

## The Effects of Regenerative Materials on Biomarkers, Clinical and Radiological Features of Healing Defects Treated by Guided Bone Regeneration

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### Abstract

**Background:** Guided Bone regeneration (GBR) has gained popularity in clinical dental practice. However, there is limited number of studies regarding the molecular and physical effects of bone regeneration materials (BRMs) on healing bone defects, which is essential for optimum clinical outcome.

**Methods and Findings:** About 24 standard alveolar bone defects in six male beagle dogs were treated by GBR using either Bio Oss® or beta-tricalcium phosphate ( $\beta$ -TCP) (experimental) whereas the control defects were left empty. The concentration of osteoprotegerin (OPG), vascular epithelial growth factor (VEGF) and matrix metalloproteinases-2 (MMP-2) in gingival crevicular fluid (GCF) were examined by immunoassay, while the computed tomography (CT) scan images were used to assess the bone density and alveolar bone height. The clinical features assessment showed no significant difference between BRMs. However, the OPG concentration registered on 3<sup>rd</sup> day was significantly higher for  $\beta$ -TCP than Control group. The VEGF concentration on 7<sup>th</sup> day was significantly lower for Bio Oss than Control and  $\beta$ -TCP. The experiment group had significantly higher MMP-2 concentration than Control on the 10<sup>th</sup> day. There was significant increase on bone density for the experiment groups compared to control at one month with higher bone height at one and two months post-operatively ( $P < 0.05$ ).

**Conclusion:** The type of BRM had fundamental effects on the pattern of healing makers (OPG, VEGF and MMP-2) concentration in GCF at some-point during healing time. Furthermore, the experimental groups had enhanced bone density one-month post-operative with more bone height gain at one and two month's post-operative.

**Keywords:** Bone regeneration; Bone substitutes; Bone dentistry; Osteogenesis; Biomarkers; Matrix metalloproteinase

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### Introduction

Bone regeneration has become a common method of managing bone defects in oral-facial surgery and orthopedics practices [1-3]. Bone regeneration is a very complex process involving various cells and signaling molecules during specific phases of the healing process under well-coordinated sequence of many biological events. A clear understanding of the molecular and cellular principles underlying bone healing with different bone regeneration materials is essential for the optimal treatment outcome of bone defects. Osteoblasts, osteocytes and osteoclasts are among the well-described cells responsible for bone

formation healing and remodeling process [4,5]. In addition, the process involves several bioactive molecules responsible for initiating and controlling biological events among the osteogenic cell community. Bone morphogenetic proteins (BMPs), vascular epithelial growth factor (VEGF), matrix metalloproteinases (MMPs), receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and osteoprotegerin (OPG) are some of the biological molecules responsible at some points during bone formation and healing [5-8].

Adequate vascularization is an absolute requirement for bone development, growth, homeostasis and repair [9]. Angiogenesis

is intimately involved in embryonic bone formation and with both endochondral and intramembranous bone formation in differentiated bone [10]. It is known that angiogenesis features three main steps: Proliferation of endothelial cells, breakdown of the extracellular matrix (ECM) and endothelial cell migration [11] in which MMPs and VEGF play great roles. MMPs are a family of enzymes that proteolytically degrade various components of the ECM. They participate in the degradation of the vascular basement membrane and remodeling of the ECM during angiogenesis.

MMP-2 along with MMP-9 have been shown to play critical roles in the "angiogenic switch", thus enhanced secretion of MMP-2 and MMP-9 stimulate angiogenesis and increase VEGF release [7]. VEGF itself is a homodimeric glycoprotein, which acts as a potent and selective endothelial mitogen, inducing rapid and complete angiogenic response [12]. Studies have shown that the development of osteoclasts from their precursors usually requires the presence of osteoblasts through a pathway that involves three members of the tumor necrosis factor (TNF) and TNF receptor families RANKL, OPG and receptor activator nuclear factor- $\kappa$ B (RANK) [8]. RANKL is produced and secreted by osteoblasts [13] and it stimulates osteoclasts differentiation through its receptor (RANK), which is expressed on osteoclasts and their precursors [13,14] OPG being a decoy receptor for RANKL can inhibit the proliferation of osteoclasts via its competitive binding against RANK, therefore impedes bone resorption [15]. RANKL and OPG have been considered relatively specific for osteoblasts [14,16] and the balance between RANKL and OPG determines osteoclast functions.

