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ORIGINAL ARTICLE

Bone density of defects treated with lyophilized amniotic membrane *versus* colagen membrane: a tomographic and histomorfogenic study in a rabbit´s femur.

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Abstract: The aim of this study was to compare bone density of bone defects treated with lyophilizated amniotic membrane (LAM) and collagen membrane (CM) at three and five weeks. Two bone defects of 4mm in diameter and 6mm deep were created in left distal femoral diaphysis of New Zealand rabbits (n=12). The animals were randomly divided into two groups. One of the defects was covered with lyophilized amniotic membrane (Rosa Chambergo Tissue Bank/National Institute of Child Health-IPEN, Lima, Peru) or collagen membrane (Dentium Co, Seoul, Korea). The second one was left uncovered (NC). The rabbits were killed after three and five weeks (three rabbits/period). The results showed a high bone density and defect repair by new bone. The tomographic study revealed that bone density of the defects treated with LAM at three weeks was equivalent to the density obtained with CM and higher density compared with NC ($p<0.05$). At five weeks, the bone density obtained with LAM was more than the density obtained with CM and NC ($p<0.05$). The histomorphometric study showed no significant differences between LAM and CM at three and five weeks ($p>0.05$). The results show that the lyophilizated amniotic membrane provides equal or higher bone density than the collagen membrane.

Keywords: *amniotic membrane dressings, bone regeneration, Cone-Beam, Dental, implant, biological dressing.*

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INTRODUCTION.

Guided bone regeneration (GBR) is a technique which promotes the osteoblastic proliferation augmentation and the synthesis of bone matrix. The whole process is controlled by complex molecular interactions acting on the mesenchymal cells and producing its proliferation and differentiation[1]. Clinical and experimental studies with GBR have shown positive results of the technique under different biological models[2,3].

Bone healing can be optimized by using a membrane as a barrier to stop undesirable cell invasion within the healing area of a bone. This technique allows the selective proliferation of specific cells in specific zones, which could regenerate the same kind of bone tissue in a particular area[4].

The amniotic membrane is a tissue of particular interest due to its special structure, biological properties and its immunological characteristics. The amniotic membrane has been already applied in medicine to treat burning lesions and in surgical wounds to cover tissue. Additionally, it has been used as a dressing or substrate for epithelial growth to manage several conditions of the ocular surface[5-7]. The amniotic membrane, or amnion, is the most interior layer of the fetus membranes. It is composed by a thin epithelial tissue, a basal membrane, and an avascular connective tissue stroma. The amniotic cells share stem cells properties with differentiation

capacity, which makes them an excellent candidate for their use in cellular therapy and regenerative medicine[8-10].

The amniotic membrane has a low immunogenicity and has been given several characteristics such as re-ephitelization, anti-inflammatory, anti-fibrotic, antimicrobial and antitumoral properties. These functions are related, in part, to its capacity to synthesize and release biologically active substances, including cytokines and signaling molecules as the tumor necrosis factor, interferon, transforming growth factor (TGF)-a, TGF-b; fibroblastic growth factor, epidermic growth factor, keratinocytes growth factor, hepatic growth factor, the interleukin-4 (IL-4), IL-6, IL-8, metal proteases, b-defensin, prostaglandins, etc[8,9,11].

The purpose of the present study was to compare bone density of the defects treated with the lyophilized amniotic membrane with the collagen membrane using Cone Beam Computed Tomography and bone histomorphometry.

MATERIALS AND METHODS.

The present study was approved by the Institutional Ethics Committee for Animal Use of the "Universidad Peruana Cayetano Heredia". The sample size calculation was performed by application of the formula to compare two mean values (Δmin=16.67) with a power test 80%, obtaining 12 rabbits which were chosen by the inclusion criteria: New Zealand white male rabbits, 4 to 6 months of age, from 2.5kg to 3kg. These were randomly divided into two experimental groups; each experimental group consisted of six rabbits.

