

# Bioactive Glass with or without Enamel Matrix Derivative in Class II Furcation Lesions: Histomorphometric Study in Dogs

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**Background:** The aim of this study was to evaluate the influence of bioactive glass (BG), with or without enamel matrix derivative (EMD) in the treatment of class II furcation lesions.

**Methods:** Experimental furcation defects were surgically created on mandibular P2, P3 and P4 of 6 mongrel dogs, and filled with gutta-percha. Four weeks later, the defects were debrided, the roots were planed and notches were placed at bone level. Each tooth was randomly assigned into BG+EMD, BG or internal control group. The animals were sacrificed 12 weeks later.

**Results:** The results for the groups control, BG and EMD+BG were, respectively: extension of new cementum (ENC),  $49.55 \pm 15.43\%$ ,  $84.01 \pm 7.87\%$ ,  $91.96 \pm 5.69\%$ ; epithelium extension (EE),  $27.00 \pm 23.89\%$ ,  $4.79 \pm 6.98\%$ ,  $1.89 \pm 2.47\%$ ; extension of connective tissue (ECT),  $23.44 \pm 9.09\%$ ,  $11.20 \pm 4.87\%$ ,  $6.15 \pm 5.19\%$ ; bone fill area (BFA),  $51.15 \pm 9.23\%$ ,  $74.97 \pm 9.02\%$ ,  $76.53 \pm 4.48\%$ ; mineralised bone (MB),  $40.15 \pm 9.95\%$ ,  $31.65 \pm 15.61\%$ ,  $37.64 \pm 16.25\%$ ; and bone marrow (BM),  $11.00 \pm 3.91\%$ ,  $32.05 \pm 14.61\%$ ,  $31.13 \pm 15.25\%$ . The area of residual bioactive glass particles in the BG group was  $11.28 \pm 6.81\%$ , and in the BG+EMD group was  $7.76 \pm 3.14\%$ . No statistical differences ( $p > 0.05$ ) were observed in any parameter between BG and BG+EMD groups, and between BG and internal control. However, comparing the BG+EMD group with the internal control group, statistically significant differences were observed concerning ENC and ECT ( $p < 0.05$ ).

**Conclusion:** Within the limits of the present study, it can be concluded that BG and BG+EMD have the potential to improve periodontal regeneration, and that the association seems to favour the cementum formation.

**Key words:** enamel matrix derivative, bioactive glass, furcation lesions, dog, periodontal regeneration

## INTRODUCTION

The ultimate goal of periodontal therapy is the regeneration of the tissues previously affected by periodontal disease. Osseous grafts and the guided tissue regeneration (GTR) technique have been employed with this aim. However, when bone grafts are used alone in the treatment of bone defects, healing results in apical proliferation of the junc-

tional epithelium between the tooth and adjacent periodontal tissues (Listgarten and Rosenberg, 1979). On the other hand, in GTR, a membrane is used to prevent the proliferation of the gingival tissues in contact with the root surfaces during healing (Nyman et al, 1982). This process may allow periodontal regeneration to occur, but there are some disadvantages: non-absorbable membranes demand an additional surgical procedure



to remove it; and absorbable membranes are potentially related to undesirable inflammatory processes, and the period of time for its complete absorption is not precise.

