

Stem cells and metformin synergistically promote healing in experimentally induced cutaneous wound injury in diabetic rats

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Abstract

Introduction. Diabetes mellitus (DM) is a serious, chronic metabolic disorder commonly complicated by diabetic foot ulcers with delayed healing. Metformin was found to have a wound healing effect through several mechanisms. The current study investigated the effect of both bone marrow-derived mesenchymal stem cells (BM-MSCs) and metformin, considered alone or combined, on the healing of an experimentally induced cutaneous wound injury in streptozotocin-induced diabetic rats.

Material and methods. Forty adult male albino rats were used. Diabetes was induced by single intravenous (IV) injection of streptozotocin (STZ). Next, two circular full thickness skin wounds were created on the back of the animals, then randomly assigned into 4 groups, ten rats each. BM-MSCs were isolated from albino rats, 8 weeks of age and labeled by PKH26 before intradermal injection into rats of Group III and IV. Groups I (diabetic positive control), II (metformin-treated, 250 mg/kg/d), III (treated with 2×10^6 BM-MSCs), and IV (wounded rats treated both with metformin and BM-MSCs cells). Healing was assessed 3, 7, 14, and 21 days post wound induction through frequent measuring of wound diameters. Skin biopsies were obtained at the end of the experiment.

Results. Gross evaluation of the physical healing of the wounds was done. Skin biopsies from the wound areas were processed for hematoxylin and eosin (H&E), Masson's trichrome staining and immunohistochemical staining for CD31. The results showed better wound healing in the combined therapy group (IV) as compared to monotherapy groups.

Conclusions. Although both metformin and BM-MSCs were effective in the healing of experimentally induced skin wounds in diabetic rats, the combination of both agents appears to be a better synergistic option for the treatment of diabetic wound injuries. (*Folia Histochemica et Cytobiologica* 2019, Vol. 57, No. 3, 127–138)

Key words: rat; stem cells, BMSCs; STZ diabetes; metformin; skin wound; healing; angiogenesis; CD31

Introduction

Diabetes mellitus (DM) is a serious, chronic endocrine disorder affecting more than 380 million people

worldwide by 2013, and is expected to rise to 592 million by 2035 [1]. The growing population of diabetic patients has increased the risk of development of complication such as nephropathy, retinopathy, neuropathy, and macroangiopathy which leads to diabetic foot ulcers with delayed healing and subsequent amputation [2]. Reduced angiogenesis and impaired production of cytokines by local inflammatory cells are crucial factors for delayed healing [3].

The clinical need to develop recent strategies of treatment to improve the healing of diabetic ul-

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cerative wounds becomes mandatory. Metformin, a biguanide, is one of the most commonly prescribed oral anti-hyperglycemic agents for treating type 2 diabetes mellitus [4]. It exerts its therapeutic effects through multiple mechanisms of actions, including inhibition of glucose production in the liver. In addition, metformin is an insulin enhancer and can increase insulin sensitivity [5]. Hence, it might be beneficial for wound healing as tissue resistance to insulin has been found to interfere with the healing of excisional skin wounds by delaying the contraction and re-epithelialization [6].

Bone marrow-derived mesenchymal stem cells (BM-MSCs) are self-renewing and expandable stem cells. BM-MSCs have several applications in regenerative medicine as they have the capacity to differentiate into different types of cells such as adipocytes, osteoblasts, chondrocytes, hepatocytes, and cardiomyocytes [7]; however, their applications, in healing and repair of skin wounds are still under research.

Due to the urgent need to develop new treatment modalities to improve the healing of diabetic ulcerative wounds, the current study was designed, as a novel research issue, to investigate the effect of combined administration of both metformin and BM-MSCs on the healing of an experimentally induced cutaneous wound injury in an animal model of streptozotocin (STZ)-induced diabetes.

Materials and methods

Animals. Forty adult male albino rats, locally bred at the animal house of Kasr El-Aini, Cairo University, Egypt, with an average weight of 200–250 g were used in the present study. The animals were housed at an ambient temperature of $25 \pm 1^\circ\text{C}$, exposed to natural daily light–dark cycles, and had free access to food and water *ad libitum*. All animal handling and procedures were followed and approved by the ethical committee and the guidelines of Kasr El-Aini animal house. All animal experimental procedures were carried out in accordance with the guidelines of National Institutes of Health for the care and use of Laboratory animals [8].

Chemicals. Streptozotocin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Metformin tablets (Glucophage 500 mg tablets) were purchased from Minapharm (Cairo, Egypt), under license of Merck Santé, Semoy, France). Metformin tablets were grinded and suspended in 0.5% carboxymethyl cellulose (CMC) according to previous work [9], and afterwards shaken to obtain a suspension form of 50 mg/ml.

Rat excision wound model. The fur on the back of the anesthetized rats was removed via light application of de-

pilatory cream and cleaned with alcohol swab. According to Dunn et al., 2013 [10], two circular 10 mm (1 cm) diameter full-thickness excision wounds were made on the dorsum of each anesthetized rat (ketamine, 100 mg/kg, intraperitoneal) [9], using sterile punch biopsy forceps. Hemorrhage, if any, was controlled by pressure application of sterile gauze. The wound size was chosen to be about 10 mm to compensate for the possible decrease in the incision diameter caused by wound contraction [11]. After wounding, the exposed raw area was covered by non-adherent gauze for about 24 hours to prevent tissue fluid loss. The rats were kept in an individual cage under specific pathogen free conditions in an animal room.

