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Article

Regenerating effect of hyaluronic acid on circumferential bone defects in Wistar albino rats.

Efecto regenerador del ácido hialurónico en defectos óseos circunferenciales en ratas albinas Wistar.

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Abstract: Objective: To determine the regenerating effect of hyaluronic acid on circumferential bone defects in albino Wistar rats. Material and Methods: An experimental type study was designed and carried out with 15 albino male Wistar rats, 4 months old and weighing between 250 and 350 grams. Two circumferential bone defects 3mm in diameter and 0.8mm deep were created in the calvaria of the parietal bone (on both sides of the midline). One defect was filled with a demineralized bone matrix (control group); while the other defect was filled with the combination of a demineralized bone matrix plus hyaluronic acid (experimental group). Five experimental rats were euthanized at 30, 60 and 90 days after surgery and they were histologically evaluated following the parameters proposed by Heiple. Results: The experimental group presented a better degree of bone regeneration at 30 and 60 postoperative days. Conclusion: Hyaluronic acid is effective in bone regeneration of circumferential bone defects.

Keywords: *Hyaluronic acid; bone regeneration; wound healing; Bone Matrix; Rats, Wistar; Biocompatible Materials.*

Resumen: Objetivo: Determinar el efecto regenerador del ácido hialurónico en defectos óseos circunferenciales en ratas albinas Wista. Material y Métodos: Se diseñó un estudio de tipo experimental y se trabajó con 15 ratas albinas Wistar (todas macho) de 4 meses de edad y con un peso entre 250 a 350 gr. Se crearon en todas 2 defectos óseos circunferenciales de 3mm de diámetro y 0.8 mm de profundidad en la calota del hueso parietal (a ambos lados de la línea media). Un defecto fue rellenado con una matriz ósea desmineralizada (grupo control); mientras que el otro defecto fue rellenado con la combinación de una matriz ósea desmineralizada más el ácido hialurónico (grupo experimental). Se realizó la eutanasia a 05 ratas de experimentación a los 30, 60 y 90 días postquirúrgicos y se evaluaron histológicamente siguiendo los parámetros propuestos por Heiple. Resultados: El grupo experimental presentó un mejor grado de regeneración ósea en los 30 y 60 días postoperatorios. Conclusiones: El ácido hialurónico es eficaz en la regeneración ósea de defectos óseos circunferenciales.

Palabras Clave: *Ácido hialurónico; regeneración ósea; cicatrización de heridas; matriz ósea; ratas wistar; materiales biocompatibles.*

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INTRODUCTION.

The human body has a limited capacity to regenerate bone tissue, which is why regenerative techniques involving different biomaterials are constantly being tested to develop a tissue that is the closest in its anatomical structure and functionality to the previous one.[1] One of the most prevalent oral diseases is periodontal disease. Due to the its high prevalence, there is consensus that the approach to treat periodontal conditions must be accompanied by a focus on public health.[2,3] Periodontal disease is a chronic inflammatory pathology that affects the supporting and protective tissues of the tooth and may result in the loss of teeth.[4]

The presence of bone defects is commonly observed both clinically and radiographically (vertical, horizontal, or circumferential) as a consequence of bone loss and the application of shear bite forces during chewing. Tooth mobility and tooth loss are frequently reported in more advanced stages of the disease. There are currently several therapeutic options for treating patients suffering from advanced periodontal disease. To reverse the problem of bone loss, the most common treatment option is bone grafts, whose main function is to ensure that the defects heal as quickly as possible. Bore grafts should be anatomically consistent, serve to guide the newly formed tissues, promote osteogenesis, and offer mechanical support.

They can provide early mechanical stability and regeneration in an area with bone defects.[5] Demineralized bone matrix is one of the most widely used biomaterials to regenerate bone tissue since it is biocompatible and osteoconductive, contributing to bone formation through osteoinduction.[6] The main disadvantage of this material is its high cost, which prevents most patients from accessing this type of treatment. Hyaluronic acid (HA) could be an alternative to regenerate bone tissue. It is a member of the glycosaminoglycan family and an important component of the extracellular matrix. It is synthesized by almost every cell in the body and performs functions such as balancing osmotic pressure and providing cell-cell and cell-matrix interactions. In addition, it also has bacteriostatic properties and can take on different functions depending on the nature of the tissue, the type of cell it secretes, and its molecular weight.[7]

HA plays a key role in bone tissue since it interacts with cells involved in bone metabolism (monocytes, fibroblasts, osteoblasts, osteoclasts, osteocytes), as well as with proteins (growth factors, type I collagen, type V collagen, fibronectin, calcitonin). It helps osteoblast cells, osteoclasts, and osteocytes, each of which plays a role in bone formation, fulfilling functions such as migration, differentiation, and proliferation.[8]

The aim of the present study was to determine the effect of hyaluronic acid on circumferential bone defects in albino Wistar rats. The goal is to find a therapeutic option so that patients can receive adequate treatment, with high-quality biomaterials, achieving success and providing an improvement in the prognosis of their teeth.

