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Biomechanical evaluation of the effect of acellular amniotic membrane loaded by autologous platelet rich plasma on bone healing in a dog model

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ABSTRACT

It may be possible to hasten the healing of bone tissues using a platelet concentrate composed of cytokines and structural glycoproteins entrapped within polymerized fibrin meshwork. Due to the availability of numerous growth factors, the amnion membrane has also attracted significant attention in the field of bone regeneration. The aim of the present study was to evaluate the effect of an amniotic membrane loaded with platelet-rich plasma on the healing of the fracture. These effects were demonstrated objectively by biomechanical testing. Fifteen mongrel dogs were used in this study. The animals were divided into three groups (n = 5 per group). A transverse osteotomy was induced in the midshaft of the left tibia of all animals, and then two groups of them were treated with bioscaffold (amniotic membrane) and platelet-rich plasma, while the third group was left without treatment. All fractured tibias are followed by a bone plate with screws as the following: The first group was treated with acellular amniotic membrane only by wrapping it around the fracture site. The second group was treated with acellular amniotic membrane accompanied by platelet-rich plasma, while the third group (control) was left without treatment. On day 56, all of the dogs had biomechanical tests done with the Universal Testing Machine, which was placed between the ends of the bones at the osteotomy site. The average maximum loads in Group (AM+PRP) were higher than in other groups (2.14 ± 0.050). The values of the (AM) group were (1.52 ±0.086). While fracture values were found to be lower in the control group than in the (AM) and (AM+PRP) groups (1.05 ± 0.050). It was found that bones recovered with AM+PRP (group) had the highest value and tibias recovered without treatment (control group) had the lowest value. Of the current study was that the application of amniotic membrane loaded with platelet rich plasma to the fracture site significantly improves the biomechanical strength of the fracture union.

1. INTRODUCTION

A partial or complete break in the continuity of hard tissues, such as cartilage and bone, is referred to as a fracture in medicine. Long bone fractures frequently occur in small animal orthopedics [1].

The ultimate goal of fracture repair is for patients to heal more quickly and walk sooner. Therefore, monitoring the rate and pattern of fracture healing is essential to identifying issues early on and implementing the appropriate interventions. According to many studies, serum biochemical analysis is one of the monitoring procedures that can detect fracture complications, fracture illnesses, and early signs of fracture healing [2].

Several treatments have been tried to improve bone healing. such as bone morphogenetic proteins (BMPs), parathyroid hormone, and bisphosphonates [3].

platelet-rich plasma, mesenchymal stem cells, Despite the fact that AM has been deemed the gold standard for treating bone abnormalities and has had positive results, The amniotic membrane (AM), a naturally occurring high-molecular-weight biological component, is composed of the basement membrane (BM), an avascular collagenous stroma, a single layer of epithelial cells, and underlying fibroblasts. AM has high levels of extracellular matrix (ECM) elements, including fibronectin, laminin, elastin, type I and type II collagen, and type IV collagen. Due to its beneficial effects on cell survival, migration, proliferation, and differentiation, the ECM is an essential component for tissue regeneration or organ creation. ECM can also offer mechanical assistance and add a variety of biophysical and pharmacological stimuli [4], Khashjoori et al., 2019).

The distinctive qualities and benefits of AM have led to its widespread usage in both scientific research and clinical practice. Fresh AM does have several drawbacks, though, like the immunogenicity of the tissue, inappropriate preservation techniques, and difficult shipping. Acellular amniotic membrane (AAM) scaffolds have been successfully created and used in tissue regeneration. AAM scaffolds are derived from fresh AM by removing the cellular components. AAM scaffolds contain several characteristics in common with fresh AM, such as a network structure, an abundance of collagen fibers, and a particularly high concentration of collagen II. AAM has been shown in some tests to lessen acute liver injury and speed bone repair [5].

(AAM) has developed into a desirable biological tissue. AM is a freely available, placenta-derived biomaterial that poses no moral dilemmas. AM is a source of growth factors and has been shown to have anti-cancer properties as well as low immunogenicity. AM has been utilized in medicine for the treatment of wounds for more than a century. Only a small number of studies, meanwhile, have examined the potential of AM in orthopedic surgery. It expresses anti-inflammatory proteins and growth factors, including VEGF and TGF-1. Lastly, several publications observed that AM had osteogenic properties [6].

