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

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ABSTRACT

Background: 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. Some of these like transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMP) and insulin like growth factors (IGF) are well conserved proteins involved in the initial response to injury and repair in soft and hard tissue.

Materials and Methods: 48 rabbits weighting an average of (2.5 - 3 kg) were used in this experimental study, and divided into 3 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), the lower incisor for each rabbits was extracted, after 2, 10, 20 and 30 days of healing periods after scarification , the socket was analysed by immunohistochemical (IHC) estimation of growth factors : TGFbeta-3 , VEGF , IGF-1R , BMP-4 .

Results: IHC findings revealed high positive expression of TGFbeta-3, BMP-4 on fibroblasts, osteoproginetor cells, osteoblasts and osteocytes,high positive expression of VEGF on endothelial cells and high positive expression of IGF-1R on endothelial cells and moderate expression on osteoblasts.

Conclusions: The inhibition of proliferation and migration of osteoblasts, or differentiation from progenitor cells, is implicated in the delay of teeth sockets healing. For this fact the results of the present study concluded that in the diabetic healing bone (rabbits of group B), the onset of cell proliferation and osteoblast differentiation were delayed and subsequently prolonged healing process when compared with the other groups (rabbits of group A,Cand D).

Key words: growth factors and tooth socket healing, experimental diabetic animals, delay socket healing. (J Bagh Coll Dentistry 2013; 25(Special Issue 1):89-95).

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 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)]. Studies of the spatial and temporal expression patterns of several growth factors **(VEGF, TGF-beta, BMP-4 and IGF-1R)** and the close correlation of their expression with local histological events showed that they play important roles in the healing process of tooth extraction sockets.

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.

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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**(Fig.1).** 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**.** Animals of group C were received daily insulin as a treatment in a dose of 0.1 mg/ kg of body weight.

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)**.

Figure 1: Alloxan injection

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).**

Figure 2: The site of operation

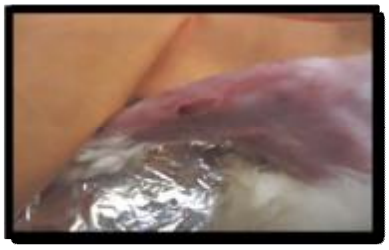

Figure 3: Skin incision

Figure 4: Dissection of the muscle

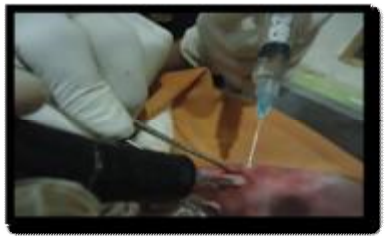

Figure 5: 1mm guide hole was made

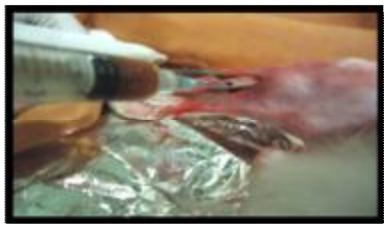

Figure 6: Aspiration of bone marrow

Figure7: Cat gut suture

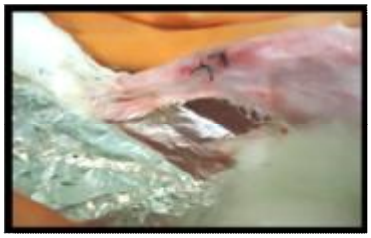

Figure 8: Skin sutured with silk suture

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**.** 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 IHC evaluations of growth factors : **TGF beta-3 , VEGF , IGF-1R and BMP-4,** statistical analysis (pearsons correlation) with scoring and positive cells counting at different healing periods was done.

RESULTS

Immunohistochemical examination of TGF-beta-3

The human placenta was used as a positive control for detection of TGFbeta-3 **(Fig.9),**the Immunostaining was detected as brown color for both cytoplasm and cell membrane. The IHC staining with TGFbeta-3 was positive in the fibroblasts, osteocytes and osteoblasts of groups A , C and D in comparison with group B animals **(Fig.10 and Fig.11).**

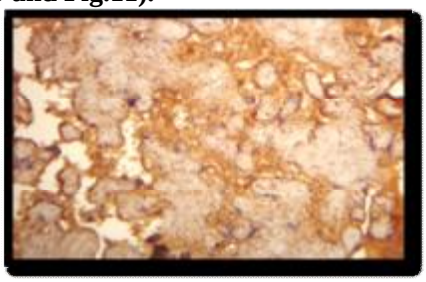

Fig.9: The human placenta was used as positive control for TGF-beta expression, DAB stains with counter stain hematoxylin, 40 X.

