

Effects of Gold Nanoparticles Combined Human β -defensin 3 on The Alveolar Bone of Experimental Periodontitis

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Research

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Abstract

Background

Nanomaterials of biomedicine and tissue engineering have been proposed in the treatment of periodontitis recently. This study aimed to investigate the effect of gold nanoparticles (AuNPs) combined human β -defensin 3 (hBD3) on the repair of alveolar bone of experimental periodontitis in rats.

Methods

A model of experimental periodontitis was established by ligating of the maxillary second molars with silk thread in rats, which were treated with or without AuNPs combined hBD3. Micro-focus computerized tomography (micro-CT) scanning, enzyme-linked immunosorbent assay (ELISA) and histological and immunohistochemical staining, including alkaline phosphatase (ALP), osteoprotegerin (OPG) tartrate-resistant acid phosphatase (TRAP) and receptor activator of NF- κ B Ligand (RANKL), were used to analyze.

Results

Micro-CT demonstrated that the alveolar bone resorption was significantly reduced after the treatment of AuNPs combined hBD3. Levels of TNF- α and IL-6 decreased markedly compared with the ligation group. HE and Masson staining showed that AuNPs combined hBD3 group had less inflammatory cell infiltration, collagen fibrosis and fracture, but higher calcification in the new bone tissue. Moreover, the administration of AuNPs combined hBD3 increased the expression of ALP and OPG (related to bone formation) expression, while decreased TRAP and RANKL (related to bone resorption) expression.

Conclusions

AuNPs combined hBD3 had a protective effect on the progress of experimental periodontitis in rats, and also played a certain role in promoting osteogenesis.

1. Background

Periodontitis is a chronic infectious disease which occurs in the periodontal supporting tissues. As one of the most common oral diseases, it will cause the destruction of the periodontal supporting tissues, and eventually cause tooth loss[1]. The purpose of periodontitis treatment is to control infection and promote tissue regeneration. However, the effect of tissue regeneration by current periodontal treatments, including scaling and root planing, periodontal flap surgery and guided tissue regeneration, is limited[2, 3]. In addition, drug therapy also has problems such as drug resistance, flora imbalance and is difficult to promote tissue repair and regeneration[4, 5]. Therefore, it is necessary to seek new treatment methods.

Human β-defensin 3 (hBD3) is one of the most broad-spectrum, antibacterial and cationic defensins in beta-defensins family[6]. A study in 2018 demonstrated that hBD3 could promote the healing of bacteria-contaminated bone defects in rat[7]. Moreover, Zhu et al. have shown that hBD3 might function as osteogenic promoter to regenerate the periodontal tissues in a dog model of periodontitis[8]. But due to its short half-life and easy-hydrolyzed characteristics,hBD3 is usually difficult to use directly. Therefore, new biocompatible materials, such as nanoparticles, have been attempted to improve the outcomes recently.

Gold nanoparticles (AuNPs), due to their ease of synthesis, characterization and surface functionalization, are recognized as an novel nanomaterials in drug delivery, diagnostic and therapy[9]. Studies had confirmed that AuNPs could be used as anti-inflammatory and anti-tumor drug[10, 11]. Moreover, AuNPs play a pivotal role in bone regeneration engineering. Recent studies demonstrated that AuNPs could promote the repair of alveolar bone defects via cell sheet technology[12]. Besides,AuNPs could regulate the macrophage phenotype to produce a microenvironment with restricted inflammatory cytokine levels and repairing cytokines, such as bone morphogenetic protein 2 (BMP-2), thereby promoting periodontal tissue regeneration and preventing the progress of periodontitis[13].

Our previous studies proved that hBD3 combined AuNPs could promote the osteogenic differentiation of human periodontal ligament cells[14]. But the effect of the AuNPs and hBD3 synergistically in vivo still remained unknown. Therefore, based on the results of previous studies, this study will continue to explore whether hBD3 combined AuNPs could reduce periodontal inflammation and alveolar bone resorption of the experimental periodontitis in rats, and further investigate the possible mechanism involved in this process.