Platelet-rich plasma (PRP) therapy is one of the alternative autologous treatments that has become more and more popular in recent years for the induction of fracture healing [7]. Studies have demonstrated that thrombocyte-derived growth factors released by PRP during the healing of a fracture can promote angiogenesis and stimulate the growth and chemotaxis of chondrocytes, osteoblasts, and mesenchymal cells. [10], [11]. The development of nonunions has been attributed to a deficiency in these growth factors, which have been shown to expedite bone repair in high concentrations. Therefore, in theory, platelet concentrates high in growth factors offer an osteoinductive therapy for the biological enhancement of fracture healing [9].

For tissue repair and regeneration, it is necessary to produce platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF beta 1 and 2), and insulin-like growth factor (IGF-1). An easy and economical technique to achieve high concentrations of these growth factors is

through autologous platelet concentrations [8].

The hypothesis of the present study was that amniotic membrane and platelet-rich plasma had good therapeutic outcomes on bone healing in terms of biomechanics.

In terms of demonstrating whether PRP and AM boost the biomechanical strength of fracture healing or not, this work is clinically valuable. This study sought to determine how platelet-rich plasma-loaded amniotic membrane affected fracture healing. Biomechanical testing was used to objectively show these impacts.

2. Materials and methods

The present study was approved by the Animal Ethics Committee of the veterinary college at Basrah University, and all experiments described in it were carried out in strict compliance with the rules for animal experiments released by the mentioned college. The animals were set up under common circumstances and in the shade. Aseptic procedures were used during the operation. Before and after surgery, blood was drawn from the cephalic vein for regular diagnostics. In addition, anti-parasitic medications (Ivermectin, Levamisole) were given, and each dog received an injection of the rabies vaccine. Manual feeding was used. Additionally, the water and diet were easily accessible as well.

2.1 Study Design

Fifteen mongrel dogs weighing 20–23 kg, aged between 1–1.5 years (irrespective of sex), were used in this study.

All dogs underwent a clinical examination and a complete blood count (CBC) to determine if they had any systemic disease. The animals were divided into three groups (n = 5 per group). A transverse osteotomy was induced in the midshaft of the left tibia of all animals and then treated with bio scaffold and platelet rich plasma, followed by fixing with a bone plate with screws as the following: The first group was treated with acellular amniotic membrane only by wrapping it around the fracture site. The second group was treated with acellular amniotic membrane accompanied by platelet-rich plasma, while the third group (control) was left free without treatment. During the experiment, dogs were housed separately in cages.

2.2 The Amniotic Membrane Preparation

The placenta was obtained from a pregnant cow after natural parturition and rinsed three times in sterile physiological saline solution in order to dislodge the clots. Immediately after being separated from the chorion, the amnion was thoroughly washed in phosphate-buffered saline (PBS) that contained 100 U/ml penicillin and 100 g/ml streptomycin.

The modified medium (Dulbecco's modified Eagle's medium) and glycerol in a volume ratio of 1:1 were used to store the amniotic membrane at -80C. The amniotic membrane was defrosted, washed three times with sterile PBS containing the antibiotic, and then sliced into roughly 10 x 10-cm pieces just before use. After two hours of incubation with 0.02 percent ethylenediamine tetra acetic acid at 37°C and gentle scraping with a cell scraper, the overlaying amniotic epithelial cells were removed. (Nakase et al., 2008).

2.3 Preparation of the Autologous PRP

Each dog in the second group (AM+PRP) had 10 ml of blood taken and placed into evacuated blood tubes containing citrate-phosphate dextrose solution before being centrifuged for 10 min at 1500 rpm. Following centrifugation, three layers were produced. After collecting the top plasma layer in a different centrifugation tube and undergoing a second centrifugation for 10 minutes at 3000 rpm, two layers—the upper layer being

platelet poor plasma (PPP) and the lower layer being PRP—were obtained. This platelet-rich plasma was used in the fracture sites of our experiment with the dogs. (Khalifa et al., 2021).

2.4 Surgical Procedure

After a 12-hour fast, each dog was given the prophylactic antibiotic cefazolin (22 mg/kg intravenously) half an hour before the surgical procedure. Regardless of the length of the operation, further dosages were given every two hours throughout general anesthesia. They were then anesthetized with xylazine hydrochloride 2% at a rate of 5 mg/kg body weight I/M and ketamine hydrochloride 10% at a rate of 10 mg/kg body weight I/M. The dogs were placed in lateral recumbency. From the level of the greater trochanter to the level of the metatarsal, the skin on the medial surface of the right hind limb was prepared by hair clipping and then scrubbed with povidone-iodine and draped for sterile surgery. The cranio-medial aspect as a surgical approach was used. An incision was made in the limb to expose the medial aspect of the diaphysis of the right tibia, and the periosteum was separated along its mid shaft by 4 cm. The tibial diaphysis's center underwent a transverse osteotomy by using a charrier bone saw. The fracture site was then flushed several times with normal saline solution (0.9% NaCl) and irrigated with Gentamycin solution. Then AM was used by wrapping it circumferentially around the site of fracture and fixing it to the surrounded tissues with multiple stitches. Then 2 ml of PRP solution, which was prepared just before the beginning of the operation, was divided into two parts. One ml was injected into the site of defect while the second ml of PRP was applied to the acellular amniotic membrane. The procedure was continued for two minutes, allowing the platelets to adhere to the tissues. The bone segments were immobilized by a five-hole dynamic compression plate with a size of 2.00 mm, which was applied to the tibia's medial surface. The screws, with a diameter of 2.00 mm, were inserted into the 4 outer holes, which were induced by drilling (with a 2.0-mm drill) into both cortices in mediolateral position, and the one screw-hole adjacent to the fracture site was left empty.

