Histological Evaluation of Effect of beta-Tricalcium Phosphate on Bone healing in Alloxan-Induced diabetes

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ABSTRACT

Background: Improved glucose level control with insulin injections have allowed for the diabetic population to live longer and healthier lives. Unfortunately diabetes remains a worldwide epidemic disease with multiple health implications. Specifically, its effects upon fracture healing are compromised in diabetics with as high as 87% recovery delay relative to "healthy" counterparts. Current medical treatments for bone injuries have been largely focused on replacing the lost bone with allogenic or autogenous bone grafts, beta-tricalcium phosphate (β -TCP), a ceramic alloplast, has interconnected system of micropores, has been widely used as a biologically safe osteoconductive bone substitute. The aim of this study was histological evaluation of effect of topical application of β –TCP on bone healing of diabetic rabbit.

Materials and methods: Sixty New Zealand rabbits used in this study were divided into three groups for four healing intervals the experimental groups were: 1-Control group(C).2-Diabetic rabbits received insulin treatment regarded as controlled diabetes mellitus (CDM)group.3-Diabetic rabbits did not receive any treatment regarded as uncontrolled diabetes mellitus (UDM)group. All animals subjected to surgical operation in right tibia, creating bone defect 3mm in depth and 4mm in diameter filled with β-Tricalcium Phosphate. Animals' scarifications were done in 5 day, 2, 4 and 6 weeks durations. Routine processing and sectioning technique was performed for histological evaluation.

Results: Histological findings indicated that bone defects in control(C) and controlled diabetes mellitus (CDM) groups showed early bone formation, mineralization and maturation in comparison to healing of uncontrolled diabetes mellitus (UDM) group. Histomorphometric analysis for all bone parameters examined in this study, showed variation in significance among all groups in different durations.

Conclusion: The study revealed that application of β-TCP was more effective in enhancement of bone regeneration and in acceleration of bone healing process in controlled diabetes as compared to the uncontrolled one.

Key words: Osteoconduction, Diabetes mellitus, Beta-tricalcium phosphate. (J Bagh Coll Dentistry 2016; 28(3):75-81).

INTRODUCTION

Research on bioactive material on bone healing is one of the thrust scientific fields to developing of a novel bioactive material, which have wide application in dentistry and biomedical fields [(1)]. Bone healing is a dynamic process that is a result of smooth progression from one healing stage to another, the phases of bone healing: Hemostasis, angiogenesis, and bone formation are all present in the drill-hole defect model [(2)]. B-tricalcium phosphate (β-TCP) is widely applied material in clinical orthopedic and due to its high osteoconductivity, lack of Histotoxicity [(3)].

Several mechanisms have been reported to explain the greater delay of healing and non-union of fractures in diabetes. These include reduction in blood supply and angiogenesis [(4)]. Severe increased inflammatory response levels of systemic inflammatory markers serum levels of both tumor necrosis factor-a and interleukin-6, a decrease in collagen synthesis, a disturbance in the mineralization process, and an imbalance between bone resorption by osteoclasts and bone deposition by osteoblasts thought affect bone healing in diabetes [(5)].

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The aim of this study was histological evaluation of effect of topical application of β –TCP on bone healing of diabetic rabbit.

MATERIALS AND METHODS

The materials used in the present study were beta - tricalcium phosphate(Septodont/France), alloxan (100 mg, England), anesthetic solution: Ketamine hydrochloride 50 mg and Xylazine 2%, formalin 10%,ethanol alcohol 96%,xylol, paraffin wax, and Hematoxylin and Eosin (H&E) stain.

Sixty New Zealand rabbits of weight 1.5 –2kg were used in this study, they were divided into 20 rabbits as control group(C) and 40 rabbits were weighted to calculate the dose of alloxan given to them**.** The rabbits were injected by a single dose (150 mg/kg B.W.) intravenously.

Five to seven days after injection; severity of the induced diabetic state was assessed by daily monitoring of blood glucose levels. After elevation of blood glucose level, the rabbits randomly divided into: Controlled diabetes mellitus (CDM) group received subcutaneous injection of insulin as a treatment in a dose of 0.1mg/kg B.W. to control the hyperglycemia, with daily monitoring of blood glucose level and Uncontrolled diabetic mellitus (UDM) group they didn't receive any treatment[(6)].

