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Effect of Autogenous Platelet-rich Plasma (PRP) on Femoral Cancellous Bone Defect Healing in Alloxan-induced Diabetic Rabbits

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Abstract: The aim of this study was to determine the effect of PRP on healing of femoral cancellous bone defect in diabetic rabbit. The experiment was conducted on 20 male adult New Zealand white rabbits. After induction of diabetes rabbits were divided into two groups of control and experiment of 10 rabbits each. After general anesthesia, a hole in size of 4×5 mm in diameter and depth was made using a dental bit between lateral and medial condyles of left femor. In control group, defect was left untreated and in experiment group, the created hole was filled with autologous PRP. Five rabbits were sacrificed at 1 and 2 month after surgery and evaluated histopathologically. Histomorphometrically evaluation was compared with healthy bone samples of 2 month after surgery. Data were submitted to statistical analysis by variance analysis (ANOVA) and Tukey test, at a significance level of 5% ($p<0.05$). In diabetic control group, within 1 month, immature granulation tissue was observed. In 2 month, some more mature lamellar bone along with areas of connective tissue could be observed. In diabetic experiment group, within 1 month, mature granulation tissue formed, within 2 month, bone formation could be observed, and bone neoformation was compact, with bone trabeculas. Differences observed in histomorphometrically findings were significant between diabetic control group and diabetic experiment group ($p=0/001$). The results of this study show that PRP provide a rapid regeneration of bone defects in femoral cancellous bone in diabetic rabbits.

Key words: Platelet-Rich Plasma, Cancellous bone, Diabetes, Rabbit, Histopathology, Histomorphometry.

INTRODUCTION

Diabetes mellitus is a systemic disease that has profound deleterious effect on bone healing, diabetes is associated with a decrease in skeletal mass and delayed healing of fractures (Lu *et al.*, 2003). Many mechanisms have been suggested how the effects of diabetes on the healing process, that causes delayed healing or non-union of fractures. These include a decrease in collagen synthesis (Gooch *et al.*, 2000), a more severe inflammatory response (Diniz *et al.*, 2008), and an imbalance between bone resorption by osteoclasts and bone deposition by osteoblasts (Suzuki *et al.*, 2005). In general, these mechanisms would be expected to affect all stages of fracture healing (Diniz *et al.*, 2008). Many studies on diabetes and bone healing is done, the researchers were constantly looking for material to be able to heal the bone in people with diabetes will accelerate. In recent years, as well as comprehensive information about growth factors that play an important in healing process is achieved, heal various tissues in the human body including bone, are carried through growth factors. This process begins by blood clots in position and continues by platelet degranulatio and causes release of growth factors (Reddi, 1997). Many growth factors have been implied on bone repair process: platelet-derived growth factors derived (PDGFs), vascular endothelium growth factor (VEGF), transforming growth factors α and β (TGF-α and TGF-β), acid and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), insulin-like growth factors I and II (IGF-I and IGF-II), cement-derived growth factor (CGF), parathyroid hormone related proteins (PTHrP), and bone morphogenetic protein 1 to 12 (BMPs

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1-12,) (Kobaiashi *et al.*, 2006). Platelet rich plasma (PRP) is called to the volume of autologous blood plasma that may contain high concentration of platelets (Pietrzak and Eppley, 2005). Studies have shown that the clinical efficiency of PRP can be at least four times increase in the normal range (Pietrzak and Eppley, 2005; Marx, 2001). Many reports suggest a positive effect of PRP on bone healing (Kassolis *et al.*, 2000; Roldan *et al.*, 2004; Jakse *et al.*, 2003; Aghaloo *et al.*, 2002), but there are few reports about beneficial effects of PRP on bones healing in animal models of diabetes. The aim of this study was to evaluate the effect of platelet rich plasma on the healing of femoral cancellous bone defect in diabetic rabbit.

MATERIALS AND METHODS

Animals: Investigations using experimental animals were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the United States guidelines (United States National Institutes for Health publication no. 85-23, revised in 1985), and our ethical committee on animal care approved the protocol. Twenty New Zealand 24 week old and weighing 3.3-3.5 kg male rabbits were used. The animals were obtained from the Central Animal Laboratory of Islamic Azad University Tabriz Branch. All animals were kept in individual cages (Dam Pars Comp. Tehran) during the whole experimental period, under similar conditions at 18-24^{oC} in a 12-hr dark-light cycle and maintained with unlimited amounts of standard laboratory pellet diet and water ad libitum. After induction diabetes animals were divided into two groups (control and experiment), with ten rabbits in each.

