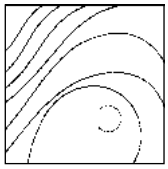
Effect of Autologous Bone Marrow Stromal Cells Transduced with Osteoprotegerin on Periodontal Bone Regeneration in Canine Periodontal Window Defects



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The purpose of this investigation was to evaluate the effect of osteoprotegerin (OPG)-modified autologous bone marrow stromal cells ($BMSCs_{OPG}$) combined with guided tissue regeneration on bone regeneration of periodontal window defects. pSecTag/2B-OPG was transduced into BMSCs by Lipofectamine 2000. The expression of OPG protein in the BMSCs was detected by immunocytochemistry and Western blotting. Periodontal window defects (4 × 4 × 3 mm) were surgically created in the buccal aspect of the mandibular premolars and randomly assigned to receive $BMSCs_{OPG}$-PLGA (cells + material + OPG), BMSCs-PLGA (cells + material), PLGA (material), or root planing only (negative control). The animals were euthanized at 6 weeks postsurgery for histologic analysis, and histologic measurements were then performed. Results showed that the height of new alveolar bone and cementum and the formation of new connective tissue were significantly greater in the experimental group than in the control groups after 6 weeks (P < .05), whereas there was no significant difference between the material control and negative control (P > .05). These findings suggest that delivery of $BMSCs_{OPG}$-PLGA may be a viable approach to promote bone regeneration of periodontal bone defects. (Int J Periodontics Restorative Dent 2012;32:e174–e181.)

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Therapeutic approaches to the treatment of periodontitis generally fall into two categories: those designed to halt the progression of periodontal attachment loss and those designed to regenerate or reconstruct lost periodontal tissues.[1] Regenerative periodontal therapy, including regeneration of new bone, new cementum, and supportive periodontal ligament, increases cells, molecules, and factors regulating formation of these tissues during development and regeneration.[2] Despite the convincing histologic evidence that some regeneration may occur in humans following a regenerative surgical approach, complete and predictable regeneration is still difficult to attain.[1]

Bone remodeling is the process by which bone is continuously repaired during adult life through coordinated cycles of bone resorption and formation.[3] Bone resorption is dependent on a cytokine known as RANKL (receptor activator of nuclear factor kappa-B ligand). RANKL, also known as TRANCE/OPGL/ODF, is expressed on the surface of

osteoblasts and plays a central role in osteoclastogenesis by providing a signal to osteoclast progenitors through the membrane-anchored receptor activator of NF-κB (RANK). Signal transduction through RANK leads to osteoclast differentiation and functional activation. The catabolic effects of RANKL are prevented by osteoprotegerin (OPG), a tumor necrosis factor (TNF) receptor family member that binds RANKL and thereby prevents activation of RANK. The osteoclast activity is likely to depend, at least in part, on the relative balance of RANKL and OPG.[4–6] Bone remodeling can be assessed by the relative ratio of OPG to RANKL. Studies in numerous animal models of bone disease show that RANKL inhibition leads to marked suppression of bone resorption and increases cortical and cancellous bone volume, density, and strength.[4,7,8] Jin et al demonstrated that OPG-Fc treatment of mice with established bone metastases resulted in an overall improvement in survival.[8] RANKL inhibitors also prevent focal bone loss that occurred in animal models with rheumatoid arthritis and bone metastasis.[4]

The protectant role of TNF in periodontal lesions has been well studied.[7] The activity of RANKL, a member of the TNF superfamily, is controlled by OPG.[9] Therefore, the authors aimed to evaluate the effect of OPG-modified autologous bone marrow stromal cells ($BMSCs_{OPG}$) combined with guided tissue regeneration on bone regeneration of periodontal defects.

Method and materials

Isolation and culture of bone marrow mesenchymal stromal cells (BMSCs)

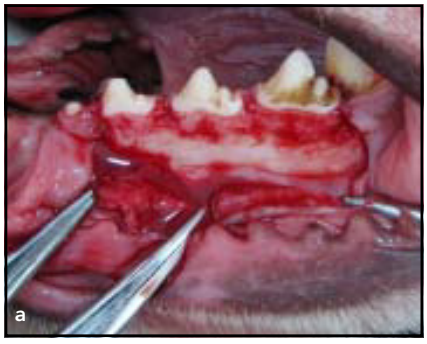
One milliliter of fresh bone marrow obtained from a Beagle dog was mixed with an equal volume of heparinized phosphate-buffered saline (PBS) and centrifuged at 800 g for 10 minutes. The supernatant was discarded, and the centrifuged cells were resuspended in normal culture medium in a T25 flask. Normal culture medium consisted of Dulbecco Modified Eagle Medium (DMEM; Gibco) containing 10% (v/v) fetal bovine serum and antibiotics. The T25 flask was kept in a humidified 5% carbon dioxide incubator at 37°C for 72 hours. The culture medium was changed every 2 to 3 days; nonadherent cells were removed by changing the medium.

