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Original Article

Enhanced periodontal regeneration and Cbfa1 expression in alveolar bone defects in dogs treated with Emdogain

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Abstract: Objectives: To provide detailed histological characteristics of newly formed periodontal tissues and determine the expression level of Cbfa1 following Emdogain (EMD) application in alveolar bone defects in beagle dogs. Methods: Eight one-year-old male beagles were used. Two untreated beagles served as blank control. Alveolar bone defects were created in the other six dogs in mesial and distal aspects of the mandibular second premolars bilaterally. The left side of the jaw was treated with EMD and the right side with open flap debridement (OFD) (control group). Then, the periodontal regeneration and Cbfa1 expression were investigated using hematoxylin-eosin staining and immunohistology methods. Results: There was significantly more periodontal regeneration in EMD-treated group than in OFD group. The intensity and number of Cbfa1-positive fibroblasts in OFD group were significantly less than those in EMD-treated group and blank control ($P<0.05$), while the difference between EMD group and blank control was not significant ($P>0.05$). Conclusion: EMD can induce significant periodontal regeneration. The osteogenic function of EMD may involve upregulation of Cbfa1 expression.

Keywords: Emdogain, periodontal regeneration, Cbfa1

Introduction

The ideal treatment for periodontitis involves the recovery of periodontal tissues that have been destroyed by diseases. Various procedures have been advocated for the regeneration of periodontium, including the placement of bone grafts or bone substitutes, root surface conditioning, or the use of organic or synthetic barrier membranes in guided tissue regeneration (GTR) [1-3]. However, periodontal regeneration is a complex process involving molecular interactions between mesenchymal and epithelial cells. The essential features of successful regeneration include cementogenesis, osteogenesis, assembly of periodontal ligaments onto the acellular cementum and arrest of apical proliferation of epithelial cells [4]. Even under guided conditions, however, treatment modalities do not always result in true regeneration due to the improper organization of the established collagen fibers within the new cementum.

Recently, a purified enamel protein matrix, Emdogain (EMD), has been introduced as an alternative substrate for periodontal regeneration [5]. EMD is prepared from developing porcine teeth, which is 90% amelogenin and contains no detectable growth factors [6]. EMD is unique in that the proteins do not provide a volumetric filling of the defect, nor do they act as a barrier to exclude certain cell types. Instead, EMD may provide “directives” for cell function through cell attachment to enamel proteins. The regenerative capacity of EMD has been demonstrated in both animal and clinical studies. In clinical trials, EMD treatment significantly improved probing depth and clinical attachment level [7, 8]. These clinical studies

suggest that EMD plays a role in the promotion of bone regeneration. In animal studies, locally applied EMD was shown to increase trabecular formation in a long bone repair rat model [9]. EMD may also promote the repair of circular defects in rat parietal bone [10]. However, EMD fails to show any significant bone formation around titanium implants in rabbit and dog models [11, 12] .

Some *in vitro* studies have unveiled the osteogenic effects of EMD underlying its tissue regenerative activities, albeit not consistently [13, 14]. The large amount of bone filling following EMD application to periodontal defects also suggests an osteogenic effect. Other studies have shown that EMD may affect the proliferation or differentiation of osteoblastic cell lines [15, 16].

Core binding factor a1 (Cbfa1), also known as Runx2, Osf2 and AML3, is a member of the runt homology domain family of transcription factors. It regulates the expression of all major genes expressed in osteoblasts and is essential for osteoblast differentiation and bone formation [17]. Cbfa1/Runx2-knockout mice do not form bone due to an absence of mature osteoblasts [18]. Furthermore, Takayama demonstrated that EMD caused a substantial increase in the expression of Cbfa1/Runx2 mRNA and protein, which preliminarily suggests that Cbfa1/Runx2 acts as a transcriptional factor in EMD [19]. In addition, Cbfa1 is shown to regulate the deposition rate of differentiated osteoblast bone matrix [20]. Thus, Cbfa1 is a critical gene not only for osteoblast differentiation but also for osteoblast function [21].

