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Histomorphological study of the bone regeneration capacity of platelet-rich plasma, bone marrow and tricalcium phosphate Experimental study on pigs

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Abstract

Introduction: Bone defects are rather common after oral surgery and may prove difficult to repair. Objective: We provide a histomorphological analysis of the bone regenerative capacity of platelet-rich plasma at different concentrations and the extraction of platelet-rich bone marrow, compared with &bgr;-tricalcium phosphate. Methodology: We performed an experimental study on 8 pigs, in which we performed trepanations of the mandible in order to place the materials to be studied. Using an electron microscope, we observed the samples obtained and took a series of photographs in order to analyze the samples through a gray-scale histogram system.
Results: Ossification phenomena were present in 96% of the charged defects, regardless of the material used to fill it. Platelet-rich plasma (PRP) and the bone marrow (M) showed an equivalent degree of osteogenesis, 12.3 and 13.4 respectively, which is greater in than the control group. The platelet-poor plasma (PPP) shows a capacity similar to the control groups (C), with an average count of 14.03 and 14.12 respectively. Tricalcium phosphate (TP) was shown to be effective as an ossification inducer, 3.03 times stronger than the control group. Conclusions: Ossification occurs in most of the charged defects. PRP and M had the greatest osteogenic capacity but PPP was no more effective than the control.

Key words: *Bone regeneration, tricalcium phosphate, platelet-rich plasma, histomorphological study.*

Introduction

In dentistry, loss of bone tissue is a complication that results after many surgical procedures (extraction, surgical prosthesis, treatment of osteolytic lesions, treatment of tumors, etc.), which causes extensive bone cavity lesions (1). These cavities cause complications such as the occurrence of pathological fractures, osteoporosis, reactivation of infections and difficulty in prosthesis restoration, among other processes. In order to avoid these complications, the placement of bone grafts is considered an option (2). The use of an allograft may not be enough to fill cavities that are very large. Moreover, the use of the allograft has been virtually abandoned due to the high rate of hypersensitivity reactions that it may cause (2). These facts have contributed to the reason why "regeneration" and bone induction techniques have been used for years, using inorganic materials that are similar to the bone matrix, especially hydroxyapatite, and in recent years, β-tricalcium phosphate, among other biomaterials (3-5). This is done in an effort to minimize the problems of edentulism, which involves a decrease of the support, thereby making rehabilitation through removable dental prosthetics either difficult or even impossible.

For example, if we choose dental extraction, we know that it produces a restorative response characterized by bone reabsorption of the alveolar surface and a deposit of new tissue in the empty alveolus. The extent of the progressive bone reabsorption depends on the interaction of several factors: anatomical, sexual, biological, mechanical and surgical, among others. There are numerous papers in the literature that evaluate the capacity for regeneration of the bone itself, either physiologically or using biomaterials, but the results are contradictory and difficult to quantify (6-8), which prompts us to conduct further research on the subject.

Given these premises, our objective is to evaluate the degree of bone regeneration that can be achieved in the mandibles of pigs, using different concentrations of platelet-rich plasma and the extraction of platelet-rich bone marrow, compared with β-tricalcium phosphate and a "non-treated" control group.

Material and Methods

- Experimentation animals

The study was carried out on 10 hemimandibles, belonging to eight male hybrid pigs aged 3 months old. Their weight is 22.5 +/- 2.5 kg. The animals are subjected to an experimental surgery. After the surgery, the animals remain in the animal facility at the Bellvitge Health Sciences Campus for two months, where the corresponding inspections and post-operative care take place. The maintenance and care of the laboratory animals is carried out following the general guidelines of the National Research Council. Guide for the Care and Use of Laboratory Animals, 1995, cited by Bayne (9), which in Spain is detailed in the Spanish Official Gazette no.252 (Royal Decree 1201/2005 of October 10th, Spanish Official Gazette no.252 of October 21, 2005). After two months, the animals are slaughtered in order to obtain the samples to be evaluated. The issues concerning the care and maintenance of the animals are specified in the report submitted and accepted by the AEEC (Animal Experimentation Ethics Committee) of the University of Barcelona, approved on June 16, 2005.

- Surgical Procedure

The surgical procedures are performed over the course of three days of surgery and the operating samples for study are obtained after the animals are slaughtered (on three different days), which occurs two months (+/- one week) after the procedure.

