Angiogenesis and bone regeneration of porous nano-hydroxyapatite/coralline blocks coated with rhVEGF$_{165}$ in critical-size alveolar bone defects in vivo

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Abstract: To improve the regenerative performance of nano-hydroxyapatite/coralline (nHA/coral) block grafting in a canine mandibular critical-size defect model, nHA/coral blocks were coated with recombinant human vascular endothelial growth factor$_{165}$ (rhVEGF) via physical adsorption ($3\mu$g rhVEGF$_{165}$ Per nHA/coral block). After the nHA/coral blocks and VEGF/nHA/coral blocks were randomly implanted into the mandibular box-shaped defects in a split-mouth design, the healing process was evaluated by histological observation and histomorphometric and immunohistological analyses. The histological evaluations revealed the ingrowth of newly formed blood vessels and bone at the periphery and cores of the blocks in both groups at both 3 and 8 weeks postsurgery, respectively. In the histomorphometric analysis, the VEGF/nHA/coral group exhibited a larger quantity of new bone formation at 3 and 8 weeks postsurgery. The percentages of newly formed bone within the entire blocks in the VEGF/nHA/coral group were 27.3$\pm$8.1% and 39.3$\pm$12.8% at 3 weeks and 8 weeks, respectively, and these values were slightly greater than those of the nHA/coral group (21.7$\pm$3.0% and 32.6$\pm$10.3%, respectively), but the differences were not significant ($P>0.05$). The immunohistological evaluations revealed that the neovascular density in the VEGF/nHA/coral group (146$\pm$32.9 vessel/mm$^2$) was much greater than that in the nHA/coral group (105$\pm$21.8 vessel/mm$^2$) at the 3-week time point ($P<0.05$), but no significant difference was observed at the 8-week time point (341$\pm$86.1 and 269$\pm$50.7 vessel/mm$^2$, respectively, $P>0.05$). The present study indicated that nHA/coral blocks might be optimal scaffolds for block grafting in critical-size mandibular defects and that additional VEGF coating via physical adsorption can promote angiogenesis in the early stage of bone healing, which suggests that prevascularized nHA/coral blocks have significant potential as a bioactive material for bone regeneration in large-scale alveolar defects.

Keywords: angiogenesis, bone regeneration, tissue engineering, block grafting, nano-hydroxyapatite/coralline, critical size, bone defect

Introduction

Clinically, large alveolar defects caused by periodontal disease, tumor, trauma, or surgery are challenging in dentistry.$^{1,2}$ Although alveolar bones have the capability to regenerate, the healing process frequently fails to progress through the normal stages of healing or is delayed when the defects reach a critical size.$^{3-5}$ Therefore, bone grafting is necessary for large defects.

Currently, autogenous grafts are considered the gold standard for bone regeneration due to their osteogenic potential, osteoinductivity, and osteoconductivity.$^{6-8}$ However, such grafts have yet to be applied clinically due to donor site limitations, morbidity, and
additional injury. In contrast, xenogenic block grafts are commercially available, free from size constraints, and result in less morbidity at the harvest site. Additionally, these grafts can easily be fabricated into suitable grafts for bone defects.

In tissue engineering, the scaffolds are of critical importance. Optimal design of scaffolds should mimic the extracellular matrix in chemical components and micro- and macrostructure and be beneficial to the promotion of cell adhesion, migration, and proliferation. Additionally, scaffolds can also act as carriers for growth factors with biological properties, such as bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF).

Coralline hydroxyapatite (CHA) is one of the synthetic porous biomaterials among numerous xenogenous grafting materials and is made by partially converting calcium carbonate from coral to a hydroxyapatite (HA) layer on the surface via a hydrothermal exchange. CHA is a composite scaffold with an outer HA layer and an inner coralline core. Numerous experiments have demonstrated that the space between CHA particles is enveloped by cells and collagen matrix and occupied by new bone. Moreover, CHA particles have exhibited good performance in increasing initial periodontal ligament cell attachment in periodontal regeneration. Therefore, CHA particles have been widely used clinically in periodontal and implant surgery due to their favorable osteogenic effects. However, predictable results have not been achieved in bone regeneration with particle grafts, due to their weakness in shape remodeling. In comparison, HA/coral blocks might be advantageous for maintaining space and rebuilding bone volume. Unfortunately, bone formation with CHA blocks offers a lower capacity for osteogenesis than that provided by particles. At present, autogenous blocks and xenogenic particles are more widely used than xenogenic blocks in the restoration of critical-size alveolar defects. Indeed, there are still few reports of CHA block grafting for alveolar defects. Therefore, the vascularization and osteogenesis of block grafts remain a key problem for dentists.

