

Tissue engineering using mesenchymal stem cells with periosteal wrap for bone defect repair in rabbits

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ABSTRACT

Aim: Mesenchymal stem cells (MSCs) are an excellent potential source of cells for bone tissue engineering due to their excellent renewal ability and osteogenic differentiation capabilities. This study was designed to evaluate the bone formation properties of a demineralized cancellous bone scaffold seeded with MSCs, with or without periosteum, in a critical size bone defect model in rabbits. **Methods:** Rabbit culture-expanded bone marrow (BM)-MSCs were seeded onto a human demineralized cancellous bone (HDCB) scaffold. Bone defects measuring 15 mm in length were created in each radius. A total of 56 bone defects in 28 rabbits were randomly assigned to one of the 4 groups for scaffold implantation: Group 1: HDCB graft only; Group 2: periosteum-wrapped HDCB graft; Group 3: HDCB graft seeded with BM-MSCs and Group 4: periosteum-wrapped HDCB graft seeded with BM-MSCs. All rabbits were sacrificed 12 weeks after surgery for gross observation, radiological assessment, histological analyses and biomechanical measurements. **Results:** New bone (NB) formation and bone healing were successfully achieved, both radiologically and histologically, on demineralized cancellous bone graft seeded with BM-MSCs. Results were improved when BM-MSCs were associated with periosteum. **Conclusion:** This study demonstrates that repair of bone defects in a rabbit model can be achieved through bone grafting using BM-MSCs, implanted on a demineralized cancellous bone scaffold. The formation of NB was optimized when combined with the preservation of periosteum at the site of injury.

Key words:

Bone defects, bone marrow, bone tissue engineering, mesenchymal stem cells, periosteum

INTRODUCTION

Despite numerous advances in orthopedic and plastic surgery, the repair of bone defects remains challenging. The

most desirable material for bone repair is autologous bone graft, due to its excellent osteoconduction, osteoinduction

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and osteogenesis properties.^[1,2] However, its limitations include additional surgical exposure required for graft harvest, limited bone supply and associated donor site morbidities.^[3,4] Vascularized bone grafts from various locations including the fibula, scapula and iliac crest may be indicated to stimulate bone formation and promote healing. However, harvest requires a complex microsurgery procedure, with the additional risk of including graft necrosis due to vessel thrombosis.^[5,6] Allografts may be a reasonable alternative, as small cancellous allografts can remodel completely. Larger grafts may be incorporated by limited intramembranous bone formation.^[1] However, allografts may increase the risk of infectious disease transmission.

Recent progress in the fields of biotechnology and tissue engineering has offered new options for the repair of traumatic and nontraumatic bone defects. Mesenchymal stem cells (MSCs), which are multipotent adult stem cells of mesodermal origin, have been shown to play a critical role in tissue engineering. MSCs are an excellent potential source of cells for bone tissue engineering due to their excellent renewal ability and osteogenic differentiation capabilities.^[7,8] In addition to the bone marrow (BM), MSCs are also derived from the periosteum. It is well known that the development and regeneration of bone depend on the presence of periosteum and BM.^[9] When transferred to the site of bone damage, MSCs multiply and differentiate into osteoblastic cells, contributing to the production of bone tissues that form a callus at the bone defect site.^[10] Alternatively, bone tissue engineering can be achieved via intramembranous ossification.^[11]

The use of MSCs with an appropriate scaffold has been demonstrated to be promising in guiding bone tissue neof ormation after implantation in the host. Cell repopulation can be achieved either by direct cell loading or indirect cell induction with osteogenic factors.^[12,13] Combining MSCs with appropriate scaffolds has been shown to improve the overall osteoconductivity of the scaffold. The search for an ideal scaffold has led to the development of reconstructive options to engineer new bone (NB) tissue. The ideal scaffold should be biocompatible, noninfectious, resorbable, osteoconductive and osteoinductive.^[14] Demineralized bone matrix (DBM), which is derived from either allogenic or xenogenic bone, is available commercially for clinical application and satisfies some of these requirements.^[4] DBM has been used for several decades in humans for the treatment of nonunion and bone defects following injury or tumor resection. The process of demineralization using hydrochloric acid destroys potential bone forming agents, but also decreases antigenic stimulation and may expose the bone morphogenic protein located within the bone matrix.^[1,4] This study is designed to evaluate the bone formation properties of a demineralized cancellous bone scaffold seeded with allogenic MSCs, with or without periosteum, in a critical sized bone defect model in rabbits.

