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# Potential Therapeutic role of Mesenchymal Stem Cell in Delayed Wound Healing of Diabetic Rats

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**ABSTRACT**

*The aim of the present work was to investigate the effect of BM-MSCs on wound closure in STZ-induced diabetic rats. Diabetic wound models were carried out by making a standard wound on dorsum of forty rats, which were divided into four groups with ten rats in each: Wound from diabetic and non-diabetic control rats were treated with PBS, while diabetic and non-diabetic treated rats were treated with BM-MSCs for 12 days. The closure rate and the ratios relative to the beta actin gene of both treated groups (diabetic and non-diabetic) were significantly increased at 7 and 12 days after wounding as compared to their corresponding controls ($P < 0.05$). Histologic analysis revealed complete reepithelialization in treated groups. Taken together, BM-MSCs mediated correction of the diabetic wound healing impairment is due to, partly, increased VEGF expression in the skin of STZ-induced diabetic rats.*

**Key words**: BM-MSCs, diabetic rats, VEGF, wound healing.

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## INTRODUCTION

There is surely no room for doubt that diabetes has become a major public health concern of the twenty-first century. numerous studies have assessed the effect of diabetes, and it appears that the numbers are developing at extraordinary rates. The International Diabetes Federation reported that more than 300 million people had diabetes in 2011 and this number will have expanded to 552 million in 2030, and that this caused 4.6 million deaths in 2011[1].

Impaired wound healing is a complication of diabetes and a significant issue in clinical practice [2]. As many as 15% of people with diabetes suffer from foot ulceration and wounds [3]. The wound healing process in healthy individual of human and all mammalian species can be divided into five phases: granulation, wound contracting, collagenation, epithelialization and cicatrization [4], [5]. Any agent that accelerate this process stimulates wound healing [5]. Diabetes mellitus delays wound healing by affecting one or more of the mentioned phases [6], [7].

Mesenchymal stem cells possess the ability of self-renewal and multilineage differentiation. Moreover, they have immunomodulatory and regenerative capacity through paracrine signalling, thereby suggesting great therapeutic potential [8], [9]. Recently, MSCs transplantation has shown significant wound healing in experimental animals as well in patients [10], [11], [12]. However, the mechanisms involved in promoting diabetic wound healing are barely

understood. In the present work, we established a delayed wound healing model in diabetic rats and evaluated the impact of BM-MSCs injection on delayed wound healing.

## MATERIALS AND METHODS

### Ethical approval

The study protocol was approved by the animal ethics committee of animal care and use at faculty of vetrinary medicine at Cairo Univerity.

### Diabetic models

Diabetes was induced according to method of **Wan et al.** [13]. In brief, rats were starved for at least 12 h before a single injection of freshly dissolved streptozotocin (STZ; 60 mg/kg body weight: Sigma, USA) in 0.1 Mm sodium citrate buffer (PH 4.4) into the peritoneum. Seven days following STZ injection, expermintal rats were screened for serun glucose level and STZ-treated rats with blood glucose levels ≥ 200 mg/dl after 2 hours of glocuse intake were concidred diabetic and selected for further studies [5].

### Culturing and characterization of mesenchemal stem cells

BM-MSCs were obtained by using previously described methods [14]. Well characterized third passage plastic adherent Mesenchymal stem cells were used for experimentation.

### Establishment of a Delayed Wound Healing Model and estimation of wound healing area

The animal model was established on 20 diabetic rats by using previously described method [15], [13], [16]. The wound healing ares was assessed at 3, 7 and 12 days after wound incision by techique has been described preiously by **Kuo et al.** [17]**.**

### Expermintal design

A total of Fourty male albino rats (*Rattus rattus*) weighing approximately 150-180 g were allocated into four groups of 10 animals each, assigned as follows: Control wounded group (I) and diabetic wounded group (II), rats were subcutaneously injected with PBS, wherease control treated group (III) and Dibetic treated group (IV), rats were treated with subcutaneous injections with BM-MSCs (6 sites, 5 mm away from the wound edge, 0.05 mL cells suspension). All expermintal animals were housed separetly after wound creation in the cages in room tempreture at 22 ± 1 $^{O}$C and relative humidity of 60-65%