Alloxan-induced diabetes model: Diabetes was induced in rabbits by a single intravenous administration in the ear vein of 100 mg/kg Alloxan monohydrate (Sigma Aldrich Chemical, Saint Louis, MO, USA) dissolved in sterile 0.9% saline. Four days after injection, blood samples were collected from the animals, blood glucose levels were determined by an enzymatic colorimetric assay procedure using UV Spectrophotometer at wavelength 505 nm. In this study, a blood glucose level of > 200 mg/dl indicated hyperglycemia.

Preparation of PRP: Under anesthesia, a 5-ml blood sample was collected of cardiac puncture in a tube, and centrifuged during 15 minutes at 1800 rpm. Red blood cells were collected deep in the tube, and plasma remained on top. Plasma was including four layers: first layer above red blood cells, was the platelet-very rich plasma (PVRP); the second layer was the platelet-rich plasma (PRP), the third layer was the plasma with moderate platelet content (PMP), the fourth layer was constituted of poor platelet content (PPP). PVRP and PRP layers were aspirated and added by 10 µl of 10% calcium chloride solution for inducing coagulation. PRP gel was ready to fill bone defect in experiment group (Kobaiashi *et al.*, 2006).

Surgical procedure: General anesthesia was induced with an IV injection of Ketamine hydrochloride (Ketamine 10%, Alfasan, Woerden-Holland, 50mg/kg) and Xylazine (Xylazin 2%, Alfasan, Worden-Holland, 5mg/kg) and left femur was routinely prepared for surgery. A 3-cm skin incision was made on the medial aspect of the distal femoral condyle. The muscle was dissected bluntly to the level of the periosteum, which was transected and lifted from the bone. The distal femoral epiphyses were exposed and a hole of 4×5mm in diameter and depth was created in the between aspect of medial and lateral condyles with low-speed dental bit, saline-cooled in a stepwise fashion. The cavity then was washed carefully with a physiological saline solution. In control group defects were left empty and the rabbits in these groups received no treatment. In experiment group defects were filled with autogenous PRP gel. The muscle attachment was repaired and skin was closed in layers. Intramuscular injection of 0.05 mg/kg dexamethasone (Vetacoid®, Aburaihan Co., Iran) and 40000 IU/kg Penicillin G, Benzedrine, Procaine and Potassium 2:1:1 (Nasr Fariman Co., Iran) was performed.

Histopathology and histomorphometry evaluation: Five rabbits were euthanized with an intravenous injection of an over dosage of thiopental sodium, causing a quick and painless death, at 1 and 2 postoperative month in each groups. The operated femur and health femur were harvested and fixed in 10% neutral buffered formalin during five days, for fixation; then dehydrated in 10% EDTA. Finally, they were embedded in paraffin. Serial sections were cut and stained with Haematoxylin and Eosin (H&E) method and used for light microscopic examination under a Nikon microscope (ECLIPSE E200, Japan) to histopathology and histomorphometry evaluation. A total of 10 cancellous bone defects were histomorphometric analyzed at 2 month postoperativly. Histomorphometry analysis were performed by linear measurements through Intersection Latticed Lines using an ocular Latticed Lens comprising 100 cross points to determine the percentage of the defect that was occupied by 1) connective tissue, 2) bone marrow, 3) woven bone and 4) lamellar bone. Thus, at a magnification of 40×, the various components were identified using a mouse cursor (Carmagnola *et al.*, 2002). Bone marrow was identified as a tissue that included adipocites, while connective tissue was defined

by the presence of fibroblasts and collagen fibers. Morphometrical analysis was performed also of right health femur, and the normal percentage of lamellar bone, woven bone and bone marrow was assessed.

Statistical analysis: Statistical evaluation of data was performed using the software package SPSS 13 (SPSS Inc., Chicago, IL). Data are expressed as the mean±SEM for each group. Statistical differences between groups were evaluated with ANOVA followed by the Tukey test to analyze histomorphometric data among groups. The significant level was set at $p<0.05$.