METHODS

Bone marrow mesenchymal stem cells isolation and expansion

Bone marrow mesenchymal stem cells (BM-MSCs) were isolated from rabbits and cultured as reported previously.^[9,11,13,15] The BM aspirates (5 mL) were obtained from the femurs of 5 rabbits that were 8 weeks old. The BM aspirates were layered over a Ficoll gradient and centrifuged at 2,000 rpm for 20 min at room temperature. Mononuclear cells at the interface were collected and cultured in Dulbecco's Modified Eagle Medium-Low glucose supplemented with 15% fetal bovine serum (FBS), 10 mM L-glutamine, 10 ng/mL epidermal growth factor, 10 ng/mL β -fibroblast growth factor and 1% antibiotic antimycotic solution (10,000 U/mL penicillin, 10 mg/mL streptomycin and 25 μ g amphotericin B) (Sigma-Aldrich, USA). Cultures were maintained in a CO₂ incubator (Shel Lab, USA) at 37 °C and 5% CO₂. The medium was replaced after 3 days and cell layers were washed twice with phosphate-buffered saline to remove nonadherent cells. The passage was carried out when cultures reached 90% confluence using 0.25% trypsin-ethylenediaminetetraacetic acid (Sigma-Aldrich, USA). Cell confluence was normally achieved after 12-14 days.^[14,16-18]

Scaffold materials

Human demineralized cancellous bone (HDCB), which is a type of DBM and has been proven to be usable as scaffold material, was used in this experiment. HDCB was supplied from the Bone Bank at the National Institute of Burns. Fresh bones were aseptically harvested within the first 12 h after being shown to be free of any infectious disease. Bones were treated with H₂O₂, a mixture of methanol/chloroform, hydrochloric acid and phosphate buffer pH 7.4. Subsequently, the bones were dehydrated for 24 h until the water content remaining in the bones was less than 5%. The bones were cut into blocks with dimensions of 1.5 cm \times 0.3 cm \times 0.5 cm. A medullary hole was made in the bone blocks with a diameter of 1.5 mm. The block was packaged and sterilized by gamma irradiation at a dose of 25 kGy. The sterilized bones were then preserved at 4 °C.

Tissue engineered bone graft preparation *in vitro*

Culture-expanded BM-MSCs were seeded evenly onto the HDCB scaffold. DHCB/BM-MSCs were cultured in T flasks (Thermo Scientific Nunc A/S, Denmark) filled with 5 mL DMEM containing 10% FBS and antibiotics. The grafts were placed in a vacuum desiccator and treated at a pressure of 100 torr for 100 s, after which they were incubated at 37 °C, 5% CO₂ for 2 weeks. The medium was replaced every 3 days.^[2,19-21]

Animals and surgical procedure

Twenty-eight male 8-week-old white New Zealand rabbits with a body weight of approximately 1.5 kg from the Experimental Laboratory of the Medical Training and Research Center, Hue Central Hospital, were used for the study. All the experimental study involving animals was approved by the Institutional Animal Care and Use

Committee and the Ethics Committee of the Medical Training and Research Center. The rabbit bone defect model was established as described previously.^[11,13] The rabbit was anesthetized with a combination of intravenous sodium pentobarbital at 20 mg/kg and intramuscular ketamine at 50 mg/kg. The anterolateral side of the forelimb was shaved and sterilized with 10% povidone-iodine. The radius was exposed through a longitudinal incision by gentle retraction of the muscles. An osteotomy gap of 1.5 cm was created in the diaphysis. Periosteum from the excised bone was preserved in the group that would later receive periosteal encapsulation of scaffolds. The ulna was left intact for mechanical stability [Figure 1]. The bone defect was created on both forelimbs of the animals. A total of 56 bone defects within the 28 rabbits were randomly assigned to one of the four groups for scaffold implantation: Group 1: HDCB graft only; Group 2: periosteum-wrapped HDCB graft; Group 3: HDCB graft seeded with BM-MSCs; and Group 4: periosteum-wrapped HDCB graft seeded with BM-MSCs. After implantation, muscle, fascia and skin were separately closed over the defect and no internal or external fixation was used. Forelimbs were postoperatively supported by a carton splint for one week. Each rabbit was administered 400,000 units of penicillin preoperatively on the 1st postoperative day to prevent infection. All rabbits from each group were sacrificed 12 weeks after surgery for gross observation of the growth of callus, radiological assessment, histological analyses and biomechanical measurements.