A pilot test was conducted to train and calibrate the researcher to perform the surgical procedures and to determine the feasibility of using cone beam tomo-graphy. The researcher was calibrated with a specialist in the area of bone histomorphometry technique. He was assessed by the intraclass correlation coefficient (ICC), a ICC of 0.960 and 0.937 between subjects. The animals used in the pilot test were not considered as part of the sample.

The effect of this new membrane was determined thro-ugh analysis of bone density of the created defects (two bone defects in each animal). For this purpose, the animals were randomly divided into 2 groups (six animals per group), obtaining a total of 24 samples (Group 1: six defects treated with amniotic membrane and six non-treated defects; Group 2: six defects treated with collagen membrane, and six non-treated ones) which were analyzed tomographically (Cone Beam CT scan) and histomorphometrically (bone histomorphometry).

In the implementation phase, a week before surgery, the animals entered to the Bioterio of the Cayetano Heredia University, Veterinary Faculty to get acclimated to the new environment. They were placed in cages under room temperature conditions and standard food regime (Conejina - PURINA®). Water was administered under demand. Water and food were suspended 12 hours before surgery. The Anesthesia was induced with intraperitoneal administration of ketamine (KET A-100) at doses of 30mg/kg. The level of anesthesia was maintained with a dose of 1ml/kg Promazil.

The surgeries were performed at the Anatomy Amphitheatre, Department of Oral and Maxillofacial Medicine and Surgery, Faculty of Dentistry of the Cayetano Heredia University. Four surgical interventions (one surgery per rabbit) were performed daily for a period of three days by a specialist in oral implantology.

The surgical site was prepared to work in the left distal femoral diaphysis. A 2cm incision was made on the distal surface of the femur extending to the periosteum with a scalpel blade No. 15 on a handle No. 3. Then, it was proceeded to lift the flap with a curette leaving the bone exposed. A progressive drilling was performed with cylindrical titanium surgical burs following the sequence by diameter (No. 2.0, 2.2, 3.0, 3.8, 4.0)[12]. A new kit of burs was used (Dentium Co, Seoul, Korea), mounted on an electric sterile motor (Saeshin, Korea) to create bone defects, for each daily intervention. In each femur, 2 bone defects were created (located at a distance of approximately 1cm, which was measured with an endodontic millimeter rule) of 4mm diameter and 6mm deep. In order to obtain the desired depth, rubber stops from the DASK system were used (Dentium Co, Seoul, Korea). After the

surgical procedure, the research materials were allocated to cover the entrance of the defects. One defect was covered with the experimental material (Lyophilized amniotic membrane or collagen), for which segments of 6x6mm were cut and measured with a millimeter endodontic rule. The other defect was the negative control (not covered with any type of membrane). All the levels were sutured, the deeper and more superficial ones, with absorbable suture of polyglactin 910 (Vicryl 3/0 - semicircular atraumatic needle). The rabbits were placed individually in their cages immediately after surgery, without mobilizing the extremity.

Analgesia was done with ketoprofen (Profenid®) at doses of 10mg/kg administered intramuscularly. Quinolaba Oral at 10% (Enrofloxacin) was used as antibiotic for 5 days. All animals were monitored daily for the first 3-5 days after surgery by the researcher, medical and veterinary technician. Then, they were monitored daily by the veterinary technician and the researcher.

The animal sacrifice was performed by administering intravenous overdose of Ketamine (KET A-100), prompting the exitus letalis by cardiac arrest. To obtain bone samples, a longitudinal lateral approach was performed over the upper region of the left posterior extremity, completely removing the femur by disarticulation. Once extracted, femurs were placed in 10% formaldehyde.

Cone beam computed tomography.

A wax stand was made. It was 3cm long, 3cm wide and 2cm height for each distal femur condyle to rest on its base, and thus performing the tomographic scan of the object under study in a vertical position. There were several captures in different positions and under different values during the pilot study in order to obtain the best image for the radiological object analysis (rabbit femur). Cone Beam tomography (Sirona, Germany), which was calibrated during the pilot test, was used to obtain an adequate image of the femur and bone density reading. This was carried out by a specialist in the field of radiology. Finally, it was determined, in each tomographic scan, to place 3 specimens, locating 2 at the back and one in front of it with a triangular shape, thus assuring that they were within the area in order to record the panoramic image. Also, a scanning time of 14 seconds, an intensity of 10mAs and a potential or voltage difference of 85kV were established.