A new perspective for the treatment of periodontal lesions was observed in a study by Karatzas et al (1999), where bioactive glass (BG), an alloplastic graft, was used in experimental defects in monkeys. Sites in which BG was used presented significantly more new cementum and less epithelial down-growth in comparison with the control. BG is composed of silica, sodium, calcium, fluorites and phosphates, and presents biocompatibility (Furusawa et al, 1997) and osteoconductive potential (Schepers et al, 1991). After implantation, particles suffer a sequence of events involving processes of lixiviation, dissolution and precipitation. As a consequence, some cracks emerge in the particles, which allow cellular penetration in direction of the centre of the graft particle, where osteoid matrix will be deposited, and subsequently, will be calcified. An osteostimulatory potential was also described (Schepers and Ducheyne, 1997), which is the ability of the BG to allow the differentiation of osteoprogenitor cells, which are at a distance from the bone walls, into osteoblasts. BG is also easily manipulated, and in contact with blood. Its particles are turned into an aggregate during the surgical procedure. This fact makes the loss of particles more difficult and contributes to local haemostasis (Schapoff et al, 1997). According to Schepers and Ducheyne (1997), among commercially available types of BGs, one composed by a narrow range particle size (300 to 355  $\mu\text{m}$ ) was chosen, because this characteristic seems to influence the process of bone formation. Particles smaller than 200  $\mu\text{m}$  may be absorbed rapidly, and cause inflammatory responses, and particles larger than 400  $\mu\text{m}$  may not be absorbed, which could impede the sequence of events that culminates in bone formation.

In the present study, BG was associated to the enamel matrix derivative (EMD), which is naturally secreted during tooth formation; thus its application is intended to mimic the processes that occur during the development of the periodontal tissues. EMD is a matrix composed of proteins, mainly amelogenins, which are extracted from porcine enamel organ, and submitted to a purification process (Heijl et al, 1997). This matrix is associated to a vehicle, the propylene glycol alginate

(PGA), which facilitates the application to the root surface.

In vitro studies report that EMD acts in epithelial cells, inhibiting their proliferation and differentiation (Kawase et al, 2000); it influences the proliferation and migration of periodontal ligament cells (Lyngstadaas et al, 2001), acting in the synthesis of extra cellular matrix by fibroblasts (Haase and Bartold, 2001); it also influences the activity of cementoblasts (Tokiyasu et al, 2000) and osteoblasts (Schwartz et al, 2000; Hattar et al, 2005), being considered an osteopromotive material (Boyan et al, 2001); and moreover, microbiological studies indicate the possibility that EMD inhibits periodontopathogen growth (Spahr et al, 2002). EMD creates a positive environment for the proliferation and differentiation of periodontal ligament fibroblasts, which is fundamental for the regenerative process (Gestrelus et al, 1997).

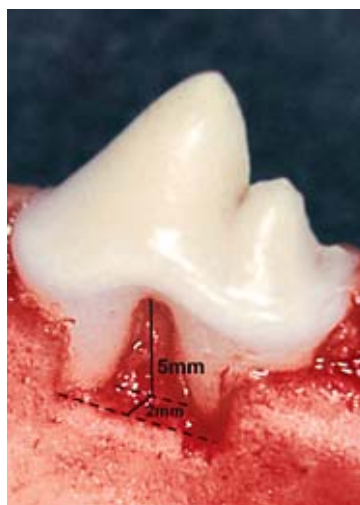
Some clinical studies (Scheyer et al, 2002; Sculean et al, 2002; Sculean et al, 2005) have used the association of EMD with a graft. However, the aim of such clinical studies was not to evaluate the quality and quantity of the newly formed tissues, since no histological analysis was presented. The association of a graft with EMD is based on the principle that space-making would be beneficial for the regeneration of periodontal lesions (Mellonig, 1999), since the creation and maintenance of space is necessary to provide a channel for the migration of progenitor cells to healing sites where they can differentiate into cementum and periodontal ligament (Minabe, 1991; Haney et al, 1993).

Thus the objective of the present study was to evaluate histomorphometrically the influence of the BG particles with or without the EMD in the regeneration of class II furcation lesions in dogs.

## STUDY DESIGN AND RESULTS

Six male mongrel dogs, weighing approximately 10 kg each, were chosen for the study. The animals had intact crowns without gross occlusal alterations, did not have buccal lesions or any fungal infections, and were healthy. The experimental protocol was in accordance with the guidelines approved by the Council of the American Psychological Society (1980) for the use of animal experiments.

