Histological evaluation of Platelet–rich plasma effect on bone healing in alloxan–induced diabetic rabbits
(Experimental study)

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ABSTRACT
Background: Diabetes Mellitus has an impact on bone healing process, Platelet-rich plasma PRP ameliorated the deleterious effect of diabetes on bone healing process so the aims of this study were to assess histologically the effects of PRP on bone healing in intrabony defect of maxillary bone in alloxan-induced diabetic and nondiabetic rabbits, and to correlate the effect of time of bone healing in two experimental groups with that of controls.

Materials and methods: Fifty rabbits were used, thirty-five rabbits rendered diabetic by the use of alloxan, five of them dead for unknown reasons, the remaining fifteen rabbits left normal. An intrabony defect was created in the maxilla of each rabbit, the defect of control group left for normal healing, while diabetic rabbits divided into two groups: First group in all the three periods postoperatively.

Results: Histological examinations showed that with the use of autologous Platelet-rich plasma in a bone of diabetic rabbit, an obvious enhancement of new bone forming and neovascularization significantly more than that of diabetic group without PRP application, the results of diabetic group treated with PRP reached the levels of normal group in all the three periods postoperatively.

Conclusions: this study illustrated that PRP has an osteopromotive activity that accelerated bone healing process in hypoglycemic animals.

Key words: - Platelet–rich plasma; a alloxan; Diabetes intrabony defect. ( J Bagh Coll Dentistry 2011;23(4):71-75).

INTRODUCTION
Diabetes mellitus is a metabolic disorder characterized by disturbances in the metabolism of carbohydrates, proteins, and lipids as a result of absolute of moderate insulin deficiency.

Diabetes impairs the bone healing process beginning with a reduction in early cellular proliferation, continuing with a delay in chondrogenesis, and ending with a decrease in the biomechanical properties of the bone callus. (1)

Alloxan experts direct cytotoxic action on pancreatic islets. thereby eliminating the production of insulin and causing severe hyperglycemia in the animal. Many of the defect in bone healing causes by diabetes are the direct result of Hyperglycemia. (2)

Platelet–rich plasma PRP is an autologous product that concentrates a large number of platelets in a small volume of plasma, PRP
functions as fibrin tissue adhesives with hemo-static and tissue sealing properties, but it differs from fibrin glue and other platelets – poor tissue adhesive because it's platelets provide a unique ability to promote wound healing and enhance osteogenesis. It's now known that platelets actively extrude several growth factors involved in initiating and sustaining wound repair. Studies showed that growth factors play an important role in bone and cartilage formation, fracture healing and repair of musculoskeletal tissue. The purpose of this study is to assess histologically the effect of PRP on bone healing in intrabony defect of maxillary and correlate the effect of time of bone healing in two experimental groups with that of controls.

MATERIALS AND METHODS

Animals
Fifty New Zealand male rabbits, weight 750–1000g were used in this study. Five of them died during study rabbits were randomly assigned to the diabetic or control group. The animal care committee at the national center of therapeutic research approved all procedures performed.

Alloxan - induced diabetes model
Thirty – five rabbits randomly selected diabetes was induced by a single intravenous injection into marginal ear vein, of 150mg/kg monohydrated alloxan.dissolved in 1cc distilled water rabbits was given a glucose solution 5% instead of tap water for 3days. After eight days, (5ml) of intracardiac blood to determine the blood glucose level, animals with the level of 220 – 500 mg/ml were considered as diabetic.