Under general anesthesia and a technique for operating on the external mandible, (10) ten fenestrations, each 8mm deep, are made using a scalpel with a 3.8 mm diameter (SDHQ4 ® cutter of the brand Biomedica Trinon S.L., Trinon Titanium GmbH, Karlsruhe, Germany, certificate registration No. 0032.01.01/0). A hemimandible is randomly chosen in six of the pigs, and in the last two pigs, two hemimandibles are used in order to broaden the sample. In order to achieve equivalent trepanations, a template is used as a surgical splint, made with Boswort Trim ® (Temporary Resin Acrylic, Chesapeake Beach, USA).

Once the various trepanations are made, they are then filled with the study materials, except two, which are not filled and are considered as the control trepanations. The operator knows the material that is used in each bone defect, but does not have access to the documentation in which the details are recorded: 1) Control group (C), no material. 2) Platelet-Poor Plasma (PPP), with a concentration of <500,000 platelets/ml. 3) Platelet-Rich Plasma (PRP), with a concentration of >500,000 platelets/ml. 4) Bone marrow (M), from the animal itself. 5) β-tricalcium phosphate (TP), fine powder of the brand Cerasorb® (Curasan AG, Lindigstraße4, 63801 Kleinostheim, Germany). All of the defects (except the control groups) are completely filled with the study material, but in the case of the tricalcium phosphate, 0.3 grams of material is used in each charged defect.

The same protocol was followed for all of the pigs and in those operated on the two hemimandibles; first the right side was operated and then the left side was operated. A marking system was implemented in order to better locate the area that was operated (3 mm titanium pins, brand IMTEC ® EC-9503, TiTac system, 3M Company, Las Alamos, New Mexico). Once the surgery is finished, the opening is closed by layers and we proceed to give an intramuscular injection (prophylactically) Terramycin 100 ®, Pfizer Laboratories (25mg/Kg). The postoperative analgesia was carried out by the administration of Meloxicam (Metamecam ® injectable solution 5 mg/ml), 5mg/20Kg/24h.

- *Collection of PPP, PRP and M*

In order to obtain the platelet-poor and platelet-rich plasmas, we adopt the criteria initially established by Marx et al. (10). A blood draw is performed (using an I.V. that was previously placed). Veinous blood is obtained (cubital vein) in 5 ml citrated tubes, 4 tubes per experimentation animal. The sample obtained is subject to centrifugation in order to obtain both the PRP platelet-rich and platelet-poor plasma. The blood sample is centrifuged at 500 g (Selecta®) for 10 minutes and the supernatant is collected, primarily the buffy-coat area (leukocytes-plasma interphase). The product obtained is the platelet-poor plasma, with an average platelet concentration of 200 x 109/l. This plasma collected is centrifuged in measurements of 500 g for 10 minutes; the supernatant is blended in and the remainder of the product corresponds to the platelet-rich plasma, with an average platelet concentration of >500 x109/l. Before placing them in the work area, the platelet-poor plasma and platelet-rich plasma are activated with 10% calcium chloride at a ratio of 1/50. In order to obtain the bone marrow (M), a puncture is made using a bone biopsy needle at the iliac crest, using local anesthesia and the bone marrow is aspirated during the same surgical procedure. Once the bone marrow has been obtained, 100g of it is centrifuged in order to separate the red blood cells and to obtain a high concentration of leukocytes. An average of 3-5 ml of bone marrow is obtained in just one puncture. The bone marrow is collected in 5 ml citrated tubes; once the supernatant is separated from the centrifuge; its coagulation is activated using 10% calcium chlorate at a ratio of 1/50.

- *Histological Study*

The samples were preserved in 10% formaldehyde and subsequently processed as directed by the Department of Anatomy at the Faculty of Dentistry, University of Barcelona, and based on the criteria established by Donath in 1985 (11). They are not subjected to a previous process of decalcification; a fixation process is carried out, followed by dehydration and preinclusion in methyl methacrylate polymers (Technovit®, Esact-Kultzer, Wahrheim, Germany). They are subsequently incorporated into a photopolymerizable product that makes it possible to obtain a rigid block (photopolymerization device: Kultzer-Exakt-light-polymerisation®, Exakta, Nordenstedt, Germany). This block is mounted and cut with a diamond edge band saw blade (Exakt micro-parallel-grinding System®, Exakt, Nordenstedt, Germany), and then eroded and polished with 1200 and 4000 grain sandpaper so that it may be studied under an electron microscope.