RESULTS AND DISCUSSION

Histopathology Results: Histologic studies performed in control group, 1 month of healing, showed the apical portion of defect to be filled with blood coagulum and inflammatory cells. Immature granulation tissue was observed in the remaining portions of the defects. Deeper portion of the defect was occupied with bone marrow. Extension of lamellar bone from the lateral aspects of the defects to the middle portions of that was prominent (Fig 1). Higher magnifications showed traces of woven bone with some marrow fibrisis, especially around the defect. Newly formed woven bones were lined with active osteoblasts evidencing active osteogenesis. These osteblasts are depositing the first lamellae on the already existing trabeculae (woven bone). The trabeculae will therefore have a core of woven bone, which is surrounded by lamellar bone (Fig 2). In 2 months of healing, apical portion of the experimental defect was occupied with blood coagulum, which harbored large numbers of inflammatory cells (Fig 3). A relatively thick layer of granulation tissue showing active angiogenesis was observed just under the coagulum (Fig 4). Some more mature lamellar bone (primitive trabecular bone) along with areas of connective tissue could be observed in central portion of the defect. Higher magnifications showed active new bone formation in healing sites (Fig 5). In histologic examination of the PRP augmented defect from experiment group, 1 month of healing, there were no remnants of coagulum and only a few inflammatory cell infiltrate was present in healing sites. The repaired construct revealed a thick layer of mature granulation tissue formed at apical portion of the defect. The fibroblasts within the granulation tissue developing into chondroblasts were forming hyaline cartilaginous nodules. Dispersed cartilaginous islets in the granulation tissue, suggesting chondrogenesis, observed in this group. In specimens taken from this group in comparison to the previously described control groups, quantity of the woven and lamellar bone was larger in the crestal and lateral portions of the defect. Lamellar bone exhibiting extensive bone surfaces give evidence of reorganization (Fig 6 and Fig 7). In sections, representing 2 months of healing, significant bone formation could be observed and almost all defects were filled with newly formed bone and bone marrow. The newly formed bone tissue had lamellar structures. However, the lesion was not completely repaired by bone tissue in this period of 2 months. In the apical portion of the healing site, a thick layer of fibrous tissue covered the entrance of the hard tissue wound. This region had its pristine saddle shape appearance (Fig 8). Multiple zones of chondrogenesis in this layer give evidence of substitution of fibrous tissue with articular hyaline cartilage (Fig 9).

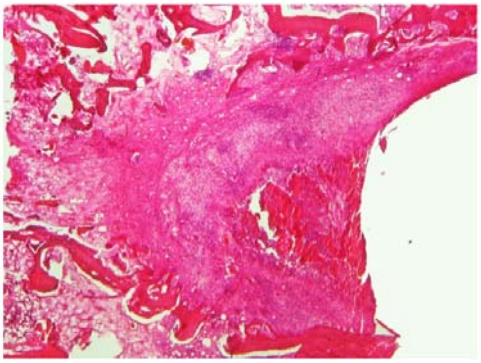

Fig. 1: Microscopic section from the healing site in diabetic control group, 1 month of healing, shows the apical portion of defect to be filled with blood coagulum and inflammatory cells. Immature granulation tissue is observed in the remaining portions of the defects. Deeper portion of the defect is occupied with bone marrow. Extension of lamellar bone from the lateral aspects of the defects to the middle portions of that is prominent. (H&E, ×40).

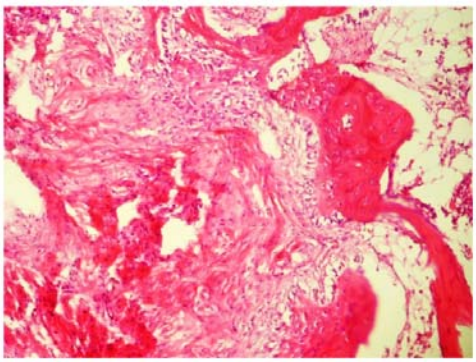

Fig. 2: Microscopic section from the healing site in diabetic control group, 1 month of healing. Higher magnification shows traces of woven bone with some marrow fibrisis, especially around the defect. The defect is filling with minimal new bone formation originating from the defect margin. Newly formed bones are lined with active osteoblasts evidencing active osteogenesis (H&E, ×100).