MATERIALS AND METHODS.

The procedures described below are in accordance with the *"Guide for the Care and Use of Laboratory Animals of the International Association for Assessment and Accreditation of Laboratory Animal Care"*.[9] It was assessed and approved by the Research Ethics Committee of Universidad Alas Peruanas Certificate 017-2018.

This research was experimental. Fifteen 4-month-old male Wistar albino rats, weighing between 250-350 grams, were used in the study. Only 15 albino rats were included since this is the number agreed to carry out this type of study. Circumferential bone defects were created in the rats on both sides of the midline. In the control group they were filled with a demineralized matrix (left side of the midline), while in the experimental group they were filled with a demineralized bone matrix combined with 3% HA (right side of the midline) in equal proportions. The 3% concentration of HA has regenerative properties, and its gel presentation facilitates its combination with demineralized bone matrix.

The demineralized bone matrix is an allograft from the bone cortex with preservation of collagen and non-collagen proteins and growth factors, prepared by extracting the mineral components using hydrochloric acid and ethanol. Its demineralization process inactivates viruses, preserving the osteoinductive activity necessary for the formation of new bone, assuring viral inactivation and osteoinductivity.[10]

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The rats were kept in separate individual cages and distributed in three groups of five rats; this in order to euthanize each group at different times: 30, 60, and 90 days after surgery, for subsequent histological analysis.

Creation of circumferential bone defects

The rats were operated under general anesthesia, using sodium thiopental as anesthetic. In addition, 3% lidocaine without epinephrine was infiltrated in the area to be operated on. The cranial area corresponding to the parietal bone of the animal was shaved, an incision was made until reaching bone tissue and then the flap was detached until access was made and the cranial vault was adequately visualized.

It was in this area where two circumferential bone defects were created on both sides of the midline, 3mm in diameter by 0.8mm deep, with the help of a previously calibrated round tungsten carbide surgical bur, and abundant irrigation with physiological serumTo fill the bone defect, a demineralized bone matrix combined with 3% HA was placed in the experimental group (right side of the midline); and the control group (left side of the midline) received a demineralized bone matrix. Finally, suturing was performed with simple stitches using 5/0 polyglycolic acid suture. After the 15 rats awoke from general anesthesia, they were frequently monitored to ensure their survival.

Histological study

A total of 30 samples were obtained: 15 samples corresponded to the experimental group and 15 to the control group. Of each group of 15 samples, 5 corresponded to days 30, 60, and 90 of the postoperative periods, respectively. The samples obtained were placed in 10% formalin and the histological study was carried out. The samples were decalcified and introduced into a tissue processing machine, then embedded in liquid paraffin. 5 µm sections were performed to finally be mounted on slides and stained with hematoxylin and eosin.

Evaluation of the effect of hyaluronic acid on bone defects

To evaluate the effect of hyaluronic acid on bone defects, the slides were observed under the light microscope and analyzed based on these 4 parameters proposed by Heiple:[11]

a) Degree of bone maturity:

0= absent, 1= presence of undifferentiated cells, 2= proliferation and differentiation of undifferentiated cells to bone-forming cells, 3= presence of isolated bone islets, 4= bone spicules joining bone islets forming a heterogeneous pattern, 5= compact mature bone.

b) Presence and quality of bone marrow:

0= absent, 1= hematopoietically active, main presence of erythrocytes, 2= decrease in the number of erythrocytes and increase in the number of adipocytes, 3= yellow bone marrow

c) Normal defect-bone continuity: 0= absence of bone formation at the edge of the defect, 1= little bone formation, 2= moderate bone formation, 2/3 of the defect without bone filling, 3= high bone formation, 1/3 of the defect without bone filling, 4= almost complete filling of the defect, 5= continuity of the defect with 100% normal bone.

d) Peripheral bone formation

(0= absent, 1= scarce, 2= moderate, 3= high).