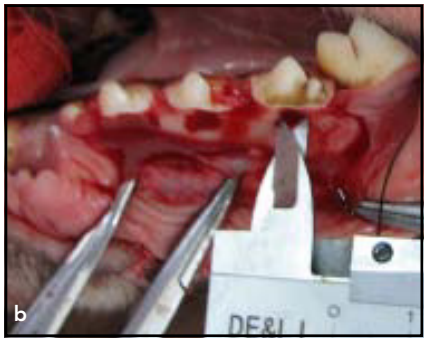
Transfection efficiency of OPG in BMSCs

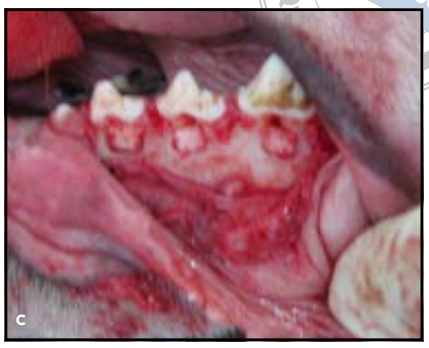
In a previous study, the plasmids (pSecTag/2B-OPG) were cloned.[10] Now, transfections were performed using Lipofectamine 2000 reagent (Invitrogen) in serum-free DMEM for 24 hours according to the manufacturer's instructions. The amount of Lipofectamine 2000 used was 1 μL for 1.2 μg of plasmid in 70% confluent cells plated onto six-well plates. Cells were assayed 24 hours after transfection. The BMSCs were transduced with pSecTag/2B as a control. The OPG protein was determined by the fluorimetric method and Western blotting.

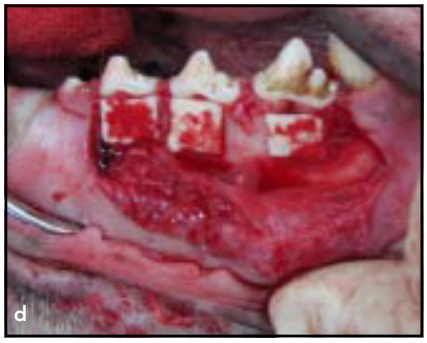
Scaffold preparation and cell seeding

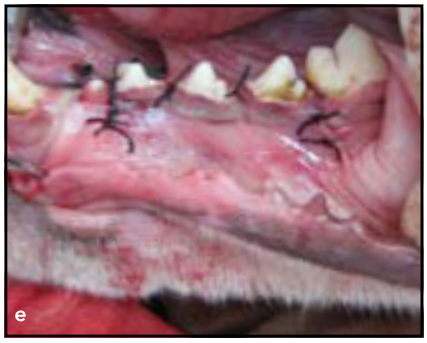
In tissue engineering, a temporary scaffold is required to serve as an adhesive substrate for implanting cells and as a physical support to induce the formation of new tissue. The temporary scaffold should be biocompatible and biodegradable.[11] Poly(lactide-co-glycolide) (PLGA) (50:50; M_w = 30,000) was purchased from Anhui Medical University, Hefei, China, which provided the appropriate pore size (range, 200 to 300 μm) and interval porosity (90% to 95%). The PLGA scaffolds were cut into 4 × 4 × 3-mm cubes, sterilized with ultraviolet light, and stored at room temperature until use. Prior to transplantation in vivo, the PLGA blocks were immersed in 70% ethanol for 10 minutes, rinsed with PBS three times, and left in normal culture medium at 37°C overnight. The following day, $BMSCs_{OPG}$ or BMSCs were suspended at a concentration of 1×10^6 cells/mL and poured onto their respective scaffolds. After 6 hours at 37°C, the medium was replaced with fresh medium and the scaffolds were maintained in a humidified atmosphere at 37°C with 5% carbon dioxide. An unseeded scaffold incubated with normal culture medium alone was used as a control.







