The Receptor Activator of Nuclear Factor-Kbligand and Osteoprotegerin Concentration in Gingival Crevicular Fluid During Orthodontic Tooth Movement on Regenerated Alveolar Bone

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ABSTRACT

Purpose: To evaluate the effects of bone regeneration materials (BRMs) and orthodontic tooth movement (OTM) on radiological features as well as receptor activator of nuclear factor-κBligand (RANKL) and osteoprotegerin (OPG) concentrations in gingival crevicular fluid (GCF).

Methods: Twenty-four alveolar bone defects in beagle dogs were treated using either Bio Oss® or beta-tricalcium phosphate (β-TCP) (experimental) with empty control defects. The concentrations of OPG and RANKL inGCF were examined by immunoassay analysis, while the tooth displacement and alveolar bone height after OTM were radiographically assessed

Results: After OTM the second premolar displacement for Bio Oss was statistically significantly lower than control group and theOPG concentration at baseline was significantly higher in the control than the Bio Oss group. The β-TCP group registered statistically higher RANKL concentration than control and Bio Oss groups at the end of orthodontic tooth movement.

Conclusions: The type of regeneration materials used determined the extent of tooth movement in regenerated alveolar bone defects. Whereas, orthodontic force as well as the type of BRMs affectedthe RANKL and OPG concentration in GCF.

Introduction

Orthodontic tooth movement is a result of osteoblastic and osteoclastic bone remodeling activities, closely coordinated and regulated by bioactive molecules [1-3]. The receptor activator nuclear factor-κBligand (RANKL) and osteoprotegerin (OPG) are the two important factors which actively participate in bone remodeling in response to orthodontic mechanical forces through OPG/RANK/RANKL signal pathway [4,5]. Bone regeneration has become a common method of managing bone defects in dental surgery and orthopedics practices. Consequently, in contemporary dentistry, it is possible to encounter a patient who needs both bone regeneration and orthodontic treatment. Regarding regeneration materials; reports have shown some significant physical, biological and chemical differences between BRMs of different origins [6-8]. In addition, the bone formed by regeneration differs in cellular and physical characteristics based on the type of BRMs used [9-11]. It is therefore assumed that the regeneration materials can have significant effects on molecular response to orthodontic force as well as the pattern of OTM.

The RANKL produced and secreted by osteoblasts [12], stimulates osteoclasts differentiation through its receptor (RANK), which is expressed on osteoclasts and their precursors [5,12], Conversely,

OPG can inhibit the proliferation of osteoclasts via its competitive binding against RANK. The binding between RANKL and RNAK can facilitate bone reabsorption via a cascade signaling pathway previous described [13,14]. Although the interaction between RANKL and RANK can be inhibited by the decoy receptor OPG, [5,12]. RANKL and OPG are relatively specific for osteoblasts [5,15,16] and the balance between them determines the osteoclast functions, while alterations of the RANKL/OPG ratio are critical in the bone remodeling and diseases pathogenesis [17].

Reduced RANKL with elevated level of OPG inhibit osteoclasts' differentiation, therefore the OPG expression peaks during bone formation phase [18]. An increased RANKL/OPG ratio favors osteoclast formation and activation; as a result, bone resorption occurs. On the other hand, a decreased RANKL/OPG ratio promotes bone formation by inhibiting osteoclastic activity.

Although allografts, xenegrafts as well as synthetic regenerative materials have shown satisfactory results in clinical applications [19], the resorption rate of regenerative material, the quality of bone formed as well as the rate of bone formation are subject to the type of materials used for regeneration [6,20-22]. Thus, there are variations inthe type and amount of cellsas well as mineral components based on the material used and time elapse post regeneration. While some bone substitutes undergo almost immediate biodegradation and resorption, others can be detected on the implant site for several years [6,7]. In addition, some materials are reported to have better osseointegration with host bone [8] and faster new bone formation with fewer inflammatory cells than others [23].

