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ORIGINAL ARTICLE

Bone neoformation in defects treated with platelet-rich fibrin membrane *versus* collagen membrane: a histomorphometric study in rabbit femurs.

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Abstract: The aim of the present research was to compare bone neoformation in bone defects treated with platelet-rich fibrin (PRF) and collagen membrane (CM) at 3 and 5 weeks. For this purpose, two bone defects with a width of 4 mm and depth of 6 mm were created in the left distal femur diaphysis of New Zealand rabbits (n=12). The subjects were randomly allocated into two groups. One of the defects was covered with a platelet-rich fibrin membrane (Centrifuged resorbable autologous blood biopolymer without biochemical modification) or a collagen membrane (gold standard - Neo Mem). The second defect was left uncovered (NC). The rabbits were sacrificed after 3 and 5 weeks (3 rabbits per period). The femur was completely removed and processed histomophometrically. The bone neformation analysis was performed using a differential point-counting method. Data was statistically analyzed (ANOVA, Tukey). The histomorphometric results showed that bone neformation in the defects treated with PRF at 3 weeks was equivalent to the CM ($p<0.05$). After 5 weeks, bone neformation obtained with PRF was higher than the control group and lower compared with the CM ($p<0.05$). The conclusion of the present study is that bone neformation in defects treated with PRF showed lower histomorphometric results compared with the one obtained with the collagen membrane and higher when compared with the control defects.

Keywords: *Fibrin, Bone regeneration, Collagen membrane.*
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INTRODUCTION.

Guided bone regeneration (GBR) is currently considered a therapy of great importance to promote bone regeneration in maxillary defects. This procedure is based on the use of resorbable and non-resorbable membranes combined with filling biomaterials such as autologous, homologous, heterologous bone or alloplastic material. Membranes act as a mechanical barrier, allow proliferation of osteoprogenitor cells and prevent the invasion of conjunctive and epithelial cells of the soft tissue[1]. Experimental and clinical studies with GBR have shown positive results in different biological models[2].

The technique for the production of platelet-rich fibrin (PRF), which was developed in France by Choukroun (2001), is a second-generation platelet concentrate widely used to accelerate healing of hard and soft tissues. Its advantages over platelet-rich plasma (PRP) include ease of preparation because, unlike PRP, it does not require anticoagulant or bovine thrombin or any other gelling agent[3].

PRF membrane is an interesting biomaterial due to its special structure, biological properties and immunologic features. It is a three-dimensional matrix of fibrin which,

in a provisional and sustained manner, releases a gradient of growth factors derived from alpha granules of platelet in which cells will migrate, proliferate and differentiate for an adequate tissue regeneration[4]

PRF membrane consists of platelets, leukocytes, cytokines and stem cells within a fibrin matrix. Leukocytes seem to strongly influence the release of growth factors, immune regulation, anti-infectious activity and remodeling during healing[5]. It has been shown that, after centrifugation, 97% of platelets and 50% of leukocytes of the original blood volume are concentrated in the PRF membrane[6].

Dohan *et al.* showed the slow release of platelet-derived growth factor (PDGF), transforming growth factor beta[1] (TGF-β1), vascular endothelial growth factor (VEGF) and glycoproteins such as fibronectin and vitronectin[7,8].

Several authors have shown that a fibrin matrix is an optimal support for transplanting mesenchymal stem cells for regeneration of bone defects. On the other hand, numerous studies in vitro and in animals seek to explain the effect of fibrin in bone regeneration[9].

The purpose of the present research was to compare bone neoformation of defects treated with platelet-rich fibrin membrane and collagen membrane using bone histomorphometry.

MATERIALS AND METHOD.

This experimental, comparative and prospective study design was approved by the Reviewer Committee of the Office of Degrees and Titles of the Dental Faculty of the Universidad Inca Garcilaso de la Vega with resolution No.01882013.

Sample size calculation was carried out by applying the formula of comparing two samples obtaining 12 rabbits which were chosen by the criteria of inclusion: New Zealand breed male rabbits between 4 and 6 months of age, and 2.5-3kg. weight[10]. They were randomly divided into two groups of experimentation. Each of the experimental groups was composed of 6 rabbits.