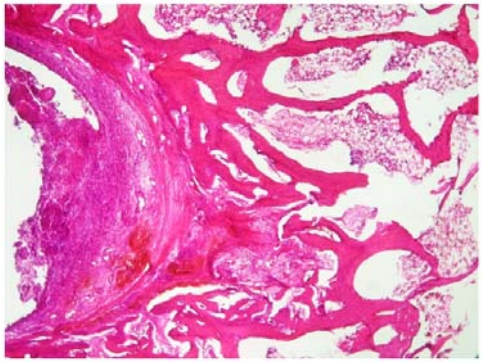

Fig. 3: Microscopic section from the healing site in diabetic control group, 2 month of healing. Apical portion of the experimental defect is occupied with blood coagulum, which harbors large numbers of inflammatory cells. Some more mature lamellar bone is extended from the lateral aspects of the defects and marrow spaces along with areas of connective tissue can be observed in central portion of the defect (H&E, ×40).

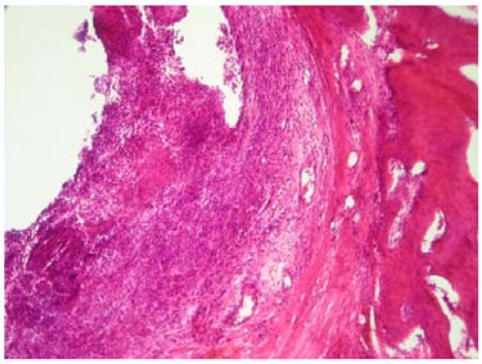

Fig. 4: High power magnification from the healing site in diabetic control group, 2 month of healing. A relatively thick layer of granulation tissue showing active angiogenesis is observed underneath the coagulum (H&E, Orig. Mag. ×100).

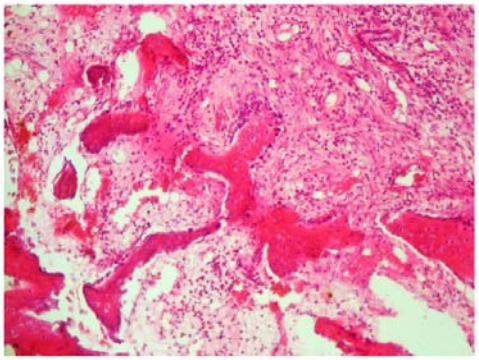

Fig. 5: Microscopic section from the healing site in diabetic control group, 2 month of healing. Higher magnification shows foci of new bone formation in healing site. Osteoblasts forming a low columnar "epitheloid layer" at sites of bone deposition show intramembranous ossification (H&E, ×100).

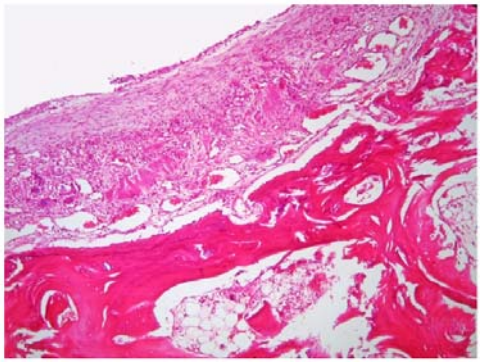

Fig. 6: Histologic section from the healing site, in diabetic experiment group, 1 month of healing. No remnants of coagulum and only a few inflammatory cell infiltrate is present in healing sites. The repaired construct reveals a thick layer of mature granulation tissue formed at apical portion of the defect with dispersed cartilaginous islets suggesting chondrogenesis in this layer. Quantity of the woven and lamellar bone is larger in the crestal and lateral portions of the defect and all defect site is almost completely briged (H&E, ×100).

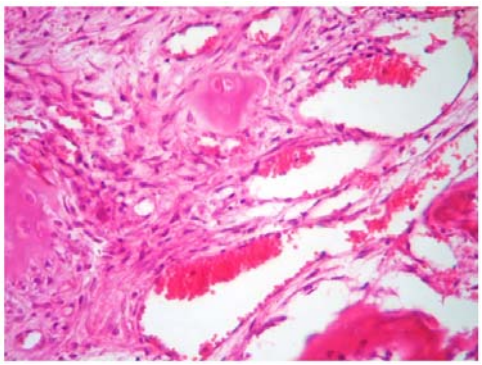

Fig. 7: Histologic section from the healing site, in diabetic experiment group, 1 month of healing. High power photomicrograph shows cartilaginous nodules and abundant capillary buds in granulation tissue layer formed in the apical portion of healing site. Considerable hyperemia is seen in this region (H&E, ×400).

