pre-treatment with CFME did not improve the increase in mean RLW ratio induced by AZA treatment, but the administration with FSME ameliorated the increase in mean RLW induced by AZA treatment.

The light microscopical examination of the liver sections from the control rats revealed normal hepatocytes

architecture [Figure 6]. The liver sections obtained from FSME and CFME treated rats showed more or less normal hepatocytes architecture, but some congested blood vessels were seen in FSME-treated rats [Figure 7] and mild inflammation around the portal tract in the CFME-treated rats [Figure 8]. In contrast, the liver sections obtained from the AZA-treated rats revealed hepatocytes disorganization, and fatty degeneration as indicated by large and microvesicular fat droplets. The hepatocytes nuclei were shrunk and pyknotic or apoptotic. There were areas of hemorrhages in blood vessels and in between hepatocytes. Hepatocytes

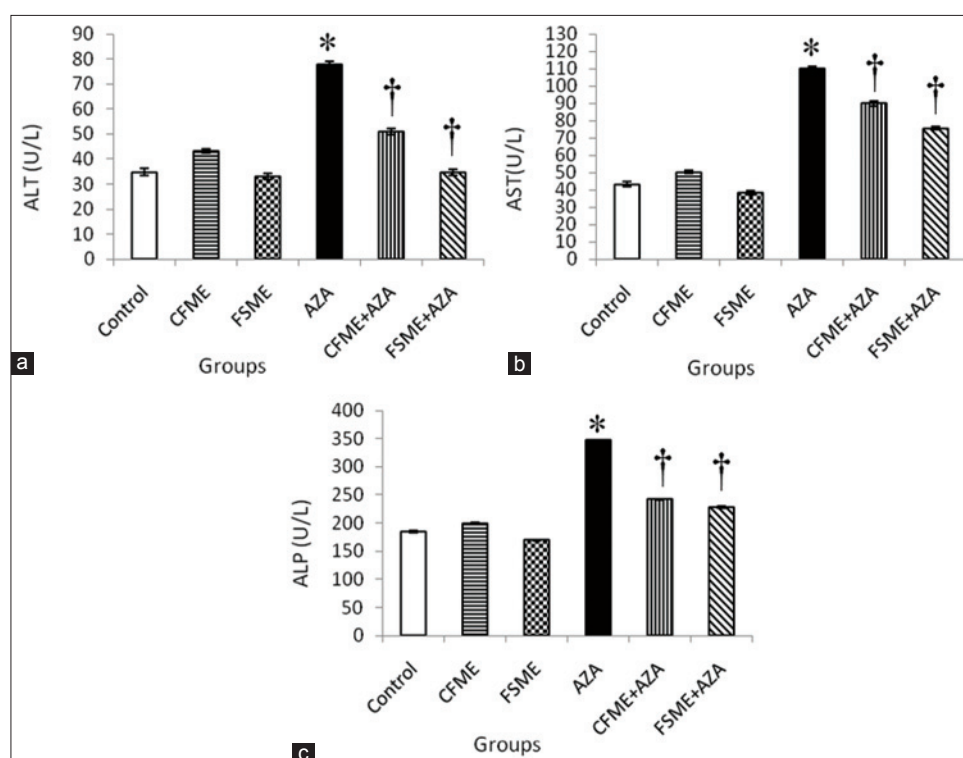


Figure 2: (a) Serum alanine aminotransferase (ALT) activity; (b) aspartate aminotransferase (AST) activity; (c) alkaline phosphatase (ALP) activity in rats treated daily for 28 consecutive days with chamomile flowers methanolic extract (CFME), fennel seeds methanolic extract (FSME), azathioprine (AZA), CFME + AZA, and FSME + AZA. Data represent the mean \pm standard error ($n = 8$). * $P \leq 0.05$ compared to normal control rats; † $P \leq 0.05$ compared to AZA-treated rats

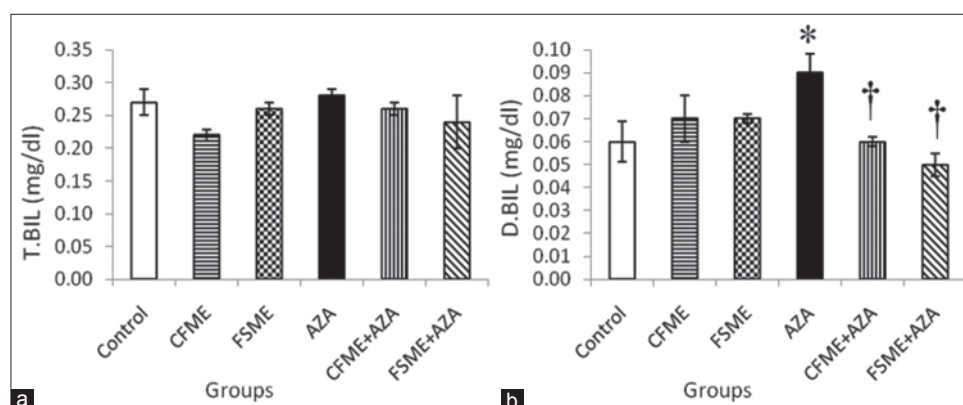


Figure 3: (a) Serum total-bilirubin (T.BIL) level; (b) direct-bilirubin (D.BIL) in rats treated daily for 28 consecutive days with chamomile flowers methanolic extract (CFME), fennel seeds methanolic extract (FSME), azathioprine (AZA), CFME + AZA, and FSME + AZA. Data represent the mean \pm standard error ($n = 8$). * $P \leq 0.05$ compared to normal control rats; † $P \leq 0.05$ compared to AZA-treated rats

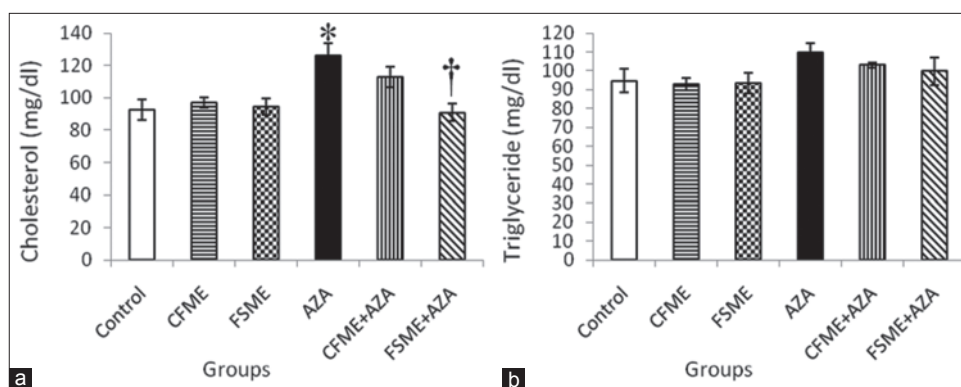


Figure 4: (a) Serum cholesterol; (b) triglyceride levels in rats treated daily for 28 consecutive days with chamomile flowers methanolic extract (CFME), fennel seeds methanolic extract (FSME), azathioprine (AZA), CFME + AZA, and FSME + AZA. Data represent the mean \pm standard error ($n = 8$). * $P \leq 0.05$ compared to normal control rats; † $P \leq 0.05$ compared to AZA-treated rats

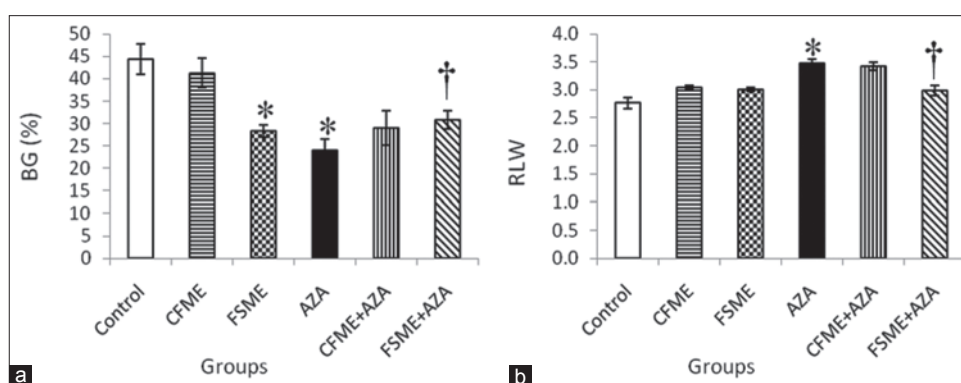


Figure 5: (a) Body weight gain (BG, %); (b) relative liver weight (RLW) in rats treated daily for 28 consecutive days with chamomile flowers methanolic extract (CFME), fennel seeds methanolic extract (FSME), azathioprine (AZA), CFME + AZA, and FSME + AZA. Data represent the mean \pm standard error ($n = 8$). * $P \leq 0.05$ compared to normal control rats; † $P \leq 0.05$ compared to AZA-treated rats

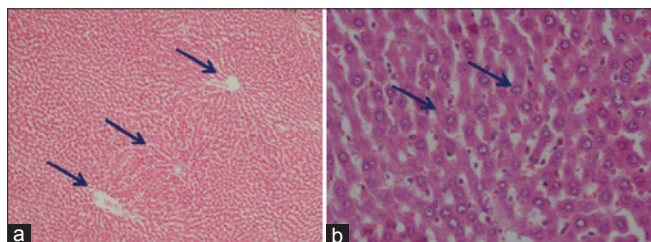


Figure 6: (a) Photomicrographs of liver sections of control rats showing the normal branching cords of hepatocytes around the portal tract and central vein separated by blood sinusoids (HE, $\times 100$); (b) high power of liver section of control rats showing the normal hepatocytes as polygonal cells having moderately acidophilic granular cytoplasm and rounded vesicular nuclei. Some cells are binucleated (HE, $\times 400$)

were seen congested and fibrosed with pyknotic nuclei, microscopical examination also revealed bile duct necrosis around the portal tract [Figure 9]. The liver sections of rats treated with FSME prior to AZA treatment showed marked improvement and regeneration in the periportal and central zone. Some hepatocytes revealed acidophilic and granular cytoplasm with central rounded vesicular nuclei [Figure 10]. The liver sections of rats administered with CFME prior to AZA treatment showed the normal hepatocytes architecture with normal central vein and portal tract, the fatty

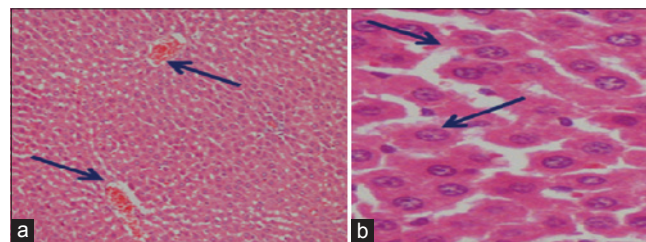


Figure 7: Photomicrographs of liver sections of fennel seeds methanolic extract treated rats showing the normal hepatocytes architecture (a: HE, $\times 100$) and congested blood vessels (b: HE, $\times 400$)

degeneration, and fibrosis or nuclear damage disappeared. Acidophilic cytoplasm with central rounded vesicular nuclei was observed [Figure 11].

DISCUSSION

AZA is a common immunosuppressant used in medicine to treat different diseases.^[25] However, AZA use has been complicated by a high incidence of hepatic injury which was found to be associated with oxidative damage.^[26] Hepatic injury is a common pathological feature which exists in many liver diseases. Liver fibrosis, cirrhosis, and even liver cancer

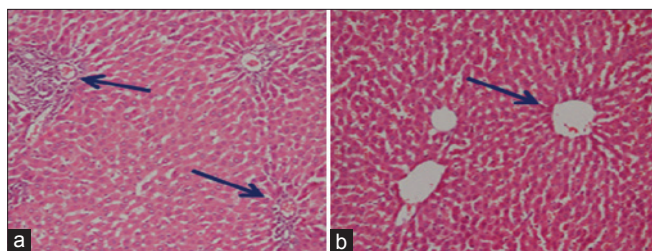


Figure 8: Photomicrographs of liver sections of chamomile flower methanolic extract treated rats showing the normal hepatocytes architecture in the central vein areas (a) and mild inflammation around the portal tract (b) (a and b: HE, $\times 100$)

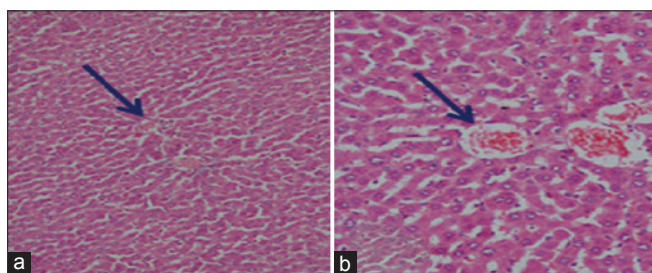


Figure 10: Photomicrographs of liver sections of fennel seed methanolic extract plus azathioprine treated rats showing the hepatocytes having acidophilic and granular cytoplasm with central rounded vesicular nuclei. Marked improvement and regeneration in the periportal (a: HE, $\times 100$) and central zone (b: HE, $\times 300$) are seen

could result from the long existence of hepatic injury.^[27] Chamomile flower and fennel seeds were reported to have antioxidant effects. Therefore, in this study, we investigated the protective effects of CFME and FSME, as natural products, against AZA-induced liver injury.

In recent years, there has been an increased interest in the possible role of reactive oxygen species (ROS) in the pathogenesis of tissue injury.^[26] Status of the oxidative/anti-oxidative profile was the mechanistic approach to assess the toxicity of AZA and/or protection to its toxic implications by using free radical scavengers.^[28] After administration, AZA is rapidly cleaved non-enzymatically within erythrocytes depending on GSH,^[29] to yield 6-mercaptopurine (6-MP) and an imidazole side chain.^[30] AZA is also metabolized in the liver by the conversion of AZA to 6-MP catalyzed largely and enzymatically by GSH S-transferase^[31] using GSH as a substrate.^[28] AZA metabolism in rat hepatocytes leads to GSH depletion, mitochondrial injury, decreased ATP levels, and cell death.^[8] 6-MP is further converted into 6-thiouric acid by xanthine oxidases (XO). It has been reported that XO has the potential to generate ROS in human hepatocytes^[32] and that the oxidation of 6-MP by XO is involved in the AZA-induced liver injury in patients with inflammatory bowel disease.^[33] Another metabolic pathway converts 6-MP into 6-thioinosine monophosphate via hypoxanthine-guanine phosphoribosyl transferase, and this intermediate is then metabolized into active 6-thioguanine

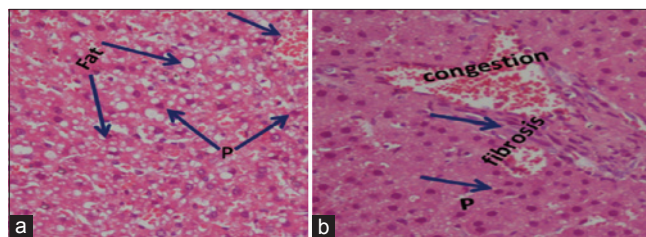


Figure 9: Photomicrographs of liver sections of azathioprine treated rats showing (a) hepatocytes disorganization, fatty degeneration indicated by large and microvesicular fat droplets. The hepatocytes nuclei are shrunk and pyknotic or apoptotic. Areas of hemorrhages in blood vessels and in between hepatocytes (HE, $\times 300$); (b) showing congestion, fibrosis and bile duct necrosis around the portal tract, the hepatocytes are disorganized with pyknotic nuclei (P) (HE, $\times 400$)

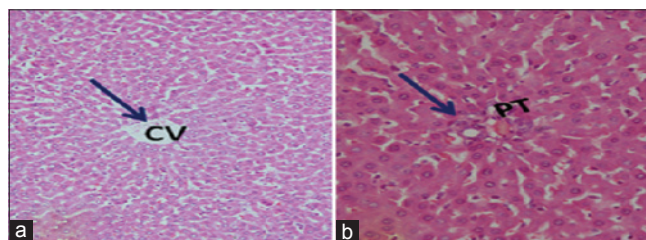


Figure 11: Photomicrographs of livers section of rats treated with chamomile flower extract prior to azathioprine treatment showing the normal hepatocytes architecture. The fatty degeneration and fibrosis or nuclear damage are disappeared and appearance of acidophilic cytoplasm with central rounded vesicular nuclei. Normal central vein (CV) (a: HE, $\times 100$) and portal tract (PT) (b: HE, $\times 300$)

nucleotides (6-TGNs).^[34] 6-TGNs are also responsible for the cytotoxic side effects.^[35]

The metabolic conversion of 6-MP into 6-thiouric acid via XO, which is a critical source of ROS, potentially leads to hepatotoxicity.^[32] It has been suggested that ROS production by mitochondria caused by thiopurines could damage the membranes and macromolecules.^[8] In the present study, the oxidative injury in AZA-treated animals was evident from the significant decline in GSH and TAC levels. The oxidative stress was further confirmed by increased lipid peroxidation and histopathological changes in the liver tissue. These findings are in agreement with previous studies, which recorded the involvement of oxidative stress and lipid peroxidation in AZA-induced liver injury.^[32,36]

In hepatocytes, GSH is consumed during the metabolism of AZA to 6-MP. The mechanism of AZA toxicity to hepatocytes involves the depletion of GSH leading to mitochondrial injury with profound depletion of ATP and cell death by necrosis.^[9] Furthermore, GSH is responsible for ROS scavenging. Therefore, the decrease in GSH induced by AZA administration may be caused by the exhaustion of GSH during ROS scavenging.^[32] Lipid peroxidation, as well as altered levels of some endogenous scavengers, are taken

as indirect *in vivo* reliable indices for the contribution of free radical generation and in turn oxidative stress.^[37] It has already been demonstrated that the depletion of GSH precedes the induction of lipid peroxidation.^[38]

In this study, AZA-induced hepatotoxicity is evidenced by significant increments in the values of serum ALT, AST, ALP, direct-bilirubin, and cholesterol that may be attributed to the liver injury and also confirmed by pathological changes in the liver of AZA-treated rats. The increase of serum ALT, AST, and ALP activities may be mainly due to the leakage of these enzymes from the liver cytosol into the blood.^[39] In necrosis or membrane damage, ALT and AST are released into circulation, and it can, therefore, be measured in serum as markers of hepatic damage.^[40] Furthermore, our results are in agreement with a study which reported the cholestatic type of liver injury in a man treated with AZA, as developed after 16 days of starting AZA therapy.^[41]

Serum ALP and total-bilirubin levels are also related to the status and function of hepatic cells. The increase in serum ALP is due to increased synthesis in the presence of biliary pressure.^[42] Bilirubin has been used to evaluate chemically-induced hepatic injury. It is one of the most useful clinical clues to the severity of necrosis, and its accumulation is a measure of binding, conjugation, and excretory capacity of hepatocytes.^[43]

Lipids concentration is determined by metabolic functions, which are influenced by the integrity of vital organs such as the liver and kidney. Therefore, lipid profile such as cholesterol and triglycerides are increased in hepatopathy. The lipid content of hepatocytes is regulated by the integrated activities of cellular enzymes that catalyze lipid uptake, synthesis, oxidation, and export.^[44]

In the current study, the observed decrease in BWG in rats treated with AZA are in agreement with the observations which reported the weight loss in rats^[45] and mouse^[46] exposed to cytotoxic agents such as AZA and methotrexate which could possibly be due to the inhibition of DNA synthesis and increased oxidative stress with consequent cellular damage of the body organs in affected rats.^[45]

Since oxidative stress has been recognized to be involved in etiology of several liver diseases and because liver is very susceptible to toxic effects, natural antioxidants, and plant extracts have been proposed as therapeutic agents to protect against liver damage.^[47] Administration of chamomile flowers and fennel seeds extracts to AZA-treated animals was potentially effective in reducing the lipid peroxidation and

enhancing antioxidant capacity in the liver of AZA-treated animals. This appeared from the amelioration of MDA to near normal level and the significant improvement of the GSH and TAC contents. These results agree with that reported for chamomile flowers extract^[48,49] and for fennel seeds extract.^[16,50]

In addition, chamomile flower and fennel seed extract could also significantly decrease serum ALT, AST, and ALP, suggesting their hepatoprotective activity. The mechanisms by which chamomile flower and fennel seed extracts offered their protective effects against AZA hepatotoxicity are based on their antioxidant abilities, which may be responsible for protecting the hepatic cells against the oxidative stress, possibly by increasing the endogenous defensive capacity of the liver to combat oxidative stress induced by AZA. This in turn improves the liver integrity and function and consequently improves the hepatic excretory function of bilirubin and also improves lipid metabolism. This improvement was more pronounced in the animals that were received fennel seed extract. Chamomile flower and fennel seed extracts also significantly ameliorated the decrease in the BWG. This may be attributed to the antioxidant effect of these extracts.

