The effect of autologous bone marrow-derived stem cells with estimation of molecular events on tooth socket healing in diabetic rabbits (Histological and histomorphometric study)

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ABSTRACT

Background: Diabetes is a metabolic disorder characterized by chronic hyperglycemia due to an inability to produce insulin. Uncontrolled or poorly controlled diabetes is clinically associated with increased susceptibility to delay healing. Many recent researches have shown that stem cell therapy can be the best choice for treatment of this disease. The aims of this research were investigating regeneration of pancreatic beta cells of diabetic induced rabbits after stem cell transplantation.

Materials and Methods: 64 rabbits weighting an average of (2.5 - 3 kg) were used in this experimental study, and divided into 4 groups as follows; group A (contains 16 healthy rabbits regarded as control group) , Group B (contains 16 diabetic rabbits not received treatment), group C (contains 16 controlled diabetic rabbits received insulin as a treatment) and group D (contains 16 rabbits received mesenchymal stem cells as a treatment) , the lower incisor for each rabbits was extracted and the socket was examined by histological and histomorphometric analysis after 2, 10, 20 and 30 days of healing periods after scarification.

Results: Histological findings showed that there was a normal healing of teeth – extracted sockets (early bone formation, mineralization and maturation) of the animals of group A, C and D when compared with group B. Histomorphometric analysis of the parameters (trabecular width (TbW), Tb Separation(TbS), Tb Number (TbNo), osteoblasts number (OBNo), osteocytes number(OCNo) and blood vessels number (BVNo) of all groups for all healing periods illustrated that there was a highly significant differences of groups A , C and D when compared with group B animals.

Conclusions: The present study concluded that there was delayed healing of teeth extracted sockets of the animals of group B (diabetic rabbits) due to the few numbers of osteoblasts (bone-forming cells) which differentiated from the fibroblasts cells and subsequent impairments in bone formation, mineralization and maturation.

Key words: Diabetic rabbits, teeth extraction, delay healing. (J Bagh Coll Dentistry 2013; 25(1):116-121).

INTRODUCTION

Diabetes mellitus is a chronic, widely spread human disease. Experimental induction of diabetes mellitus in animal models is essential for the advancement of our knowledge and understanding of the various aspects of its pathogenesis and ultimately finding new therapies and cure. Several methods have been used to induce diabetes mellitus in laboratory animals with variable success and many difficulties. Surgical removal of the pancreas is effective method; however, to induce diabetes, at least 90-95% of the pancreas has to be damaged [(1)]. Alloxan is a naturally occurring, broad spectrum antibiotic and cytotoxic chemical that is particularly toxic to the pancreas [(2)]. Induction of experimental diabetes in the rabbit using alloxan is very convenient and simple to use.

Alloxan injection leads to the degeneration of the Langerhans Islets beta cells clinically; symptoms of diabetes are clearly seen in Rabbits within 2-4 days following single intravenous or intraperitoneal injection of 100 mg/kg [(3)]. Healing of tooth extraction sockets in poorly controlled diabetic patients is often delayed and accompanied by severe infection. Current diabetes treatments just aim to lower the blood sugar through diet, exercise, medication with tablets and insulin, in recent researches, mesenchymal stem cells (MSCs) have brought to new hope ,the adult bone-marrow derived stem cells can regenerate the beta cell in diabetes animal models. These results lead to a new approach in diabetes treatment, especially type 1. Healing of a tooth extraction socket is a complex process involving tissue repair and regeneration. It involves chemotaxis of appropriate cells into the wound, transformation of undifferentiated mesenchymal cells to osteoprogenitor cells, proliferation and differentiation of committed bone forming cells, extracellular matrix synthesis, mineralization of osteoid, maturation and remodeling of bone. These cellular events are precisely controlled and regulated by specific signaling molecules [(4-10)].

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MATERIALS AND METHODS

Sixty four adult rabbits weighting an average of (2.5 - 3 kg) were used, the experimental animals were divided into four groups as follows:

Group A : contains 16 healthy rabbits regarded as control group.

Group B : contains 16 diabetic rabbits, not received any treatments.

Group C : contains 16 controlled diabetic rabbits received insulin as a treatment.

Group D : contains 16 diabetic rabbits received MSCs as a treatment.