A pilot study was conducted to train and calibrate the researcher in the procedures to be performed. For the technique of bone histomorphometry, calibration was performed by a specialist in the area and assessed using the Intraclass Correlation Coefficient (ICC); getting an ICC of 0.934 within subjects and ICC of 0.927 between subjects.

During the implementation phase, the animals entered the new environment for acclimation a week before the surgical intervention. They were placed in cages at ambient temperature and on a standard diet (Conejina, PURINA® Agribrand, Peru). Water was provided on demand[10]. Water and food were withdrawn 12 hours before surgery[10]. Anesthetic induction was performed via the intraperitoneal route by administrating ketamine (KET TO-100, Chemie, Peru) at a dose of 30mg/kg of body weight. The level of anesthesia was maintained with Promazil at a dose of 1 ml/kg of body weight (Chemie, Peru).

For obtaining platelet-rich fibrin, venous blood was taken from the specimen in 9ml test tubes without anticoagulant, approaching the dorsal vein of the atrium. Then, it was immediately centrifuged in a microcentrifuge (EBA 10, Andreas Hettich GmbH & Co. KG Föhrenstr, Tuttlingen, Germany) at a speed of 2,700 revolutions for 12 minutes[11,12].

After centrifugation, a fibrin clot was obtained in the middle of the tube, just between the red blood cells at the bottom and the acellular plasma at the top. Afterwards, the PRF clot was removed from the tube using forceps and placed in a box (PRF BOX MCT, Seoul, Korea). Finally, it was covered with a compressor and lid. This produced an inexpensive autologous fibrin membrane in approximately 2 minutes[3].

Four surgeries were performed each day for a period of three days by a specialist in oral implantology. Surgical intervention was performed with the animal in the supine position on a surgical table and the surgical field delimited by sterile surgical towels. All the instrumentation used in the surgical intervention was previously sterilized.

The surgical site was prepared to work in the left distal femoral diaphysis. A 2cm incision was made in the sur-

face of the distal femur stretching up to the periosteum with a No15 scalpel blade with a handle No3[10]. Then, the flap was lifted with a curette exposing the bone. A progressive drilling was performed with cylindrical titanium surgical drills following the diameter (Linderman No2.2, 2.9, 3.4, countersink 4.0)[13]. A new kit of drills was used (Neobiotech Co., Seoul, Korea) for each surgery. It was mounted on an Antoghyr sterile electric motor (Implanteo, France) to get the bone defects. Two bone defects of 4mm in diameter and 6mm. depth were created in each femur (they were located at a distance of approximately 1cm, which was measured with an endodontic millimiter ruler). In order to obtain the desired depth, stops from the IS Full Kit system were used (Neobiotech Co., Seoul, Korea).

After completing the surgical procedure, the experimental material was placed according to the assignment to cover the entrance to the defects. One of the defects was covered with the experimentation material (platelet-rich fibrin or collagen membrane) for which segments of 6x6mm. were measured with an endodontic millimeter ruler and cut. The other defect was the negative control (not covered with any type of membrane). Then, it was proceeded to stitch all levels, the deepest and the most superficialones, with absorbable wires of polyglactin 910 (Vicryl 3/0 with an atraumatic semi-circular needle, Pronamac, Mexico).

The rabbits were placed in individual cages immediately after the intervention without mobilizing the limb. Analgesia was done with ketoprofen (Profenid®, Chemie, Peru) at a dose of 10mg/kg of body weight administered intramuscularly. Quinolaba 10% (Enrofloxacin, Chemie, Peru) was used as an oral antibiotic for 5 days. All animals were monitored daily for the first 3-4 days after the surgery[14].

The sacrifice was carried out by administering an overdose of intravenous ketamine (KET TO-100, Chemie, Peru), which provoked the *exitus letalis* due to cardiorespiratory arrest. For obtaining the bone samples, a longitudinal lateral approach was used by making an incision on the upper region of the left hind limb, completely removing the femur by dismantling it. Once extracted, they were placed in 10% formalin.