**Fig 1 (left)** Buccal view of the furcation defects surgically created. The standardised defect measured 5 mm in the apical-occlusal direction, 2 mm in the buccal-lingual direction and extended from the middle of mesial root to the middle of distal root.



**Fig 2 (right)** Defect filled with gutta-percha.



The animals were sedated with a 2% xylazine (0.5 ml/10 kg), anaesthetised with a 25% sodium thiopental solution (1 ml/kg), and the procedure was complemented with a local infiltration of mepivacaine HCl (2%) with norepinephrine (1:100000). Ultrasonic scaling and topical application of 0.12% chlorhexidine digluconate were performed. Intra-sulcular incisions were performed on the buccal surfaces of P2, P3 and P4, followed by vertical incisions on the mesial surfaces of P2 and the distal surfaces of P4, in order to allow the release of a mucoperiosteal flap to expose the alveolar bone. Experimental class II furcation lesions were created in P2, P3 and P4: osteotomy was performed in the furcation area with high-speed diamond burs with constant irrigation, and with #1 and #2 Ochsenbein chisels. The standardised interradicular bone defect measured 5 mm in the apical-occlusal direction, 2 mm in the buccal-lingual direction, and extended from the middle of mesial root to the middle of distal root (Fig 1). The defects were filled with gutta-percha to induce an inflammatory response and to prevent spontaneous regeneration (Fig 2). Flaps were repositioned and sutured with 5.0 resorbable sutures. During the post-surgical period, animals were fed a soft diet and had water *ad libitum*.

After 4 weeks, the furcation defects were exposed by mucoperiosteal flaps, bilaterally, to perform reconstructive surgery. All remaining soft tissue adhered to alveolar bone or to the root surface was removed, careful scaling and root planing with Gracey curettes was performed, and a refer-

ence notch was placed at the base of the defect using a #33.5 round bur (Fig 3).

Avoiding contact with bone tissue, root surfaces were conditioned for 2 minutes with 24% EDTA (ethylenediaminetetra acetic acid), neutral pH in 3% carbopol gel, followed by abundant sterile saline irrigation and continuous aspiration, for complete removal of the EDTA gel. The teeth of each hemi arch were randomly assigned into internal control, BG (Biogran, Orthovita, Malvern, PA, USA) alone and BG+EMD groups (Fig 4). On the BG+EMD group, EMD (Emdogain, Biora AB, Chicago, IL, USA) was applied on the entire root surface beginning at the deepest area of the defect, followed by the placement of BG particles. The flaps were positioned coronally and sutured with resorbable sutures. Every week, until the end of the experiment, a plaque control regimen was carried out with supragingival ultrasonic points and topical application of chlorhexidine (0.12%). During the first month, intramuscular antibiotic injections (20000 IU of penicillin and 1 g/10 kg weight erythromycin) were administered every 7 days, and animals were sacrificed 12 weeks after the reconstructive surgery with a lethal dose of thiopental.

#### Histological and Histomorphometric Analysis

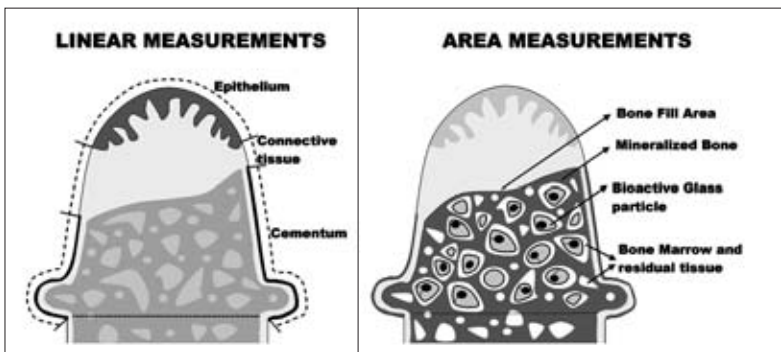
The mandibles were dissected, fixed in 10% formalin, and decalcified in multiple baths of 5% nitric acid for approximately 4 weeks. After demineralisation, samples were dehydrated and embedded in paraffin. Mesio-distal semi-serial



**Fig 3** Clinical aspect 4 weeks after chronification of class II furcation defects.



**Fig 4** The sites were randomly assigned to remain as control, BG group or BG+EMD group, in this case, P2, P4 and P3, respectively.