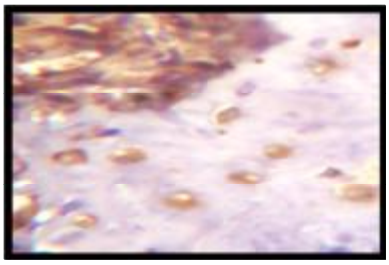

Fig.10: Positive expression of osteocytes (OC), and osteoblasts (OB) , 30 days healing period DAB stain with counter stain hematoxylin, 40 X.

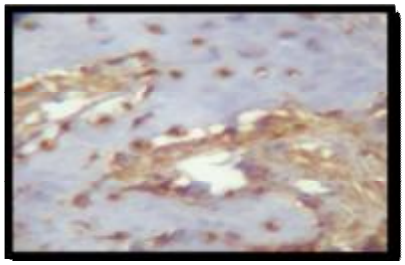

Fig.11: View for positive expression of Tgf-beta on osteocytes (OC) and osteoblasts (OB), 20 days healing period DAB stain with counter stain hematoxylin, 40 X.

Immunohistochemical examination of VEGF

The human kidney was used as positive control for detection of VEGF **(Fig.12),** The IHC staining with VEGF was positive in endothelial cells of the blood vessels **(Fig.13)**, while negative in osteocytes and osteoblasts**.** Groups A,C and D animals have great numbers of blood vessels in comparison with group B.

Immunohistochemical examination of IGF-1R

The human placenta was used as a positive control for detection of IGF-1R **,**The IHC staining with IGF-1R was highly expressed in extracellular matrix and endothelial cells in rabbits of groups B, C and D in comparison with group A animals, while moderately expressed on osteoblasts and negative expression on osteocytes **(Fig.14 and Fig.15).**

Immunohistochemical examination of BMP- 4

The human kidney was used as positive control for detection of BMP-4, the IHC staining with BMP-4 was positive in the fibroblasts, osteocytes and osteoblasts of groups A , C and D in comparison with group B animals **(Fig.16 and Fig.17).**

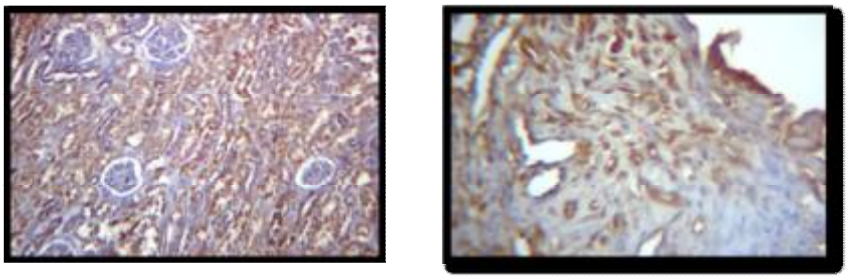

Fig.12: The human kidney was used as positive control for VEGF expression, DAB stains with counter stain hematoxylin, 40 X.

Fig.13: Positive expression of VEGF on endothelial cells of blood vessels (arrows) at 10 days healing period, DAB stain with counter stain hematoxylin, 40 X.

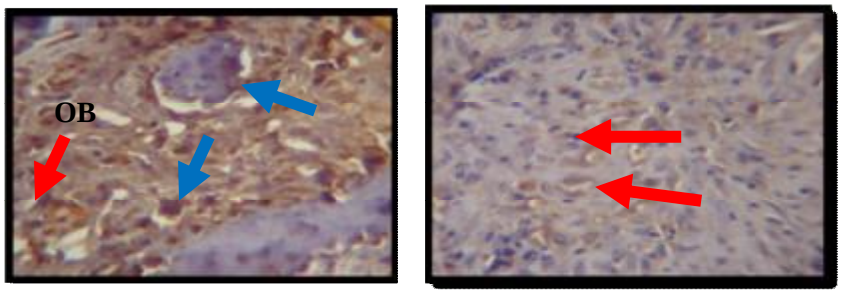

Fig.14 Positive IHC DAB stain for localization of IGF-1R on blood vessels(arrows)and osteoblasts(OB), DAB stain with counter stain hematoxylin, 40 X.

