Scand. J. Lab. Anim. Sci. 2007 Vol. 34 No. 3

Oral Bone Grafting in a Rat Model and the Use of Scanning Electron Microscopy for Tissue Morphology Evaluation

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Summary

Oral bone grafting is a procedure widely performed in current dentistry. Several biomaterials fit this purpose. The aim of this study was to use scanning electron microscopy (SEM) to evaluate the ultrastructural aspects of bone repair in a rat model, with periodontal tissues involved. Two groups (I and II) of 20 animals each were operated on to create a surgical defect with a round carbide burr (3mm) on the right side of their mandible, anterior to the mental foramen. Both groups were evenly divided with 5 animals each to receive the application of either bifasic calcium phosphate bioceramic (B), lyophilized deproteinated bovine bone (L), bifasic bioceramic associated with lyophilized deproteinated bovine bone (BL), or no biomaterial (control or C). Group I was monitored for one week and group II for three weeks prior to euthanasia. Hemi-mandibles were prepared for SEM analysis. Parameters such as exposure of incisive root surface, width of the cross-section of filiform structures and presence of mineralized-like globuli (area) were evaluated. The findings of this study suggested that surgical procedures for introduction or not of biomaterial did not cause problems with normal feeding to the animals. Both of the biomaterials used promoted a periodontal ligament involvement. Fibers (single filiform structures) could be detected in a range from 0.07 to 0.18μm of diameter, except for L that was larger – considered to be due to residual fibers of bovine origin. C bundles (groups of fibers) showed larger width of cross-section than with the use of biomaterials. Globuli areas (mineralization) were smaller to C than with the biomaterials use. B showed larger globuli areas, suggesting slow incorporation. It was concluded that the use of these biomaterials favored maintenance of tissue volume although slowing remodeling, and the combination (BL) presented the best performance.

Introduction

Bone grafting is recommended to correct defects of this tissue, which can damage the normal anatomy and, ultimately, its function. Disease or accidents can cause such defects. The bone neoformation in individuals that present considerable loss of tissue is of great importance for dentistry and medicine. For example, data reported in the year 2000 that approximately 450,000 bone grafts are performed annually in the United States of America alone. Thus, several substances have been developed with the purpose of stimulating or aiding the neoformation of the original bone, which is to promote an ideal repair in the site of interest (*Service, 2000*).

The so-called osteopromotive substances, bone substitutes, or biomaterials of bone grafting have been studied in experimental models for a long time. Already in 1965, a study showed the osteoge-

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Published in the Scandinavian Journal of Laboratory Animal Science - an international journal of laboratory animal science

nesis within intramuscular implants of decalcified bovine bone matrix (*Van De Putte & Urist, 1965*). In the mid 80s, the use of decalcified bovine bone matrix in subcutaneous implants was demonstrated (*Gendler, 1986*). More recently, positive results were found in the use of non-demineralized bovine bone applied in defects of rat tibias with a size of 3 mm (*Rodriguez et al., 1997*). Researchers have also demonstrated a model for the study of bone biomaterials in cranial defects of 6 – 5 months old rats (*Bosch et al., 1998*). And most rat oral models of bone grafting described in the literature involve the posterior parts of mandibles, with extra-oral access (*Moraes & Garcia, 2006; Zahedi et al., 1998*). Regarding the tissue evaluation, all bone repair studies with light microscopy require decalcification of specimens prior to slicing, which sometimes makes it difficult when the biomaterial particles of the grafting do not dissolve. Scanning Electron Microscopy is a technique widely used in the study of biological tissues. It allows for the observation of three-dimensional morphology without the need for slicing (*Silveira, 1998*).

The objective of this work was to describe the use of scanning electron microscopy for tissue evaluation of bone repair *in vivo,* in an experimental model with rats which had undergone surgical intraoral procedures with the application of bifasic calcium phosphate bioceramic and lyophilized deproteinated bovine bone.

Material and Methods

The protocol of this study was approved by the Committee of Ethics of Positivo University Center, Curitiba, Brazil (18/2006). All animal handling was accomplished at a conventional and pathogen-free bioterium (Positivo University Center Bioterium). There was no preoperative fasting, and no antibiotic prophylaxis was given. The rats were maintained in collective plastic cages (five rats per cage), with bedding of autoclaved Pinus sp. chips, in controlled room temperature at 20±2 ºC, 60±5% humidity, photoperiod of 12-h light/dark (7 am/7 pm), fed on pellets (conventional, Nuvital, Colombo, Brazil) and tap water ad libitum.