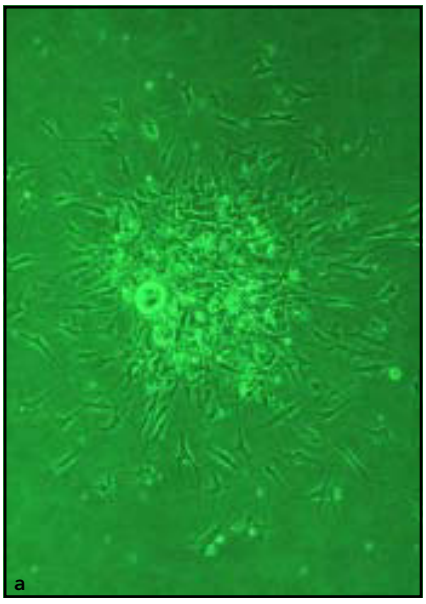




Figs 1a to 1e *Surgical protocol.* (a) *Exposed alveolar bone prepared during surgery;* (b) *periodontal window defects created surgically in the buccal aspect of the mandibular premolar;* (c) *experimental and control group defects implanted during surgery;* (d) *collagen membranes trimmed and adapted over the defects to cover 2 to 3 mm of the surrounding alveolar bone;* (e) *wounds closed with nylon sutures.*

Implantation of $BMSCs_{OPG}$ for periodontal defect repair

Four adult male Beagle dogs (weight, 10 to 14 kg) were purchased from the experimental animal center of Anhui Medical University. The protocol for the study animals was approved by the animal ethical committee of Anhui Medical University and adhered to the policies and principles established by the US National Research Council on the guidelines for care and use of laboratory animals. Food was withheld the night before surgical procedures. The animals were anesthetized using 3% pentobarbital sodium (1 mL/kg). Routine dental infiltration anesthesia (2% lidocaine) with epinephrine was used at the surgical sites. The mandibular second, third, and fourth premolars in each of the four dogs were selected for experimentation; thus, a total of 24 defects were created and randomly allocated into the following groups: *(1)* PLGA with $BMSCs_{OPG}$ (experimental group), *(2)* PLGA with BMSCs (cell control), *(3)* PLGA alone (scaffold control), and *(4)* root planing alone (negative control). The clinical defect height from the cementoenamel junction to the reduced alveolar crest was set to 4 mm, as measured using a periodontal probe. The defect width was also approximately 4 mm. The defect surfaces were instrumented using curettes, chisels, and water-cooled rotating diamonds to remove the periodontal ligament and cementum. The sizes of the periodontal window defects were $4 \times 4 \times 3$ mm. Reference notches were placed on the reduced dentin. Then, these blocks, with or without cells, were implanted into the defects. Following grafting, BME-10X Medical Collagen Membrane of Guided Tissue Regeneration membranes (Shandong Medical) were trimmed and adapted over the defects so as to cover 2 to 3 mm of the surrounding alveolar bone and to ensure stability of the graft material. Finally, the mucoperiosteal flaps were repositioned coronally and fixed with horizontal mattress sutures. The wounds were closed with nylon sutures (Figs 1a to 1e). Animals were fed a soft diet the first 14 days



Figs 2a and 2b *Cell morphologies of BMSCs. After passage, the cells were well distributed and were uniform as the fibroplastic morphology with lower refraction at the same magnification (Fig 2b, original magnification ×100).*

postsurgery. All dogs received antibiotics for 1 week. The animals were anesthetized and euthanized at 6 weeks postsurgery. Following euthanasia, teeth with the surrounding soft and hard tissues were removed en bloc, and the defects were examined by means of radiographs.

Histologic procedures

The defects and adjacent soft and hard tissues were obtained en bloc and fixed in 4% paraformaldehyde. They were placed in a series of graded ethanols (70% to 100%). Nondecalcified ground sections (30-μm thick) were produced in the mesiodistal plane so that the buccolingual plane of the teeth could be observed. Every tenth section was stained with toluidine blue for observations at 300-μm intervals.

