Article **ECORFAN Journal Republic of Guatemala**
June 2018 Vol.4 No.6 17-26

Histological study of platelet-rich plasma on lesions induced in the lab rabbit femur

Estudio histológico del plasma rico en plaquetas sobre lesiones inducidas en el fémur del conejo de laboratorio

MARTÍNEZ-AGUILAR, Pablo Isaac†*, GARCÍA-SUASTEGUI, Wendy Argelia, HANDAL-SILVA, Anabella and MORÁN PERALES, José Luis

Departamento de Biología y Toxicología de la Reproducción, Instituto de Ciencias / Benemérita Universidad Autónoma de Puebla

ID 1st Author: *Pablo Isaac, Martínez-Aguilar*

ID 1st Coauthor: *Wendy Argelia, García-Suastegui* / **ORC ID:** 0000-0001-5223-3189, **Researcher ID Thomson**: S-6831-2018, **CVU CONACYT ID**: 48932

ID 2nd Coauthor: *Anabella, Handal-Silva* / **ORC ID:** 0000-0002-6915-5655, **Researcher ID Thomson**: S-6799-2018, **CVU CONACYT ID**: 210819

ID 3rd Coauthor: *José Luis, Morán Perales* / **ORC ID:** 0000-0002-2823-2829, **Researcher ID Thomson**: S-5803-2018, **CVU CONACYT ID**: 207096

Received September 10, 2018; Accepted November 08, 2018

Abstract

We analized the effect of platelet rich-plasma (PRP) on a induced lesion (IL) over femoral diafsis in rabbits to evalued the bone recuperation time. We used 10 male New Zeland rabbits with 1.8 at 2.0 kg of body weight. The rabbits were assigned in two experimental groups: induced lesion (IL) treated with PRP group and IL control group without PRP. IL consisted in a trephine on the femoral diafisis that consisted in a 5mm diameter hole. The PRP was obtanined by blood extraction of 3 ml that was centrifuged at 1400 rpm in 7 minutes and recovered plasma and newly centrifuged at 200 rpm in 15 minutes; each PRP fraction was actived with 10% $CaCl_2$ and then was collocated over the trephine. We obtained histological samples of IL region from second to sixth week. The PRPactivated treatment on IL makes to recovered the bone structure and the speeds process of bone healt from third week in the laboratory rabbit.

New Zeland Rabbit, Platelet Rich-Plasma, Induced Bone Lesion, Femur Histology

Resumen

Se analizó el efecto del plasma rico en plaquetas (PRP) sobre una lesión inducida (LI) en la diáfisis femoral del conejo, para evaluar el tiempo de recuperación ósea. Se utilizaron 10 conejos machos de la raza Nueva Zelanda Blanco con un peso de 1.8 a 2.0 kg. Los conejos fueron divididos en 2 grupos: grupo con lesión inducida (LI) tratado con PRP y grupo testigo con lesión inducida sin PRP. La LI consistió en un trépano en la diáfisis del fémur por medio de un orificio de 5 mm de diámetro. El PRP se obtuvo por extracción de 3 ml de sangre que fue centrifugada a 1400 rpm por 7 minutos tomando el sobrenadante, que nuevamente se centrifugó a 2000 rpm por 15 minutos; cada fracción obtenida de PRP fue activada con cristales de $CaCl_2$ al 10% y se colocó directamente sobre el trepano. Se obtuvieron muestras histológicas de la región con LI a partir de la segunada semana de evolución. El tratamiento con PRP activado en la LI mejora notablemente el tiempo de consolidación del hueso y acelera el proceso de regeneración ósea a partir de la tercera semana de evolución en el conejo de laboratorio.

Conejo Nueva Zelada, Plasma Rico en Plaquetas, Lesión Ósea Inducida, Histología del Fémur

Citation: MARTÍNEZ-AGUILAR, Pablo Isaac, GARCÍA-SUASTEGUI, Wendy Argelia, HANDAL-SILVA, Anabella and MORÁN PERALES, José Luis. Histological study of platelet-rich plasma on lesions induced in the lab rabbit femur. ECORFAN Journal-Republic of Guatemala. 2018, 4-6: 17-26.

* Correspondence to Author (email: moranperales@yahoo.com.mx)
† Researcher contributing first author.