Although allografts, xenografts as well as synthetic regenerative materials have shown satisfactory results in clinical applications [17], the resorption rate of regenerative material, the quality of bone formed as well as the rate of bone formation are determined by the type of materials used for regeneration [1-3]. While some bone substitutes undergo almost immediate biodegradation and resorption, others can be detected on the implant site for several years [1,18]. Moreover, some materials are reported to have better osteointegration with host bone [19].

Events like cell migration, proliferation, chemotaxis, differentiation and synthesis of extracellular proteins taking place during bone healing process are closely related to the rate of healing which varies with the type of regenerative materials as well [20]. In fact, prominent inflammatory cells have been reported beyond two months in sites treated by regenerative materials, while less inflammation is seen in auto grafts [20]. Due to difference in cellular and mineral components of regenerated bones, it is hypothesized that all of these events are modulated when defects are treated with different BRMs. In addition, the differences in molecular and physiological activities may exist between bones formed from different regeneration materials.

Although, bone healing has been extensively studied with fracture model, the use of different BRMs and the physiological and environmental peculiarities of alveolar bone healing underline the value of the current study. The teeth supporting alveolar bone is characterized by distinctive features such as the continuous and rapid remodeling in response to mechanical force stimuli [21] lack of muscle stem cells, which plays a critical role in fracture

healing [22,23], healing without histological cartilage formation [24] as well as potential infection of healing alveolar bone due to constant oral microbial challenge surrounding the alveolar bone [25]. Therefore, the characterization of an alveolar bone healing model is important to provide specific insight on alveolar bone healing with different type of BRMs. Hence, the current study aimed at evaluating the effects of BRMs on biomarkers' trend, clinical and radiological features of healing defects treated by guided bone regeneration (GBR). The findings are expected to be useful in several clinical procedures, such as implant-based rehabilitative therapies along with improvement of alveolar bone regenerative strategies.

## 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) guide lines for the use and care of laboratory animals.

### Animal experiments

The study included six male beagle dogs aged 18 months with a mean weight of 11.8 Kg. The sample size was determined by assuming clinical significant difference of 2 mm bone height with 1 mm standard deviation at the power of 0.9 and 0.05 significant levels. The 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 GBR using synthetic  $\beta$ -TCP (Bio-lu Biomaterials Co., Ltd. Shanghai, China) or xenograft Bio-Oss<sup>®</sup> (Geistlich, Wolhusen, Switzerland) regeneration materials, whereas the control defects were left empty.

Resorbable collagen membranes Bio-Gide<sup>®</sup> (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-  $\beta$ -TCP, UL-Bio Oss, LR-Control and LL-  $\beta$ -TCP; set 2: UR-Bio Oss, UL-  $\beta$ -TCP, LR-Bio Oss and LL Control; set 3: UR-Control, LR- $\beta$ -TCP, UL-Control and LL Bio Oss). Every set was randomly assigned to two dogs; consequently, the three GBR groups ( $\beta$ -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 medially 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  $\beta$ -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 crevicular fluid (GCF) collection under general anesthesia.

### Gingival crevicular fluid (GCF) collection

The gingival crevicular fluid samples were collected from all defects on third, fifth, seventh, tenth and fourteenth day post-operatively. 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 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, MMP-2 and VEGF immunoassay process

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 °C. Further 150 µl PBS buffer was added to the supernatant followed by centrifugation. The procedure was repeated three times to obtain the supernatant aliquots for immunoassay analysis.

### The OPG and MMP-2 enzyme linked immunosorbent assay

The OPG and MMP-2 concentrations were determined using canine OPG and MMP-2 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 (Tecan, Austria). Finally, the concentrations of OPG, and MMP-2 in each of the samples were then determined by comparing the average sample optical density readings with the concentrations from the assay standard curves and the data were reported as concentrations of biomarkers in ng/ml.

### The VEGF immunoassay analysis

The Bio-Plex MAGPIX System (Bio-Rad, CA, USA) was utilized according to manufacturer's instructions and as described earlier [26] to determine the VEGF fluorescence intensity in GCF and the data was analyzed using the Bio-Plex Manager 6.0 software (Bio-

Rad, CA, USA).