Importantly, the healing ability of bone relies on the recruitment of progenitor cells and neovascularization at the injury site. Thus, the promotion of angiogenesis and prevascularization of the scaffolds achieved by coating them with angiogenic growth factors might be one of the most viable approaches for bone construction in critical-size defects. Recombinant human vascular endothelial growth factor (rhVEGF) is a potent and widely used angiogenic growth factor that is well known to induce the migration, proliferation, and differentiation of vascular endothelial cells, to control their apoptosis, and to improve new vessel formation. rhVEGF also has the capabilities to upregulate the expression of BMP-2 and enhance new bone formation.

To prevascularize block grafts and enhance the formation of new blood vessels and bones, we coated nano-hydroxyapatite (nHA)/coral blocks with rhVEGF and investigated the angiogenesis and the bone regenerative performances of bio-coated and pure nHA/coral blocks in critical-size mandibular defects in dogs. This study might provide theoretical support for the clinical application of nHA/coral block grafts. In this study, we made the following hypotheses: 1) nHA/coral blocks might act as optimal scaffolds for new bone formation and vessels in critical-size mandibular defects, and 2) nHA/coral coated with rhVEGF might improve angiogenesis and new bone formation.

Materials and methods

Scaffold characterization

The nHA/coral scaffolds used in this study were supplied by Beijing YHJ Science and Trade Co., Ltd (Beijing, People’s Republic of China). The scaffold blocks (6×9×12 mm³) were sterilised by γ-irradiation before use. The morphologies of their surfaces and cores were analyzed with JSM-6300 scanning electron microscopy (SEM) (Jeol Co., Ltd, Tokyo, Japan) at 20 kV. The dried samples were coated with gold–palladium under an argon atmosphere. SEM photomicrographs were used to analyze the pore sizes and crystal diameters.

Animals

The four male beagle dogs (age 12–15 months; mean weight 12.1±0.5 kg) used in the study were provided by the Guangdong Provincial Medical Experimental Animal Center (licence number: SYXX [yue] 2012-0081). Rabies vaccinations, vermifuge treatments, and overall examinations were completed prior to the experiments. All animals exhibited healthy and permanent dentition. In the experiment, the animals were independently housed in box-type cages (1.0×1.0×1.0 m) and fed once per day with a soft food diet and water. The experimental work began after an adaptation period of 1 week. Additionally, the ethical committee of the Guangdong Provincial Stomatological Hospital approved the animal experimental protocol. Supplemental information about the experimental animals: rabies vaccinations, vermifuge treatments, and overall examinations, including alanine aminotransferase, aspartate amino transferase, alkaline phosphatase, total protein, albumin, urea nitrogen, creatinine, glucose, total cholesterol, total bilirubin, white
blood cell, red blood cell (RBC), hemoglobin concentration, mean RBC volume, mean hemoglobin concentration of RBC, platelet, platelet distribution width, mean platelet volume, were completed prior to the experiments. Additionally, the animals in the experiment were independently housed in box-type cages (1.0×1.0×1.0 m) in general housing facilities. After the operations, milk was given for extra nutrition for 3 days.

Study design and grouping
The study was performed in two surgical phases. In the first phase, the bilateral mandibular second, third, and fourth premolars and the first and second molars (P2–M2) were extracted from each dog. After the tooth extractions, four standardized box-shaped defects were surgically created at the buccal aspect of the alveolar ridge in each mandible (n=8). Following 2 months of submerged healing, the chronic-type defects were reshaped and randomly allocated in a split-mouth design to the nHA/coral group or the VEGF/nHA/coral group. Uncoated nHA/coral blocks served as controls. Accordingly, all dogs received each treatment procedure once. The animals were sacrificed after a submerged healing period of 3 or 8 weeks. (Supplemental information about animal group allocation and study design: There were two groups in our study, the VEGF/nHA/coral group [ie, the experimental group] and the nHA/coral group [ie, the control group]. The animals were randomly chosen for the experiments. Additionally, the defects were divided into nHA/coral and VEGF/nHA/coral sites in a split-mouth design.)