However, the current knowledge regarding the characteristics of newly-formed cementum after various regenerative modalities in human, including EMD, is conflicting [22, 23]. Few studies have attempted to systematically characterize the regenerated periodontal tissue following EMD application; the available information about the mechanisms through which EMD influences cell function is relatively limited. Furthermore, there are many inconsistent opinions as to whether EMD induces the expression of Cbfa1 in periodontal regeneration. In some *in vitro* studies EMD is shown to stimulate Cbfa1/Runx2 mRNA transcription and protein expression [24, 25]; nevertheless, other studies fail to observe EMD-induced changes in the expression of Cbfa1 mRNA [26].

Therefore, the aims of the present study were to provide additional systematic histological evidence of the periodontal healing following EMD application in dogs, and to determine whether Cbfa1 is expressed in the EMD-treated periodontal tissues, in order to have better understanding of EMD-induced changes at molecular level.

Material and methods

Creation of experimental bone defects

Throughout the experiments, the animals were maintained and treated following protocols approved by the Institutional Animal Care and Use Committee, Sichuan University, Chengdu, China. All animal selection, surgery, and periodontal defect preparation were conducted by the same periodontal specialist, and these processes were blinded to other experimenters.

Eight one-year-old male beagles with intact dentition and healthy periodontium, each weighing approximately 10 kg, were chosen for this study. The animals were fed with soft diets throughout the study to reduce the chance of mechanical interference with the healing process during food intake.

Three months prior to the start of the experiment, the first and third premolars on each side of the mandible were extracted in eight beagles, and the extraction sites were allowed to heal for 3 months. Prior to extraction, the dogs were anesthetized with 0.1 mL/kg of Su-Mian-Xin II (a mix of haloperidol and DHM99 produced by the Veterinary Research Institute of Military University, China). The mouth was opened, and the mandibular quadrants were prepared by applying 0.5% chlorhexidine in 70% ethanol. Bacterial plaque and dental calculus were removed manually. For the six dogs randomly chosen from the eight dogs, full-thickness mucoperiosteal flaps were elevated at both the vestibular and oral aspects of the jaw. Under constant sterile saline irrigation, we produced approximately 5-6 mm-deep intrabony periodontal defects by using a sterile drill needle (1.0 mm in diameter) at both mesial and distal surfaces of the mandibular second premolars distal to the cemento-enamel junction

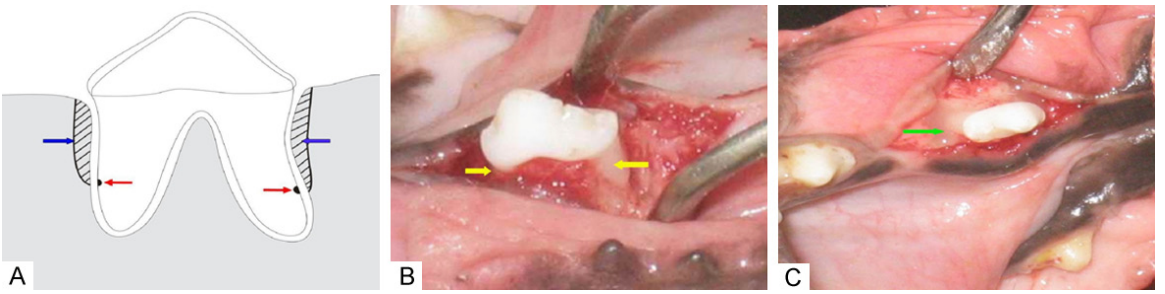

Figure 1. Creation of alveolar bone defects and application of EMD. A: Scheme diagram of the defeats; blue arrows, alveolar bone defects; red arrows, reference notches on the teeth surface; B: Surgical defects of alveolar bone (yellow arrows); C: EMD injected into bone defect (green arrow).

and reference notches, indicating the apical border of the defect. The root surfaces were scaled and planed manually to remove the entire root cementum. The smear layer on the root surface was washed away with 24% ethylenediaminetetraacetic acid (EDTA, pH7) for 2 minutes (**Figure 1A** and **1B**).