The surgical wound was closed as usual using polyglactin 910 size (0) for close approximation of the adjacent muscles by a continuous suture pattern, then S/C tissue with the same material and pattern, and finally skin was closed by an interrupted suture pattern using suture material nylon size (0). All dogs were confined to individual cages for the designed duration of the study. Following surgery, animals should receive daily wound care and dressings as well as a 4-day intramuscular course of penicillin streptomycin at doses of 10,000 IU and 20 mg/kg B.W., respectively. No external immobilization was used. The animals were allowed unrestricted weight-bearing after the procedure. The wound sutures were removed 10 days post-operatively. All dogs were subjected to biomechanical and histological examinations till the 60th day.

2.5 Biomechanical examination

Biomechanical tests were conducted for all dogs on the 56th day. After all of the animals were euthanized, the bone plates and screws were removed, and the tibias were harvested. The soft tissue was removed from them. Each tibia was divided into segments that were 10 centimeters long and contained the fracture site in the middle. To keep the environment consistently moist, the segments were then covered with wet gauze and placed in a plastic bag. Segments were provided for mechanical testing.

The Universal Testing Machine, which was positioned between the bone ends at the osteotomy site, was used to examine the tibia. The bones were positioned horizontally on two rounded supporting bars that were spaced 6 cm apart and were loaded at the midway of the diaphysis by lowering the third bar so that the fracture site was in the middle and had an equal distance from each grasp. The tibia was compressed. 10 mm/min of loading (pressure force) was applied to the bones until they fractured. At least until the tibias were fractured, the weight was increased at a rate of 2 mm per minute and the amount of force with which the bone was broken was recorded in (KN).

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The computer kept track of values received at the location where the loads were applied at each weight increment. The maximum fracture load was determined to be the final load at which the fracture occurred. [16], Rand et al., 2007).

3. Results

Three groups of tibias' average maximum loads that they could withstand before breaking are illustrated in Table 1 and Figure 1. The maximum loads average in group (AM+PRP) was highest than other groups (2.14 ± 0.050).

The values of fractures were found to be lower in the control group than in the (AM+PRP) and (AM) groups (1.05 ± 0.050). while the values of the (AM) group were (1.52 ±0.086). It was found that bones recovered with the (AM+PRP) group had the highest value, and tibias recovered without treatment had the lowest value.