**Fig 5** Schematic illustration representing the histometric parameters performed in this study.

6 µm histological sections were stained with hematoxylin-eosin, and Masson's trichromic stain. Six sections representative of the central portion of defects were selected, and observed under conventional light microscopy. Histomorphometric analysis was made with a light microscope (Eclipse E1000, Nikon, Melville, NY, USA) connected to a video camera. The image was sent to a computer with a specific processing program (ImageJ 1.23y, National Institutes of Health, Bethesda, MA, USA) for measurements according to parameters presented in Fig 5.

The following parameters were observed by carefully outlining the structures with a mouse cursor: extension of epithelium (EE: sum of linear extension of the lesion covered with epithelial tissue), extension of new cementum (ENC: sum of linear extension of the lesion covered with new cementum), extension of connective tissue (ECT: sum of linear extension of lesion not covered with epithelium or new cementum), and area of bone fill [BFA: including new mineralised bone (MB), bone-

marrow-like tissue (BM) and residual particles of BG]. In addition, the overall proportion of residual particles of BG, MB and BM were calculated by automatic selection based on contrast of the colours resulting from staining of the different tissues. This automatic selection process was carefully followed and checked with the image available at the microscope. Percentage values of all parameters were obtained in relation to total extension and total area of the experimental lesion, delimited by mesial and distal notches. Mean values obtained from the six histological sections were representative of each site, and mean values of both sites of the same group were calculated for each dog. The unit of analysis was the dog, and experimental data were submitted to non-parametric statistical analysis. The null hypothesis was based on the absence of difference between the modalities of treatment.

The histomorphometric analysis for the internal control, BG and BG+EMD groups was performed in samples from, respectively, four, five and six dogs.

**Table 1** Percentage of linear measurements (mean  $\pm$  SD).

| Group   | Cementum          | Epithelium        | Connective Tissue |
|---------|-------------------|-------------------|-------------------|
| Control | 49.55 $\pm$ 15.43 | 27.00 $\pm$ 23.89 | 23.44 $\pm$ 9.09  |
| BG      | 84.01 $\pm$ 7.87  | 4.79 $\pm$ 6.98   | 11.20 $\pm$ 4.87  |
| BG+EMD  | 91.96 $\pm$ 5.69* | 1.89 $\pm$ 2.47   | 6.15 $\pm$ 5.19*  |

\*  $p < 0.05$  in comparison with internal control group.

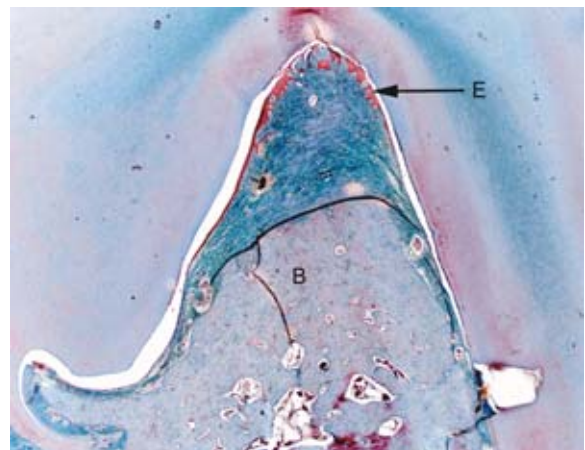
**Table 2** Percentage of area measurements (mean  $\pm$  SD).