Periodontal regeneration with EMD and tissue block harvest

Immediately after the EDTA was thoroughly rinsed from the tooth with sterile physiological saline, ready-to-use EMD (Emdogain, provided by Biora, Malmö, Sweden) was injected into the defects (3 mg/defect) and filled to the surface starting at the base of the bone defect on one side of the jaw (**Figure 1C**). The other side was used as a control and did not apply EMD. The flaps were then returned to the original position and sutured using a 4-0 nylon suture. Clindamycin (20 mg/kg, bid, po) was administered for 7 days after the procedure to prevent infection. The animals were fed with a high-calorie supplement gel (Nutri-Cal) for 3 days following surgery. Hygiene procedures were performed twice a week, including tooth brushing and a topical application of 0.2% chlorhexidine 6 weeks after surgery. After this period and for the rest of the experiment, oral hygiene measures were performed twice a week as described above.

The six dogs were sacrificed 3 months after EMD was applied and the two blank controls were also sacrificed at the same time. The dogs were perfused through the carotid arteries with a solution of 10% neutrally buffered formalin. The teeth of interest and their surrounding bones and periodontal tissues were cut and placed in a solution of 10% neutrally buffered formalin for about 48 h and decalcified for 3 months in a solution of 10% EDTA that was replaced daily.

Histological examination

The decalcified tissue blocks were dissected into 5 μm thick sections through the sagittal plane and mounted on RNase-free glass slides (Menzel, Germany) and dried at 37°C overnight. Then the sections were embedded in paraffin for 24 h and deparaffinized in xylene for 10 min, and stained with a conventional hematoxylin and eosin (H&E) and examined under light microscope.

Immunohistochemical (IHC) examination

After deparaffinization, the sections were rinsed in 0.01 M phosphate-buffered saline (PBS, 3×10 min), followed by incubation in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were rinsed again and pre-incubated with 0.1% Triton X-100 in 0.1 M sodium citrate for 10 min. Sections pre-treated with 0.1% trypsin (Dako S2012) for 30 min at 37°C were incubated with TBS containing 3% bovine serum albumin (TBS/BSA) for 30 min to avoid nonspecific background staining. Subsequently, the sections (without additional washing) were reacted with primary antibody (Cbfa1: SC12488, Santa Cruz, 1:100 dilution with 2% BSA in 0.05% Triton X-100) for 60 min at 37°C, washed with TBS plus 0.1% Tween 20, and then incubated with biotin-conjugated rabbit anti-goat IgG (E0466, Dako, 1:200) for 30 min at 37°C. After rinsing again, the sections were incubated with StreptABComplex/HRP (K0377, Dako, 1:100) for 30 min at 37°C. After another rinse, the tissue sections were stained in a 3,3'-diaminobenzidine (DAB, Sigma

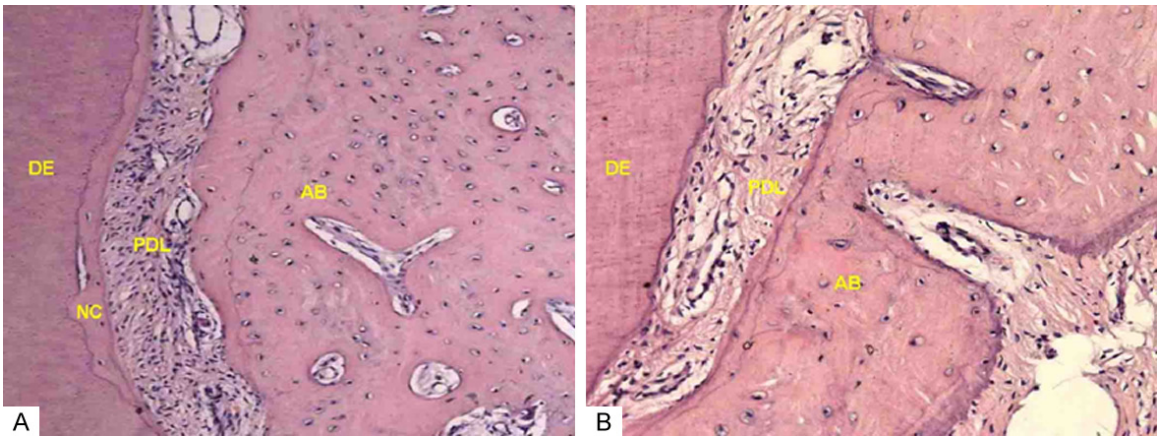

Figure 2. Histological micrographs of periodontal repair three months after treatment (HE staining, ×20). A: EMD-treated group, showing the regularity of fiber arrangement, functionally orientated collagen fibers inserted into the newly formed cementum; B: OFD-treated group, showing disordered fiber trabeculae.