The analysis of bone density scans was performed by a specialist in the area of the bone cortical, in the femoral distal diaphysis zone, who had used the Sidexis XG software (Sirona, Bensheim, Germany). Bone density was set as the average of the value of the difference gray scale in tangential, transverse and axial views. Data were recorded on an *ad hoc* card.

Bone histomorphometry.

The samples were cut horizontally creating two defects (the one covered with the membrane and control). The samples were stained with hematoxylin and eosin and then analyzed in a light microscope. Bone density was determined by placing a sheet of 1cmx1cm, with 100 vertical and 100 horizontal lines intersected between them. Photographs of the observed images were taken with a Sony DSC-W80 Cybershot (Sony Ericsson, Japan) with 7.2-megapixel lens. Differential counting points were performed by locating the vertical and horizontal lines which matched the boundaries of the defect and the counting intersections with in the trabeculae, and it was proceeded to consider a percentage of these in relation to the total.

Statistical analysis.

For the statistical analysis of the results obtained from the tomographic and histomorphometric study of the defects covered with amniotic membrane, lyophilized collagen membrane and compared with their respective controls, SPSS 18.0 Software for Windows® (Microsoft, Washington) was used. In this research, a significance level of 0.05 corresponding to a confidence interval of 95% was set. For the analytical statistics, normality tests were performed. Shapiro-Wilk test was used, for which it was considered that the variables were normally distributed when $p>0.05$. Subsequently, parametric t-student tests for related samples, t-student for independent samples and ANOVA, Tukey tests were also executed.

RESULTS.

Bone density of the bone defects treated with the lyophilized amniotic membrane, evaluated with Cone Beam Computed Tomography, was 1742.44±95.32 after 3 weeks of observation; while at 5 weeks, it was 2171.33±87.14. Statistically significant differences between mean bone density of the defects treated with lyophilized amniotic membrane evaluated at 3 and 5 weeks were found (p<0.05). Bone density of the bone defects treated with lyophilized amniotic membrane as assessed by the bone histomorphometry technique was 55.08±2.26 after 3 weeks of observation, while at 5 weeks, it was 76.41±4.27. Statistically significant differences between mean bone density of the defects treated with lyophilized amniotic membrane evaluated at 3 and 5 weeks were found (p<0.05).

Table 1 shows that bone density of the defects treated with lyophilized amniotic membrane, evaluated with Cone Beam Computed Tomography was 1742.44±95.32 at 3 weeks of observation. For its respective con-trol, it was 1536.44±21.20. Meanwhile, bone density of the defects treated with collagen membrane was 1770.44±90.61, finding statistically significant differences between mean bone density of the defects treated with lyophilized amniotic membrane, its respective control, and the collagen membrane at 3 weeks (ANOVA, Tukey - p<0.05). Bone density of bone defects treated with lyophilized amniotic membrane, evaluated with Cone Beam Computed Tomography was 2171.33±87.14 at 5 weeks of observation, their respective control was 1747.77±123.16, while bone density of the defects treated with collagen membrane was 2009.55±25.73. Statistically significant differences between the mean bone density of the defects treated with lyophilized amniotic membrane, their respective control and collagen membrane 5 weeks (ANOVA, Tukey - p<0.05) were found.

In Table 2, it is evident that bone density of bone defects treated with lyophilized amniotic membrane as assessed by histomorphometry technique was 55.08±2.26 after 3 weeks of observation; the respective control was 31.00 ±4.33, while the bone density of the defects treated with collagen membrane was 55.16±6.39. Statistically significant differences between the mean bone density of the defects treated with lyophilized amniotic membrane, its respective control, and the collagen membrane at 3 weeks (ANOVA, Tukey - p<0.05). Bone density of bone defects treated with lyophilized amniotic membrane, evaluated with histomorphometry technique was 76.41±4.27

Table 1. Comparison of bone density among all the defects treated with collagen membrane, lyophilized amniotic membrane, their respective control and the collagen membrane, using CBCT after 3 and 5 weeks.