### Semi quantitive RT-PCR

Total cellular RNA was extracted from skin tissues using the phenol–guanidine– isothiocyanate method with Trizol (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. The purity of the total RNA was assessed by the ratio of optical density 260 nm to 280 nm (acceptable values being between 1.6 and 1.9). One microgram total RNA was used for reverse transcription into complementary DNA by PrimeScript RT reagent kit under conditions recommended by the manufacturer. Gene primers for rat VEGF and beta actin (beta actin, internal control) are listed in table (1). Real-Time PCR was performed using SYBR Premix Ex Taq II (Takara, Japan). The samples were subject to the following conditions in Real-Time PCR System (Applied Biosystems, USA): after initial denaturation at 95 ◦C for 30 s, PCR amplification was performed for 40 cycles at 95◦C for 5 s and 60 ∘C for 31 s.

**Table 1: Primers used for RT-PCR in this study**

| Gene | Primers (F=forward; R=reverse) | Amplicon size (bp) |
|---|---|---|
| VEGF | F: 5' -GTCCTCACTTGGATCCCGACA-3' | 99 |
| | R: 5' -CCTGGCAGGCAAACAGACTTC-3' | |
| Beta actin | F: 5' - GCTACAGCT TCACCACCACA-3' | 156 |
| | R: 5' - ATCGTACTCCTGCTTGCTGA-3 | |

### Statistical analysis

Data were analyzed using graph pad-prism 6. Differences between groups were analyzed with one way ANOVA and student t-test. Value of $P < 0.05$ were considered significant.

## RESULTS

### Wound closure

Wounds closure rate in each groups was measured 3, 7 and 12 days after the incision figure (1). The wound closure percent in control animals was increased to 18.5, 78.13 and 84.33 at 3, 7, and 12 days respectively after experiment. This closure percent was greatly accelerated after treatment by bone marrow derived stem cells as compared to control wounded animals where the closure percent of the wound increased to 21.8, 3 days post-incision 83.8, 7 days post-incision and 96.19, 12 days post-incision. In diabetic animals, the closure rates were 13.6%, 64.43% and 69.7 % after 3, 7 and 12 days respectively, while in diabetic treated rats were 15.9%, 72.61% and 86.72 % respectively. The closure rate of both treated groups (control and diabetic) were significantly increased at 7 and 12 days after wounding as compared to their corresponding controls.