| Group   | Bone Fill Area   | Mineralised Bone  | Marrow Bone       | BG particles     |
|---------|------------------|-------------------|-------------------|------------------|
| Control | 51.15 $\pm$ 9.23 | 40.15 $\pm$ 9.95  | 11.00 $\pm$ 3.91  | –                |
| BG      | 74.97 $\pm$ 9.02 | 31.65 $\pm$ 15.61 | 32.05 $\pm$ 14.61 | 11.28 $\pm$ 6.81 |
| BG+EMD  | 76.53 $\pm$ 4.48 | 37.64 $\pm$ 16.25 | 31.13 $\pm$ 15.25 | 7.76 $\pm$ 3.14  |

Some of the samples were not used due to artifacts in the specimens. Friedman's test, followed by corrections for multiple comparisons (Bonferroni method), was used to identify statistically significant differences for more than two dependent samples, considering data from four dogs. Differences between BG and BG+EMD, concerning the amount of residual particles of BG, were tested using Wilcoxon's test, considering data from five dogs. Descriptive statistics included all data.

The surgical procedures were well tolerated by all dogs, with no observed severe inflammatory alterations or swelling during the experimental period. The values of total extension and total area, limited by the notches in the root surfaces, were not statistically different among the groups (data not presented), and all furcation lesions were completely closed. Means of the percentage values determined by the epithelium, connective tissue, cementum and bone, for each group, and also the statistical analysis are presented in Table 1 and Table 2.

Comparing the BG group with the BG+EMD group, and with the internal control group, no statistically significant differences were observed concerning any of the parameters ( $p > 0.05$ ). Comparing the BG+EMD group with the internal control group, statistically significant differences were observed concerning the extension of new cementum and the extension of connective tissue directly in contact with dentin ( $p < 0.05$ ).

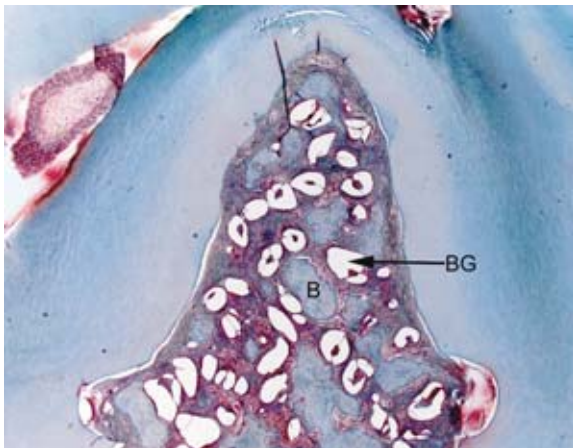


**Fig 6** Mesio-distal histological section of the internal control group with new bone formation (B) and junctional epithelium (E) along the top of the defect. (Masson's Trichromic stain, 2.5x objective).

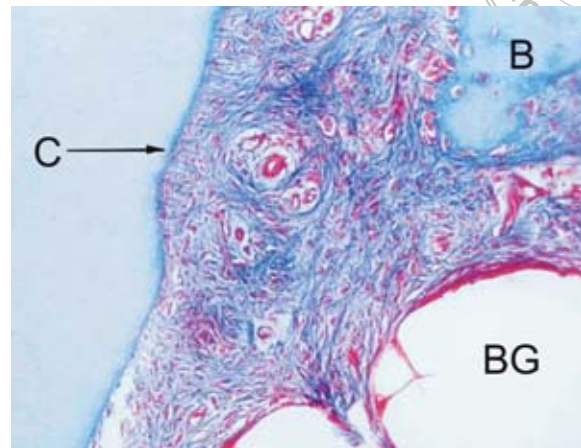
Moreover, specifically considering the bone fill area values, the Friedman's test detected a significant difference ( $p = 0.049$ ), but the multiple comparisons test was not effective in determining between which groups the difference was significant.

