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THE ROLE OF PLATELET RICH PLASMA (PRP) IN FEMORAL BONE DEFECT HEALING IN DOGS. (AN EXPERIMENTAL STUDY)

By

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ABSTRACT

To evaluate the effect of autologous platelet rich plasma (PRP) for healing of femoral bone defect in dogs. 24 adult mongrel dogs were used in this study, a 2 - cm cortical segment of the Femur was osteotomised, and then fixed with a 8-hole dynamic-compression plate. The animals were divided into two groups; group A (n=12) control group and group B (n=12) PRP group. Dogs were euthanized at 2, 4, 8 and 12 weeks. The specimens were assessed for bone union by clinical, radiographical and histological examinations. It was found that, osteoid bridging the gap at femur defect was noted in all specimens in the PRP groups at week 8 and12 while, in control group there was a minimal tissue reaction at the bony edges of the defect. In conclusion PRP increased bone healing in early stage.

Key words:

Growth factors; platelet-rich plasma (PRP); Femur; autologous, long bone defects.

INTRODUCTION

A comprehensive knowledge of fracture healing is desirable in improving treatment and management of bone fractures and defects. Although medical technologies and orthopaedic surgical techniques have been much improved, some fractures still heal poorly, others take a long time to heal (delayed unions) and some result in non-unions **(Hokugo *et al.*, 2005).** Thus, there remains a need to know more about the biology of fracture healing in order to develop strategies for ensuring normal repair of the skeleton **(Jeong *et al.*, 2013).** Successful healing of large bone defects (LBDs) is a complicated phenomenon because the body's natural ability often fails to effectively repair the LBDs. New modalities should be utilized to increase the quality and accelerate bone healing. Several strategies have been developed to enhance fracture healing clinically by either biophysical use of electromagnetic fields **(Ryaby, 1998)** and low-intensity pulsed ultrasonography **(Wang *et al.*, 1994)** or biological means including osteoinductive biomaterials. Autologous bone marrow contains a population

of mesenchymal stem cells that are capable of forming bone, cartilage, and other connective tissues **(Nečas *et al.,* 2008).** Growth factors are expressed during bone healing process **(Schliephake, 2002 and Alvarez *et al.,* 2006).** These growth factors show certain shortcomings when they are used alone **(Van den Dolder *et al.,* 2006)**. Therefore, it may be advisable to identify a source containing multiple growth factors that can accelerate bone healing. A simple way for utilization of advantages of the aforementioned growth factors can be the application of platelet concentrates such as platelet-rich plasma (PRP) **(Hakimi *et al.,* 2010 and Hernandez-Fernandez *et al.,* 2013)**. Platelets through different growth factors and cytokines in their granules can stimulate and regulate cell proliferation and differentiation, chemotaxis, adherence, and angiogenesis; all these criteria are critical especially in early stages of bone healing **(Ranly *et al.,* 2006)**. In addition to the soft tissues, platelet concentrates have extensively been used in the treatment of osteoarthritis **(Franklin and Cook, 2013)**. Oral and maxillary surgery **(Marx *et al.,* 1998)** and repair of LBDs **(Hakimi *et al*., 2010)**. Although positive effects of PRP have been reported in bone healing, there are also some controversies **(Kasten *et al.,* 2008).**

Aim of the study:

To evaluate the role of Platelet Rich Plasma (PRP) in experimentally induced femoral bone defect healing in dogs.

MATERIAL AND METHODS

Animal model:

24 healthy adult mongrel dog weighing (15-20) kg with an age of 1.5-2 years were used for the study. They were fed a balanced diet and water.

In this study, the animals were divided into 2groups (Table 1):

1- Group A (control group) (n=12) where the femoral defect left empty.

2- Group B (PRP group) (n=12) where the femoral defect was filled with PRP gel.

All animals were vaccinated against rabies vaccine (Nobivac® Rabies, Intervet, USA) and for internal and external parasites doramectine (Dectomax® :Pfizer,USA) by s/c injection and were kept in individual cages and housed in Surgery Department, Faculty of Veterinary Medicine, Cairo University.

