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RESEARCH

Autogenous dentin combined with mesenchymal stromal cells as an alternative alveolar bone graft: an *in vivo* study

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Abstract

Objective Considering the chemical and structural properties of dentin, this study was aimed at evaluating the effect of dentin matrix alone or combined with mesenchymal stromal cells (MSC) on postextraction alveolar bone regeneration.

Material and methods Wistar rats were subjected to tooth extraction with osteotomy and allocated into groups according to the graft inserted: (1) Gelita-Spon®, (2) Bio-Oss®, (3) Dentin, (4) MSC, (5) Dentin/MSC, and (6) Control. Maxillae were analyzed by means of hematoxylin and eosin (H&E) staining, immunohistochemical (IHC) analysis, microcomputed tomography (micro-CT), and scanning electron microscopy (SEM). Serum levels of calcium and phosphorus were quantified.

Results The Bio-Oss group showed less bone than Gelita-Spon and Dentin/MSC; no other significant differences were seen in H&E analysis. The Bio-Oss group showed higher expression of collagen type I compared to the Dentin and Dentin/MSC groups and also higher osteocalcin expression than the Dentin/MSC group. There was a tendency of higher expression of osteopontin in the MSC, Dentin, and Dentin/MSC groups and higher VEGF in the MSC group. On micro-CT analysis, the Bio-Oss and the Dentin/MSC groups exhibited greater bone volume than the Control. Serum calcium and phosphorus levels did not significantly differ between the groups. SEM analysis depicted particles of Bio-Oss and dentin in the respective groups, as well as significant cellularity in the MSC group.

Conclusion Autogenous nondemineralized dentin is an alternative for alveolar bone grafting, which can be improved by combination with MSC.

Clinical relevance This work provides support for the clinical applicability of dentin graft alone or combined with MSC.

Keywords Alveolar ridge · Dentin · Graft · Mesenchymal stromal cells · Stem cells · Tooth

Introduction

Autogenous bone graft is the gold standard for alveolar bone augmentation, having osteogenic, osteoinductive, and osteoconductive properties. However, the major limitation of this graft is the need of an alternative donor site and therefore the morbidity of a second surgical intervention [1]. Among the alternatives, allografts lack osteogenic potential and carry the risk of disease transmission [2], whereas xenografts and alloplastic grafts have only osteoconductive potential [3].

Dentin and bone share several chemical and structural features, including the distribution of inorganic, organic, and aqueous compounds [4–6]. It was reported that dentin has some factors that are widely implicated in osteogenesis, such as transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), fibroblast growth factor

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(FGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), bone sialoprotein (BSP), osteopontin (OPN), dentin sialoprotein (DSP), dentin matrix acidic phosphoprotein 1 (DMP1), osterix (Osx), and runt-related transcription factor 2 (RUNX2) [3, 7–10]. These properties suggest that dentin from an extracted tooth would be a good graft alternative for alveolar bone preservation. Nevertheless, this material is routinely discarded after tooth extraction. The use of nondemineralized, partially demineralized, and demineralized dentin grafts has been reported [5]. The demineralization process is time-consuming, whereas autogenous nondemineralized dentin graft is easily obtained and has demonstrated excellent biocompatibility. Although there is no standardized way of how the dentin graft is prepared, it will always demand some kind of processing, at least enamel removal and dentin crushing or cutting, since dentin can be used either in block or particulate form [5, 8]. Nondemineralized dentin particulates ranging from 300 to 1200 μm seem to be appropriate [8].

Studies in animal models and also a clinical trial [11, 12] have reported good results in bone regeneration by using stem/stromal cell transplantation. Accordingly, canine jaw defects showed improved bone formation of 20 to 40% compared to the control after systemic injection of these cells in the bone marrow [13]. Mesenchymal stromal cells (MSC) represent a heterogeneous population of multipotent cells, which were first characterized in bone marrow [14] and subsequently identified in various mature tissues, including the bone, dental pulp, and adipose tissue. These cells have a great potential for proliferation and act in the regeneration of damaged tissues, having the ability to undergo differentiation into osteogenic, chondrogenic, and adipogenic lineages under specific *in vitro* conditions [15–18]. Adipose MSC have shown some advantages compared to bone marrow MSC, especially concerning accessibility, since they can be obtained by means of less-invasive procedures such as liposuction and give a higher count yield [17, 18]. Also, differently from bone marrow MSC, adipose MSC might not be adversely influenced by age [17].

In view of dentin's potential as an effective alveolar bone graft and the properties of MSC, the aim of this study was to investigate the effects of dentin graft combined or not with MSC on alveolar bone regeneration after tooth extraction. Accordingly, we used autologous nondemineralized dentin matrix, MSC derived from adipose tissue and the techniques of light microscopy (H&E and immunohistochemistry), microcomputed tomography (micro-CT), scanning electron microscopy with energy dispersion spectroscopy (SEM/EDS), and serum analysis.

Material and methods

Sample characterization

This study followed the guidelines of the National Council for Animal Experimentation Control (CONCEA) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) [19]. The protocol was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS) (protocol #9108). The sample comprised 62 adult female rats (*Rattus norvegicus*, Wistar strain), from the Center of Experimental Biological Models of PUCRS (CEMBE-PUCRS), between 70 and 77 days old and weighing between 240 and 250 g at the beginning of the experiment. The animals were housed in microisolators with air filters and controlled humidity and temperature (23±1°C), under a 12-h light-dark cycle, with room light of 300 lux and 60 lux inside the cages. Each cage housed at most 4 animals, and food (Nuvilab-Cr1, Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*. Cleaning and change of cages were carried out according to CEMBE/PUCRS protocol. The experiments were started after a period of environmental acclimatization. For ethical reasons, we considered the number of groups (demanding a large sample), the risk of losing animals during the experiment, the need of controlling sex bias, and the nature of the variables analyzed, so we chose to use a single-sex sample. The option for females was based on the smaller size and easier handling of these animals and their use in previous reports with the same experimental paradigm [18, 20, 21]. The age of the animals was chosen to support the clinical translation of the results [18, 22].

Two of the 62 rats were used for MSC isolation. The remaining 60 animals were subjected to the extraction of the first and second upper right molars and randomly allocated into treatment groups: (1) Gelita-Spon: 10 rats that received porcine gelatin (Gelita-Spon®, Gelita Medical, Eberbach, Germany); (2) Bio-Oss: 10 rats that received the xenograft Bio-Oss® (Geistlich Pharma, Wolhusen, Switzerland); (3) Dentin group: 10 rats that received autogenous dentin; (4) Mesenchymal stromal cell (MSC): 10 rats that received adipose tissue-derived MSC loaded in a Gelita-Spon scaffold; (5) Dentin/MSC: 10 rats that received autogenous dentin combined with adipose tissue-derived MSC; and (6) Control group: 10 rats that did not receive any material at the tooth extraction site. In the MSC group, Gelita-Spon was used as a scaffold and, for this reason, the group using only Gelita-Spon was created to rule out that the effects in the MSC group were caused by the scaffold. The Bio-Oss group was selected as a comparative group (positive control), since Bio-Oss® is a xenogeneic

bone graft material widely used in clinical routine with satisfactory results and also exhaustively investigated in the scientific literature. The distribution of the animals in the individual cages was previously done by staff outside the study, who were blinded to the treatments and labeled the cages by sequential numbers. The allocation into the treatment groups followed this previous distribution.

Isolation of the murine adipose-derived MSC

The protocol was based on previous reports in the literature [23]. Two rats were subjected to euthanasia with deep anesthesia with 5% isoflurane (Isoforine®, Cristália, Itapira, SP, Brazil), washed with 70% ethanol, and handled inside a laminar flow chamber. A midline xiphopubic laparotomy gave access to the tissue of interest. Fragments of rat retroperitoneal adipose tissue were removed and digested with 1.5 mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO, USA) in DMEM (Dulbecco's Modified Eagle Medium/low glucose, Gibco, Grand Island, NY, USA) without serum for 25 min at 37°C. DMEM with 10% FBS with streptomycin (0.1 mg/mL)/penicillin (100 U/mL) was then added to block enzymatic digestion. After centrifugation at 1500 rpm for 7 min, each pellet was resuspended in DMEM with 10% FBS and placed in a moist chamber at 37°C and 5% CO_2 until cell confluence. The cells were detached by using 0.05% trypsin-EDTA. All experiments used cells at the fourth passage. It took 50 days to complete the whole process until the fourth passage.

