Evaluation of the Role of Bone Marrow-Derived Mesenchymal Stem Cells in Bone Regeneration of Dental Socket in Streptozotocin-Induced Diabetic Albino Rats.

Elham F.Mahmoud[1] and Radwa H.Hegazy[2]

[1]Oral Biology Department, Faculty of Oral and Dental Medicine, Suez Canal University. Egypt.
[2]Oral Biology Department, Faculty of Oral and Dental Medicine, Cairo University. Egypt
radhegazy@hotmail.com

Abstract: Diabetes mellitus is one of the most important public health problems worldwide. If undiagnosed or untreated, this pathology represents a systemic risk factor and offers unfavorable conditions for dental treatments, especially those requiring bone healing. **Objectives:** The purpose of this study is to evaluate histologically and histochemically the effect of bone marrow-derived mesenchymal stem cell therapy in enhancing bone repair in surgically extracted sockets of diabetic albino rats. **Design:** Thirty male Swiss Albino rats ranging from 200 to 250 g were used in this study. The experimental animals were randomly divided into three groups (10 animals each). The first lower right molar of the rats was extracted under general anesthesia. Group I acted as a control with surgical extracted sockets were left empty. Diabetes was induced by single intravenous injection of 60 mg/kg body wt streptozotocin (STZ) in the remaining 20 rats. The rats in the control group received a comparable injection of saline. The diabetic rats divided into two groups (10 animals each): group II; diabetic rats with empty sockets were left to heal spontaneously. While group III; diabetic rats in which, bone marrow mesenchymal stem cells were introduced into their sockets. Sacrification of animals was done after different time intervals. The MSCs were harvested from rat bone marrow. The two sides of each mandible were separated. The specimens were processed, decalcified and stained with hematoxylin and eosin for histological examination and Masson's trichrome for detection of collagen fibers. **Results:** MSCs accelerated bone healing in third group, while, diabetes induced delay of bone healing along the second diabetic group. This acceleration was assessed histologically by the presence of mature collagen fiber bundles and early new bone formation in the MSCs group. Histomorphometric analysis revealed an increase in the area percentage of bone trabeculae in the MSCs treated sockets compared to the control and diabetic ones. **Conclusions:** In a rat model of streptozotocin- induced diabetes, application of bone marrow mesenchymal stem cells can significantly enhance bone healing and mineralization in their sockets.
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1. Introduction

Bone defects may develop in various clinical situations such as in bone fractures caused by impact loadings or during orthopedic surgeries. Despite ongoing advances in orthopedics to enhance bone healing, about 5% to 10% of fractures still develop delayed union or nonunion **(1)**. A multitude of factors are known to play a role in causing delayed bone healing including age, smoking, medications, and certain systemic diseases. Among this array of factors, diabetes mellitus (DM) is one systemic disease that has profound deleterious effect on bone healing. Diabetes mellitus, a complex metabolic disorder, is a syndrome characterized by abnormalities in carbohydrate, lipid, and protein metabolism, that results either from a partial or an absolute insulin deficiency or from target tissue resistance to its cellular metabolic effects **(2)**. This disease is characterized by the presence of few or by the absence of functional β cells in the islets of Langerhans, and by a substantial reduction or

inexistence of insulin secretion **(3)**. Type 1 diabetes mellitus is caused by cellular-mediated autoimmune destruction of pancreatic islet beta cells, leading to loss of insulin production. It usually starts during childhood, but can occur at all ages. Type 2 diabetes mellitus accounts for 90% - 95% of all diabetes and had global prevalence estimate of 2.8% in the year 2000 and is projected to be 4.4% in 2030 **(4).** Several animal and clinical studies have documented the association between DM and impaired bone healing. Another studies showed that the fracture callus from the untreated DM rats had a 29% decrease in tensile strength and a 50% decrease in stiffness compared with non-DM rats 2 weeks after the production of a closed fracture **(5)**. The slower bone repair in diabetics is the consequence of a deficient function of osteoblasts **(6)**, a diminished production of collagen **(7)** and/or changes in the mineral metabolism **(8)**. Angiogenesis is defined as the process of formation of new blood vessels from pre-existing ones, and plays a significant role under both physiological and

pathological conditions in the process of bone healing **(9)**. Diabetes mellitus is characterized by abnormalities in angiogenesis, either excessive angiogenesis (retinopathy and nephropathy), or an attenuated angiogenic response implicated in abnormal bone or wound healing and ulcers **(10).**

Streptozotocin (STZ) is a naturally broad spectrum antibiotic and cytotoxic chemical that is particularly toxic to the pancreatic, insulin producing beta cells in mammals **(11-13).** It has been widely used to induce diabetes in animal models especially rats and mice **(12, 14)**.

In order to have a normal physiological and also mechanical function in skeletal systems, bone defects need to be restored as soon as possible. The conventional methods of bone repair, which are commonly used including autografts and allografts, have their own benefits and shortcomings **(15, 16, 17)**. Autografts are usually obtained from the iliac crest **(18)** and have been used in clinics for a long time. Autografts are limited in terms of availability of materials and may result in donor site morbidity **(19)**. Using allografts may be more desirable in some cases for bone defect regeneration. This type of bone graft can be derived from viable or sterilized non-viable (cadaver) human sources, but the possible immune reaction and infection transmission might limit their application **(20, 21)**. Xenografts obtained from nonhuman sources may be considered as the other alternative for reconstructing bone defects, but this is a last resort which may be taken because it is not an approved option in transplantation, owing to the obvious risk of viral and disease transmission, infection, toxicity and immunogenicity as well as rejection by the recipient's body **(22).**

A promising alternative bone regeneration approach was established by the identification of certain multipotential cells among the stromal cells of the bone marrow **(23, 24).** These cells have been termed mesenchymal stem cells (MSCs) **(25- 27)** or marrow stromal cells **(28)**. By culturing, they may be expanded many fold to provide nearly unlimited starting material for cellular therapeutic applications. The basic concept of utilizing mesenchymal stem cells (MSCs) and autologous bone marrow (BM) aspiration to treat bone defects has several advantages. Osteoblasts differentiation from MSCs is very well described and standardized in many protocols. Mesenchymal bone marrow stem cells (MBM-SCs) can also be isolated by means of minimally invasive procedures from BM, requires only a small amount of tissue from the patient and this approach can restore bone defects without incurring donor site morbidity **(29, 30)** . Stem cells are highly expandable in culture and have been used in the treatment of various tissues and organs. Their capacity to undergo extensive replication without losing their multi-potential capability makes them an attractive cell source for cell-based therapeutic approaches **(27)**.