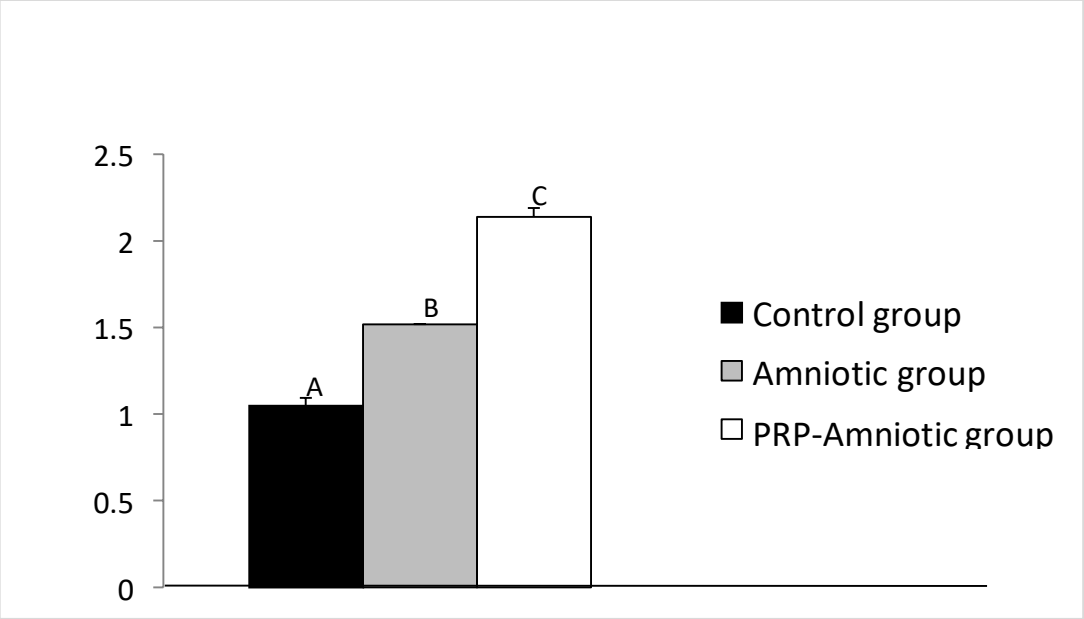

Figure 1: Biomechanical study of healed bone fractures stress forces on fracture line for Control, amniotic, PRP+AM treated groups after 2-months post-surgery. ABC Different letters indicates significant differences (P< 0.05).

Table 1: Biomechanical study of healed bone fractures for Control, AM, and PRP+AM treated groups after 2-months post-surgery. (means and standard errors).

Groups	Force
Control	1.05 ± 0.044^{A}
AM	1.52 ± 0.086^{B}
PRP+AM	2.14 ± 0.050^{C}

ABC Different letters within column indicates significant differences (P<0.05).

4. Discussion

The most notable finding of the current study was that the maximum forces to create refracture were found to be significantly better in the (AM+PRP) group than in other two groups (p<0.05). This outcome

demonstrated the enhancing biomechanical influence of (AM+ PRP) group in fracture healing [12].

wrapping AM circumferentially around the fracture line significantly promoted fracture healing. They both observed a significant higher bone formation [13].

The application of cryopreserved AM directly to the fracture site had favorable effects on bone regeneration, and these effects were more pronounced early in the healing phase.

Amniotic membrane promotes fracture healing through two distinct methods. These are: 1. AM acts as a mechanical barrier to prevent the loss of fracture hematoma, provides an ideal microenvironment for fracture healing, and serves as a source of crucial biological factors that are important in early fracture healing (paracrine effect); 2. Amnion-derived cells may differentiate into osteogenic progenitors or may produce biologic factors that support bone regeneration (cellular effect).

Amniotic membrane functions as a scaffold, and its constituent parts encourage cell growth, angiogenesis, and osteogenesis. Additionally, AM possesses antibacterial and anti-inflammatory qualities. As an osteoinductive and osteoconductive agent, AM may therefore facilitate bone healing while reducing excessive inflammation and acting as an infection barrier [14].

In bone augmentation surgery, PRP is locally applied with the goal of releasing growth factors that regulate osteogenesis processes, such as bone morphogenetic proteins (BMP), platelet-derived growth factor, transforming growth factor-h, and insulin growth factor-I. These growth factors, especially BMP, cause the osteoinduction of mesenchymal stem cells into osteoblasts, which results in the formation of new bone tissue. PRP is therefore a crucial auxiliary for facilitating quicker healing following various bone surgical techniques. [15].

The significant healing efficacy of PRP is due to the GFs and cytokines it contains, which play crucial roles in inflammation and stimulate the differentiation of marrow derived MSCs in the rebuilding microenvironment of the defect site. This differentiation may stimulate new bone formation and early revascularization [17].

Maximum load, bending stress, (AM+PRP) group showed significantly greater recovery than in the other groups at day 56^{th}, when the callus volume of the (AM+PRP) evidenced an apparent reduction.

The inter fragmentary movement that takes place in the fracture under stress is decreased by the callus tissue, which acts as a bridge between the fragments. A soft callus develops following the early stage of inflammation, which dramatically enhances its mechanical stiffness when the callus calcifies by endochondral and intramembranous ossification. The inter fragmentary movement is drastically reduced once the peripheral callus has formed a bone bridge between the fragments, allowing cortical repair to proceed. Finally, the healing area will remodel, and the callus will completely resorb [19].

Platelets as a natural source of growth factors, cytokines, and other micro and macromolecules are hypothesized to improve bone healing [16]. Vascular Endothelial Growth Factor (VEGF) is a signaling protein that stimulates angiogenesis also directly influences bone formation and stimulates osteoblast differentiation and proliferation [18].

5. Conclusion

Applying AM+ PRP is a simple, affordable, and safe way to promote general tissue healing. The implementation of amniotic membrane loaded with platelet rich plasma to the fracture site considerably improves the biomechanical strength of the fracture union, which is the most noteworthy finding of this study.

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