Surgical procedure

A hole of about 4 mm in diameter and 3mm in depth was made in the proximal tibia metaphysis of the right limb of all animals by intermittent drilling and irrigation with saline, after that the operation site was washed with a sterile normal saline to remove debris and then the dried hole was filled with β-tricalcium phosphate, suturing of soft tissues was done and prophylactic antibiotic was given to animals.

Animals were scarified by an overdose of anesthetic solution at (5 days, 2, 4 and 6 weeks) healing intervals. Removal of skin, facia and muscles at the operation site was performed; afterwards bone specimens were prepared by cutting the bone about 5 mm away from operation site with continuous irrigation with saline to avoid bone damage.

The specimens were fixed in 10% buffered formalin for 48h,decalcified with solution of formic acid and sodium citrate, then bone tissue dehydrated with alcohol and embedded in paraffin. Sections of 5µm were prepared in the usual fashion, and stained with hematoxylin and Eosin stain.

Histological evaluation was performed using light microscope to measure the number of bone cells (osteoblasts, osteocytes, and osteoclasts), trabecular area (mm^2), bone marrow area (mm^2) and trabecular number. Measurements were performed by image processing software program (ImageJ.exe)**.** Two microphotographs were taken by a camera attached to the microscope at power X4, one in upper part and other picture in lower part to cover approximately all defect area.

RESULTS

Histological evaluation

Five days duration

Control group (C): Histological findings of defect area in control group after 5 days shows deposition of bone matrix rimmed by osteoblasts (Figure1).

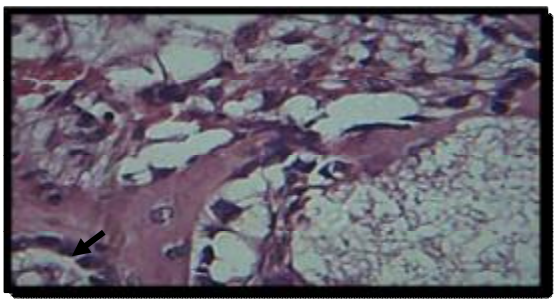

Figure 1: View of defect area in control group after 5days shows immature bone matrix and osteoblasts (OB) (arrow) at periphery. H & E X40.

Control diabetes mellitus group (CDM): Microphotograph of defect site shows osteoblasts and osteocytes (Figure 2).

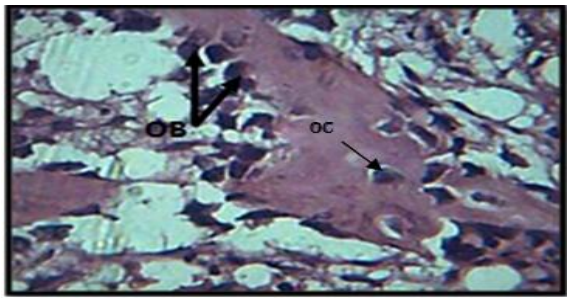

Figure 2: View of CDM group at 5days shows osteoblasts (OB), and osteocytes (OC). H & E X40.

Uncontrolled diabetes mellitus group (UDM): Histological view of defect site at 5 days healing period, shows recruitment of inflammatory cells around blood islets (Figure 3).

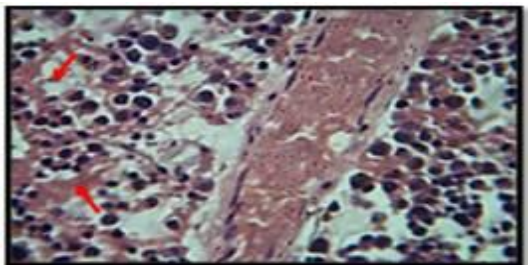

Figure 3: View of 5days duration in UDM group shows blood islets surrounded by inflammatory cells (arrows). H&E X40.

Two weeks duration

Control group(C): View of 2weeks at defect site show osteoblasts rimming bone trabeculae, and osteocytes within it (Figure 4).

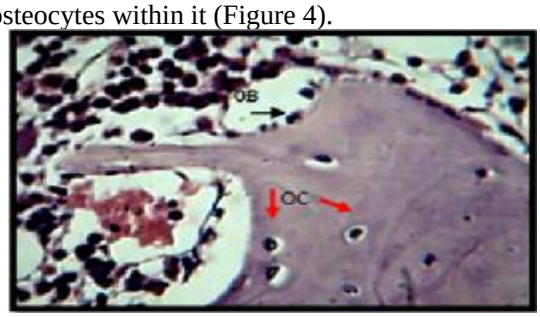

Figure 4: View in control group of 2weeks duration shows, bone trabeculae osteoblasts (OB) and osteocytes (OC). H& E X40.