Gross observation

Following sacrifice, both reconstructed radiuses were harvested and completely cleared from the soft tissues. The status of callus growth, degradation, bone healing and NB formation at the bone graft in the radius were observed.

Radiological assessment

Radius bone specimens in each group were X-rayed for the evaluation of bone formation and remodeling (Titan 2,000, COMED Medical Systems CO. Ltd., Korea). Assessment of NB formation and remodeling was based on the modified Lane and Sandhu radiological scoring system.^[1] Three experts blindly assessed the radiological scores, which were the sum of the scores of bone formation and remodeling. The score for NB formation was assigned as 0 (no NB formation), 1 (less than 25% NB formation),

2 (25-50% NB formation), 3 (50-75% NB formation) or 4 (more than 75% NB formation). The score assigned to the assessment of union was 0 (nonunion), 1 (possible union) or 2 (radiographic union). The proximal and distal unions of the bone graft were separately evaluated. The remodeling score assigned was 0 (no evidence of remodeling) 2 (intramedullary remodeling) or 4 (cortical remodeling). The maximum number of points, which could be achieved, was 10 for each reconstructed bone.

Histological analyses

Fifty-two specimens from the bone graft sites of the radius were successfully fixed with 10% paraformaldehyde, decalcified with sodium formate and embedded in paraffin. Four specimens in Group 1 experienced technical failures. Three sagittal sections were cut with a slow speed saw from each site at the distal, proximal and middle lines of the bone graft. Sections were then prepared and stained with hematoxylin and eosin. The micrographic images from the light microscope were quantified. Images from each section were taken to evaluate the bone formation ratio by a qualified pathologist blinded to the study. The NB formation ratio was calculated by the percentage area of bone tissue within the defect site, and a mean value was determined for each section.

Biomechanical analysis

The specimens of the radius of each group were loaded onto a multifunctional mechanical tester (Instron 5582 Universal Tester, USA) for the performance of a uniaxial compression test. The specimen was placed between compression plates. Force was applied to the specimens at a constant speed of 1 mm/min until fracture occurred. Compressive stress and strain were calculated and plotted. Stress value at the point of yield (load-to-failure) was determined.

Statistical analysis

The data were presented as mean and standard deviation. The Student's *t*-test was performed to compare the difference between the mean values of two groups using Statistical Product and Service Solutions version 15.0 (SPSS, Inc., USA). Differences at a level of $P < 0.05$ were considered to be statistically significant.

RESULTS

The wounds healed completely after one week and the rabbits were noted to regain full movement within two weeks. All the rabbits survived with normal behavior. No complications such as infection or necrosis were recorded prior to sacrifice.

Gross observation

At 12 weeks after surgery, radii implanted in Group 1 showed a small amount of callus and fibrous-like tissue in the interspaces between the defects and grafts. Partial degradation of the HDCB grafts was found. There was a significant amount of callus and bony union filled more than half of the defects in Groups 2 and 3. The HDCB grafts in these groups were almost degraded. In Group 4, good bony union was observed. Bone defects were almost completely remodeled with NB tissue and

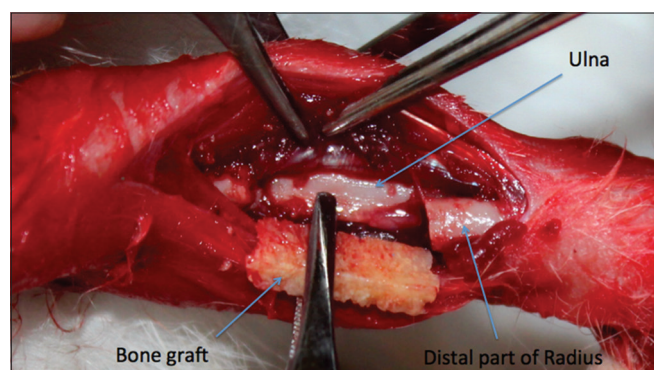


Figure 1: The procedure for the transplantation of cancellous bone graft into the segmental radial defect

the HDCB grafts were completely degraded in this group [Figure 2].