Streptozotocin-induced diabetes. Diabetes was induced by a single intravenous (IV) injection of sterile STZ in sodium citrate (0.1 mol/L, pH 4.5) to overnight-fasted animals through tail vein. STZ was given immediately once prepared, at a dose of 60 mg/kg body weight as described in previous studies [12]. After 3 days, diabetic states of rats were checked via blood samples withdrawn from the tail veins of these rats to determine fasting blood glucose level using blood glucose tests strips and meter (Accu-Check; Roche Diagnostics, Penzberg, Germany). On day 21 after injection, the animals were fasted for 8 h and blood glucose was measured. Animals with fasting blood glucose level equal to or more than 250 mg/dl were considered as established STZ-induced diabetic rats [12], and were included in the experiment (Table 1).

Wound closure analysis. The wound diameters were measured on postoperative days 3, 7, 14, and 21 (Table 1). Wound closure was assessed as a percent reduction in the wound area. Progressive decrease in the wound area was followed by tracing the wound margin on a transparent paper. After that, the tracing was placed on a graph paper and the number of squares was counted. Wound closure was expressed as percentage reduction of the original wound area and was calculated using the following formula as demonstrated in previous work [13]:

$$\% \text{ wound closure in day } N = (\text{area on day } 0 - \text{area on day } N) / \text{area on day } 0 \times 100$$

The area on day 0 is defined by the trace obtained immediately after wounding (the original wound area).

Experimental groups. Three weeks after induction of diabetes, the diabetic rats had undergone excisional wounds on their backs as described previously [10] and were randomly assigned into four groups, ten rats each (Table 1).

Group I: diabetic group (positive control). Five rats received 0.5 ml phosphate buffer saline (PBS), injected intradermally at eight different sites in the wound margins and five rats received 0.5% CMC by gastric lavage.

Table 1. Illustration of the workflow of the current study

Stages	Days	Events
Diabetes induction & monitoring	D 0	STZ injection
	D 3	Checking blood glucose & verification of the diabetic status
	D 21	<ul style="list-style-type: none"> Monitoring of the fasting blood glucose Rats with blood glucose ≥ 250 mg/dl were considered diabetic and were included
Wound creation, treatment	D 21 (D 0 post wounding)	<ul style="list-style-type: none"> Wound creation on the backs of the animals Start Metformin treatment for 21 days Start stem treatment, once intradermal injections in the wound margins Start combined therapy treatment Start vehicle treatment (positive control)
Wound Measurement & end	D 24 (D 3 post wounding)	Measurement of the wound area
	D 28 (D 7 post wounding)	Measurement of the wound area
	D 35 (D 14 post wounding)	Measurement of the wound area
	D 42 (D 21 post wounding)	<ul style="list-style-type: none"> Measurement of the wound area Euthanizing of animals End of the experiment

Group II: metformin-treated group. Ten rats received metformin (250 mg/kg/d) for 21 days after wound creation, by gastric lavage.

Group III: mesenchymal stem cell-treated group. Ten rats received single intradermal injections of MSCs (2×10^6 in 0.5 ml of PBS) at eight different sites in the wound margins [14].

Group IV: combined therapy group. Ten rats received both metformin (250 mg/kg/d by gastric lavage for 21 days) and single intradermal injections of MSCs (2×10^6) as described for Group III.

Isolation and culture of the bone marrow derived MSCs. Rat mesenchymal stem cells were isolated according to the protocol described by Hu *et al.* [15] with minor modifications. After anesthesia of 8 week-aged rats, the femur and tibia were aseptically dissected and cleared of all muscles and connective tissue. Bone marrow was collected by flushing each long bone with complete culture medium constituted of Dulbecco’s Modified Eagle’s medium (DMEM) 10% fetal calf serum (FCS), 2 mM glutamine and penicillin/streptomycin (50 U/ml and 50 mg/ml, all from Sigma), supplemented with heparin at a final concentration of 5 U/ml. Collected marrow samples were mechanically disrupted by passing them successively through 18-gauge and 20-gauge needles to obtain a single cell suspension. The cells were centrifuged and resuspended with culture medium and incubated at humidified 5% CO₂ and 95% air atmosphere at 37°C. BM-MSCs were first selected by their adherent property preferentially attaching to uncoated polystyrene tissue culture dishes [16]. The non-adherent cells were removed by 2–3 washes with PBS and adherent cells were further cultured in complete medium. For cell passaging, trypsin-EDTA solution (Sigma) was used. The medium

was changed after 2 days and twice a week thereafter. When large colonies of primary MSCs developed (80–90% confluence), they were trypsinized (trypsin-EDTA solution) for 5 min at 37°C. The resulting cell suspension was centrifuged at 1,500 rpm for 5 min, resuspended in complete culture medium. Fibroblast-like cells became the predominant cells in culture, characterized by their fusiform shape and plastic adhesiveness. The recovered cells were counted, using hemocytometer and cellular viability was quantified by the trypan blue exclusion test in which the viable cells appeared with clear cytoplasm (not stained) [17]. Up to 3 cell passages were used in this experiment.