Anesthesia protocol
Prior to the surgical procedures, the dogs were fasted overnight and handled according to the following anesthesia protocol. After intramuscular sedation with sumian xin (composition: 2,4-dimethylaniline thiazole, ethylenediamine tetra acetic acid, dihydroetorphine hydrochloride, and haloperidol; 0.2 mL/kg body weight; Animal Husbandry Research Institute, Jilin, People’s Republic of China), anesthesia was induced by intravenous injection of 3% pentobarbital solution (0.3 mL/kg body weight; Foshan Chemical Engineering Experimental Factory, Foshan, People’s Republic of China). Prior to the surgery, primacaine (0.2 mL/kg body weight; Merignac Cedex, France) was injected locally into the surgical area of the mandible. Additionally, intramuscular benzylpenicillin (Longteng pharmaceutical company, Sichuan, People’s Republic of China; 80×10⁶ U/d) was given intra- and postoperatively to each animal for 7 days.

Surgical phase 1 (tooth extraction and defect creation)
In the first surgery, after reflection of full-thickness flaps, the bilateral P2–M2 molars were removed from the mandible with minimal trauma in each dog. After tooth extraction, four standardized box-type defects (9 mm in height from the crestal bone, 6 mm in depth from the surface of the buccal bone, and 12 mm in width mesiodistally) were created in the buccal bone at a distance of 4 mm with carbide burs as previously reported. The corresponding lingual bone plates were left intact (Figure 1A). All osteotomy procedures were performed under adequate irrigation with sterile 0.9% physiological saline. The wounds were closed with interrupted mattress sutures (Foosin Medical Supplies Inc., Weihai, People’s Republic of China). All sites were allowed to heal for 2 months.

Soak loading of the nHA/coral blocks
For the soak loading of the nHA/coral blocks, the growth factor rhVEGF165 (PeproTech) was dissolved in sterile saline in aseptic conditions. Each block was incubated with 0.25 mL of 12 µg/mL rhVEGF165 solution (3 µg rhVEGF165 per block). The control samples were incubated with 0.25 mL of sterile saline as previously reported. All blocks were implanted within 30 minutes of assembly.

Surgical phase 2 (bone grafting)
In the second surgery, bilateral vestibular incisions were made, and mucoperiosteal flaps were reflected to expose the sites for block grafting in the lower jaws (Figure 1B). Prior to grafting, the chronic-type defects were reshaped (6×9×12 mm) with carbide burs, and all defects were grouped into either the nHA/coral block or the nHA/coral block soaked with rhVEGF165 group in a split-mouth design (Figure 1C). Following periosteal-releasing incisions, the mucoperiosteal flaps were repositioned coronally and closed with interrupted mattress sutures (Foosin; Medical Supplies Inc.) for a submerged healing period of 3 or 8 weeks.

Histological observation/histomorphometric analysis
After 3 or 8 weeks, the animals were sacrificed with an overdose of anesthetic. The lower jaws were detached and placed in 4% neutral buffered formalin solution. Seven days later, the jaws were dissected with a carboronum disk, and each experimental specimen contained one block and the surrounding bone tissue.
The samples were decalcified in 10% ethylenediaminetetraacetic acid, dehydrated using ascending concentrations of alcohol, and paraffin embedded. The samples were sectioned at 5 µm thickness and stained with hematoxylin and eosin (Figure 2A) and Masson trichrome. Each of the three sections per sample was evaluated separately by two blinded observers using a digital microscope (BX51, Olympus Co., Tokyo, Japan) and a digital camera (DP71, Olympus) at 10× magnification (Figure 2B). The entire scaffold and the newly formed bone within each block were then outlined (Figure 2C) and measured with the Image-Pro Plus image analysis software (Media Cybernetics, Carlsbad, CA, USA). The percentages of new bone fill (PBF = newly formed bone area/total scaffold area) were measured. Masson trichrome-stained sections were used to define the collagen secreted in the process of bone formation as an indirect index of the quantity of the newly formed bone.