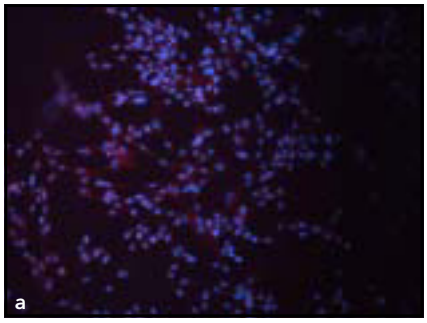
Statistical analysis

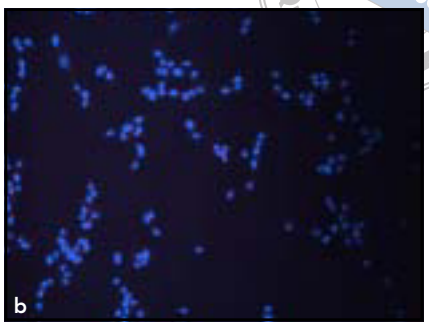
The following measurements were recorded in the mesiodistal plane for each stained section: the height of the defect (from the cementoenamel junction to the reduced alveolar crest), the height of new connective tissue formation (distance between the apical extension of the root planing and apical extension of the junctional epithelium), the height of new cementum formation (distance between the apical extension of the root planing and coronal extension of a continuous layer of new cementum or cementum-like deposit on the planed root), and the height of the new bone formation (distance between the apical extension of root planing and coronal extension of regenerated alveolar bone along the planed root). All data were statistically analyzed using the Mann-Whitney *U* test (SPSS 11.5, IBM). All measurements were normalized to the negative control, and data were expressed as means ± standard deviations of four independent experiments in each defect specimen (n = 6).

Results

Establishment of primary culture

It is generally accepted that BMSCs can be recognized as the adherent cells derived from bone marrow–capable proliferation with a fibroblastic profile.[11,12] Cells obtained from Beagle dog bone marrow were cultured in normal culture medium, and after 13 days of culture, an almost homogenous population of fibroblastic-like cells were observed throughout the flask, with little evidence of round or floating cells (Figs 2a and 2b).





Figs 3a and 3b *The expression of OPG protein in the BMSCs was detected by immunohistochemistry. The merged graphs for* (a) *the experimental group and* (b) *the control group were immunostained for the presence of OPG* (red) *and nuclei* (blue) *using DAP I (original magnification ×200).*

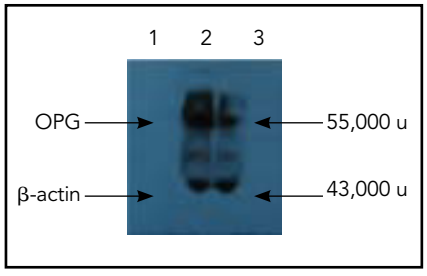
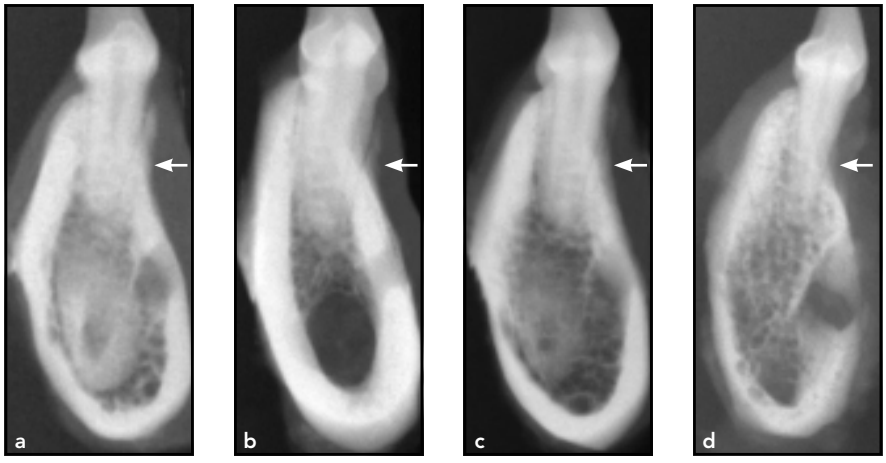


Fig 4 *Western blotting analysis of OPG and β-actin probed with respective antibodies. The OPG protein recognized by the antibodies had an apparent molecular weight of 55 kDa. The β-actin strain displayed a protein with an apparent molecular weight of 43 kDa. 1 = marker; 2 = experimental group; 3 = control group.*



