

OPTICAL COHERENCE TOMOGRAPHY QUALITATIVE ANALYSIS OF BONE REGENERATION IN RATS WITH TITANIUM REINFORCED HYDROXYAPATITE BIOMATERIAL AND PORCINE BONE-DERIVED GRAFTING MATERIAL

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ABSTRACT

Aim of the study Using efficient addition techniques and biomaterials is essential to ensure the success of implant treatment. The study aimed to evaluate the degree of bone formation of two bone augmentation biomaterials compared to natural healing using optical coherence tomography. **Materials and methods** Three study groups were made of Wistar rats, and cavities with a diameter of 3 mm were made in the parietal bone. The three groups were divided: A, a negative control group; B, a positive control group, in which a commercial porcine grafting material was used; and C, a study group, in which an experimental synthetic grafting material was used. Samples were collected at 8 and 16 weeks. The images obtained from analysing the samples using optical coherence tomography were analysed with Image J software. **Results** The results provided information related to the healing pattern of the created cavities and the degree of their filling with newly formed bone tissue. Statistically significant differences were recorded in bone density at the grafted bone defect level between the two biomaterials, compared to the control group, and between the two evaluation stages, at 8 and 16 weeks. The highest integrated bone density was recorded for the synthetic material based on hydroxyapatite reinforced with titanium particles, followed by the porcine xenograft. **Conclusions** OCT examination is a valuable tool for evaluating the osseointegration of bone grafts since their higher refractive index allows for better identification of bone regeneration patterns.

Key words: OCT, bone grafting, bone regeneration, biomaterials, animal models

INTRODUCTION

Jawbone regeneration in dentistry has become necessary with the evolution of implantology. After tooth loss, an inevitable physiological process occurs that leads to bone

resorption of the alveolar ridge, becoming a limiting factor for the placement of dental implants without prior reconstruction of the region with bone grafts [1,2]. Losses in both height and width of alveolar bone have been

reported during the healing process [3-5]. Consequently, the loss of bone volume may have negative consequences on the support of future implant-prosthetic restorations [6]. The development of techniques for preserving post-extraction alveolar bone size has become a topic of interest in dentistry in recent years. Bone augmentation procedures and biomaterials used to regenerate or maintain post-extraction bone volume for subsequent implant-prosthetic treatment are increasingly being studied by researchers in the field [7-9]. Bone substitutes play a significant role in implantology, supporting healing, reconstruction, and bone size increase [10-12]. Bone grafts should be biocompatible [13], increase bone volume in the augmented area, and be resorbed over time and replaced by native bone [14]. The success of scaffold-based bone regeneration approaches depends mainly on the performance of the biomaterial used [15].

Biomaterials used for bone grafting can be obtained from various sources, including the patient's body (autografts), animals (xenografts), or synthetic compounds called alloplastic. Each alternative has specific characteristics, making them suitable for different clinical situations [9,11,16]. Ideal bone regeneration materials must exhibit at least one of these qualities: they must be osteoconductive (provide support), osteoinductive (contain biological factors that produce cell differentiation), osteogenic (contain cells that carry out bone formation), and promote osseointegration (could join cells) [8]. Even though autografts represent the gold standard for bone regeneration [1,2,15,17,18], bovine, porcine, and equine xenografts are widely used in dentistry as alternatives to autografts [19-23], being easier to obtain, especially in Europe [20]. Synthetic materials have advantages such as the absence of contamination, good bioavailability, ease of

handling, and good patient acceptance [24].

Before being used in humans, bone grafting materials were tested on laboratory animals [25], and the grafting model on calvaria defects created in Wistar rats represented a standard [26,27]. Defects created in the calvaria are rich in osteoblast progenitor cells [28] that could be derived from the periosteum [29], capillaries [30], or dura mater [31] and that contribute to the new bone formation [32]. The size of the bone defect is essential in researching the regenerative capacity of a bone regeneration material. The bone defect must have a size that does not allow spontaneous healing during the experiment, critical defects being considered those in which bone regeneration is not achieved spontaneously, requiring grafting with material that helps the simultaneous stimulation of bone neoformation [33]. Comparative studies of different biomaterials are usually performed, including an empty defect and a clinical standard of bone regeneration, as well as commercial material, as negative and positive controls [34,35].

Optical Coherence Tomography (OCT) is an investigational technique that was initially developed for the field of ophthalmology [36-37] but has become increasingly used in the field of dentistry [38,39]. OCT has been used to study both soft and hard tissues, showing significant contrast and spatial resolution that allows the study of osteointegration over a depth of 200 μm [40-42].

Comparative studies of different biomaterials are very important for clinicians and researchers to make the right decisions for patients with different bone defects and treatment requirements. The idea of this study started from the fact that to obtain an alveolar ridge as suitable as possible for the insertion of a dental implant, at the time of tooth extraction, a decision must be made regarding the immediate insertion of the implant or the

grafting of the alveolus. The possibilities of grafting the post extraction alveolus include several types of bone addition materials, each with its advantages and disadvantages. The most studied biomaterials were xenografts of bovine origin, which have their limitations in terms of socket preservation [3-5]. In contrast, studies using biomaterials such as porcine xenograft and hydroxyapatite reinforced with titanium in socket preservation and especially the comparison between these were not identified in the literature. In addition, to our knowledge, there are few studies in the literature in which OCT has been used to assess bone regeneration [43].

The present study aimed to evaluate the bone formation in rat calvaria defects using a porcine bone graft material and an experimental synthetic graft material, compared to natural bone healing, on OCT images. The null hypothesis is that there are no differences in healing between the control group and the groups in which the two biomaterials were used.

MATERIALS AND METHODS

Study design

The present study analysed bone regeneration using two different bone grafting materials, compared to spontaneous healing after creating a bone defect. The experiment was performed on Wistar laboratory rats, in which bone defects were made in the calvaria,

in parietal bone. The results of the experiment were analysed at 8 weeks and 16 weeks. The study was performed on laboratory animals within the Animal Unit of the University of Medicine and Pharmacy of Craiova, the study protocol being approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova (Approval Number 230/28.11.2022).

Study groups

In this study, the results were analysed in parallel using the same methodology. A commercially available porcine bone graft material (THE Graft™, Purgo, Jungwongu, Korea), an experimental nanocomposite hydroxyapatite-based material reinforced with titanium-based particles material called Bony [44], and spontaneous healing were studied comparatively. Fifteen male Wistar rats were used, which were randomly distributed into three groups (Fig. 1):

(I) Group A - control group - in which the defect was created and spontaneous healing was chosen.

(II) Group B - in which porcine xenograft was used.

(III) Group C - in which synthetic bone graft material was used.

All rats were housed in standard cages, with food and water provided ad libitum. The rats used in the experiment weighed around 300 g (range: 250–350 g) and were selected to be of adult age.