Several reports demonstrated that chamomile flowers and fennel seeds extracts contain important nutrients and exhibit antioxidant functions. The results of our recently published study revealed that the methanolic extract of both plants possesses considerable amounts of phenolic compounds and radical scavenging activity,^[51] which were in agreement with those reported recently.^[52] Some phenolic compounds have the capacity to quench lipid peroxidation products, prevent DNA oxidative damage, and scavenge ROS.^[53]

Flavonoids isolated from chamomile, such as apigenin and luteolin, have been shown to possess antioxidant, anticarcinogenic, carminative, antispasmodic, and mild sedative properties.^[54] Fennel seed extract contains, by chromatographic analysis, trans-anethole, fenchone, methylchavicol, limonene, α -pinene, camphene, β -pinene, β -myrcene, α -phellandrene, 3-carene, camphor, and cisanethole.^[55] Among these, dlimonene and β -myrcene have been shown to affect the liver function. D-limonene increases the concentration of reduced GSH in the liver.^[56]

The biochemical investigations were confirmed by the histopathological results of the liver tissue. In our study, light microscopic examination of AZA-treated rats revealed hepatocytes disorganization, fatty degeneration indicated by large and microvesicular fat droplets and shrinkage,

pyknotic, or apoptotic nuclei. Furthermore, large nodules with eosinophilic cytoplasm were present. Liver mean relative weight of AZA-treated rats was significantly increased as compared to control rats. These findings are in agreement with the findings of other investigators that reported an increase in mean relative liver weight in mice treated with AZA.^[4] In addition, disorganization of the liver architecture with multiple focal areas of necrosis in AZA-treated mice and other small hepatocytes with deeply stained acidophilic cytoplasm and dark nuclei were reported.^[57] Furthermore degenerated mitochondria, dilated cisterns of rough endoplasmic reticulum and multiple lipid droplets were noticed. A study reported that AZA-induced cell death (apoptosis), hydropic degeneration, portal fibrosis, and inflammation.^[58] It has been suggested that AZA induces hepatotoxicity and mitochondrial dysfunction owing to the stimulation of stress-activated protein kinase pathways and intracellular GSH reduction.^[59] On the other hand, the experimental evidence pointed to increased lipid peroxidation leading to the induction of a necrotic or apoptotic effect in hepatocytes.^[8] Furthermore, increase in ROS (as hydroxyl radical) could be involved in AZA toxicity.^[32]

In contrast, mean relative liver weights were decreased in the rats treated with CFME or FSME before AZA treatment. Furthermore, marked improvements in the histopathological changes were noticed, due to their antioxidant abilities. Polyphenols and flavonoids in chamomile flowers extracts and apigenin-7-O-glucoside as the major constituent of chamomile which inhibited cancer cell growth were recorded.^[60] Al-Musa and Al-Hashem^[49] reported that the administration of ethanolic extract of chamomile flowers to streptozotocin-induced diabetic rats significantly ameliorated the morphological changes in the livers of treated rats. They attributed these effects to its potent antioxidant potential resulting in membrane stability. The increase in the antioxidant enzyme activity and the reduction in the lipid peroxidation by fennel methanolic extract may result in reducing a number of deleterious effects due to the accumulation of oxygen radicals, and could exert a beneficial action against pathological alterations, especially in inflammatory diseases.^[19]

From the results of the current study, it can be concluded that oral administration of either CFME or FSME has a beneficial effect in modulating liver injury induced by AZA treatment probably through their potent antioxidative and radical scavenging activity and due to their higher content of total phenolic compounds. Both plants should be considered as accessible sources of natural hepatoprotective compounds.

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Nil.

Conflict of interest

There is no conflict of interest.

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Protective effects of *Amaranthus hybridus* against aflatoxin B₁ and fumonisin B₁-induced genotoxicity in H4IIE-*luc* cells

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ABSTRACT

Aim: Protective effects of aqueous extract of *Amaranthus hybridus* against aflatoxin B₁ (AFB₁) and/or fumonisin B₁ (FB₁) on the H4IIE-*luc* cell line were determined by use of the methyl thiazol tetrazolium viability assay and disruption of DNA integrity.

Methods: H4IIE-*luc* cells were incubated with different concentrations of AFB₁ and/or FB₁ for 24 and 48 h with or without aqueous extract of *A. hybridus*. **Results:** AFB₁ decreased the viability of cells after 24 and 48 h of exposure. EC₅₀ values for AFB₁ were 10.5 and 1.8 µmol/L for the two periods, respectively. When the 48 h exposure to mycotoxin repeated with a pre-treatment of 20 and 40 µg/mL extract of *A. hybridus*, the EC₅₀ changed to 3.88 and 7.67 µmol/L, respectively. H4IIE-*luc* cells exposed to FB₁ for 24 h responded more than those incubated for 48 h. Cells treated with a combination of AFB₁ and FB₁ were less viable with a significant decrease in the greater concentration. The mixture of AFB₁ and FB₁ resulted in a significant threat to H4IIE-*luc* as indicated by the absence or appearance of new bands in random amplified polymorphic DNA analysis, which demonstrated damage to DNA. The protective effects were probably due to greater content of total phenolics, carotenoids, β-carotene, folic-, linolenic-, linoleic and palmitic acids, as well as calcium, magnesium, iron, zinc, and selenium observed in the extract. **Conclusion:** Exposure to 40 µg/mL of extract of *A. hybridus* protected cells from damage to DNA by stabilizing DNA.

Key words: Aflatoxin B₁; *Amaranthus hybridus*; cytotoxicity; DNA; fumonisin B₁; hepatoma cells; methyl thiazol tetrazolium assay

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INTRODUCTION

Mycotoxins are secondary metabolites of fungi which are associated with certain disorders in animals and humans. Contamination of grains by mycotoxins is a worldwide problem affecting staple crops such as corn (maize) and small grains (such as wheat), as well as tree nuts, peanuts, sorghum, and others.^[1] Some mycotoxins are now linked with the incidence of certain types of cancer, and it is this aspect that has evoked global concern over feed and food safety.^[2] Aflatoxins (AFs), ochratoxins, trichothecenes, zearalenone, fumonisins (FBs), tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance^[3] and are known to be hepatotoxic, genotoxic, immunosuppressive, nephrotoxic, teratogenic, and carcinogenic.^[4] The Food and Agricultural Organization of the United Nations has estimated that up to 25% of the world's food crops are significantly contaminated with mycotoxins.^[5] However, in Africa, the presence of mycotoxins in food is often overlooked due to the population's ignorance, lack of regulatory mechanisms, poor facilities for storing large volumes of food products, and the introduction of contaminated commodities into the human food chain during chronic food shortage caused by droughts, wars, and political and economic instability.^[6]

At present, the interactions between AFB₁ and FB₁ with regard to their toxic and carcinogenic properties were discussed in several reports. A synergistic effect between exposure to mycotoxins and some important diseases in Africa, such as malaria, kwashiorkor, liver cancer, and human immunodeficiency virus (HIV)/acquired immune deficiency syndrome has been suggested.^[7] Concerns about mycotoxins have increased during the last few decades because of their implications to human and animal health and productivity, as well as the economics of their management, and how they influence international trade.^[8] This has led to the development of maximum tolerated limits for mycotoxins in various countries. The European Union has legislated maximum permitted levels of 2 ng/g dry mass for AFB₁ and 4 ng/g for total AFs (B₁, B₂, G₁, and G₂) in various products.^[9] Considering the extremely potent carcinogenicity of AFs, most developed nations regulate limits of AFs as small as reasonably achievable. Several studies have shown that AFB₁ and FB₁ are cytotoxic, inhibiting the viability of different cellular models, mostly liver and kidney cells.^[10-16]

The *Amaranthus* plant has been used extensively in the rural South Africa as a traditional food and is commonly known as *morogo*. *Amaranthus* species are good sources of β -carotene, polyphenols, Vitamin C, calcium, and iron.^[17,18] Moreover, a joint publication of the United Nations Development Program and the Food and Agriculture Organization,

expressed the view that wild-growing food plants are an affordable and practical source of nutrition to improve the nutritional status of rural HIV-affected households.^[19] The present study was carried out to assess whether the aqueous extracts of *A. hybridus* can protect rat hepatoma cells against FB₁ and AFB₁ induced cytotoxicity and disruption of DNA integrity.

METHODS

Chemicals

FB₁ and AFB₁ (98% purity) and other standards were purchased from Sigma Chemicals Co (St. Louis, MO, USA). The DNA extraction kit (DNeasy blood and tissue kit) was obtained from Qiagen (Hilden, Germany). The one hundred base pair (bp) DNA ladder, polymerase chain reaction (PCR) master mix, and DNase/RNase free water were obtained from Fermentas Inc., (Glen Burnie, Maryland, USA). Supertherm *Taq* polymerase was purchased from JM Holding (UK). Forty primers were obtained from Operon Technologies (Alameda, CA, USA). All solvents were of analytical grade and were purchased from Burdick and Jackson (Muskegon, MI, USA).

Plant materials

Stems and leaves of *A. hybridus* were collected from a residential garden in the city of Potchefstroom, North-West Province, South Africa. The plant material was freeze-dried and pulverized and 1 g dry mass of the lyophilized plant powder was infused with 10 mL water for 24 h at room temperature. After centrifugation, the supernatant was freeze-dried and stored at 4 °C until used.

Determination of chemical composition of the extract

Extraction of total phenolic contents in the plant was carried out in triplicate, according to the modified method of van der Walt *et al.*^[18] and Kähkönen *et al.*^[20] Total phenolic content was expressed as mean \pm standard deviation (SD) and gallic acid equivalents were expressed in mg/100 g dm.

Total carotenoid content for the plant was extracted and analyzed in triplicate as described by Edwards *et al.*^[21] and modified by van der Walt *et al.*^[18] Total carotenoid content is presented as mean \pm SD in mg/100 g dm.

Quantification of beta-carotene

Beta-carotene was extracted according to the modified method described by Lakshminarayana *et al.*^[22] and was quantified by high-pressure liquid chromatography coupled to a photodiode array detector capable of ultraviolet-visible absorption spectrum according to the methods of de Ancos *et al.*^[23] The content was expressed as mean \pm SD in mg/100 g dm.

Folic acid

Folic acid was quantified at the South African Bureau of Standards (Pretoria, South Africa). A standard method for the microbiological assay of folic acid in foods and pharmaceutical products was followed according to Barton-Wright^[24] and AOAC.^[25]

Quantification of fatty acids

Fatty acids were identified and quantified by use of gas chromatography coupled with mass spectrometry system with split-less injection. An Agilent 6890 gas chromatograph ported to a 5973 mass selective detector (CA, USA) was used according to the method described by van der Walt *et al.*^[17]

Mineral and trace element analysis

Minerals and trace elements were quantified by use of an Agilent 7500c inductively coupled argon plasma mass spectrometer as described by van der Walt *et al.*^[18] Three separate samples were analyzed and values were reported as the mean \pm SD in mg/100 g dm.

Cytotoxicity measurements

The mammalian model was rat hepatoma cells (H4IIE-*luc*) that had been transfected stably with a firefly luciferase reporter gene under control of the dioxin response element and thus the aryl hydrocarbon receptor mechanism.^[26] These cells had originally been developed as a reporter gene assay to detect and semi-quantify the levels of certain groups of persistent organic pollutants.^[26] Since these cells are essentially still mammalian cells, they were useful to assess whether extracts of selected *A. hybridus* can be protective against AFB₁ and FB₁ or their mixture.

Cells were seeded with a density of 1.0×10^4 cells/mL media in the inner 60 wells of a 96-well microplate. Growth medium was Dulbecco's modified Eagle's medium (Sigma, D2902) supplemented with 0.044 mol/L NaHCO₃ and 10% fetal bovine serum (Gibco). The volume in each well was 250 μ L. The outer cells received 250 μ L Dulbecco's phosphate buffered saline (PBS) to create a homogenous microclimate across all wells containing cells and incubation conditions were 37 °C in a humidified 5% CO₂:air mixture. The plates were seeded and after an initial 24 h incubation medium was replaced with medium containing varying concentrations of AFB₁ (50, 25, 2.5, 0.25, 0.025 μ mol/L) and FB₁ (200, 100, 10, 1, 0.1 μ mol/L) dissolved in methanol. A combination of the already mentioned concentrations of AFB₁ and FB₁ were also tested: 50 μ mol/L AFB₁ plus 200 μ mol/L FB₁; 25 μ mol/L AFB₁ plus 100 μ mol/L FB₁; and so on. Two exposure periods 24 h and 48 h were investigated. In order to evaluate the protective effect of extracts of *A. hybridus*, this experiment was repeated with the following adjustments: After the initial 24 h incubation

period, the media was replaced with media containing either 20 or 40 μ g/mL *A. hybridus* and incubated for another 24 h which was followed by the mycotoxin exposure routine, but for only the 48 h period. The controls included (1) 11 wells with cells and nutrient medium only for the duration of the entire experiment (when media was replaced, their media was replaced with fresh nutrient medium) and (2) 6 wells with cells and plant extract containing media only. The mycotoxin exposures were dosed in triplicate.

The viability of H4IIE-*luc* cells was determined using the methyl thiazol tetrazolium (MTT) salt assay in which the mitochondria of live cells metabolize the yellow MTT into blue formazan.^[27] A final concentration of 500 μ g/mL MTT was incubated for 30 min and blue formazan crystals dissolved with dimethyl sulfoxide. The absorbance was measured spectrophotometrically at 560 nm. The amount of formazan gives an estimation of the proportion of viable cells. The percentage of viable to dead cells was calculated by comparison with a control (untreated and solvent control). The MTT assay assessed the viability of H4IIE-*luc* cells that were subjected to the two *A. hybridus* extract concentration treatments compared to the viability of cells that were not treated with *A. hybridus* extracts prior to mycotoxin exposure.

DNA extraction

Cells were harvested by first washing away non-adherent dead cells with PBS before trypsinizing (0.25% trypsin, 0.1% versene ethylenediaminetetraacetic acid) adherent cells. Enzyme activity was stopped by the addition of media. The cell suspension was centrifuged for 5 min (300 g) at room temperature. The genomic DNA was extracted from the cells, according to the Qiagen instruction manual. The concentration of DNA was determined by photometry (NanoDrop ND-1000 Spectrophotometer) and the purity of the DNA was judged by examining the ratio of absorbency at 260/280 nm.^[28]

Random amplified polymorphic DNA-polymerase chain reaction analysis

Amplification of DNA fragments was carried out on an ICycler (Bio-Rad, UK) thermal cycler using 20 primers purchased from the Operon Biotechnologies (BioCampus Cologne Nattermannalle, Germany). PCR amplification was conducted in a 25 μ L reaction volume containing 10 ng genomic DNA, 12.5 pmol Master mix (2X) (Fermentas Life Science, USA), 1.0 units of Supertherm *Taq* polymerase, and 50 pmol primer. PCR reactions were carried out in a thermocycler (Bio-Rad C1000) programmed with initial denaturation period for 5 min at 95 °C, followed by 40 cycles denaturation (95 °C for 30 s), primary annealing at 37 °C for 1 min and extension at 72 °C. Amplification was terminated by a final extension period of

72 °C for 5 min. Reaction products were stored at -80 °C prior to electrophoresis.

Gel electrophoresis

Amplified products together with a marker (100 bp DNA) were resolved by gel electrophoresis 60 V/cm for 135 min on 2% agarose gel in TAE buffer containing 0.001 mg/mL ethidium bromide. Gels were photographed by a Gel Documentation system (Gensnap) equipped with its software (Synegen, UK).

Band analysis

Gels of control and exposed DNA samples were run for each of the 20 primers [Table 1]. A DNA ladder of 100 bps was also run in each gel. Bands in PCR products were analyzed by TotalLab Quant (V11.5: TL100-LX59-7YF4-EX). The fluorimetric profiles of each amplification reaction were studied both qualitatively and quantitatively by comparing profiles from the control and DNA exposed to the extracts. Each change observed in random amplified polymorphic DNA (RAPD) profiles of the treated groups (disappearances and appearance of bands in comparison to the control RAPD profiles) was given the arbitrary score of +1. The average was then calculated for each experimental group exposed to the mycotoxins for varying time periods. Genomic stability (%) was calculated as “100 - (100 a/n)” where “a” is the average number of changes in DNA profiles and “n” is the number of bands selected in control DNA profiles.^[29]

Statistical analysis

Values for EC₅₀ and cell viability were statistically analyzed with the Graphpad Prism 4.02 Inc., (La Jolla, CA, USA). Significance of differences among treatment groups was determined with the Waller-Duncan k-ratio.^[30] All statements of significance were based on a probability of $P < 0.05$.

RESULTS

The extract was rich in polyphenols (total phenolic contents: 2181.2 mg/100 g dm, total carotenoids (113.6 mg/100 g dm) and β -carotene (18.4 g/100 g dm) [Figure 1a]. The results of the lipid profile showed significant amounts of the fatty acids, linolenic, linoleic and palmitic acids [Figure 1b]. The extract had moderate concentrations of palmitoleic, stearic, and lignoceric acids whereas behenic, arachidic, and myristic acids were found in low concentrations. The extract was rich in folic acid (72 mg/100 g dm), calcium, magnesium [Figure 1c], iron, zinc, and selenium [Figure 1d].