The surgical procedures were carried out on 40 male rats (*Ratus norvergicus*, WISTAR), divided in 2 groups, with 20 animals monitored for one week (group I = GI) and the other 20 animals monitored for 3 weeks (group II = GII). Each group was evenly divided in to subgroups of 5 animals. The subgroups were given four different treatments: no biomaterial (agglutiriatedblood vehicle only) that is the control (C); bifasic calcium phosphate bioceramic (B)(Osteosynt, Einco, Belo Horizonte, Brazil); lyophilized deproteinated bovine bone (L)(Biobone, Vianfarm, Sao Paulo, Brazil); and the association of both (BL) (Diagram 1).

Group	Animals	Treatment (see Table 1)	Monitored for (weeks)
GI	01 – 05	C	1
	06 – 10	B	
	11 – 15	L	
	16 – 20	BL	
GII	01 – 05	C	3
	06 – 10	B	
	11 – 15	L	
	16 – 20	BL	

Diagram 1. Animal distribution used.

The animals were properly weighed and anesthetized with ketamine (40mg/kg, Bayer, Germany) and xylazine (5mg/kg, Bayer, Germany) intraperitonially. The surgical area was cleaned with a povidine solution (LM Farma, Sao Jose dos Campos, Brazil). An incision at the buccal area and previous to the mental foramen of the right mandible was made with a knife (n.15, BD, Sao Paulo, Brazil). A bone defect was created by the action of a round carbide burr 3mm in diameter (Antilope, Switzerland) running at low speed (Dentec 405N, Rio de Janeiro, Brazil). The procedure was accomplished with abundant irrigation by sterile saline solution (Sandex, Itanhandu, Brazil), in order to avoid the overheating of tissues and allowing a good visualization of the root surface of the inferi-

or incisive, which was defined as the limit of the artificial lesion (Figure 1, surgical defect). The biomaterial type introduced in the bone defect followed the subgroups headings (B, L or BL), agglutinated with local blood. Afterwards, the soft tissues were sutured with silk 4-0 (Johnson & Johnson, Sao Paulo, Brazil). The same procedure was accomplished with the control animals (C), except no biomaterial application. Animals were administered postsurgically (24h) with buprenorphine (0.1-0.25 mg/kg PO, BID, Shering-Plough, USA). Euthanasia (gas chamber after general anesthesia, as described) was at 1 week (GI) and three weeks (GII) postoperatively. The animals were weighed immediately after euthanasia. Hemi-mandibles were harvested and fixed with fresh 3% glutaraldehyde (Merck, Germany) in 0.1 mol/L sodium cacodylate buffer solution (Electron Microscopy Sciences, USA) for 48 hours.

After the removal of the superficial tissues (hair, skin and part of the oral mucosa) by the same operator in all specimens, a frontal incision was made over the area of the defect dividing the soft tissue cover in the middle. One part of the cicatricial tissue was clamped and removed to allow for the SEM observation of bottom layers (part removed) and upper layers (part not removed) of the grafting area. After ethanol dehydration and critical drying (Bal-Tec, Germany), mounting on metal stubs and sputter coating with gold (SCD 030, Balzers Union, Germany), SEM micrographs were obtained (SEM 505 and XL30, Phillips, Holland). The histomorphometric parameters were set through the analysis done with UTHSCSA Image Tool 2.00 (The University of Texas Health Science Center, USA), such as areas of root surface exposure (Figure 1), width (size of the cross-section called cross-distance, d, Figure 1) of filiform structures (single structures considered as fibers in which width was taken as diameter, and group of filiform structures or fibers considered as bundles), and the selection of globuli areas (Figure 1), based on the visual identity of mineralized globuli *in vitro* (*Matsuzaka et al., 1999; Shen et al., 1993*) and *in vivo* (*Orr et al., 1992*) by SEM, with only the largest area shown in a single micrograph of each sub-group taken in the analysis of this parameter. The globuli area was manually marked on a plastic film placed over the micrographs. The marked films were scanned and the selected areas were calculated by the software.