Introduction

In the Veterinary Practice, attention to injuries of the muscular skeletal system is frequent. In order to prevent serious and even lethal consequences in domestic animals that come to the hospital or clinic, the care of fractures must be done carefully to avoid causing situations that could aggravate the picture, for this routine maneuvers are applied as they are: Diagnose the fracture, immobilize and, if necessary, perform a surgical intervention to coact the separated segments, applying implants, placing brackets or fixing the affected parts (Santoscoy, 2010).

Bone fractures in most cases can heal correctly with minimal but careful handling, for example: an immobilizer can give excellent results, as long as the animal can reduce its movement and move away from conditions that lead to relapse, aggravating the injury or injury. Therefore, the rapid restoration plays an important role for the animal to recover in the broad sense.

The rejection of implants and the lack of consolidation of fractured parts has led to the search for biomaterials to promote bone regeneration, finding a response in platelet-rich plasma (PRP) as a useful adjuvant.

This compound is the supernatant that is obtained once the anticoagulated blood is subjected to centrifugation, generating a plasma that contains more platelets than the peripheral blood. The use of PRP was proposed by Marx and Carlson in 1986, for the placement of bone grafts in oral and maxillo-facial surgery, but aroused interest in other areas of biomedicine, such as orthopedics, otorhinolaryngology and reconstructive surgery. (Carrillo et al, 2013).

The use of PRP as a surgical complement in Veterinary Orthopedics has been recommended for its modulating and stimulating action on the proliferation of stem cells derived from mesenchymal stem cells, in addition to causing the release of some growth factors such as: TGF-beta, PDGF, FGP, IGF, among others (Faudez et al, 2010). In the literature, multiple techniques on the use of PRP are described, mainly in methods of maxillofacial surgeries and spinal surgery. However, there are few control studies that determine and quantify the magnitude of the effects of PRP.

The purpose of the present study was to analyze the effect of PRP placed on an injury induced in cortical bone, assuming that the process is similar to what occurs in a fracture, using the laboratory rabbit (New Zealand White) as a study model. The process of consolidation of the bone lesion was analyzed by means of conventional histological techniques with the general purpose of promoting the use of PRP as a reducer of fracture consolidation times.

Justification

Several studies have shown that bone fractures heal successfully by using various regenerative techniques and materials that stimulate bone tissue and surrounding organs. However, according to Butterfield et al. (2004) it has not been possible to demonstrate the efficacy of PRP treatment in osteoregeneration processes in rabbits. Other studies have demonstrated their effectiveness using the model of the miniature pig and recommend it as an adequate alternative to successfully heal bone and maxillofacial lesions (Schlegel et al, 2004; Schlegel et al, 2007).

Aghaloo et al. (2004) conducted a study on rabbit parietal bone grafting a surgical defect with autogenous bone and treatment with PRP. A radiographic, histological and histomorphometric analysis showed a slight tendency to increase bone density when PRP was used. In another similar study, demineralized bovine bone was used and a higher bone density could be shown in the cases with autogenous grafted bone, with respect to the groups grafted only with demineralized bovine bone and PRP (Laguna et al, 2006).

Roldan et al. (2004) using the Wistar rat model, observed that the application of PRP did not enhance bone formation in bovine inorganic bone or in grafts with autogenous bone. In another study on the frontal bone of pigs, the preparation of the graft bed with PRP does not seem to have an influence on osseointegration. Zechner (2003), however, created mandibular defects in twelve dwarf pigs and when applying PRP and installing the implants observed a better peri-implant bone regeneration in the initial phases (6 weeks), equaling the stimulation of osteogenic cell proliferation at 12 o'clock. weeks.

ISSN-On line: 2414-8849

For all the above, we can infer the importance and differentiation in the methods of obtaining the PRP in addition to the defect to be treated. There are reports on its application in muscle, tendon injuries or in its use in maxillofacial surgery, but these are preliminary clinical studies without long-term follow-up (Laguna et al, 2006).

Problem

The use of PRP in Veterinary Orthopedic Medicine is a little used method, since it implies the use of optimal infrastructure to be able to perform the correct extraction, in addition the diffusion of its use as a surgical adjunct is poor. Although it is known that the fracture process involves the release of multiple biochemical factors that lead to platelet activation, in this work we studied the effect of PRP administration on experimentally damaged bone tissue and bone recovery will be evaluated histologically in a cortical bone like the lab rabbit's femur.