Induction of Diabetes Mellitus in Rabbits (group B, C and D rabbits)

The rabbits were injected by a single dose (120 mg/kg) intravenous injection of the pancreatic beta-cells toxin monohydrate (Alloxan), which was administered to the rabbits via the marginal ear vein**.** Severity of the induced diabetic state was assessed by daily monitoring of blood glucose levels with a calibrated glucose meter (few drops from the ear) and daily estimation of the body weight. For determination of blood glucose level, the animals whose blood glucose level was greater than 200mg / dl were indicated as hyperglycemic**.** Five to seven days after injection, Alloxan induced diabetes by destroying the beta cells of the pancreas; the blood glucose level was elevated above the 200mg/dl **(Fig.1).** Animals of group C were received daily insulin as a treatment in a dose of 0.1 mg/ kg of body weight.

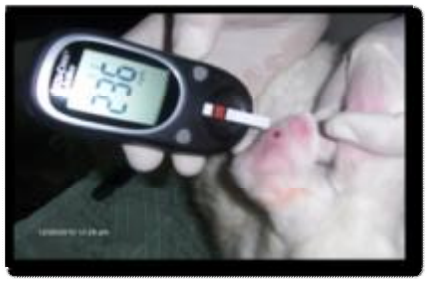

Fig.1: Elevation of blood glucose level

Isolation of MSCs from the Bone marrow (group D rabbits)

The surgery was performed under well sterilized condition and gentle surgical technique. The surgical towels were placed around the site of operation; the site chosen for operation was the proximal tibia metaphysis of the right limb **(Fig.2)**. Skin incision was done by using a sharp blade to expose the muscle **(Fig.3).** Then the muscle was dissected to expose the tibia **(Fig.4).** By intermittent drilling with (1 mm surgical drill) and continuous, vigorous irrigation with sterile normal saline, a guide hole was made **(Fig.5).** By using sterile syringe (5ml) that contains few drops of heparin (to prevent blood clotting) the bone marrow was aspirated as soon as possible **(Fig.6).** After that the area was washed very well with a sterile normal saline, the muscle was sutured with 3/0 absorbable (catgut) suture **(Fig.7).** The skin was sutured with interrupted 3/0 silk suture **(Fig.8).**

Fig.2: The site of operation

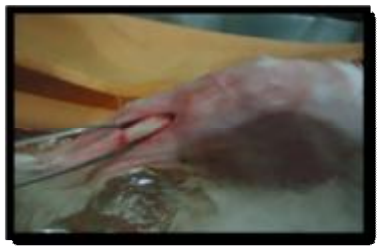

Fig.3: Skin incision

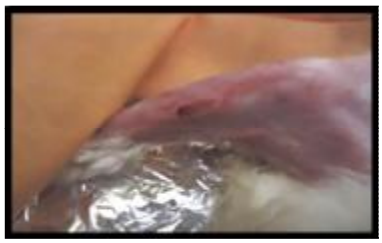

Fig.4: Dissection of the muscle

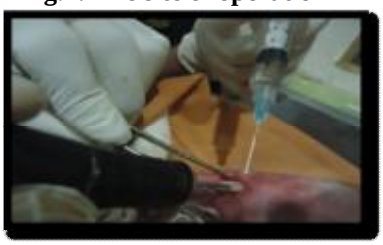

Fig.5: 1mm guide hole was made

Fig.6: Aspiration of bone marrow

Fig.7: Cat gut suture

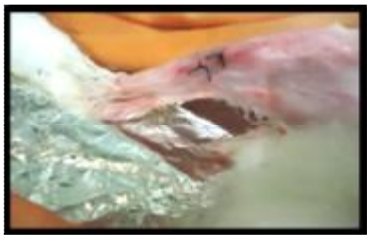

Fig.8: Skin sutured with silk suture

Inside the hood the bone marrow was inserted into two test tubes t.t.), equal volumes of phosphate buffer saline (PBS) was added to (t.t.) and shake very well ,then the two t.t. was put inside the centrifuge (2000 RPM) for 10 minutes. Inside the hood the top two thirds of the solution were removed. RPMI-culture media was added to the precipitate 1/3 of the t.t. & shake very well until the media was became homogenous, then the media was added into a well sterilized plastic falcons & covered very well by a parafilm, finally the media was incubated at (37 °C, 5% Co2 & 95% air).The cells were checked periodically under inverted microscope, the culture media was changed twice a week for two weeks. With the medium changes, almost all the non adherent cells were washed away.

Differentiation of MSCs into Insulin producing cells

1- Inside the hood about 2/3 of the medium in the falcons was removed and pre-inducing medium was added to the remaining 1/3 of the falcons, the pre-inducing medium containing low glucose–RPMI (L-RPMI) supplemented with 10 mM nicotinamide, plus 1 mM beta-mercaptoethanol and 10% of fetal bovine serum (FBS), then covered by a parafilm and incubated at (37 °C, 5% CO2 & 95% air) (for 24 hours).