The MSCs can differentiate into stroma, bone, cartilage, fat and muscle *in vitro* under defined cell culture conditions **(27)**. *In vivo* evaluation has demonstrated MSC differentiation along these lineages under local stimuli in many pre-clinical models **(31-33)**.The multi-lineage potential of mesenchymal stem cells (MSCs) and their presence in the marrow and other solid mesodermal tissues such as the periosteum, fat pad and synovium has been demonstrated *in vitro* for numerous species. However, the physiological activities of MSCs and their *in vivo* distribution are still only partially understood **(34, 35)**. Bone marrow derived MSCs are capable of differentiating into a variety of cells including osteoblasts, chondrocytes, adipocytes, tenocytes and myoblasts. The proliferative capacity of MSCs makes it possible to isolate and culture large volumes of MSCs from just a small volume of bone marrow, and their ability to maintain their functionality after culture expansion and cryopreservation has led them to be viewed as a readily available, abundant source of cells for tissue-engineering applications **(36)**. Mesenchymal stem cells (MSCs) derived from bone marrow are an obvious source of autologous stem cells, and used as seeding cells for cell therapy and tissue engineering **(31-33,37)**. MSCs have strong regeneration potential and immunosuppressive properties that are important for allografts. In fact, MSCs have already been employed for clinical trials in a number of contexts, such as the facilitation of hematopoietic and immune reconstitution after hematopoietic stem cell transplantation **(38, 39)**, construction of the vessel wall **(40)**, as well as the regeneration of cartilage using tissue engineering techniques **(41, 42)**. Furthermore, a number of studies have demonstrated that autologous MSCs cultured in scaffolds can induce new bone formation *in vivo* and lead to improved healing of critical-size defects **(43- 46)**.

Therefore, the purpose of the present work was to establish an experimental model for streptozotocin-induced diabetes in rats, to evaluate qualitatively and quantitatively the bone healing process in recently extracted bony sockets in normal and diabetic rats enhanced by bone marrow mesenchymal stem cells (MSCs). New bone formation was investigated at different intervals (3 days, 10 days, 4 weeks, 6 weeks after extraction) by histological and histochemical examination.

2. Material and Methods
Experimental Design.

The study was conducted on thirty healthy adult male Swiss Albino rats (200-250g) were selected in this study. The rats were caged separately in the animals' house of the faculty of Medicine, Cairo University. The rats were housed in polycarbonate cages with stainless-steel wire tops and maintained at 24 to 26°C with 55 to 75% humidity and a 12-h light/dark cycle, and fed a commercial rodent diet and given water *adlibitum*. All animal experiments were carried out according to the guidelines of the National Institutes of Health for the care and use of laboratory animals. The protocol was examined and approved by the institutional ethics committee at the Faculty of Dentistry, Cairo University. The experimental animals were divided into three groups (10 animals each): group I acted as control with surgical extracted sockets were left to heal spontaneously, group II in which diabetes was induced with surgical extracted socket were left empty to heal spontaneously without treatment, group III diabetic rats were administrated bone marrow derived mesenchymal stem cells into sockets immediately after extraction (MSC).

Diabetes induction:

Diabetes was induced in the animals by a single intravenous injection of 60 mg/kg body wt streptozotocin (STZ) (**Sigma Aldrich Inc**), dissolved in 0.9% NaCl & citric acid (pH 4.5) **(47)** . The rats in the control group received a comparable injection of saline. 24 hours after the induction of diabetes; blood glucose levels of the animals were measured using a colorimetric system (**Sigma glucose Kit No 115-A**). Animals of the experimental groups were considered diabetic when their serum glucose levels were above 300 mg/dl.

Bone marrow (BM) Aspiration and MSCs Expansion:

Under general anesthesia by IM injection of a compound of ketamine HCL (Ketalar^R 22 mg/kg b. wt.) and xylazine (0.2 mg/kg b.wt.), a 14-gauge needle was used to penetrate the cortex of the femur of each rat, and about 10 ml of bone marrow was drawn in a syringe containing 1500 U of heparin. BM aspirate was collected into a 50 ml tube, containing 30 ml Dulbecco's Modifed Eagle's medium (DMEM, Sigma, Australia) and heparin (100U/mL), The mixture was centrifuged at 400 g without acceleration or brake for 35 min at 20ºC on density gradient media (Ficoll-Paque; GE Healthcare, Waukesha, WI) and the top layer of fat containing plasma was discarded. Cells located at the interface between the BM sample and gradient media were collected, washed with Dulbecco's phosphate buffered saline (DPBS), (Invitrogen, Carlsbad, California, USA) and re-centrifuged at the same speed. After determination of cell viability and the number of viable cells by trypan blue staining, the cells were re-suspended in DMEM,

supplemented with 10% fetal bovine serum (FBS), (USDA, Gibco, Grand Island, NY, USA) and antibiotics (penicillin 10 000 U∕ml, streptomycin 10 000 ug∕ml, amphotericin B 25ug∕ml). The nucleated cells were plated in tissue culture flasks at 2.5 X 10^5∕cm^2 and incubated at 37°C in a humidified atmosphere containing 5% CO_2. On the fourth day of culture, the non-adherent cells were removed along with the change of medium. The culture medium was changed every 3 days until the outgrown cells reached 90% confluence. On the 18th day, the adherent colonies of cells were trypsinized counted and sub cultured at 90% confluence. Cells were passaged to the subculture 2 so that sufficient number of cells was available to continue the experiment described below. Cells were identified as being MSCs by their morphology which was fusiform, their adhesiveness, and their power to differentiate into osteocytes and neurocytes **(48- 51).**