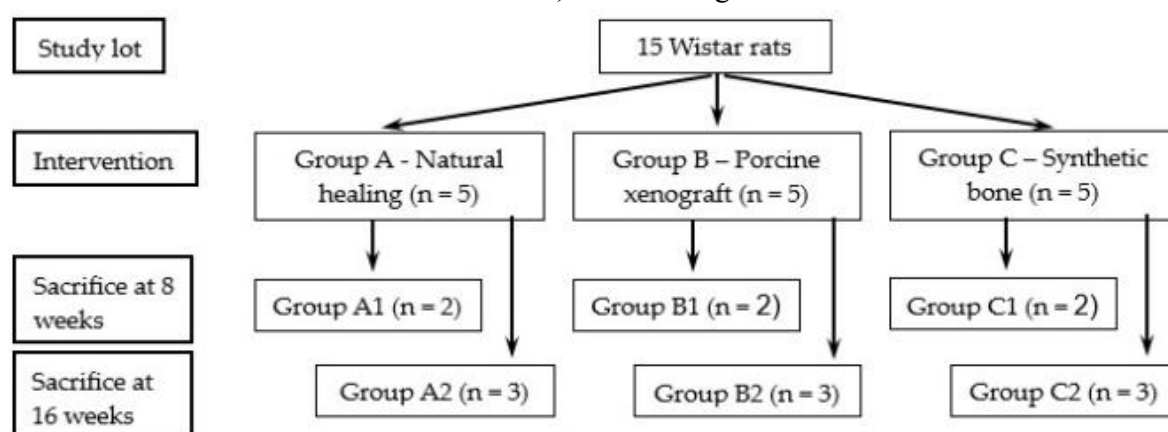


Figure 1. Study Groups Flowchart

Biomaterials

In the present study, a commercial porcine xenograft and an experimental synthetic alloplastic material based on hydroxyapatite reinforced with titanium-based particles were used. The porcine xenograft used (THE Graft™ Granules 0.25-1 mm, Purgo, Jungwongu, Korea) had 0.25g volume and 0.6cc size, suitable for minor bone augmentations and recommended for socket preservation. The material is available in spongy granules packaged in vials, sterilization being performed using gamma irradiation. The experimental material used is called Bony and is a TiO₂-HA nanocomposite, which can be used in tissue engineering due to its specific properties such as homogeneous and densified nanostructure, high stability of the hydroxyapatite (HA) component, good biocompatibility, and superior wear resistance [45]. This material is obtained through a two-stage sintering (TSS) process of a mixture of powders, a hydroxyapatite powder, and a titanium hydride (TiH₂) powder [44].

Surgical procedure

To begin the surgical procedure, laboratory animals were given general anaesthesia with Ketamine 100 mg/ml 20 U.I. (Ketabel, Belapharm GmbH & Co. KG, Vechta, Germany) and Xylazine 2% 0.3 ml (Xylazin Bio, Bioveta, Czech Republic), and Lidocaine 20 mg/ml (Xiline, Zentiva, Prague, Czech Republic) was infiltrated locally. The surgical site, namely the calvaria, was prepared by shaving the scalp and antisepticing with Betadine 100 mg/ml (Egis Pharmaceutical, Hungary). An incision was made in the anteroposterior direction, a flap was raised, and the bone defect was created using spherical burs with a diameter of 3 mm, mounted on the surgical handpiece, under continuous irrigation with sterile saline

solution. To prevent complete spontaneous healing of the bone, a calvaria defect of 3 mm in diameter was created, with great care not to damage the dura mater during the surgical procedure. The midline was followed as a reference point to establish the exact position of the bone defects, with defects created in the parietal bone on either side of the midline.

After the creation of the bone defects, natural healing was chosen for the control group, porcine xenograft was added for study group B, and synthetic bone graft material was added for study group C. After the addition procedure was completed, the periosteum was sutured with 6-0 sutures (Supramid, SMI sutures, Sankt Vith, Belgium), and the scalp was sutured using 3-0 sutures (Supramid, SMI sutures, Sankt Vith, Belgium). The incisions were covered with Betadine to avoid secondary infections (Fig. 2).

After surgery, the animals were returned to their cages, which were well ventilated, with a 12-hour light/dark cycle and at a temperature of $25 \pm 1^\circ\text{C}$. The animals were fed standard food, granulated combined feed, and water ad libitum. All surgeries followed the same protocol. The healing period was uneventful and without complications; no animals died due to the experiment. The animals were divided into groups according to two healing periods (8 weeks and 16 weeks) applied after the surgical procedure, as follows:

- Group A1 – in which natural healing was expected, and sacrifice was performed at 8 weeks.
- Group B1 – in which porcine xenograft was used, and sacrifice was performed at 8 weeks.
- Group C1 – in which synthetic bone grafting material was used and sacrifice was performed at 8 weeks.
- Group A2 – in which natural healing was expected, and sacrifice was performed at 16 weeks.

- Group B2 – in which porcine xenograft was used, and sacrifice was performed at 16 weeks.
- Group C2 – to which experimental synthetic grafting material was added and sacrificed at 16 weeks.

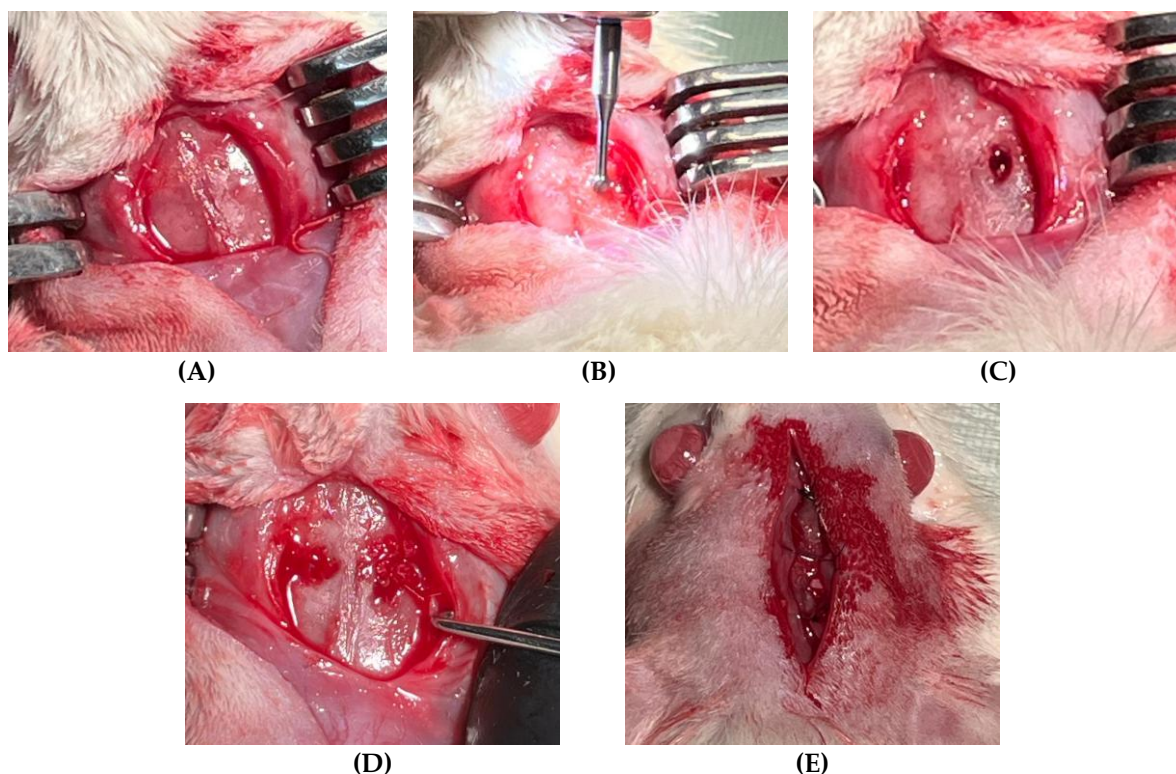


Figure 2. Stages of the surgical intervention. (A) Flap elevation. (B) Creation of the defect. (C) Created defect. (D) Defect filled with bone granules. (E) Suture.