### Computed tomography scan

All animals were subjected to CT scanning before and after OTM using a Cone Beam Computed Tomography (CBCT) machine (DCT Pro; Vatech & EWO 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. [27]. A 0.5 cm<sup>2</sup> area was selected in the density measurement options, to determine the bone density 1 mm mesial to the second premolar's mesial root and 8.5 mm from imaginary apical plane (a line connecting the canine and the first molar mesial root's apices). The alveolar bone height was determined by the length of the line drawn perpendicular to the apical plane to the nearest alveolar crest level mesial to the second premolar. Both laboratory technician and the radiologist were blinded of the type of BRMs used for each bone defect sample.

### 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  $p < 0.05$ . With sphericity-Mauchly's test at  $p > 0.05$ , the repeated-measures ANOVA was used to evaluate the biomarkers concentration of different GBR modes at every experimental time point. The statistical package for social sciences (SPSS) software version 19.0 (IBM SPSS Inc., Chicago, IL, USA) was used with statistician's guide.

## Results

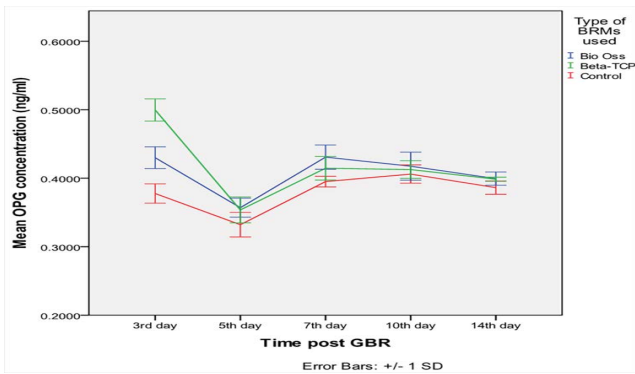
### Clinical features assessment

In the current study, all defects healed without pus or regeneration material discharge. However, on the third day post operation, bleeding was observed in 5 (20.8%) of the Bio Oss group defects compared to 3 (12.5%) and 1 (4.2%) of  $\beta$ -TCP and Control groups respectively. On 5<sup>th</sup> day the bleeding had stopped, except for one Bio Oss defect. Some swelling of different size was evident on all surgical sites on the 3<sup>rd</sup> and 5<sup>th</sup>. On most of the surgical sites, the swelling had subsided on the 7<sup>th</sup> day except for 5 (20.8%) of Bio Oss and  $\beta$ -TCP with only 1 (4.2%) of the control group. On day 10, the swelling had subsided from all defects. All differences observed by clinical variables were not statistically significant.

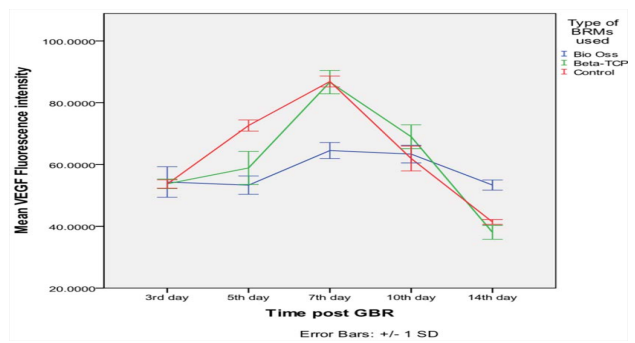
### The trend of OPG, MMP-2 and VEGF concentration in GCF during bone healing

The OPG concentration showed quite similar patterns in the three GBR groups (Bio Oss,  $\beta$ -TCP and Control) with high values on 3<sup>rd</sup> and 7<sup>th</sup> day post-operative for Bio Oss and  $\beta$ -TCP (**Figure 1**). The OPG concentration registered on 3<sup>rd</sup> day for  $\beta$ -TCP was significantly higher than Control group ( $P < 0.05$ ) (**Table 1**).

The VEGF concentration for Bio Oss group was significantly different from the two groups ( $\beta$ -TCP and Control). More details are shown in **Figure 2**. The VEGF concentration for Bio Oss group on 5<sup>th</sup> day was significantly lower than the Control, while the Bio Oss concentration on 7<sup>th</sup> day was significantly lower than Control and Beta-TCP (**Table 2 and Figure 2**).



**Figure 1** The linear mean plot of OPG concentration in GCF according to different BRMs measured by ELISA test.



**Figure 2** The linear mean plot of VEGF fluorescence intensity in GCF according to different BRMs measured by ELISA test.