2. Results

2.1. Effects of AuNPs combined hBD3 on alveolar bone resorption in the SD rats with experimental periodontitis

The absorption of alveolar bone in SD rats was detected by micro-CT. Micro-CT results showed that the alveolar bone of the NaCl + ligation group was significantly absorbed after 14 days, indicating that an experimental periodontal disease model was successfully established. As shown in Fig. 1a, the degree of alveolar bone resorption of the maxillary second molars in the AuNPs-hBD3 + ligation group was significantly lower than that in the NaCl + ligation group, hBD3 + ligation group and AuNPs + ligation group. In addition, as shown in Fig. 1b, the bone mineral density (BMD), bone volume (BV) and relative bone volume fraction (BV/TV) analysis showed that the AuNPs-hBD3 + ligation group were significantly higher than the other groups, and the tissue volume (TV) of the AuNPs-hBD3 + ligation group was significantly lower than the other groups.

2.2. Effects of AuNPs combined hBD3 on serum inflammatory factors in SD rats

In order to further prove the role of hBD3 combined AuNPs in experimental periodontitis, the concentration of TNF- α , IL-6 and IFN- γ in serum were detected by ELISA. As shown in Fig. 2a and 2b, the expression of TNF- α and IL-6 in the NaCl + ligation group was significantly up-regulated, indicating that inflammation stimulated the secretion, while the AuNPs-hBD3 + ligation group significantly reduced the serum TNF- α and IL-6 levels. However, in Fig. 2c, the expression of IFN- γ in the NaCl + ligation group was down-regulated, while in the hBD3 + AuNPs + Ligation group, this was significantly up-regulated.

2.3. Histological examination of rat maxilla treated by AuNPs combined hBD3

Based on H&E and Masson staining of the rat maxilla, the effects of AuNPs combined hBD3 on experimental periodontitis was further verified from the histological point of view.

H&E staining (Fig. 3a) showed that when compared with the NaCl group, the soft tissue around the second molars in the NaCl + ligation group was obviously receding, the alveolar bone was resorbed, local epithelial hyperplasia was observed and the infiltration of inflammatory cells in the lamina propria significantly were significantly increased. While, the AuNPs-hBD3 + ligation group showed that the infiltration of inflammatory cells was reduced, the elastic fibers and collagen fibers were well-organized and the epithelial nail process hyperplasia was reduced.

Masson staining (as shown in Fig. 3b) showed that in the NaCl group, the trabecular bone structure was tight, the muscle fibers were stained deep and obvious, the Havers system structure was obvious and the bone maturity was high. In the NaCl + ligation group, there were more blue collagen fibers, indicating new bone with low calcification. The Havers system was still in the initial formation stage. However, these damages in the NaCl + ligation group were apparently recovered via combination treatment with AuNPs and hBD3. The Havers system in the new bone tissue was more mature and similar to that of a normal jaw bone tissue structure.

2.4. TRAP and ALP staining of rat maxilla treated with AuNPs combined hBD3

The effect of hBD3 combined AuNPs on experimental periodontitis was also determined by observing the number of osteoclasts and osteoblasts.

As shown in Fig. 4a, there were a large number of cells with positive TRAP staining in the alveolar bone of the NaCl + ligation group, indicating active osteoclasts, while there were relatively few TRAP-positive cells in the AuNPs combined hBD3 group on the surface of the alveolar bone, suggesting that the bone-

breaking activity was weaker. On the contrast, the ALP expression of AuNPs combined hBD3 group was markedly increased in the periosteum of the alveolar bone, while was weak in the control group (as shown in Fig. 4b).

2.5 OPG and RANKL staining of rat maxilla treated with AuNPs combined hBD3

Last but not least, we evaluated the OPG and RANKL expression to further confirm the interventional effects of AuNPs combined hBD3 on experimental periodontitis.

For OPG (bone formation), positive cells could be visualized in light brown in the AuNPs combined hBD3 group (as shown in Fig. 5a). While for RANKL (bone resorption), number of positive cells could be found in the NaCl + ligation group, but few in the AuNPs-hBD3 + ligation group (as shown in Fig. 5b).