Dental surgical procedure:

All animals were fasted twelve hours before the operation to avoid aspiration of gastric contents during general anesthesia. Extraction was performed under sterile conditions, and general anesthesia. The animals were anesthetized by intraperitoneal administration of a 4:1 solution of ketamine/xylazine at a dose of 0.15 ml per 100 g of body weight **(52)**. Depth and maintenance of anesthesia were confirmed by loss of eye blinking reflex and relaxation of skeletal muscles at surgical site. The operative site was scrubbed with betadine (Providine Iodine 7.5% w/v). The first lower left molar of rats was extracted atraumatically according to the technique described by **Guglielmotti and Cabrini (53).** Extraction was performed using a curved hemostat with a gentle buccolingual movement. Extraction sockets in animals of the groups I and II were left empty, while that of Group III were received autogenous bone marrow-derived stem cells (BMSCs) that were cultivated for 48 hrs before implantation. The margins of the wounds were sutured with (coated vicryl 4/0 (Ethicon, Edinburgh, UK). Much care should be taken to remove all blood and debris of the rat's mouth.

Postoperative Care:

After surgery, a single dose of 30,000 IU penicillin-G benzathin was administered intramuscularly. In addition, the animals were given 1 ml of methyl prednisolone acetate (Depomedrol 40 mg/ml, Egyptian International pharmaceutical) and Diclofenac Sodium (Voltaren 75 mg/3ml, Novartis) intramuscularly to reduce postoperative edema and pain. During the first week after tooth extraction, animals were fed with regular pressed soft food to avoid post surgery trauma. The rats were sacrificed

by cervical dislocation at different intervals (3days, 10days, 4weeks and 6 weeks) after tooth extraction.

Specimen Preparation and Histopathological Examination:

Each mandible was dissected free and soft tissue was immediately removed. The right and left mandibular segments were dissected and the specimens were processed. Specimens were fixed in neutral buffered formaldehyde solution for 72 hours. The samples were then decalcified by immersing the specimen in decalcifying working solution of EDTA for 30 days. The decalcified specimen was then dehydrated with graded ethyl alcohol washes (50%, 70%, 85% then 100%) and imbedded in paraffin wax with an orientation that provide sectioning along the sagittal plane of the jaw. Sections of 4μm thickness were cut sequentially from the lateral to the medial side till the socket center was reached. The sections were stained with hematoxylin and eosin for routine examination and Masson trichrome stain for detection of collagen fibers.

Histomorphometric analyses:

The data were obtained using Leica Qwin 500 image analyzer computer system (England). The image analyzer consisted of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer programme into actual micrometer units.

The area and the area percentage of bone trabeculae were measured using an objective lens of magnification 20_ (total magnification of 200). Ten fields were measured from each specimen. Using the colour detect, areas were masked by a blue binary colour. The area percentage was calculated in relation to a standard measuring frame of area 118476.6 mm2. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research **(54).**

3. Results

Clinical observations of experimental extraction sites

All experimental sites healed uneventfully; showed signs of soft tissue inflammation; swelling and redness during the first two weeks of healing .At the end of the study period, the mucosa covering the extraction sites and the gingival tissues at adjacent teeth appeared to be clinically healthy without depicting any signs of inflammation.

Histopathological observations of experimental extraction sites

Qualitative histopathological observations were demonstrated from the hematoxylin and eosin and Masson's sections while quantitative results were revealed from the H&E sections only.

At 3 days after extraction:

The animals from the control and experimental groups did not show any difference in what sockets healing was concerned. This period was characterized by the sockets was almost filled with the blood clot (erythrocytes, platelets and leukocytes) entrapped in dense vascular fibrous network containing immature collagen fibers and small proliferation of fibroblasts. The formation of a granulation tissue is observed, which substitutes partially, blood coagulum. Osteoblastic activity with beginnings of osseous formation is very clear at the sockets (**Figs. 1a and b**).

At 10 days after extraction:

The socket of control group (group I) was shown that the contracted clot was replaced by compactly packed granulation tissue composed of immature collagen fibers, fibroblasts and blood vessels. Evidence of bone formation was detected. Unconnected spicules of bone were interspersed within the granulation tissue. Blood vessels were dilated. Areas of woven bone and osteoblastic rimming showed in some cases. Angiogenesis increased markedly reflecting the persistent attempt of the tissues to heal **(Figs. 2a and b).**

The socket of diabetic rats (group II) showed a great quantity of granulation tissue substituting the blood coagulum. The quantity of osseous tissue which was formed was smaller than that of the control. This osseous tissue appeared as immature and scattered throughout the socket with wide medullary spaces **(Figs. 3a and b**).

The socket of (group III) contained, large, interconnected bone trabeculae, filling almost the majority of the socket. Granulation tissue is entrapped inbetween the trabeculae. The bone matrix as well as the cells appears well organized **(Figs. 4a and b)**.

At 4 weeks after extraction:

The socket of the control group showed increased thickness of woven bone with wide osteocytic lacunae. Extensive fibrous and mesenchymal activity was also demonstrated. Osteoclasts occurred in discrete areas at the surface of woven bone trabeculae and were consistently present in Howship's lacunae. Angiogenesis increased reflecting the persistent attempt of the tissues to heal **(Figs.5a and b).**

The socket of diabetic rats (group II) showed observable decrease in angiogenesis and bone remodeling than that of the control. Remaining blood

coagulum areas and granulation tissue were still observed (**Figs. 6a and b**).

The socket of (group III) was occupied by mature, compact osseous trabeculae with small medullary spaces, little cellularized and discrete osteoblastic activity. The central part the socket filled with immature woven bone with wide osteocytic lacunae (**Figs.7a and b**).