Controlled diabetes mellitus group (CDM):

Histological view shows bone trabeculae numerous osteocytes trapped inside matrix and reversal line (Figure 5).

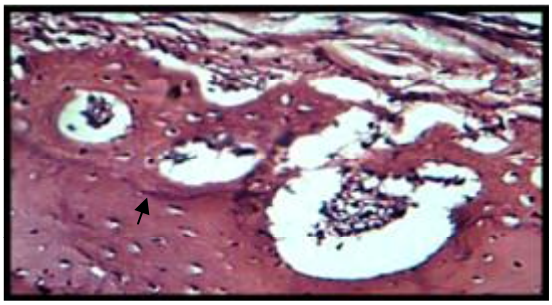

Figure 5: View of 2weeks duration in CDM group shows reversal line (arrow). H&E X20.

Uncontrolled diabetes mellitus group (UDM): Histological view shows defect area filled with β-TCP material surrounded by numerous bone trabeculae, osteoblasts seen at their periphery, and osteocytes in bone matrix **(Figure 6).**

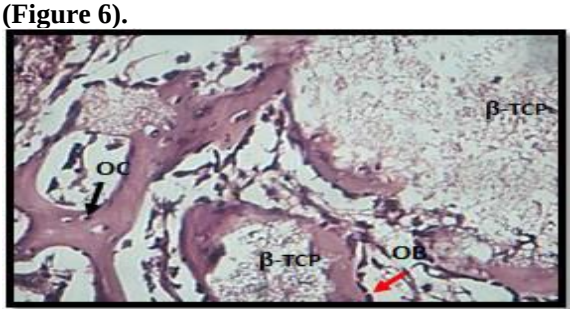

Figure 6: View of defect site in UDM group after 2 weeks shows osteoblasts (OB), rimming newly formed trabeculae. H & E X20.

Four weeks duration:

Control group (C): View of defect area in control group shows bone trabeculae, new and old bone are separated by reversal line, osteoblasts and numerous irregularly arranged osteocytes, and osteoclasts indicate bone remodeling (Figure 7) .

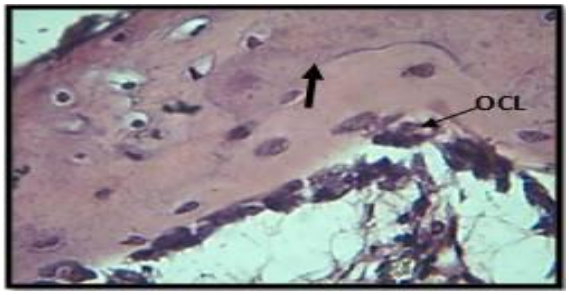

Figure 7: View of 4weeks shows bone trabeculae, osteoblast, osteocyte and osteoclast, (OCL) and reversal line (arrow). H&E X40.

Controlled diabetes mellitus group (CDM): Histological view illustrates bone trabeculae at defect area, osteocytes trapped in the bone matrix, reversal line between new and old bone (Figure 8).

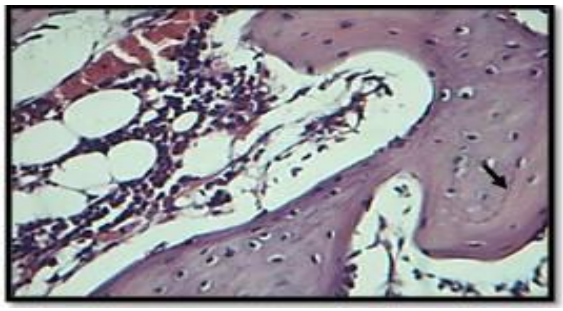

Figure 8: View of 4weeks duration in CDM group shows numerous irregular osteocytes (OC) trapped in bone trabeculae (BT), and reversal line (arrow). H &E X20.

Uncontrolled diabetes mellitus group (UDM): Histological view shows β-TCP material is surrounded by newly formed bone, osteoblast at the periphery, numerous scattered osteocytes in the bone matrix (Figure 9).