3. Discussion

In recent years, several studies have shown that the destruction of periodontal tissue closely related to the immune response[15]. With the in-depth understanding of the pathogenesis of periodontitis, it has been found that the interaction between the host immune response, bacteria and microorganisms and the environment is crucial in the occurrence and development of periodontitis and the regeneration of periodontal tissues[16]. In the periodontal inflammation microenvironment, lipopolysaccharide (LPS) interacts with monocytes to produce a variety of cytokines which involved as classic cytokines of periodontitis bone destruction, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6), play important roles in the progression of periodontitis. Studies have confirmed that the expression of TNF- α and IL-6 is positively correlated with the severity of periodontitis, which is significantly increased at the site of periodontal inflammation, and its level is significantly reduced after treatment, so they can be used as the evaluation index for the degree of periodontal tissue damage[17–19]. In this study, the concentration of TNF- α and IL-6 in rat serum was determined by ELISA experiment. The results showed that the expression levels of TNF- α and IL-6 in serum increased significantly by ligating. After treatment with AuNPs combined hBD3, the levels decreased significantly compared with the ligation group, indicating that AuNPs combined hBD3 can significantly reduce periodontal inflammation.

The clinic-pathological features of chronic periodontitis mainly include the formation of periodontal pockets, alveolar bone resorption, tooth mobility, and tooth loss in the terminal stage. In this study, Micro-CT was used to detect the effect of alveolar bone resorption in the rat maxillary second molars. The results showed that there were obvious alveolar bone resorption images at the root furcation of the second molars in the ligation + NaCl group, and the alveolar bone resorption was significantly reduced after the treatment of AuNPs and hBD3. The analysis of bone parameters related indicators, including bone mineral density (BMD), bone volume (BV) and relative bone volume fraction (BV/TV), indicated that AuNPs combined hBD3 had contributed to bone formation.

HE staining showed that compared with the control group, AuNPs combined hBD3 group had less inflammatory cell infiltration, less collagen fibrosis and fracture. Masson staining depicted that in AuNPs combined hBD3 group had obvious red staining and higher calcification in the new bone tissue. What's more, ALP and TRAP play a critical role in osteogenesis as key enzymes involved in the process of bone matrix deposition and resorption[20]. ALP is unique to osteoblasts and preosteoblasts, whereas osteocytes do not secrete ALP while TRAP is secreted by osteoclasts[21]. In our study, the administration of AuNPs combined hBD3 increased the expression of ALP, while decreased TRAP expression. Similar effects were observed in MG-63 cells and MC3T3E-1 treated by human beta-defensins and gold nanoclusters respectively[22, 23].

Further research we continue to explore the mechanism by which AuNPs combined hBD3 protects periodontitis. During the process of bone reconstruction, osteoclasts and osteoblasts maintained a certain number to restrict each other and renewed to keep balance[24]. Osteoprotegerin (OPG) and receptor activator of NF- κ B Ligand (RANKL) are one of the most critical pair of cytokines in this regulation. RANKL is the main regulator of bone resorption. Various cytokines, inflammatory mediators and hormones indirectly make osteoclasts mature and activate mainly by promoting the secretion of RANKL. While OPG was the receptor of RANKL. By binding to RANKL, it could hinder the functional activities of RANKL, and could also promote the generation of osteoblasts and the apoptosis of osteoclasts[25, 26]. Therefore, the expression of OPG and RANKL in periodontal tissues played an important role in regulating alveolar bone resorption[27]. Park et al. had shown that HBD3-C15 could inhibit RANKL-induced osteoclast differentiation and disrupted the formation of RANKL-induced podosome belt[28]. Studies have also shown that AuNPs could not only suppressed pre-osteoclast fusion induced by RANKL and macrophage colony stimulating factor (M-CSF), but also brought about significant down-regulation in gene expression of RANKL and RANKL/OPG ratio[29, 30]. Our results demonstrated that AuNPs combined hBD3 could inhibit the secretion of RANKL and increase the expression of OPG, which may reduce the absorption of alveolar bone.

In this study, we mainly focus on the effect of AuNPs combined hBD3 of periodontitis and osteogenesis, but the specific regulatory mechanisms still need to be further explored.

4. Conclusions

Based on the development of nanomaterials and tissue engineering technology, the combination of AuNPs with hBD3 had a protective effect on the progress of experimental periodontitis in rats, and also played a certain role in promoting osteogenesis. These findings suggested that AuNPs with hBD3 might be a novel biomedical material for promoting the repair of alveolar bone defects.