Fig.15 Positive IHC DAB stain for localization of IGF-1R on blood vessels (arrows), DAB stain with counter stain hematoxylin, 40 X.

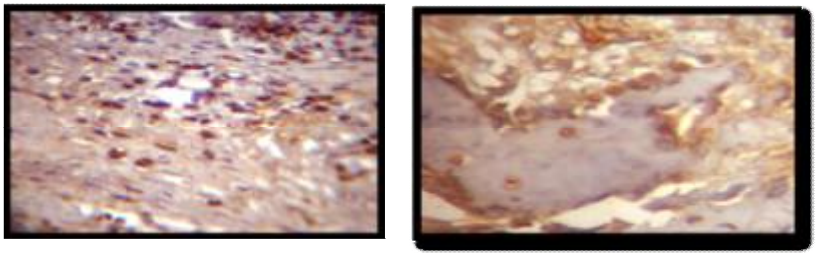

Fig.16: Positive IHC DAB stain for localization of BMP-4 on fibroblasts (arrows), DAB stain with counter stain hematoxylin, 40 X.

Fig.17: Positive IHC DAB stain for localization of BMP-4 on osteoblasts (red arrows) and osteocytes (black arrows),DAB stain with counter stain hematoxylin, 40 X.

IHC scoring of TGF beta-3

Table 1: The positive cells percentage counting of TGF beta -3 at all healing periods

periods	Group A	Group B	Group C	Group D
2 Days	73%	52%	70%	71%
10 Days	65%	20%	63%	62%
20 Days	52%	23%	48%	47%
30 Days	45%	18%	43%	44%

IHC scoring of VEGF

Table 2: The positive cells percentage counting of VEGF at all healing periods

periods	Group A	Group B	Group C	Group D
2 Days	70%	45%	65%	67%
10 Days	68%	23%	65%	67%
20 Days	45%	35%	44%	43%
30 Days	20%	45%	22%	23%

IHC scoring of IGF-1R

Table 3: The positive cells percentage counting of IGF-1R at all healing periods

periods	Group A	Group B	Group C	Group D
2 Days	20%	35%	30%	31%
10 Days	17%	30%	24%	22%
20 Days	17%	23%	21%	20%
30 Days	14%	20%	18%	19%

IHC scoring of BMP-4

Table 4: The positive cells percentage counting of BMP-4 at all healing periods

periods	Group A	Group B	Group C	Group D
2 Days	48%	40%	45%	46%
10 Days	65%	40%	62%	60%
20 Days	62%	35%	61%	63%
30 Days	45%	23%	42%	41%

Statistical correlations of all markers among groups at all healing periods

Statistical correlations of all markers in group A at all healing periods

According to the Pearson correlation **(table 5)**, there was a significant correlation between TGF-beta and VEGF. In addition to a significant correlation between TGF-beta and BMP-4. While showed a non-significant correlation between the other markers.

Statistical correlations of all markers in group B at all healing periods

According to Pearson correlation **(table 6),** there was a significant correlation between TGF-beta and IGF1R. In addition to a significant correlation between IGF1R and BMP-4. While showed a non-significant correlation between the other markers.

Statistical correlations of all markers in group C at all healing periods

According to the Pearson correlation **(table 7),** there was a significant correlation between TGF-beta and VEGF, between TGF-beta and IGF1R. In addition to a significant correlation between VEGF and IGF1R. While showed a non-significant correlation between the other markers.

Statistical correlations of all markers in group D at all healing periods

According to the Pearson correlation **(table 8),** there was a significant correlation between TGF-beta and VEGF, between TGF-beta and IGF1R. In addition to a significant correlation between VEGF and IGF1R. While showed a non-significant correlation between the other markers.