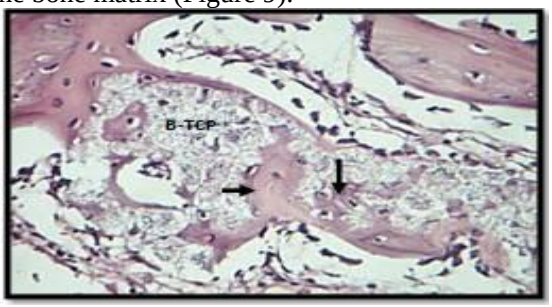

Figure 9: View of defect site after 4weeks in UDM group shows β-TCP material, surrounded by new bone trabeculae (arrows). H & E X20.

Six weeks duration

Control group(C): The histological view in control group at 6 weeks duration at defect site shows bone trabeculae, osteocytes and osteoblasts (**Figure** 10).

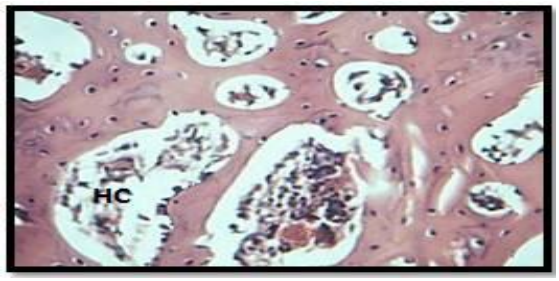

Figure 10: View of control group after 6 weeks shows bone trabeculae enclosing

Haversian canals (HC) lined by osteoblasts, surrounded by osteocytes (OC) H&E X20.

Controlled diabetes mellitus group (CDM): After 6 weeks view of defect site shows mature dense bone trabeculae and osteocytes appear regularly arranged around haversian canals which are lined by osteoblasts (Figure11).

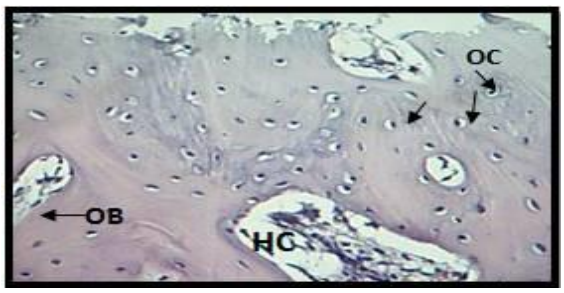

Figure 11: Microphotograph view at defect site of 6weeks duration in CDM group shows haversian canals lined by osteoblasts (OB) and surrounded by osteocytes (OC). H & Ex 20.

Uncontrolled diabetes mellitus group (UDM): Histological view shows defect site filled with trabeculae, with numerous scattered osteocytes inside bone matrix, osteoblasts seen at areas occupied by β-TCP material (Figure12)

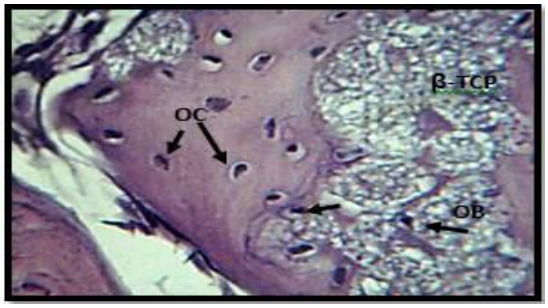

Figure 12: View in UDM group after 6weeks shows bone trabeculae (BT), with scattered osteocytes (OC), osteoblasts) and areas of remnants of β-TCP material. H & Ex 40.

Inflammatory cells parameter

The results of the present study showed that mean values of inflammatory cell count were decreasing with time in each studied group (C, CDM, UDM),and the highest mean values recorded were in UDM group at 5days healing period (Figure 13).

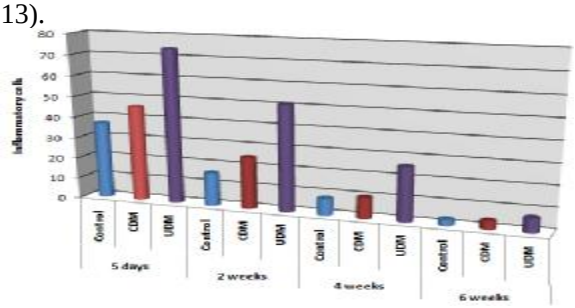

Figure 13: Comparison of mean values of inflammatory cells among studied groups in different durations.