Table (1): The animal's distribution according to the groups and the follow up period.

Groups / Period	A (control)	B (PRP)
2 weeks	3	3
4 weeks	3	3
8 weeks	3	3
12 weeks	3	3
Total	12	12

Preparation of platelet-rich plasma (PRP):

A blood sample was drawn using Acid Citrate Dextrose A (ACD-A) as anticoagulant. Gradient density centrifugation was made to obtain the platelet rich plasma layer and added to calcified thrombin for activation and preparation. **(Andia, Sánchez, and Maffulli, 2012)**.

Anesthesia:

All dogs were premedicated with intramuscular injection of Atropine sulphate 0.04 mg/kg (Atropine sulphate®:1mg/ml, Adwia, Egypt). Anesthesia was induced immediately through intravenous injection of mixture of Ketamine 10mg /kg (ketamine® HCL 10%: El Yosr, Egypt) and Xylazine Hcl 1mg/kg (xylaject ® 2%: Adwia, Egypt) in the cephalic vein. The anesthetic depth was maintained with Thiopental sodium (Thiopental® : 2.5mg/kg, Epico, Egypt) administered by intravenous route.

Operative procedures:

All dogs were restrained in lateral recumbancy.

Craniolateral approach skin incision was carried out over the Midshaft of the femur with minimal separation of attached soft tissues and gentle retraction of the muscles to expose the femur. In the middle of the femur diaphysis, the periosteum was removed and a standardized 2 cm full thickness defect was created with an embryotomy saw under irrigation with 0.9% sterile saline solution. The defect was stabilized with a dynamic compression plate (stainless steel) with three proximal and three distal screws in each end. The PRP-gel was placed in the defect site in PRP group while in the control group the defect was left empty. Muscles, fascia and skin were separately closed over the defect using absorbable suture (vicryl). Immediately after the operation, the animals were allowed freedom of movement in individual galvanized cages. A prophylactic antibiotic coarse consist of Ceftriaxone 1000 mg. (22mg/kg, I/M) for 7 consecutive days.

Postoperative follow up examination:

1-General condition

Checking for the general health conditions and body temperature every day till 7 days postoperatively.

2-Assessment of soft tissue swelling and wound healing

Any changes of the operated limbs like swelling of surrounding soft tissues, wound infections and sutures dehiscence must be recorded.

3-Lameness grading was carried out according to **(Sumner-Smith, 1993).**

4-X-ray analysis:

Conventional X-rays in two planes were obtained immediately after surgery and every two weeks until the end of experiment. Radiographs were taken with a standard 30 x 40 cm x-ray film with radiographic factors 70 cm FFD, at 48 to 56 kVp and 15-30 mAs.

Postmortem examination:

The dogs were euthanized at 2, 4, 8 and 12 weeks after surgery by using barbiturate over dose through cephalic vein.

The femur bones were disarticulated. The plate and screws and soft tissues in the operation site were removed carefully to minimizecallus disruption.

Macroscopic examination:

Bone sample were tested by manual clinical assessment of bone union.

Radiographic examination:

Contact x-ray was performed after extraction of operated femur.

Histological evaluation:

The bone pieces were washed thoroughly with normal saline and were fixed in 10% formalin for 15 days. Subsequently bones were decalcified in Goodling and Stewart's fluid containing formic acid 15 ml, formalin 5 ml and distilled water 80 ml solution; it was stirred daily and changed once in three days. The sections were checked regularly for the status of decalcification. They were considered as completely decalcified when sections became flexible, transparent and easily penetrable by pin. The decalcified tissues were processed in a routine manner and 4 -5 µm sections were cut and stained with Haematoxylin and Eosin. The stained sections were observed for the status of the bone implant and cellular response of host bone to the implant.

RESULTS

In the present study, all dogs tolerated the surgical procedures. They were apparently healthy throughout the study period.