At 6 weeks after extraction:

The socket of the control group was occupied by mature, compact osseous trabeculae with small medullary spaces, little cellularized and discrete osteoblastic activity. The collagen architecture has been changed to be mature bone (**Figs. 8a and b**).

Concerning the dental sockets of animals from the diabetic group (group II) revealed areas of mature lamellar bone with wide medullary spaces, interlacing with immature woven bone with large irregularly arranged osteocytes. The rest of the socket on the other hand, contained mature and immature collagen fibers were blended together (**Figs. 9a and b**).

The socket of (group III) was occupied by more mature, compact osseous trabeculae with narrow medullary spaces, little cellularized and discrete osteoblastic activity. The newly formed bone showed slight changes in its collagen architecture, where changed to be almost as the mature bone (**Figs. 10a and b**).

Histomorphometric analysis:

Using computer image analysis, the mean area percent of bone trabeculae was calculated in extraction sockets of the control and experimental groups. Group III had the greatest mean area percent of bone trabeculae (95.537 ± 3.501), while group II had the lowest value (67.337 ± 6.021). Using Analysis of variance (ANOVA) test, the difference between the groups was found to be extremely statistically significant ($p < 0.0001$), (Table 1, Histograms 1-3). Unpaired Student's t test was used for pair wise comparison of mean area percent of bone trabeculae in extraction sockets of the control and experimental groups. The difference between each two groups was found to be extremely statistically significant (Table 2).

Table (1) Mean area percent of bone trabeculae in extraction sockets of the control & experimental groups and statistical significance of the difference using ANOVA test:

|	Control (Group I)	Group II	Group III	F value	p value
Mean	84.695	67.337	95.537	53.2923	<0.0001***
±Std Dev	2.906	6.021	3.501		
±Std Error	1.678	3.476	2.022		
Max	88.203	75.646	98.096		
Min	81.087	61.567	90.586		
2-s Range	11.624	24.085	14.006		

***extremely statistically significant

Table (2) Pairwise comparison of mean area percent of bone trabeculae in extraction sockets of the control and experimental groups using unpaired Student's t test.

| Comparison	95% confidence interval of the difference:	±standard error of difference	t value	p value
Control versus group II	10.463293 to 24.252707	2.990	5.8056	0.0004***
Control versus group III	-15.534235 to -6.149765	2.035	5.3283	0.0007***
Group II versus group III	-35.382711 to -21.017289	3.115	9.0536	0.0001***

***extremely statistically significant
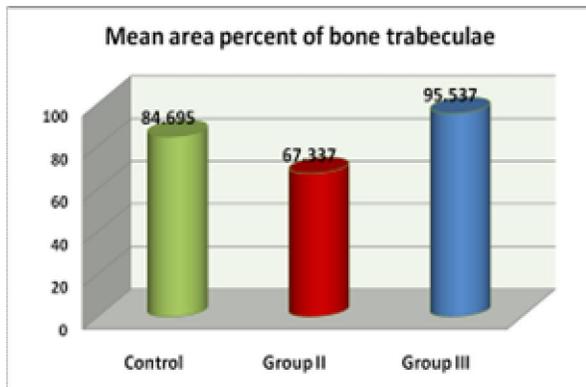
Histogram 1: Mean area percent of bone trabeculae in extraction sockets of the control and experimental groups.
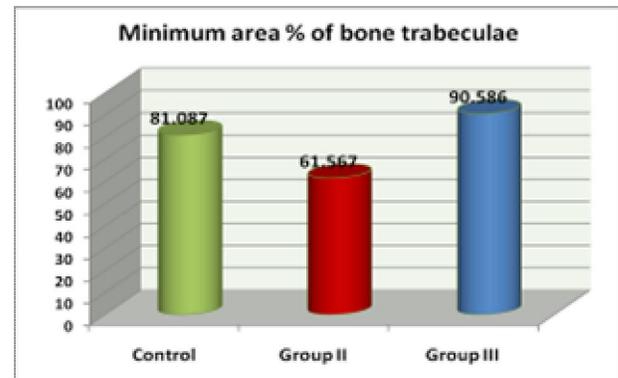
Histogram 2: Minimum area percent of bone trabeculae in extraction sockets of the control and experimental groups
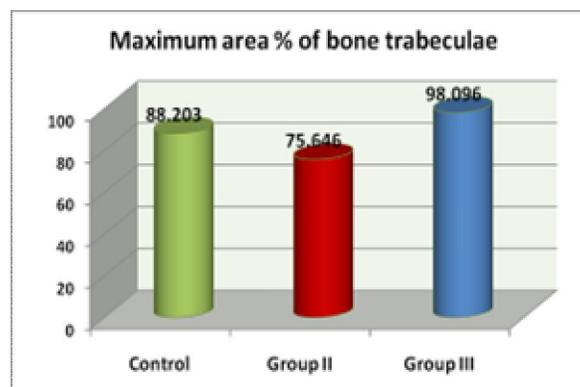
Histogram 3: Maximum area percent of bone trabeculae in extraction sockets of the control and experimental groups.
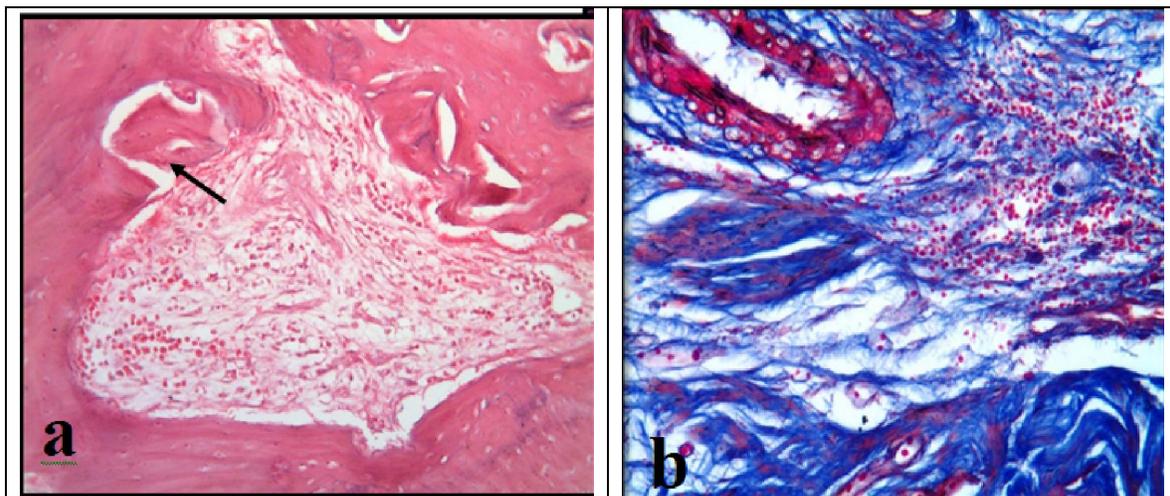