2- The medium was changed with fresh inducing medium; containing serum free high glucose–RPMI (H-RPMI) , supplemented with 10 mM nicotinamide , plus 1 mM beta-mercaptoethanol , then covered by a parafilm and incubated at (37 °C, 5% CO2 & 95% air) (for 10-12 days).

Detection of Insulin producing cells

The insulin producing cells can be detected by dithiazone (DTZ) stain. DTZ is a zinc-chelating agent known to selectively stain pancreatic beta cells because of their high zinc content**.**

Inside the hood about 2/3 of the medium was removed from the falcon, then 2 ml of DTZ solution was added for the remaining 1/3 of the medium in the falcon that containing the MSCs, the cells were incubated at (37 °C, 5% CO2 & 95% air) for 30 minutes and examined under inverted microscope.

Reimplantation of MSCs

5 ml of the medium was reimplanted to the rabbits by subcutaneous injection.

Under sterile condition and gentle surgical technique, the lower incisor of each rabbits for all groups was extracted **(Fig.9).** 2 days after extraction 4 rabbits from each group were killed; 10 days after Extraction another 4 rabbits from each group were sacrificed; 20 days after extraction another 4 rabbits from each group were sacrificed; 30 days after extraction the remaining 4 rabbits from each group were sacrificed; the sockets blocks were immediately fixed in 10% formaldehyde solution and processed for histomorphometric and histological evaluations.

Fig.9 Extraction of the incisor tooth

The histomorphometric parameters currently used for the description of trabecular bone microarchitecture are all based on Parfitt's principles of the ''plate and rod'' model [(11)], these microarchitecture descriptors are: a.Trabecular number (TBNO), b.Trabecular width (TBWID,in microns),c.Trabecular separation (TBSEP,in microns), d.Osteoblast cell number (OB/mm2), e. Osteocyte cell number (OC/mm2) and f. Blood vessels number (BVNO).These parameters are derived from microscopic two dimension (image measurements analyzer software program, the magnification power lense X40).

Statistical Analysis

The following statistical data analysis approaches were used in order to analyze and assess the results of the study:

a. Mean (M) , b. Standard deviation (SD) , c. ANOVA test : for the comparison among the groups.

RESULTS

Histological Examination

For description purpose each block (the site of extraction) for each healing period of each group was divided into three regions: Cervical, middle and apical regions. The histological examination was performed under a light microscope.

Histological Findings of 2 days:

The histological findings of all regions showed that formation of blood clot with fibrinous network connection which infiltrated by inflammatory cells in groups A ,C and D, while in group B the histological finding showed blood clot formation with few numbers of inflammatory cells**.**

Histological Findings of 10 days:

The histological findings showed the proliferation of cellular connective tissue with formation of woven bone, deposition of osteoid tissue, and presence of proliferative osteoblasts and fibroblasts in groups A ,C and D, while in group B the histological findings showed the formation of granulation tissues which were infiltrated by mononuclear cells, there were no signs of osteiod tissues deposition or woven bone formation **(Fig.10) , (Fig. 11).**

Histological Findings of 20 days:

Histological findings showed that formation of new bone trabeculae, some of them were elongated to form rod-like surrounded by osteoblasts with numerous numbers of osteocytes and blood vessels in groups A, C and D while in group B, the histological finding showed sparse, thin osteoid tissue deposition surrounded by a basal bone **(Fig. 12) , (Fig. 13).**

Histological Findings of 30 days:

Histological findings showed thick well formed bone trabeculae that almost filled the entire sockets with numerous osteoblasts and osteocytes in groups A, C and D while in group B, the histological finding showed thin scattered bone trabeculae surrounded by socket bone with deposition of immature woven bone and numerous blood vessels in between**(Fig. 14) , (Fig. 15).**

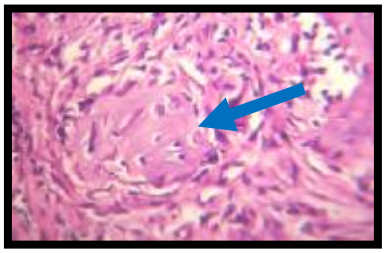

Fig.10: 10 days group A showing osteoid tissues.

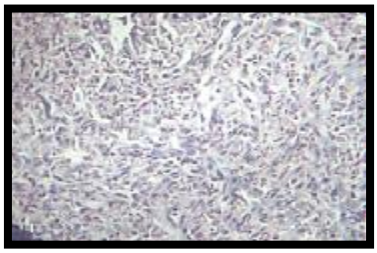

Fig.11: 10 days group B showing granulation tissues.