Clinical evaluation:

All animals showed non-weight bearing on the operated limb either in standing or slow and fast motion situations. These signs were observed from the day after operation up to one week. Group A, showed a non - weight bearing and persistent lameness till 2weeks postoperatively then progress to partial lameness. The full weight bearing was not observed even at 12 weeks. In Group B, the animals gait changed to partial weight bearing at 2 weeks P.O. The full weight bearing was achieved within 6 - 8 weeks P.O.

X-ray evaluation:

All animals developed mineralization within the defect area in the PRP group. Ossification leading to a connection of the femur segments could be seen in the defect area after 4 weeks Fig. (2-c). However, the animals of the control group showed only a cloudy mineralization in the defect area without reconnection between the ends of the femur segments Fig. (1-c). By 12 weeks after surgery, most of the defect area filled with cancellous ossification Fig. (2-e) whereas none of control group did so Fig. (1-e).

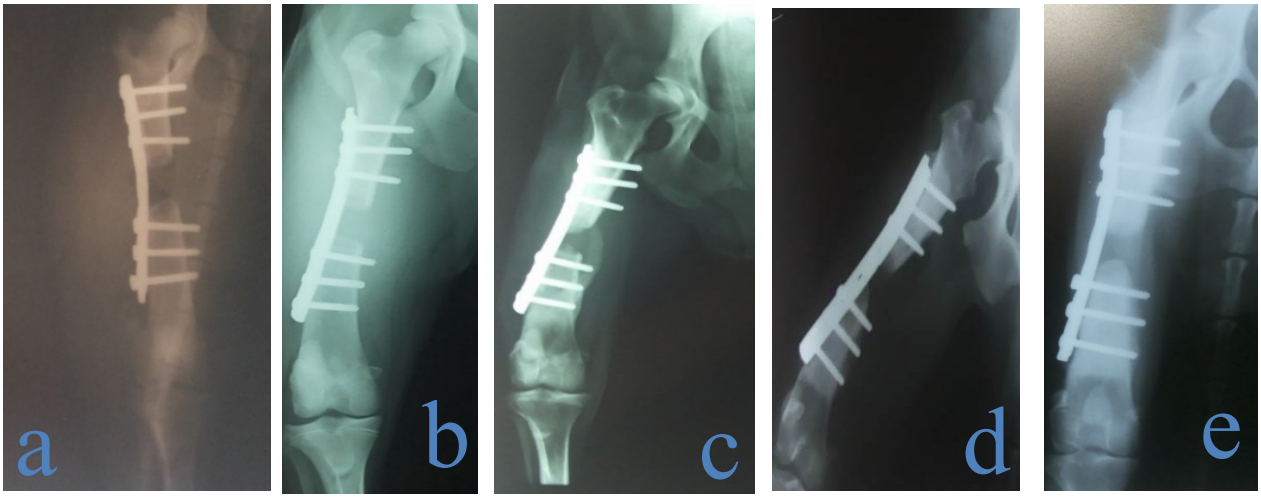

Fig. (1): x-ray of group A (control group), a) x ray immediately postoperatively to show the alignment of the plate and bone defect. b) X-ray after 2 weeks postoperatively showing minimal callus formation at the defect site. c) X-ray 4 weeks postoperatively showing minimal tissue reaction at bone edges of the defect. d) X-ray 8 weeks postoperatively showing minimal increase tissue reaction at bone edges of the defect. e) X-ray 12weeks post operatively showing increase tissue reaction in the defect site between bones edges but fail to bridge the defect.