The data obtained were collected in a laboratory observation file, to later be recorded in a systematization matrix for subsequent statistical analysis. Fisher's exact statistical test was performed to obtain greater statistical precision in the results due to the qualitative nature of the variable and sub-variables (Heiple's parameters) and to the reduced number of observations in each study group.

RESULTS.

Degree of bone maturity

It was observed in the experimental group that at 30 days after the surgery, 80% of the sample presented isolated bone islets (Table 1). At 60 days, 100% of the samples presented bone spicules joining the islets of bone forming a heterogeneous pattern. At 90 days, all the samples presented mature bone. With respect to the control group, at 30 days after surgery, 60% of the samples presented proliferation and differentiation from undifferentiated cells to bone-forming cells. At 60 days, 80% of the samples presented isolated bone islets; and at 90 days, 100% of the samples presented mature bone.

When comparing both study groups, it was found that at 30 and 60 days the experimental group presented a greater degree of bone maturity compared to the control group (Figure 1). However, at 90 days after the surgery, both groups showed the same result

obtaining the maximum degree of bone maturity (mature bone). Figure 1A shows the presence of isolated bone islets from the experimental group and Figure 2B shows the proliferation of undifferentiated cells from the control group at 30 post-surgical days. Bone spicules are observed joining the bone islets forming a heterogeneous pattern in both groups at 60 days post-surgery: Figure 1C (experimental group) and in Figure 1D (control group).

Presence and quality of bone marrow

Regarding the Presence and Quality of Bone Marrow (Table 1) in the experimental group at 30 days postoperatively, 60% of the sample presented a hematopoietically active marrow with a high presence of erythrocytes. At 60 days, 100% of the samples showed a decrease in the number of erythrocytes and an increase in the number of adipocytes. At 90 days, 100% showed a yellow bone marrow.

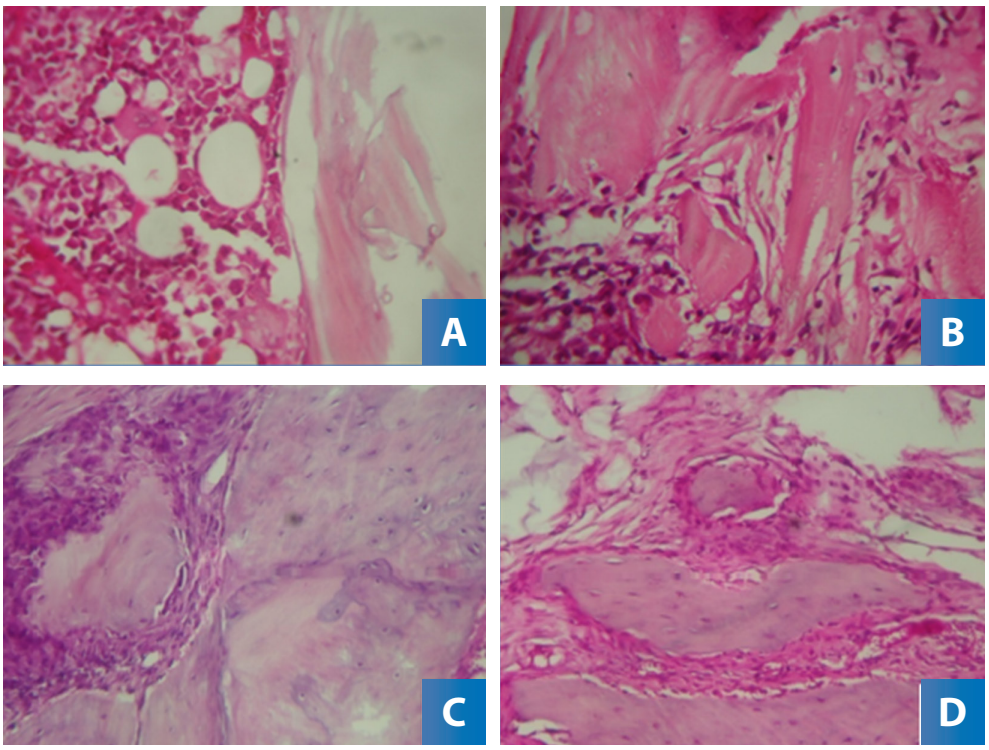
In the control group at 30 days, 60% of the samples presented absence of bone marrow, at 60 days 40% had a hematopoietically active marrow and 40% presented a decrease in erythrocytes with an increase in adipocytes. At 90 days 80% of the samples already had yellow bone marrow.

When comparing both study groups, at 30, 60, and 90 days, more bone presence and higher quality was observed in the experimental group since it presented a more active bone marrow with a greater number of erythrocytes. This did not happen in the control group (Figure 2).