Cytotoxicity of AFB₁, FB₁, and mixture with or without the extract of *A. hybridus* on H4IIE-*luc* cell line as measured by the tetrazolium dye-based MTT assay are shown in Figure 2a-d. There was a significant difference in viability between cells treated with 20 μ g/mL *A. hybridus* and those not treated before

exposure to AFB₁ for 48 h [Table 2]. Other combinations did not have any statistically significant difference. Percentage inhibition of the cells incubated for 24 h with FB₁ showed more cytotoxicity than those incubated for 48 h. On the other hand, FB₁ at the concentration of 200 μ mol/L decreased cell viability to 41.6% [Figure 2b]. The protective effect of 20 μ g/mL *A. hybridus* extract was decreased by increasing FB₁ dose to 100 μ mol/L [Figure 2b]. *A. hybridus* extract at 40 μ g/mL was more efficient at protection against all concentrations of FB₁.

Overall, AFB₁ was more cytotoxic than FB₁ for both exposure periods [Figure 2a and b]. Exposure of H4IIE-*luc* cells to AFB₁ led to a dose-and time-dependent decrease in cell viability. At 25 μ mol/L AFB₁, viability was inhibited to 58.7% and 96.1% for the 24 and 48 h exposure periods respectively. Pre-treating the cells to 40 μ g/mL *A. hybridus* had a more protective effect than pre-treatment of 20 μ g/mL [Figure 2a].

The combination of AFB₁ and FB₁ was more cytotoxic than AFB₁ alone which indicated that FB₁ increased the cytotoxicity. This was true for both exposure periods [Figure 2a-c]. However, this general trend was not corroborated by the EC₅₀ values (EC₅₀ = concentration by which viability was declined to 50%) [Table 3]. They were in fact slightly greater for the combined mycotoxins than exposure to AFB₁ alone, meaning that 50% effect was reached at a greater mycotoxin concentration. Extract of *A. hybridus* alone (20-100 μ g/mL) had no significant influence on the viability of cells [Figure 2d].

Table 1: Sequences of the primers used to amplify cell line of hepatoma (H4IIE-*luc*) cells

Primer	Sequence 5'-3'	Primer	Sequence 5'-3'
D01	ACCGCGAAGG	D11	AGCGCCATTG
D02	GGACCCAACC	D12	CACCGTATCC
D03	GTCGCCGTCA	D13	GGGGTGACGA
D04	TCTGGTGAGG	D14	CTTCCCAAG
D05	TGAGCGGACA	D15	CATCCGTGCT
D06	ACCTGAACGG	D16	AGGGCGTAAG
D07	TTGGCACGGG	D17	TTTCCACGG
D08	GTGTGCCCCA	D18	GAGAGCCAAC
D09	CTCTGGAGAC	D19	CTGGGGACTT
D10	GGTCTACACC	D20	ACCCGGTCAC

Table 2: Summary of the P values of the Wilcoxon matched pair tests to compare viability of cells exposed to FB₁, AFB₁, and their mixture and those treated with *A. hybridus* extracts prior to 48 h mycotoxin exposure

Mycotoxins	<i>A. hybridus</i> extract	
	20 μ g/mL	40 μ g/mL
FB ₁	0.04*	0.69
AFB ₁	0.5	0.08
FB ₁ + AFB ₁	0.69	0.2

* $P < 0.05$. FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; *A. hybridus*: *Amaranthus hybridus*

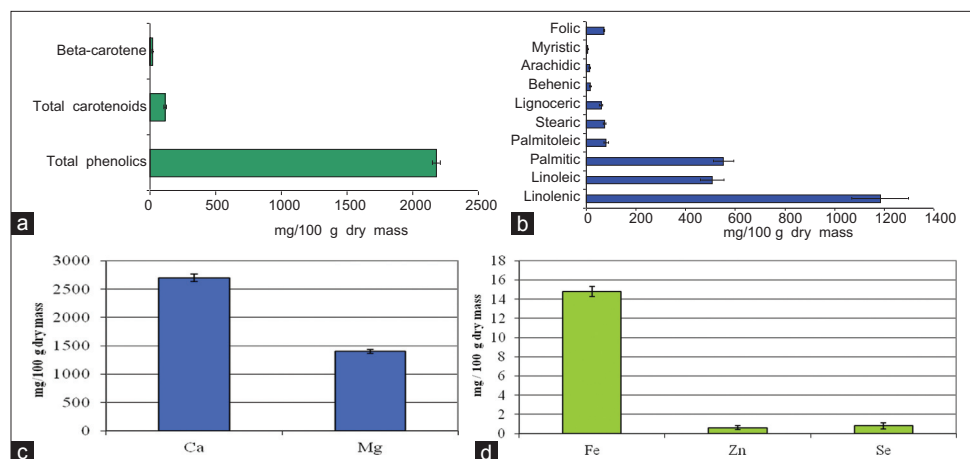


Figure 1: (a) Total phenolics, total carotenoids, and β -carotene content; (b) fatty acid profiles and folic acid content; (c) calcium and magnesium concentration; and (d) trace elements (iron, zinc, and selenium) concentration of *Amaranthus hybridus* extract

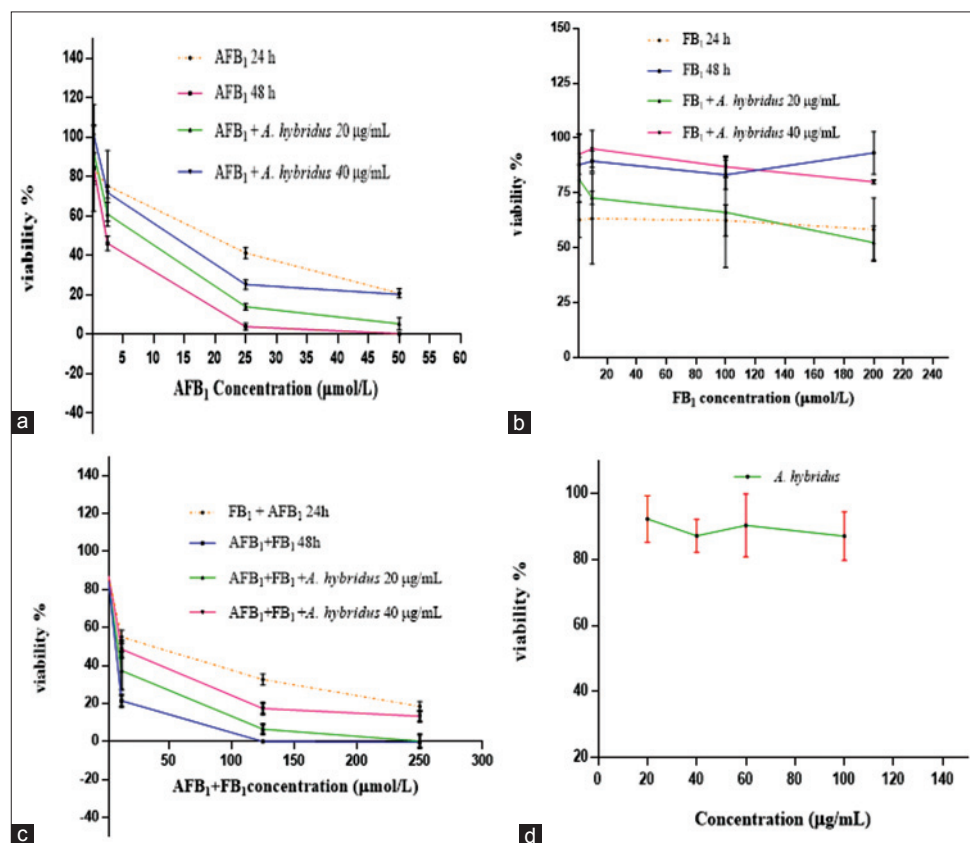


Figure 2: The cytotoxic effects of AFB₁ at different concentrations (μ mol/L) without/with *Amaranthus hybridus* extract: (a) FB₁ at different concentrations (μ mol/L) without/with *Amaranthus hybridus*; (b) AFB₁ plus with FB₁ without/with *Amaranthus hybridus*; (c) *Amaranthus hybridus* extract only; and (d) on proliferation of H4IIE-luc cell line determined by MTT bioassay. FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; MTT: methyl thiazol tetrazolium

EC₅₀ values for AFB₁ were 10.5 and 1.8 μ mol/L after 24 and 48 h of exposure, respectively. When the plant extract was added at 20 and 40 μ g/mL, the EC₅₀ values were 3.88 and 7.67 μ mol/L after 48 h of exposure, respectively. On the other hand, the EC₅₀ for the combined mycotoxins (AFB₁ + FB₁) was 24.02 and 5.86 μ mol/L after 24 and 48 h, respectively. While the 50% inhibition of proliferation for AFB₁ plus FB₁ with plant extract were 7.30 and 14.0 μ mol/L after the

addition of 20 or 40 μ g/mL *A. hybridus* extract, respectively. No discernible cytotoxicity was observed in cells, based on the MTT assay, when cells were exposed to FB₁ at lesser concentrations. Whereas, at greater concentrations sufficient cell mortality was exhibited in the MTT assay that indicates cytotoxicity at dosages of 200 μ mol/L but the quantities tested were still substantially less than those required to obtain EC₅₀.

Genetic variability among treated cells was evaluated using 10 oligonucleotide primers. Only five primers, D07, D09, D13, D15, and D16, gave positive and detectable bands [Figure 3]

Table 3: The EC_{50} -values of AFB₁ and/or FB₁ alone or in combination with *A. hybridus* at two exposure periods using the H4IIE-*luc* cell line

Mycotoxin/ plant extract	Pre-treatment concentration of <i>A. hybridus</i> (μg/mL)	Mycotoxin exposure time (h)	EC_{50} (μmol/L)
FB ₁	-	24	ND
	-	48	ND
	20	48	ND
	40	48	ND
AFB ₁	-	24	10.55
	-	48	1.84
	20	48	3.88
	40	48	7.67
FB ₁ + AFB ₁	-	24	24.02
	-	48	5.86
	20	48	7.30
	40	48	14.00

A. hybridus 5592 μg/mL

FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; ND: not detectable; *A. hybridus*: *Amaranthus hybridus*

since they amplified a total of 69 different bands ranging from 144 to 2000 bp. All 69 bands were “polymorphic” given 100% polymorphism for control cells, FB₁, AFB₁, AFB₁ plus FB₁, plant extract at 40 μg/mL, 40 μg/mL plant extract plus FB₁, 40 μg/mL extract plus AFB₁ and 40 μg/mL extract plus 0.025 and 50 μmol/L AFB₁, respectively, for all primers used. Of the 69 scorable bands, 18 (26%) were similar “monomorphic” to the control and the 40 μg/mL *A. hybridus* treatment (D09-700, D09-525, D09-363, D13-363, D15-1080, D15-869, D15-646, D15-547, D15-447, D15-325, D15-229, D15-176, D16-183, D16-1267, D16-813, D16-679, D16-536, and D16-417; 1 band (1.4%) was similar for control and *A. hybridus* extract at 40 μg/mL in all treatments after the addition of the extract at 40 μg/mL for all treatments (D16-646).

Quantitative analysis of these bands, expressed as a percentage of band loss, shows a time-dependent relationship. The increase in band loss is related to the increase in time period [Table 3 and Figure 4]. Similarly, in case of band loss at the short exposure period (24 h), 26 out of 66 bands (39.4%) disappeared [Figure 4a]. At the 48 h exposure period, 44 out of 65 bands vanished which representing 75.4% [Figure 4b].

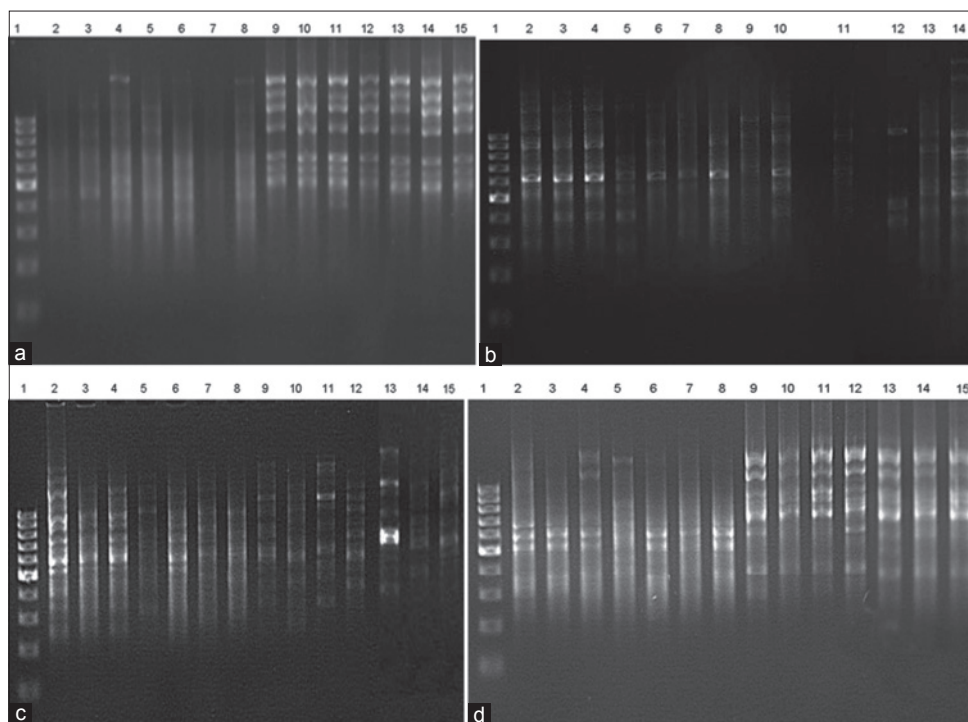


Figure 3: (a-d) RAPD profiles of genomic DNA from cell line of hepatoma (H4IIE-*luc*) of rats following exposure to FB₁ and/or AFB₁ for various time periods. Four pictures represents PCR products with primer OPD07 (a), OPD09 (b), OPD15 (c), and OPD16 (d), respectively, at less and greater concentration. The DNA marker (100 bp) in lanes 1 and 2 represents cells only; lane 3 represents cells plus FB₁ (0.1 μmol/L); lane 4 represents cells plus FB₁ (200 μmol/L); lane 5 represents cells plus AFB₁ (0.025 μmol/L); lane 6 represents cells plus AFB₁ (50 μmol/L); lane 7 represents cells plus the mixture of FB₁ and AFB₁ (0.1 + 0.025 μmol/L); lane 8 represents cells plus the mixture of FB₁ and AFB₁ (200 + 50 μmol/L); lane 9 represents cells plus *Amaranthus hybridus* extract (40 μg/mL); lane 10 represents *A. hybridus* extract (40 μg/mL) plus FB₁ (0.1 μmol/L); lane 11 represents *Amaranthus hybridus* extract (40 μg/mL) plus FB₁ (200 μmol/L); lane 12 represents *Amaranthus hybridus* extract (40 μg/mL) plus AFB₁ (0.025 μmol/L); lane 13 represents *Amaranthus hybridus* extract (40 μg/mL) plus AFB₁ (50 μmol/L); lane 14 represents *Amaranthus hybridus* extract (40 μg/mL) plus the mixture of FB₁ and AFB₁ (0.1 + 0.025 μmol/L), and lane 15 represents *Amaranthus hybridus* extract (40 μg/mL) plus the mixture of FB₁ and AFB₁ (200 + 50 μmol/L). FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

A protective effect of the extract at 40 µg/mL was observed for the short exposure period and 14 out of 69 bands (20.29%) disappeared, as compared to the 28 out of 69 bands (40.58%) for the longer period which disappeared.

Meanwhile, bands also appeared at the short exposure period, 43 new bands out of 66 bands were amplified which represents 65.2%. In the same trend, at the longer exposure period, 31 out of 65 appeared which represents 47.7% [Figure 4c]. The protective effect of the 40 µg/mL plant extract was observed as the production of new bands appeared at the short exposure period (24 h) since 22 out of 66 bands emerged which represents 33.3% while at the long exposure period, 6 out of 66 bands occurred and represented 9.09% [Figure 4d].

Profiles of RAPD-PCR and the number of bands that appeared or disappeared in the DNA of hepatoma H4IIE-*luc* at various exposure periods are shown in Table 4. A maximum of 9 bands vanished in the mixture of FB₁ at 200 µmol/L and AFB₁ at 50 µmol/L plus the plant extract-treated cells for 48 h with OPD 15 primer. Whereas, the maximum appearance of 7 new bands were observed in the AFB₁ at 50 µmol/L plus plant extract-exposed cells at 48 h with OPD 15 too.

The percentage of DNA template stability in the treated cells in comparison to the controls at various concentrations is presented in Figure 5. The results showed that there was a significant difference in the DNA template stability between the control and all the treated groups, no significant difference was observed in the DNA template stability between the

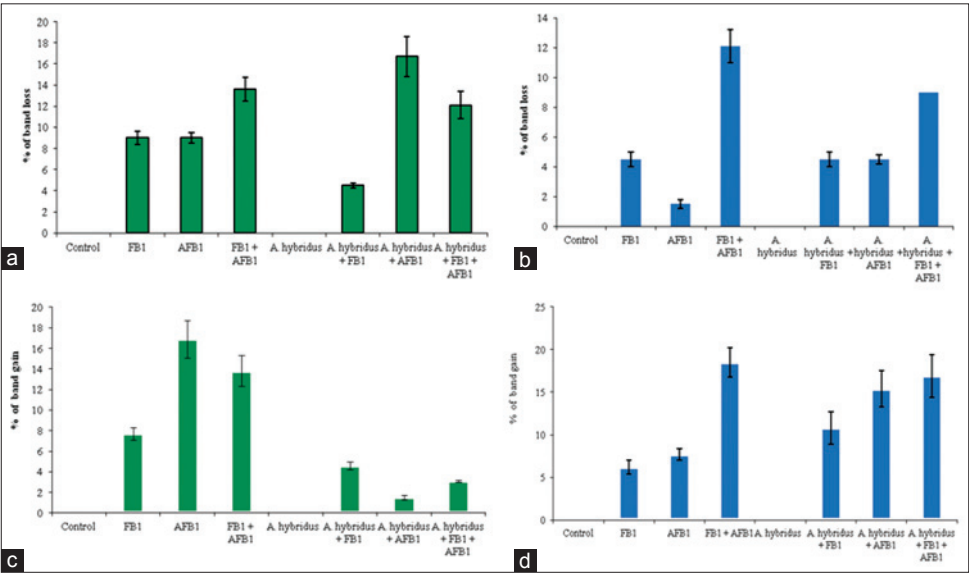


Figure 4: Genomic damage. The percentage of altered bands in each treatment of low and high concentration of FB₁ and AFB₁ detected by RAPD-PCR. (a) Average band loss after 24 h; (b) average band loss after 48 h; (c) average band gains after 24 h; and (d) average band gain after 48 h. FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

Table 4: Frequency of appearance and disappearance of bands in the RAPD profiles of genomic DNA from cell line of hepatoma (H4IIE-*luc*) of rats following exposure to FB₁ and/or AFB₁ for various time periods

Name of primer	Change in RAPD profile	Control	FB ₁ (LC)	FB ₁ (HC)	AFB ₁ (LC)	AFB ₁ (HC)	AE (LC)	AE + FB ₁ (LC)	AE + FB ₁ (HC)	AE + AFB ₁ (LC)	AE + AFB ₁ (HC)	AE + FB ₁ + AFB ₁ (LC)	AE + FB ₁ + AFB ₁ (HC)
D7	A	0	3	5	4	4	0	6	0	2	1	0	1
	D	0	0	0	0	0	0	0	1	0	0	0	0
D9	A	0	1	0	0	0	0	0	0	0	1	5	0
	D	0	0	0	1	3	3	2	0	6	3	0	2
D13	A	0	0	0	0	3	3	0	1	0	3	0	2
	D	0	0	3	0	0	0	2	0	0	0	2	0
D15	A	0	0	0	1	4	6	3	0	1	7	0	0
	D	0	3	1	0	0	0	0	0	0	5	6	9
D16	A	0	2	0	1	0	0	0	0	0	0	0	1
	D	0	0	0	0	2	5	8	2	1	2	3	0

RAPD: random amplified polymorphic DNA; FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; AE: *Amaranthus* extract (40 µg/mL); LC: low concentration (1 µmol/L for FB₁ and 0.25 µmol/L for AFB₁); HC: high concentration (200 µmol/L for FB₁ and 50 µmol/L for AFB₁); A: appeared; D: disappeared

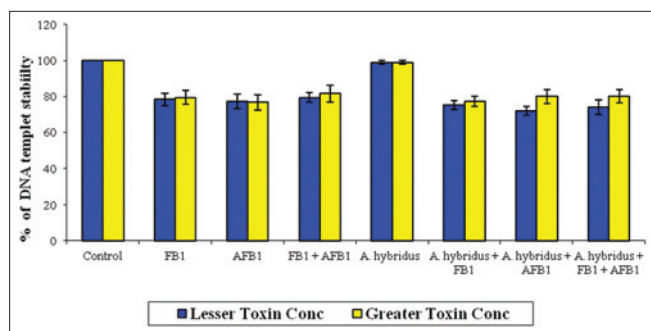


Figure 5: Template DNA stability (%) in cell line of hepatoma (H4IIE-*luc*) of rats following exposure to FB₁ and/or AFB₁ for various time periods evaluated in RAPD-PCR. FB₁: fumonisin B₁; AFB₁: aflatoxin B₁; RAPD: random amplification of polymorphic DNA; PCR: polymerase chain reaction

control and cells treated with 40 µg/mL *A. hybridus* extract. The protective effect of the extract at 40 µg/mL appeared as marked in the stability of DNA in all treatments.