Flow cytometry. Extended effort was afforded for immunophenotyping of the separated, purified MSCs using flow cytometry (Accuri, San Jose, CA, USA). The cell suspension was incubated with different fluorescently labeled monoclonal antibodies against rat CD45, CD29, and CD90 molecules [18]. The BM-MSCs were positive for the MSCs markers (CD29 and CD90) and negative for the hematopoietic-lineage marker, CD45.

Histological procedure. On day 21 after wound creation, the rats were euthanized with an overdose of ether. Skin tissue samples were collected including the wounded tissues and the surrounding normal skin. The tissues were immediately fixed in 10% neutral-buffered formalin and processed for paraffin sections. Five micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E) for morphological observations, and Masson’s trichrome staining for the evaluation of collagen disposition, as based on previously stated protocols [19]. Immunohistochemical (IHC) reaction was done, according to previously mentioned protocols [20], for the detection of expression of CD31 (PECAM-1) by a specific antibody, mouse monoclonal antibody (Labvi-

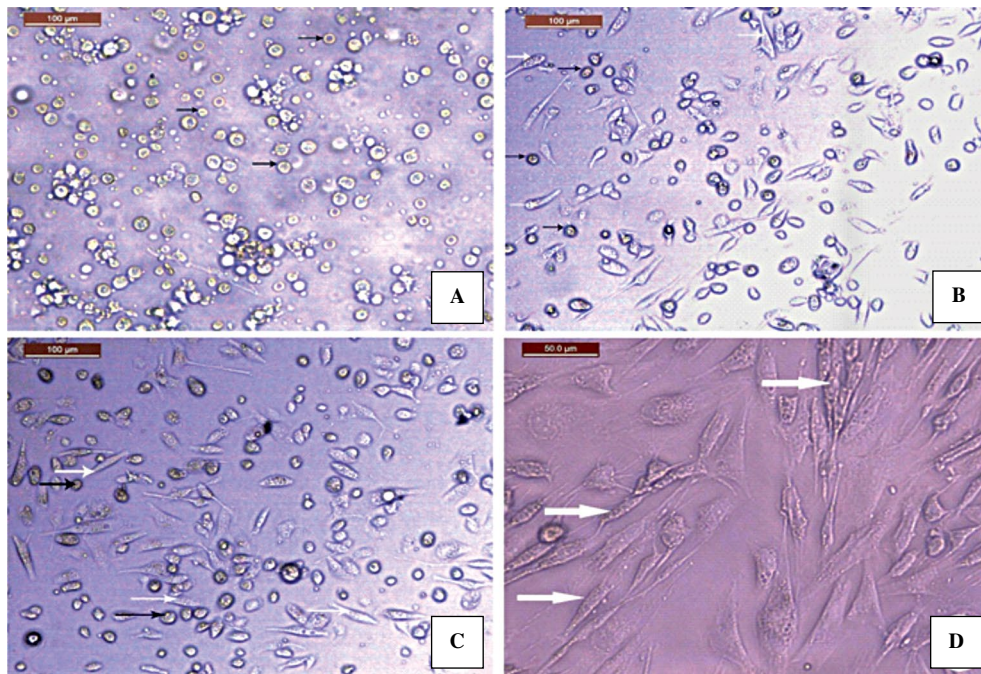


Figure 1. Inverted microscope photomicrographs of primary cultures of bone-marrow-derived mesenchymal stem cells (MSCs) on days 2 (**A** and **B**), 3 (**C**), and 7 (**D**). Panel (**A**) shows rounded nonadherent refractile cells (black arrows). Panel (**B**) shows few spindle-shaped cells (white arrow) between rounded nonadherent refractile cells (black arrow). Panel (**C**) shows many spindle cells (white arrow) between rounded nonadherent refractile cells (black arrow). Panel (**D**) shows many purer spindle shaped cells (white arrow).

sion corporation, Fremont, CA, USA). It was supplied as a prediluted antibody ready for staining formalin-fixed and paraffin-embedded tissues. The sections were incubated with the primary antibody diluted to a concentration of 1:100 in PBS for one hour, followed by a reaction with biotinylated secondary antibody. After conjugation with streptavidin–biotin–peroxidase complex, 3,3'-diaminobenzidine (DAB) was used as a chromogen, and hematoxylin solution was used as a counterstain. The reaction gives brownish discoloration in the plasma membrane of capillary endothelial cells in addition to macrophages and fibroblasts [21]. Initially, immunofluorescence detection of PKH26-labeled MSCs was done by fluorescent microscope in unstained paraffin sections for tracking of stem cells.

Morphometric measurements. Quantitative morphometric measurements of the mean area percent of CD31 immunostained skin sections were done using the image analyzer computer system (Leica Qwin 500, Leica, Cambridge, England). The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. For each group, ten measuring fields in each specimen were randomly selected, using the high power magnification ($\times 400$).

Statistical analysis. All the data obtained were presented as mean \pm standard deviation (SD). The normal distribu-

tion of the values in the different groups of the study was checked and the evaluation of differences between groups was performed using one-way analysis of variance (ANOVA) and *post hoc* LSD test with SPSS 19.0 software (IBM SPSS Statistics for Windows, Armonk, NY, USA). Results with P-value of less than 0.05 were considered statistically significant.