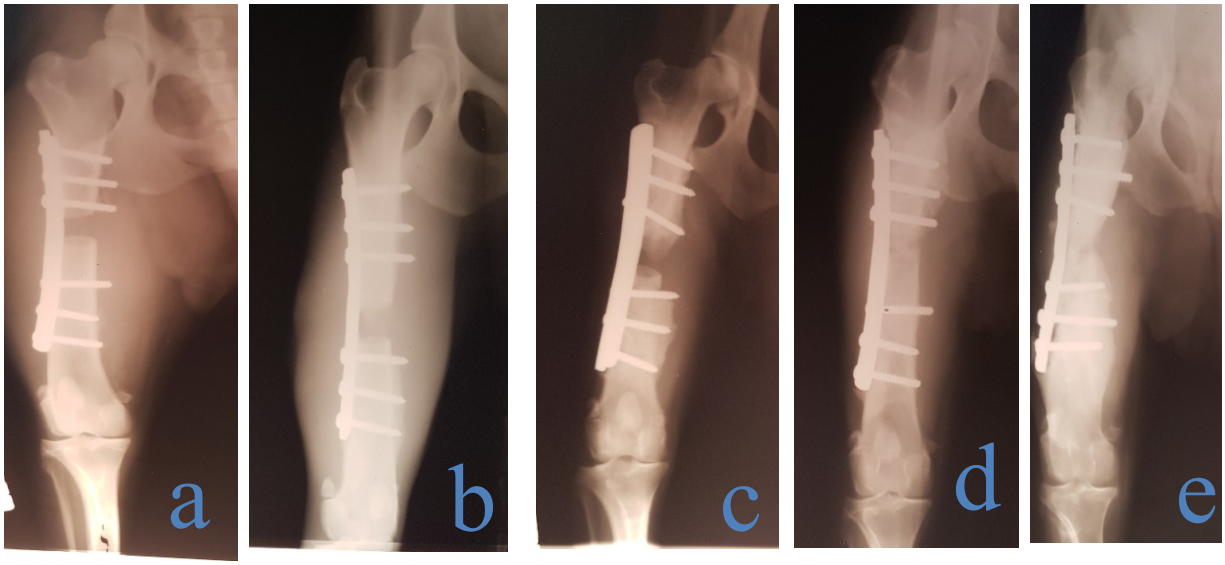

Fig. (2): x-ray of group B (PRP group). a) X-ray immediately postoperatively to show the alignment of the plate and bone defect. b) X-ray after 2 weeks postoperatively showing callus formation at the bone edges of defect site. c) X-ray 4 weeks postoperatively showing increase tissue reaction at bone edges of the defect. d) X-ray 8 weeks postoperatively showing tissue reaction and bone formation bridging bone edges of the defect site. e) X-ray 12 week's post operatively showing complete bone formation and bridging of the defect site.

Histological evaluation:

Histological analysis of the tissue filling the bone defect at the control group specimens showed that after 2 weeks the gap was filled with immature and unorganized fibrous connective tissue and inflammatory cells while the bony edges showed hyper-thickening and hyper-cellularity**.** After 4 weeks, the fibrous connective tissue became organized as blood vessels began to appear; the fibrous network was hypercellular containing large deeply stained cells with large nucleus (mesenchymal like cells). After 8 weeks, a fibrocartilage began to form with large chondrocytes separated by lacunae at the bone edges while the center of the gap showed a large network of collagen bundles and blood vessels with low cellularity. And after 12 weeks, small areas of ossification began to appear within the formed fibrocartilage which were separated and unorganized. A large number of osteoblasts began to appear at the edges of the bone while the center of the gap remained filled with collagen bundles Fig. (3).