Bone samples preparation

Laboratory animals were euthanized according to current standards by anaesthesia overdose. Bone samples were harvested and prepared to include the defect along with the surrounding tissues. After harvesting, the samples were washed and stored in tubes with 10% formalin (Bio-Optica, Milano). Before the OCT examination, the samples were washed under running water and carefully dried (Fig. 3).

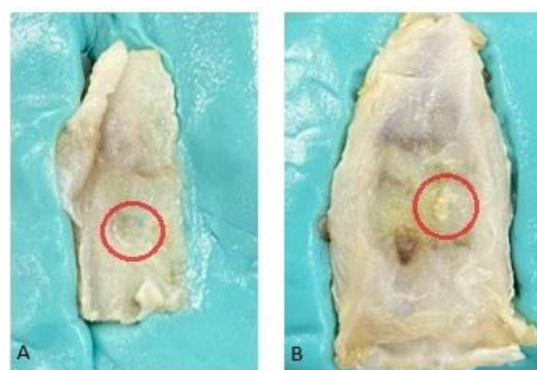


Figure 3. Samples collected. (A) Naturally healed defect. (B) Defect filled with bone graft.

OCT system

We used Optical Coherence Tomography (OCT), an imaging technique characterized by high spatial resolution and non-invasive detection, to evaluate the newly formed bone

tissue. The OCT study was performed at the University of Medicine and Pharmacy of Craiova, using an SSOCT device (THORLABS, OCS1300SS; Munich, Germany). The laser source is a swept laser (55 kHz), operating at a central wavelength of 1325 nm (average power ≈ 12 mW). The system allows 2D and 3D scans. The axial resolution is approximately 12 μm , and the lateral resolution is approximately 15 μm . The optical power of the sample is 5 mW. The image configuration used was X-1024 pixels, width 15 mm; Z- 512 pixels, width 1.92 mm, contrast 1.8 and brightness 54 (Fig. 4).

OCT analysis was performed for each harvested sample, and approximately 600 images were obtained over an interval of 1.6 mm. From the 600 images resulting from the OCT analysis, a set of 10 images was selected for each sample, thus ensuring complete coverage of the entire defect. For the OCT analysis, different signal strengths were considered. Therefore, many photons scattered back from the surface means a weaker signal from the deeper layers.

These can be influenced by the focus position and shading from the upper layers, which can cause a separation of tissue types based on signal intensity. The disadvantage of the method is the curvature of the surface.

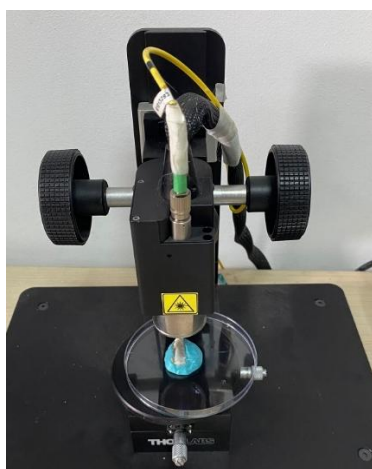


Figure 4. OCT sample analysis.

To minimize errors, the bone samples were positioned so that the surface analysed by OCT was as flat as possible.

ImageJ analysis

After obtaining the images from the OCT analysis, they were analysed using the Image J software (National Institutes of Health (NIH)), which is based on the possibility of counting pixels in previously selected areas of interest. The Image J software quantified the results as a table showing the pixel density in the selected area of interest.

Depending on the use or non-use of an augmentation material, the areas analysed, and the time elapsed since the enlargement, 12 groups of samples resulted for the analysis of the results. Finally, from the 10 OCT images, one image from the initial part of the defect, one image from the centre of the defect, and one image from the final part of the defect were selected.

For each image, three areas of interest were selected and measured: one from the native bone, one from the defect area, and one from the newly formed bone. The areas of interest were selected in this way because the density of a bone sample is influenced by these three types of bone [46] and by the refractive index of the biomaterial particles used for grafting [43]. The integrated density is the sum of the pixel values in the image or region of interest [47]. In addition, skewness and kurtosis were calculated for each region of interest. Skewness represents the third-order moment of the mean, and kurtosis represents the fourth-order moment of the mean. Darker, glossier surfaces tend to have more positive skew values than lighter, matte surfaces. Kurtosis refers to a measure of the noise and resolution of an image. A high value of kurtosis means low resolution noise [48].

Statistical analysis

The obtained data were grouped and analysed using Microsoft Excel 365 (San Francisco, CA, USA) and Statistical Package for Social Sciences (SPSS), version 26 (IBM Corp., Armonk, NY, USA). Continuous variables were reported as minimum and maximum values and mean $\pm$ standard deviation (SD). Statistical tests included Shapiro-Wilk's test for data normality analysis, Levene's test of equality of variances, One-Way ANOVA for group comparisons (followed by Tukey or Games-Howell post hoc analysis) and paired-samples t-test for repeated measurements. For this study, the value $p < 0.05$ was considered statistically

significant.

RESULTS

Initially, the biomaterials were analysed separately. The OCT image of the porcine xenograft showed a weaker light intensity and a more disordered particle distribution, which could be explained by the structure like that of natural bone. The OCT image of the synthetic material showed a stronger light intensity, showing a semi-crystalline, denser, more compact, and more homogeneous structure, which could be explained by the reflectivity of the incorporated titanium particles (Fig. 5).

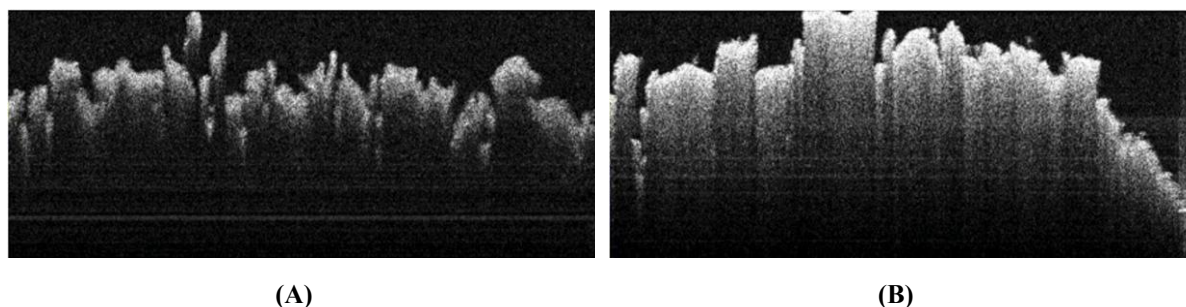


Figure 5. OCT images. (A) Porcine xenograft. (B) Synthetic bone graft.