Orthodontic forces generate cellular and molecular responses to induce bone remodeling for tooth movement. Previous studies have shown that the efficiency of tooth movement is related to the number and activities of osteoclasts [2,3]. which are modulated by OPG/RANK/RANKL signal pathway [4,5]. Due to the differences in cellular and mineral components of regenerated bones, the difference in molecular and physiological activities may exist between bones formed from different regeneration materials with probable disparities in RANKL and OPG expression as well as potential impact on OTM on regenerated bone. Therefore, the aim of the present study was to investigate the effects of BRMs and OTM on radiological features as well as OPG and RANKL concentration in GCF collected fromalveolar bone treated with different type of BRMs.

Methods
Ethical considerations
The study was approved by the Ethics Committee of Fujian Medical University. All animal handling and surgical procedures were conducted according to the Institutional Review Board (IRB) guidelines for the use and care of laboratory animals.

Animal experiments
The study used six male beagle dogs aged 18 months with a mean weight of 11.3 Kg. Data were collected by intraoral clinical examination computed tomography (CT) scan image and immunoassay analyses. Twenty-four alveolar bone defects were created by extending the first pre-molar extraction socket. The experimental defects were treated by guided bone regeneration (GBR) using synthetic β-TCP (Bio-lu Biomaterials Co., Ltd. Shanghai, China) or xenograft Bio-Oss® (Geistlich, Wolhusen, Switzerland) regeneration materials, whereas the control defects were left empty. Resorbable collagen membranes Bio-Gide® (Geistlich, Wolhusen, Switzerland) were used in both experimental and control defects. The regeneration materials were equally allocated to the maxillary right and left (UR and UL) as well as to the mandibular right and left (LR and LL) defects by randomizing three pre-determined sets of defect managements to the six experimental animals (i.e set 1: UR- β-TCP, UL-Bio Oss, LR-Control and LL- β-TCP; set 2: UR-Bio Oss, UL- β-TCP, LR-Bio Oss and LL Control; set 3: UR-Control, LR- β-TCP, UL-Control and LL Bio Oss). Every set was randomly assigned to two dogs; consequently, the three GBR groups (β-TCP, Bio Oss and Control) were equally distributed to the right and left of maxillary and mandibular jaws. The set randomization also allowed for every GBR group to be assigned to eight defects.

Surgical procedure
Under general anesthesia, the maxillary and mandibular first premolar extraction sockets were extended mesially from the second premolar using cylindrical tungsten bur to create standardized artificial defects measuring 5 mm deep, 7 mm long (mesial-distal) and 5 mm wide on each quadrant of the animal's jaws. Depending on the GBR group allocation, the defects were filled with β-TCP or Bio Oss mixed with animal's blood collected during defect preparation. The mixture was packed into the artificial defects to the natural alveolar height level whereas, the control defects were left empty. The filled experimental and the empty control defects were all covered by resorbable collagen membranes Bio Gide® followed by wound closure using 3/0 nylon sutures which remained in the site for two weeks.

Clinical assessment
A standardized clinical data sheet was used to collect clinical features of all defects during two weeks healing stage. The features clinically observed at this stage included local swelling, bleeding on gentle touch, pus discharge, BRM discharge and Membrane exposure. The assessment was done on second, third, fifth, seventh, tenth- and fourteenth-day post-operative. For convenience, the assessment was simultaneously done with gingival crevicula fluid (GCF) collection under general anesthesia.

Orthodontic appliance design and computed tomography scan analysis
The second premolar was moved to the mesial side by the application of a 150 g force as measured by a tension gauge (Aidebao, Leqing, China), using NiTi close coil spring (Ormco, Orang, County, CA, USA) for two months according to. The force was activated at a two-weeks interval.

All animals were subjected to CT scanning before and after OTM

using a Cone Beam Computed Tomography (CBCT) machine (DCT Pro; Vatech& EWOO Group, South Korea). The images were used to extract digital information on alveolar bone height and second premolar displacement using EZ 3D 2009 software (Vatech, Hwaseong-si, Korea) as previously described by Machibya et al [24]. The alveolar bone height was determined by the length of the line drawn perpendicular to the apical plane (a line connecting the canine and the first molar mesial root's apices) to the nearest alveolar crest level mesial to the second premolar. The distance between the second and third premolar cusps were measured digitally before and after OTM to determine the second premolar displacement. Both laboratory technician and the radiologist were blinded of the type of BRMs used for each bone defect sample.