DISCUSSION

Polyphenols are a class of phytochemicals that contribute to the total antioxidant capacity of dark green leafy vegetables.^[31] They have aromatic rings and achieve their antioxidant activities mainly through the donation of hydrogens.^[32] In the current study, *A. hybridus* extract was found to be enriched in phenolic compounds in amounts comparable to those of conventional and commercially-grown non-conventional vegetables.^[17] Total phenolic concentrations reported herein were similar to those reported in commercial spinach.^[33] Total phenolic concentrations in leaves of commercially-produced *Ipomoea batata*, which is also eaten as *morogo* in South Africa were similar.^[34]

Carotenoids are pigment molecules responsible for the color of many fruits and vegetables, have important functions in photosynthesis and are abundant in plant leaves. Carotenoid and beta-carotene concentrations reported in the current study were comparable with that of baby spinach reported previously.^[35] Bioavailability of carotenoids in dark green leafy vegetables is reduced by the leaf matrix.^[36] Notwithstanding this limitation, and distinct from being Vitamin A precursors, carotenoids also exhibit considerable antioxidant capacity based on their symmetrical linear 40-carbon tetraterpene structure, which features alternating double and single carbon-carbon bonds.^[23,34] Folic acid concentration in *A. hybridus* reported in the current study was similar to that reported previously in African vegetables.^[118] In the present study, six saturated fatty acids, one monounsaturated fatty acid, and two poly-saturated fatty acids were isolated. These results were in accordance with those reported by Weather^[37] who suggested that dark green leafy vegetables generally

contained small amounts of fat predominantly in the form of polyunsaturated fatty acids.

Calcium (Ca²⁺) plays a vital role in regulating cellular transmembrane trafficking of elements and molecules.^[38] Dark-green leafy vegetables, therefore, are primary sources of minerals and trace elements.^[39] In the present study, the extract was enriched in calcium and magnesium, and trace elements iron, zinc, and selenium. Mineral and trace element content of plant leaves is a function of the environment and in leafy vegetables would be strongly influenced by the chemical composition of the soil and the climate.^[40,41] The current results were similar to those reported previously,^[42] which suggests that wild *morogo* should be considered an important source of calcium, magnesium, iron, and zinc, particularly for households that are not in a position to access conventional vegetables, whether for economic or demographic reasons.

Exposure of H4IIE-*luc* cells to AFB₁ resulted in the death of cells in a concentration and time-dependent manner. H4IIE-*luc* cells were more sensitive to AFB₁, and AFB₁ plus FB₁ mixture as compared to the control and FB₁ alone. The results showed that treatment with the plant extract 24 h prior to mycotoxin exposure succeeded to blocks the AFB₁ toxicity in H4IIE-*luc* cells line. This may be associated with the content of vitamins, antioxidants and minerals in the plant extract. This result suggested that natural vitamins, provitamins, carotenoids, chlorophyll, phenolics, and synthetic compounds with antioxidant properties could potentially be effective against the toxic consequence of these mycotoxins.^[43]

Toxic effects of FB₁ were more pronounced after 24 h than 48 h exposure. However, the cytotoxic effect of FB₁ was eliminated at lesser concentrations, suggesting the rapid metabolism of this mycotoxin.^[44] These results were similar to those observed during an *in vivo* study that proved the elevation of sphinganine was reversible after short-term exposure.^[45] Disruption of sphingolipid metabolism as a specific cytotoxic response to FB₁ exposure and sphingosine reached its maximum concentration after 48 h.^[46,47] Several reports indicate that FB₁ inhibited cell proliferation in different cell lines H4TG, MDCK, NIH3T3, and LLC-PK1.^[47,48] Among 15 mammalian cell lines, MDCK and H4TG were found to be the most sensitive to FB₁ with EC₅₀-values of 2.5 and 4 µg/mL, respectively, after 4 days exposure.^[49]

AFB₁ is a well-known genotoxicant able to alter the genetic constitution of an organism by inducing insults of various types. Changes in profiles were observed between control and all mycotoxin treatments. Differences in profiles of bands between the control and treated samples might be due to AFB₁ and/or FB₁-induced point mutations and/or base

modifications elicited in the genome.^[50] All primers used in this study could detect changes in all treatments that might be due to a latent phase required for the appearance of adequate number of cells with genetic damage.

Alterations observed in the present study included the absence and/or presence of bands in all treatment groups. The appearance and disappearance of bands might be associated with genetic rearrangements or clastogenic effects of the toxicant. Such alterations in the genome might subsequently interfere with binding of primers or amplification step.^[51] Increases in band intensity and appearance of new PCR products have been attributed to conformational changes in DNA,^[52] which might improve the access of primer(s) to the binding site(s). Furthermore, enhancement and reduction of signal intensity of an amplified DNA fragment might be related to localized over- or under-amplification of that gene locus in the genome, which could result from changes at the chromosome level.

Instability in template DNA was observed in all treatments which may be due to DNA damage. Although RAPD appears to be instrumental in observing definitive changes, it requires enough time and sufficient theoretical knowledge for initial standardization to obtain reproducible and unambiguous results. Interpretation of molecular events responsible for differences observed in the RAPD pattern is not easy since different DNA alterations may induce similar types of changes. The RAPD is known to produce non-reproducible bands, but once established and standardized, there are certain additional benefits to using this method for early genotoxicity studies other than being fast.

Differences in sensitivity were observed, depending on the primer sequence. This observation suggests the mode of action of FB₁ and/or AFB₁. The five primers used showed a greater alteration after the treatment and the appearance of new bands in all the extracts-treated groups were produced from those primers. The mechanism by which these toxins affect the sequence of DNA has been extensively supported in the literature.^[53] Some of the AFB₁ adducts have been shown to be capable of inducing base substitution, frameshifts, insertions and deletions at specific loci of the DNA. For example, AFB₁ adduct induces G > T transversion at specific loci within p53.^[54,55] The resulting alterations in DNA can induce changes in the DNA sequence at specific places generating different annealing primer-template sites.^[56] This is probably the reason why altered bands were always the same in most of the concentrations and the exposure periods in both the qualitative and the quantitative analysis.

Generation of new annealing primer-template sites would be in accordance with the presence of new bands in the

amplification profiles. The nature of the RAPD reaction, where the final products are the result of an exponential multiplication of the most abundant and stable fragments co-amplified in the first cycles is the cause of the differences in the concordance among replicates. In other words, it is necessary that new annealing sites appear in a high proportion of the cell population to get a high reproducibility. The first new bands appeared at the high concentration of FB₁ and/or AFB₁ (D-7₁₄₆₆, D-7₅₂₅) suggested that the proportion of cells with a new annealing primer-template was increased at the greater concentration. The RAPD assay is able to detect mutation only if they occur in at least 2% of the DNA.^[56] A concentration-dependent effect was observed when the same chemical and the same cells were used. Similar results were previously demonstrated a dose-dependent effect of the genotoxic action of mycotoxin when measured by micronuclei induction.^[57]

The combined use of *in vitro* systems and the RAPD technique permits detection of alterations in DNA caused by multiple mechanisms with a sufficient degree of sensitivity. Alterations were detected in an unspecific form by losses and/or gains of bands and variations in the amplification intensity. Nevertheless, when the objective is to establish the existence of DNA damage, that is, for hazard identification in risk assessment studies, the presence in the fingerprint of any of these abnormalities would be enough to identify a genotoxic effect. For example, the presence of one or both of the two new bands in DNA extracts of cells treated either with a chemical or with an environmental sample can be considered as a suitable genotoxicity biomarker of chronic exposure.

The protective effects of *A. hybridus* extract against FB₁ showed that the extract was more effective at its greater dose than at the lesser dose. In addition, the ability of the extract to eliminate the cytotoxic effects induced by AFB₁ appeared less effective compared with that induced by FB₁. The difference between AFB₁ and FB₁-induced cytotoxic effects may be due to the stronger oxidative stress caused by AFB₁ even at a lower concentration than FB₁. Several studies showed the benefits of antioxidant compounds in the diet against the toxicity of mycotoxins.^[58] The inhibition of DNA and protein synthesis induced by AFB₁ and FB₁ were decreased by pre-treatment of the CaCo-2 and Hep G2 cell lines with the antioxidant cyaniding-3-0-β-glucopyranoside.^[58] The ability of *A. hybridus* extract to inhibit the cytotoxic effects induced by the mixture of AFB₁ and FB₁ was more pronounced at the higher concentration of the extract (40 μg/mL) than the lower concentration (20 μg/mL). Moreover, this protective effect was smaller in the case of the mycotoxin mixture compared to that of FB₁ only. These results were similar to those reported by Guerra *et al.*^[59] who suggested that the inhibitory action of

cyaniding-3-O- β -glucopyranoside on AFB₁ and OTA-induced toxicity is likely to be attributed to its antioxidant power.

Previous studies showed that the aqueous extract of *A. hybridus* has a significant immune-stimulating effect and its stem extract has been credited with antimalarial activity and these effects are attributed to the presence of amaranthine, isoamaranthine, hydroxycinnamates, quercetin, kaempferol glycosides, amaranthoside, amaricin, and stigmasterol glycoside.^[60] These authors concluded that the hepatoprotective activity of *A. hybridus* might be due to antioxidant defence factors and phenolics might be the main constituents responsible for the activity. Isolated polyhydroxylated nerolidols which have antiradical and reducing capacities and could act as antilipoperoxidants.^[61]

In conclusion, *A. hybridus* extract has a high content of total phenolic, total carotenoids, β -carotene, folic acid, linolenic, linoleic, palmitic, calcium, magnesium, iron, zinc, and selenium. AFB₁ or FB₁ alone or in combination induced toxic effects on rat hepatoma cells. However, a mixture of the two mycotoxins was most potent. The H4IIE-*luc* cells showed a weak antagonistic effect when exposed to the mixture of the two mycotoxins as compared to the single toxin exposures. The viability of cells was decreased by increasing concentrations of mycotoxins. Moreover, the binary mycotoxin mixture posed a significant threat to the treated hepatoma cell line as indicated by the absence/appearance of new bands beside the severe DNA damage. Combined treatment with *A. hybridus* extract and mycotoxins resulted in significant improvement in cell viability accompanied with a significant decrease in DNA damage and genotoxic effects. This improvement was more pronounced in the individual toxin-treated cells and a dose dependent manner of the extract.

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Conflict of interest

There is no conflict of interest.

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Synergistic curative effect of chicory extract and cisplatin against thioacetamide-induced hepatocellular carcinoma

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ABSTRACT

Aim: Hepatocellular carcinoma (HCC) is the dominant form of primary liver cancer and is histologically and etiologically distinct from other forms of primary liver cancer. The objective of this study was to elucidate the synergistic effect and the role of chicory extract [inulin (IN)] as a chemo-sensitizer for cisplatin (CIS) treatment of HCC. **Methods:** Five groups of rats were treated for 4 months. These groups consisted of the control group, a group receiving thioacetamide (TAA) (200 mg/kg b.w) in drinking water, a group injected intraperitoneally with a single dose of CIS (7.5 mg/kg b.w) in addition to TAA for 4 months, a group receiving oral doses of IN (10 mg/kg b.w) in addition to TAA for 4 months, and a group injected intraperitoneally with a single dose of CIS (7.5 mg/kg b.w) and IN (10 mg/kg b.w) plus TAA for 4 months. **Results:** The current data exhibited increment of serum and liver enzyme (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, creatine kinase, and lactate dehydrogenase) activity, serum lipid profile levels (total lipids, total cholesterol, triglycerides, low-density lipoprotein, and very low-density lipoprotein), and a significant increase in α -fetoprotein and bilirubin, accompanied with reduced total serum protein and albumin levels in a HCC rat model. Histopathologically, numerous alterations were detected in hepatic tissues of HCC rats, such as lymphocytic cell infiltration, damage of hepatocytes, dilated congested central vein with degenerated endothelial cells, and congested blood sinusoids in addition to Masson's trichrome staining blue collagen fibers in hepatocytes and central vein indicating hepatic fibrosis. Treatment of HCC rats with CIS or IN improved such deleterious effects, where IN is more effective than CIS, and the best effect can be observed in rats that received both CIS and IN. **Conclusion:** It could be concluded that IN in chicory extract acts as a chemo-sensitizer to CIS for treatment in an HCC rat model.

Key words: Chicory; cisplatin; hepatocellular carcinoma; inulin

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INTRODUCTION

Cancer is one of the most anxiety-inducing diseases and it is presenting with further prolongation and increasing occurrence.^[1] The most common and recurrent primary malignancy of the liver is recognized as hepatocellular

carcinoma (HCC), a major malignancy worldwide and is increasingly associated with cancer-related death.^[2] HCC represents the most common primary malignancy of the liver. According to epidemiological surveys, the prevalence

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of HCC ranks sixth among all cancers. Under normal physiologic conditions, the liver ensures homeostasis of lipid and lipoprotein metabolism.^[1] Accordingly, hepatic cellular damage impairs liver function and can lead to alterations in lipid metabolism, and subsequently, may play a vital role in the development of HCC. Moreover, lipids are also involved in cellular signaling. In particular, lysophosphatidic acid is a powerful cellular signaling agent and acts as a potent mitogenic molecule.^[1] However, according to recent reports, the incidence of HCC has increased sharply in the last decade, especially in Egypt, where there has been a doubling of the incidence rate during the last 10 years. This sharp rise has been attributed to several factors including hepatitis B and C virus infections, endemic infections in the community as schistosomiasis, as well as environmental factors.^[3] Cisplatin (CIS) is extensively used as a chemotherapeutic agent for the treatment of HCC. A major problem with CIS treatment of HCC is the development of CIS chemoresistance.^[4] Revealing the underlying mechanism for the development of chemoresistance is indispensable for developing effective chemotherapeutic agents.^[4] Cellular proliferation, an important prerequisite for tumorigenesis, imparts metabolic challenges.^[5] The constant doubling of proteins and lipids requires uptake of nutrients in excess of that normally required. In addition to increasing nutrient uptake, proliferating cells also increase metabolic pathways to support biosynthesis.^[6] Chicory (*Cichorium intybus* L.) is one of the most promising novel candidates among carbohydrates with potential for both nutrient and non-nutrient utilization. It has been implemented in folk medicine from North Africa to South Asia for more than 100 years.^[7] Fresh chicory typically contains 68% inulin (IN), 14% sucrose, 5% cellulose, 6% protein, 4% ash, and 3% other compounds, while dried chicory contains approximately 98% IN and 2% other compounds.^[8] Chicory IN is a natural linear molecule of fructose with 9-(2-I) glycosidic linkages that are not digested in the upper part of the gastrointestinal tract but fermented in the cecocolon.^[9] IN, a naturally occurring fermentable chicory fructan, has been shown to stimulate the growth of bifidobacterium, which are regarded as beneficial strains in the colon and have been found to inhibit colon and liver carcinogenesis in laboratory animal models.^[10] IN is fermentable dietary fiber, resistant to hydrolysis by pancreatic amylase and saccharidases in the upper gastrointestinal tract. Previous studies have demonstrated that IN is produced enzymatically from sucrose and that supplementing IN to high-fat diets for 12 weeks reduced body weight and serum and hepatic levels of triacylglycerols in rats.^[11] IN in human nutrition is noted for its prebiotic effect, that is, the specific stimulation of growth and/or activity of a limited number of colonic bacteria beneficial to the host, as well as its inhibition in growth of pathogens and

harmful microorganisms.^[12] The combination of prebiotics and probiotics has given rise to so-called “synbiotics”, with promising healthy properties.^[13] Experimental data demonstrates that IN and oligofructose affect processes and parameters involved in lipid metabolism, thus producing a beneficial effect on diseases related to lipid disorders such as atherosclerosis.^[14]

In light of this, this study was carried out to elucidate the effective role of IN on HCC-linked adverse effects, and to evaluate its activity as a chemo-sensitizer for CIS treatment.

METHODS

Experimental animals

This study was performed on adult male Wistar rats; weighing 120-150 g. Rats were obtained from the Institute of Ophthalmic Disease Research (Cairo, Egypt). They were housed in stainless steel cages in an automatically illuminated and thermally controlled room (12 h light/dark cycle at 22-25 °C) at the Animal House, Faculty of Science, Mansoura University, Mansoura, Egypt. Rats were permitted an adequate standard diet (60% ground corn meal, 15% round beans, 10% bran, 10% corn oil, 3% casein, 1% mineral mixture, and 1% vitamin mixture) purchased from Meladco Feed Company (Aubor City, Cairo, Egypt), and given water *ad libitum* for an adaptation period of 1 week prior to the experimental work. This experimental study was approved by review board of Mansoura University.

Chemicals

Thioacetamide (TAA) and chicory extract (IN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). CIS was purchased from Tarshouby Pharmacy (Mansoura, Egypt).