For all analysed samples, it was considered that all three types of bone were present: native bone, grafted bone, and newly formed bone (Fig. 6). New bone formation was evaluated comparatively between the three groups at 8 and 16 weeks. The OCT images recorded for samples collected from rats in the three groups highlight the differences in density between the samples (Fig. 7). Comparing the three batches, differences in the healing process were highlighted depending on the type of

grafted bone and natural healing (Fig. 8).

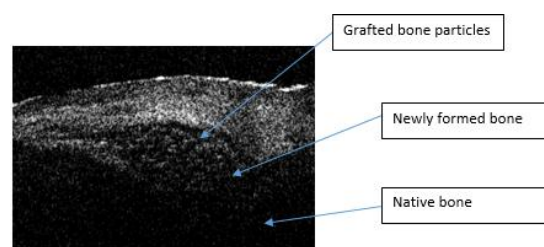


Figure 6. Different bone layers - native bone, grafted bone particles, newly formed bone.

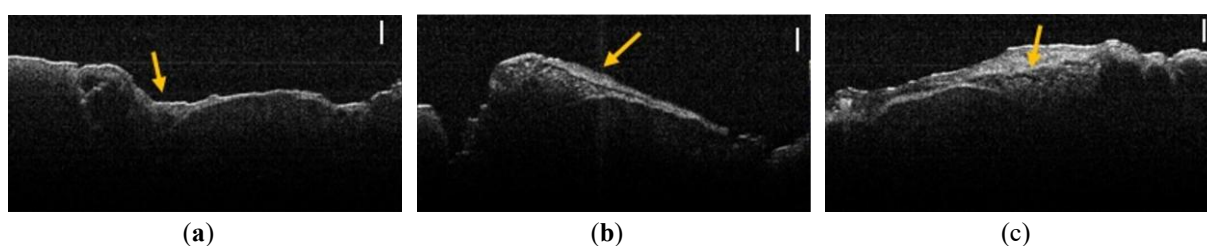




Figure 7. OCT images- yellow arrows mark defect areas (a) Group A1- negative control group- sample collected at 8 weeks. (b) Group A2 - negative control group - sample collected at 16 weeks. (c) Group B1 - positive control group to which the porcine xenograft was added - sample collected at 8 weeks. (d) Group B2 - positive control group to which the porcine xenograft was added - sample collected at 16 weeks. (e) Group C1 - study group to which the experimental synthetic bone was grafted - sample collected at 8 weeks. (f) Group C2 - study group to which the experimental synthetic bone was grafted - sample collected at 16 weeks (scale bar 100 μ m).

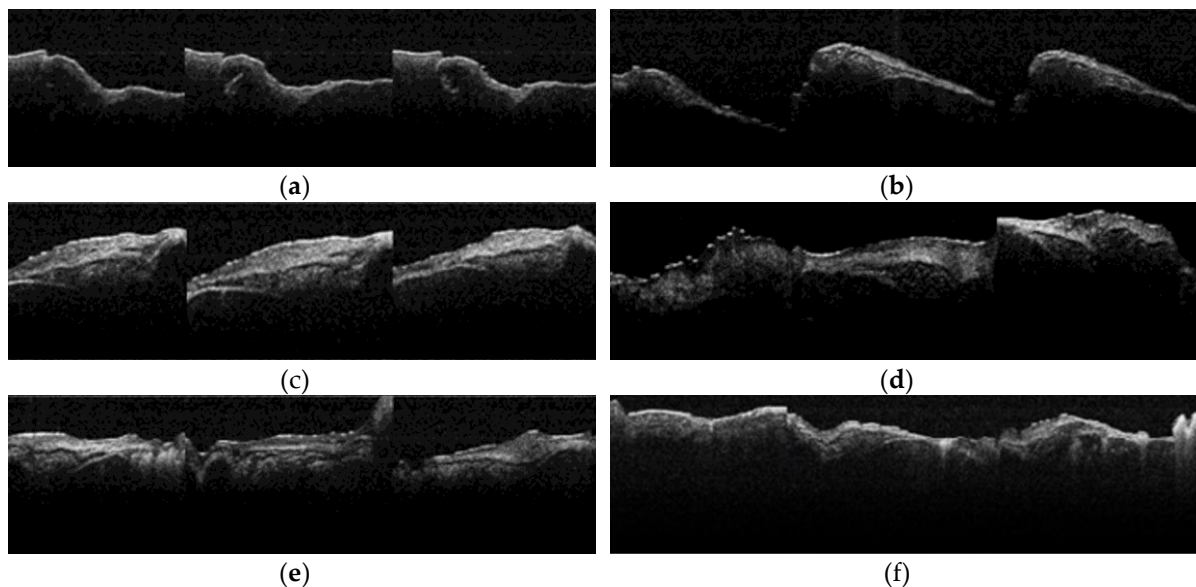


Figure 8. Comparative OCT images at 8 and 16 weeks, with bone defects of the 3 study groups: (a) Group A1 - negative control group - sample collected at 8 weeks; (b) Group A2 - negative control group - sample collected at 16 weeks; (c) Group B1 - positive control group to which the porcine xenograft was added - sample collected at 8 weeks; (d) Group B2 - positive control group to which the porcine xenograft was added - sample collected at 16 weeks; (e) Group C1 - study group to which the experimental synthetic bone was grafted - sample collected at 8 weeks; (f) Group C2 - study group to which the experimental synthetic bone was grafted - sample collected at 16 weeks.

Analysis of the evolution between 8 weeks and 16 weeks, by group and bone type

For each group, a comparison was made between the results obtained after 8 and 16

weeks (Table 1). Thus, for the negative control group, statistically significant differences were observed between the bone density values inside the defect at 8 weeks, compared to those at 16 weeks, the bone at 16 weeks presenting a

lower value. Similar results were also observed in the case of newly formed bone, the bone at 16 weeks having lower integrated density values. Regarding native bone, the results showed no statistically significant differences (Fig. 9).

In the case of the positive control group, in which porcine xenograft was used, statistically significant differences were observed in all three types of bone, between 8 weeks and 16 weeks, in all cases the lower values of

integrated density being present at 16 weeks (Fig. 10).

In the study group, in which the experimental synthetic bone was used, statistically significant differences were observed in the case of the bone inside the defect, as well as in the case of the newly formed bone, the bone at 16 weeks having lower values than that at 8 weeks (Fig. 11). Regarding the native bone, the differences were not significant (Table 1).