Radiological outcomes

At 3 months postoperatively, there was a small amount of callus formation at the defect gaps in Group 1. NB formation was found to account for over half of the material at the reconstructed bone in Groups 2 and 3. Bone regeneration in the radius in Group 4 was observed to be the best, where callus formation was greatest in comparison to the other groups [Figure 3]. With the radiological score results, the mean score in Group 4 was 8.58 ± 0.64 , which was significantly higher than the other three groups ($P < 0.05$). There was a significant difference between Groups 2 and 3 ($P < 0.05$). The mean scores in Groups 2 and 3 were significantly higher than those in Group 1 ($P < 0.05$) [Table 1].

Histological observations

Inflammation was not observed in the grafted bone segment. Poor NB formation and capillary network were found at the interface between the graft and radius in Group 1. Both ends of the original radius were united with newly regenerated bone in Groups 2 and 3, while the HDCB scaffold was mostly degraded and cortical bone was only observed at the center of the defects. A larger amount of NB was generated along the entire scaffold structure and more capillaries were formed in the area of NB in Group 4. Group 4 showed superior bone union, cancellous bone, cortical bone, marrow formation and capillary formation in comparison to the other groups. Cortical bone was also found along the entire gap of the bone defect, bridging adjacent native bone [Figure 4]. The newly formed bone area in Group 4 increased to $80.5\% \pm 4.96\%$, which was significantly higher when compared with Group 3 ($64.12\% \pm 11.31\%$), Group 2 ($49.79\% \pm 11.69\%$) and Group 1 ($29.6\% \pm 8.33\%$) ($P < 0.05$) [Table 1]. Statistically

significant differences were found between Groups 2 and 3 ($P < 0.05$), while both groups were statistically superior as compared to Group 1 ($P < 0.05$) [Table 1].

Biomechanical testing results

Radii of rabbits with partial or complete union were subjected to biomechanical testing. Results of the biomechanical tests are summarized in Table 1. Group 4 showed the highest compressive strength ($P < 0.05$). Group 3 of HDCB grafts seeded with BM-MSCs showed significantly higher compressive strength than both Groups 1 and 2 ($P < 0.05$). The difference between Groups 1 and 2 was statistically significant ($P < 0.05$) [Table 1].

DISCUSSION

This study demonstrates the presence of NB formation and bone healing, as shown both radiologically and histologically, on demineralized cancellous bone graft seeded with BM-MSCs. Results were improved when BM-MSCs were associated with periosteum.

MSCs, periosteal cells and osteoblasts have all been successfully used for bone tissue engineering.^[4,18] In particular, BM-MSCs play a major role in the repair of bone defects.^[22-25] They are capable of self-replication and differentiation into osteocytes in appropriate culture conditions and can contribute to the regeneration of mesenchymal tissues such as bone.^[3,26] BM-MSCs can be rapidly expanded *ex vivo* without loss of their multi-lineage differentiation potential.^[13] They are readily available and amenable to genetic manipulation. BM-MSCs can, therefore, be viewed as a viable alternative for bone tissue engineering.^[8,11,27,28]

The anatomy of the periosteum, its nutrient transport and its osteoinductive and osteoconductive capacities have been well described.^[29] Periosteum plays a primary role in bridging callus formation and participating in endochondral and intramembranous ossifications in fracture healing.^[30] Previous studies have shown that the