Bone histomorphometry

The samples were cut horizontally at the level of the two defects (both covered with membrane as the control). The samples were stained with hematoxylin eosin and later analyzed in a light multi head microscope (BX50F4, Olympus Optical Co., Ltd., Japan). Bone neoformation was determined by placing a sheet of 1cmx1cm with 100 vertical lines and 100 horizontal lines that intersected each other. Photographs were taken of what has been observed with a Sony Cybershot DSC-W80 7.2 megapixels camera with (Sony Ericsson, Japan)

The differential count of points was done through the location of the vertical and horizontal lines that match the limits of the defect and counting the intersections within the trabeculae. Then, the percentage of these was calculated in relation to the total.

Statistical Analysis

For the statistical analysis of the histomorphometric results obtained from the defects covered with platelet-rich fibrin membrane, a collagen membrane and the comparison with their respective control, we used SPSS® v.20.0® for Windows (IBM®, USA). For this research, a significance level of 0.05 was set. Normality tests were carried out for the analytical statistics; the test used was the Shapiro-

Table 1. Comparison of bone neoformation in bone defects of the experimental group with PRF membrane, their respective control and the experimental group with collagen membrane at 3 and 5 weeks using bone histomorphometry.

Periods of observation /Material	Bone neoformation (%) X ± SD	ANOVA, Tukey
3 Weeks later		
Platelet rich-fibrin	52.78 ± 0.69	
Collagen Membrane	75.97 ± 8.75	0.001
Control	43.85 ± 0.65	
5 Weeks later		
Platelet rich-fibrin	59.50 ± 2.18	
Collagen Membrane	78.98 ± 3.77	0.000
Control	45.61 ± 1.09	

Test Anova, Tukey.

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Wilk in which it was accepted that the variables had a normal distribution when p>0.05. Subsequently, parametric t-student tests for related samples, student t-test for independent samples and ANOVA, Tukey were run.

RESULTS.

Table 1 shows bone neoformation at 3 and 5 weeks for the different membranes used and the negative control. There are statistically significant differences between the groups.

DISCUSSION.

Bone is a dynamic tissue in constant formation and resorption, since it is the only tissue capable of regeneration in the body. Currently, a large quantity of materials is marketed with the aim of stimulating bone regeneration, but it is clear that these materials available are not equal in terms of their biological effects, host response and clinical outcomes.

The PRF is a new regenerative material. It contains a large amount of growth factors, cytokines and leukocytes and collects all the constituents of a blood sample which are favorable for healing and immunity in a fibrin clot[15]. In addition, this biomaterial can be used in together with graft material to accelerate bone regeneration, but there is much controversy as there are few studies in this regard[6,16-19]. Also, PRF can be used as a membrane. The potential of the fibrin membrane was proved when used as a barrier in the process of bone regeneration and, since no studies have done so yet, this would be the first research to address it.

With this purpose, bone neoformation using PRF membrane as biological barrier and the membrane of collagen (gold standard) was compared in the present investigation to evaluate the formation of the new bone as filling for the bone defects which were created in the bone tissue of the left distal femoral diaphysis.

The chosen rabbits were New Zealand breed due to their size, docility and ease of handling. They were males between 4 to 6 months old and 2.5-3kg. because of the bone maturation degree (adult individuals with their already grown cartilage not compromise nearby tissues in bone neoformation) in order to get more reliable results. The area to intervene was the distal femoral diaphysis because of the femoral cortical thickness and because it is a bone of greater size, being ideal for creating the defect since it decreases the risk of complications.

Bone neoformation of the defects treated with platelet-rich fibrin membrane was lower than the neoformation obtained with a collagen membrane at 3 and 5 weeks but greater than the neoformation of the control defects. These results are consistent with the study by Bolukbasi *et al.*[6] who histomorphometrically evaluated the effectiveness of platelet-rich fibrin in bone regeneration in tibial defects of sheep at 3 weeks. In this case, neoformation for the PRF group was 53.78% and 42.85% for the control. It should be noted that they used different experimental animals, but the same protocol described by Choukroun for obtaining the platelet-rich fibrin[16].