The Control registered peak of MMP-2 concentration on 7<sup>th</sup> day while Bio Oss and  $\beta$ -TCP had peaks on 10<sup>th</sup> day (**Figure 3**) and the MMP-2 concentration in Control group on 7<sup>th</sup> day was significantly lower than Bio Oss, while both Bio Oss and  $\beta$ -TCP groups had significantly higher concentration than Control on the 10<sup>th</sup> day (**Table 3**).

### Radiological assessment

The CT scan analysis done at one and two-month post GBR

**Table 1** The mean and standard deviation of OPG concentration in GCF according to different BRMs measured by ELISA test (ng/ml) [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].

Type of BRM	Means (SD) of OPG concentration on respective days post GBR				
	3 <sup>rd</sup>	5 <sup>th</sup>	7 <sup>th</sup>	10 <sup>th</sup>	14 <sup>th</sup>
Bio Oss	0.415 (0.016) <sup>a</sup>	0.357 (0.13)	0.431 (0.018)	0.418 (0.020)	0.399 (0.009)
Beta-TCP	0.500 (0.018) <sup>b</sup>	0.354 (0.19)	0.415 (0.017)	0.413 (0.013)	0.399 (0.003)
Control	0.378 (0.013) <sup>a</sup>	0.332 (0.02)	0.395 (0.008)	0.406 (0.013)	0.386 (0.010)

**Table 2** The mean and standard deviation of VEGF fluorescence intensity in GCF according to different BRMs measured by immunoassay [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].

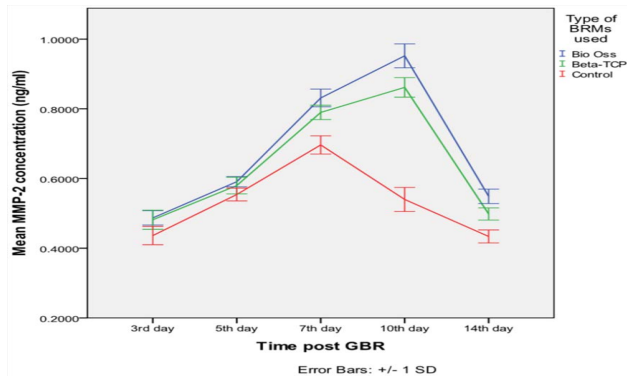
Type of BRM	Means (SD) of VEGF fluorescence intensity on respective days post GBR				
	3 <sup>rd</sup>	5 <sup>th</sup>	7 <sup>th</sup>	10 <sup>th</sup>	14 <sup>th</sup>
Bio Oss	54.34 (4.95)	53.32 (2.97) <sup>a</sup>	64.52 (2.60) <sup>a</sup>	63.37 (2.86)	53.35 (1.63)
Beta-TCP	53.76 (1.54)	58.89 (5.35) <sup>ab</sup>	86.68 (3.78) <sup>b</sup>	68.99 (3.87)	38.04 (2.24)
Control	53.69 (1.37)	72.62 (1.81) <sup>b</sup>	86.87 (1.76) <sup>b</sup>	61.94 (4.03)	41.40 (0.78)

**Table 3:** The mean and standard deviation of MMP-2 concentration in GCF according to different BRMs measured by ELISA test (ng/ml) [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].

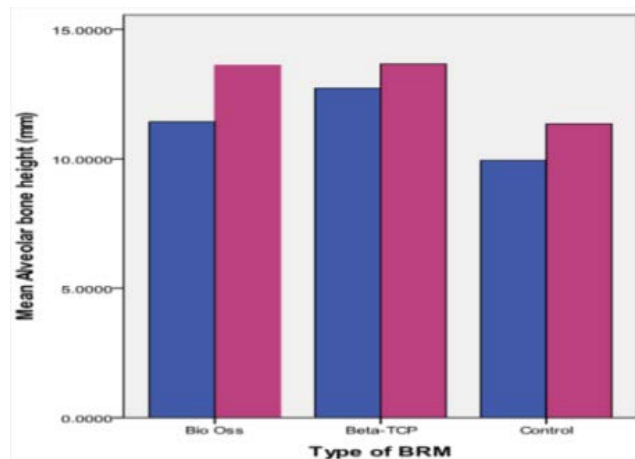
Type of BRM	Means (SD) of MMP-2 concentration on respective days post GBR				
	3 <sup>rd</sup>	5 <sup>th</sup>	7 <sup>th</sup>	10 <sup>th</sup>	14 <sup>th</sup>
Bio Oss	0.487 (0.018)	0.528 (0.014)	0.831 (0.026) <sup>a</sup>	0.952 (0.034) <sup>a</sup>	0.549 (0.020)
Beta-TCP	0.465 (0.026)	0.633 (0.035)	0.810 (0.025) <sup>ab</sup>	0.914 (0.028) <sup>a</sup>	0.533 (0.018)
Control	0.269 (0.008)	0.457 (0.18)	0.771 (0.026) <sup>b</sup>	0.581 (0.034) <sup>b</sup>	0.367 (0.009)