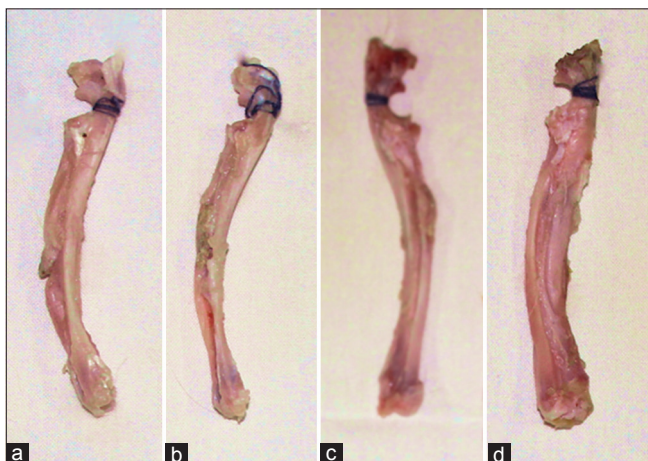


Figure 2: Gross observations of the reconstruction of radius at 3 months after surgery. (a) Small amount of callus and fibrous-like tissue in the interspaces between defect and human demineralized cancellous bone graft in Group 1; (b) callus formed in the defect repair by periosteum-wrapped human demineralized cancellous bone graft in Group 2; (c) significant amount of callus and bony union filled in the defect repair with the human demineralized cancellous bone graft seeded with mesenchymal stem cells in Group 3; (d) complete bone healing in the defect repair by periosteum-wrapped human demineralized cancellous bone graft seeded with bone marrow mesenchymal stem cells in Group 4

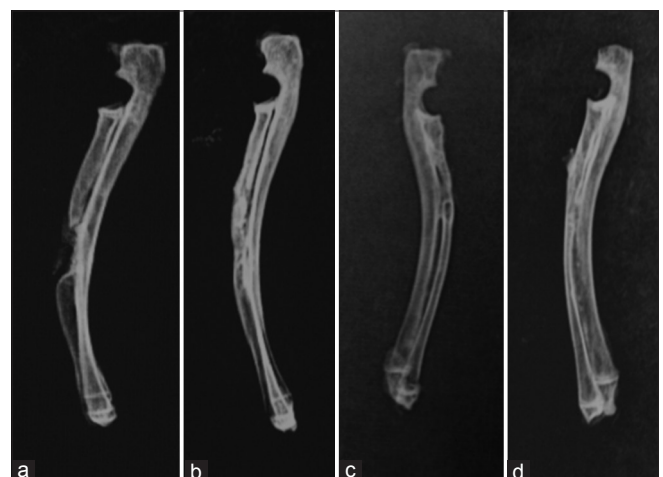


Figure 3: Results of X-ray at the 3 months postoperation. (a) A few calluses at the defect gap in Group 1; (b) significant new bone information at the reconstructed bone in Group 2; (c) more new bone formation between graft and bone tissue in Group 3; (d) almost remodeling of new formed bone along the entire gap of the bone defect in Group 4, and the cortical bone bridged to the adjacent native bone

Table 1: Modified Lane and Sandhu radiological scores, mean new bone formation in Histology (%), and mean compressive strength (MPa) of the rabbit's radius in each group at 3 months after surgery

Group	Scaffold implantation	Mean radiological scores	Mean compressive strength (MPa)	Mean new bone in Histology (%)
1	HDCB only	2.95 ± 0.58	31.14 ± 6.72	29.60 ± 8.33
2	Periosteum-wrapped HDCB	5.57 ± 0.51	73.00 ± 7.20	49.79 ± 11.69
3	HDCB/BM-MSCs	6.41 ± 1.03	80.57 ± 8.50	64.12 ± 11.31
4	Periosteum-wrapped HDCB/ BM-MSCs	8.58 ± 0.64	129.31 ± 5.99	80.50 ± 4.96

HDCB: Human demineralized cancellous bone, BM-MSCs: Bone marrow mesenchymal stem cells

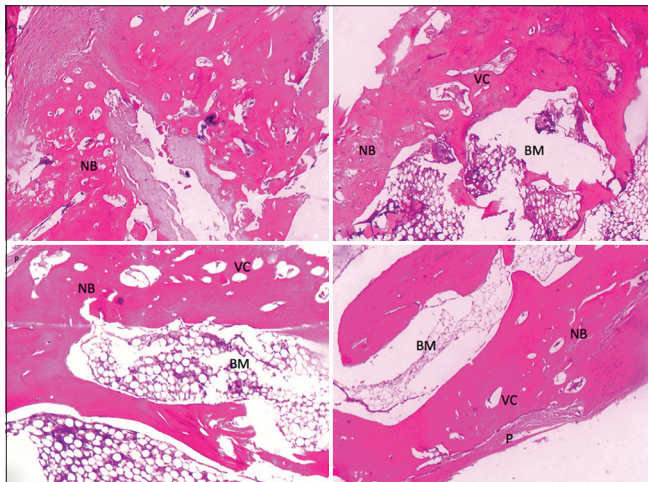


Figure 4: HE stained histological sections from the grafted bone of four groups at 3 months after implantation (original magnification, ×40). NB: New bone, VC: Vascular cavity, BM: Bone marrow, P: Periosteal membrane