Fig. (1): A photomicrograph showing: the socket of (group I) (3 days after extraction) almost filled with granulation tissue (containing hematoma, soft tissue and extravasated RBCs). Notice small fragment of newly formed bone (arrow) (a) (H&E X 200), (b) Masson trichrome (X400).
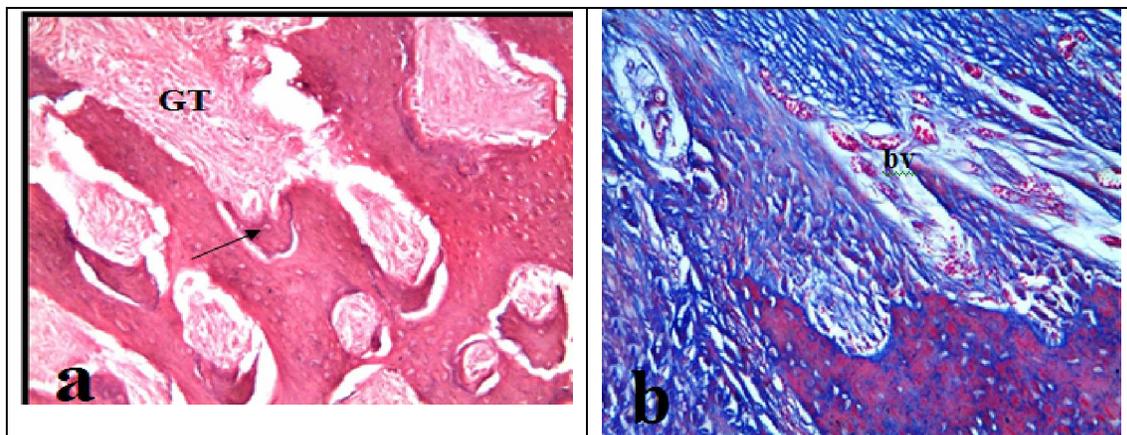
Fig. (2): A photomicrograph of the socket of control group (10 days after extraction) showing formation of the new bone trabeculae (arrow). Osteoblastic rimming of marrow cavities, remnants of granulation tissue (GT) was evident in between the trabeculae. Note: multiple blood vessels (bv) denoting angiogenesis. (a) (H&E X200) & (b) Masson trichrome (X200).
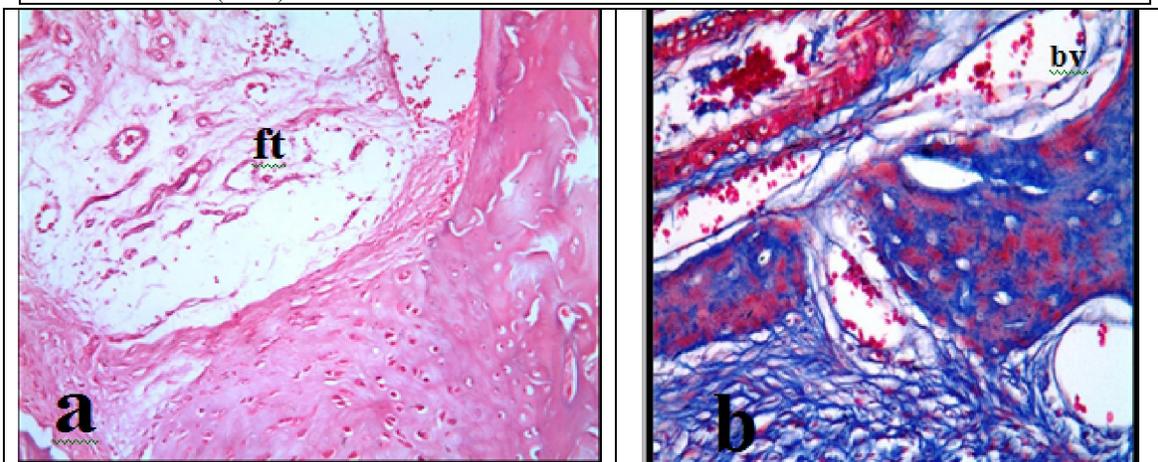

Fig. (3): A photomicrograph of the socket of the group II (diabetic rats) after 10 days of extraction showing disturbance in mineralization. The socket appeared to be filled with loose fibrous tissue (ft). (a) (H&E X200). (b) Masson trichrome (X400) Note: presence of dilated blood vessels (bv).
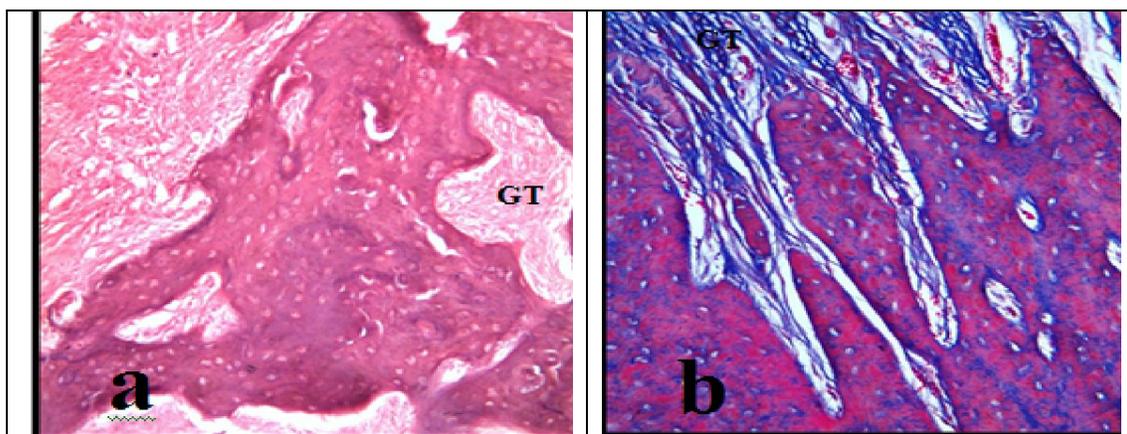