#### Cementum

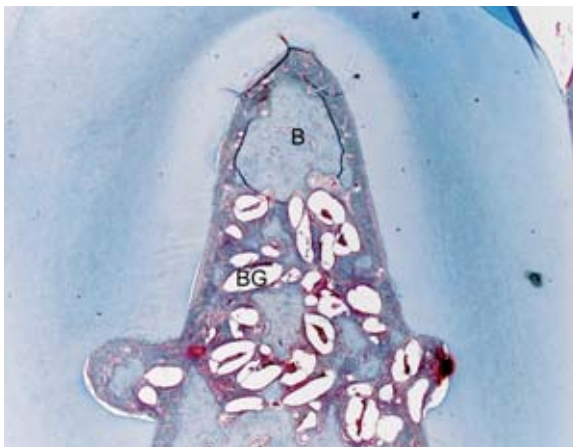
The extension of new cementum (Fig 6) in the internal control group corresponded to 49.55  $\pm$  15.43% (ranging from 28.32 to 65.07%) of the



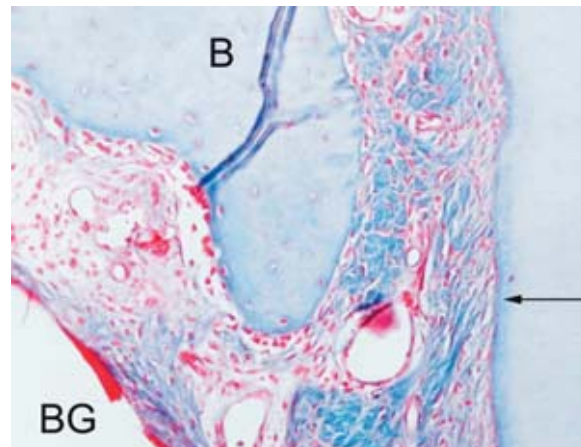
**Fig 7** Mesio-distal histological section of the BG group, with new bone formation (B) and bioactive glass particles (BG). (Masson's Trichromic stain, 2.5x objective).



**Fig 8** Part of Fig 7, with resorption of the dentin that is filled by new acellular cementum with extrinsic fibres (C). There are also new bone (B) and bioactive glass particles (BG). (Masson's Trichromic stain, 20x objective).



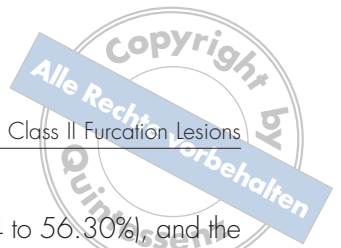
**Fig 9** Mesio-distal histological section of the BG+EMD group, with new bone formation (B) and bioactive glass particles (BG). (Masson's Trichromic stain, 2.5x objective).



**Fig 10** Part of Fig 9, with acellular cementum with extrinsic fibres (C), new bone (B) and bioactive glass particles (BG). (Masson's Trichromic stain, 20x objective).

dentin surface facing the defect, characterised as a thin and acellular tissue, and composed mainly by intrinsic and/or extrinsic fibres. In the BG group (Figs 7 and 8), the new cementum formation did not follow a pattern in relation to thickness and incorporation of cementocytes, and some interrupted areas of resorption were observed along the dental surface. In this group, mean extension of cementum was  $84.01 \pm 7.87\%$  (ranging from 70.82 to

91.44%), and it was not statistically different from the internal control or from the BG+EMD group ( $p > 0.05$ ). In the BG+EMD group (Figs 9 and 10), few areas of dentinal absorption were distributed along the root surface and frequently filled with cementum. The extension of cementum was  $91.96 \pm 5.69\%$  (ranging from 84.82 to 99.57%) and was considered statistically different from the internal control group ( $p = 0.02$ ).



### Epithelium

The extension of epithelium in internal control was  $27.00 \pm 23.89\%$  (ranging from 5.64 to 59.98%); in the BG group, EE was  $4.79 \pm 6.98\%$  (ranging from 0.00 to 17.95%) and in the BG+EMD group, this was  $1.89 \pm 2.47\%$  (ranging from 0.00 to 5.37%). No statistically significant differences were observed among groups ( $p > 0.05$ ).