Table 1. Comparative analysis of the integrated density values for the three groups at 8 and 16 weeks depending on bone area

Sample		Integrated density							
		8 weeks			16 weeks			Paired differences (16 weeks – 8 weeks)	
		Min	Max	Mean $\pm$ SD	Min	Max	Mean $\pm$ SD	Mean $\pm$ SD	p *
Negative control group	Native bone	5.074	6.041	5.351 $\pm$ 0.324	4.198	5.844	4.943 $\pm$ 0.618	-0.248 $\pm$ 0.761	0.073
	Defect area	27.386	31.519	28.488 $\pm$ 1.532	17.008	21.914	19.029 $\pm$ 1.627	-8.878 $\pm$ 2.306	< 0.0005
	Newly formed bone	14.033	17.902	15.61 $\pm$ 1.342	9.262	14.502	11.467 $\pm$ 1.444	-3.324 $\pm$ 1.337	0.0001
Positive control group	Native bone	5.283	6.644	5.944 $\pm$ 0.559	5.101	5.787	5.302 $\pm$ 0.236	-0.653 $\pm$ 0.812	0.025
	Defect area	50.159	68.452	58.979 $\pm$ 6.327	33.069	40.957	37.533 $\pm$ 2.398	-20.906 $\pm$ 8.937	0.0005
	Newly formed bone	20.123	21.697	20.854 $\pm$ 0.601	13.022	15.019	14.073 $\pm$ 0.594	-6.723 $\pm$ 1.242	< 0.0001
Study group	Native bone	5.321	6.091	5.673 $\pm$ 0.298	5.158	6.167	5.824 $\pm$ 0.356	0.173 $\pm$ 0.505	0.212
	Defect area	76.625	95.821	83.48 $\pm$ 6.01	53.496	70.818	62.309 $\pm$ 4.733	-22.352 $\pm$ 8.410	< 0.0005
	Newly formed bone	16.441	18.975	17.793 $\pm$ 0.905	15.004	17.824	16.24 $\pm$ 0.804	-1.460 $\pm$ 1.608	0.005

* Paired-samples t-test, comparative analysis within the groups at 8 and 16 weeks depending on the bone area.

Study group analysis

A one-way ANOVA was conducted to determine if the integrated density differed for the three bone types within each study group.

For all three groups, the analysis of the values measured after 8 weeks revealed that there were no outliers, as assessed by a boxplot; data was normally distributed for each bone type, as assessed by Shapiro-Wilk test ($p > 0.05$); there was no homogeneity of variances, as assessed by Levene's test of

homogeneity of variances ($p = 0.002$ for the positive control group, $p = 0.045$ for the study group, and $p = 0.018$ for the negative control group), thus, the results are expressed based on the Welch ANOVA. The integrated density was statistically significantly different for the three types of bone for all three groups, Welch's $F(2, 7.303) = 603.381$, $p < 0.0005$ for the negative control group, Welch's $F(2, 8.898) = 889.115$, $p < 0.0005$ for the positive control group, and Welch's $F(2, 7.326) =$

741.771, $p < 0.0005$ for the study group. Games–Howell post hoc analysis revealed that the multiple group comparisons were statistically significant (Table 2).

For the values measured after 16 weeks, for the negative control group, the analysis revealed that there were no outliers, as assessed by a boxplot; data was normally distributed for each bone type, as assessed by Shapiro-Wilk test ($p > 0.05$); there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances ($p = 0.140$). The integrated density was statistically significantly different for the three types of bone, $F(2, 24) = 232.942$, $p < 0.0005$. Tukey's post hoc analysis revealed that the multiple

group comparisons were also statically significant (Table 2). For the positive control group and the study group, the analysis revealed that there were no outliers, as assessed by a boxplot; data was normally distributed for each bone type, as assessed by Shapiro-Wilk test ($p > 0.05$); there was no homogeneity of variances, as assessed by Levene's test of homogeneity of variances ($p < 0.0005$ for the positive control group, and $p = 0.003$ for the study group). The integrated density was statistically significantly different for the three types of bone for both groups, and Games–Howell post hoc analysis revealed that the multiple group comparisons were also statically significant (Table 2).

Table 2. Integrated density variation between different types of bone for each study group — overall result and multiple group comparisons.

Group	Bone type	8 weeks		16 weeks	
Negative control group	ANOVA / p	(F(2,7.303)) 603.381 / < 0.0005 ##		(F(2,24)) 232.942 / < 0.0005 #	
	Multiple group comparisons	Variation	p **	Variation	p *
	Native bone — Defect area	23.137	< 0.0005	14.082	< 0.0005
	Defect area — Newly formed bone	12.877	< 0.0005	7.561	< 0.0005
	Native bone — Newly formed bone	10.259	< 0.0005	6.520	< 0.0005
Positive control group	ANOVA / p	(F(2,8.898)) 889.115 / < 0.0005 ##		(F(2,12.180)) 1343.177 / < 0.0005 ##	
	Multiple group comparisons	Variation	p **	Variation	p **
	Native bone — Defect area	53.034	< 0.0005	32.231	< 0.0005
	Defect area — Newly formed bone	38.125	< 0.0005	23.460	< 0.0005
	Native bone — Newly formed bone	14.909	< 0.0005	8.770	< 0.0005
Study group	ANOVA / p	(F(2,7.326)) 741.771 / < 0.0005 ##		(F(2,12.410)) 1037.482 / < 0.0005 ##	
	Multiple group comparisons	Variation	p **	Variation	p **
	Native bone — Defect area	77.807	< 0.0005	56.484	< 0.0005
	Defect area — Newly formed bone	65.686	< 0.0005	46.068	< 0.0005
	Native bone — Newly formed bone	12.120	< 0.0005	10.416	< 0.0005

One-way ANOVA test. ## Welch's ANOVA test. * Tukey post hoc analysis. ** Games–Howell post hoc analysis.

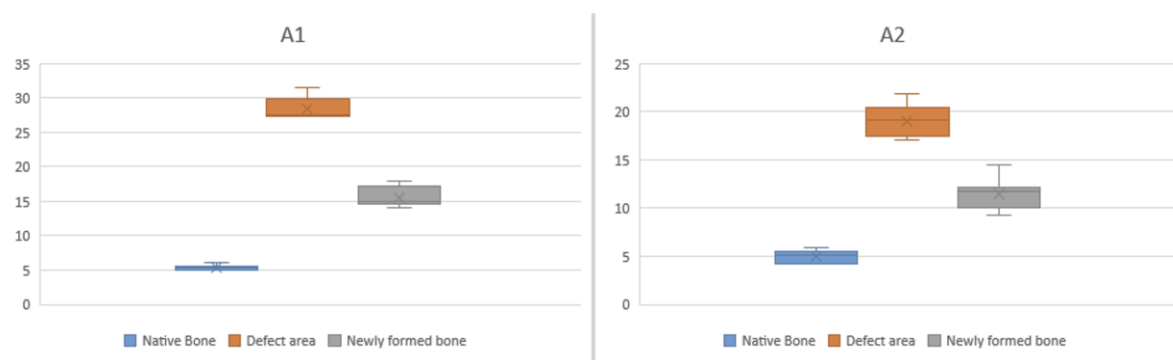


Figure 9. The ratio of integrated density values (the sum of the pixel values in the region of interest) resulting from OCT analysis of groups A1 and A2 - comparative analysis at 8 and 16 weeks of negative control groups.

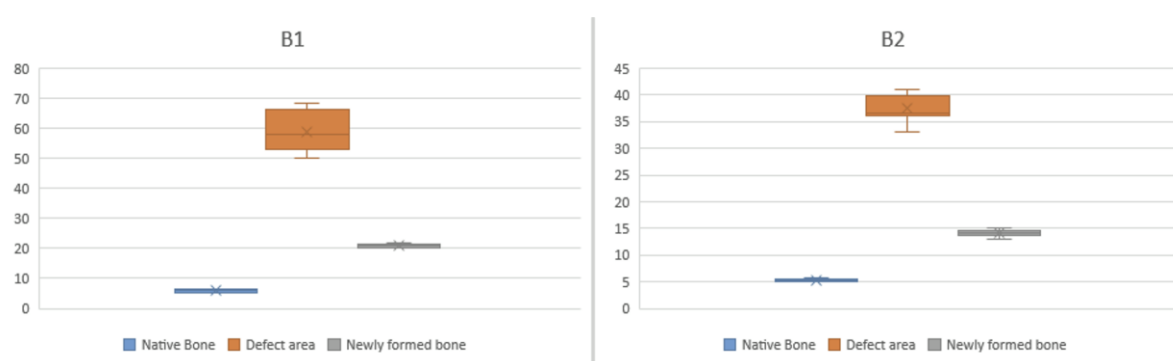


Figure 10. The ratio of integrated density values (the sum of the pixel values in the region of interest) resulting from OCT analysis of groups B1 and B2 - comparative analysis at 8 and 16 weeks of positive control groups.