Figure 2A shows the hematopoietically active experimental group (majority presence of erythrocytes) 30 days after surgery, and Figure 2B shows the control group 30 days after surgery with absence of bone marrow. In figures 2C and 2D it is shown that both the experimental group and the control group at 60 days post-surgery showed a hematopoietically active marrow but with a decrease in the number of erythrocytes and an increase in adipocytes compared to the samples on day 30 post-surgery. Finally,

Figure 2E and Figure 2F show that both the experimental group and the control group at 90 days post-surgery presented a yellow marrow, but the control group (Figure 2F) still does not have bone filling the totality of the defect.

Figure 1. Degree of bone maturity at 30 and 60 days after surgery.



A: The presence of isolated bone islets from the experimental group is shown.
B: Shows the proliferation of undifferentiated cells from the control group at 30 days post-surgery.
C: The experimental group is shown.
D: Shows the control group 60 days after surgery where bone spicules are observed joining the bone islets forming a heterogeneous pattern.

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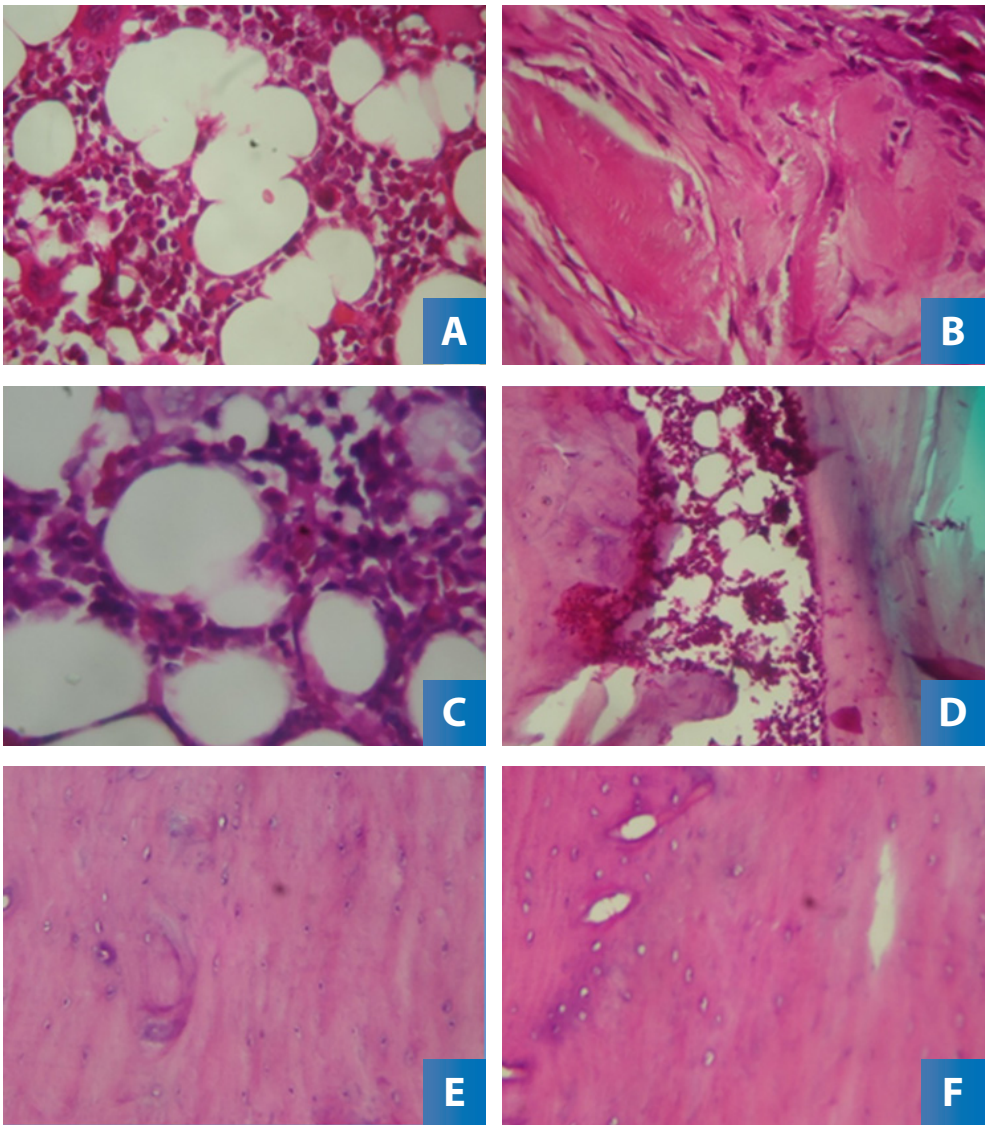
Normal defect-in-continuity