Results

Characterization of bone marrow-derived MSCs in culture

Using inverted microscope, MSCs were identified in culture as spindle-shaped cells between rounded cells (Fig. 1). On day 2 of primary culture of MSCs presented as rounded nonadherent refractile cells (black arrows) (Fig. 1A, B) and few spindle-shaped cells (white arrows) (Fig. 1B). On day 3 (Fig. 1C) of primary culture many spindle cells (white arrows) were visible between rounded nonadherent refractile cells (black arrows). On day 7 (Fig. 1D), numerous spindle-shaped MSCs (white arrows) were seen.

Evaluation of wound healing

Wound areas of each diabetic rat in the studied groups were measured on day 3, 7, 14 and 21 post wounding (Table 2 and Fig. 2). The area percentage of wound

Table 2. Changes in the wound areas of the studied groups of diabetic rats at different days post-wounding

Groups of rats	Wound surface area (cm ²) in post excision days			
	3rd day	7th day	14th day	21st day
Control (Gr. I)	0.905 ± 0.01 (9.5%)	0.852 ± 0.12 (14.8%)	0.550 ± 0.01 (45%)	0.305 ± 0.11 (69.5%)
Metformin-treated (Gr. II)	0.848 ± 0.14 ^c (15.2%)	0.705 ± 0.15 ^c (29.5%)	0.405 ± 0.01 ^c (59.5%)	0.260 ± 0.10 ^c (74%)
MSC-treated (Gr. III)	0.846 ± 0.11 ^b (15.4%)	0.75 ± 0.01 ^b (24.7%)	0.347 ± 0.02 ^b (65.4%)	0.251 ± 0.2 ^b (74.9%)
Metformin- and MSC-treated (Gr. IV)	0.501 ± 0.19 ^a (49.9%)	0.402 ± 0.01 ^a (59.8%)	0.203 ± 0.01 ^a (79.7%)	0.061 ± 0.11 ^a (93.9%)

Diabetes was induced by streptozotocin injection of rats as described in Methods. Data express wound area in cm² as mean ± SD, n = 10 in each group. The approximate percentages of wound closure are demonstrated in brackets. ^aSignificantly different (P < 0.05) when compared with metformin- (Group II) or MSC-treated rats (Group III); ^bP < 0.05 when compared with non-treated wounded diabetic rats (Group I); ^cP < 0.05 when compared with non-treated groups (control). MSCs, bone-marrow-derived mesenchymal stem cells.

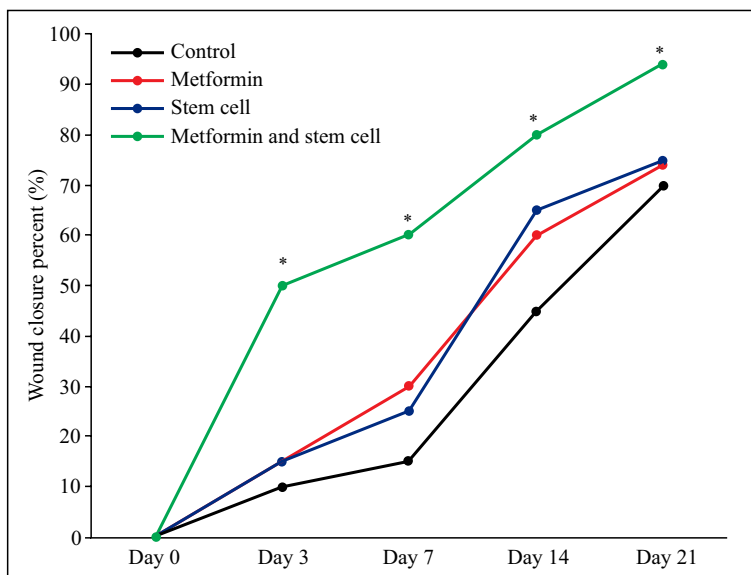


Figure 2. Rough estimation of the wound closure progress expressed as the percentages of reduction in the wound area at different time intervals (days 0, 3, 7, 14, and 21) in diabetic rats (control, metformin-, stem cells- or metformin and stem cell-treated). *Significant difference (P < 0.05) when compared at the same time-intervals with metformin- and mesenchymal stem cell-treated groups (Groups II and III, respectively). n = 10 in each group.

closure increased significantly in the combined therapy group (MSCs plus metformin) compared to the metformin (p < 0.05) and MSCs (p < 0.05) groups at 3, 7, 14 days after wounding. On day 21 after surgery, 94% wound closure was reached in the combined therapy group, while non-treated, metformin-treated and stem cell-treated wounds reached 69.4%, 74%, and 75% closure, respectively.

Morphological study of MSCs and wound sections

Using fluorescent microscopy, examination of unstained paraffin sections was carried out to track PKH26-labeled bone marrow-derived MSCs. The

PKH26-labeled stem cells emitted red fluorescence (Fig. 3).