Pripatnamont *et al.*[12] histomorphometrically evaluated the effect of platelet-rich fibrin for bone regeneration in cranial defects in New Zealand rabbits at 8 weeks. In this case, neoformation for the PRF group was 19.81% and 6.24% for the control. This is similar to the result obtained by Yilmaz *et al.*[18] in bone regeneration in tibial defects of pigs at 12 weeks. They found neoformation for the PRF group was 28% and 18.7% for the control. It should be noted that the protocol described by Dohan was used for obtaining PRF in both studies[12].

In the present study, PRF membrane showed a greater bone neoformation than the obtained in the defects that were not treated with the membrane; and lower than with the collagen membrane, which acted as the gold standard because it has been studied and proven its effectiveness in several studies due to its marketing[20,21]. Now, there are some studies in the field of dentistry evaluating PRF membrane for treating gingival recession[22-24], the effect of the membrane as a biomaterial in periodontal defects[25,26], or as graft material for lifting the floor of the maxillary sinus[16,27,28]. In all these cases,

the PRF membrane had a successful performance and proved to be a safe technique for inducing rapid epithelization, collagen formation and reducing inflammation, thereby accelerating the healing process at the level of soft and bone tissue.

These results showed that the PRF membrane allows repairing bone defects and therefore has a positive effect on the process of guided bone regeneration. It should be noted that this material is cheaper than the materials that are currently on the market; because it is fully autologous since it is produced after centrifuging the patient's own blood. Also, it can be used as an alternative barrier when the defects to regenerate are small or combined with collagen membrane thus providing biological properties to accelerate the bone regeneration process.

Extrapolating results obtained from experimental animals to human species is always debatable, especially when the bone regeneration process has proven to be faster in these animals[10].

The importance of this work lies not only in the results, but also in the methodology used; being purely quantitative with a high scientific value; using the bone histomorphometry technique.

It is concluded that bone neoformation obtained in defects treated with platelet-rich fibrin membrane showed lower results than the neoformation of the defects treated with the collagen membrane; and higher in comparison with the neoformation obtained in the negative control defects.

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Neoformación ósea de defectos tratados con membrana de fibrina rica en plaquetas versus membrana de colágeno: un estudio histomorfométrico en fémur de conejos.

Resumen: El objetivo de la investigación fue comparar la neoformación ósea de defectos óseos tratados con membrana de fibrina rica en plaquetas (PRF) y membrana de colágeno (CM), a las 3 y 5 semanas para lo cual se crearon dos defectos óseos de 4 mm de diámetro y 6 mm de profundidad en la diáfisis femoral distal izquierda de conejos Nueva Zelanda (n=12). Los animales fueron divididos aleatoriamente en 2 grupos. Uno de los defectos fue cubierto con membrana de fibrina rica en plaquetas (Biopolímero Reabsorbible Autólogo de sangre Centrifugada sin modificación bioquímica) o membrana de colágeno (Gold estándar-Neo Mem,) mientras que el segundo defecto se dejó sin cubrir (NC). Los conejos fueron sacrificados después de 3 y 5 semanas (3 conejos/periodo), se les extrae el fémur por desarticulación, se procesa y evalúa histomorfométricamente. El análisis de la neoformación ósea histológica fue realizado mediante el conteo diferencial de puntos. Los datos fueron analizados estadísticamente (ANOVA, Tukey). Los resultados histomorfométricos mostraron que la neoformación ósea de los defectos tratados con PRF a las 3 semanas fue equivalente a la neoformación obtenida en el grupo control y menor comparado con la neoformación del CM ($p<0,05$). A las 5 semanas la neoformación ósea obtenida con PRF fue mayor a la neoformación del grupo control y menor a la CM ($p<0,05$). Concluyendo que la neoformación ósea obtenida en los defectos tratados con PRF mostraron resultados histomorfométricos menores a la neoformación obtenida con colágeno y mayores en comparación a la neoformación de los defectos controles.

Palabras clave: *Fibrina, Regeneración ósea, Membrana de colágeno.*

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