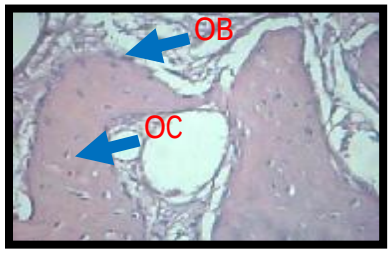

Fig.12: 20 days group D showing osteoblasts (OB) & osteocytes (OC).

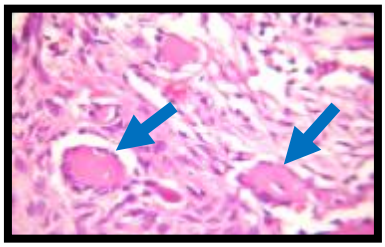

Fig.13: 20 days group B showing osteoids.

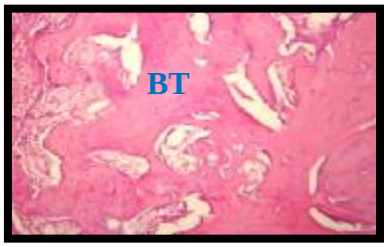

Fig.14: 30 days group A showing bone trabeculae (BT).

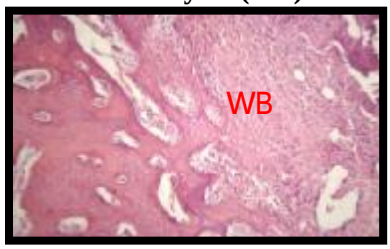

Fig.15: 30 days group B showing woven bone (WB).

Histomorphometric analysis

Data was collected by two dimension image analysis , **table1** showed descriptive statistics (M, SD and ANOVA test) of group A at different healing periods for total measurements, **table2** showed descriptive statistics (M, SD and ANOVA test) of group B at different healing periods for total measurements, **table 3** showed descriptive statistics (M, SD and ANOVA test) of group C at different healing periods for total measurements and **table 4** showed descriptive statistics (M, SD and ANOVA test) of group D at different healing periods for total measurements.

Figures (16 and 17) showed the correlation of the mean of variables measured at different healing periods (10, 20 and 30 days) for all groups.

Table 1: Descriptive statistics of group A at different healing periods for total measurements

Variables	10 Days		20 Days		30 Days		Sig.
	Mean	SD	Mean	SD	Mean	SD	
TbW	1.6667	1.37060	3.7500	.75378	5.8333	1.58592	.000
TbSep	16.7500	2.52713	13.1667	4.40729	11.4167	1.62135	.003
TbNo	2.5000	1.08711	8.3333	2.74138	13.5000	1.73205	.000
OBNo	9.1667	1.58592	20.3333	3.14305	15.5000	2.64575	.001
OCNo	3.5833	1.31137	8.0000	2.00000	11.8333	3.18614	.000
BVNo	9.7500	1.28806	12.9167	1.16450	5.5000	.90453	.000

Table 2: Descriptive statistics of group B at different healing periods for total measurements.

Variables	10 Days		20 Days		30 Days		Sig.
	Mean	SD	Mean	SD	Mean	SD	
TbW	0.0834	.28865	1.0835	.28862	2.6667	.65134	.000
TbSep	21.9167	2.31432	24.5000	2.90767	21.4167	2.57464	.015
TbNo	.5000	.52223	1.5833	1.16450	2.4167	.79296	.009
OBNo	.8333	1.19342	4.4167	1.56428	4.5833	1.72986	.017
OCNo	.5000	1.16775	2.0000	.85280	3.5000	1.44600	.000
BVNo	1.5000	52223	2.3333	.49237	1.3333	.49237	.648

Table 3: Descriptive statistics of group C at different healing periods for total measurements.

Variables	10 Days		20 Days		30 Days		Sig.
	Mean	SD	Mean	SD	Mean	SD	
TbW	1.0933	1.07309	2.3333	.65134	5.6667	2.41536	.000
TbSep	15.4167	2.90637	13.5833	2.02073	12.9167	1.24763	.103
TbNo	1.7500	1.21543	6.7500	1.42223	12.0000	1.27841	.000
OBNo	9.1667	1.26730	18.4167	2.53909	15.0833	1.88092	.001
OCNo	3.2500	1.71232	7.9167	1.44332	10.8973	1.93324	.000
BVNo	8.9167	1.00932	10.4167	.61493	4.9442	.87695	.000

Table 4 Descriptive statistics of group D at different healing periods for total measurements.