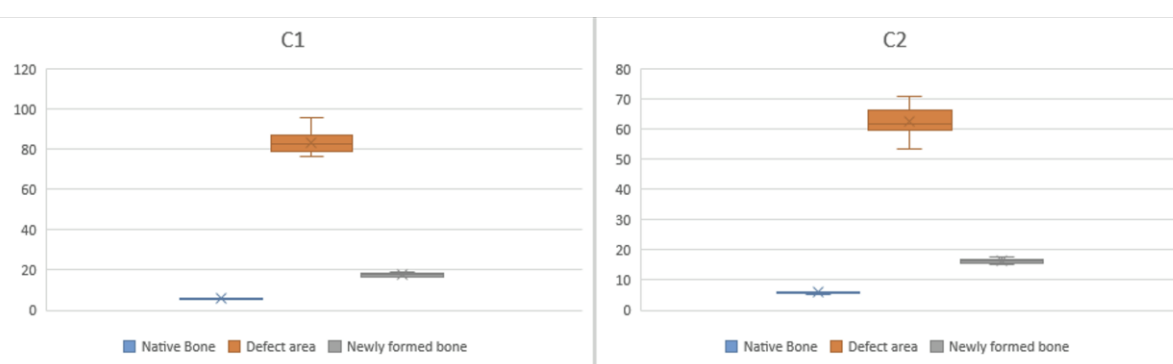


Figure 11. The ratio of integrated density values (the sum of the pixel values in the region of interest) resulting from OCT analysis of groups C1 and C2 - comparative analysis at 8 and 16 weeks of the study groups.

Bone type analysis

A one-way ANOVA was conducted to determine if the integrated density differed for the three study groups for each type of bone. The 8-week and 16-week analyses revealed

that there were no outliers for all three types of bone, as assessed by a boxplot; data was normally distributed for each bone type, as assessed by the Shapiro-Wilk test ($p > 0.05$).

For the native bone, there was no

homogeneity of variances at 8 weeks ($p = 0.014$), nor 16 weeks ($p < 0.0005$). The integrated density was not statistically significantly different between the study groups for the native bone at 8 weeks, Welch's $F(2,9.551) = 2.380$, $p = 0.145$, so no multiple group comparisons were performed. However, for the values measured at 16 weeks, the integrated density was statistically significantly different between different study groups, Welch's $F(2,14.385) = 8.110$, $p = 0.004$. The integrated density increased from the negative control group (4.943 ± 0.618) to the positive control group (5.302 ± 0.236) to the study group (5.824 ± 0.356), in that order (Table 3, Fig. 12). Subsequent post-hoc analysis for the native bone at 16 weeks revealed that the multiple group comparisons were also statically significant (Table 3).

The defect area's variances were

homogeneous at 8 weeks ($p = 0.146$). The integrated density was statistically significantly different between the study groups $F(2,15) = 145.028$, $p < 0.0005$, and the post-hoc analysis revealed that the multiple group comparisons were also statically significant (Table 3). Lower integrated density values were observed in the negative control group, followed by the positive control group and, finally, the study group (Table 3, Fig. 13). Similar results were obtained following the analysis of values after 16 weeks. Variances were homogeneous ($p = 0.101$). The integrated density was statistically significantly different between the study groups $F(2,24) = 367.407$, $p < 0.0005$. The order of means remained the same between the study groups. The post-hoc analysis revealed that the multiple group comparisons were statistically significant (Table 3).

Table 3. For each type of bone — overall result and multiple group comparisons- integrated density variation between different study groups.

Bone type	Group	8 weeks		16 weeks	
Native bone	ANOVA / p	(F(2,9.551)) 2.380 / 0.145 ^{##}		(F(2,14.385)) 8.110 / 0.004 ^{##}	
	Multiple group comparisons	Variation	p ^{**}	Variation	p ^{**}
	Negative control — Positive control	0.593	-	0.356	0.248
	Positive control — Study	0.271	-	0.522	0.061
	Negative control — Study	0.322	-	0.878	0.001
Defect area	ANOVA / p	(F(2,15)) 145.028 / < 0.0005 [#]		(F(2,24)) 367.407 / < 0.0005 [#]	
	Multiple group comparisons	Variation	p ^{***}	Variation	p ^{***}
	Negative control — Positive control	30.490	< 0.0005	18.505	< 0.0005
	Positive control — Study	24.501	< 0.0005	24.775	< 0.0005
	Negative control — Study	54.991	< 0.0005	43.281	< 0.0005
Newly formed bone	ANOVA / p	(F(2,15)) 34.870 / < 0.0005 [#]		(F(2,24)) 44.395 / < 0.0005 [#]	
	Multiple group comparisons	Variation	p ^{***}	Variation	p ^{***}
	Negative control — Positive control	5.243	< 0.0005	2.605	< 0.0005
	Positive control — Study	3.060	0.001	2.167	0.001
	Negative control — Study	2.182	0.009	4.773	< 0.0005

[#] One-way ANOVA test. ^{##} Welch's ANOVA test. ^{*} Tukey post hoc analysis. ^{**} Games-Howell post hoc analysis.

For the newly formed bone, at 8 weeks, variances were homogeneous ($p = 0.116$). The integrated density was statistically significantly different between the study groups $F(2,15) = 34.870$, $p < 0.0005$. At 8 weeks, the comparison of the mean values

between the negative control group and the positive control group, as well as between the negative control group and the study group, reflected that lower values of the integrated density were observed in the negative control group; in the case of the comparison between

the positive control group and the study group, the bone with lower values of integrated density was observed in the study group, thus the order of values was different compared to the other types of bone. The post-hoc analysis revealed that the multiple group comparisons were statistically significant (Table 3). At 16 weeks, in the case of the comparison between the negative control group and the positive control group and between the negative control group and the study group, it was observed that the bone that presented lower values of the

integrated density was present in both cases in the negative control group. In the case of the comparison between the positive control group and the study group, the bone with lower integrated density values was observed in the positive control group (Table 3, Fig. 14). The integrated density was statistically significantly different between the study groups $F(2,24) = 44.395$, $p < 0.0005$. The post-hoc analysis revealed that the multiple group comparisons were statistically significant (Table 3).