Immunohistological staining
After decalcification, hydration, paraffin embedding, and sectioning (5 µm), slides from independent samples harvested at 3 and 8 weeks were immunostained for the von Willebrand factor (vWF), which is a protein present in large quantities in subendothelial matrices such as blood vessel basement membranes. All processes were performed following the manufacturer’s protocol using a rabbit antidog vWF primary antibody (1:1,500) and biotinylated antirabbit immunoglobulin G from a commercial kit (Abcam, Cambridge, UK) and followed by counterstaining with hematoxylin. All slides were imaged with an Olympus BX51 light microscope and an Olympus DP71 digital camera. The positively stained blood vessels were counted manually at 200× magnification within the scaffold area, and blood vessel density was calculated using the Image-Pro Plus Software (Media Cybernetics) as previously reported.

Statistical analyses
All data are expressed as the mean ± the standard deviation (n=8). The statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Independent-sample t-tests were used to analyze the significance of the differences between groups and between the different time points.
Results

Scaffold presentation and morphology

The nHA/coral blocks were three-dimensionally porous generally (Figure 3A). The porous structures were visualized with SEM (Figure 3B). The pore sizes ranged from 57 µm to 164 µm. All macropores inside the block were in spatial communication via interconnected pores. The diameters of the interconnected pores varied from 107 µm to 550 µm. The hydroxyapatite (HA) crystals were well distributed on the surface and had diameters that varied from 71 nm to 99 nm (Figure 3C).

Histological observations/ histomorphometric analysis

In general, the postoperative healing was uneventful in all animals. No complications such as swelling, premature exposure of the grafting sites, and infections were observed throughout the entire study period.

In both groups, the neovascular tissues and newly formed bone were observed on the surface and in the macropores at the four peripheral parts of the blocks at 3 weeks after...
surgery. Additionally, the newly formed bone and blood vessels had grown toward the centers between macropores through the interconnecting paths. Moreover, the lines of the active osteoblasts and collagen were observed in the macropores at the peripheral parts of the scaffolds; new bone was seldom found in the centers of the blocks in either group. The shapes of the blank areas and the newly formed bone were regular. Furthermore, histomorphometric analyses revealed that the PBFs were 21.7%±3.0% and 27.3%±8.1% in the nHA/coral group and VEGF/nHA/coral group, respectively. The quantity of newly formed bone in the VEGF/nHA/coral group was slightly more than that in the nHA/coral group; however, no significant increase with respect to PBF was observed after coating the nHA/coral blocks with VEGF (P>0.05, Figure 4).

Eight weeks after surgery, the macropores at the periphery adjacent to the host bone and the center were filled with interconnected newly formed bone in both groups. Compared with the sample taken at 3 weeks, the structure of the trabecular bone in the peripheral parts had increased in width, and considerable amounts of woven bone and new blood vessels were present in the peripheral parts adjacent to the host bone. The shapes of the blank areas and the newly formed bone were still regular. Additionally, at 8 weeks, the PBFs were 32.6%±10.3% for the blank sample and 39.3%±12.8% for the VEGF sample. Compared with

Figure 3 Cross-sectional view of the porous nano-hydroxyapatite/coralline block, scale bar: 5 mm (A). The interconnected pore system of the scaffold was visualized and measured with scanning electron microscopy (B). Nano-scaled hydroxyapatite crystals were found on the surface by scanning electron microscopy (C).
week 3, the PBFs at week 8 were significantly increased in both the VEGF/nHA/coral ($P<0.01$) and nHA/coral groups ($P<0.01$). Additionally, the quantity of newly formed bone in the VEGF/nHA/coral group was greater than that in the nHA/coral group; however, no significant increase in PBF was to be observed after coating the nHA/coral blocks with VEGF ($P>0.05$; Figure 4).

From the Masson staining images, collagen expression could be observed in terms of blue-stained tissue. At 3-week time point, collagen as the most important component of bone matrix was secreted copiously, with collagen fibers connected together to form the tabular structure as the matrix of mineralization in both groups. In addition, lines of active osteoblasts were observed around the tabular bone and a large number of neovascular channels can be easily found, especially in VEGF/nHA/coral group (Figure 5A). As the time point increased to 8 weeks, collagen progressively increased along with the new bone formation. Moreover, the mineralization degree of newly formed tabular structures increased significantly in both groups (Figure 5B).