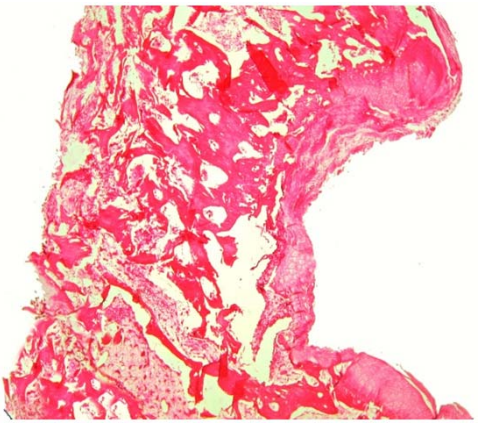

Fig. 8: Histologic section from the healing site, in diabetic experiment group, 2 month of healing. Almost the defect is filled with newly formed trabecuar bone and bone marrow (H-&E, ×10).

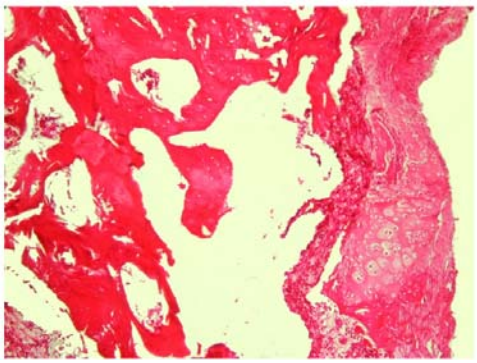

Fig. 9: High power magnification, from the healing site in diabetic experiment group, 2 month of healing. Chondrogenesis in fibrose layer give evidence of gradually substitution of granulation tissue with articular hyaline cartilage (H&E, ×40).

Histomorphometry Results:

Histomorphometry evaluation results obtained, two months after surgery, indicating that amounts of lamellar bone formed in experimental group significantly more control group and is less than healthy bone ($p<0/05$). Amount of immature bone, bone marrow and connective tissue in experimental group significantly lower than control group, respectively ($p<0/05$). In terms of the amount of lamellar bone formation, immature bone, bone marrow and connective tissue in comparisons between the groups studied there was a significant difference ($p<0/05$). Mean and error bar than lamellar bone, immature bone, bone marrow and connective tissue among the studied groups, are presented in Fig. 10.

Fracture healing is a process of restoring the structural and biological properties of injured bone. Clinical and experimental studies indicate that diabetes delayed healing fractures by effect on one or more stages of the healing process and can be lead to delayed union or non-union. Nowadays, many researchers are trying to find materials that can improve bone healing and that is very important in diabetic patients. An ideal material for bone healing should have osteoinductive and osteogenic properties (Mousavi *et al.*, 2010). It is widely accepted that growth factors play a central role in the healing process and tissue regeneration (Sampson *et al.*, 2008). In an animal study by Gandhi et al., show that diabetes can be reduced growth factors (Ghandi *et al.*, 2006). Platelet rich plasma is called to a high concentration of plasma that is preparation of autologous blood and contains large amounts of growth factors (Sampson *et al.*, 2008). Some authors have reported improved bone formation with PRP.