Results

The monitoring of the animal weights did not demonstrate significant differences between the beginning (in = initial) of the experiment (presurgical) and immediately after euthanasia (fi = final) for the two groups. In GI the animals had initial mean weight of 315g and final mean weight of 317g, and for GII; the initial mean weight was 313g and the final mean weight was 325g. The increase in the total weight in GI (37g) was smaller than in GII (252g). GI also presented less standard deviation (14.3g) comparing to GII (24.4g), but not statistically significant different by the *t*-test (α=5%). Animal 3 in GII demonstrated a big weight alteration (from 414g to 334g), although no pathological sign of the graft area was detected, as well as no behavior disturbance in the animal. The weight variation of the remaining animals of GII was similar to the one of GI. No infection indications were noted. In relation to the defect-exposed area, having tissue more adhered to the dental root surface could result in minor root exposure during the clamping procedure (*Karimbux et al., 1995*). Although in the present study, results form the removal of the cicatricial tissues did not show a standard pattern for this analysed characteristic (Table 1), the presence of biomaterial suggested an inhabitation of this adherence.

The degree of root exposure area was evaluated within the groups, with only one sample of each subgroup exhibiting this pattern (Figure 1 and Table 1). Exposure was calculated as percentage of root exposed area in relation to defect exposed area produced by clamping. The global standard deviation for this parameter was 2.04. Although this may seem a relatively high value, results showed that only C and BL decreased root exposure (1C =

11.47% to 3C = 3.14%, and 1BL = 12.50% to 3BL = 3.40%), while L increased and B maintained root exposure areas. Clinically, it was also possible to observe that with the use of biomaterials, tissue volume was higher than in control samples.

Table 1 exhibits the width of the cross-section (d) of fibers and bundles, selected from all micrographs showing filiform structures, with a total of 213 analyzed fields. The single filiform structures with lowest width were considered as fibers, which

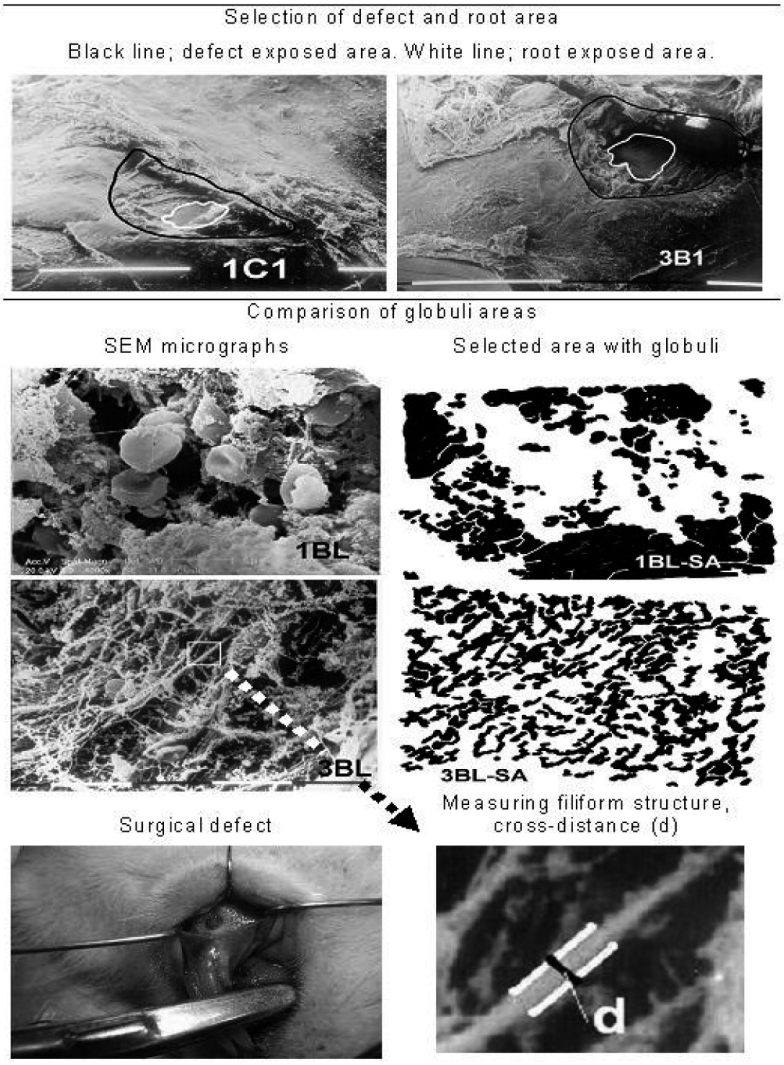

Figure 1. Example of Defect exposed area, Comparison of globuli areas, surgical defect and Measuring filiform structure. 1C1; control 1st. week animal 1 (bar = 1mm). 3B1; bioceramic 3rd. week animal 1 (bar = 1mm). 1BL; bioceramic + lyophilized bone 1st. week (bar = 5µm).1BL-SA; bioceramic + lyophilized bone 1st. week selected area. 3BL; bioceramic + lyophilized bone 3rd. week (bar = 10µm). 3BL-SA; bioceramic + lyophilized bone 3rd. week selected area.