Gingival crevicula fluid (GCF) Collection
The gingival clavicular fluid samples were collected from all defects at baseline (one day before OTM) and at the end of OTM (few minutes before termination of OTM). Prior to GCF collection, the animals were anaesthetized cleaned in the mouth and washed with normal saline. A methylcellulose paper strip was gently inserted in the gingival sulcus on the mesial aspect of second premolar and was left in for 30 seconds. Afterwards, the paper strips were placed into Eppendorf tubes and preserved at -80°C. To quantify the GCF collected, the Eppendorf tubes with strips and those with paper points were weighed before and after sampling.

The OPG and RANKL Enzyme linked immunosorbent assay
For immunoassay analysis, the samples were sent to Shanghai Biotechnologies, Inc. for protein extraction and immune assay process. Before analysis, the frozen GCF samples were thawed at room temperature for 1 hour, followed by addition of 200 µl Phosphate-buffered saline (PBS) and centrifuge at 10000 RPM for 15 minutes at 4□. Further 150 µl PBS buffer was added to the obtained supernatant followed by centrifugation. The procedure was repeated three times to obtain the supernatant liquor for immunoassay analysis. The OPG and RANKL concentration were determined using canine OPG and RANKL enzyme linked immunosorbent assays (ELISA) kit (MyBiosource, CA, USA.) according to the manufacturers' instructions and the optical densities were determined at 450 nm using Tecan® Infinite F50 microplate reader.

The concentration of OPG, and RANKL in each of the samples was then determined by comparing the average sample optical density readings with the concentrations from the assay standard curve and the data were reported as concentrations of biomarkers in nanogram per milliliter (ng/ml) and pictogram per milliliter (pg/ml) for OPG and RANKL respectively.

Statistical analysis
The means and standard deviations were calculated for each group. The data showed a normal distribution tendency; hence, we applied parametric statistical analysis, with the level of statistical significance set at p<0.05. The repeated-measures ANOVA was used to evaluate the biomarkers concentration of different GBR modes at BL and end of OTM, while the Pearson's correlation coefficient was employed in measuring the association between biomarkers concentration and radiological variables. The statistical package for social sciences (SPSS) software version 19.0 (IBM SPSS Inc., Chicago, IL, USA) was used with statistician's guide.

Results
The results showed that the second premolar displacement (3.93 ± 0.054) mm registered for Bio Oss was statistically significantly lower than control group (5.02 ± 0.031) mm (p>0.05). While the 4.41 ± 0.044 mm displacement registered for β-TCP was not statistically significant different compared to Bio Oss and Control groups.

The bone level change after OTM showed some specific characteristics between the groups with Bio Oss registering small gain (0.38 ± 0.098) mm while the control group showed alveolar bone loss (resorption) (-0.08 ± 2.08) mm and β-TCP had neither gain nor loss (0.00 ± 1.074) mm. However, the differences across the groups were not statistically significant.