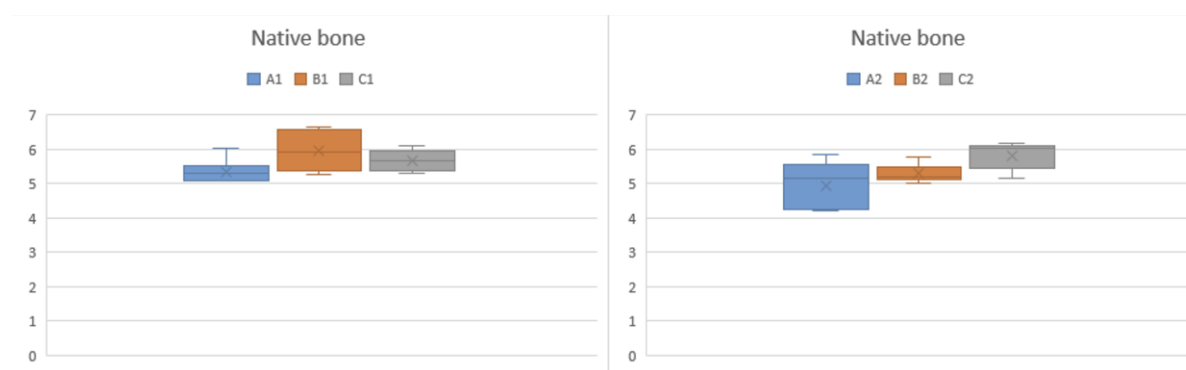


Figure 12. The ratio of values of the integrated density (the sum of the pixel values in the region of interest) resulting from OCT analysis of native bone, compared between the three groups at 8 and 16 weeks

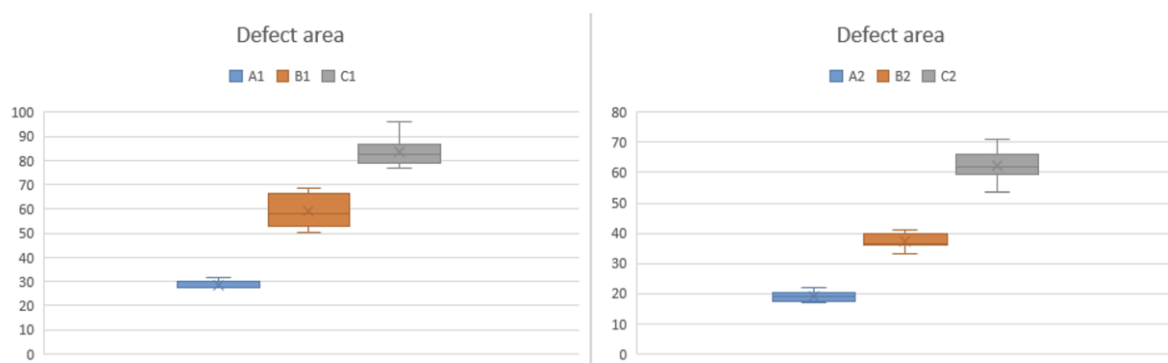


Figure 13. The ratio of integrated density values (the sum of the pixel values in the region of interest) resulting from OCT analysis of defect areas, compared between the three groups at 8 and 16 weeks.



Figure 14. The ratio of integrated density values (the sum of the pixel values in the region of interest) resulting from OCT analysis of newly formed bone, compared between the three groups at 8 and 16 weeks.

DISCUSSIONS

The present study was conducted on the calvaria of adult male rats, with the aim of comparing the osteointegration of two biomaterials, a porcine xenograft and a synthetic nanocomposite based on hydroxyapatite reinforced with titanium particles. The study also had a temporal component, with evaluations carried out at 8 weeks and 16 weeks, in order to observe the changes produced over time in the area of osteointegration. From the point of view of the optical qualities highlighted by OCT analysis, the newly formed bone in the case of using the two biomaterials, presented a higher density than that of the newly formed bone in the case of the control group, this difference being statistically significant, both at 8 weeks and at 16 weeks. It should be mentioned, however, that at 16 weeks, the optical properties of the samples were much closer to those of the control group in the case of newly formed bone for both types of biomaterials. In the case of defect areas, the integrated bone density was significantly higher in the groups using the two biomaterials at 8 and 16 weeks. Although the density improved at 16 weeks compared to 8 weeks, it remained lower in the control group, being almost 2 times lower than in the positive control group (porcine xenograft) and 3 times lower than in the study group (titanium-

reinforced synthetic hydroxyapatite).

Currently, in regenerative dentistry, there is a wide range of biomaterials used for grafting bone defects, which can be divided into two broad categories, namely, natural materials and synthetic materials [49]. Bone grafts should be able to provide support for the formation of new bone of sufficient quality and quantity to support future implants [50-53]. Xenografts and alloplastic materials are the biomaterials widely used in Europe for jaw bone regeneration [54]. In order to be used in humans, new biomaterials must be tested in animals, and the rat calvaria is widely accepted for experimental studies of the osteogenic, osteoconductive, and osteoinductive properties of different bone graft materials [25,34,55]. Xenografts are produced by removing organic materials by deproteinizing bones of animal origin. Their main advantage is represented by their porous structure, like human cancellous bone, presenting the property of osteoconduction, slow absorption helping to maintain space. The disadvantage is given by the weaker bone formation capacity than autologous or allogeneic bones [56]. The porcine xenograft used in this study is a porous bone mineral matrix, which is produced by removing all organic components from porcine bone and which, due to its natural structure, compares the physical and chemical

aspects of the mineralized matrix of human bone [56]. When used for grafting bone defects, it is gradually reabsorbed and is replaced by natural bone during the healing process [57]. Over time, numerous in vivo and in vitro studies have been performed that have demonstrated the effectiveness of this biomaterial [56-59]. No statistically significant differences between porcine and bovine xenografts have been reported in the literature [60]. In contrast, a study by Fernandez et al. evaluated the use of a porcine bone graft compared to two bovine bone grafts in rat calvaria bone defects. Their analysis showed that the porcine bone graft performed better than the two bovine grafts [61]. Synthetic hydroxyapatite is a relatively chemically stable substance that dissolves and remains in the area of the bone defect for a long time [62].

The differences observed between the two groups in which grafting materials were used, in terms of integrated density, can be explained by the mechanisms underlying the processes of osteointegration and osteogenesis. OCT provides us with data related to the presence of mineralized bone particles within the defects and in the newly formed bone area. Thus, it is observed that the porcine xenograft has a structure closer to native bone than the material based on hydroxyapatite reinforced with titanium particles. Comparing the evolution of the defect grafted with porcine bone with that of the defect grafted with the experimental synthetic biomaterial, it was observed that the alloplastic material is maintained at the defect level for a longer time, which is reflected by higher values of the integrated density. This shows that the experimental material has a more scaffolding role and can be used more in augmentations than in socket preservation. It maintains itself longer as a resistance structure at the defect level. At the same time, the porcine bone, probably due to its structure, is more like that

of native bone and is taken up more quickly by osteoclast cells and then reused in the creation of new bone, thus being more suitable for the graft function in socket preservation. On the other hand, the resorption of biomaterials and the local inflammatory response can influence tissue integration and bone remodelling, thus contributing to variations in the observed integrated density. The results of the study showed that the porcine xenograft required a shorter integration period compared to the experimental synthetic material due to its organic composition and porous structure, which favoured osteoconduction and bone remodelling. The synthetic material required a longer time for integration due to the slower resorption rate. These findings are consistent with other studies in the literature, which suggest that synthetic biomaterials require a longer osteointegration period compared to animal xenografts but may provide long-term benefits in terms of the stability and strength of the regenerated tissue [63,64].