The samples are analyzed using scanning electron microscopy with secondary electrons (SEM) or backscattered electrons (SEM-ES): LEO200®, Cambridge, United Kingdom, (Scientific-Technical Service, University of Barcelona). All of the samples obtained are observed at different magnifications in order to assess the quality of bone repair based on the presence of different bone tissues.

- *Quantitative Analysis*

After observing the samples, pictures are taken in order to perform a quantitative study using conventional techniques for digital image processing, using the Matlab language (12). The images are obtained using the SEM software, which provides images of 1.024x1.024 pixels with a useful central area of 1004x753 pixels. Each pixel is coded on a scale of 256 levels on the range [0, 1]. The images obtained at a 20 magnifications are the ones that we use for the analysis. These images are obtained with a resolution of 152 pixels per mm horizontally and 114 pixels per mm vertically. This difference is due to the 4x3 image aspect ratio. Three parts are analyzed in each image. The first part analyzes the two concentric circles of 4 mm and 1 mm that coincide with the subjective geometric center of the charged defect in the hemimandible (the circles transform into ellipses in the image due to the difference of horizontal and vertical resolutions). The third part corresponds to the rest of the image, that is, to the periphery of the trepanation (Figure 1). We also analyzed 10 photographs corresponding to a hemimandible that was not treated, in order to obtain an average pattern.

- *Statistical Analysis*

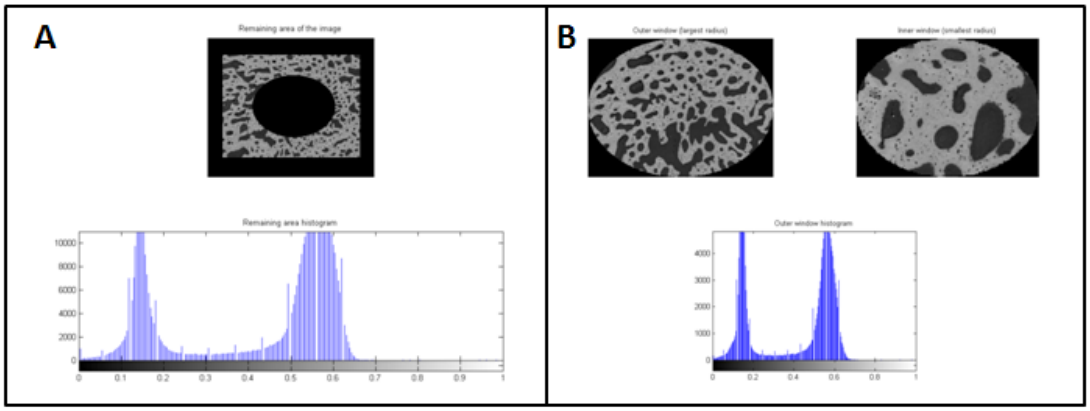
The average between the differences was compared using ANOVA between the different groups studied. We proceeded to make the post hoc comparisons using the Sheffe test. The significance level was set at $p<0.05$.

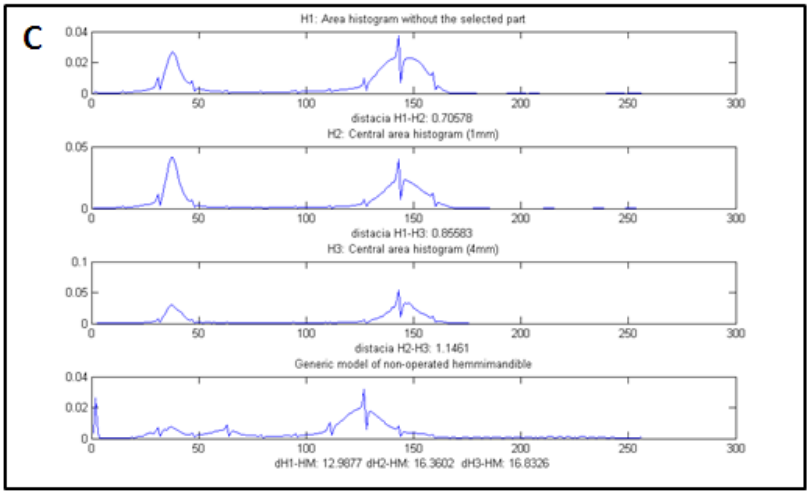
Results

Of the eight pigs used in the study, one of the animals died due to postoperative complications (animal number five). None of the pigs presented keloids or hypertrophic scars in the operative area. Only in the hemimandible operated on in pig number four presented a macroscopically observable enlargement with respect to the hemimandible control group.