Surgical procedures

The rats were anesthetized with a mixture of 70 mg/kg ketamine hydrochloride (Syntec, Cotiá, SP, Brazil) and 7 mg/kg xylazine hydrochloride (Syntec) administered intraperitoneally (IP) (Luvizuto et al., 2010). Extraction of the first and second upper right molars was performed with an adapted 3s carver (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Edlo, Canoas, RS, Brazil) adapted to the size of the teeth [24, 25]. Immediately after tooth extraction, a groove osteotomy was made at the extraction site with a 1.8-mm diameter ball bur under water irrigation, obtaining a standardized bone defect (1.8 mm x 1.8 mm x 6 mm). The bone defect was then filled with different materials, according to the experimental group.

The porcine gelatin (Gelita-Spon®) was inserted soaked in saline; the bovine bone graft (Bio-Oss®, Geistlich) was used as particles ranging from 0.25 to 1 mm; dentin particles of crushed nondemineralized dentin were obtained from the extracted teeth and applied in the Dentin group and Dentin/MSC group. The MSC group received MSC loaded in Gelita-Spon®, and the control group had no material inserted.

The teeth from which the autogenous dentin matrix was obtained were the extracted first and second right upper molars. The enamel was removed with a diamond bur at high speed under water irrigation, and the dentin was ground by using a mortar and pestle until obtaining granules up to 1 mm in diameter. Each animal received dentin from their own teeth. After grafting, the soft tissues in all groups were sutured with resorbable sutures (Vicryl 6-0, Ethicon, Somerville, NJ, USA).

Euthanasia and specimen processing

At 35 days after the surgical procedures, the animals were anesthetized with IP administration of ketamine hydrochloride (70 mg/kg) and xylazine hydrochloride (7 mg/kg) and subjected to exsanguination by cardiac puncture followed by an overdose of ketamine (210 mg/kg) and xylazine (21 mg/kg). The blood samples collected were centrifuged after clot formation, and the serum was stored at −20°C. Maxillae were dissected, and the site of the tooth extractions was cut in a coronal direction by using an extra fine diamond disk (KG Sorensen, Cotia, SP, Brazil) and divided into two fragments, both showing the area of interest on the cut surface [24, 25]. The specimens were immersed in 10% buffered formalin for 24 h. One fragment from each animal was prepared for histological processing and the other for microcomputed tomography (micro-CT) and one specimen from each group for scanning electron microscopy (SEM) (Fig. 1).

Histological processing and analysis

The specimens were decalcified in 10% nitric acid for 8 h, embedded in paraffin, and processed for hematoxylin and eosin staining (H&E) and immunohistochemical (IHC) analysis.

IHC

Immunostaining was performed in Dako Autostainer Link 48 (Agilent-Dako, Santa Clara, CA, USA) with Dako EnVision FLEX+ detection system (Dako, Glostrup, Denmark). The protocol included deparaffinization, heat-induced antigen retrieval with EnVision FLEX Target Retrieval Solution, and endogenous peroxidase blocking with EnVision FLEX Peroxidase-Blocking Reagent. The samples were incubated for 35 min at room temperature with the primary antibodies: collagen type I (polyclonal, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) at 1:100 dilution, pH 6; osteopontin (OPN, AKm2A1: sc-21742, Santa Cruz Biotechnology, Dallas, TX, USA) at 1:600, pH 6; vascular endothelial growth factor (polyclonal, VEGF, Thermo Fisher Scientific) at 1:200, pH 9; and osteocalcin (OCN, G-5: sc-365797, Santa Cruz Biotechnology) at 1:400, pH 6.

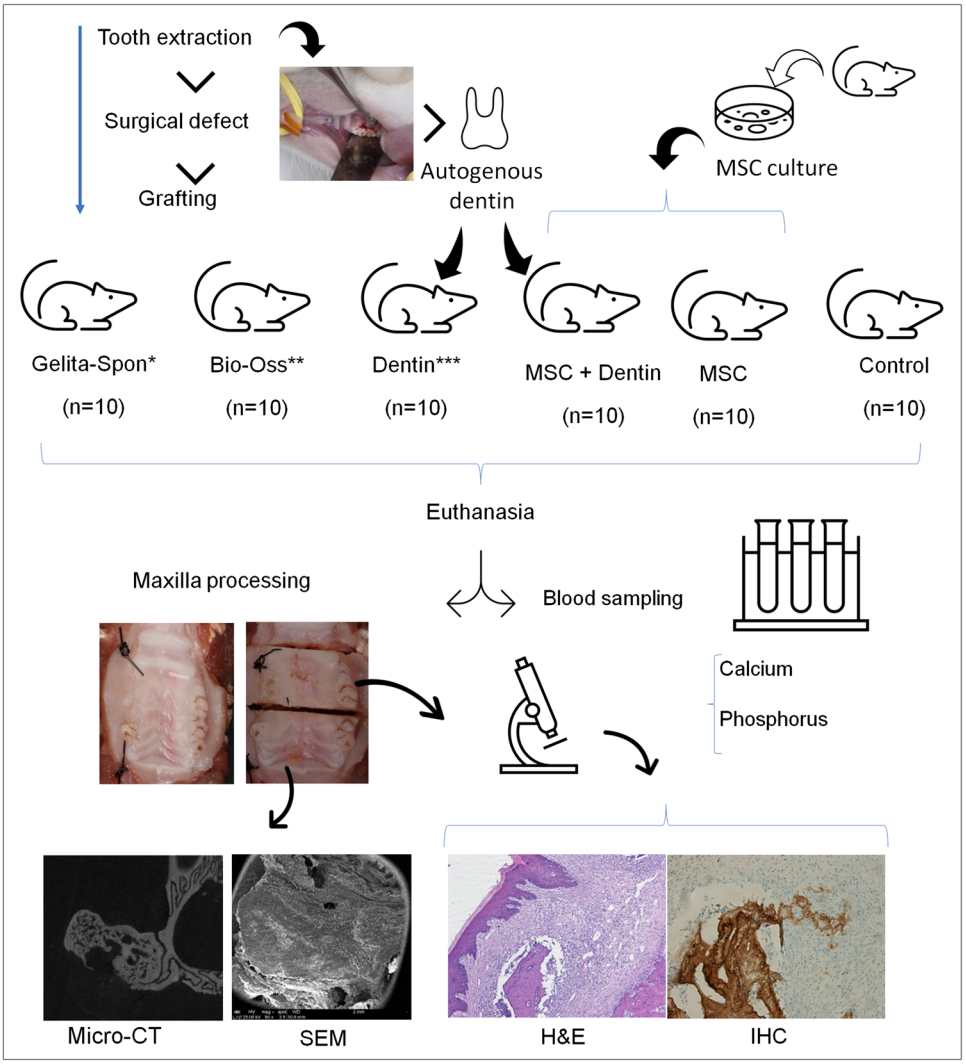


Fig. 1 Flow diagram of the study. *Gelita-Spon®(Gelita Medical, Eberbach, Germany); **Bio-Oss® (Geistlich Pharma, Wolhusen, Switzerland); ***autogenous dentin; H&E=hematoxylin and eosin; IHC=immunohistochemistry; MSC=mesenchymal stromal cell; Micro-CT=micro-computed tomography; SEM=scanning electron microscopy. The MSC group used Gelita-Spon® as a scaffold

Labeled polymer FLEX/HRP was applied (20 min), and the substrate FLEX DAB + chromogen (3,3′-diaminobenzidine tetrahydrochloride hydrate) (Dako) revealed the complex. Slides were counterstained with hematoxylin and coverslipped with Entellan (Merck Millipore, Darmstadt, Hesse, Germany). Positive controls were samples of skin, gall bladder, placenta, and rat maxilla, respectively, for collagen type I, OPN, VEGF, and OCN.