Study design

After the acclimation period, rats were divided into five groups (6 rats each). The first group was a normal control group fed a normal diet without any treatment. The second group consisted of normal rats fed a normal diet while receiving TAA daily in drinking water at a dose of 200 mg/kg b.w for 4 months.^[15] The third group consisted of normal rats fed a normal diet receiving CIS (7.5 mg/kg b.w) as a single intraperitoneal dose in addition to TAA for 4 months.^[16] In the fourth group, rats orally received IN (10 mg/kg b.w) in addition to TAA for 4 months.^[17] The fifth group of rats were administered CIS (7.5 mg/kg b.w) as a single intraperitoneal dose and IN orally (10 mg/kg b.w) in addition to TAA for 4 months.

Blood and liver sampling

At the end of the experimental period, rats were sacrificed by cervical dislocation, and blood samples from each rat were collected into clean centrifuge tubes and centrifuged at

860 g for 20 min. The separated sera were frozen at -20 °C for further analysis. The rats were dissected and the livers were immediately excised, rinsed with ice-cold saline, blotted dry, and accurately weighed. They were then minced and homogenized in ice-cold buffered saline (10% w/v). The homogenates were centrifuged at 860 g for 10 min at 4 °C. Finally, the supernatants were subjected to biochemical analysis. Other samples of the liver tissue were stored in 10% neutral formalin for histopathological studies.

Biochemical analysis

Total lipid, total cholesterol (TC), triglycerides (TGs), and high-density lipoprotein cholesterol (HDL-C) levels were quantified using kits supplied by Spinreact S.A. (Sant Esteve de Bas, Spain).^[18-21] Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) levels were calculated according to the following equations, $LDL-C = TC - HDL-C - TG/5$ ^[22] and $VLDL-C = TG/5$.^[23]

Alpha-fetoprotein (AFP) levels in serum was estimated by the method described previously through kits purchased from the Diagnostic Products Company (Los Angeles, CA, USA).^[24] Total protein, albumin, and total bilirubin levels were quantified, using kits from Bio-Diagnostic Co., (Dokki, Giza, Egypt) as described previously.^[25-27] Aspartate transaminase (AST) and alanine transaminase (ALT) activity was quantified using kits supplied by Spinreact S.A. (Sant Esteve de Bas, Spain) according to Young,^[28] and Belfield and Goldberg,^[29] respectively. Alkaline phosphatase (ALP) activity was quantified using kits supplied by ABC (Cairo, Egypt) according to Belfield and Goldberg.^[29] Creatine kinase (CK) and lactate dehydrogenase (LDH) activity was determined using kits supplied by Pinreact S.A. (Sant Esteve de Bas, Spain) according to Tietz^[30] and Goldman *et al.*,^[31] respectively.

Histopathological studies

Liver specimens were carefully fixed in neutral formalin solution (10%), dehydrated in ascending grades of ethanol, cleared in xylene, embedded in a paraffin wax, sectioned at 5-7 µm, and stained with hematoxylin and eosin (HE). The stained sections were examined and photographed under a light microscope to detect its histopathological properties.^[32] In addition to the routine H and E stain, Masson's trichrome stains (a three-color staining protocol used in histology, where connective tissue is stained blue, nuclei are stained dark red/purple, and cytoplasm is stained red/pink) were employed for identification of collagen fibers, a good marker for various diseases such as fibrosis.^[33]

Statistical analysis

Results were expressed as a mean ± standard error of mean (SEM). Statistical significance was calculated using one-way analysis of variance followed by Duncan's multiple range test.^[34] All of the statistical analyses were carried out with the use of SPSS 12.00 software, SPSS (Hong Kong) Ltd, Quarry Bay, Hong Kong. Differences were considered significant at $P \leq 0.05$.

RESULTS

As shown in Table 1, the obtained data showed a significant increase in the lipid profile of serum (TL, TC, TG, LDL-C, and VLDL-C), AFP, and bilirubin, accompanied with significant decrease in total protein and albumin levels in HCC rats compared to the control group. In contrast, administration of IN or CIS to HCC rats showed a significant amelioration of the tested parameters, in which IN is more effective than CIS. Moreover, the results shown in Table 2, recorded a significant increase in serum enzymes (AST, ALT, ALP, LDH, and CK) activity accompanied with a significant decrease in liver enzyme (AST, ALT, and ALP) activity in HCC rats compared to control group. However, administration of IN or CIS to

Table 1: Serum biochemical parameters in control and treated rat groups

Parameters	Animal groups				
	Control	TAA	TAA + CIS	TAA + IN	TAA + IN + CIS
Total lipids (mg/dL)	452.52 ± 4.81 ^a	599.39 ± 6.86 ^b	550.39 ± 8.73 ^c	552.18 ± 8.54 ^c	504.37 ± 4.86 ^d
Cholesterol (mg/dL)	143.80 ± 1.63 ^a	203.50 ± 3.01 ^b	157.20 ± 2.55 ^c	168.50 ± 4.18 ^d	151.10 ± 1.74 ^a
Triglyceride (mg/dL)	121.25 ± 3.88 ^a	214.30 ± 5.49 ^b	148.42 ± 4.35 ^c	180.21 ± 1.98 ^d	130.33 ± 2.66 ^e
HDL-C (mg/dL)	48.50 ± 0.31 ^a	23.70 ± 1.86 ^b	29.90 ± 0.93 ^c	27.90 ± 0.74 ^c	39.50 ± 0.36 ^d
LDL-C (mg/dL)	71.05 ± 1.63 ^a	136.94 ± 3.01 ^b	97.62 ± 2.55 ^c	104.56 ± 4.18 ^d	85.54 ± 1.74 ^e
VLDL-C (mg/dL)	24.25 ± 0.08 ^a	42.86 ± 0.49 ^b	29.68 ± 0.35 ^c	36.04 ± 0.98 ^d	26.06 ± 0.16 ^a
AFP (pg/mL)	26.65 ± 1.30 ^a	59.46 ± 3.20 ^b	42.05 ± 1.07 ^c	36.58 ± 0.99 ^d	31.84 ± 0.65 ^e
Tp (mg/dL)	9.75 ± 0.42 ^a	3.95 ± 0.60 ^b	6.86 ± 0.43 ^c	7.15 ± 0.32 ^c	8.99 ± 0.17 ^a
Albumin (mg/dL)	5.28 ± 0.45 ^a	1.36 ± 0.13 ^b	2.70 ± 1.65 ^c	2.15 ± 0.09 ^c	3.81 ± 0.26 ^d
Bilirubin (mg/g)	1.30 ± 0.24 ^a	7.95 ± 0.92 ^b	3.90 ± 0.20 ^c	3.35 ± 0.20 ^c	2.71 ± 0.17 ^c

Results are expressed as a mean ± SEM, with each row. Values superscripts with different letters (a-e) express the significant change at $P \leq 0.05$. Values superscripts with similar letters were non-significant. Means with different letters were significant ($P \leq 0.05$, $n = 6$). TAA: thioacetamide; IN: inulin; CIS: cisplatin; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; VLDL-C: very low density lipoprotein cholesterol; AFP: alpha-fetoprotein; SEM: standard error of mean

Table 2: Serum and liver enzyme activity in control and treated rat groups

Parameters	Animal groups				
	Control	TAA	TAA + CIS	TAA + IN	TAA + IN + CIS
Serum AST (U/L)	35.00 ± 3.20 ^a	200.76 ± 3.33 ^b	123.06 ± 1.19 ^c	118.81 ± 2.35 ^c	86.96 ± 2.23 ^d
Serum ALT (U/L)	56.53 ± 1.85 ^a	199.00 ± 2.89 ^b	96.16 ± 3.50 ^c	77.08 ± 1.80 ^d	60.53 ± 2.62 ^a
Serum ALP (U/L)	6.44 ± 0.30 ^a	11.77 ± 0.33 ^b	8.95 ± 0.24 ^c	8.14 ± 0.19 ^c	7.32 ± 0.39 ^d
Serum CK (U/L)	241.16 ± 0.97 ^a	482.81 ± 7.05 ^b	308.91 ± 4.39 ^c	278.28 ± 2.72 ^d	259.96 ± 1.04 ^e
Serum LDH (U/L)	218.33 ± 10.77 ^a	468.33 ± 9.36 ^b	297.83 ± 16.03 ^c	291.33 ± 0.18 ^c	279.50 ± 10.44 ^c
Liver AST (U/g)	8.94 ± 0.16 ^a	0.09 ± 0.42 ^b	2.21 ± 0.38 ^c	3.96 ± 0.15 ^d	5.43 ± 0.23 ^e
Liver ALT (U/g)	4.65 ± 0.31 ^a	0.63 ± 0.06 ^b	1.55 ± 0.15 ^c	1.93 ± 0.12 ^c	2.68 ± 0.35 ^d
Liver ALP (U/g)	50.97 ± 1.93 ^a	23.19 ± 1.43 ^b	36.11 ± 1.29 ^c	39.76 ± 0.51 ^c	48.35 ± 1.41 ^a

Results are expressed as a mean ± SEM, with each row. Superscripts with different letters (a-e) express significant change with $P \leq 0.05$. Superscripts with similar letters were non-significant. Means with different letters were significant ($P \leq 0.05$, $n = 6$). TAA: thioacetamide; IN: inulin; CIS: cisplatin; AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase; CK: creatine kinase; LDH: lactate dehydrogenase; SEM: standard error of mean

HCC rats succeeded in inducing a significant improvement in these changes.

Histopathological examination revealed that the liver of the control group was composed of classical hepatic lobules of normal central veins lined by endothelial cells, radiating hepatic cells, and hepatic sinusoids. Hepatocytes were polyhedral in shape, with sharply defined boundaries. They had an acidophilic cytoplasm and central rounded nuclei. Frequently seen were von Kupffer cells [Figure 1, pictures C and C1]. The examination of the liver in HCC rat groups exhibited severe hepatic damages. Inflammation (lymphocytic cell infiltration), significant damage of hepatocytes, dilated congested central vein with degenerated endothelial cells, and congested blood sinusoids were observed [Figure 1, pictures HCC and HCC1]. HCC rats treated with CIS showed hepatocytes with little damage, dilated congested blood sinusoids, degenerated endothelial cells, and congested central veins [Figure 1, pictures HCC + CIS and HCC + CIS1]. However, HCC rats administered with IN showed normal hepatic lobules similar to the control group, indicating its hepatoprotective effect [Figure 1, pictures HCC + IN and HCC + IN1]. HCC rats treated with CIS and IN maintained the hepatic architecture, with minimal damage in lymphocytic cell infiltration [Figure 1, pictures HCC + CIS + IN and HCC + CIS + IN1]. These findings were evidenced by Masson's trichrome staining blue collagen fibers in hepatocytes and the central vein as a good marker for more fibrous collagen deposition, developed extensive fibrosis in the periportal area, and damaged hepatocytes in HCC rats [Figure 2, picture HCC]. Whereas, small fibrotic lesions were detected in the liver of HCC + IN, HCC + CIS and HCC + IN + CIS treated groups compared to the control rat group [Figure 2].

DISCUSSION

HCC is the most common and lethal of all cancers. There exists a diversity of dietary,^[35] endogenous, and environmental^[36]

stimuli that mediate hepatocarcinogenesis. Carcinogenesis may arise as a result of chemical or biological damage to normal cells in a multistep process that involves changes at the initiation level followed by promotion and progression, which leads to malignancy.^[37]

The increment in the concentrations of serum total lipid, TC, TGs, and LDL-C with a reduction in the level of HDL-C in the TAA treated rats may reflect impairment of liver function, particularly in lipid metabolism.^[38] The long-term regimen of TAA led to a significant decrease of hepatic markers; total protein and albumin levels indicating acute hepatocyte damage.^[39] The alterations in lipid profiles and protein content in malignant tissues are of importance due to their effect on membrane integrity, fluidity, regulation, altered internal viscosity, and the internal chemical composition of cellular processes related to growth and cell survival.^[40] Under normal physiological conditions, the liver ensures homeostasis of lipid and lipoprotein metabolism. HCC impairs this process, leading to alterations in lipid and lipoprotein patterns.^[41] Many tumor markers have been described in the hope of finding a blood test for cancer, and some have found their way into widespread but indiscriminate clinical use. Classically, a marker is synthesized by the tumor and released into circulation, but it may also be produced by normal tissue in response to invasion by cancer cells. The ideal tumor markers should be produced by the tumor cell and be readily detectable in body fluids.^[42] The continuing improvement of tumor-associated markers may help approaches in cancer treatment and diagnosis.^[42] In addition, TAA-induced abnormal lipid synthesis or defective degradation of lipids is implicated in a pathological condition like cancer. Peroxidation of lipids in biomembranes and tissues causes the leakage of these lipids into circulation and consequently leads to hyperlipidemia. Hyperlipidemia has been shown to increase the risk of metastasis in several cancers.^[43] Hepatoma is usually associated with hyperlipidemia, as well as a notable

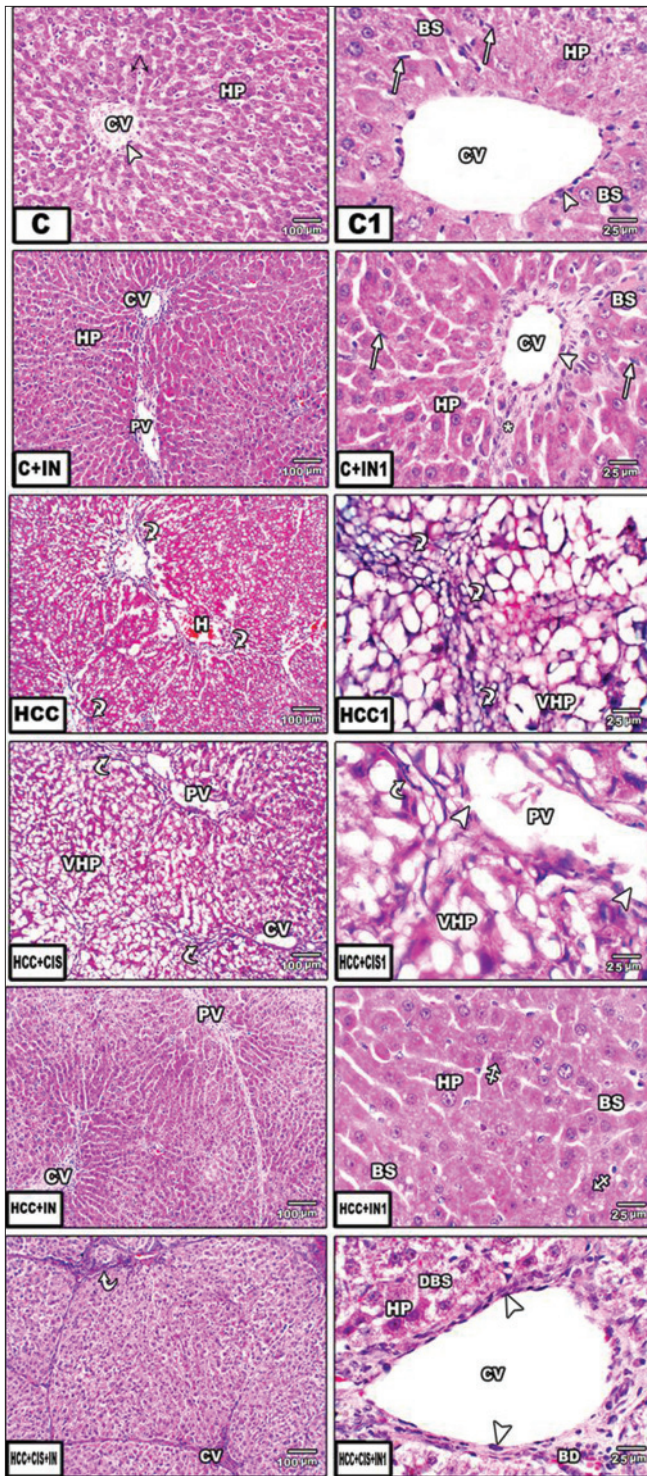


Figure 1: Photomicrographs of liver sections stained with hematoxylin and eosin (HE, left row $\times 100$, right row $\times 400$). (C) Control rat showing normal hepatocytes architecture; (HCC) liver of HCC rat treated with CIS, showing damage hepatocytes with lymphocyte cells infiltration; (HCC + CIS) liver of HCC rat treated with CIS showing congested central vein and blood sinusoids; (HCC + IN) liver of HCC rat that received IN similar to control group; (HCC + CIS + IN) liver of HCC treated with both CIS + IN showing maintained hepatic architecture, with minimal damage. C: control; IN: inulin; CIS: cisplatin; HCC: hepatocellular carcinoma; CV: central vein; HP: hepatocytes; BS: blood sinusoids; VHP: vascular hepatocytes; PV: portal vein; H: hepatocytes; white arrow: blood sinusoids; arrowhead: Kupffer cells; black arrow: endothelial cells; *Lymphocytic cell infiltration

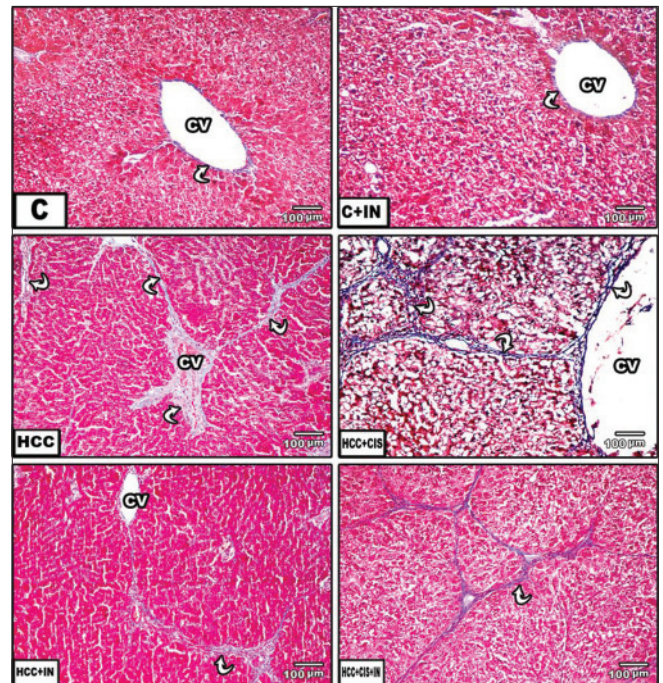


Figure 2: Photomicrographs of liver sections stained with Masson's trichrome staining ($\times 100$). (C) Liver section of control rats, fibrosis, and collagen fibers could not be seen; (HCC) liver section of HCC rat treated with thioacetamide, increased fibrosis, and blue collagen fibers are seen among hepatocytes and central vein; (HCC + CIS) liver section of HCC rat treated with CIS, strands of blue collagen are less seen among hepatocytes; (HCC + IN) liver section of HCC rat that received IN, similar to the control rat group; (HCC + CIS + IN) liver section of HCC rat treated with both CIS + IN, blue collagen fibers among hepatocytes are less seen. C: control; IN: inulin; CIS: cisplatin; HCC: hepatocellular carcinoma; CV: central vein; white arrow: a curved short arrow on the figure indicating blood sinusoids

decrease in the HDL fraction and an enormous increase in the VLDL and LDL fractions through resulting increase lipid peroxidation (LPO) during oxidative stress.^[44] Furthermore, albumin in the human body transports essential fatty acids from adipose tissue, otherwise known as fat, to muscle tissue; it is made by the liver. Consequently, decreased albumin levels may be associated with liver disease.^[45] Moreover, the increase in the level of AFP in HCC rats indicates an HCC.^[46] It was reported that elevated serum concentrations of AFP can be observed in rats due to exposure to hepatotoxic agents or hepatocarcinogens and are frequently associated with HCC.^[47] Its serum concentration can be used to confirm hepatocarcinoma and the diagnosis of tumor response to therapy. More than 90% of patients with hepatitis, cirrhosis, and hepatic cancer have increased serum AFP levels.^[47]