Histological examination of H&E-stained skin sections from control diabetic rats (Group I) showed skin defect covered by poorly regenerated interrupted epidermis. The underlying papillary dermis looked loose and irregular (Fig. 4A). In both Group II and III (metformin- and MSC-treated groups, respectively), signs of partial healing were observed, such as formation of large amount of dermal granulation tissue filling the wound defect area, thin regenerated epidermis creeping over the granulation tissue, formation of new blood capillaries (Figs. 4B and

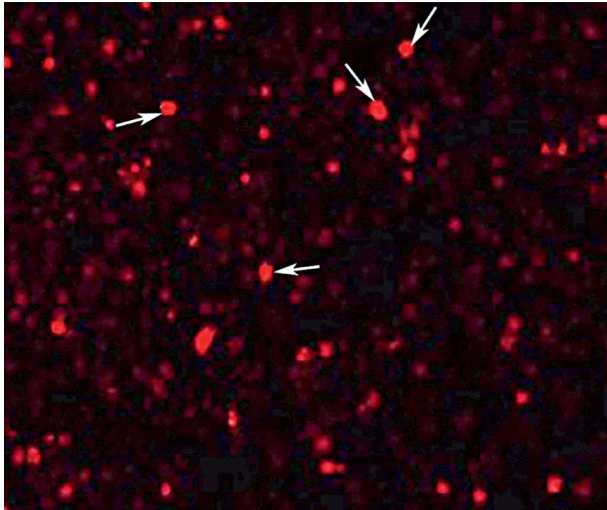


Figure 3. Fluorescence microscopy photomicrograph of a section in the skin wound area of a diabetic rat from Group III (MSC-treated) demonstrating presence of several red fluorescent PKH26-labeled bone marrow-derived mesenchymal stem cells (white arrows). Magnification: $\times 400$.

4C, respectively). In Group IV (combined therapy group), proper healing was observed in the form of the contraction of the wound area, complete re-epithelization of the wound surface, accumulation of huge amount of granulation tissue under the re-epithelized wound surface, differentiation of the epidermal cells, re-organization (remodeling) of the collagen fibers within the granulation tissue, and formation of new more functioning (containing red blood cells) blood capillaries with good regeneration of skin appendages (Figs. 4D, E).

Trichrome stained sections showed marked prominent deposition of collagen fibers in Group IV (stem cells plus metformin-treated animals). The collagen fibers appeared deeply stained, coarse and arranged in a network like different directions (Fig. 5D). However, in Group I (positive control), collagen fibers were lightly stained and scarce (Fig. 5A), and increased gradually in metformin- (Group II) (Fig. 5B) and stem cell-treated (Group III) rats (Fig. 5C).

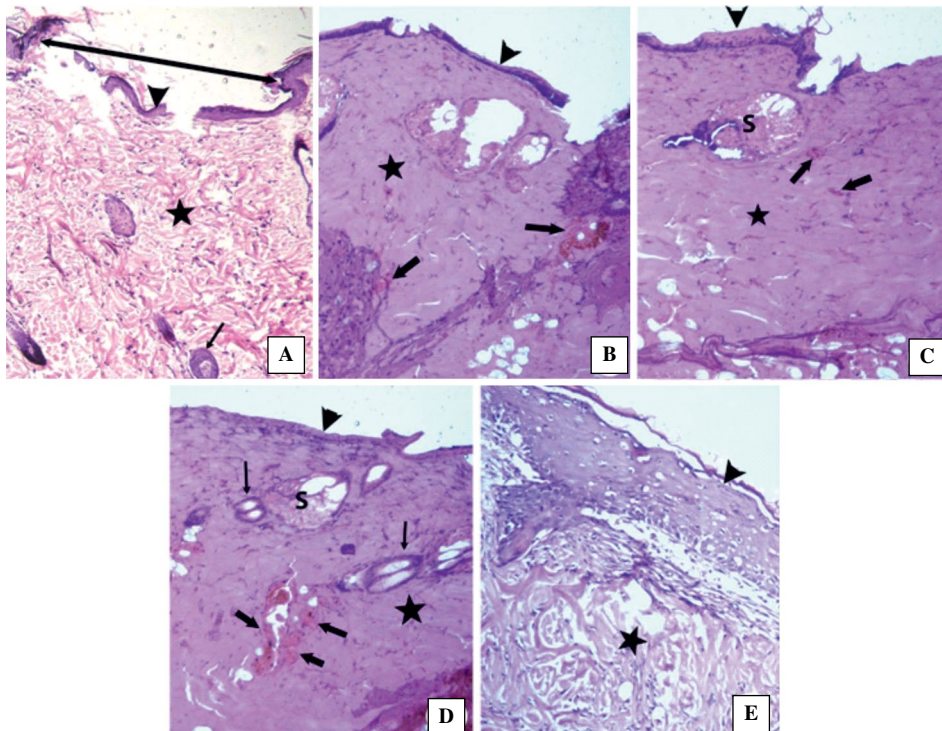


Figure 4. Photomicrographs of hematoxylin and eosin-stained skin sections of diabetic rats from different experimental groups. (A). Rat from the control group presents the wound defect area (double headed arrow) with thin poor healing epidermis (arrowhead) and less cell-dense connective tissue underlying dermal granulation tissue (star). (B). Skin of metformin-treated rat (Group II) discloses more cellular granulation tissue (star) and congested blood vessels (thick arrows). Regenerating thin epidermis (arrowhead) is seen overlying the granulation tissue. (C) Mesenchymal stem cell-treated rat (Group III) shows the dermal granulation tissue (star). Thin regenerated migratory epidermis (arrowhead) is seen extending to cover the granulation tissue. Small sized blood vessels are seen. (D) and (E). Rat treated with metformin and MSCs (Group IV) shows almost complete re-epithelization of the wound surface (arrowhead), dermal granulation tissue formation (star). Clear differentiation of the epidermal cells and re-organization of the granulation tissue are seen in some sections. Congested blood vessels and formation of new small-sized blood capillaries (thick arrows) are evident. Skin appendages (sebaceous gland (S) and hair follicles (thin arrows)) are well formed. Magnification: A–D $\times 100$, and E $\times 400$.