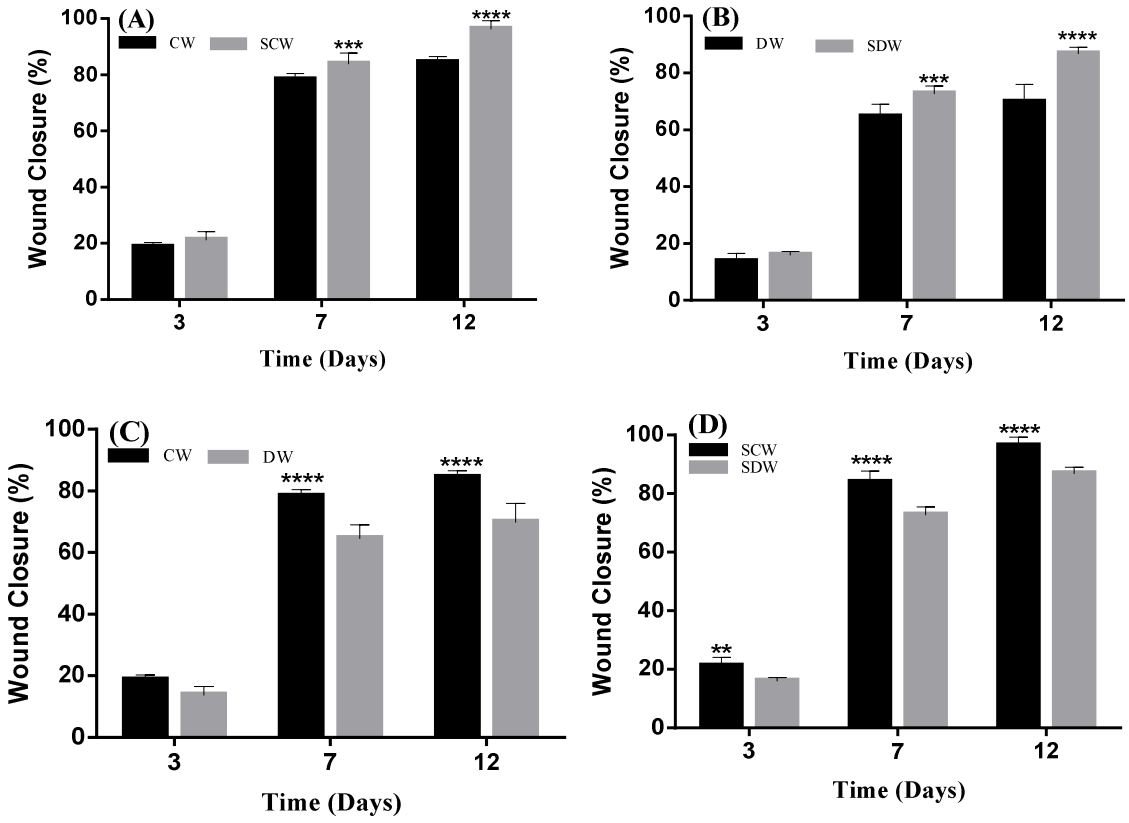


**Figure (1): comparison of wound closure rates expressed as percentage of its initial wound area after wounding (n=10; ***P = 0.0001 and ****P < 0.0001 (A), CW vs. SCW while (B) DW vs. SDW)**

### Levels of expression of VEGF mRNA in different studied groups

Vascular epithelial growth factor (VEGF) mRNA was expressed in all the studied groups figure (2). In the control wounded group, the mean ± S.D. values of the ratio relative to the beta actin gene were 1.00 ± 0.06, 1.55 ± 0.08 and 1.12 ± 0.04 after 3,7 and 12 days of wounding respectively, while in control treated group the ratios were 1.12 ± 0.09, 2.01 ± 0.19 and 4.25 ± 1.21 respectively. In the control diabetic wounded group, the ratios were 0.43 ± 0.05, 0.54 ± 0.04 and 0.56 ± 0.05 after 3,7 and 12 days of wounding respectively, whereas, in diabetic treated group the ratios were 0.62 ± 0.06, 1.11 ± 0.10 and 2.36 ± 1.02 respectively. The ratios relative to the beta actin gene of both treated groups (control and diabetic) were significantly increased at 7 and 12 days after wounding as compared to their corresponding controls.