Fig. (4): A photomicrograph of the socket of the group III (10 days after extraction) showing formation of thick newly formed bone trabeculae with large osteocytes. Granulation tissue (GT) is entrapped inbetween the trabeculae. The bone matrix as well as the cells appears well organized (a) (H&E X200) & (b) Masson trichrome (X200).
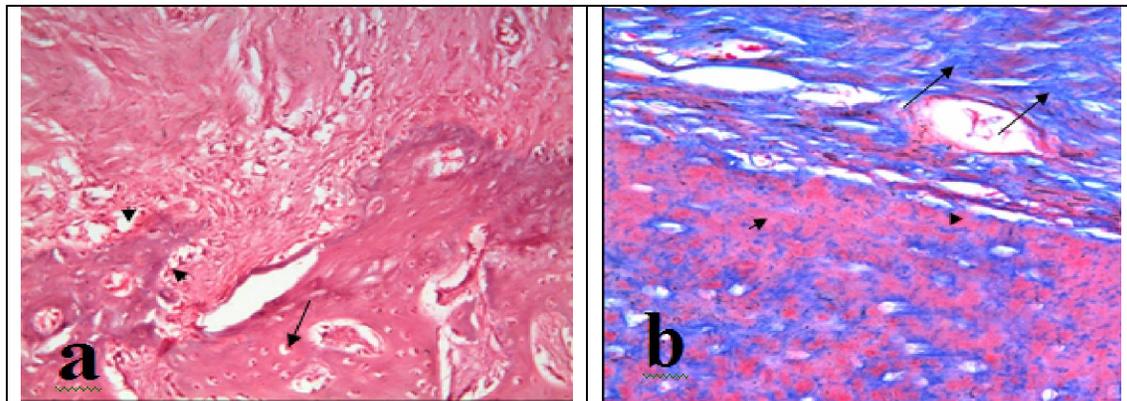
Fig. (5): A photomicrograph of the socket of the group I (control) (4 weeks after extraction) showing woven bone with wide osteocytes (arrow). Osteoclasts occurred in discrete areas at the surface of woven bone trabeculae and were consistently present in Howship's lacunae (arrows heads). (a)(H&E X200). (b) (Masson trichrome X200) Note: presence of vascularized fibrous tissue with mature collagen fibers (arrow) on top of newly formed bone with immature collagen fibers (arrow heads).
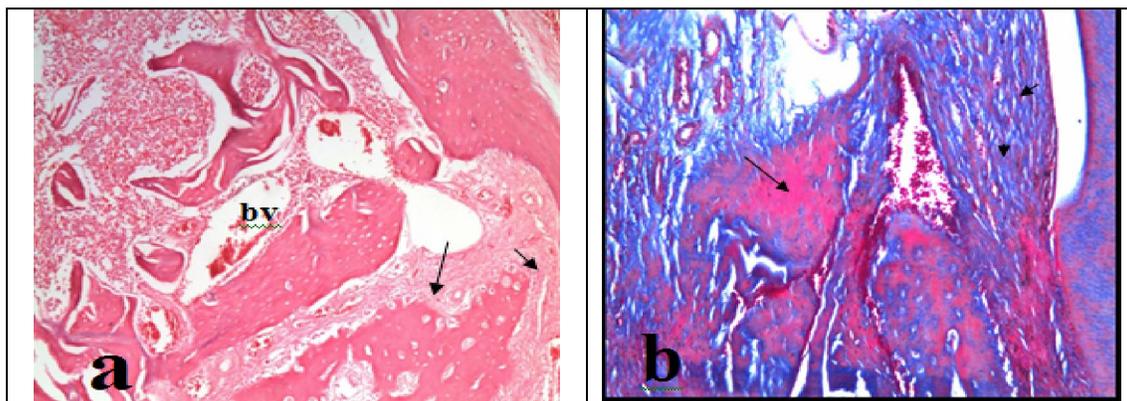

Fig. (6): A photomicrograph of the socket of the group II (4 weeks after extraction) showing formation of few delicate bone trabeculae surrounded by granulation tissue, extravasated RBCs and dilated blood vessels (bv). Osteoclasts as well as resorption lacunae can be observed (arrows) (H&E X200). (b) Masson trichrome (X200). Note: newly formed bone at the boundaries of the socket with mature (arrow heads) and immature collagen (arrow) fiber bundles blended together.
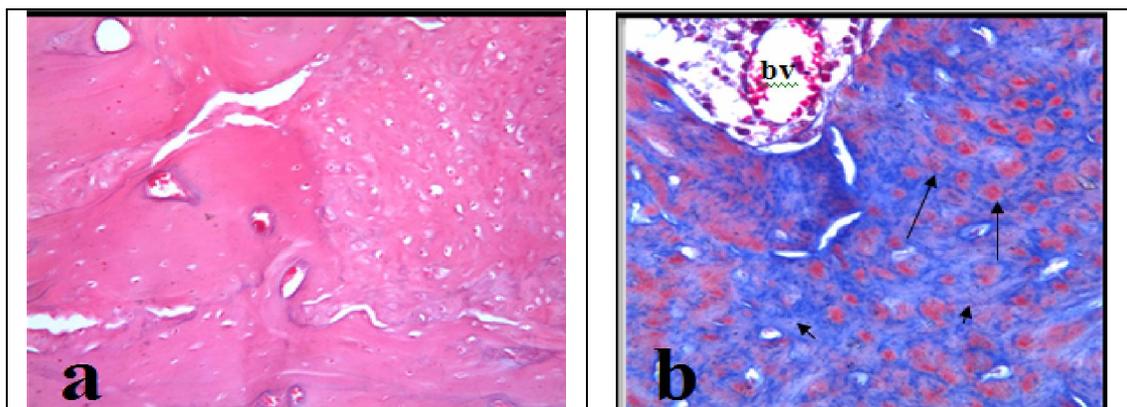