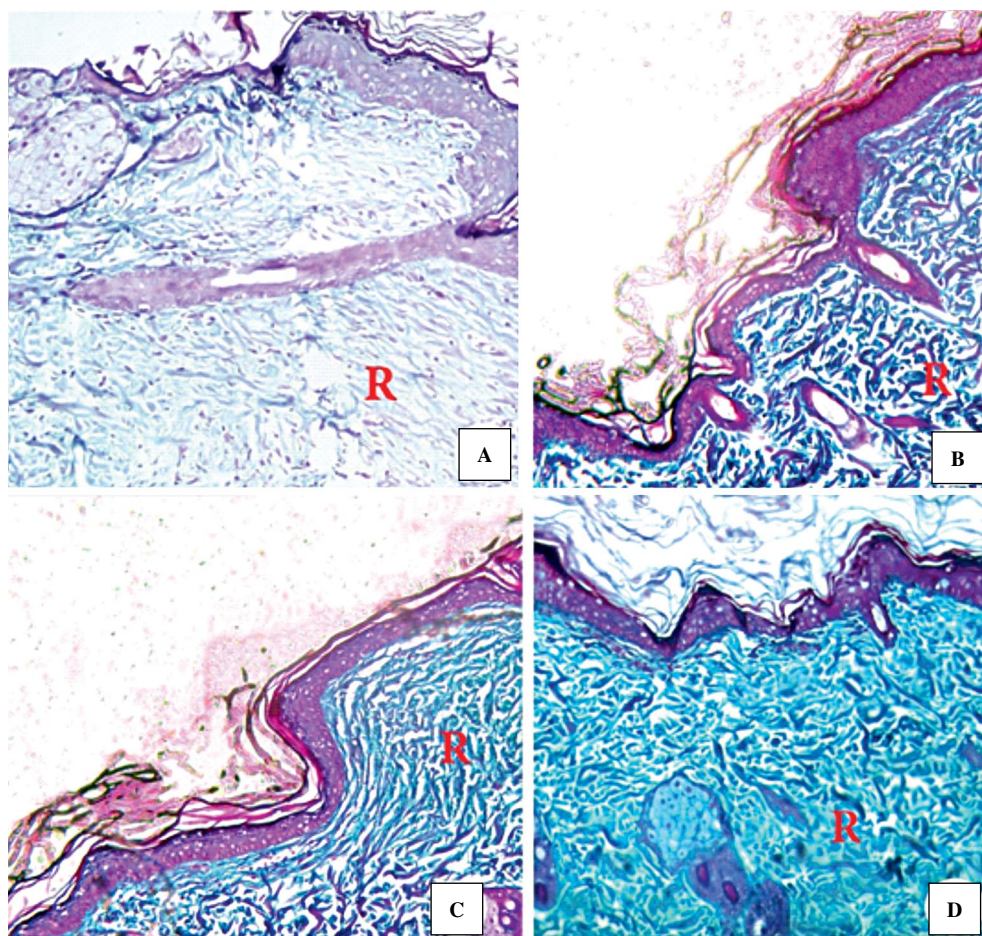


Figure 5. Masson's trichrome stained skin sections of the different groups of diabetic rats. (A). Rat from Group I (control) shows lightly stained collagen bundles. (B). Rat from Group II (metformin-treated) shows disarranged collagen bundles partially filling the wound defect area. (C). Rat from Group III (mesenchymal stem cell-treated) shows the dermal granulation tissue with rich collagen bundles which is disorganized under the wound area. (D). Rat from Group IV (combined therapy group) shows marked deposition of darkly stained, heavily distributed, coarse collagen fibers. Magnification: A–D $\times 100$.

Immunohistochemical detection of CD31 localization during skin wound healing

Immunohistochemical staining of skin sections of diabetic rats of Group I (control) revealed moderately-intense expression of CD31 and was limited to the endothelial cells of blood vessels (Fig. 6A). However, in Groups II and III (metformin- and mesenchymal stem cell-treated animals, respectively), diffuse CD31 immunoreaction was noticed in the cells of the reticular layer of dermis such as fibroblasts and macrophages (Fig. 6B, C). In Group IV (metformin- and stem cell-treated rats), the CD31 immunoreaction was diffuse and marked with the existence of large number of CD31 positive cells in dermal connective tissue (Fig. 6D).

Histomorphological evaluation of the area percent of CD31 immunostaining

The data, demonstrated in Figure 7, revealed significant increase ($P < 0.05$) in the mean area percent

of the CD31 immunostaining in combined therapy group (Group IV) when compared to either Group II (metformin-treated) or Group III (mesenchymal stem cell-treated). Compared to Group I (control), the CD31 area was significant higher for Groups II–IV ($P < 0.05$), while no difference was observed between Group II and III.