inner cambium layer is highly cellular and populated with cells, which influence bone formation and bone repair, including adult mesenchymal skeletal progenitor cells.^[29,31] These progenitor cells proliferate and differentiate into osteoblastic and chondroblastic cells, driving the process of bone repair via either direct intramembranous bone formation or indirect endochondral mechanisms, respectively.^[32] On the contrary, the absence of periosteum reduced by 75%, the number of osteoblasts on devitalized bone graft, which correlated with the poor remodeling activity of the bone graft.^[33] These features indicate that periosteum should be considered to be a structure with regenerative capacity. This suggests the need to restore the essential osteogenic activity of periosteum on bone graft in combination with grafting of MB-MSCs. This approach assists in the early induction of a reparative response by an increase in the formation of a cortical shell around the grafted bone.^[34,35] Agata *et al.*^[34] have also shown that periosteal cells act as progenitor cells with the ability to proliferate and expand. Thus, periosteum-derived cells are another suitable source for bone tissue engineering.

Based on clinical observation, radiologic examination, histological analyses and biomechanical measurements, the current study supports the essential role of periosteum in the process of bone repair. In addition, the regenerative effect of combining BM-MSCs with periosteum showed better outcomes in both the quantity and quality as compared to BM-MSCs alone. Furthermore, the MB-MSCs used in the current study are derived from an allogenic

source, which is more convenient for isolation and expansion when compared with periosteum-derived cells. To further enhance the current bone tissue engineering strategies, a successful cellular replacement for periosteum or tissue-engineered periosteum should be investigated. Zhang *et al.*^[11] previously reported successful regeneration of segmental bone defects in rabbit ulnas using periosteum encapsulated scaffolds seeded with MSCs, with an increase in the newly formed bone area to 80.1% ± 9.6%. This result is compatible with the results of the current study at 80.5% ± 4.96%.

Xenogeneic demineralized cancellous bone grafts, which have the advantages of favorable cellular compatibility and histocompatibility as a scaffold, have widely been used for the repair of short bony defects showing the induction of NB formation and good mechanical properties. Osteoinductive structures in demineralized bone graft include a series of low-molecular-weight glycoproteins with bone morphogenetic proteins. These proteins promote chondroblastic differentiation of mesenchymal cells and create NB formation via endochondral osteogenesis.^[1,31,35] The bone formation process increases when decalcification of cortical bone exposes osteoinductive growth factors buried within the mineralized matrix. However, bone grafting has not been successful in the repair of large bone defects.^[13] BM-MSCs, which can be seeded to the HDCB graft for construction of the tissue engineered bone graft, has been suggested as an effective option for the reconstruction of large bone defects.

In the group repaired by periosteum-wrapped HDCB graft seeded with BM-MSCs, bone healing and union were significantly accelerated as compared to the other three groups. Increased density at the graft site and early fusion of cortical bone were observed. In addition to NB formation demonstrated histologically, a significant amount of regenerated capillary vasculature between the NBs was also being observed in a high proportion of grafted bone pores. Zhang *et al.*^[11] reported similar results when incorporating MSCs and periosteum-loaded poly scaffolds. However, our findings have notable differences from the results of Zhang *et al.*^[11] as HDCB/BM-MSCs grafts were significantly superior to periosteum-wrapped HDCB grafts in terms of union rates and capillary density.

For improved biochemical analysis for bone regeneration, a three-point bending test should be performed to evaluate the degree of scaffold integration with the host bone.

In conclusion, this study demonstrates that repair of bone defect in a rabbit model can be achieved through bone grafting using BM-MSCs implanted on a xenogeneic demineralized cancellous bone scaffold. NB formation was optimized with the preservation of the periosteum at the site of injury. The combination of biocompatible material, the ability for self-renewal and differentiation of MSCs with the augmenting effects of periosteum may prove to be an extremely promising approach in the fields of orthopedic and plastic surgery.

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Conflicts of interest

There are no conflicts of interest.

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