Image capture and histological analysis

The images were captured with an Olympus BX-43 light microscope (Olympus, Tokyo, Japan) with an Olympus DP-73 digital camera (Olympus). The captures were performed in a standardized manner (clockwise). For H&E, five fields from each slide were captured by using the 10x objective. The IHC images were captured using a 20x objective, totaling three fields per slide, also clockwise. The images were stored in TIFF format (uncompressed). Analyses were performed in Image Pro-Plus software (Media Cybernetics, Bethesda, MD, USA) by means of a manual point-counting technique for H&E and semiautomated segmentation technique for IHC [26]. A quantitative analysis considered the amounts of epithelium, connective tissue, bone, inflammatory infiltrate, amorphous material, and root fragment. A point-grid of 520 points was overlaid on each image, and each point was counted according to the matching morphological structure. In IHC images, positive-staining areas for collagen type I, OPN, VEGF, and OCN were quantified by using the semiautomated segmentation technique. The images were analyzed by a calibrated blinded observer who did not know the group to which each image belonged. Before the analysis, intraobserver calibration was performed by evaluating 25 images in each technique twice at two

different moments. The agreement of these two analyses was tested by the intraclass correlation coefficient, which showed r=0.9. After finishing the quantitative analysis, a qualitative whole slide evaluation was conducted.

Microcomputed tomography (micro-CT)

Micro-CT images were acquired in a Micro CT Skyscan 1173 (Bruker, Billerica, MA, USA). Samples were scanned in the three spatial planes. The scanning was standardized with an isotropic voxel size of 9 µm, 85 kV, 75 µA, at 360°, rotation angle of 0.2°, 1 mm aluminum filter, with exposure time of 700 ms. Images of each maxilla were reconstructed with Bruker's Micro CT Skyscan software (Bruker), with pixel and voxel of 7.44 µm, resulting in 134 tomographic slices per mm. The images were positioned in three dimensions using DataViewer software. Coronal sections were analyzed using the CTVol® Micro-CT Surface Rendering Software, and in each specimen, the images anterior to the right maxillary third molar were selected by an anterior extension of 1 mm (134 tomographic sections). In these images, the region of interest (ROI) was selected, in the shape of a cylinder, with a diameter of 2 mm and a depth of 1 mm (ROI has a volume of 3.14 mm^3). The ROI was positioned in a standardized way within the alveolar bone in front of the right maxillary third molar of the rats. The gray scale (0 to 255) was adjusted in its parameter from 60 to 255 to recognize only radiopacity compatible with hard tissue. The CTVol® software calculated the absolute volume and the percentage of the ROI filled with hard tissue.

Scanning electron microscopy (SEM)

Specimens were dehydrated with gradually increasing concentrations of acetone, critical-point dried, and subsequently mounted on stubs and coated with a gold layer to be analyzed in an XL 30 scanning electron microscope (Phillips, Eindhoven, Holland). The topography of the entire tooth extraction area of one sample of each group was scanned at 80x magnification. Next, the tooth extraction area was scanned at 500x magnification in a standard manner, and the areas of interest were subsequently scanned at higher magnifications up to 10,000x. Chemical analysis of each sample was performed by means of energy dispersive spectroscopy (EDS).

Serum analysis

Serum samples were digested in 2% nitric acid for 120 min and analyzed by means of inductively coupled plasma mass spectrometry (ICP-MS) in a mass spectrometer Agilent 7700 ICP-MS (Agilent Technologies, Tokyo, Japan) to measure the levels of calcium and phosphorus. The operating conditions were with plasma RF power of 1550 W, argon carrier gas of 1.3 L/min, and helium collision gas flow of 4 L/min. The isotopes monitored for calcium were 42Ca, 43Ca, and 44Ca and phosphorus as 31P, with a dwell time of 0.3 s. The level of oxide species (CeO/Ce) was below 1.5%, and doubly charged (Ce2+/Ce) was below 3%. The quantification was performed with external calibration curves constructed at concentrations of 10, 100, 250, 500, and 1000 µg/L in both gas (He) and no gas modes. The analysis was performed in triplicate.

Statistical analysis

Data were analyzed by means of descriptive and inferential statistics. The Shapiro-Wilk test was applied to analyze data distribution and determined the use of Kruskal-Wallis complemented by Dunn's multiple comparison test in the H&E and IHC analyses, and ANOVA complemented by the Tukey test in the micro-CT and serum analyses. Data were processed in SPSS 21.0 (IBM Corp., Armonk, NY, USA) at a significance level of 5%.

Results

Four animals were lost within 24 h of the postoperative period, and the final n in each group was 10 rats in Gelita-Spon, 10 rats in Bio-Oss, 10 rats in MSC, 9 rats in Dentin, 9 rats in Dentin/MSC, and 8 rats in the Control group.

H&E—quantitative analysis

The Bio-Oss group showed significantly less bone than Gelita-Spon group and Dentin/MSC group; there were no other significant differences between the groups for this variable. Epithelium, connective tissue, inflammatory infiltrate, amorphous material, and root fragment did not significantly differ between the groups (Fig. 2, Kruskal-Wallis complemented by Dunn's multiple comparison test, α=0.05).

H&E—Qualitative analysis

Overall, H&E examination showed that the Gelita-Spon group and the Control apparently exhibited a more stable mature bone and less cellular activity in the adjacent connective tissue. Meanwhile, dentin and Bio-Oss particles were found in their respective groups, either incorporated