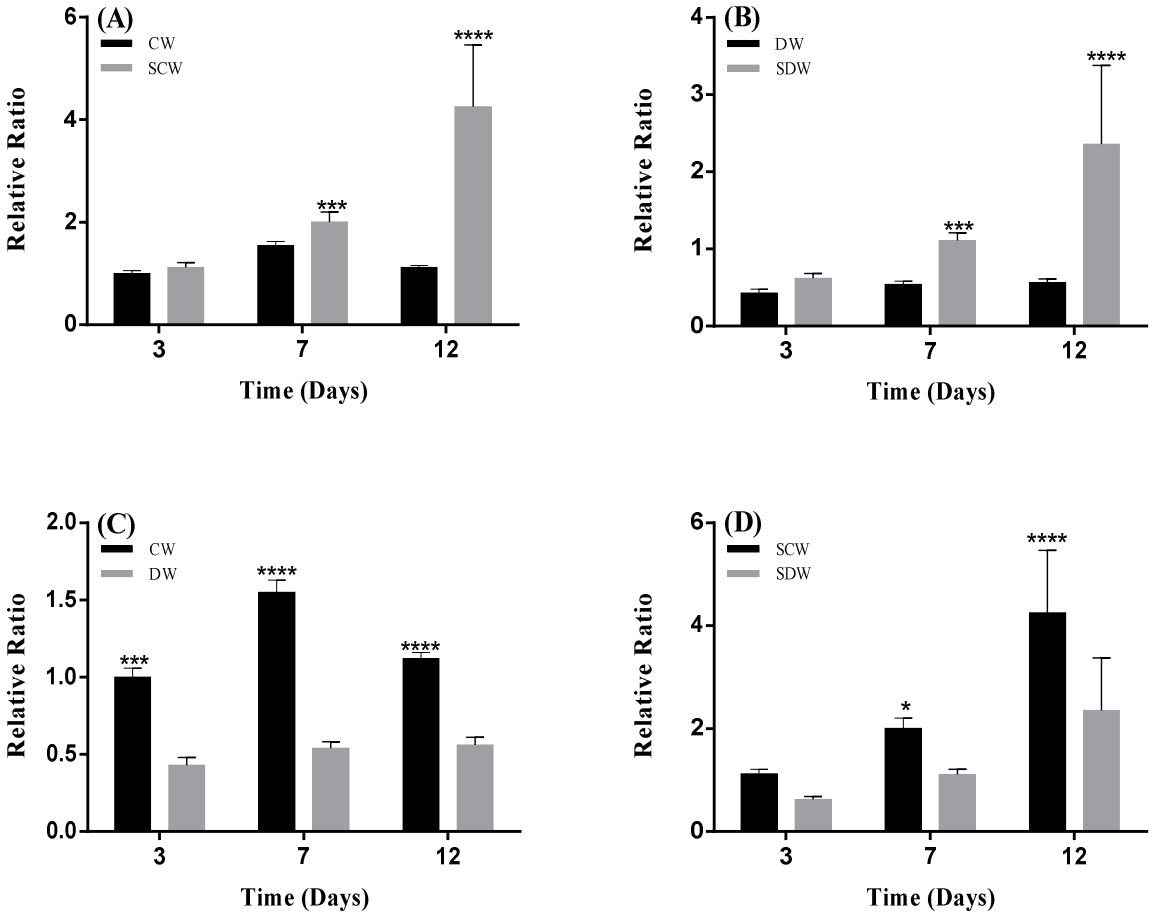


**Figure (2): The level of expression of VEGF of control with treated control group(A) and diabetic control with treated diabetic group (B), normalized as the ratio relative to the amount of b-actin. The bands were scanned and the density and the width of each PCR product were measured using the software package. We calculated the ratio of each product of VEGF mRNAs to b-actin mRNA**

**Histological analyses**

The histopathological discoveries of skin are shown in Figures. 3 and 4.

**Three days after incision**

In both wounded non-diabetic and diabetic animals that had been wounded but not treated, the skin showed wound gap with fibrin clot, however, necrotic muscles were observed in wounded non-diabetic animals and numerous bacterial colonies in non-treated diabetic rats. In wounded treated animals, the skin showed fibrin clot within the wound gap and entrapped intense leukocytes in diabetic animals, while in non-diabetic animals the skin showed hemorrhage , serofibrinous edema and leukocytes.

**Seven days after incision**

The wound site was diminished due to deep contraction but was still lined by granulation tissue and covered with crusts. The skin cellularity increased may be due to proliferation of fibroblasts and new matrix deposition. In control non-diabetic rats the skin showed leukocytes, few fibroblasts and blood capillaries. The skin in non-treated diabetic wounded rats showed serofibrinous exudates, leukocytes and plumped fibroblasts, while in treated non-diabetic animals the epidermis showed thickness on the margin of the wound (hyperplasia) and the scab formation was observed with few leukocytes and fibroplasias. The fibrin clot formation within the wound gap and entrapped intense leukocyte were observed in the skin of treated diabetic animals.

**Twelve days after incision**

At day 12, the skin showing hyperplasia in the prickle cell layer, as well as fibrovascular tissue in the dermis of untreated control rats. However, the skin of diabetic non-treated rats showed extensive scab with slight proliferation in the epidermal keratinocytes , intensive aggregation of leukocytes and organized thrombus and mild fibroplasias. In both treated animal the epidermis showed complete re-epithelialization.