The OPG concentration registered at BL and end of OTM in experimental group was statistically significant different (p< 0.05). Nevertheless, the control group showed limited increase in OPG concentration at the end of OTM (P = 0.09) (Figure 1). Before OTM the control's OPG concentration was significantly higher than the Bio Oss group (p< 0.05). Although the β-TCP group registered higher concentration than both control and Bio Oss groups atthe end of OTM, the difference fell short of statistical significance (Table 1). The OPG concentration at the end of OTM showed no significant correlation with alveolar bone level change as well as total tooth displacement for both experimental and control groups.
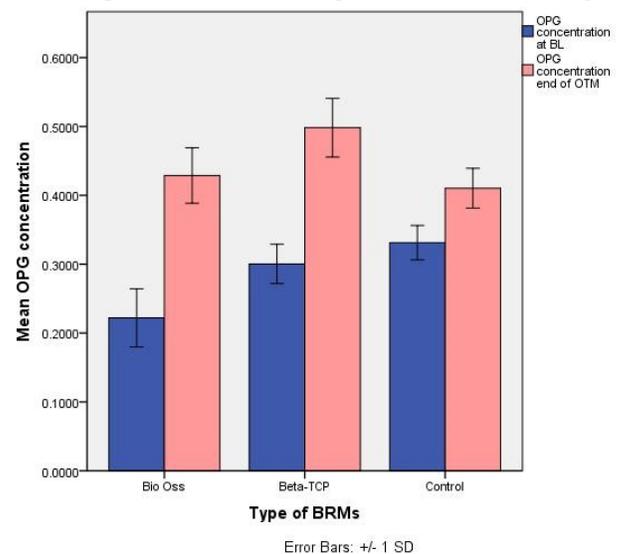
Figure 1: Bar chart displaying the OPG concentration in GCF according to different BRMs measured by ELISA test.
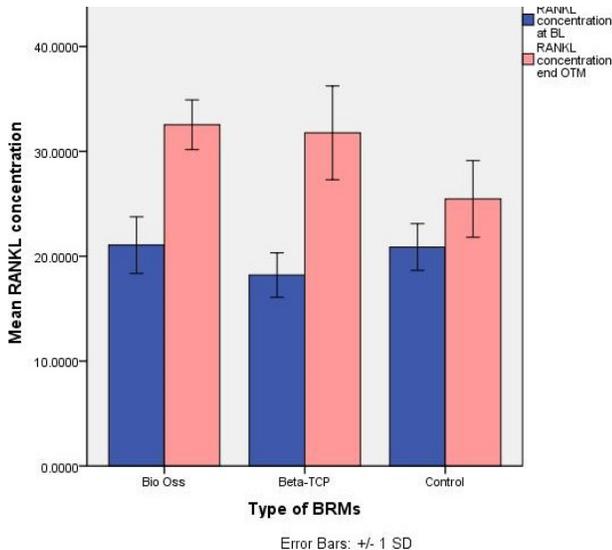
The RANKL concentrations registered at BL and end of OTM within groups were statistically significant different in all

groups (p< 0.01) and the Control group had consistently lower concentration than experimental groups at BL and end of OTM (Table 1 and Figure 2). There was no RANKL concentration difference between groups at BL, but the β-TCP group had statistically higher concentration than control and Bio Oss groups at the end of OTM (p< 0.05) (Table 1). The RANKL concentration at the end of OTM was weakly correlated to the alveolar bone level change (r = 0.22; p< 0.05) for β-TCP. The RANKL concentration for both experimental and control groups had no significant correlation with total tooth displacement.

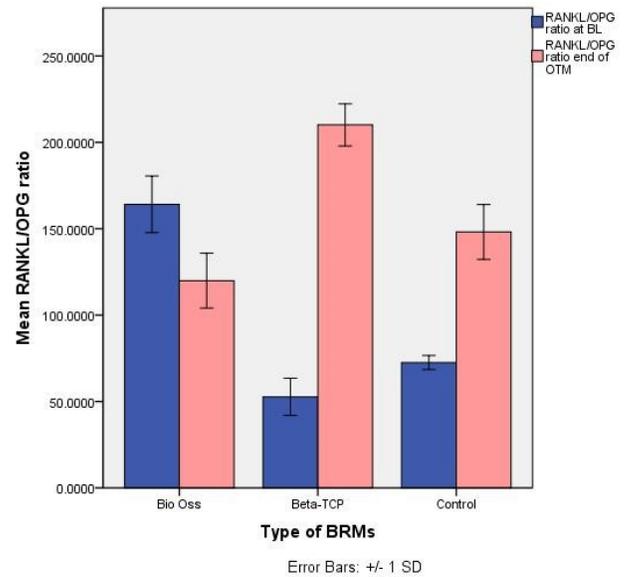| Type of BRMs	OPG Concentration Mean (Std)		RANKL Concentration Mean (Std)	
	At BL	End of OTM	At BL	End of OTM
Bio Oss	0.222 (0.109)[a]	0.416 (0.167)	19.654 (7.931)	30.586 (20.565)[a]
β-TCP	0.300 (0.106)[ab]	0.498 (0.276)	19.232 (9.866)	38.390 (15.572)[b]
Control	0.331 (0.196)[a]	0.410 (0.231)	18.373 (7.419)	26.992 (16.074)[a]

Table 1: The mean and standard deviation of OPG and RANKL concentration in GCF according to different BRMs measured by ELISA test (ng/ml for OPG and pg/ml for RANKL).