**Immunohistochemical analysis**

Immunohistochemical analysis revealed that the formation of new blood vessels was characterized by the vWF staining of numerous endothelial cells located in the vessel basement membranes (Figure 6A). The uneven distribution of the newly formed blood vessels was easily observed. The neovascular structure at the periphery, adjacent to the host bone, was more evident than that at the center of the sample. At the 3-week time point, compared with the nHA/coral block group (105±51.8 vessel/mm$^2$), an increase in new blood vessel density was evident after in the combined nHA/coral block with VEGF group (146±32.9 vessel/mm$^2$, $P<0.05$).

The neovascular density within the scaffolds in the VEGF/nHA/coral block group was significantly increased ($P<0.05$). At the later 8-week time point, the neovascular densities of both groups had significantly increased ($P<0.01$). At the 8-week time point, the neovascular densities increased to 269±50.7 vessel/mm$^2$ in the nHA/coral block group and 341±86.1 vessel/mm$^2$ in the VEGF/nHA/coral block group, and there was no significant difference between the two groups ($P>0.05$) (Figure 6B).

**Discussion**

Bone healing in critical-size alveolar defects requires different grafting approaches to greatly improve volume and

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**Figure 4** Histomorphometric analysis of the newly formed bone. The columns indicate the mean values, and the error bars represent the corresponding standard deviations (n=8).

**Note:** **P<0.01.

**Abbreviations:** nHA/coral, nano-hydroxyapatite/coralline; VEGF, vascular endothelial growth factor.

**Figure 5** Histological observation of the Masson staining.

**Notes:** Representative images showing the expression of collagen (stained in blue), newly formed blood vessels (nv), new tabular bone (nb), and lines of osteoblasts (black arrows) within the scaffolds of both groups at different time points. (A) Represents the masson stained section at the timepoint of 3 weeks; (B) represents the masson stained section at the timepoint of 8 weeks.
shape. The data presented in our study support the notion that optimal scaffold grafting can result in good performance in terms of new bone regeneration, and that combinatorial approaches involving the local delivery of angiogenic growth factors from scaffolds can provide significant therapeutic benefits.

In bone tissue engineering, the pore sizes and interconnectivities of the pores in the scaffold play important roles in blood vessel growth, the migration of cells, and bone formation. Some studies have reported that the aggregated space between the pores acts as the passageway for the metabolism of cells and the transportation of nutrition that promote the ingrowth of blood vessels. Yoshikawa et al suggested that the pore size in grafts used for bone healing should be at least 50 µm. Larger pore sizes result in greater quantities of nutrition and oxygen being transported into the inside of the scaffold. Moreover, scaffolds with larger pore sizes provide more superficial area and improve cell adhesion.

Figure 6 Staining for vWF of the decalcified tissues treated with nHA/coral blocks.
Notes: (A) Representative images of the nHA/coral and VEGF/nHA/coral groups showing circular, dark brown vessels (black arrows) in the scaffolds. The VEGF/nHA/coral group displayed greater densities of new blood vessels than the nHA/coral group at each time point. (B) The blood vessel densities were calculated based on the immunohistochemical staining. The VEGF released from the scaffolds promoted the formation of new blood vessels. The columns indicate the mean values, and the error bars represent the corresponding standard deviations (n=8). *P<0.05; **P<0.01.
Abbreviations: vWF, von Willebrand factor; nHA/coral, nano-hydroxyapatite/coralline; VEGF, vascular endothelial growth factor.
and proliferation due to full contact between the graft and body fluids.\textsuperscript{31,33,34} However, Grynpas et al\textsuperscript{35} noted that oversized pores not only reduce mechanical strength but also influence cell function and new bone formation. High levels of interconnectivity between the pores enhance the porosity and enlarge the superficial area and consequently increase the numbers of endothelial cells and active osteoblasts.\textsuperscript{35,36} It is important to note that our histological evidence revealed obvious cell migration from the peripheries to the cores of the nHA/coral blocks at the 3-week time point; collagen matrices were also readily observable in the centers. There were more newly formed blood vessels and greater quantities of bone tissue present at the 8-week time point. These results indicate that the nHA/coral blocks had excellent characteristics in terms of biocompatibility, pore size, and pore interconnectivity and were able to provide appropriate interconnected paths for cell migration and bone regeneration.