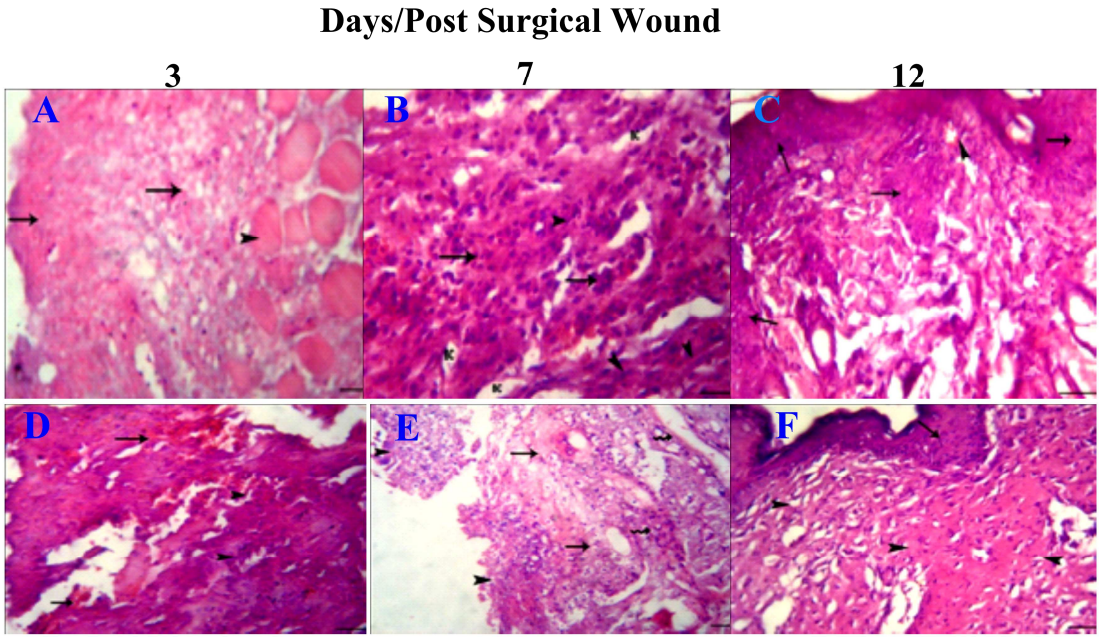


**Figure 3: Histomorphological observation of skin rats in CW (A, B and C) and SCW (D, E and F) groups at 3, 7 and 12 days post surgery**