Moreover, the observed alterations in serum AST, ALT, ALP, CK, and LDH activity and total bilirubin levels are good indicators for hepatic injury resulting from cellular leakage and the loss in functional integrity of the cell membrane in liver.^[48] Cellular damages are identified by increases in serum ALP, ALT, and AST levels in that these enzymes are in the cytoplasm and

after cellular damage, enters blood circulation.^[49] This is due to the increased permeability of the plasma membrane or cellular necrosis leading to leakage of the enzymes into the blood stream.^[50]

ALP is used as a specific tumor marker during diagnosis in the early detection of cancer.^[51] It is involved in the transport of metabolites across cell membranes, protein synthesis, secretory activities, and glycogen metabolism. It is a membrane bound enzyme and its alteration is likely to affect membrane permeability and induces derangement in the transport of metabolites.^[52] The increase of ALP in HCC rat groups found in this study may be due to the disturbance in secretion activity or due to altered gene expression. The development of a tumor results in tissue damage that leads to the release of ALP into circulation and the liver tissues of the tumor-bearing animals. Elevation of ALP is one of the signs suggesting space-occupying lesions in the liver.^[53] The rise in the activity of ALP in cancer-bearing animals may be due to a disturbance in secretory activity, the transport of metabolites, or may be due to the altered synthesis of certain enzymes in these conditions. In addition, GGT is overexpressed in tumor cells.^[54]

CIS is one of the most potent chemotherapy drugs widely used for cancer treatment. It was a keystone that triggered the interest in platinum (II) and other metal-containing compounds as potential anticancer drugs.^[55] The related action of CIS activated nucleases and acid phosphatase lead not only to the breakdown of nucleic acids but also to the further dephosphorylation of mononucleotides, thereby leading to the acceleration of the processes of cell degeneration.^[56] In histology, the main hallmark of HCC is its resemblance to the normal liver both in its plate-like growth and its cytology.^[57]

HCC is usually a hypervascularized tumor showing different degrees of hepatocellular differentiation, ranging from well to poorly differentiated, based on the architectural and cytologic features. Different histological patterns may be seen: (1) The trabecular pattern of growth in which tumoral hepatocytes are arranged in plates of various thickness, separated by sinusoid vascular spaces; (2) the acinar or pseudoglandular pattern showing gland-like dilatation of the canaliculi between tumor cells (lumens can contain bile) or central degeneration of trabeculae (lumen containing mainly fibrin); and (3) the compact or solid pattern composed of thick trabeculae compressed into a compact mass that causes liver damage.^[57]

Cytologically, tumoral hepatocytes are polygonal, displaying an eosinophilic granular cytoplasm, rounded nuclei, and

prominent nucleoli. The importance of cell pleomorphism varies according to the degree of differentiation. Several variants of HCC are described regarding the cytological aspect of the hepatocellular proliferation. The clear cell variant is made of clear cells that may contain fat or glycogen. In the scirrhous HCC, tumor cells are generally smaller in size, showing a granular eosinophilic cytoplasm, vesicular nuclei, and conspicuous nucleoli.^[58] Sarcomatoid HCC is characterized by a sarcomatous-appearing component of spindle-shaped or giant tumor cells.^[59]

In this study, IN has a prebiotic effect indicated by a decrease in the lipid profile (total lipids, cholesterol, triglyceride, LDL-C, and VLDL-C) but increased HDL-C and AFP, as well as total protein and albumin. Experimental data demonstrate that IN affect processes and parameters involved in lipid metabolism and thus produces a beneficial effect on diseases related to lipid disorders such as reductions in TG, while showing modest reductions in total, LDL-C and VLDL particles^[14] due to the inhibition of fatty acid synthesis.^[60] The lipid-lowering action of this natural product may be mediated through the inhibition of hepatic cholesterol biosynthesis, increased faecal bile acid excretion, enhanced plasma lecithin, cholesterol acyltransferase activity, and the reduction of lipid absorption in the intestine.^[61] The IN extracted from chicory contains some fructooligosaccharides (FOSs) in addition to polysaccharides, which may provide another mechanism of action.^[62] It is well established that FOSs, besides their effect on the gastrointestinal tract, are also able to exert systemic effects by modifying the hepatic metabolism of lipids in many animal models.^[63] Colonic fermentation of FOSs results in the synthesis of short-chain fatty acids, which influence lipid metabolism in humans.^[64] In addition, IN is soluble in water and not hydrolyzed by human digestive enzymes; it is expected to behave like a soluble fiber and to have a hypolipidemic effect.^[61,65] IN lowers serum cholesterol when added to the diet of rats, and may decrease cholesterol synthesis by inhibiting hydroxymethylglutaryl-coenzyme reductase. A mechanism of action of oligofructose was associated with the modulation of *de novo* cholesterol synthesis by short-chain fatty acids produced by gut microflora during the fermentation process.^[66] The current study is in agreement with a previous study suggesting that chicory extract can minimize liver enzymes (AST, ALT, ALP, CK, and LDH) activity. This finding may be attributed to chicory extracts having hepatoprotective and antioxidant effects that were effective in reducing serum liver enzymes toward or even below the normal value.^[67,68] Regarding the present histopathological examination of the liver sections of HCC rats that received IN showed that the administration of IN protected rat livers from inflammation, necrosis, fibrosis, and steatosis. This was indicated by the efficiency of chicory

extract to retain the normalization of the hepatic tissue as neither necrosis nor fatty accumulation were observed and the central vein clearly appeared.^[69]

In conclusion, the current data indicated the efficacy of chicory extract (IN) supplementation as an anti-HCC in addition to its ability as a chemo-sensitizer for CIS treatment. This is mediated by intracellular pathways, involving improvement of the alterations in liver functions, as well as other aspects of HCC, the suppression of oxidative stress, and modulation of antioxidant defense mechanisms. Thus, supplementation with chicory extract may help in the safe application of cancer technology in medicine, as well as in many other aspects of everyday life. Fractionation guided evaluation could help in the development of an ideal anticancer treatment in the near future.

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Conflict of interest

There is no conflict of interest.

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Expectations for partial splenic arterial embolization simultaneous transcatheter arterial chemoembolization for hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is frequently complicated by cirrhosis, and it is not unusual for treatment options to be limited as a result of pancytopenia due to hypersplenism. Partial splenic embolization (PSE) has been performed for thrombocytopenia resulting from hypersplenism. However, the safety and efficacy of concurrent transcatheter arterial chemoembolization (TACE) with PSE for HCC remain unclear. Thrombocytopenia has been improved, and treatment continued using concurrent PSE. In addition, the hepatic functional reserve could be maintained even after treatment for HCC. Concurrent TACE and PSE for HCC with thrombocytopenia can be expected to help maintain a hepatic reserve, and it may contribute to improving the prognosis of HCC. Hence, PSE could lead to an asplenic state. The appearance of Howell-Jolly bodies on a peripheral blood smear is reported useful for assessing splenic function. The appearance of Howell-Jolly bodies is associated with an increased risk for post-splenectomy sepsis/overwhelming post-splenectomy infection in patients with reduced splenic function. These bodies are frequently observed in peripheral erythrocytes after PSE, and when they are present, it is appropriate to administer the pneumococcal vaccine to prevent severe infection. The expectations for PSE combined with TACE for the treatment of HCC associated with cirrhosis are reviewed.

Key words: Hepatic functional reserve; hepatocellular carcinoma; partial splenic embolization; thrombocytopenia; transcatheter arterial chemoembolization

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INTRODUCTION

Patients with cirrhosis develop hypersplenism (splenomegaly) and decreased platelet counts as their liver fibrosis progresses.^[1] Within this clinical context, hepatocellular carcinoma (HCC) is frequently associated with cirrhosis. In addition to surgical procedures such as hepatic resection, liver transplantation,^[2] transcatheter arterial chemoembolization (TACE),^[3]

percutaneous ethanol injection therapy (PEIT),^[4] and radiofrequency ablation (RFA)^[5] are all reported to be effective in the treatment of HCC. There are various other therapeutic alternatives including systemic chemotherapy,^[6] but thrombocytopenia is a major obstacle in the treatment of HCC. In other words, HCC associated with cirrhosis is often characterized by pancytopenia due to hypersplenism, so it is not uncommon for treatment options to be limited.

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Historically so far, splenectomy^[7] and partial splenic embolization (PSE)^[8] have achieved prolonged improvement for thrombocytopenia due to portal hypertension. Sugawara *et al.*^[9] reported that patients with hypersplenism who underwent combined treatment with splenectomy had improved hepatic functional reserve and became eligible for hepatectomy with post-operative 3- and 5-year survival rates among patients with HCC associated with cirrhosis reaching 72.3% and 38.9%, respectively. However, whether splenectomy should be performed simultaneously with HCC treatment is controversial. They recommended that simultaneous splenectomy is appropriate for patients whose HCC could be easily resected while simultaneously performing splenectomy and who had a relatively stable general condition including hepatic functional reserve, while patients who did not satisfy these criteria should first undergo splenectomy followed by assessment of the change in hepatic functional reserve and only then be considered for secondary hepatectomy.

Splenectomy is believed to be more effective in increasing platelet counts than PSE. However, overwhelming post-splenectomy infection (OPSI) and portal vein thrombosis (PVT) are major complications of splenectomy.^[10,11] OPSI has a high mortality rate and poor prognosis, especially for patients with HCC.

Hence, Maddison^[12] first reported splenic embolization as a treatment for hyper-splenectomy, in 1973; but its use was initially limited by severe complications such as splenic abscess and pneumonia with sepsis. Spigos *et al.*,^[8] however, described a PSE procedure for a limited infarct area in 1979, leading to improved safety and enhanced clinical applications. Like splenectomy, PSE is reported to increase platelet counts and improve hepatic functional reserve and portal hypertension. In the present review, the significance and usefulness of PSE in the treatment of TACE for HCC are discussed.

HCC TREATMENT OF PATIENTS WITH THROMBOCYTOPENIA

HCC is associated with severe complications in patients with cirrhosis or chronic hepatitis who have severe fibrosis.^[13]

Although the treatment outcome of HCC has improved recently, intra-hepatic recurrence occurs at a high rate of 10-25% annually despite radical treatment, and in many patients, HCC recurrence leads to fatal consequences.^[14] We previously reported that the combination of total hepatic artery infusion of a powdered formulation of arterial cisplatin (IA-Call; DDP-H) with TACE to treat Stage I/II HCC reduced intra-hepatic distant recurrence^[15] and improved

survival rates in HCC patients with a Japan Integrated Score of 0-1.^[16] However, the exacerbation of thrombocytopenia associated with arterial infusion of anticancer drugs in patients with cirrhosis often restricts subsequent treatment options.

Since both PSE and splenectomy are expected to improve the hepatic functional reserve, there are hopes that HCC treatment combined with PSE represents a valuable treatment modality for patients with thrombocytopenia.

We performed TACE combined with PSE and investigated whether these procedures, when performed simultaneously, could prevent thrombocytopenia, whether there were any complications, and whether there was a secondary effect in the form of improved hepatic functional reserve.^[17] In the simultaneous PSE group, platelet count ($\times 10^4/\mu\text{L}$) increased from 6.54 ± 2.60 before TACE to 10.23 ± 3.93 at 2 weeks after TACE, even though anti-cancer drugs were administered during the TACE procedure. Meanwhile, in the TACE without PSE group, the platelet count ($\times 10^4/\mu\text{L}$) decreased from 6.89 ± 3.21 before TACE to 4.47 ± 1.55 at 2 weeks after TACE. In the simultaneous PSE group, the increased platelet count made it possible to perform loco-regional treatments such as PEIT and RFA. Moreover, assessment of hepatic reserve based on the Child-Pugh score showed that the PSE group experienced temporary worsening from 7.04 ± 1.05 to 7.21 ± 0.99 at 2 weeks after TACE/PSE, but their scores later improved to 7.00 ± 1.77 at 2 months and 6.70 ± 1.16 at 6 months after TACE/PSE.

PROBLEMS WITH REDUCED SPLENIC FUNCTION AFTER PSE

The occurrence of post-splenectomy sepsis (PSS) or OPSI after splenectomy or during reduced splenic function is thought to be associated with a fatality rate of more than 70%.

The appearance of Howell-Jolly bodies in peripheral erythrocytes has drawn attention as an indicator of reduced splenic function. Howell-Jolly bodies are erythrocyte inclusions shown by May-Giemsa staining while they do not appear in healthy individuals, they are apparent in certain blood diseases and in functional asplenia following splenectomy. When we examined the incidence of Howell-Jolly bodies in patients who underwent PSE at our department, we found that they were present in as many as 17 of 95 treated patients (17.89%).^[18]

Comparison with the group that was negative for Howell-Jolly bodies did not reveal any significant differences in residual spleen volume or the splenic infarction rate after PSE.

However, recent advances in automated analyzers have resulted in a decline in microscopic observation, so there is a tendency for Howell-Jolly bodies, which can only be confirmed visually to be overlooked, and to be less emphasized in clinical settings. Nevertheless, the appearance of Howell-Jolly bodies is associated with an increased risk of PSS/OPSI in patients with reduced splenic function, so the fact that these bodies were frequently observed in peripheral erythrocytes after PSE without any relationship to residual spleen volume or the splenic infarction rate emphasizes the need to visually determine the presence or absence of these entities and when they are present to administer pneumococcal vaccine to prevent severe infection.

Moreover, PSE and splenectomy sometimes induced PVT. PVT is a severe, potentially fatal complication.^[19] Some predictive factors of PVT are reported for early detection.^[20,21] Early detection of PVT and prompt anticoagulation are effective to avoid serious consequences of PVT.^[22] It is necessary to perform PSE recognizing to these problems with reduced splenic function after PSE.

CONCLUSION

Compared to splenectomy, some advantages of PSE are that it is minimally invasive, can preserve splenic function, and only rarely causes OPSI and PVT. However, PSE also has the risk of complications such as fever, abdominal pain, vomiting, and ascites/pleural effusion, as well as serious symptoms including splenic abscess, and peritonitis, so the decision as to whether to perform the procedure should be a carefully considered one. Myelosuppressed patients receiving anticancer drugs or immuno-suppressants are even more susceptible to the risk of infection. HCC is a tumor-bearing condition, so it is believed to be essential to attempt to minimize the patient's susceptibility to infection due to the asplenia or significantly reduced splenic function. There is no clear evidence to suggest whether PSE should be performed simultaneously with HCC treatment or at a different time. The decision will obviously be influenced by various aspects of the treatment strategy including the location and size of the HCC and any additional therapies, and by the condition of the patient's cirrhosis. Still, simultaneous TACE combined with PSE represents a safe and effective approach in patients who cannot undergo concomitant RFA therapy. Furthermore, TACE combined with PSE is capable of maintaining a hepatic functional reserve. This finding suggests that TACE combined with PSE may represent a treatment strategy for HCC associated with portal hypertension and a multidisciplinary treatment modality for HCC associated with cirrhosis characterized by thrombocytopenia.

In the future, a prospective study of a large patient population is needed to determine whether HCC with portal hypertension should be treated with simultaneous splenectomy or simultaneous PSE.

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Conflict of interest

There is no conflict of interest.

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Microwave coagulation therapy of hepatocellular carcinoma

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ABSTRACT

Microwave coagulation therapy (MCT) is a relatively new method of tumor ablation compared to other minimally invasive local therapies for hepatocellular carcinoma (HCC). It is a thermal ablation modality based on the application of heat, potentially leading to larger ablation zones. In recent years, there is a steady increase in the application of this modality to the treatment of HCC because it offers several advantages in the management of tumors larger than 3 cm in diameter. This article reviews the advances in MCT for the treatment of HCC in recent years including its brief history, basic principles, main technical parameters, safety issues, current status in clinical application, limitations, and future perspectives.

Key words: Coagulation; hepatocellular carcinoma; microwave; treatment

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INTRODUCTION

Microwave is an ultra-short and high-frequency electromagnetic wave with a wavelength range between 1 and 1,000 mm, and a frequency range between 300 and 300,000 MHz.^[1,2] The most common frequency of microwave used medically is 2,450, 915, and 433 MHz.^[3,4] Hepatocellular carcinoma (HCC) is a common tumor with a dismal prognosis and development of novel therapies were needed for improving the treatment of this disease.^[5] Microwave coagulation therapy (MCT) is a relatively novel method of tumor ablation compared with other minimally invasive local therapies for HCC such as high-intensity focused ultrasound, irreversible electroporation, laparoscopic liver resection, percutaneous ethanol injection, radiofrequency ablation (RFA), and stereotactic body radiation therapy.^[6] This treatment method offers an easy-to-perform alternative option either in a percutaneous, laparoscopic or open surgical

procedure.^[7,8] In recent years, MCT has attracted increasing interests from clinicians for the treatment of HCC because this method provides the advantages of minimal invasiveness and safety in humans.^[9]

This paper reviews the advances in using MCT for the treatment of HCC in recent years, with emphasis on the basic principles, and perspectives of this treatment modality for future research.