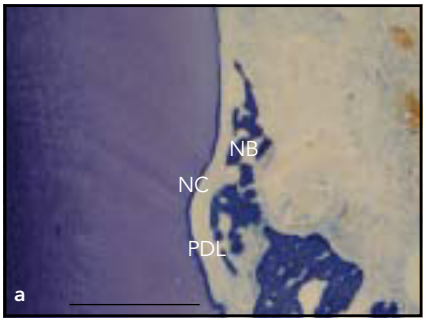
Figs 5a to 5d *Following euthanasia, teeth and the surrounding soft and hard tissues were removed en bloc, and the defects were examined by means of radiographs. The notch, signifying the bottom of the periodontal window defect, was indicated with black striping* (arrows). (a) *In the BMSCs$_{OPG}$-PLGA group, a significant amount of new bone and cementum were observed.* (b) *In the BMSCs-PLGA group, the alveolar bone and cementum underwent slight regeneration. Compared with the two groups containing BMSCs, less hard tissue regeneration was observed in the* (c) *PLGA group and* (d) *negative control group.*

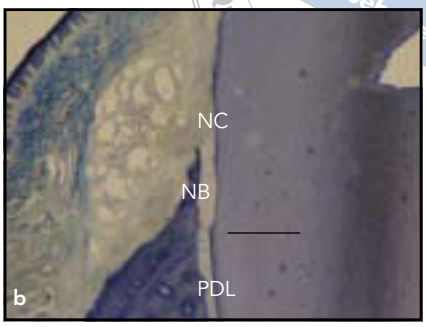
Immunohistochemistry and Western blotting analysis

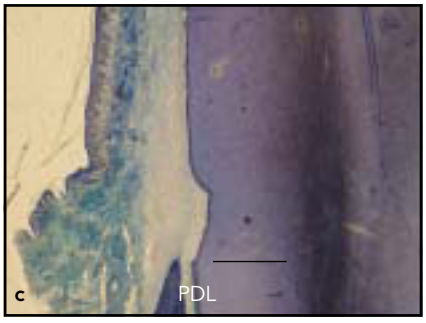
The results of immunohistochemistry and Western blotting showed that the expression of OPG protein in the BMSCs$_{OPG}$ group was statistically higher than that in the control group (P = .05) (Figs 3a, 3b, and 4).

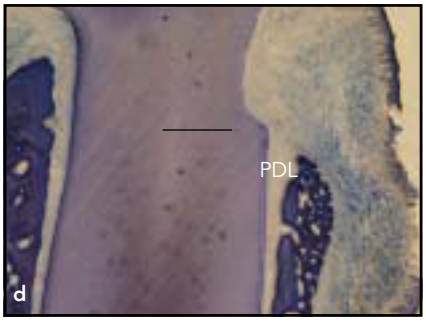
Radiographic analysis

In the experimental group, new bone formation was observed in the defect. The height of the newly formed bone was approximately that of the original bone crest, and there was close fusion between the old and new bone. In the cell control group and scaffold control group, the height of the newly formed bone was not as good as that in the experimental group. In the negative control, there was virtually no new bone formation (Figs 5a to 5d).









Figs 6a to 6d *Photomicrographs of the periodontal window defect. Histologic examination of periodontal tissue regeneration (toluidine blue) in different groups over 6 weeks postsurgery.* (a) *In the BMSCs$_{OPG}$-PLGA group, a significant amount of new bone and new cementum were observed. The denuded root surface almost separated the new bone from cementum, and newly formed cementum covered the surface. The height of the new alveolar bone and cementum and the formation of new connective tissue were significantly greater in the experimental group than in the control groups (P < .05).* (b) *In the BMSCs-PLGA group, the alveolar bone and cementum underwent slight regeneration. Compared with the two groups containing BMSCs, less hard tissue regeneration was observed in* (c) *the PLGA group and* (d) *the negative control group. There was no significant difference between the PLGA group and negative control group (P > .05). The height of new connective tissue formation was not significantly different between the four groups. NC = new cementum formation; NB = new bone formation; PDL = periodontal ligament cell; black line = notch (original magnification ×10.).*

Table 1 Histologic measurements of the different groups over 6 weeks postsurgery (means ± standard deviations)

	Defect height (mm)	NB (mm)	NC (mm)	CT (mm)
BMSCs$_{OPG}$-PLGA	3.82 ± 0.45	2.02 ± 0.11*	2.02 ± 0.10*	3.34 ± 0.14*
BMSCs-PLGA	4.44 ± 0.60	1.12 ± 0.12*	1.02 ± 0.05*	2.12 ± 0.11*
PLGA only	4.08 ± 0.10	0.67 ± 0.14†	0.11 ± 0.11†	2.73 ± 0.09†
Negative control	4.23 ± 0.76	0.33 ± 0.09	0.44 ± 0.04	2.12 ± 0.05

NB = height of new bone formation; NC = height of new cementum formation; CT = height of new connective tissue formation.
*$P < .05$, significant compared to the values of the negative control group.
†$P < .05$, significant compared to the values of the negative control group.