Histomorphometrical analysis of bone architecture parameters

All measured parameters had increased mean value with time except bone marrow area was decreased in all groups, the highest mean value of all measured parameters were recorded in(C) group except bone marrow area and osteoclast in (UDM)group. The mean values of bone marrow area, trabecular area, osteoblasts and osteocytes showed high significant difference in all groups with different duration except bone marrow area showed significant difference at 6weeks, while trabecular No. mean value showed non significant difference at 2 and 4weeks but at 6weeks duration showed significant difference only (Table 1).

Table 1: Descriptive statistics and group differences in each duration of bone parameters

Duration	Variables	Groups	Descriptive Statistics						Group difference (d.f.=14)	
			N	Mean	S.D.	S.E.	Min.	Max.	F-test	p-value
2 weeks	Trabecular No.	Control	5	6.20	1.92	0.86	4	9	2.396	0.133 (NS)
		CDM	5	5.20	1.92	0.86	3	8		
		UDM	5	3.80	1.30	0.58	2	5		
	Trabecular area	Control	5	0.96	0.17	0.08	0.759	1.22	23.137	0.000 (HS)
		CDM	5	0.75	0.05	0.02	0.697	0.793		
		UDM	5	0.52	0.02	0.01	0.497	0.553		
	Bone marrow area	Control	5	1.16	0.11	0.05	1.01	1.3	29.446	0.000 (HS)
		CDM	5	1.32	0.07	0.03	1.251	1.412		
		UDM	5	2.01	0.30	0.13	1.674	2.391		
	Osteoblasts	Control	5	6.00	1.54	0.69	4.5	8.5	11.499	0.002 (HS)
		CDM	5	5.45	1.18	0.53	3.5	6.5		
		UDM	5	2.10	1.43	0.64	0.5	4		
	Osteoclasts	Control	5	0.50	0.35	0.16	0	1	3.263	0.074 (NS)
		CDM	5	0.60	0.22	0.10	0.5	1		

		UDM	5	1.10	0.55	0.24	0.5	2		
	Osteocytes	Control	5	9.25	1.44	0.64	7.25	11	12.584	0.001 (HS)
		CDM	5	7.75	2.32	1.04	5.5	11.5		
		UDM	5	4.10	0.96	0.43	3	5.5		
4 weeks	**Trabecular No.**	Control	5	7.80	1.48	0.66	6	10	0.902	0.431 (NS)
		CDM	5	7.20	1.30	0.58	6	9		
		UDM	5	6.40	2.07	0.93	4	9		
	Trabecular area	Control	5	1.82	0.10	0.05	1.689	1.915	57.723	0.000 (HS)
		CDM	5	1.46	0.06	0.03	1.386	1.534		
		UDM	5	1.07	0.15	0.07	0.867	1.233		
	Bone marrow area	Control	5	0.73	0.08	0.03	0.626	0.81	23.865	0.000 (HS)
		CDM	5	0.83	0.04	0.02	0.781	0.887		
		UDM	5	1.07	0.11	0.05	0.939	1.212		
	Osteoblasts	Control	5	8.50	2.06	0.92	6	11	8.455	0.005 (HS)
		CDM	5	6.15	1.50	0.67	4.75	8		
		UDM	5	4.00	1.58	0.71	2	6		
	Osteoclasts	Control	5	0.40	0.55	0.24	0	1	0.563	0.584 (NS)
		CDM	5	0.40	0.55	0.24	0	1		
		UDM	5	0.70	0.45	0.20	0	1		
	Osteocytes	Control	5	12.30	1.48	0.66	10	14	20.047	0.000 (HS)
		CDM	5	11.10	2.46	1.10	9	15		
		UDM	5	5.60	1.14	0.51	4	7		
6 weeks	**Trabecular No.**	Control	5	12.20	3.11	1.39	9	16	6.454	0.013 (S)
		CDM	5	10	2.74	1.22	7	14		
		UDM	5	6.60	1.14	0.51	5	8		
	Trabecular area	Control	5	2.38	0.19	0.09	2.143	2.65	28.472	0.000 (HS)
		CDM	5	2.12	0.17	0.08	1.887	2.316		
		UDM	5	1.46	0.23	0.10	1.114	1.66		
	Bone marrow area	Control	5	0.54	0.07	0.03	0.469	0.636	4.174	0.042 (S)
		CDM	5	0.66	0.05	0.02	0.591	0.724		
		UDM	5	0.86	0.29	0.13	0.54	1.21		
	Osteoblasts	Control	5	6.30	0.82	0.37	5.25	7.5	21.573	0.000 (HS)
		CDM	5	5.15	0.96	0.43	4	6.25		
		UDM	5	2.75	0.83	0.37	2	3.75		
	Osteoclasts	Control	5	0.30	0.45	0.20	0	1	0.438	0.656 (NS)
		CDM	5	0.40	0.55	0.24	0	1		
		UDM	5	0.60	0.55	0.24	0	1		
	Osteocytes	Control	5	16.50	2.50	1.12	13	20	18.166	0.000 (HS)
		CDM	5	15.85	1.75	0.78	13.5	18		
		UDM	5	7.50	3.39	1.52	4	12		