Table 1. Analysis of Root exposure area in relation to defect area, width of cross-section (diameter) of fibers or bundles and Globuli area observed by SEM.

Root exposure area				
Animal			Relative exposure (%)	
1C1			11.47	
1B1			12.33	
1L3			6.16	
1BL3			12.50	
3C2			3.14	
3B1			15.06	
3L3			20.10	
3BL1			3.40	
Overall samples	Width of fiber or bundle*		Globuli area#	
Subgroup	Minimum (fiber, μm)	Maximum (bundle, μm)	Selected area (SA), μm^2	Globuli area, %
1C	0.07	55.82	349.45	14.33
1B	0.15	28.56	1934.14	69.24
1L	0.40	13.01	746.69	18.38
1BL	0.18	5.37	329.41	44.70
3C	0.07	49.14	340.8	17.74
3B	0.18	19.57	2549.93	33.84
3L	0.13	17.59	403.21	28.26
3BL	0.13	5.62	669.80	41.97

*Micrographs from all samples that showed filiform structures. # Largest areas of samples with visual identity to mineralized globuli *in vitro* and *in vivo*.

C = control (no biomaterial applied). B = bioceramic, HA/β tricalcium-phosphate®. L = lyophilized deproteinated bovine bone®. BL = mixture of bioceramic and lyophilized bone in equal parts (v/v).

1C1 = control 1st. week animal 1. 3C2 = control 3rd. week animal 2. 1B1 = bioceramic 1st. week animal 1. 3B1 = bioceramic 3rd. week animal 1. 1L3 = lyophilized bone 1st. week animal 3. 3L3 = lyophilized bone 3rd. week animal 3. 1BL3 = bioceramic + lyophilized bone 1st. week animal 3. 3BL1 = bioceramic + lyophilized bone 3rd. week animal 1.

reached values ranging from 0.07 to 0.18 μm, except for 1L that presented a diameter of 0.40 μm. On the other hand, the group of filiform structures with highest diameters were considered as bundles, which had a great variation of thickness (1BL = 5.37μm to 1C = 55.82 μm). The highest values were observed for controls (1C = 55.82μm and 3C = 49.14μm), while the lowest values were observed for BL (1BL = 5.37μm and 3BL = 5.62μm).

According to Table 1, the smallest globuli areas were exhibited by controls of 1 and 3 weeks, while the largest areas were observed with the use of bioceramic isolated or not, mainly in 1B (69.24%). This fact may be expected once the bifasic bioceramic has an inorganic, compact and dense composition, with slow absorption. Even so, at the third week a reduction was observed to less than half (3B = 33.84%). 1L (18.38%) presented a globuli area slightly different form 1C (14.33%) while 3L showed a greater difference when compared to 3C (3C = 17.74% and 3L = 28.26%). The association of the biomaterials showed a similar pattern along with time (1BL = 44.70% and 3BL = 41.97%).

Discussion

The choice of the rat as a biological model of study was confirmed as having several advantages: easy handling, small costs and fast tissue response.

Regarding the creation of defect, a round burr is described (*Bohning et al., 1999; Gendler, 1986; Rodriguez et al., 1997*) as the option for the simulation on bone, in some studies considered better than the trephine burr (*Bartee & Carr, 1995*). This choice was attributed to the production of non-transosteal defects, once the action of the burr was conveniently limited by the root surface of the animal's right incisive. A suitable defect for the insertion of biomaterials was easily obtained, at a region already well described (*Greene, 1959*).

Also, in relation to the anatomical site chosen for the surgery, other factors influenced in the positive sense, this choice: 1) the study of bone neoformation aided by biomaterials in the oral cavity is of great interest for dentistry, as the masticatory movements and salivation can be important aspects that need to be considered; 2) the use of an anterior area of the oral cavity allows for an intraoral access, just as done in the dental clinic (*Zahedi et al., 1998*); 3) the involvement of the periodontal ligament is frequent in oral surgery, and it represents an abundant source of indifferentiated cells and other factors responsible for the repair of the periodontium, as reflected by the scientific community's growing interest in this tissue (*Brett et al., 2002; Shimazu & Morishita, 2003*). In our study, no sign of necrosis or infection was observed and no statistical or significant differences were observed in the weight of the animals ("t" test, $\alpha = 5\%$), suggesting that the experimentation did not impair normal feeding.