The present work aims to show that the PRP is a useful resource for the acceleration in the regeneration of fractures or injuries in long bones, which require a surgical intervention to ensure the immobilization of the affected limb. The data obtained may be used in cases of surgical intervention in veterinary clinical management in the orthopedic area, in order to assess the time and effectiveness of bone recovery.

Hypothesis

The use of PRP in an induced fracture in the femur of the rabbit will reduce the time of bone repair with respect to the use of the conventional surgical technique used for the management of fractures in cortical bones.

General objective

To analyze the use of PRP on a fracture of the femur induced in the experimental model of the rabbit and bone reconstruction by means of conventional histology.

Theoretical framework

In the red bone marrow originates different phases of progenitor cells, which differ in cells of the erythrocytic, granulocytic, megakaryocytic and agranulocytic series.

The final result of this production is the emission of erythrocytes, leukocytes and platelets Megakaryocytopoiesis begins with the development and formation of megakaryoblasts, these are a large cell with a single nucleus, this cell is progenitor of promegakaryocytes, which are cells Larger than megakaryoblasts and with a multilobed nucleus, the precursor cells of the promegacariocytes are megacaricitos, which are cells with a single nucleus and abundant cytoplasm (Geneser et al, 2000).

Platelet formation takes place in the cytoplasm of the megakaryocytes through the formation of a structure known as proplaqueta, which suffers a fragmentation process resulting in small cells of discoidal and anucleated form, these are marked as platelets. Platelets are cells that fulfill different functions and have a specific structure (Reagan et al, 1999).

Platelets form a major role during the hemostasis process, after a vascular lesion initially seals the vascular defects and generates contact with the exposed extracellular matrix elements such as collagen and Von Willebrand Factor, in addition they provide a surface on which Active coagulation factors are recruited and grouped. The participation of platelets in the processes of hemostasis and thrombosis depends on the action of 3 events: platelet adhesion, change in shape and platelet aggregation (Milagros et al, 2000).

The bone is a specialized form of dense connective tissue, the extra cellular components suffer a calcification, providing the main function to the bones, being a supporting device, since they insert the muscles; In addition to providing protection to the thoracic viscera and the central nervous system, the bone system is of great importance for the regulation of the metabolism of calcium and phosphorus in the blood, which enables its homeostasis, bone growth and repair of the bones. fractures (Geneser et al, 2000).

Bone histology is diverse and functionally complex. The main cell types of the bone are: Osteoprogenitor cells: These are stem cells of pluripotent mesenchymal origin, present in the vicinity of the entire bone surface. In the bones of adults it retains its osteoprogenitor capacity that can be manifested through stimuli (such as a fracture). Osteoblasts:

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These are bone-forming cells derived from osteoprogenitor cells. It is disposed on the osteoid border, these cells synthesize, transport and organize the numerous proteins of the bone matrix and are in charge of initiating the mineralization process due to the accumulation of phosphate and calcium granules in their mitochondria, this content will pass to the calcification vesicles that will intervene directly in calcification and osteoregeneration.

Osteocytes: These are osteoblasts incorporated into the calcified bone matrix. These cells communicate with each other and with the cells of the bone surface through a wireless network of cytoplasmic processes that pass through tunnels in the bone matrix, called canaliculi, allowing the transcellular transport of substances captured by cells of the bone covering, in addition to the existing diffusion by means of the extracellular fluid that surrounds the extensions in the canaliculi. What is extremely important to start the remodeling of the bone tissue.

Osteoclasts: These are the cells responsible for bone resorption, these are the cause of bone destruction in bone remodeling. Bone is formed where mesenchymal connective tissue or cartilage previously exists. If the ossification takes place from mesenchymal connective tissue it is called intramembranous ossification, because it develops between conjunctive membranes that will give rise to the membranous bones such as the cranial vault. If the ossification takes place from the cartilage it will be called endochondral ossification and it is the one that originates the axial skeleton, the appendicular and the chondrocranium.