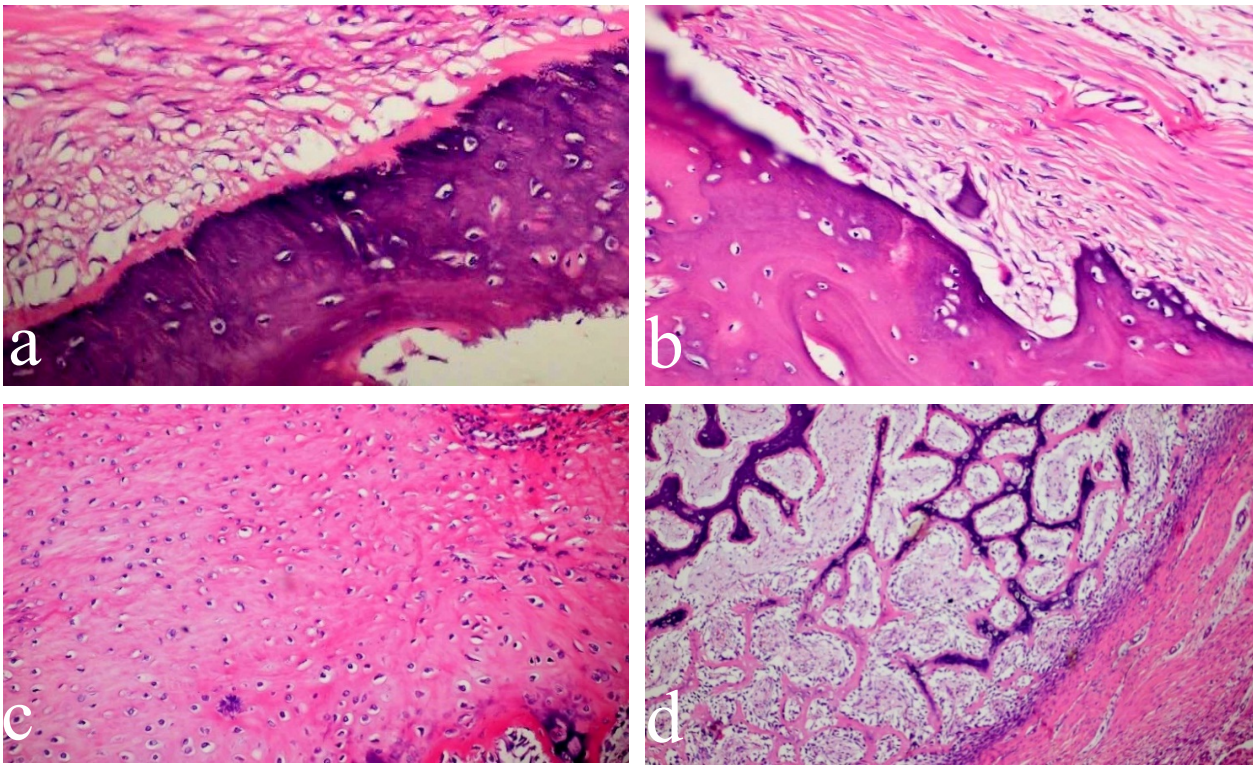

Fig. (3): showing the histological analysis of the control group where a) shows the bony edge with deeply stained matrix and fibrous connective tissue formations on the gap site after 2 weeks, H&E 400X. b) shows the fibrous connective tissue formed above the bony edge which is condensed and organized containing deeply stained largely nucleated cells after 4 weeks, H&E 400X. c) Shows the formation of hyaline like cartilage formation with chondrocytes separated by lacunae in collagen like matrix after 8 weeks, H&E 200X. d) Shows newly formed osseous tissue with small trabeculae and calcium deposition on the left while the other side shows collagen bundles with blood vessels after 12 weeks, H&E 100X.The histological analysis of the tissue filling of the bone defect of the PRP group showed that After 2 weeks, the bone edges were surrounded by a hypercellular network of mesenchymal like cells in condensed and organized fibrous tissue with large centers ossification, while the center of the gap was filled by a hyaline like cartilage with mature chondrocytes separated by lacunae with a deeply stained matrix. After 4 weeks, all the bone edges began to form bony trabeculae which are hypercellular and deeply stained and surrounded by a large number of osteoblasts, the center of the gap is still a hyaline like cartilage with calcium deposition in many centers. After 8 weeks, the newly formed bony trabeculae showed more organization and condensation with few gaps in between. The lower parts showed similar staining affinity to the underlying bone while near the center of the gap they appeared more deeply stained and hypercellular.

The center of the gap showed ossified cartilage containing osteonal canals and surrounded by bone trabeculae.And after 12 weeks, the newly formed bone showed more compacted appearance with the same staining affinity of the normal bone, the center of the gap showed more developed osteonal canals with large number of osteoblasts replacing chondrocytes Fig. (4).