Given that one of the pigs died, a total of 9 hemimandibles were obtained for the study (five came from the hemimandibles of the five animals of the first group and the remaining four were from the two hemimandibles of the two from the second group). Five samples corresponding to the non-operated hemimandibles of the first five pigs are also collected.

A total of 100 trepanations are made (10 trepanations in each hemimandible). As animal no. 5 died, it is not included in the study; therefore, 90 fenestrations remain to be studied (18 for each of the filler materials) (Fig. 2). Once the hemimandibles obtained have been processed for their study, we find that we are unable to locate 15 of the fenestrations for the microscopic studies: nine





D

---------- SUMMARY OF SELECTION 1 ----------

Mean:		
region: 95.58131	central area: 98.22498	
Standard deviation:		
region: 55.35396	central area: 53.16162	
Smoothness:		
region: 0.04500	central area: 0.04165	
Histogram symetry:		
region: -2.21084	central area: -2.76688	
Uniformity:		
region: 0.06067	central area: 0.07082	
Entropy:		
region: 5.53306	central area: 4.79498	

--SUMMARY OF UNSELECTED AREA ---
Mean: 120.42039
Standard deviation: 21.65900
Smoothness: 0.00716
Histogram symetry: -0.27263
Uniformity: 0.03196
Entropy: 5.58964

Fig. 1. Data obtained in each photograph magnified 20x, the photo corresponds to a trepanation filled with bone marrow. A) shows the histogram for the area remaining after extracting the 4 mm circle (oval) (H1). B) shows the histogram corresponding to the 4 mm circle (H2), exterior window and 1mm (H3), interior window. C) shows the comparison between histograms, difference between H1-H2 (center and outer area 4 mm) between H1-H3 (center and outer area 1 mm), H2-H3 (1 mm area and 4 mm area) and H1-HM, H2-HM, H3-HM (differences between the different areas and a standard histogram produced with 10 photographs of the non-operated hemimandible. The tables show the mathematical values obtained by the program for each area.

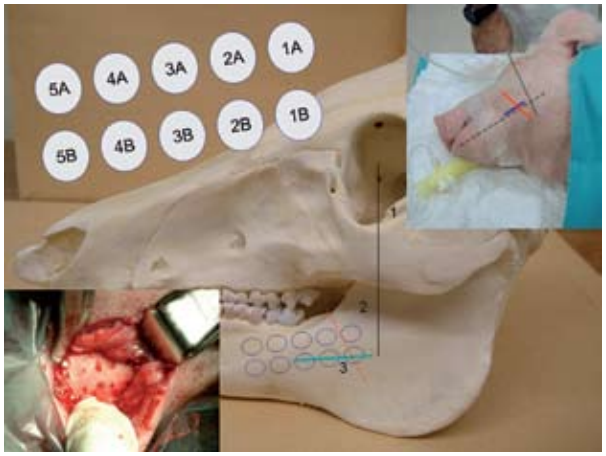


Fig. 2. Drawing showing the distribution of trepanations made in the hemimandibles. Each one is filled with a material (except two, which are considered part of the control group). 1) pupillary midline, 2) anterior insertion of the masseter; 3) surgical entry. The image in the top right and bottom left shows the simulation and surgery corresponding to animal number 4. Diagrams 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B, 5A and 5B correspond to the five materials used in the study (C, PPP, PRP, M and TP).

from pig no.1 (all are lost except one, which corresponds to TP), one from pig no.2 (corresponding to M), three from pig no.3 (corresponding to TP, C and PPP); and two from pig no.6 (corresponding to PRP and PPP). In this manner, there are 75 charged defects to be studied using electron microscopy. The remaining 75 trepanations are distributed into: 15 C, 16 TP, 14 PPP, 15 PRP and 15 M (Table 1).