**Table 4** CT Scan, The mean and standard deviation of alveolar bone height and density according to different BRMs measured by CT scan analysis [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].

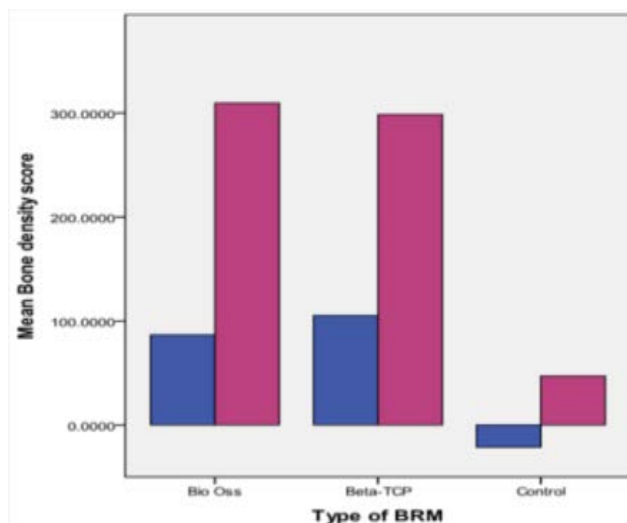
Type of BRM	Bone height one month after GBR	Bone height two month after GBR	Bone density one month after GBR	Bone density two month after GBR
Bio Oss	11.43 (1.54) <sup>a</sup>	13.61 (0.79) <sup>a</sup>	86.7 (53.56) <sup>a</sup>	312.13 (30.91)
Beta-TCP	12.73 (1.57) <sup>a</sup>	13.66 (0.94) <sup>a</sup>	105.23 (60.14) <sup>a</sup>	304.79 (43.26)
Control	9.94 (0.41) <sup>b</sup>	11.35 (1.03) <sup>b</sup>	-21.4 (34.84) <sup>b</sup>	47.18 (38.86)



**Figure 3** The linear mean plot of MMP-2 concentration in GCF according to different BRMs measured by ELISA test.



**Figure 4** Bar chart displaying the Bone height bar chart according to BRMs at one and two months post GBR.



**Figure 5** Bar chart displaying the bone density according to BRMs at one and two months post GBR.

post GBR, while the bone density difference across the groups at two months post GBR was not statistically significant (**Table 4 and Figure 5**).

## Discussion

The current study's findings show that the regeneration materials modulate the molecular activities and influence the clinical as well as the radiological features of the healing bone defects. Similar findings have been reported by previous studies [6,28]. The clinical features assessed during early healing stage elicited more bleeding on gentle touch among Bio Oss group on the third day post operation, compared to  $\beta$ -TCP and Control and more swelling on the surgical site was observed among Bio Oss defects than other GBR group on the 7<sup>th</sup> day.

Although, the differences observed by clinical variables were not statistically significant, the tendency to more adverse effects in defects regenerated with xerographic and synthetic materials has been reported by previous studies in the form of prominent inflammatory cells than autograph and empty control defects [2,29]. This can be attributed to the immune body response to trauma and presence of foreign body (BRMs) in the healing bone defects. Autografts have less adverse effects because they are reported to be more compatible than other BRMs [2,29], while the slow rate of Bio Oss resorption [1] may be the reason for prolonged adverse effect in the group.

### The OPG, MMP-2 and VEGF concentration in GCF during bone healing

In general, the ELISA test results demonstrated that OPG concentration decreased with time in the first week following GBR, while VEGF and MMP-2 increased. The clinical appearance and pattern of biomarkers expression can be inferred to the known three phases of bone healing, namely inflammation, repair and remodeling [30]. It is not surprising therefore to detect an increase in VEGF responsible for angiogenesis and MMP-2 for ECM degradation during inflammatory stage. The OPG presented quite similar concentration patterns in the three GBR groups (Bio Oss,  $\beta$ -TCP and Control) with peaks on third and seventh day post-operative. The study by Pellegrini et al. [28] which compared OPG expression between regenerative surgery and open flap debridement (OFD) in healing human periodontal defects found that OPG was at peak before surgery followed by a decline to the 7<sup>th</sup> day and the trend was similar between the regenerative and the OFD groups.