5. Methods

5.1. Experimental animals and groups

After being fed adaptively for 1 week, 25 female SD rats at the age of 5 weeks old were randomly divided into five groups ($N = 5/\text{groups}$) as follows: 1) NaCl group, NaCl group with no ligation; 2) NaCl + ligation group, rats with ligature-induced periodontitis un-treated; 3) hBD3 + ligation group, rats with ligature-induced periodontitis were treated with hBD3; 4) AuNPs + ligation group, rats with ligature-induced periodontitis were treated with AuNPs and 5) AuNPs-hBD3 + ligation group, rats with ligature-induced periodontitis were treated with AuNPs combined hBD3.

5.2. Reagents configuration

hBD3 was commercially available from Peprotech (Rocky Hill, NJ, USA), and AuNPs with a diameter of 45 nm were synthesized by adopting a chemical reduction method. The concentration of AuNPs and hBD3 were used according to our published data[13, 14].

5.3. Rat ligature-induced experimental periodontitis model

The rats were anesthetized with pentobarbital sodium (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) based on 50 mg/kg. The silk threads were soaked in the medium of *P. gingivalis* for 2 h ahead of time, and then were then used to ligate the bilateral maxillary second molars of the SD rats. The control group was treated with no ligation and the ligated groups with sterile silk thread. According to the groups, 100 μL of 5 $\mu\text{g/mL}$ hBD3, 10 μM AuNPs or 0.9% NaCl solution were injected into the mesial, central and distal sides of the maxillary second molars respectively and repeated every 3 days. At the study endpoint, when the rats were 7 weeks old, the rats were euthanized and the blood was collected by eyeball removal, and the maxillary bone, gums and other tissues were taken and fixed in a 4% neutral paraformaldehyde solution.

5.4. Micro-CT scanning

The maxillary samples of the SD rats were soaked in a 4% paraformaldehyde solution, and then were scanned by micro-CT with a Skyscan 1176 scanner (Bruker, Karlsruhe, Germany). The scanning layer thickness was 18 μm , the X-ray exposure time was 404 ms, the tube voltage was 70 kV, and the tube current was 353 μA . After scanning, 3D volume rendering technology from CTvox software was used to convert the 2D CT tomography images into 3D, proportionally, and to reconstruct the images to measure the bone density (BMD), bone volume (BV) and tissue volume (TV).

5.5. Detection of serum inflammatory factors

After the rats were anesthetized, the hair around the eyes was cut off, the eyeballs were harvested and the whole blood was placed in an EP tube. After standing at room temperature for 2 h, 2000 xg and centrifuged at 4 °C for 10 min, the serum was transferred to a new centrifuge tube. ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to detect the concentrations of TNF- α , IL-6 and IFN- γ .

5.6. Histological and immunohistochemical analysis

The maxillary samples were soaked in 10% Ethylenediamine Tetraacetic Acid (EDTA) for 4 weeks, dehydrated with the ethanol gradient, embedded into paraffin sections, and then haematoxylin and eosin

(H&E) staining, Masson staining, tartrate-resistant acid phosphatase (TRAP) staining, osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) were performed.

5.7. Statistical analysis

In our study, all statistical computations were performed using GraphPad Prism 6.0 software and the experimental data of each group are expressed as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) was used for the comparison of multiple sample means. When the P value < 0.05 , the difference was considered statistically significant.

6. Abbreviations

AuNPs, gold nanoparticles; hBD3, human β -defensin 3; micro-CT, Micro-focus computerized tomography; ELISA, enzyme-linked immunosorbent assay; ALP, alkaline phosphatase; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of NF- κ B Ligand; TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6.

7. Declarations

7.1. Ethics approval and consent to participate

The study was approved by the Ethics Committee of Nanjing University.

7.2. Consent for publication

Not applicable

7.3. Availability of data and materials

All data generated or analysed during this study are included in this published article.

7.4. Competing interests

The authors declare that they have no competing interests.

7.5. Funding

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7.6. Authors' contributions

ZJ and YFH designed the study. YWR synthesized the AuNPs. ZJ did the model of experimental periodontitis with LLJ, CD and XXT. LLJ had contributed to the data analysis as well. ZJ edited the

manuscript and was responsible for the integrity of the data and the accuracy of the data analysis. YFH was involved in the revision of the manuscript. All authors read and approved the final manuscript.