### Connective Tissue

The extension of connective tissue in the internal control group was of  $23.44 \pm 9.09\%$  (ranging from 11.70 to 31.79%), which was not statistically different from the results observed in the BG group ( $11.20 \pm 4.87\%$ , ranging from 2.62 to 16.14%) ( $p > 0.05$ ). The differences between the BG and the BG+EMD groups were not statistically significant ( $p > 0.05$ ). In the BG+EMD group, the extension of the connective tissue was of  $6.15 \pm 5.19\%$  (ranging from 0.00 to 11.53%), and it was significantly lower than the internal control group ( $p = 0.02$ ).

### Bone Fill

In the internal control group, histomorphometric analysis revealed a percentage of bone formation of  $51.15 \pm 9.23\%$  (ranging from 41.06 to 59.13%), which consisted of woven bone, with a mineralised area of  $40.83 \pm 9.95\%$  (ranging from 27.53 to 51.15%), and an area of bone marrow of  $10.16 \pm 4.56\%$  (ranging from 6.68 to 16.82%). The majority of the defect was filled by dense connective tissue, which in some cases exhibited a mononuclear inflammatory process in focal areas.

The mean value of the bone fill area in the BG group was of  $74.97 \pm 9.02\%$  (ranging from 63.05 to 85.91%), the area of mineralised bone was  $30.57 \pm 15.60\%$  (ranging from 11.15 to 50.54%) and the area of bone marrow was  $34.22 \pm 14.46\%$  (ranging from 12.87 to 51.19%). The percentage of residual particles of BG in the BG group was  $11.68 \pm 6.81\%$  (ranging from 2.57 to 21.54%). These were embedded in vascularised connective tissue, and did not present characteristics of encapsulation. In the inner side of the BG particles early stages of bone formation was observed.

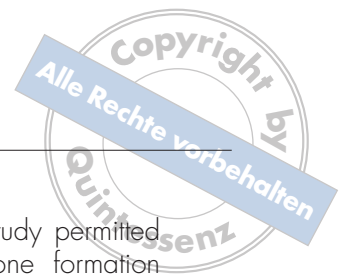
Moreover, the area of bone fill in the BG+EMD group was  $76.53 \pm 4.48\%$  (ranging from 72.17 to 81.77%), with a mineralised area of  $35.53 \pm$

$16.25\%$  (ranging from 12.94 to 56.30%), and the area of bone marrow of  $34.17 \pm 15.52\%$  (ranging from 19.55 to 57.54%). The amount of residual particles of BG in the BG+EMD group ( $7.76 \pm 3.14\%$ , ranging from 5.07 to 11.76%) was lower than that of in the BG group, but this difference was not statistically significant ( $p > 0.05$ ). The stage of incorporation of these particles was similar to the one observed in the BG group.

The only statistically significant difference observed among groups concerned the bone fill area ( $p = 0.049$ ). However, the multiple comparisons test was not effective in determining between which groups this difference occurred.

## DISCUSSION

In the present study it was observed that the use of BG with or without EMD provided improvements in hard and soft tissues in class II furcation lesions in dogs. However, statistical differences were not detected between the sites treated only with BG (BG group) in comparison with the internal control, and between the BG group and the group in which BG was associated with EMD (BG+EMD group). Nevertheless, statistically significant differences ( $p > 0.05$ ) were observed when the BG+EMD group was compared with the internal control group. The best regenerative result was observed in the BG+EMD group, and can be represented by the statistically significantly higher values of the extension of new cementum (91.96%, in contrast to 49.55% in the control) and the lower value of the extension of connective tissue directly in contact with dentin (6.15%, compared with 23.44% in the control). A tendency was identified in favour of the BG+EMD group when compared with the BG group: there was a numerically lower epithelium down-growth, and higher connective tissue attachment and new cementum formation when the association was proposed. The values for hard tissues were also different in the BG + EMD compared with the BG group, especially the conversion rates of residual particles into new bone. Generally, descriptive statistics revealed differences among groups that could not be confirmed by statistical analysis, probably because of the small sample size of the present study. In a study developed by Regazzini Filho et al (2004), class II furcation lesions were created in