In both types of ossification the bone tissue that is deposited will be of the immature type, later this tissue will be organized and will become laminar, forming first all the spongy type bone and subsequently the one that will be compact. In any type of ossification, calcification of the extracellular matrix is essential. This calcification is granted by the secretion of the ossification vesicles (Geneser, 2000). Tissue regeneration occurs through a series of events at the cellular and molecular level, which are regulated by signal proteins. These substances with paracrine actions are released by the surrounding tissues damaged and emit signals to the cells of the hematopoietic system.

Platelets assume control of vascular regeneration when the subendothelium comes into contact with the plasma and induces a series of chain reactions that lead to the formation of a platelet plug and a blood clot, in addition to the secretion of biologically active proteins which they are secreted by the platelet structure (Barret et al, 2010). In the platelets there are alpha granules, which contain numerous proteins that influence healing or known as growth factors derived from platelets. The alpha granules also secrete proteins that will function as defense against external pathogens, through signal proteins that will attract macrophages, in addition to the leukocyte cells of the plasma. The platelets activate these proteins around 10 minutes after clot formation, reaching 95% activation in about 1 hour. After this the platelets synthesize and secrete additional proteins while they remain alive between 5 and 10 days (Morales, 2014).

When the platelets begin to diminish their action, the macrophages that arrived through the bloodstream stimulated by the platelets assume the responsibility of the regulation of the healing establishing the place of the regeneration by hand of the platelets (Maczy et al, 2012; Schlegel et al. al, 2004).

There are chemical signals that tissue cells produce and release to have paracrine effects on surrounding organs, which allow us to establish all the physiological conditions for the maintenance and regeneration of organs. Several studies have shown that bone fractures heal successfully by using various regenerative techniques and materials that stimulate bone tissue and surrounding organs. However, according to Butterfield (2004) it has not been possible to demonstrate the efficacy of PRP treatment in osteoregeneration processes in rabbits. Other studies have demonstrated their effectiveness using the model of the miniature pig and recommend it as an adequate alternative to successfully heal bone and maxillofacial lesions (Schlegel et al, 2004; Schlegel et al, 2007).

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Research Methodology

Biological material

Ten male rabbits of the New Zealand White breed with a weight of 1.8 to 2.0 kg were used. The rabbits were divided into 2 groups: group with induced fracture treated with PRP (Group FI + PRP; N = 5) and control group with fracture induced without PRP (FI; N = 5).

Anesthesia Procedure and Surgical Technique

The surgical technique and post-surgical accommodation were carried out in accordance with the guidelines set by the CICUAL-BUAP and NOM-062-ZOO-1999. All animals were subjected to the anesthesia protocol recommended by the Institutional Committee on Animal Use and Care of Cornell University (Gourdon, 2010, Martin & Kirsipuu, 2017).
For the process of sedation and anesthesia, the marginal vein was canalized using a No. 4 catheter, in order to avoid rupturing the vein or obstruction by coagulum. During the entire surgery 10 ml / kg / hour 0.9% NaCl was infused in each patient.

The anesthesia of the animals was performed with a Ketamine-Xylazine mixture (20 mg / kg / ketamine + 5 mg / kg / xylazine; iv) (Martin & Kirsipuu, 2017). Once the animal was anesthetized, the area to be incised with lidocaine was marked and blocked to reduce the pain, where the dermis and the epidermis were incised with a # 24 scalpel, to be able to approach the thick fascia lata muscle, which was incised to find the aponeurosis of the biceps femoral and vastus lateralis muscles; this tissue was debrided to expose the femoral diaphysis.

To avoid affecting the biomechanics of the bone, a trepano of small dimensions was performed in the femoral diaphysis with a manual drill of Jacob, until forming a hole of 5 mm in diameter and entering the medullary canal and touching the bone in its contralateral side.

Obtaining Plasma Rich in Platelets

On the day of surgery, five animals were assigned to the group with FI + PRP to obtain the PRP fraction. For this, the following steps were carried out, according to the Maczy and collaborators technique (2012):

Venous puncture: puncture was performed in the area of the neck in the jugular vein since this area is the least traumatic for the patient, blood must be obtained 10 minutes before surgery, the amount of blood required will depend on the weight, size of the patient and the defect to be treated. In the present work 3 ml of blood was extracted in each patient.