[a-b]Means in a column without a common superscript letter differ (P < 0.05) as analyzed by repeated measures ANOVA and the Tukey's test.

Figure 2: Bar chart displaying the RANKL concentration in GCF according to different BRMs measured by ELISA test.

Regarding RAKNL/OPG ratio; the Bio Oss registered significantly higher ratio than both control and β-TCP at BL. The RAKNL/OPG ratio at the end of OTM was statistically significantly higher than at BL for β-TCP (p< 0.01), but it decreased in Bio Oss group (Table 2, Figure 3). The RAKNL/OPG ratio difference between BL and end of OTM in Control group was statistically significant (p< 0.05) and was significantly higher in β-TCP compared to Bio Oss group at the the end of OTM. For the β-TCP group the RAKNL/OPG ratio at the end of OTM was correlated to the alveolar bone level change and total tooth displacement (r = 0.43; p< 0.05 and r = 0.33; p< 0.05, respectively).

Figure 3: Bar chart displaying the RANKL/OPG concentration ratio in GCF according to different BRMs.

Type of BRMs	RANKL/OPG Ratio Mean (Std)	
	At BL	End of OTM
Bio Oss	164.121 (62.892)[a]	119.655 (161.406)[a]
β-TCP	57.766 (18.009)[a]	206.765 (238.578)[b]
Control	73.060 (54.926)[a]	148.137 (198.358)[ab]

Table 2: The mean and standard deviation of RANKL/OPG ratio in GCF according to different BRMs measured by ELISA test.

[a-b]Means in a column without a common superscript letter differ (P < 0.05) as analyzed by repeated measures ANOVA and the Tukey's test.

Discussion

The study identified some radiological and molecular differences between different types of BRMs. The tooth displacement in Bio Oss was statistically significantly lower than control after OTM probably due to the slow resorption of the Bio Oss residual materials reported in the previous studies [6,7] and possible physical obstruction of tooth movement by regeneration materials in Bio Oss compared to the empty control group. There was no statistically significant difference in alveolar bone height changes based on BRMs following two-month OTM, however the Bio Oss group tended to resist bone resorption than other groups perhaps due to differences in the rate of BRMs resorption and the variation of cellular component of regenerated bone as previously reported [9,10]. Several studies have shown significant association between OTM and cellular (osteoblasts and osteoclasts) remodeling activities coupled with OPG/RANK/RANKL signal pathway [4,5,25,26]. In the current study the differences observed in RANKL and OPG concentration was based on the type of BRMs as well as the orthodontic force (BL and end of OTM). There was statistically significant difference in OPG concentration between BL and end of OTM in experimental (Bio Oss and β-TCP). But the control group showed limited increase in OPG concentration at the

end of OTM (p = 0.09) (Figure 1). The findings in the control group are in agreement with a study by Grant et al [25] which reported non-significant OPG increases 4 hours after orthodontic force application atcanine sites of both tension and compression and Otero et al. [26] who found no statistically significant differences in OPG level on comparing force magnitudes and experimental teeth with those in the control teeth. The findings in the current study's experimental group differ from the control group (Table 1 and Figure 1) and those of previous studies [25,26]. The plausible explanation for the disparity is the difference in cellular and molecular components of regenerated bone and natural (non-regenerated) bone [4, 25,26]. Shahoon et al [27] in a histological study reported a gradual reduction of inflammation along with an increase in new bone formation in both Human Bone Matrix Gelatin (HBMG) and autograft groups on 7, 14, 28 and 60 days after surgery, while autograft registered less giant inflammatory cells consistently. Although the actual molecular pathways involved in the remodeling is beyond the scope of this study, it is worth noting that the probable biological and chemical differences of bones regenerated by different BRMs could affect the OPG/RANK/RANKL signal pathway leading to the differences in OPG expression in the current study. The β-TCP group registered higher concentration than both control and Bio Oss groups at the end of OTM, however the difference fell short of statistical significance. The analysis also showed no significant correlation between OPG and alveolar bone level change as well as total tooth displacement for both experimental and control groups. The findings are similar to a report by Otero et al [26].