Preparation of platelet – rich plasma (PRP)

PRP Preparation 10ml of intracardiac blood drawn from each diabetic rabbit was combined with 1cm³ of anticoagulant citrate dextrose phosphate (ACD – A) to prevent coagulation. The blood was centrifuged at 1500rpm for 10min to separate the plasma containing the platelets from the red cells – the plasma was drawn off the top, mixed with 0.4ml of ACD–A anticoagulant, and centrifuged for an additional 10min at 3000rpm to separate the platelets. The platelet – poor plasma (PPP) was separated from the PRP along with the Buffy coat. The buffy coat and PRP, approximately 1 – 1.5ml was gently aspirated with a micropipette and placed in a sterile tube. Before application the concentrate should be activation by the addition of 0.5ml of 10% calcium chloride which inhibits the blood – thinning effect of (ACD – A). After activation, PRP turned into a gel – like solution with adhesive properties (with in 30mintes) and freshly used by inserting it inside a syringe for application.7

Surgical procedure
Animals were anesthetized by using a general anesthesia with ketamine hydrochloride 10% (50mg / kg) and xylazin 2% (5mg / kg) while preoperative antibiotic (oxytetracycline 20% 1ml / kg) i.m. gave for diabetic rabbits only and the same dose give.

An infrbony defect of 2 – 3mm depth was created in the right side of maxilla, fifteen of the diabetic rabbits their defects filled with PRP then a piece of hematic agent placed to close the hole to avoid seepage of PRP into the surrounding tissue, the wound was closed with black silk suture, while with the other fifteen diabetic rabbits and the normal fifteen rabbits just closed with apiece of hematic agent the rabbits recovered from anesthesia without complications. They were given postoperative narcotic pain medication.

Specimen preparation
The control (N=15) and diabetic (N=30) groups were sacrificed at 1,3,6 weeks (5 rabbits from each group at each time). The specimens were fixed in 10% buffered formalin for 48h, decalcified with solution of formic acid and sodium citrate for 2 – 4 weeks then bone tissue dehydrated with grated alcohol and embedded in paraffin. They sectioned at 5µm with a steel knife. The histological specimens were prepared in the usual fashion with hematoxylin and eosin staining. Histological evaluation was performed at 4,10,20,40x magnification. The specimens were examined regarding the number of bone forming cells (osteoblasts, osteocytes, and osteoclasts), the number inflammatory cells (lymphocytes, polymorphnuclear leukocytes, and macrophages),and thickness of new bone trabeculae were measured by using a special graduated microscopic lens at power 40X.in different intervals.Microscopic analysis was performed by two pathologists in a blind manner.

Statistical analysis
Data reported as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was used to determine the groups differences. Multiple comparisions (like LSD least significant difference) were used between each two groups separately. All analyses were made using (SPSS) statistical soft ware package.
RESULTS

Histological evaluation

Figures below showed the histological evaluation of all groups at 1, 3, 6 weeks intervals.

One week interval

Both control and diabetic treated with PRP groups, showed with slight differences between them, granulation tissue with large number of inflammatory cells and fibroblasts. Bundles of collagen fibers were detected with new blood vessels. While diabetic group without PRP revealed a small size of granulation tissue with less number of inflammatory cells and fibroblasts as well as collagen fibers.

Three weeks interval

Both control and diabetic treated with PRP groups showed fibrocartilagenous tissue with prominent new bone trabeculae, with osteocytes trapped within lacunae and osteoblasts lined the surfaces of new bone trabeculae, with less number of osteoclasts. The diabetic group expressed a fibrous connective tissue with small size of new bone trabeculae. Both osteocytes and osteoblasts were less in number than those of control and diabetic treated with PRP groups, while the osteoclasts number are much more than it's number in the other two groups.
Six weeks interval

Area of matured new bone was observed in both control and diabetic with PRP groups, with large number of osteocytes and less number of osteoblasts than those of the same groups at 3 weeks interval. Diabetic group showed a thin area of new bone trabeculae and a fibrous connective tissue revealed new vascularity with small new bone trabeculae scattered within it that indicated the process of bone formation still continuous.