7.7. Acknowledgements

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Figures

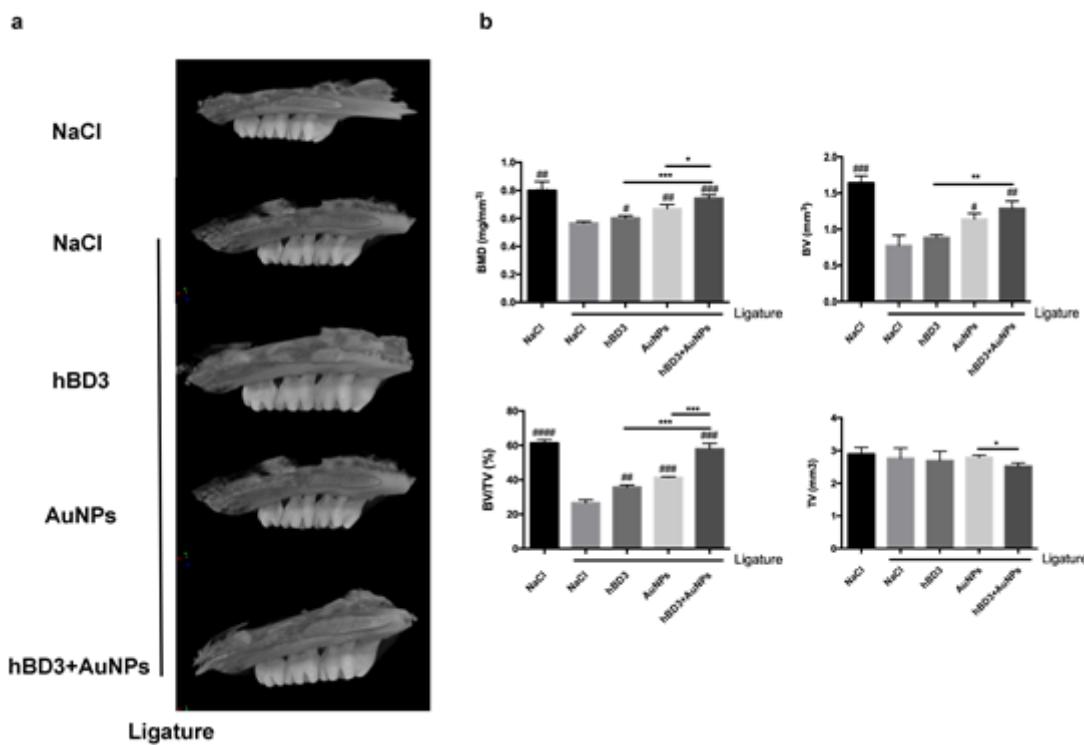


Figure 1

Effects of AuNPs combined hBD3 on alveolar bone loss. (a) The view of the alveolar bone level was showed by micro-CT through three-dimensional reconstruction images; (b) The BMD (Bone Mineral Density), BV (Bone Volume), bone fraction (BV/TV) and TV (Tissue Volume) in the interradicular regions of the second maxillary molars of each group ($n=5$ per group). #P < 0.05, ##P < 0.01, ###P < 0.001, #####P < 0.0001, compared with the NaCl +ligation group. *P < 0.05, **P < 0.01, ***P < 0.001.