Variables	10 Days		20 Days		30 Days		Sig.
	Mean	SD	Mean	SD	Mean	SD	
TbW	1.8333	1.12523	2.6658	1.07986	5.5987	2.45361	.000
TbSep	15.6667	3.25431	12.9587	1.88298	11.5417	1.96873	.210
TbNo	1.9998	.87634	5.0896	2.14321	11.3542	2.13245	.000
OBNo	8.7989	2.34521	19.8765	2.40932	16.7865	1.78259	.000
OCNo	3.3300	1.71226	7.8697	1.87695	9.4624	1.76382	.000
BVNo	7.1667	1.12453	10.6667	.59845	5.1023	1.00139	.000

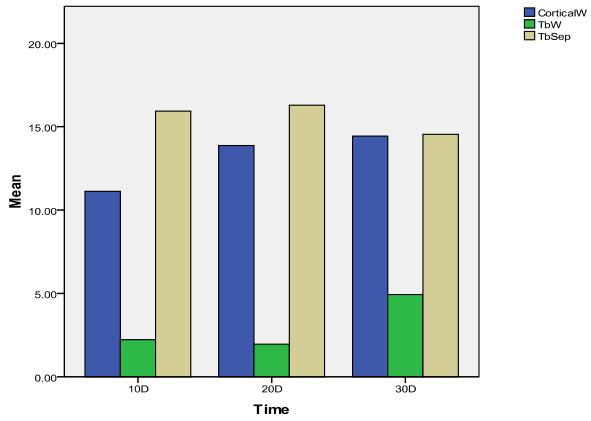

Fig.16 Comparison the Mean of TbW, and TbSep with time.

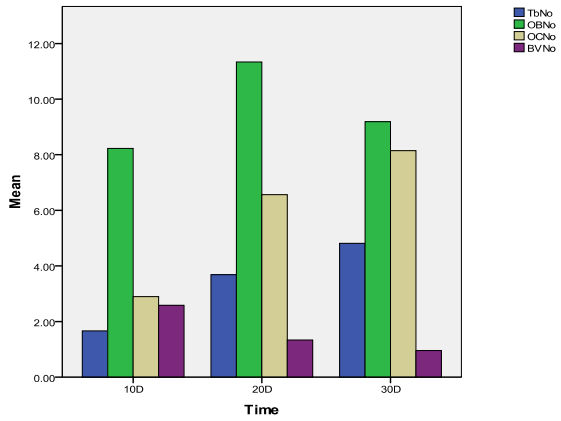

Fig.17 Comparison the Mean of TbNo, OBNo, OCNo and BVNo with time.

DISCUSSION

Histological evaluation

The results of the present study showed early detection of osteiod tissues formation in cervical region at 10 days of healing periods for groups A,C and D and increased deposition of osteiod tissues in the middle and apical areas. While in group B there was no evidence of osteiod tissues deposition in cervical and middle areas which were restricted for granulation tissue formation with heavy infiltration of mononuclear cells, a sign of extracellular matrix deposition of osteiod tissues were detected only in the apical area of group B. These histological observations were in agreements with Sloan [12 , 13] who suggested that in uncontrolled, insulin-dependent diabetes; the formation of the osteiod tissues in the tooth extraction socket is inhibited, resulting in delayed healing and increased alveolar destruction.

At 20 and 30 days of healing periods more osteoblsts , osteocytes and blood vessels was detected in groups A,C and D, when compared with group B. This result agreed with Luu [14,15] who demonstrated that diabetes leads to the decreased bone formation, because of decreased proliferation and differentiation of osteoblasts when compared with the control animals**.**

Histomorphometric evaluation

In particular, histomorphometry, based on the use of new computerized methods allow the acquisition of more sophisticated measurements by means of a digitizer have been introduced to integrate the use of the Microscope. These methods supply information on cortical width, osteoblasts, osteocytes ,trabecular width, blood vessels as well as on its distribution and on the organization of the trabeculae in the marrow space [16]. The equality of means and variance of all parameters tested for micro architecture records between all study groups illustrated a high value in groups A, C and D than those of group B, this result can be explained on a fact of early enhancement and recruitment of the fibroblasts and osteoprogenitor cells to be differentiated into osteoblasts (bone formative cells) and enhancement of osteiod tissue formation which need more supplements and more blood vessels. Osteocytes formation was happened by entraption of osteoblasts within their matrix; more osteoblasts resulted in more number of osteocytes and as a result of more and faster building of bone matrix.

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