Regarding normal defect-in-continuity, it can be observed that in the experimental group, at 30 postoperative days, 80% of the sample presented a moderate bone formation with 2/3 of the defect without bone filling (Table 1). At 60 days, 40% presented high bone formation with 1/3 of the defect without filling, and another 40% presented an almost total filling of the defect. At 90 days, 100% of the sample presented 100% continuity of the defect with normal bone.

On the other hand, in the control group, 80% of the sample both at 30 days and 60 days postoperatively presented moderate bone formation with 2/3 of the defect without bone filling. Only at 90 days 80% of the sample presented 100% defect-in-continuity with normal bone.

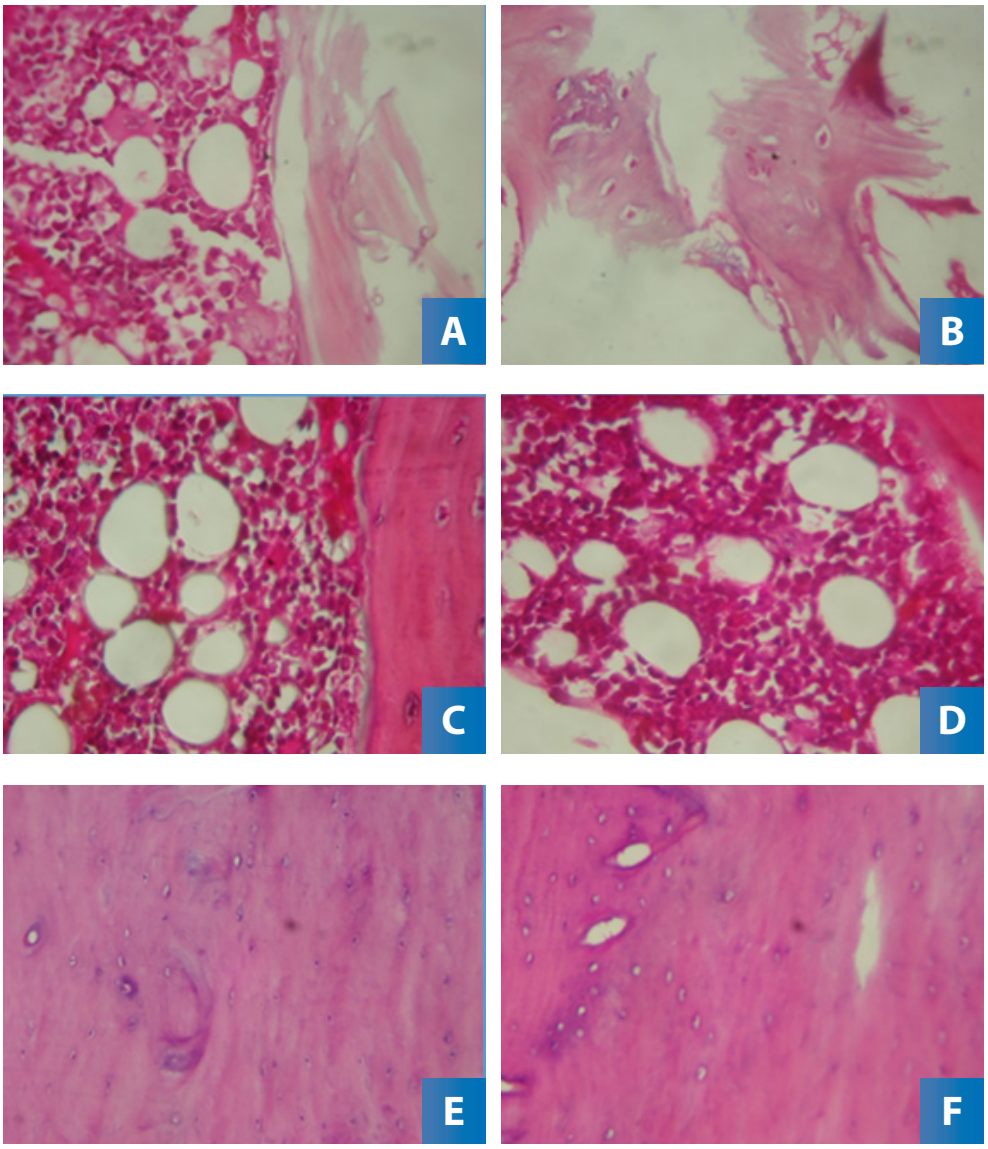
When comparing both study groups, it was found that the experimental group obtained a better normal defect-in-continuity at 30, 60, and 90 days compared to the control group (Figure 3). In Figure 3A the experimental group is observed 30 days after surgery showing moderate bone formation and moderate peripheral bone formation, while Figure 3B shows the control group 30 days after surgery with less bone tissue filling of the bone defect and minor peripheral bone formation. In the samples 60 days after surgery, an almost total bone filling of the bone defect is