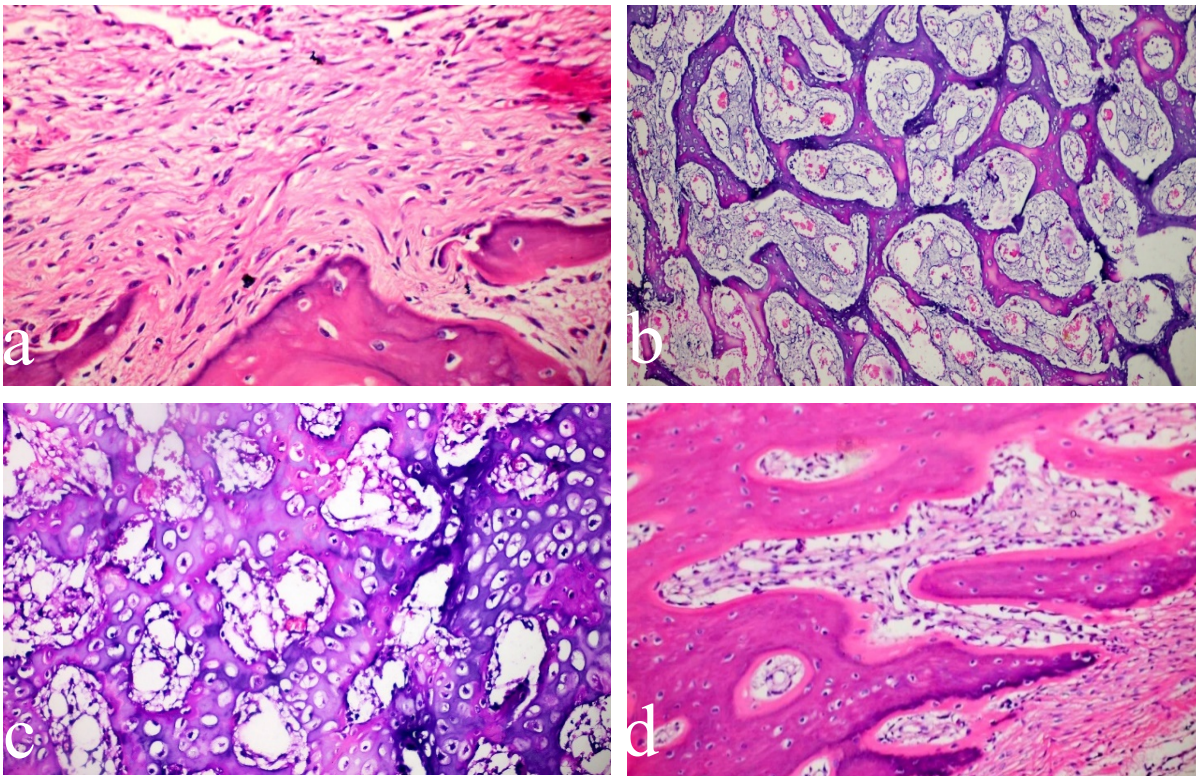

Fig.(4): showing the histological findings of the PRP group where a) shows a dense hypercellular network at the bony edges filled with mesenchymal like cells after 2 weeks, H&E 200X. b) shows the newly formed trabecular bone with large osteonal canals and blood vessels after 4 weeks, H&E 100X. c) shows the transition from chondral to osteoid tissue, osteonal canal formation between cartilage and large number of osteoblasts replacing chondrocytes after 8 weeks, H&E 400X.d) shows the increase in compact bone mass formation which is more dense and organized after 12 weeks, H&E 200X.