Chemical Co., USA) solution, rinsed and counterstained with Mayer's hematoxylin. The resulting slides were dehydrated and coverslipped for light microscopy analysis.

Semi-quantitative image assessment and statistical analysis

Five sections of each periodontal defect were taken on a random basis for the immunohistochemical staining and light microscope observation. Five fields of each stained section were selected randomly and photographed at the same luminous density and magnifying power (×400). All images were analyzed using the Image-Pro® plus image analysis software (Mediacybernetics, Silver Spring, MD). Area of positive sites (brown colored), mean optical density and integral optical density (IOD) of each vision were calculated to represent the degree of antigen expression.

Statistical analysis

Data were expressed as mean ± SD and analyzed by SPSS 17.0 software. For comparison between groups, the data were analyzed by ANOVA followed by paired or unpaired Student's t test. A value of $P<0.05$ was considered statistically significant.

Results

An oral inspection of the dogs revealed that the sutures in the mucosal flap could be removed 1 week after surgery in both EMD and OFD-treated groups and that there was no infection of the surgical areas in all the animals.

Histological observation of the eight dogs was performed for all sites of interest. We did not observe acute inflammation or foreign-body giant cell reaction in any of the specimens, nor did we note ankylosis or root resorption.

Histological examination

EMD-treated sites

Periodontal regeneration occurred to various extents in the defects treated with EMD (**Figure 2A**). The newly formed cementum was of an acellular type in lower portion of the defects, whereas in the more coronal portion of the defect, we observed a mixed cellular and acellular cementum. Moreover, functionally oriented collagen fibers were seen inserting into the newly formed cementum, oriented perpendicularly to the tooth surface and connected to the newly formed bone.

OFD-treated sites

The continuous layer of new cellular cementum that formed on the denuded root surface was limited to the apical and coronal ends of the defects. The new cementum and epithelium were similar to those observed in the EMD-treated sites; however, the cementum was rather thin. Periodontal regeneration was limited to the bottom of the defects. Artifacts (splits between the new cementum and the dentin

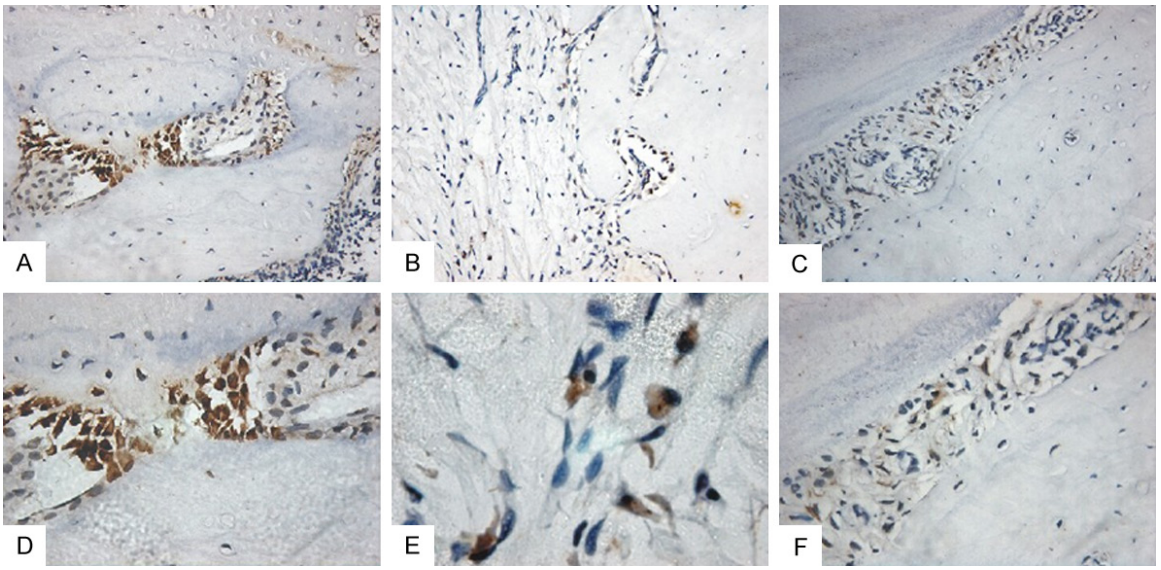

Figure 3. Immunohistochemical analysis of Cbfa1 expression periodontal tissue. A, D: EMD-treated (×20 and 40); B, E: OFD-treated (×20 and 40); C, F: Normal periodontal tissue (×20 and 40).