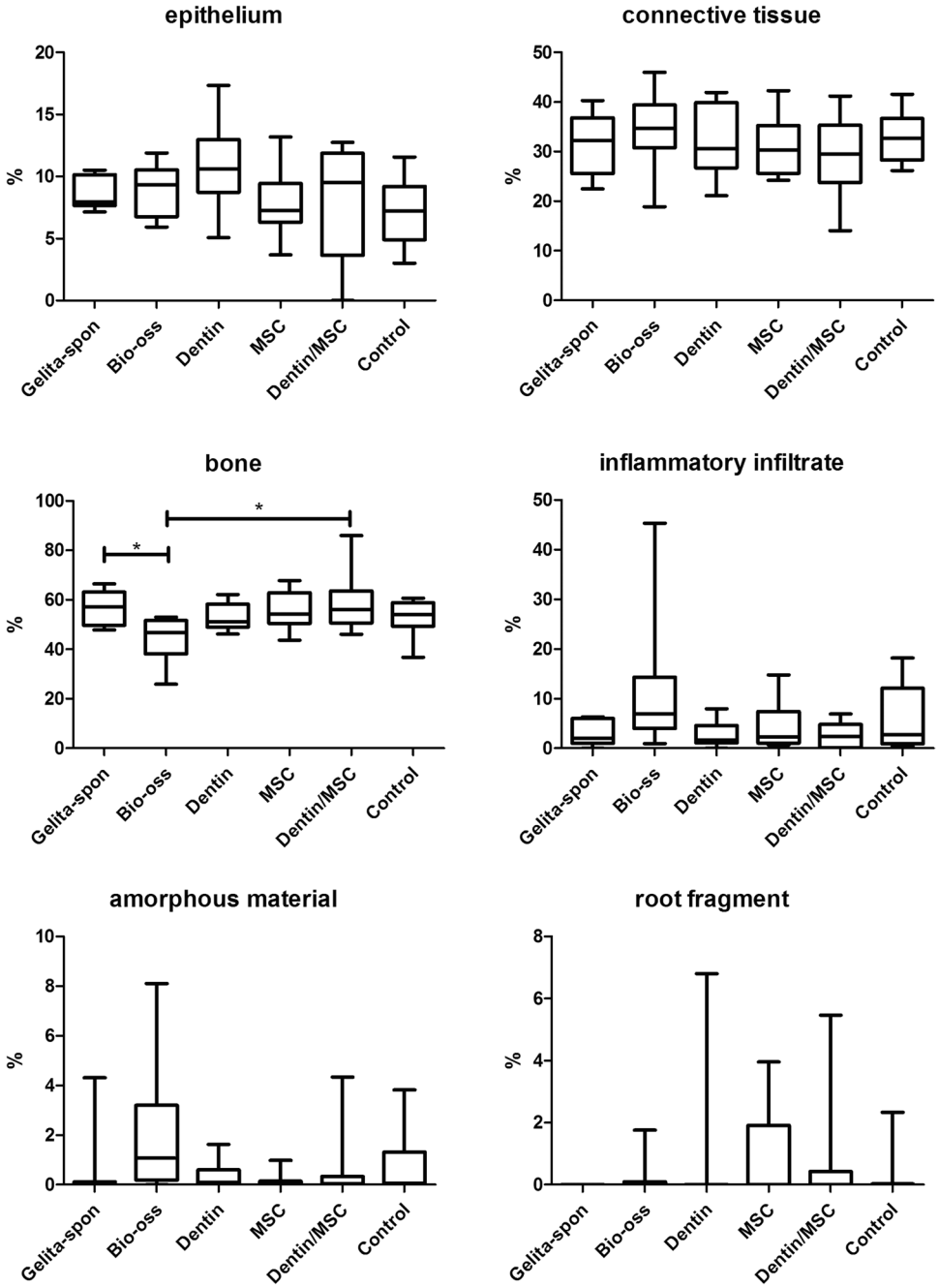


Fig. 2 Histological analysis in H&E: proportion (%) of the histological features at the tooth extraction site according to the treatment. *$P \leq 0.05$, Kruskal-Wallis complemented by Dunn's multiple comparison test. MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold

within the healing bone or dispersed in the connective tissue. In general, Bio-Oss particles were larger than dentin ones. When dispersed in the connective tissue, both promoted substantial cellular activity with some adjacent foci of bone matrix deposits. Nevertheless, when located in the proximity of the alveolar bone, they were incorporated by this tissue without any inflammatory response. MSC groups in turn seemed to promote even more cellular activity and neovascularization than the aforementioned groups (Figs. 3 and 4).

IHC—quantitative analysis

The Bio-Oss group showed significantly higher expression of collagen type I than the Dentin and Dentin/MSC groups and also of OCN compared to the Dentin/MSC group. Even though not statistically significant, there was a tendency of higher OPN expression in the MSC, Dentin, and Dentin/MSC groups and higher VEGF expression in the MSC group (Table 1, Kruskal-Wallis complemented by Dunn's multiple comparison test, α=0.05).

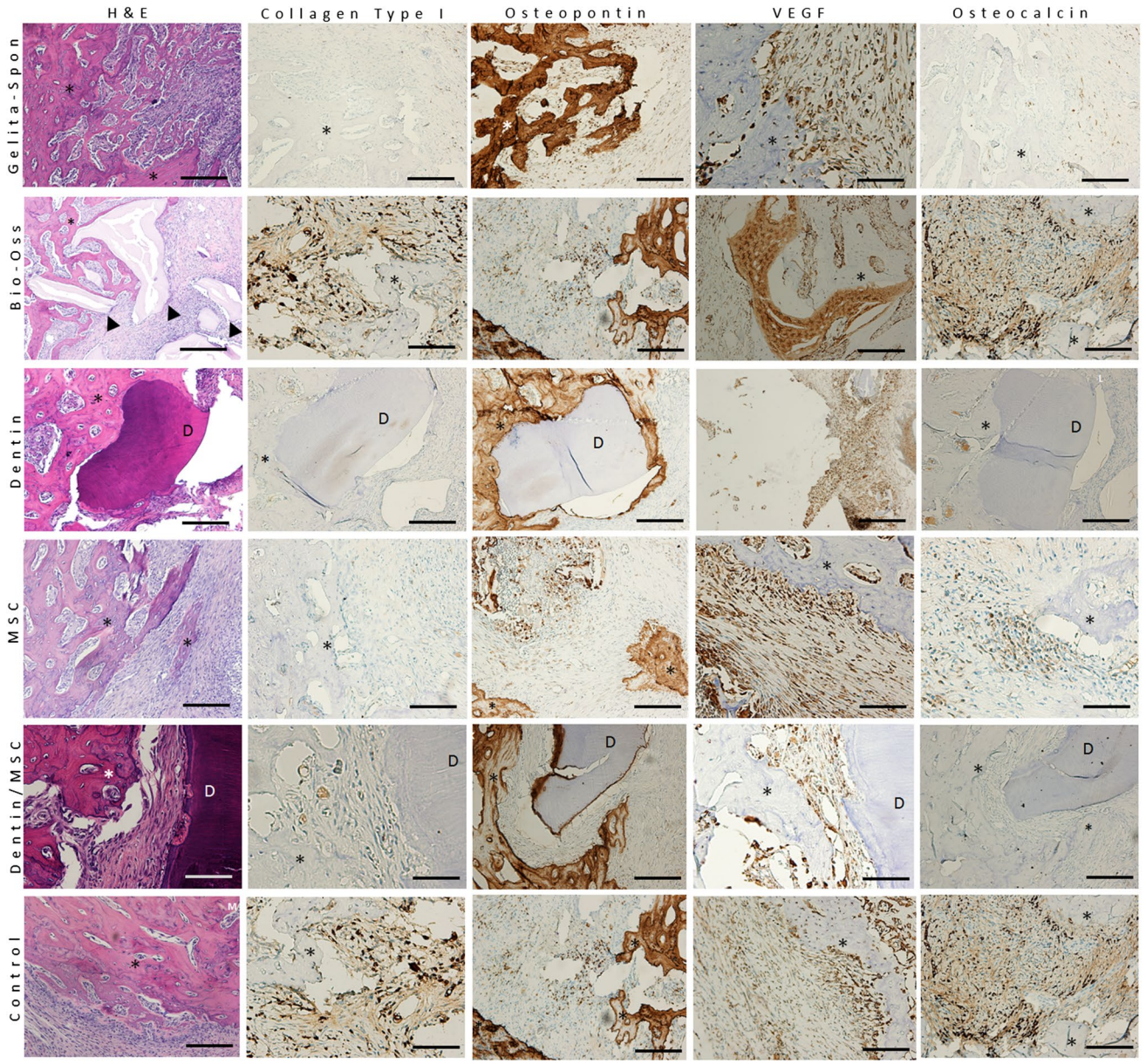


Fig. 3 Representative illustration of the hematoxylin & eosin (H&E) and immunohistochemical analyses (collagen type I, osteopontin, VEGF, and osteocalcin) in the Gelita-Spon, Bio-Oss, Dentin, MSC (mesenchymal stromal cell), Dentin/MSC, and Control groups. Bone (*), dentin (D), Bio-Oss (arrow heads). Scale bar=100 µm. MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold

IHC—qualitative analysis

Interestingly, we found Bio-Oss particles wholly stained for collagen type I, OPN, VEGF and OCN. Dentin was not stained for collagen type I, nor OCN or VEGF; but it was sparsely stained for OPN, especially within the dentinal tubules. The alveolar bone showed complete staining for OPN, strong staining in areas of woven bone and mild staining in areas of lamellar bone. OCN staining was found in the connective tissue and in Bio-Oss fragments and sparse areas of lamellar bone as well. Some aspects of this analysis are represented in Figs. 3 and 4.

Micro-CT analysis

The Bio-Oss and the Dentin/MSC groups showed greater bone volume than the Control (P=0.021); there were no significant differences between the other groups (Table 2, Fig. 5, ANOVA complemented by Tukey's multiple comparison test, α=0.05).