DISCUSSION

Platelet-rich plasma (PRP) is included within the field of Regenerative Medicine, because the preparation process is rapid and requires minimal specialized equipment and safe storage are not required. Furthermore, because it is autologous, PRP does not provoke an immune response in the patient and is therefore perceived to have a high margin of therapeutic safety

(Torricelli *et al.,* 2011). PRP can be used alone or in conjunction with bone grafts in the surgical site **(Kurikchy *et al.,* 2013)**. It is prepared with the intention of influencing soft and hard tissue repair and/or regeneration **(Parizi *et al*., 2012).** In this study, PRP was used alone in the form of gel mixed with bovine thrombin and calcium chloride while most of studies used PRP in conjugation with other biomaterials like autogenous bone graft **(Hakimi *et al.,* 2010),** allograft **(Nather *et al.,* 2012),** mesenchymal stem cells **(Yamada *et al.,* 2004),** TCP (**Rai *et al.,* 2007).** An important parameter for the performance of PRP is the method of its preparation, since this can significantly influence the concentrations of platelets and growth factors **(Weibrich *et al.,* 2003)**, and consequently their osteogenic capacity **(Wiltfang *et al.,* 2004).** In the present study, the platelet concentrations in PRP range from three to five times its original concentration in whole blood this was in agreement with **(Marx *et al.,* 1998; Roldán *et al.,* 2004)**. Moreover, **Dugrillon and Klu¨ter (2002).** Added that, higher platelet concentration do not necessarily correlate with higher levels of growth factors. Addition of thrombin in the presence of calcium chloride was used in the present study. The degranulation of PRP and the release of the growth factors can be achieved by the addition of thrombin in the presence of calcium chloride **(Marx *et al.,* 1998).** In the present study, the course of healing was monitored using radiographic evaluation and histological analysis. At 3rd month of follow-up period, the radiographic evidence of bone formation was clear in PRP group in contrast to control group. Nearly the same findings were in agreement with those of **Kim *et al.,* (2002). Aghaloo *et al.* (2002) and Freymiller and Aghaloo (2004),** where they observed increased tendency of bone formation at 1 and 2 months on rabbit cranial defects. The same observations, in terms of radiographic changes in the grafts from radiolucency to radio-opacity, have been reported by **(Silva *et al.,* 2005).** In this study, trabecular pattern of bone formation and large number of osteoblasts at 1 month follow up was seen in PRP group compared to control group. At 3 months, the histological appearance of the gap showed more developed osteonal canals with large number of osteoblasts replacing chondrocytes that indicate the positive effect of PRP on bone defect healing. This indicates that PRP enhances new bone formation by its early healing potential, nearly the same observation was mentioned by **(Gerard *et al.,* 2006).** Early bone formation in PRP is a result of the effect of different growth factors which are secreted by platelets, which cause mitogenesis, angiogenesis, fibroblastic and osteoblastic activity, macrophage activation, maturation of bone and osteoclast mediated resorption **(Gilberto and Mariano, 2005)**.The vast majority of

published clinical studies suggested that, PRP accelerated bone healing **(Marx *et al.,* 1998; Roldán *et al.,* 2004; Hokugo *et al.,* 2005; Sugimori *et al.,* 2006; Kroese-Deutman *et al.,* 2008; Souza *et al.,* 2012; Smyth *et al.,* 2012 and Zhang *et al.,* 2013).** While other authors have reported different results **(Cornell *et al.,* 1992; Cheung *et al.,* 2000; Watabe *et al.,* 2009; van Buul *et al.,* 2011; Lippross *et al.,* 2011; Browning *et al.,* 2012 and Baksh *et al.,* 2013**. The beneficial effect of PRP on bone healing could not be demonstrated in combination with every bone substitution material and every animal model as it failed to improve bone healing in critical size forehead bone defect in goat treated by PRP and autogenous cancellous bone **(Mooren *et al.,* 2007)**, in pigs using bovine cancellous blocks **(Wiltfang *et al.,* 2004)** and in using PRP with hydroxyapatite **(Plachokova *et al.,* 2007**).

CONCLUSION

In conclusion this study demonstrated that, PRP could promote bone regeneration in bone defects with high regenerative capacity. This allows PRP to become an attractive alternative for the reconstruction of diaphyseal defects in long bones.

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