Histologic examination

All animals tolerated the operation very well, and no animal was excluded from the study or died during anesthesia. Tissue specimens were stained with toluidine blue. The height of the defect, the height of the new bone formation, the height of connective tissue formation, and the height of new cementum formation were examined with a light microscope and measured. The histologic findings are shown in Figs 6a to 6d. The histologic analysis of the four groups is shown in Table 1. Significantly more tissue regeneration for the scaffolds with BMSCs$_{OPG}$ was noted compared with the other groups.

Discussion

The objective of this study was to evaluate the role of OPG as an independent promotion factor of alveolar bone regeneration in a periodontal defect model. For this purpose, periodontal window defects from four young adult Beagle dogs were analyzed. The animals were euthanized at 6 weeks postsurgery. Histometric analysis assessed the vertical regeneration of alveolar bone and cementum at the base of the defect. The results suggest that delivery of $BMSCs_{OPG}$-PLGA may be a viable approach to promote the regeneration of periodontal bone defects. Bioactive molecules incorporated directly into a bioresorbable scaffold are generally released by a diffusion-controlled mechanism that is regulated by the pore sizes such that different pore sizes affect the tortuosity of the scaffold and thereby control the release of protein.[13] Wei et al incorporated human parathyroid hormone into biodegradable PLGA microspheres and demonstrated that it could be released in a controlled fashion.[14]

Increased knowledge of the genetic and cellular mechanisms of human diseases allowed the development of a new therapeutic approach for congenital and acquired diseases via gene therapy. Although initially designed to permanently correct a single gene in monogenetic disorders, gene therapy methods have included modification or elimination of malignant cells, modulation of host defenses, and reengineering of diseased organs or tissues.[15] With this approach, the potential to genetically modify the cells to express the required growth factors for bone regeneration and, more specifically, periodontal regeneration is increased.[16] In fact, the host modulation therapies are promising alternatives for the treatment of periodontal diseases.[7] In general, gene delivery can be approached by using viral or nonviral vectors. Nonviral vectors include plasmid DNA (eg, pSecTag/2B-OPG) and synthetic vectors that consist of complexes of plasmid DNA with cationic lipids and polymers known as lipoplexes and polyplexes, respectively. They present improved safety and are more easily manufactured than viral vectors; nonetheless, they have low gene-transfer efficiency and, in some cases, in vivo instability.[17,18] The most commonly employed vectors are retrovirus, lentivirus, adenovirus, and adeno-associated virus.[15] Kunze et al demonstrated that using self-complementary AAV vectors dramatically enhanced transduction efficiency.[18] Therefore, more viral vectors and in vivo studies are necessary to promote transduction rates of autologous BMSCs with a high safety profile.

Gene therapy has several potential advantages over the use of recombinant proteins. Via gene therapy, it may be possible to achieve sustained high concentrations of growth factors at a regenerative site without systemic effects.[19] In this study, OPG was used to modify BMSCs. OPG has a protective effect against bone resorption, which occurs via a reduction in the number of osteoclasts.[20] In relation to periodontitis, mice bearing human peripheral blood lymphocytes from patients with periodontitis have been challenged with *Actinobacillus actinomycetemcomitans*, a common causative agent of this disease, resulting in alveolar bone destruction. Treatment of these mice with OPG was found to inhibit this alveolar destruction and decreased osteoclast population.[21] Recent evidence suggests that more signaling molecules probably control the cells and factors involved in periodontium development and regeneration.[22] Gene-induced periodontal regeneration translated into therapeutic applications has yet to be determined.[23]

Conclusion

This study demonstrated the potential of a PLGA scaffold combined with OPG-modified autologous bone marrow stromal cells ($BMSCs_{OPG}$) as a good substrate candidate for periodontal tissue regeneration.

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