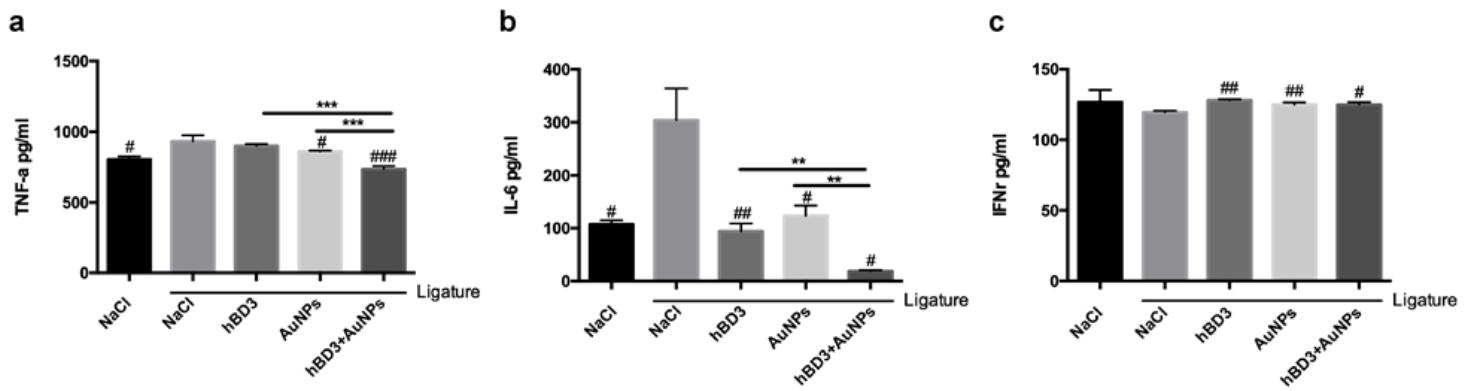


Figure 2

Effects of AuNPs combined hBD3 on inflammation profile. ELISA kits were used to measure the serum levels of TNF- α (a), IL-6 (b) and IFN- γ (c). #P < 0.05, ##P < 0.01, ###P < 0.001, compared with the NaCl +ligation group. **P < 0.01, ***P < 0.001.