Figure 2. Presence and quality of bone marrow at 30, 60, and 90 days after surgery.



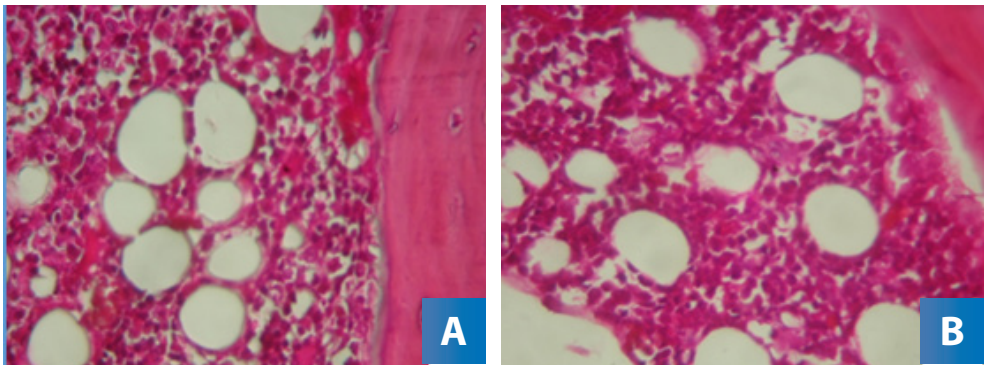
A: The experimental group is shown
B: Shows the control group at 30 days after surgery.
C: Shows the experimental group.
D: Displays the control group at 60 days after surgery.
E: Shows the experimental group.
D: the control group is observed at 90 days post-surgery.

Figure 3. **Normal defect-in-continuity 30 days after surgery to 30, 60, and 90 days after surgery.**



A: The experimental group is observed.
B: The experimental group at 30 days post-surgery.
C: Shows the experimental group.
D: The control group at 60 days post-surgery.
E: Shows the experimental group.
F: The control group at 90 days post-surgery.

Figure 4. **Peripheral bone formation 30 days after surgery.**



(A) T the experimental group is shown, and the control group in (B).

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Table 1. Systematization matrix for samples at 30, 60, and 90 days after surgery according to Heiple's parameters.

Heiple's parameters	Experimental Group	Measurement					
		30 days		60 days		90 days	
		N	%	N	%	N	%
Degree of Bone Maturity	Absent	0	0.0	0	0.0	0	0.0
	Undifferentiated cells	0	0.0	0	0.0	0	0.0
	Cell proliferation	0	0.0	0	0.0	0	0.0
	Presence of islets	4	80.0	0	0.0	0	0.0
	Bone spicules	1	20.0	5	100.0	0	0.0
	Mature bone	0	0.0	0	0.0	5	100.0
Control Group	Absent	0	0.0	0	0.0	0	0.0
	Undifferentiated cells	0	0.0	0	0.0	0	0.0
	Cell proliferation	3	60.0	0	0.0	0	0.0
	Presence of islets	2	40.0	4	80.0	0	0.0
	Bone spicules	0	0.0	1	20.0	0	0.0
	Mature bone	0	0.0	0	0.0	5	100.0
Presence and quality of bone marrow	Absent	2	40.0	0	0.0	0	0.0
	Hematopoietically active	3	60.0	0	0.0	0	0.0
	Decreased erythrocytes	0	0.0	5	100.0	0	0.0
	Yellow bone marrow	0	0.0	0	0.0	5	100.0
Control group	Absent	3	60.0	1	20.0	0	0.0
	Hematopoietically active	2	40.0	2	40.0	0	0.0
	Decreased erythrocytes	0	0.0	2	40.0	0	20.0
	Yellow bone marrow	0	0.0	0	0.0	0	80.0
Total		**5**	**100.0**	**5**	**100.0**	**5**	**100.0**
Normal defect-in-continuity	Experimental	0	0.0	0	0.0	0	0.0
	Absence of formation	0	0.0	0	0.0	0	0.0
	Little formation	0	0.0	0	0.0	0	0.0
	Moderate formation	4	80.0	1	20.0	0	0.0
	High formation	1	20.0	2	40.0	0	0.0
	Almost full filling	0	0.0	2	40.0	0	0.0
	Defect-in-continuity	0	0.0	0	0.0	5	100.0
Total		**5**	**100**	**5**	**100**	**5**	**100**
Control group	Absent	0	0.0	0	0.0	0	0.0
	Undifferentiated cells	0	0.0	0	0.0	0	0.0
	Cell proliferation	5	100.0	5	100.0	0	0.0
	Presence of islets	0	0.0	0	0.0	0	0.0
	Bone spicules	0	0.0	0	0.0	1	20.0
	Mature bone	0	0.0	0	0.0	4	80.0
Peripheral Bone Formation	Absent	0	0.0	0	0.0	0	0.0
	Little	1	20.0	0	0.0	0	0.0
	Moderate	3	60.0	5	100.0	0	0.0
	High	1	20.0	0	0.0	5	100.0
Control group	Absent	0	0.0	0	0.0	0	0.0
	Little	3	60.0	0	0.0	0	0.0
	Moderate	2	40.0	5	100.0	0	0.0
	High	0	0.0	0	0.0	5	100.0