The selection of the biomaterials was based on their possible mechanism of action. The bioceramic (B) used in this work, with HA/phosphate β-tricalcium, is an alloplastic product (synthetic graft biomaterial) with osteoconductive properties (*LeGeros et al., 2003*), which is able to fill the bone defect, without direct contribution to cell biological activity, and could be absorbed by the organism. And the lyophilized deproteinated bovine bone (L) is a xenogenic product (graft biomaterial with different species origin) with probable osteoinduction mechanism shown by similar biomaterials (*Edwards et al., 1998*), able to induce the cell activity, such as osteoblast's, accelerating bone neoformation.

As the operator that prepared the samples prior to SEM was always the same, it can be assumed that the results from a smaller root exposure area during the removal of the cicatricial tissue were due to a better root surface adherence of the cicatricial tissue. The comparison among samples suggested the presence of an initial cicatricial stage in the ligament, and as consolidation of the repair grows the development of a mature tissue connection should be established with the dental root (*Karimbux et al., 1995*). Figure 1 demonstrates this situation. Results of the isolated biomaterials (B and L) suggested a slower repair (Table 1) with a higher exposure ever at the three-week period. This could be explained by the two different methods of action. L may con-

tain molecules that initially stimulate remodeling positively, but it can also contain molecules foreign to the species, which may delay the tissue repair. B can supply important ions for bone structure, but at first seems to fill the site more densely than really contribute to the production of bone tissue. This can hinder cell diffusion and lack vascularization. If on one side this is negative, the advantage of maintaining the tissue volume that allows for the recovery of height and thickness of maxilla/mandible bones justifies its use in these cases.

The association of the two biomaterials seemed to create a beneficial synergism. L could facilitate a larger biological activity and its xenophobic reaction could be compensated by the abundance of ions of hydroxyapatite composition in B, boosting the organism's action in regenerating the tissue. This is probably the same process that happens in the control: it has the incentive molecules for bone production as well as calcium and phosphate present in highly vascular tissue, facilitating high cellular and molecular diffusion, such as of oxygen and carbohydrates. Thus, the control may have the precursory cells of the involved tissues (osteoblasts, fibroblasts and cementoblasts), the agents of regeneration incentive (growth factors), the ions to produce the inorganic portion (phosphate and calcium) and the molecules for production of energy and source of carbon. The clinical problem of the control system (agglutinated blood) is its inability to maintain the remodeling space, which causes a loss of tissue volume. Therefore, the insertion of some biomaterial can be considered crucial when volume is necessary to the recovery of appearance, for example.

According to some observations the proliferation of osteoblasts *in vitro* can be influenced by the presence of elements such as calcium and phosphate, depending on the amount. These elements can activate differentiation and expression in osteogenic cells, which could lead to faster bone neoformation (*Hulshoff et al., 1998*). Therefore, depending on the amount of ion clearance showed by the grafted bioceramic, it could also have osteoinduction properties. It is already demonstrated *in vitro* that the β-tricalcium phosphate, present in the bioceramic of this study, facilitates osteoblast growth and the secretion of extracellular matrix, and that large amounts of these ions can also inhibit cell growth, for example, in the use of $CaKPO_4$ bioceramic (*Knabe et al., 2000*). Others demonstrate that the degradation of biomaterial particles increases proportionally to the increase of the number of pores in the surface of the biomaterial (*Benhayoune et al., 2000*).