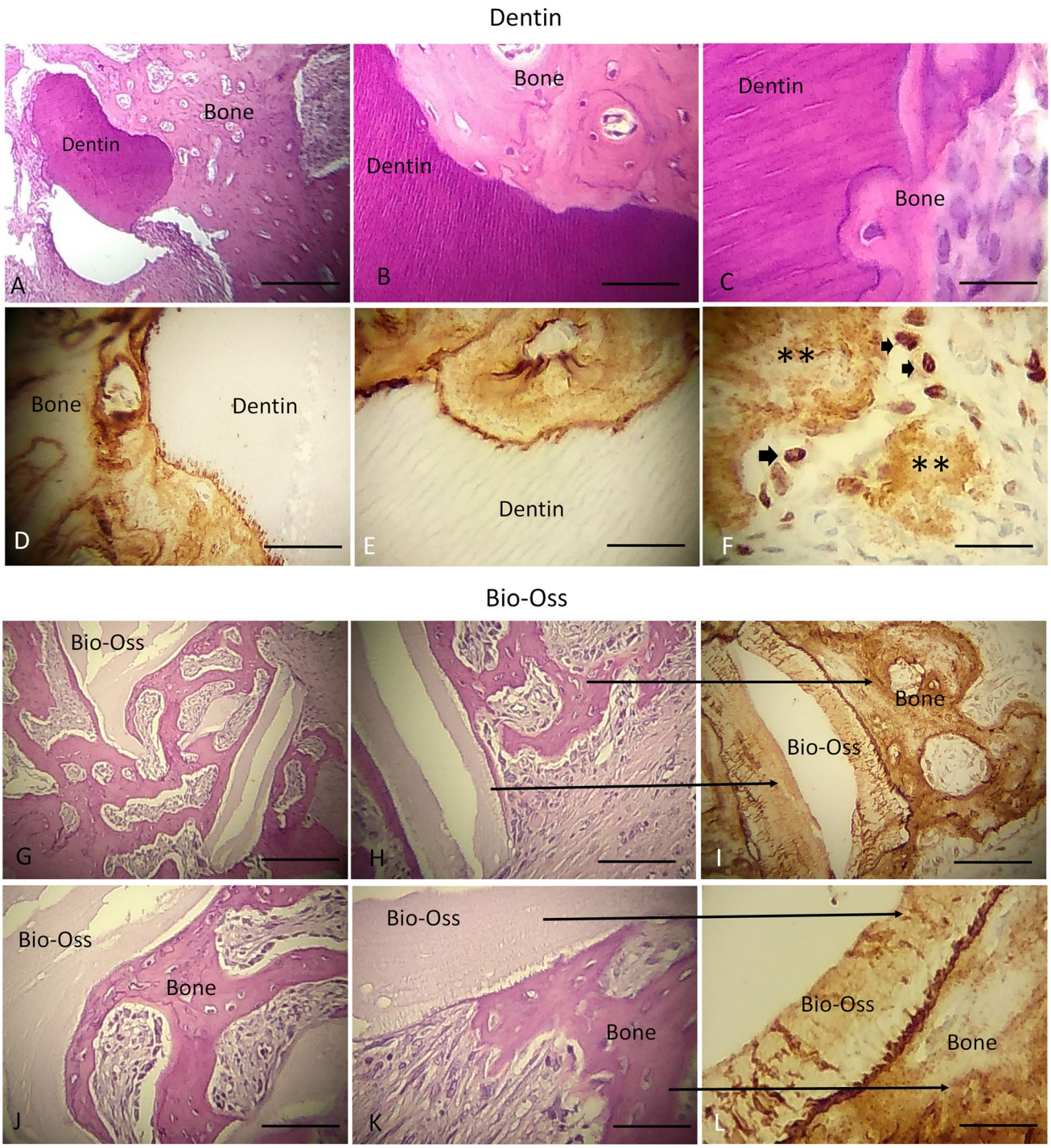


Fig. 4 Interface bone/dentin and bone/Bio-Oss [H&E and immunohistochemistry (osteopontin, OPN)]. Dentin fragment incorporated by bone forming dentin/bone complex, without any sign of immune reaction (A, H&E 100x, scale bar=100 µm), which can be seen in higher magnification (B, 200x, scale bar=50 µm and C, 400x, scale bar=25 µm). C shows an osteoblast being entrapped within the bone matrix. D, E and F show details of the OPN staining, where the whole bone is stained, whereas dentin shows positivity in the interface. In F, deposits of bone matrix (**) and osteoblasts (arrows). G (H&E, 100x, scale bar=100 µm), H (200x, scale bar=50 µm), J and K (400x, scale bar=25 µm) show Bio-Oss incorporated in the bone. In I (scale bar=50 µm) and L (scale bar=25 µm), the structures are immunostained for OPN. MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold

Table 1 Immunohistochemical analysis: expression (μm^2) of collagen type I, osteopontin (OPN), vascular endothelial growth factor (VEGF) and osteocalcin (OCN) at the tooth extraction site according to the treatment

Marker	Group							
		Gelita-Spon	Bio-Oss	Dentin	MSC	Dentin/MSC	Control	*P**
Collagen I	Mean	430.748	15,048.598	320.314	500.984	268.428	803.099	0.003
	SD	598.219	60,534.248	575.483	846.585	360.676	1078.348	
	Median	242.640	579.910	107.732	162.666	60.174	276.318	
	P25	97.298	102.831	40.375	61.485	16.305	43.287	
	P75	580.638	2512.056	427.629	539.874	472.857	1343.014	
	MR	86.38 ^{AB}	**111.07^{A}**	**68.80^{B}**	82.57^{AB}	**62.91^{B}**	93.31 ^{AB}	
OPN	Mean	34,512.638	34,098.278	43,335.602	39,272.680	50,910.743	34,023.065	0.057
	SD	24,196.841	21,764.020	23,132.026	21,247.858	28,912.328	17,600.219	
	Median	28,965.735	32,711.133	42,770.133	32,782.955	42,804.488	30,945.487	
	P25	16,799.890	16,110.063	26,282.229	23,614.446	29,786.539	19,938.735	
	P75	45,567.049	44,458.325	59,309.281	54,982.417	63,853.449	45,246.327	
	MR	72.23	74.30	95.56	85.57	105.74	74.92	
VEGF	Mean	3575.827	4293.202	1783.072	3928.814	2173.382	16,267.812	0.062
	SD	3571.875	6471.593	1243.253	3176.160	1594.162	70,210.320	
	Median	1959.952	2123.977	1503.788	3250.411	1778.651	1643.743	
	P25	995.214	788.726	635.329	954.398	719.768	752.233	
	P75	5554.767	5187.992	2777.941	5121.314	3100.556	2927.456	
	MR	90.53	88.67	67.59	104.20	77.07	74.50	
OCN	Mean	782.195	1889.716	538.000	530.651	243.553	1385.266	0.026
	SD	1099.936	4228.518	903.012	637.117	420.536	2489.616	
	Median	312.909	410.644	196.441	249.531	95.697	340.861	
	P25	49.644	134.616	63.474	135.781	31.640	92.882	
	P75	1212.765	1014.042	775.672	793.773	325.914	1138.226	
	MR	87.07^{AB}	**99.58^{A}**	77.63^{AB}	88.77^{AB}	**58.13^{B}**	94.50^{AB}	

**P* value for Kruskal-Wallis

SD standard deviation, *MR* mean rank, *MSC* mesenchymal stromal cell

Bold mean rank values followed by different superscript letters in the row differ significantly from each other. In collagen analysis, Bio-Oss (A) differed from Dentin (B) and from Dentin/MSC (B); in OCN analysis, Bio-Oss (A) differed from Dentin/MSC (B); Dunn's multiple comparison test; α=0.05. The MSC group used Gelita-Spon® as a scaffold

Table 2 Microcomputed tomographic analysis: bone volume at the tooth extraction site, according to the treatment

Group	Bone volume				
	Mean	SD	MD	Minimum	Maximum
Gelita-Spon	42.281^{AB}	12.503	43.676	16.296	62.842
Bio-Oss	50.479^{A}	9.616	48.940	35.916	62.356
Dentin	44.914^{AB}	11.108	42.072	29.178	59.646
MSC	42.700^{AB}	7.856	44.086	27.534	51.102
Dentin/MSC	50.744^{A}	8.353	49.312	41.452	66.507
Control	35.303^{B}	8.480	35.851	16.472	42.627
P	0.021*				