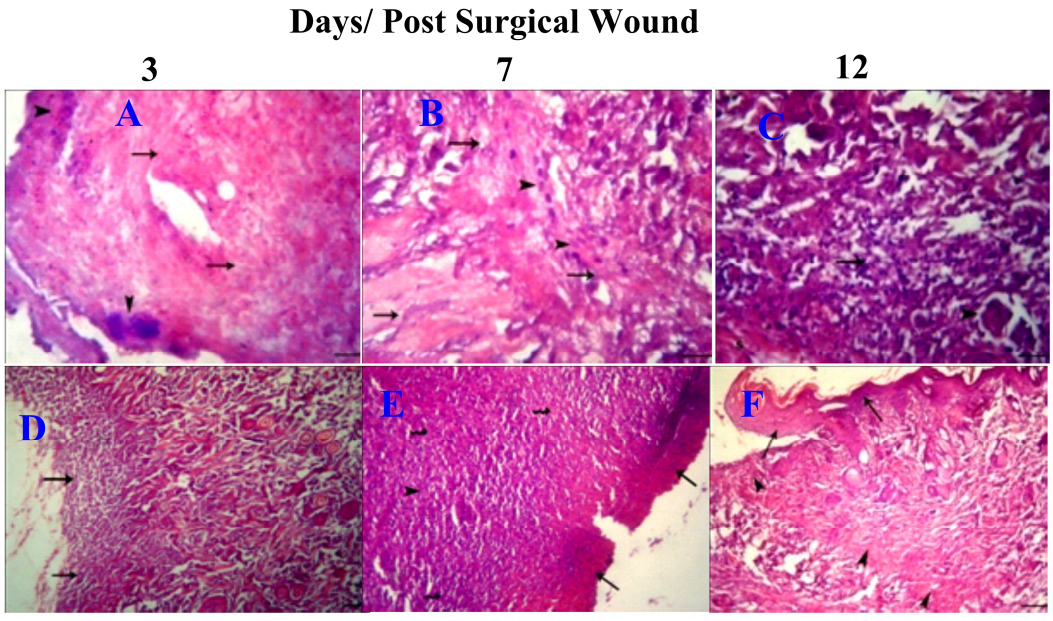


**Figure 4: Histomorphological observation of skin rats in CDW (A, B and C) and SDW (D, E and F) groups at 3, 7 and 12 days post surgery**

## DISCUSSION

There is surely no space for doubt that creative treatment for the prevention, mitigation, and/or total cure of the diabetic wound are in high demand. Examination of the wounds in the present work demonstrated that although diabetes mellitus slows wound healing due to presence of bacteria in the wound which may increases pro-inflammatory mediators and decreases growth factor levels, treatment with BM-MSCs after wounding hastens wound closure in both diabetic and nondiabetic rats. Consistent to our results, albeit with different sources of mesenchymal stem cells, including those umbilical cord blood [18], adipose tissue [19], or same source from bone morrow [20], [21], also promoted wound healing in diabetic animals. Wolter et al. [22] found that hBM-MSCs increased wound closure rate by increasing the in vitro migration of fibroblasts and kiratinocytes. Similar findings were reported by Smith et al. [23], who found that the accelerated wound closure in the presence of murine bone morrow-derived MSCs was due to increased dermal fibroblast migration.

Neovascularization, the formation of new blood vessels which is important to maintain the newly created granulation tissue and survival of keratinocytes, is considered as one of the essential procedures in wound healing [24], [25], [26]. Shrestha et al. [27] demonstrated that MSCs- treated wound in diabetic animals had enhanced capillary density, suggesting that MSCs induce angiogensis. Vascular epidermal growth factor (VEGF) is one of angiogenic factors may be involved in neovascularization. It functions as an endothelial cell mitogen [28], chemotactic agent [29] and inducer of vascular permeability [30]. Wound healing angiogensis involves multiple steps including vasodilation, basement membrane degradation, endothelial cell migration and endothelial cell proliferation [31]. Subsequently, capillary tube formation occurs, followed by anastomasis of parallel capillary spouts and finally new basement membrane formation VEGF plays a role in several of these processes. Semi-quantitative PCR rather than northern blot analysis has been widely accepted as a highly sensitive and specific method to examine mRNA expression, particularly for analysis of rare transcripts in small amount of tissue or cultured cell [32]. By using this method in the present study, we examined VEGF mRNA expression in the skin tissue of wound in all studied groups. the ratios relative to the beta actin gene of both treated groups (diabetic and non-diabetic) were significantly increased at 7 and 12 days after wounding as compared to their corresponding controls ($P < 0.05$). These findings suggest that VEGF in the injured tissue may play an extensive role in physiology of normal vasculature or the cellular regeneration in skin regardless of wound angiogenesis. Moreover, BM-MSCs were promoted VEGF mRNA expression in treated groups, subsequently, led to promote tissue regeneration in experimental rats. Our findings consistent with other studies conducted by Broun et al.[33] and Nissen et al. [34]. They found that VEGF mRNA and protein increased at early time points after injury in the skin, and VEGF protein levels increased and remain elevated in wound fluid for at least a week in surgical wounds. Moreover, Zhang et al. [35] observed high levels of growth factors, including vascular epidermal growth factor (VEGF) in MSCs in vitro. The findings in the present study, suggested that BM-MSCs promote wound epithelialization and neovasvascularization by means of increased expression of VEGF and stimulation of epithelialization in experimental treated rats. Taken together, our results demonstrated that BM-MSCs mediated correction of the diabetic wound healing impairment is due to, partly, increased VEGF expression in the skin of STZ-induced diabetic rats and accelerate wound healing in the controls.

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