dogs, and treated with EMD either associated or not with GTR, with the aim of maintaining an adequate space for bone formation. In their control group, similarly to the present study, a thin and acellular new cementum and low bone formation in comparison with the experimental groups was observed. However, the amount of new tissues was different when both studies are compared. This could be explained by differences in the methodology, such as the chronification period and size of the defects. Moreover, the individual characteristics of the animals and their response to the inflammatory process related to the chronification of the lesions should be considered.

In contrast with the internal control group, which presented predominantly acellular new cementum, both experimental groups (BG and BG+EMD) presented variable characteristics concerning the presence of cells, thickness and type of fibres that were incorporated. The fibres were deposited without a specific pattern, and this observation is not in accordance with Hammarström (1997), who related the formation of acellular cementum after the use of EMD. The BG+EMD group presented values of new cementum extension (91.96%) that were higher than the BG group (84.01%), but the differences were not statistically significant.

In spite of the higher mean values of mineralised bone and also the lower values for bone marrow in the internal control group, the area of bone fill was significantly higher in BG+EMD group. Thus the distribution of new tissues was different in each of the groups, probably because of the presence of the biomaterials, and the quantity of BG residual particles present in the experimental groups at sacrifice. Possibly, with a longer experimental period, BG would be further converted into new bone. The total area of BG residual particles was slightly lower in the BG+EMD group (7.76%) than in the BG group (11.28%), however the difference was not statistically significant. Tadjoeidin et al (2000) followed the healing dynamics of BG resorption in humans, evaluating the period of time necessary to replace completely the particles by bone tissues. It was found that, 4 months after its use, a small amount of bone was present within the particles and that the bone formation was not related directly to the surrounding bone. Additionally, at 16 months, the particles were completely absorbed, and new mature bone formation was evident. The healing period of 12

weeks established in the present study permitted the observation of a discrete bone formation along the periodontal lesion. This formation was not limited to the apical portion of the periodontal lesion, as could be expected for other grafts, since each particle of BG acts as an 'island' of bone formation. In the present study, the particles were not completely embedded in the new bone, but mainly surrounded by a well-vascularised connective tissue with no signs of encapsulation, which seems to be a previous step before complete particle absorption, and is part of the osteostimulatory process (Cordioli et al, 2001). In general, the particles from the BG and the BG+EMD groups presented evidence of rupture and the formation of osteoid tissue within the particles.

Clinical studies based on the same principles of this study, involving the use of EMD and a graft, have been published. In one of these studies, Sculean et al (2002) treated intrabony defects with BG alone or BG associated with EMD. Significant improvements were found (t test,  $p < 0.0001$ ) concerning reduction of probing depth and gain of clinical attachment level. In addition, no statistically significant differences were observed between sites in which BG was used alone or in association with EMD. This behaviour was also observed in the study by Scheyer et al (2002), in which EMD was associated with an inorganic cancellous bovine-derived bone xenograft (BDX), and as consequence, clinical improvements ( $p < 0.001$ ) were observed in probing depth reduction, gain in clinical attachment levels, and re-entry measurements resulted in 3.0 mm (67.0%) and 3.2 mm (63.3%) in bone fill for the BDX groups and BDX+EMD respectively. These clinical studies cited above demonstrated the applicability of this treatment protocol; however, histological studies are necessary to clarify the percentages of different tissues formed during healing, improving the understanding of the behavior of these biomaterials.

Within the limits of the present study, it can be concluded that BG and BG+EMD have the potential to improve periodontal regeneration, and that the association seems to favour the cementum formation.

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