Extraction of blood: blood was collected in a tube with sodium citrate, as an anticoagulant to have a better activation process, since sodium citrate promotes platelet aggregation because it acts as a calcium scavenger and prevents the premature activation of platelets.

Platelet separation: this phase of the separation must be carried out with the appropriate equipment and ensure obtaining the highest concentration of platelets per unit volume, avoid rupture thereof and avoid premature activation of platelets. When the anticoagulated blood was centrifuged, 3 fractions were obtained according to their density: 1) Lower layer: density of 1.09, composed of red blood cells. 2) Medium layer: density of 1.06, composed of white blood cells and platelets (PRP). 3) Top layer: density 1.03, composed of plasma.

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This protocol is based on the separation of the formed elements from the blood and is carried out as a function of the density from highest to lowest. In this study the double centrifugation protocol was used, which consists in carrying out the first centrifugation process at 1300 RPM for 7 minutes, achieving the separation of the whole blood in a lower strip of red blood cells and a yellowish upper plasma, which will be platelet poor plasma (PPP). In order to obtain the plasma fraction with greater purity, the upper plasma fraction was extracted with a micropipette and centrifuged again at 2000 RPM for 15 minutes to obtain the fraction with the highest content or concentration of platelets (Beca et al, 2007; Martínez et al, 2002).

Activation of Plasma Rich in Platelets

Each sample of PRP was extracted with another micropipette and placed in another sterile tube, until the time of activation with 10% calcium chloride crystals to reverse the coagulation, add a proportion of 0.05ml of calcium chloride per 1ml of PRP, this mixture was later deposited directly in the trephine of each exposed femur of the experimental group (Figure 1) (Danche, 2006).

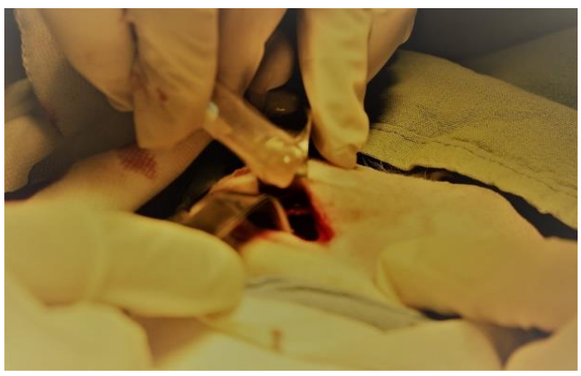

Figure 1 Form in which the PRP was applied; platelet activation with calcium chloride 10% produces a reaction transforms the PRP into a gel of easy application

Postoperative care

The 10 animals submitted to the surgery received isolation in the Claude Bernard Bioterio for their recovery. Hosted in individual cages, they received water and balanced feed ad libitum; the cleaning of the cages was done every day. All the animals were monitored daily until their sacrifice. During their lodging, all the animals received antibiotic therapy and postoperative analgesia with Enrofloxacin (5 mg / kg; s.c.) and meloxicam (3.5 mg / kg; s.c.), for five days.

The wound was subjected to daily cleansing with 1% chlorhexidine gluconate solution, for ten days until the healing was completed.

Autopsy and Obtaining Samples

To obtain samples of the femur, one animal from each group was transferred to the Neuroendocrinology Laboratory of the Department of Biology and Toxicology of Reproduction, of the Institute of Sciences of the BUAP, where euthanasia was applied to the 2nd, 3rd, 4th, 5th and 6 weeks with sodium pentobarbital (60 mg / kg, ip), to obtain samples of the left femur in each interval of postoperative evolution. The bone samples were obtained by cutting the femoral shaft with a saw. In all cases, the right femur was dissected as a control. The samples were fixed in 10% formaldehyde for 24 hours, before being processed for demineralization (Kemmerman et al, 1995), paraffin block assembly and histological section (Luna, 1975).

Histology of the Bone Tissue Samples

The demineralization process was carried out in the space of four weeks per sample, removing the acid on a daily basis and checking with the chemical methods every third day. From the fourth week, the bone tissue samples were ready to be included in paraffin. Strips were formed with 6 consecutive cuts and were left to float in the tub until the strip was extended avoiding creases, and were mounted on a slide with 3% inclusion gelatin to adhere the tissue cut to the glass.