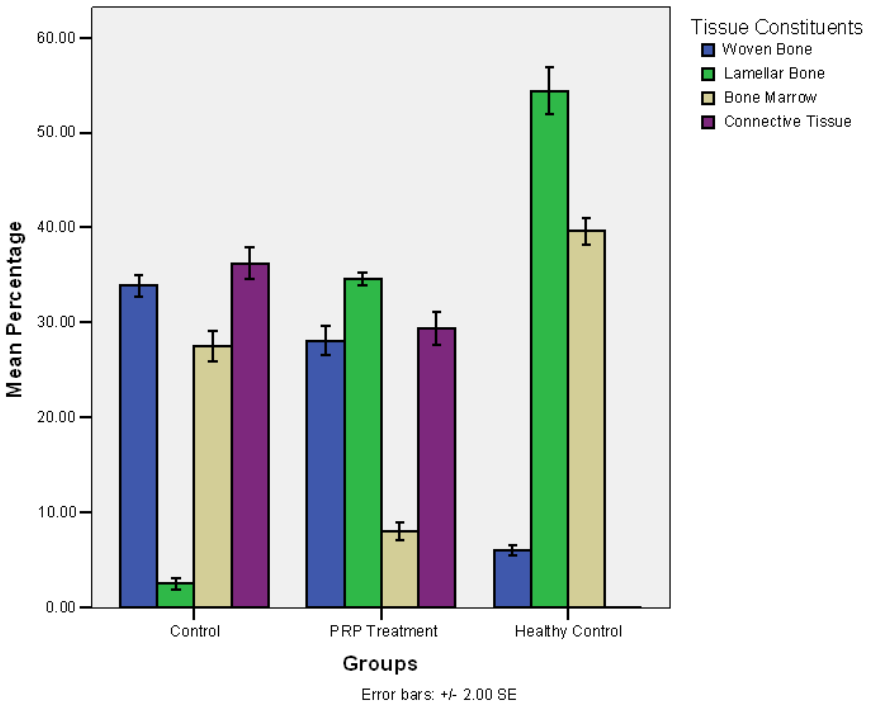

Fig. 10: Chart component repair bone tissue status between the study groups- In bone samples obtained 2 months after surgery

The results in this study shows that, because the experimental defect had a limited diameter, classic response of bone fractures due to trauma, did not exist. Osteoprogenitor cells of periosteal layer not active and external callus (fibrocartilage callus) was not created. Healing was done only with activation of ancestral cells that can become osteoblasts and there are endosteal layer and endosteal Osteoprogenitor cells are known. Irregular and immature bone formation and it also with time, Changes in spicule of immature bone emerged and with collagen fibers become organized than lamellar bone were converted; this type of osteogenesis, Intramembranous Ossification called (Link *et al.*, 2006; Liacouras *et al.*, 2006). Bone defect in the diabetic control group that was empty, due to the absence of osteoinduction or osteoconduction substance, exchange of connective tissue to bone and bone healing process of the extent and intensity was not high. At the end of 60 days after surgery, the diabetic control group had a lot of connective tissue in the defect, while the amount of fibrous tissue in the diabetic experimental group was much lower and rather large amount of undifferentiated mesenchyme tissue, there was bone union; and varying amounts of bone had seen that had filled the defect area. According to the histopathologic results, due to being provocative platelet rich plasma in diabetic bone tissue and accelerate tissue reaction, two months after surgery, in experimental group, organized regular trabeculae were formed. Compared with the diabetic control group, defect in diabetic experimental group within two months filled with new bone cells and this prevents the formation of fibrous tissue at the defect. Assessment of histomorphometry results, two months after surgery, show that the majority of the defect in the diabetic control group was filled by immature bone and connective tissue and immature bone and connective tissue was significantly more than diabetic experimental group and healthy bone, the amount of lamellar bone in the diabetic control group is very low, however, the lamellar bone in diabetic experimental group was significantly higher than the diabetic control group, that represents an active osteogenesis in this group than the control group. The results obtained in this study show that platelet rich plasma causes increases bone induction and accelerate osteogenesis in diabetic rabbits. Seems to increase osteogenesis by platelet-rich plasma, due to the effect of growth factors in PRP on osteoblast cells, mesenchymal cells and endothelial cells that are involved in bone regeneration (Kobaiashi *et al.*, 2006).

In a study show that PRP is an autologous preparation without antygenicity, this allows to eliminate biological concerns such as immunogenic reactions (Czuryszkiewicz-Cyrana and Banach, 2006). Several studies reported some beneficial effects in osseous healing from the use of PRP in oral and maxillofacial procedures. A significant benefit from using PRP was reported by Butterfield et al. and satisfactory results such as reduced

healing period and increase bone density achieved in orthopedic surgery (Butterfield *et al.*, 2005). The current result of the effect of PRP on defect in the cancellous bone of rabbits confirmed the previous literature data on these defects (Kassolis *et al.*, 2000; Roldan *et al.*, 2004; Jakse *et al.*, 2003; Aghaloo *et al.*, 2002). Siebrecht and *et al*., reported in rabbits, PRP increases gap healing in combination with bovine cancellous bone in a calvarial defect model (Siebrecht *et al.*, 2002). Kobaiashi, et al. showed that PRP increased amounts of osteoid and osteoblasts (Kobaiashi, *et al.*, 2006). Research was conducted to compare with this study show that the results in this study are consistent with most other research and PRP as a biologic autogenous bone graft increases bone induction and osteogenesis.

Conclusion:

The results show that platelet rich plasma can increase the bone healing in femoral cancellous bone defect in diabetic rabbit.

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