DEFECTS	MEDIA	SD	STATISTICS OF LEVENE	ANOVA, TUKEY
3 WEEKS				
Lyophilized amniotic membrane	1742.44	95.32	---	---
Collagen membrane	1770.44	90.61	0.064	0.019
Amniotic membrane control	1536.44	21.20	---	---
5 WEEKS				
Lyophilized amniotic membrane	2171.33	87.14	---	---
Collagen membrane	2009.55	25.73	0.044	0.000
Amniotic membrane control	1747.77	123.16	---	---

CBCT: Cone Beam Computed Tomography; **SD:** Standartd Deviation; ANOVA, Tukey - (p<0.05).

Table 2. Comparison of the bone density of defects treated with the lyophilized amniotic membrane, its respective control and the collagen membrane using bone histomorphometry from 3 to 5 weeks.

DEFECTS	MEDIA	SD	STATISTICS OF LEVENE	ANOVA, TUKEY
3 WEEKS				
Lyophilized amniotic membrane	55.08	2.26	---	---
Collagen membrane	55.16	6.39	0.171	0.001
Amniotic membrane control	31.00	4.33	---	---
5 WEEKS				
Lyophilized amniotic membrane	76.41	4.27	---	---
Collagen membrane	73.16	5.43	0.110	0.001
Amniotic membrane control	54.66	1.46	---	---

SD: Standartd Deviation; ANOVA, Tukey - (p<0.05).

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at 5 weeks of observation, their respective control was 54.66±1.46, while the bone density of the defects treated with collagen mem-brane was 73.16±5.43. Statistically significant differences between the mean bone density of the defects treated with lyophilized amniotic membrane, their respective control and collagen membrane to 5 weeks (Anova, Tukey - p<0,05) were found.

DISCUSSION.

The analysis of bone density scans was performed with the program Sidexis XG (Sirona, Bensheim, Germany), through which it was possible to make a close observation of the defects in three dimensions, including an overview and three views: tangential, radial and axial. In the diagnostics section of the program, there is the icon which shows the value of the gray scale of the defect analysis. The tomographic results showed a high bone density and bone defect repair. Bone density of the defects treated with lyophilized amniotic membrane after 3 weeks was comparable to the density obtained with collagen membrane and higher density compared with control defects (p<0.05), whereas at 5 weeks, it was greater than the density of the defects treated with collagen membrane defects and controls (p<0.05). Statistically significant differences were seen between the groups, as in the parametric ANOVA, Tukey test.

The methodology used in this study was quantitative. A software adapted to Cone Beam Computed Tomography (Sirona, Bensheim, Germany), which is significantly more accurate in its measurements of intraoral radiographs, was used. In a study, Grimard *et al*.[13] compared measurements with digital intraoral and cone beam images (CBCT) to measure 35 internal defects, concluding that CBCT measurements were significantly more accurate than intraoral radiographs, and comparable with direct surgical measurements. This is because the three-dimensional image can obtain a proper orientation in three views and measuring changes occurring after bone grafting procedures (defect filling and resolution) with better accuracy and similarity to direct measurements. Other studies support these findings. For example, Misch *et al*.[14] evaluated changes in bone level defects created in mandibles of dry skulls, concluding that CBCT dimensional capacity has a significant advantage over conventional methods, because all defects are detected and quantified in interproximal areas, buccal and lingual. Also, Kehl *et al*.[15] evaluated the marginal bone level in three dimensions around dental implants. The use of CBCT allowed a quantitative analysis and accurate bone loss, regarding the radiography (two dimensions) which generates difficulty for these analyzes. In literature, this is an early study meant to assess bone density of defects in three dimensions, using the average valuation of grayscale registered at the center of the bone defect, in three views; tangential, transverse and axial.