Table 5: Pearson correlation between all markers in group A at all intervals

Markers	Rabbits no. of each group	Pearson correlation sig. (2-tailed)
TGF-beta VEGF	16	0.908**
TGF-beta BMP-4	16	0.533*

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 6: Pearson correlation between all markers in group B at all intervals

Markers	Rabbits no. of each group	Pearson correlation sig. (2-tailed)
TGF-beta IGF1R	16	0.689**
IGF1R BMP-4	16	0.789**

**. Correlation is significant at the 0.01 level (2-tailed).

Table 7: Pearson correlation between all markers in group B at all intervals

Markers	Rabbits no. of each group	Pearson correlation sig. (2-tailed)
TGF-beta VEGF	16	.891**
TGF-bet IGF1R	16	.843**
VEGF IGF1R	16	.790**

**. Correlation is significant at the 0.01 level (2-tailed).

Table 8: Pearson correlations among all markers in group D at all intervals

Markers	Rabbits no. of each group	Pearson correlation sig. (2-tailed)
TGF-beta VEGF	16	0.901**
TGF-beta IGF1R	16	0.835**
VEGF IGF1R	16	0.684**

**. Correlation is significant at the 0.01 level (2-tailed).

DISCUSSIONS

The morphology and physiology of the rabbit oral apparatus are well known and the mechanical properties of its incisors and periodontal ligaments have been thoroughly examined , therefore it is suggested to be the experimental animal of choice in the present study [12 , 13 , 14]. Osteoblast growth and differentiation is determined by a complex array of growth factors and signalling pathways. The following three families of growth factors influence the main aspects of osteoblast activity and induce, mediate or modulate the effects of other bone growth regulators: TGF-β , IGFs and BMPs [15,16,17]. Furthermore, other growth factors, such as the VEGF, as well as platelet derived growth factor (PDGF) are involved in bone formation. Many growth factors involved in the natural process of bone healing have been identified and tested as potential Therapeutic candidates to enhance the regeneration process [18 , 19].Bone-related growth factors, including TGF beta-3, VEGF, IGF-1R and BMP-4 were selected in this experimental study. The current results showed that the fibroblasts, osteoproginator cells and osteoblasts had a strong positive expression with TGF-b monoclonal antibody at early stages of socket healing (10 days), while started to be decreased to become moderately expressed at 20 and 30 days, these findings agreed with **Spinella** who demonstrated (Regarding the effect of TGF-beta on the differentiation of Osteoprogenitor cells, most studies agree that TGF-b have a positive function in the early differentiation stage but an inhibition effect on differentiation in the terminal stage) [20] , The results showed that the endothelial cells and endothelial progenitor cells were highly expressed with VEGF at early healing periods (2 and 10 days), while decreased in expression at 20 and 30 days of healing periods , also the current result showed that the number of blood vessels and endothelial progenitor cells which positively expressed with VEGF was higher in groups A and C than those of group B. This data suggests that diabetes delays wound healing of the tooth extraction socket by inhibiting angiogenesis; these results were in agreement with **Cross et al** [21] , The results also showed that At 2 and 10 days of healing period the immunohistochemical staining, with IGF-1R was positive in the epithelial cells, and endothelial cells of groups B and group C while group A showed a negative expression in the epithelial cells and weak expression in endothelial cells, At 20 and 30 days of healing periods the IGF1R was highly Expressed in Endothelial cells , adipose cells , while moderately expressed on osteoblasts (Groups B and C), while in group A there was a weak positive expression on endothelial cells and negative expression on osteoblasts and osteocytes. Also the results showed that there was a significant difference in positive cell expression in rabbits of group Band C at all healing periods when compared with rabbits of group A, the previous results are in agreement with **Markopoulos** and **Katz et al** [22] , the current study showed that the BMP-4 has a positive expression on osteoblasts and osteocytes ranging from intensely stained (at 10, 20 days of healing periods) to moderately stained (at 30 days of healing period) depending on the numbers of these cells in all groups , this result is in agreements with **Jason et al**. whom evaluated the expression patterns of several members of the BMP family (2,4, and 7) in bone defect which was made in a mandible of 28 rats and studied the healing process after 1,2,3, and 4 weeks . They found that the BMP-4 had a strong positive expression on osteoblasts,osteocytes and osteoclasts at 2 and 3 weeks of healing periods, while the immunostaining of BMP-4 protein return to moderate expression at 4 weeks of healing period which was coincided with the histological appearance of mature lamellar bone [23] .

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