SD standard deviation, *MD* median, *MSC* mesenchymal stromal cell,

**P* value for ANOVA. Means followed by different superscript letters in the column differ significantly from each other [Bio-Oss (A) significantly differed from Control (B)]; Tukey's multiple comparison test, α=0.05. The MSC group used Gelita-Spon® as a scaffold

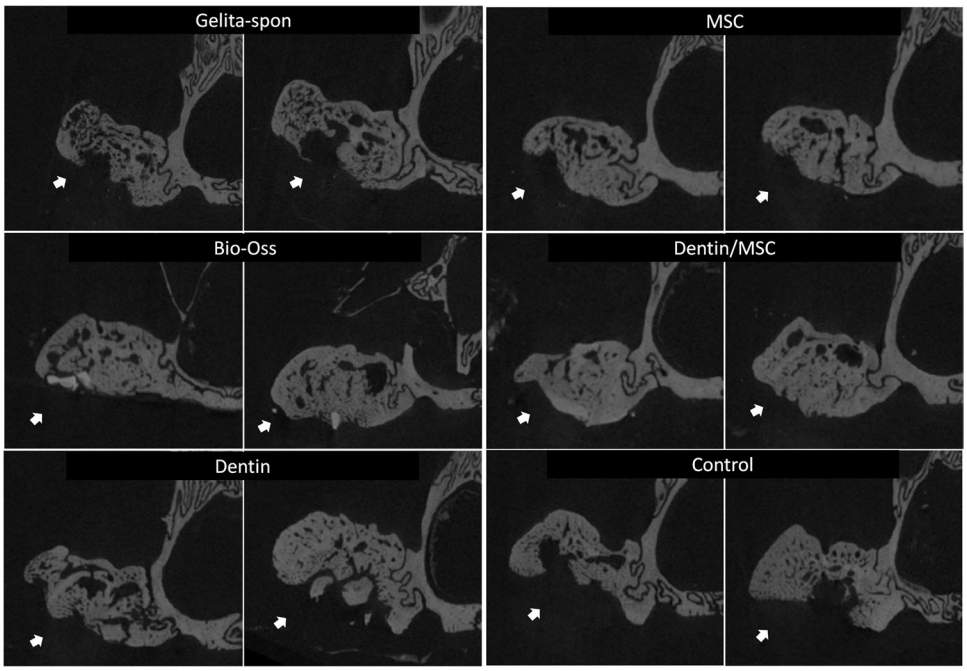


Fig. 5 Representative images of the micro-CT in the grafted area. Gelita-Spon shows alveolar bone with two areas of defect; in the Bio-Oss group, homogeneous cancellous bone can be seen without any defect; the Dentin group shows images compatible with nonincorporated dentin graft; MSC and Dentin/MSC have homogeneous cancellous bone with no defects. In Dentin/MSC, it is possible to identify a fragment with higher density compatible with dentin graft incorporated within the alveolar bone. In the Control, alveolar bone shows two areas of bone defect. MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold.

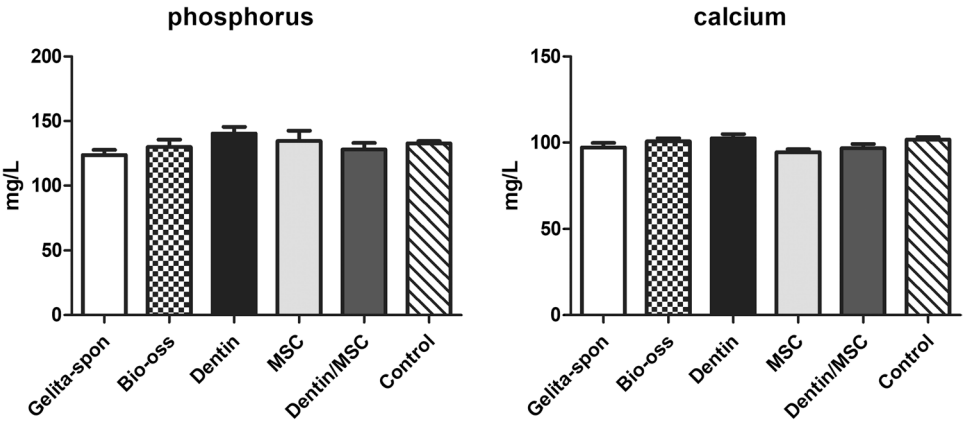


Fig. 6 Serum calcium and phosphorus levels (mean) by means of ICP-MS (inductively coupled plasma mass spectrometry). There was no significant difference between the groups, ANOVA, $P>0.05$; MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold

Serum analysis

Calcium ($P=0.086$) and phosphorus ($P=0.352$) serum levels did not significantly differ between the groups (Fig. 6, ANOVA, $\alpha=0.05$).

Scanning electron microscopy analysis (SEM analysis)

SEM analysis corroborated some findings of the H&E examination. Particles of Bio-Oss and dentin were visualized in the respective groups, as well as significant cellularity in the MSC group. Figure 7 depicts some morphological aspects of this analysis, and Fig. 8 presents the EDS results, which confirmed calcium and phosphorus as the major inorganic components of the samples in the area of interest

Discussion

We investigated here the performance of autogenous dentin graft, with or without MSC, in alveolar bone regeneration, and compared it to Gelita-Spon, Bio-Oss, MSC, and Control by means of light microscopy, micro-CT, SEM, and serum parameters. There was no significant difference between the grafted groups and the Control in the H&E and IHC analyses, suggesting that the materials used did not impair normal alveolar bone healing. Accordingly, 55.56% of animal studies found no significant difference between tooth-bone graft and control [27]. Such results would depend on, among other factors, some characteristics of the animal model, including its species and age [28]. Our sample comprised healthy adult female Wistar rats with normal bone physiology and therefore predictable normal bone healing in the Control