The vWF immunostaining results indicated a significant increase in neovascularization due to the use of the nHA/coral blocks combined with VEGF at 3 weeks and revealed no significant improvement in vascular density at 8 weeks; these findings demonstrated the excellent effect of VEGF local therapy in terms of angiogenesis at an early stage of bone repair. Interestingly, the areas of newly formed bone in the VEGF/nHA/coral group at 3 and 8 weeks were slightly greater than those of the blank scaffold group; however, there were no significant differences in PBF between the two groups. Some previous studies have demonstrated that bioactive glass combined with VEGF can participate in bone healing through indirect processes, and these findings accord well with the well correlated results of the present study. Specifically, the enhancements of angiogenesis and bone maturation are involved in these processes, whereas osteoprogenitor differentiation and bone formation are not.\textsuperscript{25} At the 8-week time point, the quantities of new bone tissue had continuously increased in the peripheries and centers of the blocks in both groups. A slightly greater amount of new bone formation was observed in the VEGF/nHA/coral group, but there was no significant difference in PBF between the two groups. The results from our study demonstrated that local VEGF therapy delivered by combining nHA/coral blocks and VEGF might only improve neovascularization in the early stage and not significantly increase bone regeneration.

In previous experiments, several methods have been developed to coat scaffolds with growth factors for bone tissue engineering. Fu et al\textsuperscript{37} utilized CHA and poly(lactic-co-glycolic acid) microspheres encapsulated BMP-2, that were fabricated with the water-in-oil-in-water (w/o/w) double-emulsion method as carriers for BMP. Shiels et al\textsuperscript{38} reported that proteins and drugs are rapidly released from HA based on an in vitro study, and this speed of release was unsatisfactory. In our immunohistochemical and histomorphometric analyses, only angiogenesis in the early stage was improved by the physical resorption of VEGF onto the nHA/coral block. No significant increase in osteogenesis was noted in the VEGF/nHA/coral group, which demonstrated that the function of VEGF was not continuously and completely developed. At 3 and 8 weeks, uneven distributions of new bone and blood vessels between the peripheries and cores of the scaffolds were observed in both groups. These findings are signs of insufficient blood, oxygen, and nutrient supplies in the cores of scaffolds. Previous experiments have revealed that uneven nutrition and oxygen supplies are likely to occur to some degree when the volume of the defect is increased and the oxygen concentrations in the cores of large-scale blocks are significantly decreased in the cell culture.\textsuperscript{41} The gradient diffusion of oxygen and nutrients might influence cellular proliferation and differentiation within block grafts and consequently influence bone formation.

Therefore, many types of composite scaffold systems, such as HA/gelatine and nHA/chitosan, have been developed and successfully applied in bone tissue engineering to achieve sustained release.\textsuperscript{42,43} Some experiments have demonstrated that different doses and dual delivery of VEGF and BMP-2 might lead to different effects on bone regeneration.\textsuperscript{44} A previous study reported that the combination of gradient VEGF with BMP-2 and Wnt released from a three-dimensional bio-degradable porous calcium phosphate scaffold can promote
angiogenesis and bone formation more readily than a single factor alone.45

Hence, in bone tissue regeneration, local therapies with different doses of single or combinations of growth factors result in complicated in vivo cascades that involve the presence of clots containing large numbers of mesenchymal cells followed by proliferative and metabolic levels in osteoblasts and macrophages and the formation of vascular structures and woven bone. Hence, in our ongoing work, we plan to develop a multilayer biodegradable composite scaffold and to optimize the growth factor dose, other possible cytokine combinations, and their controlled release.

Conclusion

Our results indicate that the nHA/coral block is an optimal scaffold for block grafting with an ideal mesoporous structure and nanosized HA crystals. The characterizations performed in vivo demonstrated that porous nHA/coral blocks coated with rhVEGF165 promoted neovascularization in the early stage of bone healing but failed to enhance bone formation. VEGF/nHA/coral blocks might serve as potential biological scaffolds for improving bone regeneration in orthopedic and implant surgeries.

Acknowledgments

This work was supported by the Guangdong Provincial Stomatological Hospital (Southern Medical University, People’s Republic of China), grants from the Natural Science Foundation of China (No 81170998), and the Fund of Guangdong Province Medical Science Research (C2012034).

Disclosure

The authors report no conflicts of interest related to this work.

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