Fig. (7): A photomicrograph of the socket of the (group III) after 4 weeks showing different patterns of bone formation separated by Reversal lines. (a) (H&E X 200). Well vascularized fibrous tissue (bv), areas of immature collagen fibers (arrow) blended with mature collagen fibers (arrow heads). (b) Masson trichrome (X200).
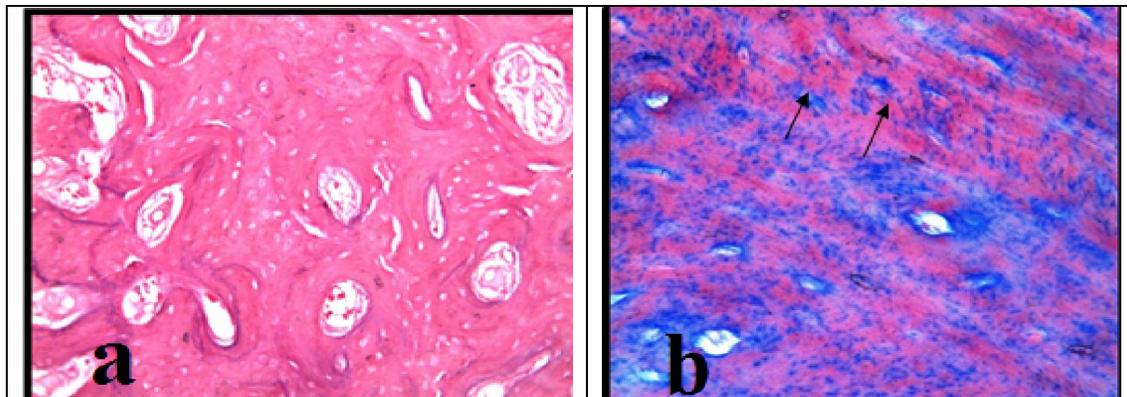
Fig. (8): A photomicrograph of the socket of the group I (control) after 6 weeks of extraction showing formation of thick bone trabeculae consisting of mature lamellar bone with small marrow spaces in between. Notice, fibroblasts and RBCs fill the medullary spaces (a) (H&E X200). Mature collagen fibers between well formed bone (arrows) (b) Masson trichrome (X200).
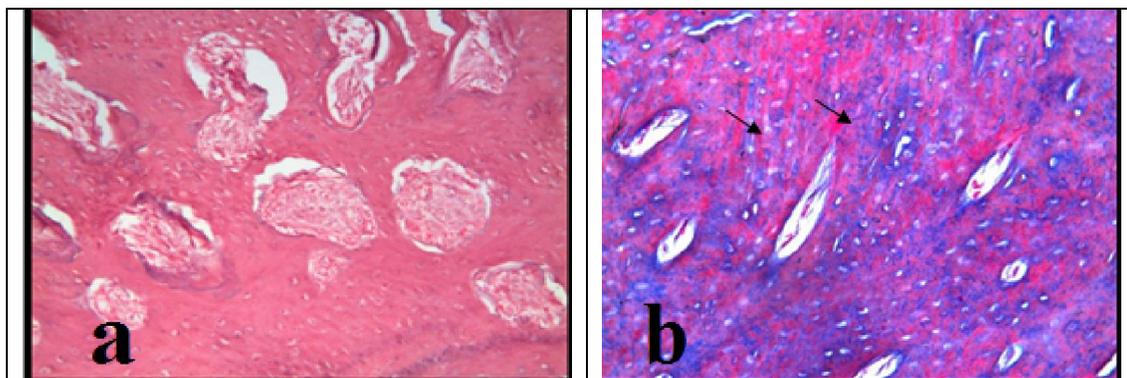

Fig. (9): A photomicrograph of the socket of the group II(diabetic) after 6 weeks of extraction showing areas of mature lamellar bone interlacing with immature woven bone with large irregularly arranged osteocytes. Notice, numerous reversal lines are evident, Remnants of granulation tissue &blood clot entrapped within lamellar mature bone (a) (H&E X200). Immature collagen fibers still entrapped between lamellar bone (arrows). (b) Masson trichrome (X200).
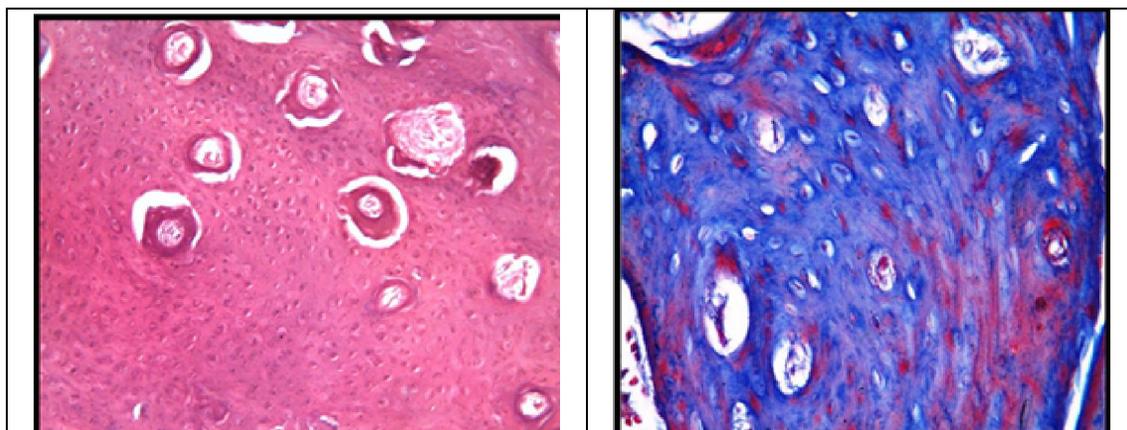