Once assembled the tissue sections were allowed to dry at room temperature and placed in a closed chamber with formaldehyde vapors for 24 hours, before being dyed. All the dry sections were subjected to a stain with the hematoxylin-eosin technique (Luna, 1975).

Results

The results of the present study confirm the efficacy of PRP treatment for healing fractures or bone lesions. In general, after three weeks of evolution, a high proliferation of differentiated osteoblasts of progenitor cells was found in the histological sections of the injured bone. Similarly, large amounts of osteoprogenitor cells related to the endosteum and osteoblasts were found in areas with tissue formation.

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In addition, it was very common to find more collagen fibers in the group treated with PRP than what was observed in the animals of the control group. There were also signs of greater formation of canaliculi in the animals treated with PRP with respect to the control.

Figure 2 shows the appearance of an intact bone compared to the appearance of a lesion (induced fracture) in the second week of evolution. The animal with lesion without treatment with PRP still shows clear signs of tissue alteration characterized by the presence of leukocytes; however, no signs of necrosis were observed.

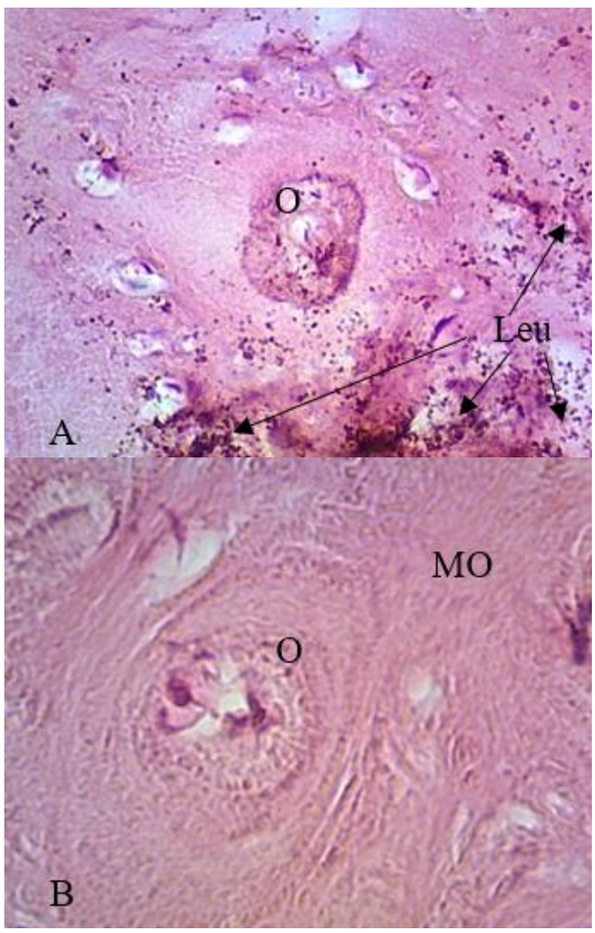

Figure 2 Images at 100X that show the appearance of the femoral shaft with the induced lesion (A) with respect to the diaphysis of a bone without fracture (B) at the second week of evolution. In A, the dense infiltration of leukocytes visible in the third postoperative week can be observed, even in areas of bone consolidation. The intact bone shown in photograph B allows us to observe the region around an osteone in healthy bone. O: Osteona; Leu: Leucócitos; MO: Bone matrix

From the third week of evolution, there is a visible regeneration of the mineralized bone matrix in the group of animals treated with PRP with apparent signs of regeneration clearer than in a control animal without treatment.

In both groups, the process of bone matrix formation is evident; however, in the animals treated with PRP, a greater degree of consolidation could be observed in the areas of bone formation, which was not observed in the control group (Figures 3 and 4).