96% of the trepanations studies (N=75) show a process of bone neoformation by membranous ossification. Various amounts of chondroid tissue is observed in the charged defects, which is replaced by fibroreticular bone and then by laminar bone. The numerous visible cementing lines show the abundant osteoclastic activity and the change in the system of forces (cutting cones) (Fig. 3).

The three defects in which there does not appear to be any bone formation activity correspond to two control fenestrations and one fenestration treated with platelet-poor plasma; this aspect is proven by the absence of repair tissues and by having maintained diameters similar to the original diameters of the wounds.

Of the 18 trepanations into which β-tricalcium phosphate is inserted, two of them cannot be located, and in 3 of the 16 remaining trepanations, we observe traces of the material used (18.75%).

As described in the methodology, Figure 1 shows how the data for each one of the photographs (magnified 20 times) of the charged defects is obtained. A histogram is obtained for each one of the portions analyzed and they are then compared with each other. If we analyze the averages of the differences between histograms H1-H2 (peripheral region less central region of 4 mm) for each one of the filler materials, we can observe: 14.1 for the C; 4.64 for TP; 14.03 for PPP; 1.105 for M; and 1.14 for PRP. If we group the data associated with the comparison between the image and the theoretical histogram obtained with ten photographs of a mandible that was not operated on (HM), we see that there is a match of data between H1-H2, H1-H3 and H2-H3 for each of the materials used. This match is no longer maintained when comparing the standard pattern (H1-HM, H2-HM, H3-HM) (Table 2).

Table 1. This table shows data from the 100 trepanations made. The 10 trepanations from pig number 5 (animal that died) are not taken into account; 75 of the remaining 90 are found to be effective for use in the study (83.3%). They are distributed as follows: 15 controls, 16 β-tricalcium phosphate, 14 platelet-poor plasma, 15 platelet-rich plasma and 15 bone marrow.

	1A	**2A**	**3A**	**4A**	**5A**	**1B**	**2B**	**3B**	**4B**	**5B**
Pig 1	C	M	PRP	TP	M	C	PPP	PRP	TP	PPP
Pig 2	M	PRP	C	M	TP	PPP	C	TP	PRP	PPP
Pig 3	TP	PRP	PRP	M	TP	PPP	M	C	C	PPP
Pig 4	C	TP	PRP	PPP	M	TP	C	PRP	PPP	M
Pig 5 (RIP)	M	TP	TP	C	C	PPP	PPP	M	PRP	PRP
Pig 6	PPP	PRP	C	TP	M	PPP	PRP	C	TP	M
Pig 7 (right)	C	PRP	PPP	M	TP	C	PRP	TP	PPP	C
Pig 7 (left)	PPP	TP	M	PPP	PRP	M	C	PRP	C	TP
Pig 8 (right)	TP	M	C	PRP	PRP	TP	M	PPP	PPP	C
Pig 8 (left)	C	PPP	PRP	TP	M	M	PPP	PRP	TP	C

(We have shaded the fenestrations that we were unable to locate after the procedure, whether it was because the animal died or due to complications with the procedure itself)

-C: Control group, no material; -PPP: Platelet-Poor Plasma, with a concentration of <500,000 platelets/ml; -PRP: Platelet-Rich Plasma, with a concentration of >500,000 platelets/ml; -M: Bone marrow from the animal itself; -TP: ß-tricalcium phosphate