BRIEF HISTORY

In the late 1970s, some surgeons began to use microwave coagulation for the purpose of intra-operational hemostasis and tissue cutting when they found that as soon as the temperature in the target area exceeded 60 °C the heat will cause tissue solidification followed by cellular death.^[10] In 1988, the first experiment of microwave coagulation

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for tumor inactivation was conducted successfully in the Second Department of Surgery, Kurume University Hospital in Fukuoka City, Japan.^[11] Soon, successful use of microwave coagulation was reported throughout Japan, with large patient cohorts, multiple heating sessions, treating tumors with larger diameters, combination use with other ablation modalities, and review and management of side-effects.^[12] A recent study reported the long-term efficacy results of surgical MCT for the treatment of 60 cases of unresectable HCC in the Kyushu Medical Center, National Hospital Organization, Fukuoka, Japan. The 1-, 3-, and 5-year survival rates were 93.9%, 53.8%, and 43.1%, respectively, almost the same as the results of palliative surgical resection.^[13] In April 1996, MCT was included in the coverage list of Japanese Medical Insurance, which contributed to the advancement in the research on MCT for the treatment of HCC in Japan.^[14]

In the 1990s, MCT was gradually accepted in other regions of the world for the treatment of HCC.^[8]

BASIC PRINCIPLES

The fundamental principle of MCT is based on the facts that tumor tissues are more sensitive to heat than the adjacent normal tissues, and that microwave heat can be controllably delivered to the tumor tissues.^[15] Compared with normal tissue, tumor tissues have low blood flow and extra-cellular pH value, as proven by experiments showing that the blood flow volume in murine HCC tissue is only 5% of that in normal liver tissue.^[16] When a tumor is irradiated with microwave energy, the heat will make the blood flow stagnant at the local area.^[17] When the temperature in tumor tissue goes up to 42-45 °C, the blood flow in the tumor stops, cancer cells become anoxic, mitochondria in cancer cells show vacuolation, resulting in the cessation of intra-cellular respiration, apoptosis, heat fixation, and death of cells.^[12,18,19]

When the local temperature reaches 60 °C and above, tumor tissues will be immediately coagulated and damaged by the effects of thermal treatment.^[20] A controllable temperature range of 60-120 °C can be presented rapidly to the target area by the treatment system.^[21] During the procedure of MCT, a specially made needle is initially inserted into the center of tumor. The tip of the needle contains a point acting as an emission antenna.^[22] The system includes an electric device that produces microwave energy forming a magnetic field around the point of the antenna.^[23] The magnetic field makes nearby molecules spin and vibrate in very high velocity with resultant hyperthermia in the local area.^[24,25] This is called the thermal effect of microwave antenna radiation.^[26] Because energy density is very high in the area, protein will be coagulated by the heat swiftly in the contacted tissues

leading to immediate degenerative necrosis of local tissue, which can be better evaluated using the histochemical method.^[27] MCT is non-carbonizing, non-spattering, minimally invasive, and wound controllable.^[28] With these advantages, this method is now widely used in coagulation therapies including dermatology, gynecology, stomatology, and otorhinolaryngology.^[29,30]

Cool-tip microwave therapy apparatus incorporates a microcomputer, which automatically operates the system.^[31] Electrical resistance, current, and power of the system are regulated automatically during the entire course of treatment.^[32] The technique of cool recycling solved the problems of inappropriately high temperature in the rod antenna and a limited coagulation area that the conventional microwave systems had.^[4] The new systems enhanced the operational safety, expanded therapeutic indications, improved the patients' quality-of-life, and post-operational survival.^[17,33]

MCT for the treatment of tumors is now being investigated its potential to enhance host resistance to malignancies. This was resulted from reports of incidental observations showing spontaneous regression of remote metastasis after thermal ablation.^[34] On one hand, microwave coagulation results in irreversible coagulation necrosis of tumor cells through the thermal effect;^[35] on the other hand, the coagulated tumor tissue that retained in the body will stimulate the host to enhance its systemic immunity against cancer with consequent resistance to viable allogenic and heterogenic cancer cells.^[36] Animal experiments demonstrated that in mice model of H22 implanted advanced HCC, intra-tumoral CD8+ cells increased remarkably in the MCT group compared to that in the surgery alone group. This provided supporting evidence for the immune enhancement by the coagulated tumor tissue that retained in the local area.^[37,38] Studies showed that the local density of lymphocytes infiltration is closely related to the prognosis of patients with some cancer, that is, a higher density of intra-tumoral and peri-tumoral lymphocytes infiltration, like CD8+, is predictive of a better patient outcome.^[39]

EQUIPMENT

Microwave ablation system

At present, the MCT systems with water-cool shaft or antenna are more commonly used in the clinic for the treatment of HCC.^[4,25] All systems are equipped with flexible low-loss power cables and a surface coating microwave antenna of 15 gauges.^[7,40] The tip of the needle-like antenna is not insulated and is exposed to the tissue to be ablated.^[7] Electromagnetic microwaves are emitted by the microwave

generator and will stimulate the water molecules in the neighboring tissue to induce heat and abrasion, resulting in coagulation necrosis of tissue.^[12,19]

The technique of cool recycling makes the temperature in the antenna shaft below 37 °C to avoid pain at the puncture site of the patient undergoing MCT.^[41] Coagulation temperature in the ablation area is precisely controlled by monitoring devices.^[25] Two modes of thermometry are used simultaneously during the procedure. Thermometers are installed both in and outside the rod antenna. A warning temperature is set by the operator to ensure the safety of the patient.^[42]

For ablation of larger tumors, two to three microwave antenna needles can be used at the same time to produce maximum coagulation area.^[43] Simultaneous use of two antenna needles could not be simply regarded as a linear superposition of two solitary thermal fields, because the interaction between two thermal fields constitutes a spherical coagulation area that is significantly greater than the coagulation area when two needles are used separately with the same power and time period.^[44] The mode of impulse transmission enhances the depth of microwave penetration and contributes to the increase in coagulation area and the decrease in tissue carbonization.^[45]

Parameters

Most systems use 2,450 MHz as the working frequency for tumor destruction.^[4,12] For MCT, the higher the frequency, the more powerful the instant energy, but the weaker the penetrating power.^[46] Thus, the 2,450 MHz microwave is usually used for MCT because of the advantage of its powerful instant energy while the 915 and 433 MHz microwaves with strong penetration are used for thermotherapy of other diseases.^[3,4] The novel dual-band systems have two working frequencies including 2,450 and 915 MHz.^[47] The 915 MHz microwave creates a remarkably larger area of ablation and is suitable for one-time ablation of large tumors *in-situ* because this frequency produces double depth of penetration in tissues compared with that produced by the 2,450 MHz microwave.^[48] The output power for ablation is usually set at 40-80 W.^[4] Microwave with high power and high frequency (135 W, 2.45 GHz) has been used in the animal study for producing large ablation areas in short time periods.^[28] Power and time period for coagulation are determined on the basis of tumor size.^[9] With tumor sizes ranging from 3 to 5 cm in diameter, microwave power for ablation is set at 50-60 W for 5-15 min;^[4] with tumors over 5 cm in diameter, two needles are used simultaneously.^[25]

Image guidance modalities

MCT for the treatment of HCC can be used in an open surgery procedure or in a percutaneous approach.^[7] The surgical use of MCT for HCC is performed under the monitor of direct visualization or image guidance modalities like ultrasound Type B.^[13] After verification of the tumor dimensions, the rod antenna is inserted into the tumor for coagulation.^[17]

When a percutaneous approach is taken, there are more choices regarding image guidance modalities including ultrasound, X-ray fluoroscopy, computed tomography, and magnetic resonance image (MRI).^[48] Among them, ultrasound and MRI offer radiation-free alternatives to image guidance, and MRI provides high-resolution and multi-planar images.^[49] However, MRI-compatible microwave electrode and accessories must be prepared.^[50]

CURRENT STATUS IN CLINICS

Cool-tip MCT for the treatment of HCC has several beneficial characteristics in clinical application, as well as microwave systems, with an electrode with saline passing through and injected continuously into the target area.^[51] Direct puncture of lesions makes the operational procedure relatively simple and easy.^[17]

General anesthesia is necessary in a surgical MCT procedure while in a percutaneous approach local anesthesia is commonly used with venous analgesics such as pethidine and sedatives as additional pain-killers.^[52] Under the guidance of a selected image modality, the tumor is localized, and the needle is directly inserted into the tumor with the needle tip placed at a calculated point.^[11] The ablated area is assessed through real-time images to find tumor tissues that are still viable.^[40] Repeated ablation procedures are performed to ensure no viable tumor tissue remained at the site.^[53]

MCT is different from RFA, which has a longer history and has acquired a broad acceptance as a first-line treatment option for early HCC.^[54] However, RFA has the limitations associated with treating large tumors and tumors at high-risk locations.^[22] MCT has the advantage of treating larger tumors and is regarded as a valuable alternative to RFA. Compared with other available modalities and devices for thermal ablation, MCT offers the advantages of greater volume of tumor ablation, consistently greater temperatures in the ablation area, better analysis of heat transfer, and shorter ablation sessions.^[7]

A recent single-center study reported the treatment outcome of MCT for the treatment of 719 consecutive HCC patients in more than 15 years. The 1-, 3-, 5-, 7-, and 10-year overall

survival rates of all 719 patients were 97.7%, 79.8%, 62.1%, 45.3%, and 34.1%, respectively. One-third of the patients had Child-Pugh Class B cirrhosis, and a portion of them had multiple tumors. Compared with another group of 34 patients treated with hepatic resection during the same period, no significant difference was found in overall survival, disease-free survival or local recurrence rates between the two groups. Based on the results of this study, the researchers proposed that MCT should be considered as one of the first options for the treatment of HCC.^[55]

ADVERSE EFFECTS

The adverse effects of MCT for the treatment of HCC are various and can be divided into mild, moderate, and severe categories.^[9] Mild adverse effects include slight local pain at the puncture site, sensation of heat, bodily uneasiness felt during the coagulation process, and slightly abnormal results from a blood test such as mild elevation of blood urea nitrogen and creatine levels.^[12] Post-ablation syndrome can be mild to moderate and is characterized by fever, chills, malaise, local pain, and nausea.^[56] Moderate adverse effects comprise bacterial infection, diaphragmatic muscle injury, skin burns, tumor implantation in needle pass, pleuritis, hydrothorax, hemothorax, continuous discharge of necrotic tissue, local implantation of tumor cells, and hematoma under the hepatic capsule.^[57,58]

However, severe adverse effects happen occasionally during and after the procedures of MCT such as anesthetic accident, colonic leakage, severe arrhythmia, damage to the biliary tract, abdominal bleeding, diaphragm injury, acute renal failure, generalized intra-peritoneal seeding of HCC, and serious infection.^[59-63] Severe adverse effects can be fatal and need emergency treatment to save the patient.^[63]

The rate of side-effects do not differ significantly from other interventions, but significantly more treatment sessions are needed with percutaneous microwave coagulation to achieve complete tumor ablation, which theoretically increases the risk of potential side-effects.^[62] Most adverse effects can be controlled with timely and careful management. Safety precautions must be taken to avoid or reduce the occurrence of adverse effects including cautious indication selection, well-designed puncture route, the appropriate extent of coagulation, and sufficient peri-operational management.^[59,61]

LIMITATIONS AND PERSPECTIVES

It is now proven that MCT is a safe and effective method for the treatment of HCC and the condition of spontaneous

rupture of HCC tumors.^[64] With the advancement of techniques and equipment for clinical application of microwave, MCT will be promoted more widely and used more extensively than before, through the approaches of laparoscopy or image-guided percutaneous puncture.^[20] Regrettably, intra-hepatic recurrence of HCC is common because MCT is indicated as a substitute for surgical resection for patients with advanced liver cirrhosis.^[53] Nevertheless, MCT can be readily repeated when there is a recurrence of HCC.^[53] Overall, MCT is great for cost-effectiveness compared with other treatment for HCC like sorafenib.^[65]

The characteristic feature of MCT for the treatment of HCC is the conversion of energy by tumor tissue from the microwave into heat with resultant coagulation necrosis of the tissue.^[25,26] MCT will be more promoted because it not only effectively kills HCC cells, but also preserves normal liver tissue to a great extent.^[59]

Image artifacts must be recognized and carefully distinguished from anatomical structure to ensure the accuracy of the location of the needle tip.^[66] Some issue will be further investigated like the impact of MCT on the systemic immunity of the HCC patient.^[34] At present, there are very few reports on the association of MCT used in the treatment of HCC and its modification of patients' systemic immunity. Further investigations into this topic are warranted.

CONCLUSION

Microwaves can produce very high temperatures in very short time intervals. MCT is increasingly used in the treatment of HCC because it offers several advantages such as greater efficacy, minimal invasiveness, easy conduction, wider indications, and less adverse effects compared to other invasive methods. Overall, MCT for the treatment of HCC is a very promising technique to develop further. Sufficient pre-operative preparation, mastering the techniques of operation, and good collaboration between doctors, nurses, and patients are essential for enhancing therapeutic outcomes of HCC and reducing the incidence of side-effects. Investigations should be carried out to determine the modulation by MCT of both innate and adaptive immunity. Although MCT is still in its infancy, it has great promise for future use, especially with further improvements in the clinical implementation and technical developments.

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Conflict of interest

There is no conflict of interest.

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“PERISH” flowchart for selection of the patients with resectable hepatocellular carcinoma

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ABSTRACT

A selection of patients with hepatocellular carcinoma (HCC) for surgical resection is crucial and algorithms/staging systems help surgeons to decide on a standard treatment for each patient and each HCC stage. However, there are always difficulties in remembering and/or recalling the contents of the algorithms/staging systems. Moreover, most algorithms/staging systems do not include data about the extent of hepatectomy, intra-hepatic distribution of tumor(s), and technical feasibility of resection, all of which are vital in the surgeons' decision-making process. Here, we aimed to present a simple and handy mnemonic acronym for selecting resectable HCCs in surgical practice. This was reproduced from the existing well-known staging systems. The designed mnemonic acronym is a phrase “PERISH” and it includes asking for Performance of patient, Extra-hepatic disease, Reserve of the liver, Intra-hepatic distribution, Stratifying risk factors, and Hepatectomy size in order. Performance based on whether the patient is mostly bedridden or not should be the first step of evaluation. Next, asking for suspicious metastasis as bone pain and radiological evaluation of abdomen/thorax is mandatory. The calculation of Child-Pugh score is only the third step. Good candidates for surgical resection should be Child-Pugh “A” with normal bilirubin levels. Technical feasibility of resection, according to the intra-hepatic distribution of tumor(s) should be done later, and the candidates preferably should not have portal hypertension (no splenomegaly, no thrombocytopenia). If the patient fulfills all the previous steps, the surgeon may perform indo-cyanine green clearance test. Consequently, following the PERISH flowchart may prevent “perish” of the surgeons while selecting the appropriate resectable HCCs.

Key words: Hepatectomy; hepatocellular carcinoma; surgery; surgical education

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the primary malignancy of the hepatocytes accounting for nearly 90% of all the liver cancers, which is the fifth most common cancer around the world.^[1] HCCs are most common in damaged liver parenchyma. Chronic hepatitis B virus (HBV) and hepatitis C virus, alcohol and steatohepatitis-induced hepatocyte

damage are the most common predisposing etiological factors for HCCs.^[2] HBV is the main etiology responsible for most of the HCC cases observed in Africa and the Asian parts of the world due to its high prevalence. However, due to the global utilization of vaccination programs the HBV-associated HCC incidences are getting lower.^[3] One-third of the cirrhotic population develops an HCC throughout their lives, with an annual incidence of 3-5%.^[3]

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TREATMENT DECISION

There are numerous classifications and staging systems for planning the treatment of HCCs, but every HCC and every patient must be evaluated individually. Treatment options for HCC have a wide range of modalities that change according to the stage of the tumor and the patient specific factors.

In 70% of the HCC cases, the treatment modalities used are not for curative intent. When diagnosed, 50% of HCC patients are suitable only for some palliative treatment modalities such as transarterial chemoembolization, chemotherapy, and sorafenib, which are proven to be effective on the HCCs by giving a median survival of 11-12 months at max. For the remaining 20% of the patients, symptomatic and supportive treatments can be used within a very limited survival period of < 3 months.^[4] The curative intent treatment modalities vary among a simple curative resection of the HCC, ablative methods, and liver transplantation, and these methods are usually suitable in patients with small size HCCs. However, these curative treatment options may be possible only for 30% of all the HCCs with 5-year survival rates of 40-70%.^[4] Liver transplantation itself has an advantage over other curative therapies by eliminating both the HCC and the underlying cirrhosis. However, resections and ablations for a curative intent should also be preferred because of the limited shortage of donors. The incidence of the HCCs is increased in years from 2.8 per 100,000 in 1979 up to 4.6 per 100,000 in 1999. Every year over 571,000 new cases are diagnosed with HCCs, most of them (44%) from China. Annually, 552,000 deaths due to HCCs or HCC related consequences are seen worldwide.^[5] It is estimated that nearly 20,000 liver transplantations are annually performed all around the world for all indications. When compared to the annual cases diagnosed and yearly deaths seen due to HCCs, this overall transplantation number is more than insufficient. That's why these resections and local ablative methods must be performed much more frequently in every HCC case possible to improve survival rates at different stages if possible.

HOW TO DECIDE: WHICH WAY TO GO? WHAT TO DO?

HCCs' stage evaluation is essential to decide on the treatment modality to be chosen. The stage of the disease and the status of the patient give an idea about the resectability, the prognosis and the therapy to be chosen for the HCCs. There are various staging systems applied for staging of the HCCs in the literature such as Barcelona Clinic Liver Cancer (BCLC), Cancer of the Liver Italian Program, Group Study, and Treatment of Hepatocellular Carcinoma, Chinese University Prognostic Index, Japan Integrated Staging, and Okuda or Tokyo.^[6] From these staging systems, the BCLC classification

has been generally proposed as the backbone of the HCCs treatment that has been approved by the European Association for the Study of the Liver and American Association for the Study of Liver Diseases.^[7] The system encounters the size, number, extrahepatic dissemination, and vascular invasion of the tumor. It also uses the Child-Pugh score system for the liver function, examines the presence of portal hypertension, and the Eastern Cooperative Oncology Group (ECOG) classification for the health status of the patient.^[8] Every detail and finding about the HCC and the patient is important in deciding the right stage, treatment choice, prognosis, and outcome of the disease. Hence, evaluation and the content of the staging systems must be well-known by the surgeons to avoid a misdiagnosis or an overtreatment that is in-effective and un-necessary for the patients. The only handicap of the staging systems, scores, grading systems, classifications, algorithms, charts is the difficulty in memorizing, remembering, and/or recalling the contents of them. Moreover, most of these systems do not include any data about surgical details such as extent of liver resection, intra-hepatic distribution of the tumor(s), and the technical feasibility of the resection that are all vital for the surgeons in deciding the surgical resections of HCCs.

TRICKS AND TREATS TO LEARN, REMEMBER AND RECALL

There have been numerous learning techniques and strategies described in time to simplify learning, to make memorizing processes easy, and to ease recalling of them from memory when they are needed. When there is a list of items, steps or words to be remembered in a sequence, the first letters of these words is written one after the other and that formed word or the phrase is called a mnemonic acronym. This acronym can be used to remember the right words in the right order. It is proven that this strategy makes the learning, memorizing, and recalling processes much more easier.^[9,10]

The mnemonic acronym is defined as an invented combination of letters for this purpose. The acronym formed from the initials may be a meaningful word, sentence, phrase, or a nonsense phrase for example like "PVT TIM HALL". This is a mnemonic acronym used by the medical students to remember the essential amino acids. These amino acids are Phenylalanine, Valine, Threonine, Tryptophan, Isoleucine, Methionine, Histidine, Arginine, Leucine, and Lysine. Sometimes the mnemonic acronyms formed may be a meaningful word or phrase related to the original sentence, which is much more favorable due to the ease of recalling and remembering the acronym too. The example for this can be the total parenteral nutrition (TPN) indication, which is the phrase "MISIPPI Burning". Here, the word is composed of the

first letters of the following indications of the TPN, and the result is a phrase that can be much more easily remembered: Major visceral injury, Inflammatory bowel disease, Sepsis, Ileus, Post-operative, Paralysis, Intestinal fistulas, and Burns. These mnemonics are being used with increasing frequency in the medical education due to the need to learn, memorize, remember, and recall a lot of things.^[9,10]

A MNEMONIC ACRONYM TO DECIDE AND CHOOSE THE RESECTABLE HCC: “PERISH”

This phrase “PERISH” is designed to help practically and easily the surgeons in choosing the HCCs to be resected in an algorithm while evaluating a patient. It is a mnemonic acronym designed for: Performance of patient, Extra-hepatic disease, Reserve of the liver, Intra-hepatic distribution, Stratifying risk factors, Hepatectomy size. All these factors have a great importance in the patient selection that will be eligible for the surgery.