The RANKL concentrations registered at BL and end of OTMwere statistically significantly different in both experimental (Bio Oss and β-TCP) and control groups. Other studies [25,26,28] have observed increase in RANKL during resorption phases on pressure zone during OTM. Grant et al [25] registered an increased in RANKL from day 7to 42nd days on pressure zone of teeth under orthodontic force. The control group in the current study had consistently lower concentration than experimental groups at BL and end of OTM (Table 1 and Figure 2). Although the difference was not statistically significant, the observation may suggest lowered osteoclastic activities in control group than experimental group, particularly with β-TCP group. There was no RANKL concentration difference between groups at BL, but the β-TCP group registered statistically higher concentration than control and Bio Oss groups at the end of OTM. The presence of the material in the healing defect may have provided an ideal scaphoid for faster bone formation with adequate cellular component. Contrary to Bio Oss known for slow rate of material resorption, [9,10] the β-TCP material might have undergone degradation and resorption at the end of OTM giving ideal biological environment for bone cells to respond effectively to the orthodontic mechanical force through OPG/RANK/RANKL signal pathway. The RANKL concentration at the end of OTM was weakly correlated to the alveolar bone level change (r = 0.22; p< 0.05) for β-TCP. The findings are in keeping with a previous study by Grant et al. [25] which reported significant association between bone metabolism indicators (RANKL and OPG) in GCF with orthodontic force application.

Many studies have demonstrated significant association between OTM and RANKL/OPG ratio, whereby the ratio tend to increase on pressure zone of teeth loaded with orthodontic forces [26,28-30]. The RAKNL/OPG ratio in the current study increased for β-TCP and control groups but the Bio Oss group registered lower ratio at the end of OTM than BL. Bio Oss registered statistically higher ratio than both control and β-TCP at BL. Since osteoclasts are involved in bone remodeling during bone regeneration [31,33], the high RAKNL/OPG ratio observed in Bio Oss at BL may be due toactive osteoclastic remodeling process as part of bone healing process even before OTM commencement. Some studies have suggested osteoclasts involvement in BRMs residual degradation during healing [34-37]. Active osteoclasts have been suggested to be part of the multinucleated giant cells (MNGCs) association with different types of regenerative materials during regenerative bone healing [34-37]. The low RAKNL/OPG ratio in β-TCP and control may be due to the lack of significant amount of residual material due to the relatively faster rate of resorption for β-TCP and the lack of regeneration materials in the empty control group. The RAKNL/OPG ratio at the end of OTM was significantly higher in β-TCP compared to Bio Oss group. The difference was mainly due to increase in OPG concentration in Bio Oss group as well as significant increase in RANKL concentration in β-TCP at the end of OTM. The role of osteoblasts and osteoclasts in both bone healing and OTM is the probable explanation for the differences. The cellular and mineral component as well as morphological structure of regenerated bone varies with time and type of regeneration materials [11,27,38] which could affect the pattern of molecular expression of RANKL and OPG during OTM along regenerated bone defects.

The rate of wound healing is reported to be significantly faster in dogs than in human [39], therefore the current study's findings cannot be directly inferred into clinical practice. Due to difference in bone turnover, the time correlating to the observed events can vary in clinical situation, although similar pattern of observations may be expected.

Conclusion
The type of regeneration materials used determined the extent of tooth movement in regenerated alveolar bone defects. Additionally, the orthodontic mechanical force as well as the type of BRMs used significantly affected the RANKL and OPG concentration in GCF.

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