DISCUSSION

Histological evaluation

According to the present findings deposition of organic matrix of bone was detected at 5days in (C) and (CDM) groups , as reported by previous studies, [7,8] that could be due to role of β-TCP in accelerating healing more effectively by promoting vascularization and ossification, due to it's similarity in composition to the mineral component of bone and presence of micropores which have been proven important in the bone induction process. Micropores enlarge the surface area, which facilitates ion exchange and bone-like apatite formation, and/or accompanied binding of endogenous bone inducing proteins to the surface, all of which may positively affect the adhesion, proliferation, and differentiation of the bone forming cells thus can precipitate new bone growth onto the cement surface as reported by Zhang *et al.* [9]. The findings of the present study indicated accelerate healing process after controlling of hyperglycemia by insulin, where the highest mean values of inflammatory cells that infiltrated into bone defect sites were recorded at 5 days and especially in UDM group, it was obviously decreased during the 2, 4 and 6weeks periods in agreement with the findings of Cardaropoli *et al.* [10]. Highest mean values of inflammatory cells were seen in UDM group which could be due to severe inflammatory response levels of systemic inflammatory markers both tumor necrosis factor-a and interleukin-6 as

reported by Suzuki *et al.* [5]. At 4 weeks duration, immature bone trabeculae surrounding β-TCP were seen with signs of bone remodeling, in accordance with the findings reported by Ogose *et al.* who evaluated the histologic characteristics of β-TCP in the human femur and observed a considerable amount of newly formed bone on β-TCP particles and found osteoclast-like giant cells surrounding β-TCP particles [11]. The remodeling process lasts for 3 to 6 months in humans and 6 weeks in rabbits [12].

A marked reduction in mean number of osteoblasts, osteocytes, and trabecular area and increased in bone marrow area was noticed in the present results in the animals of UDM group than that of animals of other groups. This result agreed with Luu *et al.* [13]**,** Regarding UDM, the findings of this study, they could be explained according to:

1- Botolin *et al.* who revealed that decreased numbers of osteocytes and osteoblasts, serum alkaline phosphatase and osteocalcin levels suggesting reduced bone formation [14].
2- Diabetes decreases osteoclastogenesis reduces bone formation and enhances apoptosis of osteoblastic cells in bacteria stimulated bone loss [15,16]**.** They affirmed that diabetes may cause a net loss of bone because the suppression of bone formation is greater than the suppression of bone resorption.
3- Confirmed by other studies that evaluated fracture healing at 2, 4, and 6 weeks suggesting that diabetes decreases the anabolic aspect of fracture healing by affecting osteoblasts in terms of formation, function, and bone deposition [17]**.**

At 4 and 6weeks of healing periods more osteoblasts and osteocytes were detected in (C) and (CDM) groups and marked increased in trabecular area, indicating progression of bone deposition and maturation and being denser with narrowing of marrow tissue regions. when compared with UDM group, in agreement with results of an experimental study conducted by Soares *et al.* who revealed that at 4 weeks the specimens showed the defect either partially or completed filled by newly formed interconnecting trabecular bone of varied thicknesses [18]. Results obtained concerning CDM groups could be explained according to the fact that insulin has been postulated to elicit an anabolic role in bone, it has been suggested that insulin signaling pathways may mediate communication between metabolic control and appropriate bone remodeling as demonstrated by Fulzele *et al.* by means of an in vitro model that insulin administration suppresses an inhibitor of osteoblast development [19].

As conclusion; the study revealed that application of β-TCP was more effective in enhancement and in acceleration of healing process of bone defects in healthy animals and in controlled diabetic as compared to the uncontrolled ones.