Performance of patient

These patients with HCC, if eligible for a resection will be candidates for one of the major surgeries in the general surgery practice. This may be a small size resection or a major hepatectomy, if the HCC and the patient are suitable. Hence, even if there is a chance of surgical resection as a cure for the disease, there are patient factors that are as important as the HCCs status when the surgeon is making a surgery decision. The age of the patient, debilitating, and co-morbid diseases (cardiovascular, renal, pulmonary, *etc.*) are the important factors that help the surgeon in making the evaluation.

The age of the patients is an important factor affecting the outcomes of the surgical interventions. During the years 1991-1995, in USA, the HCC incidence increased significantly in 40-60 years old patients up to 2.4 per 100,000 from 1.4 per 100,000.^[11] Furthermore, a more objective criteria, the ECOG classification for the health status is used in the BCLC staging of the HCCs [Table 1].^[5] Here, the patient's performance in doing their daily routines and taking care of their own needs are taken into consideration. The ECOG Class 0, Class 1, and sometimes the Class 2 can tolerate the surgical treatment. However, ECOG Class 3 and Class 4, due to their debilities cannot be candidates for surgical intervention whatever the HCC status is. The end stage HCC (BCLC Stage D) accounting for the 20% of HCCs also includes ECOG Class > 2 and/or Child-Pugh Class C patients. These are directly classified as the terminal stage patients that are only candidates for supportive treatments with a survival period of < 3 months [Figure 1].^[4] Poor patient performance is an early indicator for the treatment decision without a need

for a further investigation in ECOG > 2 patients who are symptomatic and in bed > 50% of the day.

Extra-hepatic disease

Here, the extra-hepatic dissemination of the HCCs are evaluated, which is an important finding in the advanced stage HCC diagnosis (BCLC Stage C) and helps in the differential diagnosis of the intermediate stage HCCs (BCLC Stage B). The portal vein invasion, lymph node positivity (N1), and distant metastasis (M1) are the pathognomonic findings of the advanced stage HCCs. These HCCs tend to be mostly locally advanced cancers that have a high affinity to make lymph node metastasis (30%). The distant metastasis are seen less frequently (13.5%); most commonly to lungs, bones, peritoneum, and the adrenal glands.^[11-13] There are some authors suggesting surgical resection for the distant metastasis to lungs or adrenal glands for a better prognosis in HCCs with up to three pulmonary lesions.^[14] However, this is not generally accepted. The major vascular invasion that cannot be reconstructed also leads to the HCCs advanced stage. The vessel invasion is more common in extra-hepatic disseminated HCCs.^[12,13] In these situations, only sorafenib treatment, and even in some, only the supportive treatment modalities can be used.

Reserve of liver

Since 80% of these HCCs originate from the cirrhotic livers, resection of these tumors is a much more complicated issue. The pitfalls of liver surgery in these patients are inadequate functional remnant, decrease in liver regeneration capacity, and increase the probability of hemorrhage due to portal hypertension. When inadequate liver remnant is left behind, this may lead to hepatic insufficiency and failure, which is the most common cause of death in this group. Calculation of liver reserve should be the third step of the evaluation. There are several staging methods to determine the hepatic reserve, such as Model for the End-Stage Liver Disease, Indo-cyanine Green (ICG) Retention Test, Metabolism of Lidocaine to the Metabolite, and Arterial Body Ketone Ratio.^[15] However, the most widely accepted staging system is Child-Pugh classification as A, B, and C [Table 2]. Previous studies clearly demonstrated that liver resection in cirrhotic patients accompanied with exacerbated transient hepatic dysfunction,

Table 1: ECOG performance scoring system

Performance status	Definition
1	No symptoms; normal activity level
2	Symptomatic, but able to carry out normal daily activities
3	Symptomatic; in bed less than half of the day; needs some assistance with daily activities
4	Symptomatic; in bed more than half of the day

ECOG: Eastern Cooperative Oncology Group

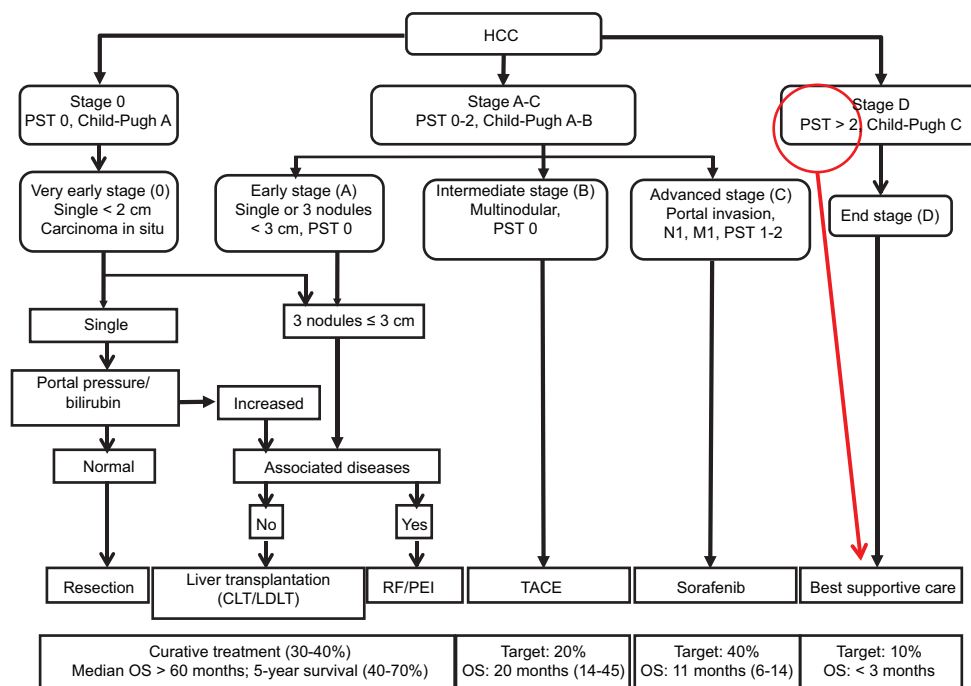


Figure 1: Barcelona Clinic Liver Cancer staging system. HCC: hepatocellular carcinoma; PST: performance status of the patient; OS: overall survival; CLT: cadaver liver transplantation; LDLT: living donor liver transplantation; RF: radiofrequency; PEI: percutaneous ethanol injection; TACE: transcatheter arterial chemoembolization

Table 2: Child-Pugh scoring system

Parameter	Points assigned		
	1	2	3
Ascites	Absent	Slight	Moderate
Hepatic encephalopathy	None	Grade 1-2	Grade 3-4
Bilirubin $\mu\text{mol/L}$ (mg/dL)	< 34.2 (< 2)	34.2-51.3 (2-3)	> 51.3 (> 3)
Albumin g/L (g/dL)	> 35 (> 3.5)	28-35 (2.8-3.5)	< 28 (< 2.8)
Prothrombin time			
Seconds over control	< 4	4-6	> 6
INR	< 1.7	1.7-2.3	> 2.3

Child-Pugh score classification - Child A: Score 5-6 (well-compensated); Child B: Score 7-9 (significant functional compromise); Child C: Score 10-15 (de-compensated). INR: international normalized ratio

impaired regeneration, increased risk of operative bleeding, post-operative ascites, and bleeding varices, high portal flow in non-compliant vascular bed, and liver failure.^[16] These risks and post-operative mortality rates are closely related with the reserve of the liver. Nagasue *et al.*^[17] reported the results of major hepatectomies (more than two segments) in cirrhotic patients with the mortalities for Child-Pugh score A, B, and C as 16%, 33%, and 100%, respectively. As a result, the candidates for a surgical resection should be preferably in Child-Pugh Class A.

Intra-hepatic distribution

The curative resection is the only modality that can achieve survival benefits in HCC treatment. However, size and number of the tumors are not the only determinant for the selection of the resectable HCCs. In case of difficult tumor locations, the size of the tumor cannot be the main determinant for

the decision of surgical resection. Contrary, patients with peripherally located large HCCs could be good candidates for a surgical resection [Figure 2].

Stratifying risk factors

In an optimal HCC patient, with a good patient performance, no distant metastasis, a well-compensated liver reserve and a technically feasible tumor for a resection, it has been shown that bilirubin levels and portal hypertension are additional independent survival predictors. It was shown that in Child-Pugh Class A patients, without a portal hypertension and with bilirubin levels < 1 mg/dL compared with the patients with a portal hypertension and bilirubin levels > 1 mg/dL; 5-year survival rates were 74% and 25%, respectively [Figure 3].^[18] In other words, patient who is a good candidate for surgical resection should be in stage Child A and moreover, they should be in a “better” Child A subgroup with a normal bilirubin level and without portal hypertension. The indicators of portal hypertension as splenomegaly, thrombocytopenia, and esophageal varices should be checked.

Hepatectomy size

In a normal healthy non-cirrhotic liver parenchyma, liver resections up to 70% are well-tolerated due to the intact regeneration capacity of the hepatocytes.^[19] The size of the hepatectomy must be as small as “oncologically” possible in HCCs. “The Makuuchi criteria” is an important algorithm for the HCC treatment in cirrhotic patients. These criteria use the

presence of ascites, total serum bilirubin levels, and the ICG disappearance rate for deciding the eligibility of the patients for a resection and the type of the surgical resection. In patients with uncontrolled ascites, bilirubin levels above 2 mg/dL, any type of hepatectomy is contraindicated. The ICG uptake rate in “Makuuchi criteria” is used as objective criteria for deciding the extent of the resection that can be safely performed. According to the ICG uptake resections, that can be safely performed are classified as, major hepatectomy (ICG < 10%), segmentectomy < 1/3 of liver (10% < ICG < 19%), subsegmentectomy < 1/6 of liver (20% < ICG < 29%), and a limited resection (ICG > 30%) [Figure 4].^[20]

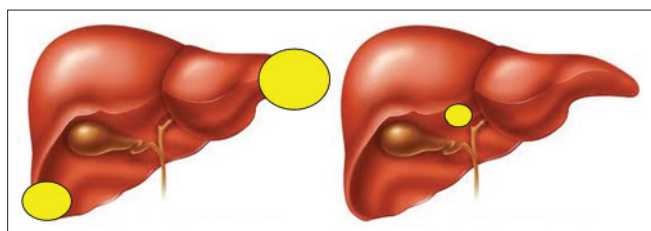


Figure 2: Location may be as important as the number and size of the tumors for technical feasibility of surgical resection

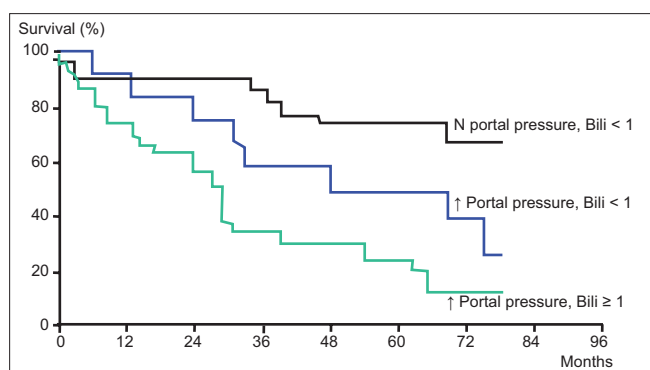


Figure 3: Resection of < 5 cm tumors in Child-Pugh A patients according to the bilirubin and portal hypertension (adopted from Llovet *et al.*^[18])

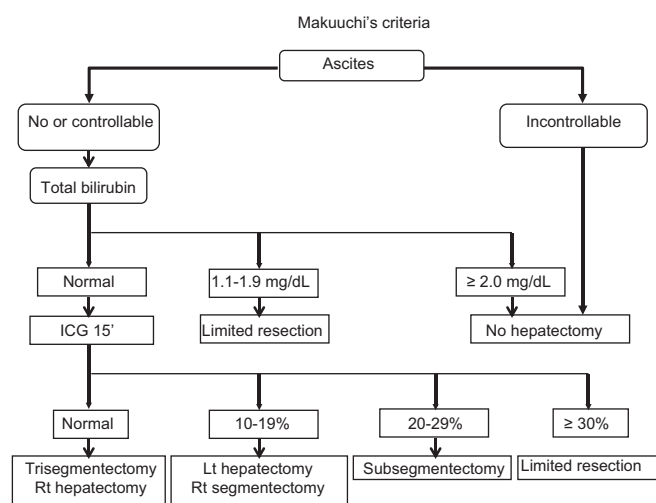


Figure 4: Makuuchi criteria for safe hepatectomy. ICG: Indo-cyanine Green; Lt: left; Rt: right

We have to say that while deciding a surgery for a patient with HCC, we use the BCLC and the other algorithms, as well. To avoid a misunderstanding, we have to highlight that we do not try to create an alternative system to the well-known systems (such as BCLC and others) to evaluate the HCC patients. This mnemonic flowchart may only help in assessing a systematic check of this important clinical decision-making process. Here, we just want to re-read the BCLC and other algorithms from another direction, but more simply and practically in the daily life. In our clinical practice, lots of HCC patients are referred to our department for the aim of resection from other cities by several clinicians. We observed that most clinicians (surgeons, but not an expert on liver surgery, oncologists, gastroenterologists, or internists) focus only on the size or number of the tumors in the liver while they were referring their patients. However, the general condition of the patient (mostly bedridden or not), bone pains (the possible metastasis), platelet counts, or presence of esophageal varices, *etc.*, can be overlooked before the transfers of the patients. Sometimes the simplest points are missing in the complex algorithms. “PERISH” flowchart can be used as a simple checklist in the clinical evaluation of the patients with HCC. This mnemonic flowchart could be more useful for the clinicians who are not experts on HCC. We believe that an easy learning method for the selection of the most appropriate candidates for surgical resection can create a charm among the non-expert clinicians on HCC, as well. This mnemonic can make the evaluation of the HCC patients more attractive due to its simplicity.

CONCLUSION

Asking for the patients’ general condition, that is, whether the patient is symptomatic and in bed > 50% of the day, should be the first question to select the correct cases for the resectable HCCs. Following this, asking for suspicious metastasis as bone pain and radiological evaluation of the abdomen and thorax is mandatory. Calculation of the Child-Pugh score is only the third step of the evaluation. Good candidates for a surgical resection should be Child-Pugh “A” but a better subgroup “A” with normal bilirubin levels should be preferred. Technical feasibility of the resection according to the intra-hepatic distribution of the tumor(s) should be done radiologically, and the patients preferably should not have portal hypertension. If the patient fulfills all the previous steps, the surgeon can perform the ICG clearance test, if necessary [Table 3].

As a result, following the “PERISH” flowchart in the treatment of any HCCs may prevent “perish” of the surgeons while deciding the appropriate treatment of HCCs.

Table 3: PERISH flowchart

PERISH flowchart

Performance of the patient (ECOG)
Extra-hepatic disease (metastasis)
Reserve of the liver (Child-Pugh score)
Intra-hepatic distribution (CT)
Stratifying risk factors (portal hyper-tension and bilirubin)
Hepatectomy size (Makuuchi-ICG)

ECOG: Eastern Cooperative Oncology Group; CT: computed tomography;
ICG: Indo-cyanine Green

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Nil.

Conflict of interest

There is no conflict of interest.

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Additional data and information can be uploaded as Supplementary Material to accompany the manuscripts. The supplementary materials will also be available to the referees as part of the peer-review process. Any file format is acceptable, such as data sheet (word, excel, csv, cdx, fasta, pdf or zip files), presentation (powerpoint, pdf or zip files), image (cdx, eps, jpeg, pdf, png or tiff), table (word, excel, csv or pdf), audio (mp3, wav or wma) or video (avi, divx, flv, mov, mp4, mpeg, mpg or wmv). All information should be clearly presented. Supplementary materials should be cited in the main text in numeric order (e.g., Supplementary Figure 1, Supplementary Figure 2, Supplementary Table 1, Supplementary Table 2, *etc.*). The style of supplementary figures or tables complies with the same requirements on figures or tables in main text. Videos and audios should be prepared in English, and limited to a size of 500 MB or a duration of 3 minutes.

2.4 Manuscript Format

2.4.1 File Format

Manuscript files can be in DOC and DOCX formats and should not be locked or protected.

2.4.2 Length

There are no restrictions on paper length, number of figures, or amount of supporting documents. Authors are encouraged to present and discuss their findings concisely.

2.4.3 Language

Manuscripts must be written in English.

2.4.4 Multimedia Files

The journal supports manuscripts with multimedia files. The requirements are listed as follows:

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The video or audio files should be limited to a duration of 3 min and a size of up to 500 MB.

Please use professional software to produce high-quality video files, to facilitate acceptance and publication along with the submitted article. Upload the videos in mp4, wmv, or rm format (preferably mp4) and audio files in mp3 or wav format.

2.4.5 Figures

Figures should be cited in numeric order (e.g., Figure 1, Figure 2) and placed after the paragraph where it is first cited;

Figures can be submitted in format of tiff, psd, AI or jpeg, with resolution of 300-600 dpi;

Figure caption is placed under the Figure;

Diagrams with describing words (including, flow chart, coordinate diagram, bar chart, line chart, and scatter diagram, *etc.*) should be editable in word, excel or powerpoint format. Non-English information should be avoided;

Labels, numbers, letters, arrows, and symbols in figure should be clear, of uniform size, and contrast with the background; Symbols, arrows, numbers, or letters used to identify parts of the illustrations must be identified and explained in the legend;

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All non-standard abbreviations should be explained in the legend;

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Tables should be cited in numeric order and placed after the paragraph where it is first cited;

The table caption should be placed above the table and labeled sequentially (e.g., Table 1, Table 2);

Tables should be provided in editable form like DOC or DOCX format (picture is not allowed);

Abbreviations and symbols used in table should be explained in footnote;

Explanatory matter should also be placed in footnotes;

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2.4.7 Abbreviations

Abbreviations should be defined upon first appearance in the abstract, main text, and in figure or table captions and used consistently thereafter. Non-standard abbreviations are not allowed unless they appear at least three times in the text. Commonly-used abbreviations, such as DNA, RNA, ATP, *etc.*, can be used directly without definition. Abbreviations in titles and keywords should be avoided, except for the ones which are widely used.

2.4.8 Italics

General italic words like *vs.*, *et al.*, *etc.*, *in vivo*, *in vitro*; *t* test, *F* test, *U* test; related coefficient as *r*, sample number as *n*, and probability as *P*; names of genes; names of bacteria and biology species in Latin.

2.4.9 Units

SI Units should be used. Imperial, US customary and other units should be converted to SI units whenever possible. There is a space between the number and the unit (i.e., 23 mL). Hour, minute, second should be written as h, min, s.

2.4.10 Numbers

Numbers appearing at the beginning of sentences should be expressed in English. When there are two or more numbers in a paragraph, they should be expressed as Arabic numerals; when there is only one number in a paragraph, number < 10 should be expressed in English and number > 10 should be expressed as Arabic numerals. 12345678 should be written as 12,345,678.

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Equations should be editable and not appear in a picture format. Authors are advised to use either the Microsoft Equation Editor or the MathType for display and inline equations.

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