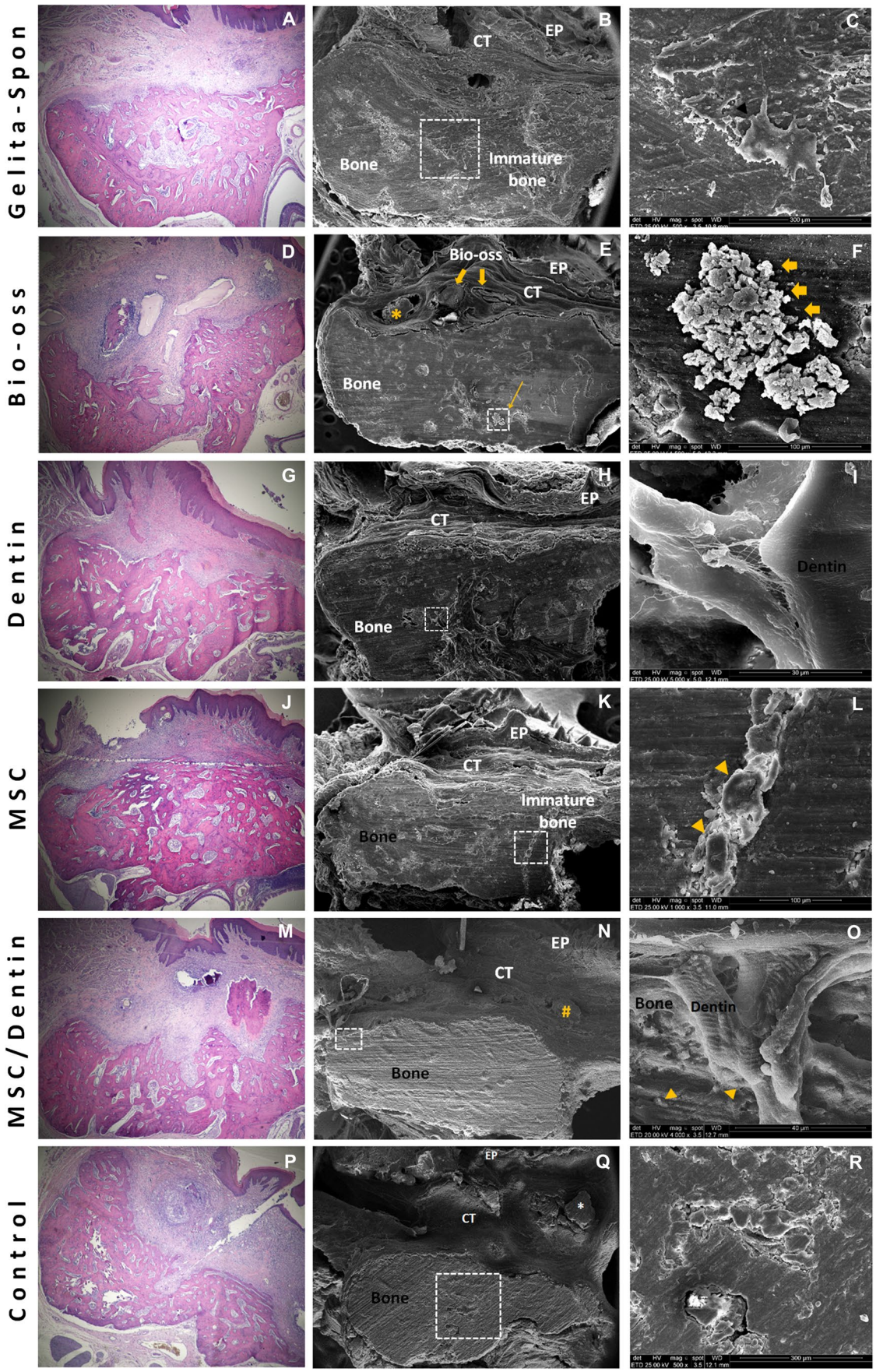
group. On the other hand, we found some significant differences between the grafted groups. Interestingly the Bio-Oss group showed, on H&E examination, less bone tissue than the Gelita-Spon group and the Dentin/MSC group. It is important to recall that Gelita-Spon is a gelatin sponge of porcine origin, which is completely biodegraded within 21 days [29] whereas Bio-Oss corresponds to anorganic bovine bone particles (0.25 to 1 mm) [30, 31], which last much longer [31, 32]. Accordingly, Gelita-Spon was not visualized on microscopic analysis, which is consistent with the time of euthanasia of our sample (35 days). Otherwise, particles of Bio-Oss were observed; some of them completely incorporated within the healing bone, but some also dispersed in the connective tissue associated with intense cellularity. In fact, there are reports of Bio-Oss particles found, either within the bone or in the connective tissue, not only at 90 days [31, 32] but also at six months [33], or even after some years of grafting [32, 34]. According to some authors, the process of hard tissue healing is apparently delayed in the Bio-Oss-augmented sockets. Nevertheless, it has been assumed that with longer healing time the amount of Bio-Oss-associated new bone formation will increase [33].

The Dentin/MSC group had significantly more bone than the Bio-Oss group, whereas the Dentin group and the MSC group did not. This finding suggests the effect of dentin graft was potentiated by MSC, which would migrate to the area of bone defect and differentiate into osteoblasts, enhancing bone regeneration [13]. Similarly, rabbits whose bone critical defects were treated with MSC loaded in a hydroxyapatite scaffold showed better results in bone volume compared to those receiving only the scaffold [11]. Still, a clinical trial comparing ossification of mandibular fractures found higher ossification rate in the group treated with autologous MSC [12]. On the other hand, one could reasonably point out that if Bio-Oss was combined with MSC, its results could also be better. Our study focused on dentin, combined or not with MSC; meanwhile, Bio-Oss was used as a comparative group on the basis of its acceptance and satisfactory clinical performance.

The connective tissue at the tooth extraction site of the groups receiving Bio-Oss, MSC, and/or dentin graft exhibited considerable cellularity with persistent activity of fibroblasts/osteoblasts and bone matrix deposition. This finding suggests that these grafts led to the persistence of osteogenesis at the healing site, which did not happen in the Control and Gelita-Spon groups. Maybe an analysis at a later period would have depicted greater deposition of mature bone in the Bio-Oss, MSC, and Dentin groups. Accordingly, clinical studies evaluating dentin graft at a later period verified greater amounts of bone and progressive resorption of the graft [35]. We chose the 35-day period for analysis considering that ossification of tooth extraction healing in rats is completed in 28 days [36]. However, there are reports on the ossification rate of alveolar critical-sized defects in this animal model reaching a plateau at eight weeks after surgery [28].

The Bio-Oss group showed significantly higher expression of collagen type I compared to either the Dentin or Dentin/MSC group and also had significantly higher OCN expression than the Dentin/MSC group. Collagen type I is the major collagen protein of bone matrix, whereas OPN and OCN are important noncollagen ones. These proteins represent osteogenic activity, which also demands VEGF upregulation [37]. Bio-Oss is a bovine bone classified as anorganic because the extremely high temperatures used during its processing eliminate the protein/organic component [32]. Despite that, we found Bio-Oss particles positively stained by all the IHC markers tested (collagen type I, OPN, VEGF, and OCN). This probably contributed to the collagen I and OCN results, since dentin did not stain with the markers, except for sparse staining with OPN. The transition of preosteoblasts to mature osteoblasts is characterized by an increase in the secretion of bone matrix proteins such as OCN, BSP I/II, and collagen type I [37]. Therefore, the greater expression of collagen type I and OCN in the Bio-Oss group compared to dentin groups (Dentin and Dentin/MSC) could also be explained by the longer resorption time of Bio-Oss compared to dentin [38], which would mobilize osteoblasts and osteogenic mediators for a longer time. Still, considering that demineralization of dentin is capable of releasing growth factors [39], the nondemineralized aspect of our dentin graft could be responsible for the lack of immunostaining in this structure. Since dentin demineralization is time-consuming, whereas autogenous nondemineralized dentin graft is easily obtained and has demonstrated excellent biocompatibility [8], and also because the rats were undergoing tooth extractions, we chose to use nondemineralized dentin.

OCN is the most abundant noncollagenous bone matrix protein, preferentially expressed by osteoblasts [40]. Anyway, we found the neoformed bone completely stained for OPN but not OCN. The exact role of OCN in bone is not completely understood [40]. Even though it plays an important role in osteogenic differentiation, nucleation of hydroxyapatite, and recruitment of osteoclasts, OCN deposition in bone matrix is most likely a hallmark of mature bone with low metabolic activity; and during bone development, OCN production is very low and does not reach maximal levels until late stages of mineralization [41]. So OCN is often used as a late marker for bone formation, being identified as a negative regulator of the process [40]. Maybe an analysis at a longer period after the surgical procedure would have given us some different results such as greater amounts of mature bone with higher OCN expression in all the grafted groups. Or even at a later stage, the results would be the same considering the constant high metabolic


Gelita-Spon
Bio-oss
Dentin
MSC
MSC/Dentin
Control
A
B
CT
EP
Bone
Immature bone
C
D
Bio-oss
E
EP
CT
Bone
F
G
H
EP
CT
Bone
I
Dentin
J
K
EP
CT
Immature bone
Bone
L
M
N
EP
CT
Bone
O
Bone
Dentin
P
Q
EP
CT
Bone
R

◂**Fig. 7** Representative sampling of the right maxilla in each group. A, D, G, J, M, P: H&E staining, 40X. B, C, E, F, H, I, K, L, N, O, Q, R: scanning electron microscopy (SEM) showing structural surface of bone tissue, connective tissue (CT) and epithelium (EP), dentin (in I and O), particles of Bio-Oss (arrows in E and F), cells (arrow heads in C, L, O), root fragment (#), and necrotic bone (*). Square indicates detail shown at higher magnification. Magnification: 80x (B, E, H, K, N), 100x (Q), 500x (C, R, Q), 1000x (L), 1500x (F), 4000x (O), and 5000x (I). MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold

activity of the alveolar bone and the dual behavior of the bone markers.

Although not statistically significant, there was a tendency of higher OPN expression in the Dentin, MSC and Dentin/MSC group and higher VEGF in the MSC group. On the basis of the *P* values found ($P=0.057$ for OPN; $P=0.062$ for VEGF), it is possible that a larger sample size would have shown significance for these results, which in turn, would represent the cellular activity determined by these grafts in the connective tissue, mobilizing osteogenic factors, and demanding angiogenesis. Moreover, the high values of OPN and VEGF in the MSC group is corroborated by the reports on MSC expressing the OPN receptor CD44 and OPN ability to regulate VEGF and angiogenesis [41].