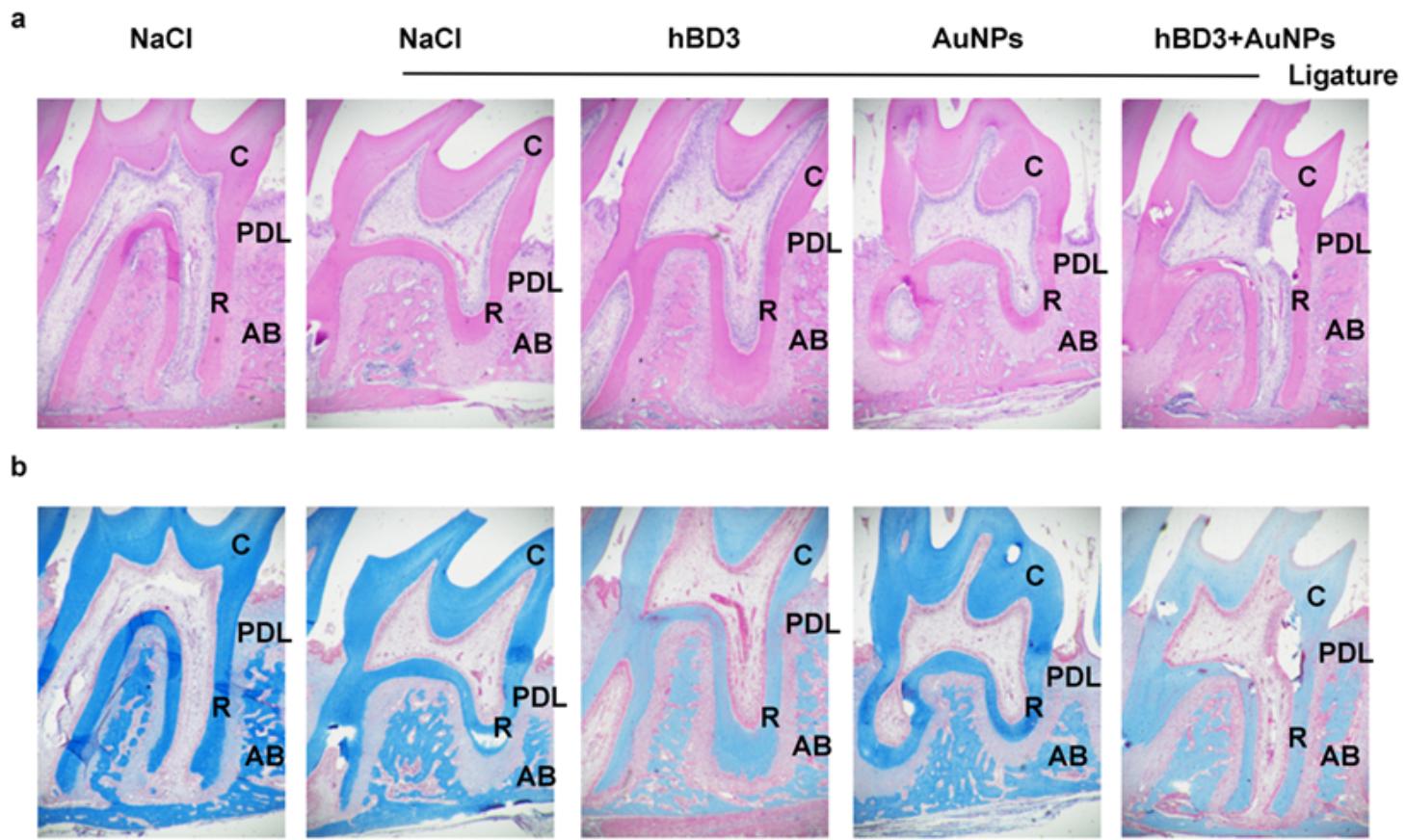


Figure 3

Histological examination of periodontal tissues measured by AuNPs combined hBD3. (a) H&E staining (x40), (b) Masson staining (x40). (C, crown; R, root; AB, alveolar bone; PDL, periodontal ligament.)

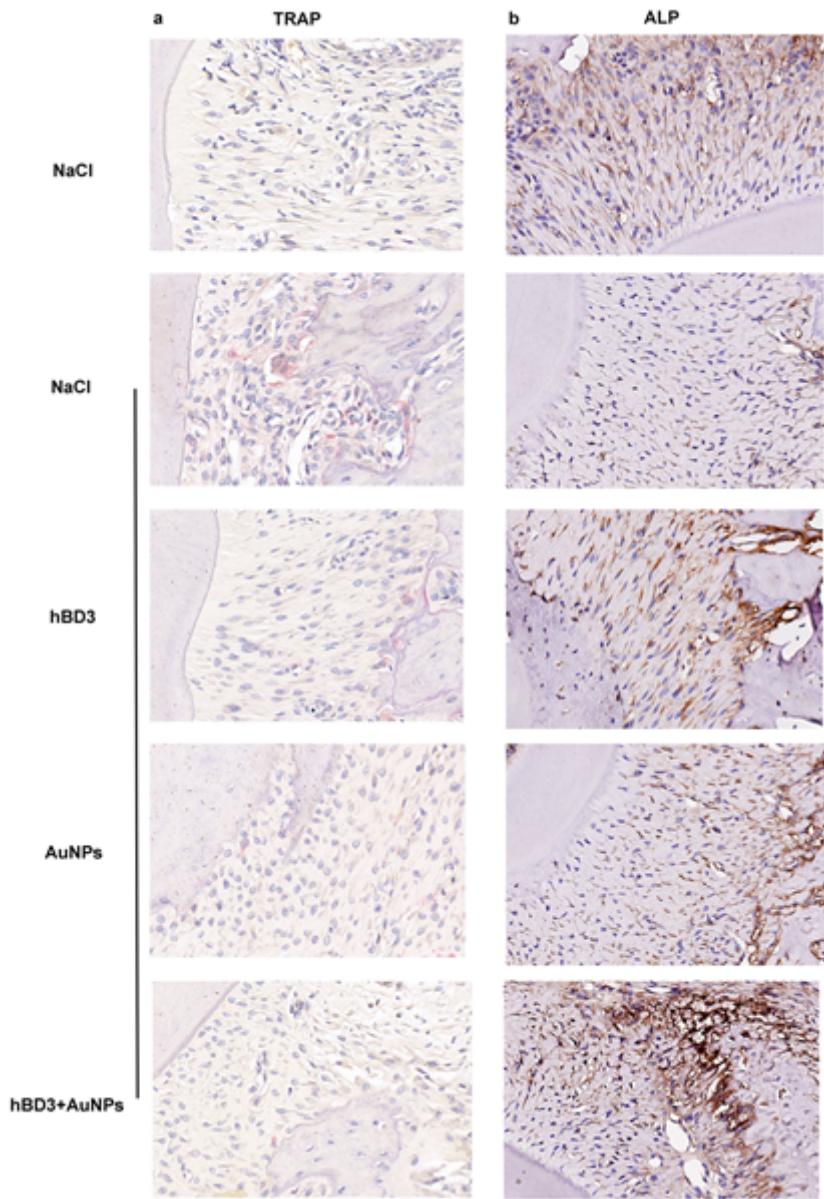


Figure 4

ALP and TRAP staining of periodontal tissues measured by AuNPs combined hBD3. (a) TRAP staining (x 400), (b) ALP staining (x 400).