Discussion

Diabetic foot ulcer with impaired wound healing is a common complication of uncontrolled diabetes mellitus. Untreated neglected foot ulcers could be complicated by gangrene and amputation with its bad psychological impact on these patients [22, 23]. Introduction of new treatment modalities is mandatory to improve health status of diabetic patients. Bone marrow-derived stem cells are a promising stem cell source for regenerative medicine and wound repair. They can differentiate into other cell types within the

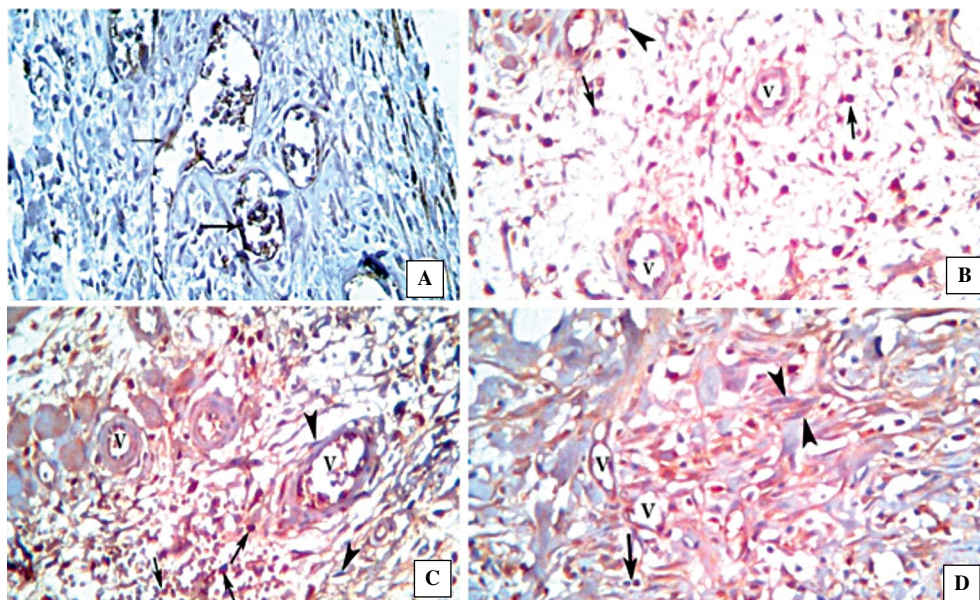


Figure 6. CD31-immunostained skin sections of diabetic rats from the studied experimental groups. (A). Rat from Group I (control) reveals scarce CD31 immunoreactivity limited to the endothelial cells of blood vessels (V). (B). Rat from Group II (metformin-treated) shows moderate CD31 immunostaining of the endothelial cells as well as dermal connective tissue cells, macrophages (arrows) and fibroblasts (arrowheads). (C). Rat form Group III (mesenchymal stem cell-treated) shows moderate CD31 immunostaining of the endothelial cells, fibroblasts (arrowheads) and macrophages (arrows). (D). Rat from Group IV (combined therapy group) shows marked CD31 positive immunoreaction including endothelial cells of blood capillaries (V) and dermal connective tissue cells. Magnification: A–D ×400.

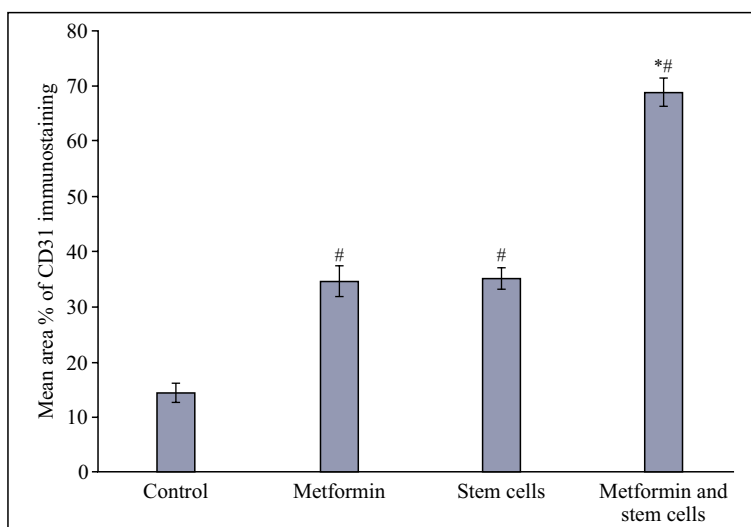


Figure 7. The mean values ± SD of the area percent of CD31 immunoreactivity in the different groups of diabetic rats, the control group, metformin-treated, mesenchymal stem-cell-treated groups, and both metformin- and MSCs-treated-group. n = 10. *Significant difference (P < 0.05) when compared to metformin- and bone marrow-derived stem cell-treated groups. #significant difference (P < 0.05) when compared to the control group.

injured tissue to promote repair and regeneration of the skin [7, 24]. Metformin is a well-known, commonly prescribed oral hypoglycemic drug [4], and was found in healthy animals to have a wound healing effect

against cutaneous skin injuries [25]. For this reason, the current study was designed to investigate the effect of metformin and stem cells treatment on skin wound healing in diabetic rats.