On micro-CT analysis, the Bio-Oss and Dentin/MSC groups showed higher bone density than the Control group. The discrepancy between the results of this analysis compared to H&E could be explained by the fact that in H&E dentin and Bio-Oss can be distinguished from bone (they were classified as amorphous material during the blinded analysis), whereas in micro-CT, some particles of the grafts can be misinterpreted as bone because of having similar density. This is perfectly acceptable since in some samples it was possible to observe dentin and Bio-Oss grafts completely incorporated in the healing bone, without any immune reaction in the interface. That is, the grafts were already making part of the bone structure forming dentin/bone and Bio-Oss/bone complexes.

Our serum analysis showed no significant differences between the groups, reinforcing the idea that the events involving alveolar bone grafting did not have systemic repercussions, at least regarding serum calcium and phosphorus levels. Differently, some systemic immune responses to bone grafts have been reported [42]. SEM analysis in turn corroborated light microscopy results, especially regarding the evidence of Bio-Oss and dentin particles in the respective groups as well as the intense cellularity in the MSC group.

Even though autogenous bone graft is still considered the gold standard for alveolar bone augmentation [32, 43], our results support autogenous nondemineralized dentin as an alternative with promising perspectives, including the possibility of improved performance when combined with MSC. In this work we chose MSC from adipose tissue considering their reported advantages and good results over MSC from other sources. Harvesting adipose-derived MSC requires less-invasive methods and renders higher cell count yield. Moreover, sometimes in clinical routine, it is a waste material such as in buccal fat pad surgical excision and submental liposuction. Cell transplantation/tissue engineering has evolved greatly, but some limitations do exist especially concerning technical challenges and costs related to processing under good manufacturing practice guidelines [17]; specific features of cell preparation need to be clarified and standardized, and risk and cost/benefit aspects need to be considered as well. Eventually, concerning the clinical translation potential of dentin and dentin/MSC grafting, limitations such as the source and amount of available graft material still need some working out, which seems a worthwhile matter for further research.

Conclusion

Autogenous nondemineralized dentin is an alternative for alveolar bone grafting, which can be improved by combination with MSC.

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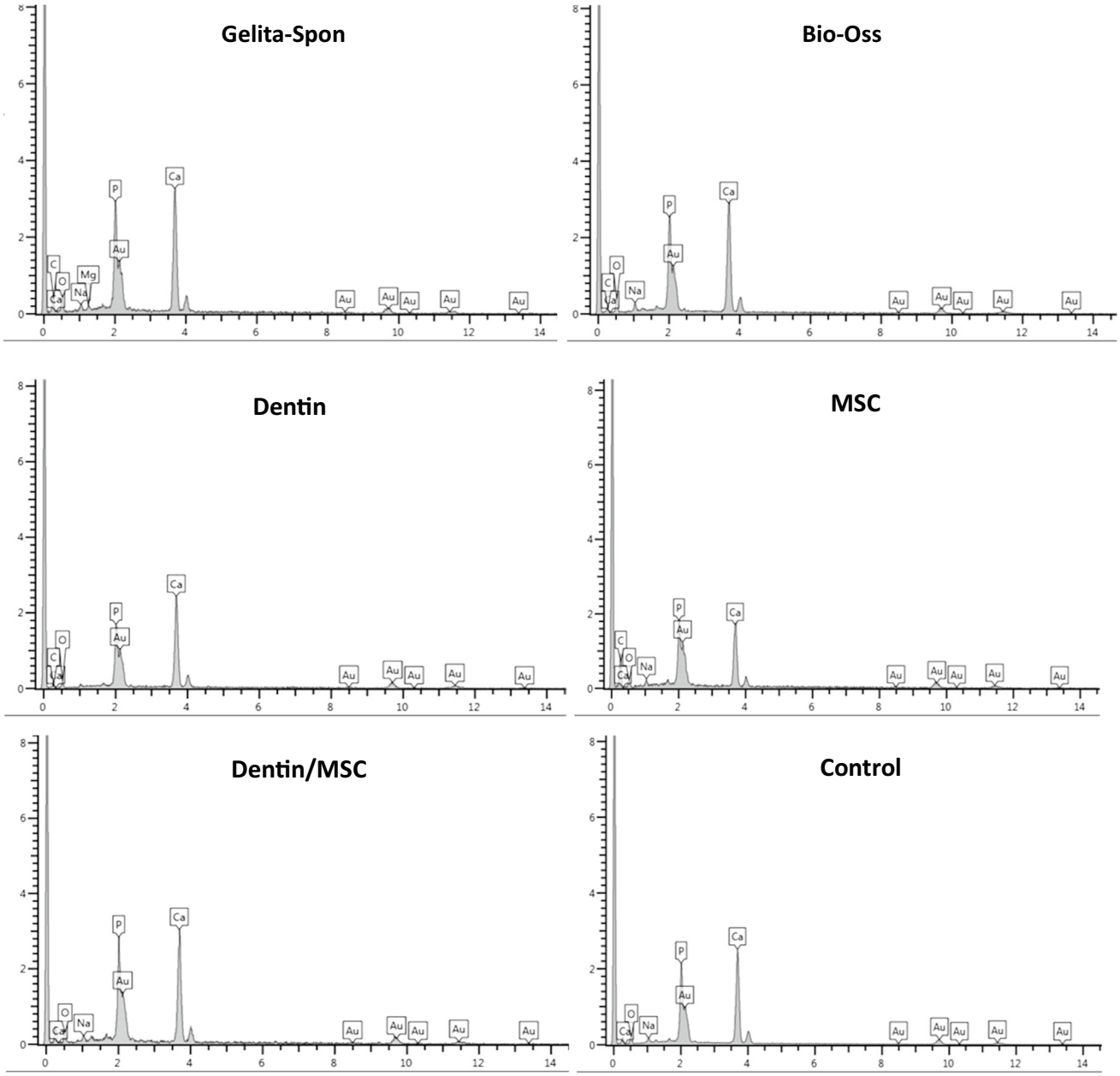
Author contribution Bernardo Ottoni Braga Barreiro is responsible for conceptualization, investigation, methodology, data curation, writing—original draft, review and editing, and project administration. Valesca Sander Koth performed investigation, methodology, data curation, formal analysis, and writing—review and editing. Patrícia Sesterheim performed investigation and contributed to conceptualization, methodology, and writing—review and editing. Fernanda Gonçalves Salum performed investigation and writing—review and editing. Gabriel Rübensam performed investigation and contributed to conceptualization, methodology, and writing—review and editing. Adolpho Herbert Augustin performed investigation and contributed to conceptualization, methodology, and writing—review and editing. Karen Cherubini performed investigation and contributed to conceptualization, formal analysis, writing—original draft, review and editing, project administration, and funding acquisition.



Group	Chemical element (%)					
	C	O	Na	Mg	P	Ca
Gelita-Spon	38.17	29.23	1.67	0.66	11.38	18.89
Bio-Oss	19.59	37.97	3.27	0	14.58	24.60
Dentin	22.44	31.12	0	0	14.68	31.75
MSC	60.18	18.77	1.63	0	7.19	12.23
Dentin/MSC	0	44.80	3.20	0	19.38	32.62
Control	0	40.21	2.95	0	20.99	35.84

Fig. 8 Energy dispersive spectroscopy (EDS). C: carbon; O: oxygen; Na: sodium; Mg: magnesium; P: phosphorus; Ca: calcium; MSC: mesenchymal stromal cell; the MSC group used Gelita-Spon® as a scaffold

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Declarations

Competing interests The authors declare no competing interests.

Ethics approval This study was approved by the Ethics Committee on Animals Use of the Pontifical Catholic University of Rio Grande do Sul (PUCRS, protocol #9108). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Consent to participate For this type of study, informed consent is not required.

Conflict of interest The authors declare no competing interests.

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