The difference in time for the lowest readings between the current and the aforementioned study [28] may be due to difference in bone turnover in the subjects assessed as well as the regenerative materials and surgical procedures used between the two studies. The previous studies [31,32] indicated that the multinucleated osteoclast-like cells exhibit resorption signs on both the bone and the regeneration material sides. The immediate OPG decrease following GBR is probably associated with more osteoclast resorption activities during inflammatory stage of bone healing coupled with ECM degradation and angiogenesis processes facilitated by MMPs and VEGF respectively. It is now believed that bone defects like extraction socket wall proceeds through a phase of resorption before regeneration [33,34]. Osteoclasts are reported to be involved in resorption and eventually sloughing off the exposed bone in fresh bone defects, leaving a soft connective-tissue surface that can be defended against bacterial invasion [35,36].

The VEGF concentration trend for Bio Oss group was different from the two ( $\beta$ -TCP and Control). The  $\beta$ -TCP and Control registered peak on 7<sup>th</sup> day, whereas Bio Oss had more or less steady concentration throughout healing time. The current findings are in general agreement with other studies which reported an increase in VEGF level immediately after surgical procedure [6,28]. VEGF is an angiogenic factor released by fibroblasts and endothelial cells to induce formation of new blood vessels in hypoxic hematoma. VEGF also works as a potent chemotactic stimulus for inflammatory cells, and a major stimulus for the migration and proliferation of MSCs and osteoblasts [37]. Its restricted expression in Bio Oss group may be associated with a slow rate of angiogenic activities in the group compared to the  $\beta$ -TCP and Control which can be attributed to the limited rate of material resorption [1].

The Control registered peak of MMP-2 concentration on 7<sup>th</sup> day while  $\beta$ -TCP and Bio Oss had peaks on 10<sup>th</sup> day. Similarly, the study by Vieira et al. [9] reported an up regulation of MMP-2 in the bone healing defects in comparison with the untreated control in mice extraction socket. The study reported MMP-2 expression peak on day 14, followed by a decrease to the lowest level on 21<sup>st</sup> day. The expression of MMPs is important in the initial healing stages. Since, they play active roles in the migration of inflammatory cells, degradation and remodeling of extracellular matrix proteins as well as angiogenesis processes [38,39], essential for bone healing.

### Radiological assessment

Radiological evaluation has been used by several researchers to examine the progress of bone healing in clinical and animal experiments [40,41]. The current study findings support the previous reports suggesting that the use of BRMs promotes bone defect healing. The CT scan analysis done at one and two-month

time post GBR showed some differences between the GBR groups regarding the alveolar bone height gain and bone density.

The Control group recorded significantly lower bone height than Bio Oss and  $\beta$ -TCP at one- and two months post GBR. On the other hand, the Control group had significantly lower bone density than Bio Oss and  $\beta$ -TCP only at one-month post GBR, while the bone density difference across the groups at two-month post GBR was not statistically significant. Sun et al. [40] however, reported an enhanced bone mineral density, trabecular thickness, and angiogenesis when comparing regenerative defects to the control groups at 1<sup>st</sup>, 3<sup>rd</sup> and 6<sup>th</sup> month post-trauma in rats' calvarial. The variations between the two studies may be due to the different BRMs and the experimental models used in the studies.

The results of the current experiment should be cautiously applied in clinical practices because it involved canine subjects who are fundamentally different from human physiological and anatomical features. However, the assessed healing markers and radiological variables have elicited some important characteristic impacts of different BRMs on healing bone defects worth considering in clinical settings.

### Conclusions

The type of BRM had fundamental effects on the pattern of healing marker (OPG, VEGF and MMP-2) concentration in GCF at some-point during healing time. Additionally, the BRMs (Bio Oss and  $\beta$ -TCP) groups had enhanced Bone density one-month post-operative, while bone height gain was significantly higher for experimental defects than empty control defects at one and two months post-operative.

### Conflict of Interest

The authors have no conflict of interest in the products described in the article.

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