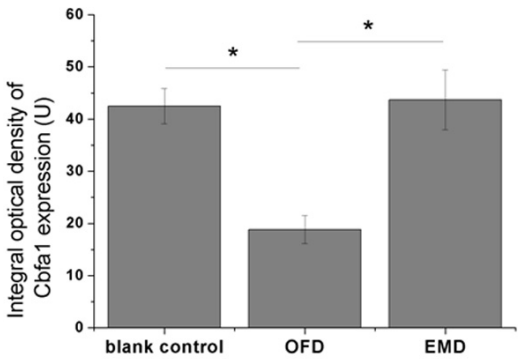

Figure 4. Immunohistochemistrial measurement of Cbfa1 expression in blank control, EMD and OFD group. The intensity of staining was quantified using Image Pro Plus software. Data were expressed as mean ± SD. *P<0.05 between the two groups.

surface) were observed in nearly all specimens (**Figure 2B**).

To sum up, there was obviously more periodontal regeneration in EMD-treated than in OFD-treated group.

IHC examination

Cbfa1-positive signals were detected in the fibroblasts and the extracellular matrix (ECM) in the periodontal ligament (PDL) of all the three groups (**Figure 3**). In EMD group, the number of positive stained osteoblasts was quite abundant. Cells located near the edge of the bone marrow had hyperchromatic cytoplasm and ovoid nuclei, indicating that they were extremely active. Moreover, Cbfa1 protein was found in the cytoplasm. In the control group, Cbfa1 protein was also observed in the osteoblasts near the alveolar bone, but there were much fewer stained cells and a lower intensity of staining compared to the EMD-treated group.

The IOD of Cbfa1 expression in OFD group was 18.83 ± 2.68, significantly less than 43.71 ± 5.73 in EMD group and 42.47 ± 3.36 in blank control (P<0.05), while the difference between EMD group and blank control was not significant (P>0.05) (**Figure 4**).

Discussion

EMD has been a focus of periodontal regeneration since Heiji first found that the new cementum formed by the procedure consisted of acellular cementum in a patient in 1997 [5]. Commercial EMD is prepared from the tooth germ of 6-month-old pigs and marketed as Emdogain®. The rationale for using EMD is based on the observation that enamel matrix proteins (EMP), synthesized and secreted by Hertwig's epithelial root sheath cells, can induce differentiation of dental follicle cells into cementoblasts. These cells are possibly responsible for the formation of acellular extrinsic fiber cementum (AEFC), which participates in tooth anchorage. In the previous studies,

newly formed cementum appeared to be similar to AEFC, and artifacts were either absent or only sparsely observed [27, 28].

Our investigation, however, showed that acute surgical defects treated with EMD resulted primarily in acellular cementum. This observation was similar to that of previous studies, which found that newly formed cementum was of acellular type, regardless of the periodontal regeneration therapy used in clinical practice [27, 29, 30]. The results of our study are also in line with recent findings from a study on the association between Hertwig's epithelial root sheath cells, EMP, and cementogenesis in porcine teeth. In that study, researchers did not demonstrate a direct link between EMP and the formation of AEFC [29]. Similar results were documented in several comparable human studies, with "mixed cellular cementum" or "cellular cementum with inserting fibers" used to describe the newly formed cementum instead of AEFC [23, 31].

Additional studies have shown that the AEFC increases in thickness by an average of several micrometers per year, indicating that the formation of AEFC is extremely slow [32]. In contrast to the slow formation of AEFC, the formation of cellular cementum is rapid [33]. The speed of cementum formation may depend on incorporation of cementocyte into the reformed cementum. This suggestion is supported by the results of our study, in which a large amount of cellular cementum was formed in a relatively short period of time (i.e., 3-6 months). This study, together with data from previous research, demonstrates that AEFC does not form predictably after EMD application in periodontal regeneration.