Fig. (10): A photomicrograph of the socket of the (group III) showing mature well organized lamellar bone with relatively small Haversian canals. Some of these canals contain remnants of granulation tissue. The osteocytes are well organized within the bone matrix (a) (H&E X200). Mature collagen fibers between well formed lamellar bone (b) Masson trichrome (X200).

4. Discussion:

The present experiment was conducted to study the influence of bone marrow mesenchymal stem cells (BMSc) on the bone healing in diabetic rats. Healing was evaluated histologically and histochemically. Histological results revealed that BMSc has obviously accelerated bone healing, while on the other hand, diabetes induced delay of bone healing along the diabetic experimental group of the ongoing study.

A control group was allowed to heal normally following teeth extraction without the application of any treatment in this study. Therefore, the purpose of the inclusion of a control group was not to assess the normal healing process of the extraction sockets but to investigate whether the streptozotocin or the use of BMSc in the other two groups would enhance or delay the healing process in comparison to the non-treated control group.

The healing of an extraction socket involved a series of events including the formation of a coagulum that was replaced by provisional connective tissue, woven bone, lamellar bone and bone marrow (**55**). The histological and histochemical results of the present study showed a retarded dental socket healing in the diabetic rats induced by streptozotocin compared to the control. This study demonstrated that the formation of new osseous tissue is fundamentally influenced by the diabetic state, since exhibited a much more pronounced disturbance of the process of bone healing. These findings were similar to those explained by **Shyng et al. (56**) who found that the bone formation in the femur was greatly reduced in the Streptozotocin-induced diabetic model. These observations are also consistent with published reports of impaired osteoid formation (**57**), and decreased synthesis of both collagen (**58, 59**) and proteoglycan (**60**), in Streptozotocin-induced diabetes. Since osteoclast numbers are also reduced in diabetic rats (**8, 57**) an overall reduction in bone turnover, rather than excessive bone resorption is implicated in the pathogenesis of diabetic osteopenia (**61**).

A relatively reduced angiogenic response in diabetic group has been attributed to several factors such as impaired release of endothelial progenitor cells from the bone marrow as well as defective function of progenitor cells (**62**) Studies have shown that levels of vascular endothelial growth factor A (VEGF A) in the plasma as well as in various tissues are higher in diabetics compared to controls. VEGF A has been shown to be an endogenous inhibitor of angiogenesis in other tissues (**63**).

Histological results in-group III (3 days,10 days, 4 weeks and 6 weeks post operatively) of the current study strongly indicated that MSCs accelerated the formation of new bone formation, maturation of the new bone tissue, increase amount of bone deposition and a highly vascularized connective tissue. Such findings are in line with other studies, in **2009**, **Granero-Molto et al. (64)** stated that at the injury site, MSCs could help in repair in two ways; first by differentiating into tissue cells in order to restore lost morphology and function and second by secreting a wide spectrum of bioactive factors that help creating a repair environment owing to their anti-apoptotic and immune-regulatory properties by stimulating the proliferation of endothelial progenitor cells. Hence, we can deduce that the local microenvironment and surrounding tissues may have provided the nutrients, growth factors and extracellular matrices necessary to support differentiation of the transplanted MSCs.

On the other hand one can attribute the improved results observed in the experimental sites of group III in the present study to the bone forming capacity of MSCs and its migration capacity in response to signals produced by an injured bone, a conclusion that was also conducted by (**65**).

In a study conducted by **Nasiff et al. (66**) stated that MSCs transplantation was also shown to promote peri-implant bone regeneration and it was suggested to use this approach in clinical settings to enhance bone regeneration and healing in patients with poor bone quality.

Histological examination of the current study revealed a considerable concentration of collagen fibres within the MSCs-treated sockets, unlike those in the control ones. Utilizing the Masson's trichrome stain in the current research was useful in demonstrating the presence of collagen, as well as providing improved photographic contrast when compared to the H&E stain. Collagen is an important component of the extra-cellular matrix of bone, and increased amounts may indicate a positive effect of MSCs on bone healing, it is necessary to consider that large numbers of collagen fibres will represent new bone formation after mineralization of the matrix.

In the current study, we observed that MSCs had a positive effect on the periods adopted in this study. The ongoing statistical data for the regenerated area across the healed sockets for all experimental groups illustrated in (Histograms 1-3) using analysis of variance (ANOVA) and unpaired Student's-t test revealed that, the differences between all groups were statistically significant. An increase in the area percentage of bone trabeculae in the MSCs treated sockets of group III compared to the diabetic group. This increase was extremely statistically significant ($p < 0.0001$).

The higher percentage of new bone obtained in the MSCs treated sockets in the present study is consistent with previous studies, who used MSCs in enhancing bone regeneration in various animal models and confirm the positive bio stimulatory effect of MSCs which depends mainly on the ability of the tissue to respond including, promotion of angiogenesis, collagen production, osteogenic cell proliferation and differentiation (**67**).

Conclusion

It may be possible to develop new therapeutic approach for improving bone repair in diabetic patients. In this in vivo experiment the histological analysis showed that application of MSCs as adult stem cells are able to efficiently differentiate along the healing site. Currently, several clinical trials are being performed on problematic human bone lesions, including nonunion fractures, delayed union, bone cysts, and bone neoplasm, depending on MSCs characteristics in bone engineering and regeneration.

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Conflict of Interest

No conflict of interests.

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