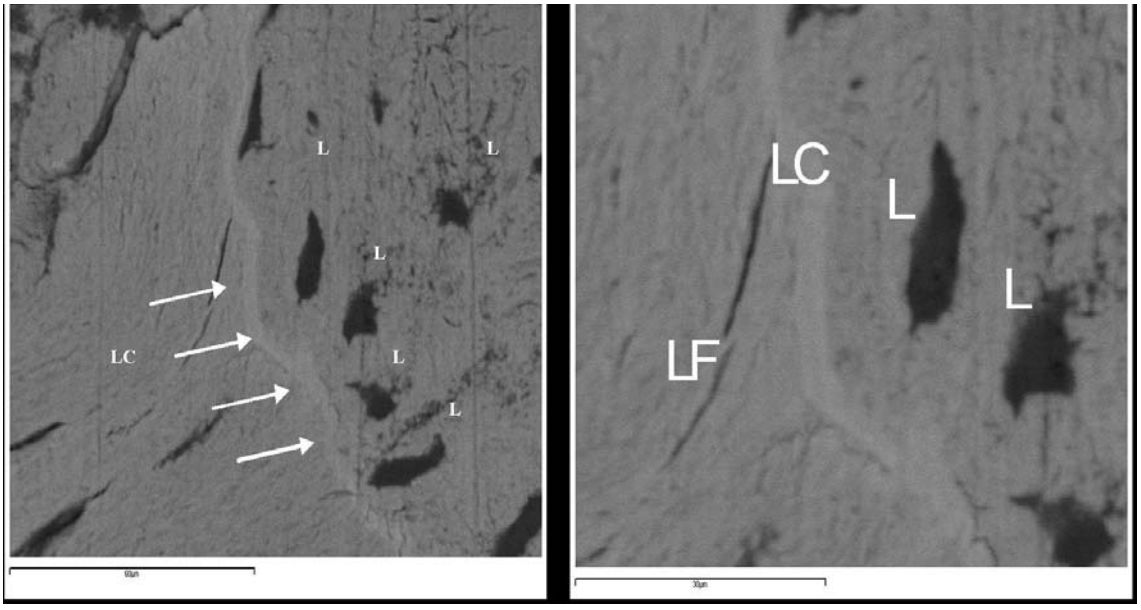


Fig. 3. The image on the left (1000x) shows the morphological aspect of the cementing line (arrows). We also see the gaps in the Woven Bone (WB), which are polygonal, large and scattered, typical of the fibroreticular bone (L). The image on the right shows the same area magnified at 2000x, where we can see the cementing line (CL) and also assess the morphological difference between a fracture line produced when handling the sample (LF) and the cementing line.

Table 2. Comparison between the groups and averages of the differences.

GROUP		H1-H2	H1-H3	H2-H3	H1-HM	H2-HM	H3-HM
C	Average	14.1280	22.7713	7.5800	41.6393	44.1540	44.0893
	N	15	15	15	15	15	15
	SD	0.80786	3.53930	0.65406	2.08239	1.90501	2.23374
TP	Average	4.6444	11.2713	3.5075	19,127.4850	29.0406	33.8969
	N	16	16	16	16	16	16
	SD	0.36809	1.83495	0.53503	4,306.67246	4.99153	2.87947
PPP	Average	14.0350	27.0636	8.0714	34.8843	37.8771	42.8764
	N	14	14	14	14	14	14
	SD	1.34210	3.22313	1.24103	4.42012	11.12248	10.65474
M	Average	1.1033	1.8527	1.3347	30.7313	30.5107	31.7327
	N	15	15	15	15	15	15
	SD	0.47036	0.73499	0.05436	1.73479	1.06888	1.27737
PRP	Average	1.1460	1.6860	1.7133	27.8400	29.6720	33.5100
	N	15	15	15	15	15	15
	SD	0.07327	0.64409	0.16487	2.48115	2.08232	0.84636
Total	Average	6.8861	12.7184	4.3805	4,107.0840	34.1331	37.1013
	N	75	75	75	75	75	75
	SD	5.94590	10.65515	2.92486	8,109.83819	7.98840	7.06381

-SD: Standard deviation; C: Control group, no material; PPP: Platelet-poor plasma, with a concentration of <500,000 platelets/ml; PRP: Platelet-rich plasma, with a concentration of >500,000 platelets/ml; M: Bone marrow derived from the animal itself.
-TP: β-tricalcium phosphate
-H1-H2: Diagram of grays of the outer area, subtracting the 4 mm center
-H1-H3: Diagram of grays of the outer area, subtracting the 1 mm center
-H2-H3: Diagram of grays of the center areas (4 mm and 1 mm)
-H1-HM: Diagram of grays of the outer area, subtracting an average value described above
-H2-HM: Diagram of grays of the 4 mm center area, subtracting an average value described above
-H3-HM: Diagram of grays of the 1 mm center area, subtracting an average value described above

As a result of the statistical analysis, we can determine that for H1-H2, the averages are different from those of the control groups in all of the groups except for in the platelet-poor plasma. As for the control groups, the averages for H1-H3 are different in all the groups. For H2-H3, the averages are different from those of the control groups in all of the groups except for in the platelet-poor plasma. For H1-HM, the averages of the control groups are only different corresponding to the tricalcium phosphate group. For H2-HM, the averages are different from those of the control groups in all of the groups, except in the platelet-poor plasma and for H3-HM; the averages are different from those of the control groups in all of the groups, except for the platelet-poor plasma.