Some investigators hypothesize that artifacts between root surfaces and the newly formed cementum result from the decalcification process during the histological preparation of the specimens [34]. However, we observed artifacts in only one specimen in the EMD group, whereas no artifacts were seen in all specimens in the OFD-treated group. Thus, we speculated that the artifacts likely resulted from a weak attachment between the newly formed cementoblast-like tissue and the treated root surface. As it has been proposed that these histological artifacts are inferiorly regenerated tissues, the absence of artifacts can be interpreted as additional evidence of the superior quality of EMD-induced cementum (compared to other regenerative techniques such as GTR). It remains to be seen whether the presence or absence of these artifacts influences treatment outcomes.

In this study, we also observed that PDL collagen fibers in the EMD treated group ran parallel to each other and inserted into the newly formed cementum. Nevertheless, the PDL collagen fibers in the control group were relatively sparse and irregularly distributed, which may be explained by decreased synthesis of collagen or ECM proteins. EMD may have the ability to promote collagen synthesis and increase protein secretion in PDL cells. Gestrelius found that EMD promotes protein production and mineralized nodule formation in PDL cells [35]. In addition, He postulated that EMD acts as a matrix enhancement factor, creating a positive environment for osteoblast and cementoblast proliferation, differentiation, and matrix synthesis [26]. The effect of EMD on matrix synthesis was investigated using cultured periodontal fibroblasts. EMD significantly affected mRNA levels of matrix proteoglycans (two mRNAs were upregulated and one was downregulated) and stimulated hyaluronic acid synthesis [36]. These findings suggest that EMD has the potential to significantly modulate matrix synthesis *in vitro*, consistent with the changes noted in tissues undergoing repair and regeneration.

EMD has been widely used to promote periodontal tissue regeneration. Its osteogenic activities have been shown to repair periodontal intrabony defects in the alveolar bone and various experimental bone defects in animal models [9, 37]. The mechanisms underlying EMD-derived osteogenic activities remain largely unknown. Schwartz et al. examined the osteoblast response to EMD in tissue cultures at three stages of osteogenic maturation and concluded that EMD affects early states of osteoblastic maturation by stimulating proliferation [16]; however, as cells mature in the lineage, EMD enhances differentiation. Boyan et al. suggested that EMD is not osteoinductive but instead osteopromotive due to its osteoconductive properties at a threshold concentration [38]. He et al suggested that blocking of osteoblast apoptosis by inflammatory cytokines is one of the mechanisms through which EMD promotes bone regeneration [39]. There-

fore, EMD's ability to increase the osteoblast population during bone regeneration might be derived from its capacity to stimulate osteoblast proliferation and prevent apoptosis, subsequently leading to increased bone formation.

Cbfa1 is a master transcription factor driving osteoblast differentiation and plays a crucial role in osteogenesis. The results of our study showed that in both experimental and control groups, Cbfa1 protein was expressed in osteoblasts near the alveolar bone and in fibroblasts in PDL. This observation was coincided with that of Wendell's research that Runx2 expression was increased in EMD-treated human periodontal ligament fibroblasts [40]. Periodontal regeneration, including osteogenesis is a complex process involving many cells and processes that must function accurately. The exact progenitor cells that contribute to this process are not fully characterized. Periodontal fibroblasts was also postulated to be potential osteoprogenitor cells [40]. As expected, we found that the signal of Cbfa1 protein in the EMD group was stronger than that in the control group. Moreover, the type of newly-formed cementum in OFD group was similar to those observed in the EMD group while the cementum was rather thin and periodontal regeneration was limited to the bottom of the defects. Together, these results indicate that EMD promotes Cbfa1 expression in osteoblasts and fibroblasts, resulting in the commitment of these potential osteoprogenitor cells to osteogenic linages, thus, cementum formation and alveolar bone regeneration ensues.

In conclusion, this study histologically analyzed healing events and Cbfa1 expression in periodontal tissues of six EMD-treated beagles and sheds light on the osteogenic action of EMD. Instead of AEFC formation, a partially mineralized connective tissue containing many embedded cells without extrinsic fibers was observed to form in periodontal defects in EMD group, the quality of which was superior to that in the OFD group. However, this is only a pilot semiquantitative study with limited number of animals employed. Future studies are needed to investigate the mechanisms involved in increased ECM/connective tissue formation by EMD, and verify the effect of EMD on the behaviors of osteoblasts.

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Disclosure of conflict of interest

None.

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