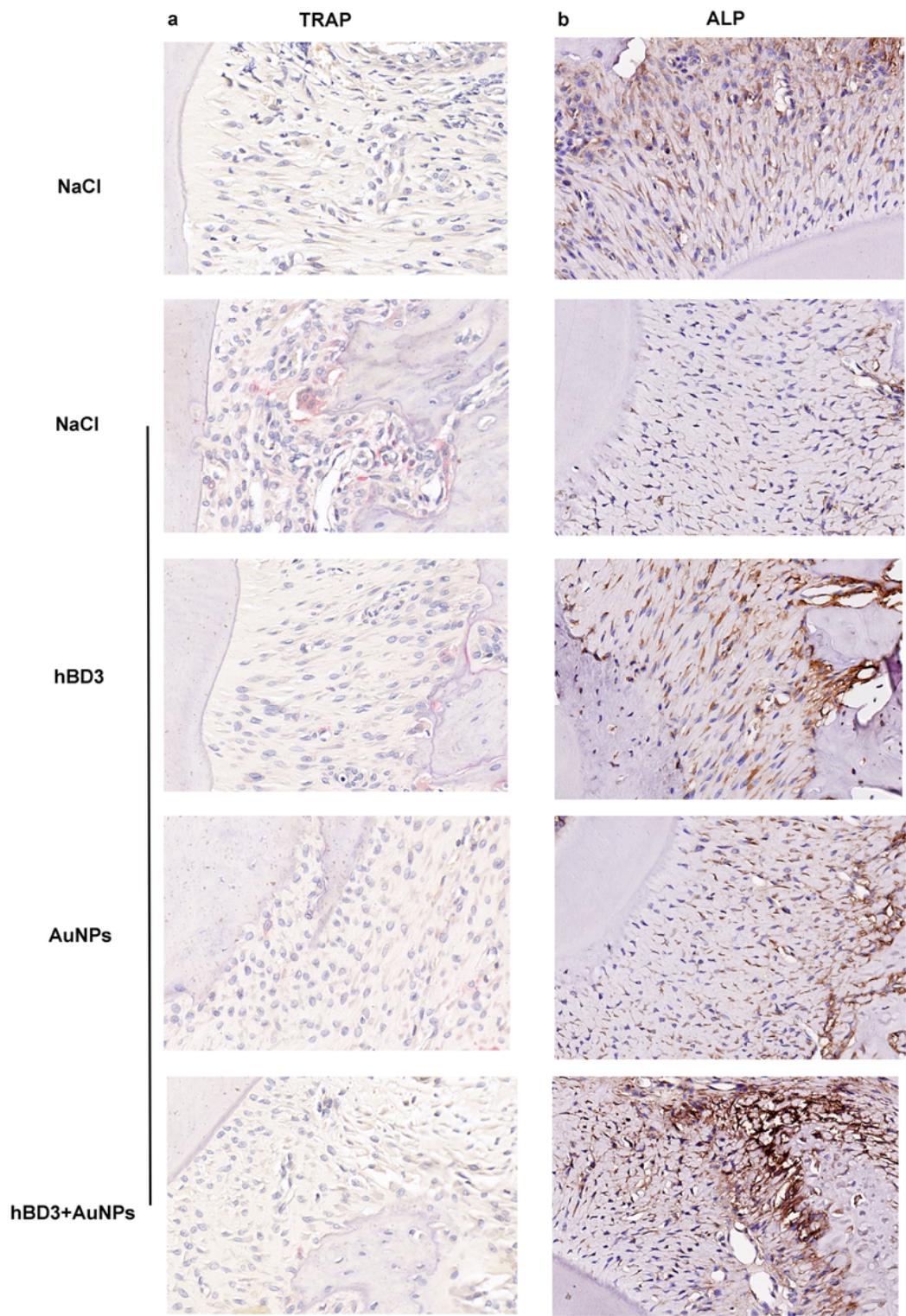


Figure 5

OPG and RANKL staining of periodontal tissues measured by AuNPs combined hBD3. (a) OPG staining (x 400), (b) RANKL staining (x 400). (The red arrows point out the OPG-positive cells.)