REFERENCES

1. Mauth C, Huwig A, Graf-Hausner U, Roulet J. Topics in Tissue Engineering 2007; 3: 1-30.
2. Mueller M, Schilling T, Minne HW, Ziegler R. A systemic acceleratory phenomenon (SAP) accompanies the regional acceleratory phenomenon (RAP) during healing of a bone defect in the rat. J. Bone Miner Res 1991; 6(4): 401-10.
3. Kobayashi K, Shimoyama K, Nakamura K, Murata K. Percutaneous vertebroplasty immediately relieves pain of osteoporotic vertebral compression fractures and prevents prolonged immobilization of patients. Eur Radiol 2005; 15: 360-7.
4. Dib SA, Russo EMK, Chacra AR. Tratado de endocrinologia clínica. São Paulo: Editora Rocca; 1992.
5. Suzuki K, Kurose T, Takizawa M, Maruyama M, Ushikawa K, Kikuyama M, et al. Osteoclastic function is accelerated in male patients with type 2 diabetes mellitus: the preventive role of osteoclastogenesis inhibitory factor/osteoprotegerin (OCIF/OPG) on the decrease of bone mineral density. DiabetesRes Clin Pract 2005; 68: 117-25.
6. Wang J, Wan R, Mo Y, Zhang Q, Sherwood LC, Chien S. Creating a Long-Term Diabetic Rabbit Model. Experimental Diabetes Research 2010; Article ID 289614, 10 pages
7. Ghareeb R. The Role of topical application of bone morphogenetic protein 7 (BMP7) as a biomaterial on bone healing. A master thesis. College of Dentistry, University of Baghdad, 2014.
8. Fadhil E. Histological and immunohistochemical evaluation of the effect of local exogenous application of VEGF on bone healing. A master thesis. College of Dentistry, University of Baghdad, 2014.
9. Zhang W, Li G, Deng R, Deng L, Qiu S. New bone formation in a true bone ceramic scaffold loaded with desferrioxamine in the treatment of segmental bone defect: a preliminary study. J Orthopaedic Sci 2012;17(3): 289–98.
10. Cardaropoli G, Araujo M, Lindhe J. Dynamics of bone tissue formation in tooth extraction sites. An experimental study in dogs. J Clinical Periodontol 2003; 30(9):809-818.
11. Ogose A, Hotta T, Hatano H, Kawashima H, Tokunaga K, Endo N, et al. Histological examination of beta-tricalcium phosphate graft in human femur. J Biomed Mater Res 2002; 63(5): 601–4.
12. Rokn AR, Moslemi N, Abadi HK. Histologic Evaluation of Bone Healing Following Application of Anorganic Bovine Bone and β-tricalcium Phosphate in Rabbit Calvaria. J Dentistry, Tehran University of Medical Sciences, Tehran, Iran 2012; 9(1): 35-40).
13. Luu H, Kraut D, Graves D, Gerstenfled L. Diabetes interferes with the bone formation by affecting the expression of transcription factors that regulate the

osteoblasts differentiation. Endocrinol 2003; 144: 352-64.
14. Botolin S, McCabe LR. Chronic hyperglycemia modulates osteoblast gene expression through osmotic and non-osmotic pathways. J Cell Biochem 2006; 99: 411–24.
15. He H, Liu R, Desta T, Leone C, Gerstenfeld LC, Graves DT. Diabetes causes decreased osteoclastogenesis, reduced bone formation, and enhanced apoptosis of osteoblastic cells in bacteria stimulated bone loss. Endocrinol 2004; 145(1): 447-52.
16. Diniz SF, Amorim FPLG, Bocca AL, Batista AC, Simm GEPM, Silva TA. Alloxan-induced diabetes delays repair in a rat model of closed tibial fracture. Braz J Med Biol Res 2008; 41(5): 373-9
17. Graves DT, Paglia DN, Lin S .Impact of Diabetes on Fracture Healing. J Exp Clin Med 2011; 3(1): 3-8.
18. Soares LG, Marques AM, Guarda MG, Aciole JM, Pinheiro AL, dos Santos JN. Repair of Surgical Bone Defects Grafted with Hydroxylapatite + β-TCP and Irradiated with λ=850 nm LED Light. Braz Dent J 2015; 26(1): 19-25.
19. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, Faugere MC. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. Cell 2010; 142(2): 309-19.