The histomorphometric study showed statistically significant differences between bone density of the defects treated with lyophilized amniotic membrane and its respective control (p<0.05) at 3 and 5 weeks of observation. However, it showed no significant diffe-rences when compared to the density of bone defects treated with collagen membrane at 3 and 5 weeks (p>0.05), according to the parametric test ANOVA, Tukey. This method has been widely used in various studies[16] due to the simplicity of the technique and its precision, since it is obtained a quantitative value of the number of newly formed trabeculae. Studies on the application of lyophi-lized amniotic membrane grafts as barriers in the process of bone regeneration have been made known to the scientific community in the field of ophthalmology[16].

This studied lyophilized amniotic membrane showed a higher bone density than that obtained in the defects which were not treated with membrane, and better or equal results than with collagen membrane, which acted as gold standard, because the market has studied it and it has proven to be effective in several studies. Currently, there are some studies already released in the field of dentistry, either evaluating gingival wound epithelialization in rabbits[17], the effect of membrane as a biological dressing in oral mucositis[18], as graft material for repair of oronasal fistula mid palate[19], or as an aid in healing and wound

healing after dental implant surgery[20]. In all cases, the amniotic membrane had a successful performance and it is seen as a safe technique to induce rapid epithelialization, collagen formation and for reducing inflammation, there by accelerating the healing process at soft and osseous tissue level.

This can be explained because the amniotic membrane provides a basal membrane which promotes cell migration and differentiation[21], reduces inflammation in the area below the membrane[21] and acts as a seal preventing the biological pass of different kinds of microorganisms into the wound, and various types of tissue with rapid regeneration capacity (epithelial and connective) entering into the bone defect and prevent the osteogenic potential thereof. These results show that the lyophilized amniotic membrane allows the repair of bone defects and therefore has a positive effect on the guided bone regeneration process. It is worth noting that this material is economically more accessible than the current materials on the market. Also, extrapolation of the results obtained in experimental animals to the human species is always debatable, especially when it is proven that the process of bone regeneration is faster in these animals[14].

According to the results, it has been shown that lyophilized amniotic membrane helps to reduce the period for bone regeneration and can be used as a barrier membrane in those patients requiring prosthetic pre-surgical treatments. The importance of this work lies not only in the results, but on the used methodology, due to the nature of the variables and the accuracy of the techniques. Moreover, in literature, there are not other studies reporting the association of these techniques to assess bone regeneration.

CONCLUSION

It is concluded that the obtained bone density in the defects treated with lyophilized amniotic membrane and the histomorphometric and tomographic results showed comparable or higher density than that bone defects treated with collagen membrane, and higher density compared to the defects obtained in controls (treated without membrane).

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Densidad ósea de defectos tratados con membrana amniótica liofilizada *versus* membrana de colágeno. Un estudio topográfico e histomorfogénico en el fémur de un conejo.

Resumen: El propósito de este estudio fue comparar la densidad ósea (DO) de defectos óseos tratados con membrana amniótica liofilizada (MAL) y membrana de colágeno (MC), a las 3 y 5 semanas. 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 amniótica liofilizada (Banco de tejidos Rosa Chambergo/ INSN-IPEN, Lima, Perú) o membrana de colágeno (Dentium Co, Seoul, Korea). El segundo se dejó sin cubrir (NC). Los conejos fueron sacrificados después de 3 y 5 semanas (3 conejos/periodo). Los resultados mostraron una alta DO y reparación del defecto por hueso neoformado. El estudio tomográfico reveló que la DO de los defectos tratados con MAL a las 3 semanas fue comparable a la densidad obtenida con MC y mayor comparado con la densidad de NC ($p<0,05$); mientras que a las 5 semanas fue mayor a la densidad de MC y NC ($p<0,05$). El estudio histomorfométrico no mostró diferencias significativas entre MAL y MC a las 3 y 5 semanas ($p>0,05$). Los resultados muestran que la membrana amniótica liofilizada brinda densidad ósea comparable o mayor que la membrana de colágeno.

Palabras clave: *Apósito de membrana amniótica, regeneración ósea, Cone Beam, implante dental, apósito biológico.*

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