Table 2. Fisher's exact test applied to the control and experimental groups at 30, 60, and 90 days after surgery.

Post-surgical days	Degree of Bone Maturity	Presence and quality of bone marrow	Normal defect-in-continuity	Peripheral bone formation
30	0.167	1.000	1.000	0.524
60	0.048	0.167	0.048	-------
90	-------	-------	-------	-------

observed in the experimental group (Figure 3C), while in the control group (Figure 3D) there is moderate bone formation with 2/3 of the defect filling. At 90 days after surgery, it is observed that both in the experimental group and in the control one (Figure 3E and Figure3F) there is an almost total bone filling of the bone defect.

Peripheral bone formation

It can be observed that at 30 days postoperatively in the experimental group, 60% of the sample presented moderate bone formation, while at 60 days, 100% presented moderate peripheral bone formation (Table 1). At 90 days, 100% presented high peripheral bone formation. In the control group, at 30 days, 60% of the sample presented little bone formation. At 60 days, 100% of the sample showed moderate peripheral bone formation, and at 90 days, 100% presented high peripheral bone formation.

When comparing both study groups, it is observed that only at 30 days postoperatively the experimental group presented better peripheral bone formation (Figure 4); while at 60 and 90 days the results obtained were the same as the control group.

Statistical analysis

Fisher's exact statistical test (Table 2) indicates that there is a statistically significant difference (p=0.048) in the degree of bone maturity parameter at 60 days after surgery when comparing the experimental group with the control group; that is, at 60 days after surgery, there was a greater degree of bone maturity in the experimental group compared to the control group. Likewise, there is also a statistically significant difference (p=0.048) in the parameter normal defect-in-continuity at 60 days post-surgery, which means that in this period in the experimental group an almost total filling of the created bone defect was observed in comparison to the control group.

However, in the parameter presence and quality of bone marrow, no statistically significant difference was observed at 30 (p=1,000) and at 60 days after surgery (p=0.167). Similarly, in the peripheral bone formation parameter, 30 days after surgery, there is no statistical significance (p=0.524) between both study groups. At 90 days post-surgery, in both experimental and control groups, very similar results were observed, that is: the formation of mature bone, the total filling of the created bone defect and the presence of a yellow bone marrow, which might indicate the beginning of a new phase: bone remodeling.

DISCUSSION.

The results obtained in the present study suggest that hyaluronic acid promotes bone regeneration of circumferential bone defects, which were evaluated based on four parameters proposed by Heiple.[11] In the case of the bone maturity parameter, a differentiation of the type of tissue formed was confirmed histologically, from a tissue in the process of mineralization (osteoid) to a mature bone tissue. The experimental group experienced greater and faster bone maturity than the control group.

This suggests that hyaluronic acid accelerates the degree of bone maturity, which may be due to the fact that it is the most abundant organic component of the bone extracellular matrix. Additionally, it performs physiological and structural functions, including cellular and extracellular interactions, growth factor interactions, regulation osmotic pressure and tissue lubrication.[12] The parameter presence and quality of bone marrow was another very important criterion

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to verify the efficacy of bone neoformation. The desirable factor is the presence of an active marrow because it guarantees a constant regenerative cellular activity.