A SEM study (*Kihara et al., 1998*) of embryonic bone development reports that before collagen fibers form regular bundle arrangements an irregular net of fibers is observed. Although our study deals with bone repair, with different signals than the ones found in embryology, it was possible to observe the presence of some regular bundles in an irregular net, in the area near the root surface of the 3BL sample (Figure 1). It is also demonstrated by SEM (*Suda et al., 1999*) that the mechanical stress affecting bone also influences the arrangement of collagen fibers, and therefore the patterns can be different from those found in fixed bones (e.g. calvaria). Table 1 shows that 1B (0.15 μm), 1L (0.40 μm) as well as 1BL (0.18 μm) had cross-section widths much larger than 1C (0.07 μm). At third week postoperatively all biomaterials samples demonstrated similar patterns of filiform structures metabolism, presenting fiber width of about 2-3 times larger than 3C and 1C. However, with the bundles, the control samples had about 2-10 times larger widths. Thus, it can be suggested that the insertion of the biomaterial may definitely have influenced the synthesis and modeling of fibrillar proteins. This aspect was more intense for BL. Neither B nor L alone approached the C bundle widths. Although the manufacturer declared deproteination, it might be there were some residual (xenogenic) proteins present in L prior to application, which seemed to delay the synthesis of autogenous fibrillar proteins for substitution of xenogenic ones. Researchers agree that local and systemic factors may differently influence bone repair (*Ohlsson et al., 1998; Wikesjo et al., 1999*).

According to previous reports, before new bone, appears a layer of calcified globules (called globular accretions) are seen; afterwards collagen fibers are secreted and then become calcified (*Orr et al., 1992*). In Figure 1, 1BL showed a large amount of these globular accretions; likewise, 3BL showed many globules adhered to filiform structures, suggested to be collagen fibers. Although the globuli area analysis done in this study was not as specific as others previously reported by immuno-SEM (*Shen et al., 1993*), its findings may be of some morphological interest. The percentile (Table 1) of globuli area for control in GI and GII (1C = 14.33% and 3C = 17.74%) were relatively similar. Larger values are expected once mineralization of the healing increases along with time, and to stabilize according to biomechanical demands and dynamics of cellular activity for bone remodeling. That happened more clearly with L (1L = 18.38% and 3L = 28.26%), while with the association of the bioceramic the globuli area kept relatively high and almost steady (1BL = 44.70% and 3BL = 41.97%). At first, it may seem that when bioceramic was used alone, the clearance of ions that may contribute to the formation of globuli, or globular accretions, was very high (1B = 69.24%), diminishing with time (3B = 33.84%). Regarding this aspect, in this present study, the association of the biomaterials (BL) seemed to deliver a more stable condition for tissue repair.

Summarizing, considering root exposure, the smallest areas, therefore best condition for this parameter, were 3C and 3BL (approximately 3% for both). Taking into account the maintenance of tissue volume, it is suggested that the association of biomaterials (BL) was the best option at three weeks postoperatively. The diameter of fibers was lowest and width of bundles was highest for the controls (GI and GII). This may suggest that the synthesis of fibers is prevalent in natural bone repair. Thus, the fibers are formed, but in a more organized way in the control groups, which has the appropriate environment for the production of more voluminous bundles formed by fine fibers. The presence of biomaterial seemed to disturb the organized production of fibers. Fibers may be produced thicker to counteract the biomechanical forces to which the tissue is subordinated; this may also hinder the organized aggregation to produce the thicker bundles. The highest globuli area (1B) can resemble the best situation, but in this case, the difficulty of mineral absorption from the isolated bioceramic, can create the false impression that B alone is the best graft.

The results obtained from experimental models like this can aid in the search for the best conditions in clinical applications of biomaterials of bone grafting (*Cavaliere et al., 2001*).

Conclusions

SEM provided morphological tissue information to evaluate bone-grafting success. Root exposure area in relation to defect area was reduced from one to three weeks for control and BL (bifasic calcium phosphate bioceramic and lyophilized deproteinated bovine bone), while it was similar or higher for B (bifasic calcium phosphate bioceramic) and L (lyophilized deproteinated bovine bone), respectively. Fiber diameter observed at one week was similar to three weeks, except for L that reduced (attributed to bovine origin). After three weeks, all fibers showed similar diameters. Control (C) bundles (groups of fibers) had larger width than biomaterial specimens and were similar along time (1 and 3 weeks). The BL combination probably inhibited the growth of bundles. The globular accretion (globuli area of mineralization) was higher for B specimens, compatible with its slow tissue absorption; C had slightly smaller area than L; BL demonstrated the best profile in this characteristic. Thus, considering overall tissue repair, BL seemed to demonstrate the best condition for bone grafting in this study. All used biomaterials promoted periodontal ligament involvement, favored maintenance of tissue volume, although slowing remodeling, and their combination in equal parts seemed to augment the desired repair. The findings of this study suggest that the biological model used simulated the oral clinical conditions well.

Acknowledgements

This article was supported by research grants from Federal University of Parana (UFPR) and Positivo University Center. Special thanks to Electron Microscopy Center of UFPR.

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