Figure 7: Control group at 6th weeks interval showed, oc., osteocytes, ob., osteoblasts, b.t., bone trabeculae, b.v., blood vessel, fb., fibroblasts, cf., collagen fibers, lym., lymphocytes. (H&E, 10X)

Figure 8: Diabetic group at 6th weeks interval showed, RL., reversal line, oc., osteocytes, ob., osteoblast, n.b.t., new bone trabeculae, o.b., old bone, f.c.t., fibro connective tissue, fb., fibroblasts, cf., collagen fibers, b.v., blood vessele. (H&E, 10X)

Figure 9: Diabetic group without PRP at 6th weeks interval showed, n.b.t., new bone trabeculae, oc., osteoblasts, ob., osteoblasts, f.c.t., fibrous connective tissue, fb, fibroblasts, cf., collagen fibers, RL., reversal line, b.v., blood vessel, o.b., old bone.(H&E, 10X)

DISCUSSION

Histological Evaluation

1-At first week interval

Diabetic-group with PRP showed higher number of inflammatory cells than that in the (DM-group) (P<0.001), while their numbers approaching the numbers of the control group of the same interval. That led to improving early cell proliferation rates in the defect area. This agreed with (Rick et al.), who showed that macrophages arrived due to the vascular ingrowth stimulated by the platelets and regulate bone healing by secreting some of the same growth factors plus additional ones. Platelet-rich plasma has an added anti microbial effect since high concentration of leukocytes present within it. (8)

The mean number of fibroblasts in (DM-group with PRP) was higher than in (DM-group), but reaching the level of controls. this finding agreed with (Liberman et al.), they showed that transforming growth factor-beta (TGF-β) released by platelets during inflammatory stage of fracture healing affects all stages of healing process. (PRP) able to release (TGF-β) that stimulate indifferentiated meusenchymal stem cells to proliferate. (4)

Collagen fibers arranged more obviously in (DM-group with PRP) than that in (DM-group), but mostly the same as in control. This result agreed with (Marx RE), who showed that TGF-β activates fibroblasts to induce collagen formation, which indicates that (PRP) able to stimulate the synthesis of collagen fibers. (9)
New vascularization were seen in DM-group with PRP much more that in the (DM-group). This is in agreement with Devescovi et al, they showed that platelets in (PRP) on activation release growth factors (particularly) vascular endothelial growth factor (VEGF) that serve to accelerate angiogenesis. (10)

2-At 3 week interval
Both osteoblasts and osteocytes numbers in DM-PRP group are higher than those of the DM-group, but they are similler to those of control groups. This result agreed with Mehta and Watson, they showed that (PRP) can potentially enhance the recruitment and proliferation of stem cells. TGF-β stimulates the proliferation of osteoblasts precursor cells, and modulates bone matrix synthesis; regulate cellular proliferation, differentiation and apoptosis. (1)

Osteoclasts number higher in DM-group than those in the (DM-group + PRP), which indicated an increase of osteoclastic activity that contribute to smaller bone formation than those of other two groups. This agreed with Kayal et al, they showed an increase in osteoclast number in diabetic group which was generally higher across the fracture callus compared to normal. (11) While the number of osteoclasts in (DM group with PRP) was nearly the normal level. This agreed with Grageda, who suggested that TGF-β decreases bone resorption by inducing apoptosis of osteoclasts. (12)

Thickness of new bone trabeculae
Diabetic group with PRP showed prominent bone trabeculae at the defect site with mean thickness nearly the same as in control group, while DM-group showed the least amount of new bone formation than the other two groups. This is in agreement with Gandhi et al; they showed that in diabetics, the loss of cartilage contributes to the reduced bone formation. (13)

3-At 6 week interval
In (DM-group with PRP) revealed high mean thickness of new bone trabeculae than that of (DM-group) and about the same level of the control group. Bone formation continued and healing process of the area reach nearly the end stage, represented by thick bone trabeculae formation with osteocytes trapped with in lacunae. While in (DM-group) revealed a long, thin area of new bone trabeculae observed that indicated an early bone formation, with large area of fibrous connective tissue in the middle.

The formation of bony callus in DM-group was delayed, but not inhibited. This result agreed with Diniz et al, who stated that this result attributed to the facts that persistence of a large cartilaginous callus, together with trabecular bone with sparse spacing, suggested delayed healing in diabetics. (14)

REFERENCES