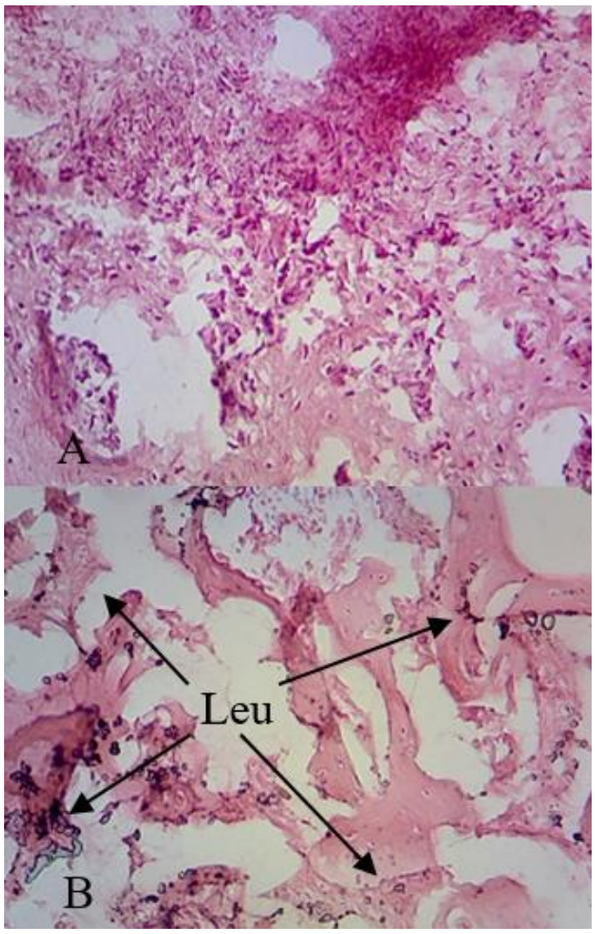

Figure 3 Images at 40 X that show the injured areas at the second week of evolution. A: treatment with PRP; B: bone of the control animal. Compared to the control, a better consolidated bone matrix is observed in the animal treated with PRP and virtual absence of leukocytes in the field

From the fourth week on, no great differences in bone regeneration between the experimental groups are observed; however, it was possible to notice the difference in the bone matrix since in the group treated with PRP, the process of mineralization of the matrix is more advanced and almost reaches its totality in comparison to the control group where the matrix remains largely cartilaginous (Figure 4).

The process of regeneration, restructuring and bone remodeling is favorable in the reduction of healing time in animals treated with PRP. It was possible to compare the presence of a zone of high cell proliferation in the group treated with PRP, which would reduce the bone healing time.

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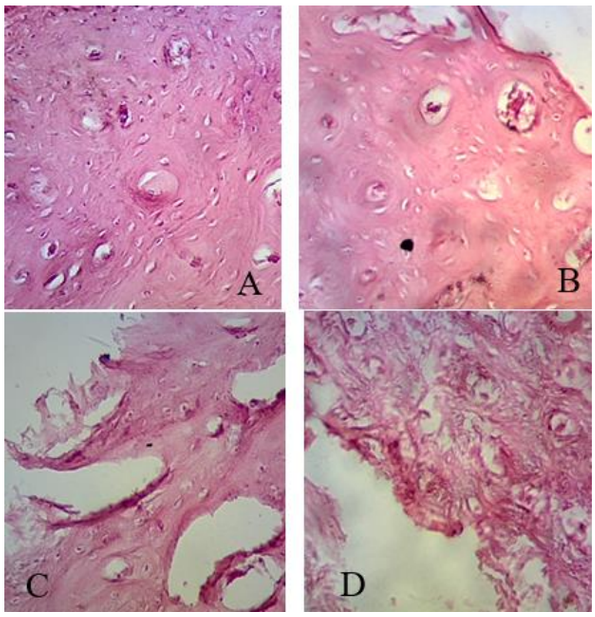

Figure 4 100X images showing the appearance of the bone matrix (MO) in animals treated with platelet-rich plasma (PRP) (Right)) and their controls (Left) by the third week. In the photographs of the control animals (B and D), a process of consolidation of the MO is apparently slower than in the animals treated with PRP, where the cartilaginous matrix (MC) in the regeneration zone is less dense than in the animals. the animals treated with PRP. In the animals that received the treatment with PRP (A and C), a greater quantity of osteocytes (OC) is noted and differentiated cells converted into osteoblasts (OB) can be found; however, in the control group, areas with a large population of osteoclasts (OT) predominate; CA: capillaries; ZM: Spinal Zone

Discussion of results

After assessing the effectiveness of bone regeneration after an induced lesion treated with PRP, the histological sections analyzed showed effective changes, particularly in areas of high bone regeneration that were more evident than in the animals that did not receive the treatment. The apparent reduction of time to heal the induced fracture could be a sign of the stimulus induced after the application of PRP.