However, the results obtained contradict the theoretical foundations since in the first 30 and 60 days, the experimental group and control group presented an active bone marrow, which would indicate that hyaluronic acid and demineralized bone matrix bio-stimulated the undifferentiated cells, transforming these into fibroblasts and osteoblasts. However, with the passage of time the medullary activity decreased until at 90 days both groups presented a yellow bone marrow, which would indicate the end of new bone formation and perhaps the beginning of the bone remodeling process.

Regarding the parameter defect-in-continuity with the surrounding bone, this was observed histologically in a large part of the sample of the experimental and control groups. Bone formation and bone filling of the defect occurred faster in the experimental group than in the control group. This could have a direct relationship with the degree of bone maturity, only that in this parameter the mineral apposition was located on the margins of the created defect.

Finally, the parameter of peripheral bone formation had a strong association with continuity since the peripheral bone formation of the defect area allows the closure of the defect. In this research, when the biomaterial was implanted within the bone bed, neoformation occurred in two directions, firstly from the peripheral cell layer towards the center (intramembranous) and almost simultaneously from the biomaterial (which acts as a scaffold that guides the regeneration process) such as it occurs in endochondral ossification. In both study groups, a moderate to high peripheral bone formation was observed in the first 30 postoperative days.

At 60 and 90 days, both groups obtained the same results, that is, high peripheral bone formation. The results obtained are similar to those reported by Diker *et al*.,[13] and Agrali *et al*.,[14] since they found that the healing parameters related to bone formation (new bone formation, closure of defects and immature bone formation) were induced by the action of HA[13],and that HA[9] supports the formation of new bone.[10]

It should be noted that HA combined with a demineralized bone matrix was used in the present research because there are studies that indicate that HA only promotes the formation of new bone but does not contribute significantly to bone regeneration,[13] and that if HA is combined with other biomaterials there is positively an improvement in bone defects.[15]

In the research carried out by Taz *et al*.,[16] they studied the efficacy of the injectable granule of biphasic calcium phosphate (BCP) supplemented with hyaluronic acid (HA) to promote bone regeneration, obtaining as a result an improvement in bone formation after 4 weeks of implantation and better handling characteristics for BCP-HA than for BCP.

Likewise, Cai *et al*.,[17] conducted a study where they studied the efficacy of the Chinese medicine magnoflorine combined with hyaluronic acid (HA)-gel to promote subchondral bone (SCB) regeneration and attenuate cartilage degeneration in osteoarthritis (OA). They concluded that their study clarified the potential benefits of HA-gel + magnoflorine in promoting SCB regeneration and revealed a protective effect of stimulating the recovery of SCB integrity in attenuating cartilage degradation to prevent the progression of OA.

Similarly, Matheus *et al*.,[18] evaluated the potential of different formulations of hyaluronic acid to improve the formation of new bone in critical- sized calvaria defects combined with a deproteinized bovine graft material (DBG), demonstrating that the combination of HA in a high viscosity crosslinking agent with DBG enhances the bone repair process and increases the amount of newly formed bone. These studies show, like the present study, that hyaluronic acid combined with another biomaterial improves the bone regeneration process.

Within the limitations of the present study, it can be concluded that hyaluronic acid is effective in the treatment of circumferential bone defects. In the four parameters proposed by Heiple[9] studied histologically, hyaluronic acid was shown to positively improve the bone regeneration process, accelerating it, and enabling complete regeneration of the circumferential defect. In addition, its physical characteristics, relative low cost, and ease of handling would be additional benefits in its clinical use. The use of demineralized

bone matrix also demonstrated good properties as a graft material, completing in the same way, the bone regeneration process of the circumferential defect. Hyaluronic acid by itself is perhaps not a biomaterial with osteoconductive properties, but by interacting with a demineralized bone matrix it improves its regenerative properties.

Conflict of interests: The authors declare that they have no conflict of interests.

Ethics approval: This research was approved by the Research Ethics Committee of the Alas Peruanas University (Constancia 017-2018).

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Authors' contributions: All authors participated during the development of the research, review of results, writing of the scientific article and validation of the manuscript. Bueno-Beltrán C: Coordinated with other institutions for the development of the research. Bueno-Beltrán C, Budiel-Salguero Y and Palacios-Bustamante S participated in the performance of the surgical act. Budiel-Salguero Y and Palacios-Bustamante S participated in the selection of study units and post-surgical care and monitoring.

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