Discussion

For the surgical procedure, a unique technique is used, which limits the postoperative effects and makes it possible to systemize the study (Fig. 2). The macroscopic study of the operative samples provides little data; only in animal number four was it possible to observe an enlargement of the operated mandible with respect to the mandible of the control group. We believe that because the animals are very young, the surgical procedure causes little distortion in these experimental animals. The loss of 15 trepanations for the subsequent histological study is attributed to the complexity of the technique used for the preparation of the samples (11). We are including cutting and polishing a block in which the operated sample is included, but the external references are gradually lost as the process is carried out, such that the handling of the first references produces more errors.

The microscopic observation of the various samples allows us to evaluate the different degrees of ossification, an aspect in which no noticeable differences in the charged defects are observed. The vacuum effect occurs in three of the 75 trepanations (4%) and the organism reacts negatively. We must consider that the ossification process is similar, regardless of the trauma caused. Initially there is a humoral response in which they play an essential role for growth factors (13), with significant osteoclastic activity reflected by the presence of the cementing line, which is then followed by the formation of new bone lining by the lining cells (Fig. 3). These lining cells do not always form laminar bone, but may form chondroid tissue in the form of thin trabeculae. It is a tissue with thin irregular trabecular formations that have a heavily calcified aspect, with very little matrix and sometimes streaked with grooves that correspond to the insertion of the collagen fibers. The cellular gaps are large, irregular and typically confluent. The next step in repairing the defect or fracture is the apposition of the fibroreticular bone (woven bone), on the trabeculae of the chondroid tissue. It is a more regular bone tissue, with a less calcified extracellular matrix and cell gaps that are not too large, polygonal and isolated, located on the trabeculae of preexisting chondroid tissue. An intense remodeling then occurs based on the very strong vascular presence, in which we observe numerous vascular elements, some from the preexisting cortical and others from the periosteal callus, resulting in the subperiosteal reaction, initially described as cutting-filling-cones by Roberts et al. in 1992 (14). These cones usually have a group of osteoclasts on the end, followed by a row of bone-forming cells (lining cells). These structures are what enable the fracture callus to remold itself with the formation of laminar-bone.

β-tricalcium phosphate is widely recommended as one of the ideal materials for filling bone defects, both due to its biocompatibility, as well as its capacity for bone induction, degradation and replacement by informed bone tissue in just a few months (15, 16). In our study, only in three of the 18 trepanations in which we used it (actually 16, given that we were unable to locate two of them), we can see traces of the β-tricalcium phosphate after two months, indicating the complete reabsorption of the material in the rest of samples.

In analyzing the samples at 20x magnification, we obtain a 7.5 x 6 mm working window. This is the magnification that we use for obtaining photographs of each of the defects, in order to analyze them afterwards. The analysis is based on the study of the range of grays, and as we described in the methodology, it uses the Matlab programming system (12). Different authors have used techniques in biomedicine based on this programming system (17,18). In analyzing the samples, the comparison between the regions is made using the histogram of the portions. The mean squared error of the histograms previously smoothed with a low-pass filter is used as a measure of similarity. The range of error is between 1 and 100, hence the smaller the error, the greater similarity between the samples, or when the same, there is more similarity between the areas with fill material and its environment. By examining the portions analyzed, we make comparisons between different areas of the same photograph in order to determine how much it resembles the area in which the fill material has been placed with its environment. One would expect greater similarity between the environment and the 4 mm circle, as opposed to that of the 1mm circle, which can be observed in the data. By comparing the result with a possible standard model created with 10 photographs taken of a hemimandible that was not operated on, we obtain mixed results. The analysis of these results suggests that the system used is useful for comparing areas within the same photograph, but suggest that the generalization made from other photographs (that is, different samples) may not be accurate.

We arrive at the following conclusions:

-Ossification occurs in 96% of the charged defects, re-

gardless of the material used to fill it, including for the control group (C).
-Compared with the control groups, there is twelve times more neoformation based on the study of the gray histograms (H1-H2) in the defects filled with PRP and M.
-Based on the gray histograms (H1-H2), our study shows tricalcium phosphate to be three times more effective as an ossification element than that of the control groups.
-Our study shows the PPP to be ineffective as a method of bone regeneration, compared to the control groups.

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