Experimental data report that the use of PRP induces acceleration in bone regeneration in the first weeks after an injury or surgery in maxillofacial surgery (Danche, 2002, Fontana et al, 2004, Roldán et al, 2004). These works coincide in that effective results can be observed up to the fourth week with respect to the controls. However, as of the fourth week, a decrease in the speed of bone regeneration is noticeable, to the point of being minimally exceeded by the control group at 8 weeks.

Zechner (2003) suggests a PRP action dependent on time. In a study conducted in pigs, he affirms that PRP favors osteorregeneration around the sixth week; in his work he describes that, from that moment the effect of the PRP ceases in such a way that there are no structural differences in the regenerated bone with respect to what was observed at 12 weeks.

Several studies suggest that platelets significantly increase the proliferation of bone cells in adult individuals, stating that platelets act as local regulators in posttraumatic bone regeneration, possibly because of the growth factors released by platelets and attracted by means of chemotaxis (Gruber et al, 2002; Soffer 2004).

It has also been observed that the effect of PRP at the site of application lasts 5 days, but its effects on the acceleration of bone regeneration can be observed up to 4 weeks after its administration (Weibrich, 2004, Marx and Carlson, 1998). We chose to follow the technique proposed by Maczy and collaborators (2012), since it is an efficient, low cost method and preserves the autologous procedure since the activation is carried out with calcium chloride. However, it should be mentioned that there is no method that can be called "standardized", although there are comparative studies between the different methods of obtaining the PRP, none seems to be better than another, because apparently everything depends on the experimental conditions in the moment of its application. It has already been described that there are determinant variables such as the type of bone, its approach and even, the properties of the surrounding tissues where it is applied and the activation process (Beca et al, 2007).

Recent studies have estimated the concentrations of growth factors and cytokines contained in the PRP with various cellular concentrations and it has been observed that the amount of these elements is lower compared to the amount of plasma. Similarly, these studies indicate that low centrifugation speeds produce PRP with low purity and suggest that red blood cells release free radicals in large quantities, which could generate damage to the tissue that is desired to stimulate (Montesinos et al, 2017). The protocols analyzed and compared between different researchers allow us to suggest the use of the PRP obtaining technique that was used in this work, as long as the fracture model is similar.

ISSN-On line: 2414-8849

The rabbit as an experimental animal seems to be adequate because the process of bone formation and remodeling is three times faster than in other species (Roberts et al, 1988). In humans the remodeling process lasts between 6 and 9 months; This period of time is known as sigma. Thus, the sigma for the dog lasts 3 months and for the rabbit 6 weeks (Danche et al, 2006). The choice of trepan size (5mm diameter) was to wait for the prompt regeneration of the organism without PRP treatment, in addition to avoiding possible affections and unnecessary suffering for rabbits. On the other hand, the choice of femoral diaphysis as a test tissue for induced fracture was based on the fact that ossification is endochondral, where cartilage is formed first and then bone, which is important for the interpretation of the regeneration process bone with orientation to the reduction of fractures surgically.

It is important to note that the manipulation of blood is accompanied by possible changes in the quality of blood cells; These imponderable changes can be significant alterations that can affect the functioning and the interaction of the cells. The objective of the activation of the PRP before its application is to inhibit these alterations and obtain an optimal functioning of the PRP (Carrillo et al, 2013; Saez et al, 2007).

Conclusion

The treatment with activated PRP in bone lesions significantly improves the time of bone consolidation and accelerates the process of bone regeneration after a lesion from the third week of evolution in the laboratory rabbit.

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Agradecimientos

Nuestro Cuerpo Académico (CA-090) agradece al MVZ Francisco Ramos Collazo, director del Bioterio *Claude Bernard* de la Benemérita Universidad Autónoma de Puebla, médico asignado al cuidado del bienestar de nuestros animales de experimentación, todas las facilidades y atenciones para el desarrollo del proyecto. También al MVZ Martín Herrera Pelaez, jefe de quirófanos de la Facultad de Medicina de la BUAP, por sus valiosas enseñanzas y atenciones para la realiación de este trabajo.