Optical Coherence Tomography (OCT) was used as an evaluation method. Unlike other traditional techniques such as microcomputed tomography (μ CT) or histomorphometry, OCT has the advantage of being a non-invasive and easy-to-use (no sample preparation is required, so no destructive process is involved), radiation-free, and non-ionizing three-dimensional imaging technique that produces two-dimensional cross-sectional images of two-dimensional tissue samples [65,66]. This method uses low-power IR laser radiation [43,65,67], the limitations of the technique being related to the analysis of samples at greater penetration depths, which is currently not possible, the poor contrast of mineralized tissues, difficulties in volumetric analysis of bone regeneration, where micro-CT analysis is superior, and the impossibility of identifying cellular details, which can be achieved by

histomorphometry [65,67,68]. However, with the help of OCT, an analysis of bone formation can be performed on defects created in the calvaria of rats [46]. Hard tissues can be studied using the low-coherence interferometry signal to assess mineral changes [69] in humans and animals [70,71].

The level of osseointegration was evaluated with the help of Image J software, which allowed the possibility of measuring the number of pixels in the areas of interest. In the case of samples harvested at 8 weeks, the created defects were still clinically detectable, with OCT examination showing the existence of cavities inside the newly formed bone. In the case of samples harvested at 16 weeks, clinical examination revealed with difficulty the location of the created defects, both materials facilitating the filling of the created cavities with newly formed bone tissue. The refractive index of the commercial biomaterial being higher than the index of the adjacent natural bone structures may allow some observations, following the analysis with optical coherence tomography [72].

In 2023, Khijmatgar et al. [65] published an article in which they used OCT to assess bone mineral content in laboratory rats, as well as the depth of light penetration into the bone. The study's results showed that measurements made using OCT demonstrated significant differences in the depth of light penetration into mineralized bone compared to demineralized bone. Demineralized bone showed significantly greater light penetration depths than bone with normal mineralization. The greater penetration was due to the increased porosity, allowing light to penetrate the sample deeper. The lower the porosity and the denser the bone, the lower the light penetration depth. The authors observed a 64% difference between the light penetration values of demineralized bone compared to bone with normal mineralization. The working principle

of OCT is explained by polarized light scattering. In bone, as in teeth, mineralized structures composed of hydroxyapatite nanocrystals provide properties such as anisotropy, birefringence, and depolarization to the hard bone tissue. These properties of bone underlie the action of OCT in detecting minor variations in bone mineral content. According to Khijmatgar et al., the OCT technique can be a reliable method for differentiating bone density between bone samples as a function of light penetration depth. Since several studies have observed differences in bone mineralization between sexes, with females presenting less mineralized bone than males [65], all rats used in this study were male.

In our study, the positive control group, where porcine xenograft was used as grafting material, tended to transform into native bone more quickly than the titanium-reinforced hydroxyapatite-based synthetic bone used as grafting material in the study group. The values of the measurements taken at 16 weeks of the porcine xenograft transformed into native bone were like the values of the newly formed bone of the negative control group, in which spontaneous healing was achieved at 8 weeks. Thus, the negative control group recorded a delay in the healing process of 2 months. The values measured for the synthetic biomaterial showed a delay in bone formation of more than 2 months. Therefore, the speed of new bone formation in this case was much slower. The synthetic biomaterial maintains its scaffold function for a more extended period. This property can be used when it is necessary to create a rigid wall that maintains its shape for a more extended period (e.g., sinus lifting procedure). It is not recommended to use this material to maintain the size of the post-extraction edentulous ridge, where scaffold materials should have a transformation rate as close as possible to that of natural bone [73].

Healing of the calvaria bone defect in one month was described by Develioglu et al. in 2009. In the control group, a dense collagen tissue was observed, while in the study group, the xenograft particles were surrounded by fibrous tissue [74]. In addition, the results of their study showed new bone formation at the edge of the defect and no bone formation in the central area. After 4 weeks, the xenograft particles were not resorbed but only encapsulated by fibrous tissue [74]. In the study conducted by Draghici et al., a common pattern of evolution of graft biomaterials was found, with differences in the formation speed of new bone tissue [70]. In the study by Obadan et al., OCT evaluation allowed for surface and subsurface evaluation of the areas of interest. OCT images of the colla-gen-based material revealed the degree of defect filling by the lack of refraction of the graft material, having a homogeneous appearance with some gaps in the samples collected at two months. In the case of the synthetic hydroxyapatite-based biomaterial, OCT images revealed a higher refractive index of the graft material compared to native bone, resulting from the persistence of residual particles in the defect [75].

In the in vitro study by Genina et al., the transmission and reflection spectra of human and porcine cranial bone were measured using OCT analysis [76]. The refractive index of cranial bone depends on the stage of mineralization, being between 1.555 and 1.564 [77]. The tissue components presented the following refractive indices: apatite above 1.623, hydrated type 1 collagen 1.43, and lipids approximately 1.45 [78-80]. Water has a refractive index of 1.33 [76]. The refractive index of rat calvaria is between 1.4 and 1.6, varying depending on its composition and structure (degree of mineralization and collagen content) [43]. The OCT technique, based on the refractive index of biological

tissues, provides valuable results in understanding biological systems at the structural and cellular level [81]. Although the refractive index of natural bone is quite variable, the refractive index of synthetic material is usually higher and can be an advantage in evaluating the presence of material particles in a bone cavity [43].

The porcine xenograft used in this study had granules 0.25-1 mm, suitable for minor bone augmentations and recommended for socket preservation. The synthetic material had irregular granulation, with larger dimensions than the porcine xenograft. The size of the bone graft granules is essential in bone healing [82]. It has a significant effect on bone formation and mineralization, as well as on the bone density in the defect area [72]. According to Leiblein et al., bone granules with sizes between 0.5 and 1.0 mm were more useful for complete repair than bone granules that presented larger sizes [83].

The first limitation of the study was that the bone samples evaluated came from different rats. Therefore, some variation, given the specificity of the healing process of each animal, is inevitable. Considering several rats in each group and averaging the results was the method used to reduce errors produced by individual variations. Another limitation is related to the potential for reduced penetration into bone, poor contrast for mineralized tissue, and difficulties in analysing bone regeneration with OCT. Another limitation of the study is related to the extrapolation of the results from rats to humans.

CONCLUSIONS

The study results showed, in general, a good integration of the materials used, and the porcine xenograft provided a more reproducible healing pattern than the synthetic bone graft. The comparative study of the two types of bone grafting materials compared to

natural healing revealed significant differences in the process of osteointegration and osteogenesis. The porcine xenograft, having a structure closer to native bone, integrated more quickly due to its porosity and organic composition. In contrast, the experimental synthetic material, characterized by slower resorption, offered prolonged structural stability at the defect level, suggesting an optimal use in bone augmentation. The choice of the ideal grafting material must be adapted according to the

clinical objective pursued: rapid integration and efficient remodelling or long-term stability. That's why a porcine xenograft could be more suitable for socket preservation and the synthetic biomaterial based on hydroxyapatite reinforced with titanium particles for sinus lifting procedures. Therefore, more experimental studies are needed to evaluate the clinical efficacy of bone substitutes and support the choice of ideal grafting material.

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