Skin has a remarkable accelerated capacity for spontaneous wound healing. Sex hormones, in particular estrogens, play an important role in the regulation of biological processes involved in tissue regeneration and wound healing [26, 27]. In the current study, the male gender of the experimental animals was preferred to minimize the accelerating effect of endogenous estrogens on wound healing [26]. Experimental diabetes was induced to evaluate the therapeutic effects of new agents in rats in which the cutaneous wound healing was impaired by the underlying disease [28]. In order to mimic the clinical manifestations of human DM, streptozotocin was chosen to induce diabetes in the experimental animals as it selectively destroys the beta cells of the pancreatic islets [12]. Each rat was later exposed to experimental creation of a full thickness excisional wound using a sterile punch biopsy. Given that wound healing was accelerated in partial thickness skin injury, the full thickness excision was preferred as an experimental skin wound model, rather than partial thickness skin injury to abolish the effect of endogenous stem cells located within hair follicles and sebaceous glands [29].

In the present work, there were no great gross or histological differences between the wounded skin of rats injected with PBS or those which received orally streptozotocin solvent (CMC) in the diabetic control group, so that these groups were considered the same.

Regarding the gross evaluation of wound healing and closure of the wound defect area at different time intervals throughout the experiment (days 3, 7, 14, and 21), it was found that approximately complete reduction in the wound defect area was reached in combined therapy group (Group IV) on day 21. There were no significant differences between metformin treatment (Group II) and BM-MSCs transplantation (Group III) in wound closure area. However, the combination of metformin and BM-MSCs showed significantly better curative effect over the single treatment modality. The current result indicated that stem cells, combined with metformin improved wound healing which was in accordance with findings of Seo *et al.* [30] who reported better effects on wound healing when they utilized stem cells, in combination with another antidiabetic agent, exendin-4.

Histological analysis of the H&E stained skin sections obtained from the diabetic non-treated rats (Group I) revealed poor wound healing, delayed closure of the wound area. Poorly regenerated interrupted epidermis was also noted covering the wound floor. The papillary layer of the dermis showed less dense, deficient underlying dermal granulation tissue, which was evidenced by the lightly stained scarce collagen fibers visualized by trichrome staining. Fur-

thermore, it was correlated with gross wound gapping and poor healing in the same group. Similar to the current findings, other investigators [31] revealed delayed cutaneous wound healing in non-treated diabetic rats. These findings could be supported by the explanation of Serra *et al.* who demonstrated the effect of diabetes on the different phases of wound healing: the inflammatory phase (with compromised immune system reactivity), the proliferative phase (suppressed collagen deposition and formation of new blood vessels) and the remodeling phase in which re-organization of collagen occurs to restore the tissue structural integrity [32].

In the treated diabetic groups of rats we noted that both metformin and stem cells improved healing process in experimentally induced skin wounds. In metformin-treated and stem cell-treated (Group II and Group III, respectively), histological examination revealed similar findings in the form of the accumulation of granulation tissue partially filling the wound defect area with thin regenerated creeping epidermis and formation of new blood vessels. Masson's trichrome stain showed more abundant collagen bundles than in injured control diabetic rats. However, the combination of both stem cells plus metformin (in Group IV) showed nearly complete re-epithelialization of the wound surface in comparison to either agent alone, formation of more cellular granulation tissue and formation of numerous blood capillaries. As well, darkly stained coarse abundant collagen fibers were evident in Masson's trichrome stained skin sections. Collagen is an important extracellular matrix component that provides integrity and structure of skin and other tissues [33]. This notion supported the finding of granulation tissue formation and collagen deposition in H&E and trichrome stainings, respectively. The finding of combined therapy group suggested better curative effect not only through increasing rate of wound healing, but also ensured normalization of the wound through effective re-epithelialization. These findings were in agreement with the results of Elsharawy *et al.*, (2011) [34], who reported better epidermal healing and accelerated revascularization of the wound when they utilized umbilical cord blood-derived CD34+ stem cells injected into the wound bed. Thus, MSCs from various sources can accelerate healing of skin wounds in diabetic rats, which offers a new possibility of diabetic ulcers treatment in humans.

To our best knowledge the application of bone marrow-derived stem cells, in combination with metformin, in the current study, is the first such attempt aimed at the improvement of the health status of diabetic patients through aiding diabetic wound repair and regeneration.

Different mechanisms were postulated to explain the action by which BM-MSCs transplantation leads to tissue repair. One of these mechanisms was the differentiation of the stem cells into mature endothelial cells leading to angiogenesis, or the paracrine stimulation of neovascularization through the release of angiogenic growth factors and chemical mediators [35]. Differentiation into healing tissue components is also an important mechanism. BM-MSCs can differentiate into fibroblasts and myofibroblasts so that better healing and contraction of the wound defect area can be achieved. Moreover, there was evidence suggesting that BM-MSCs could recruit more fibroblasts and stimulate their migration from the surrounding tissues *via* chemotaxis [36, 37]. Immunohistochemical staining of CD31, in the present work, seems to confirm these mechanisms. In stem cell-treated animals (Group III), significant difference was found when compared to control group (Group I). Furthermore, the CD31 immunoreaction was more intense in the combined therapy group when compared to metformin- or stem cell-treated groups, where the positive reaction was observed in the endothelial cells of the blood capillaries and large number of fibroblasts and macrophages. This observation could support the angiogenic mechanisms and differentiating potential of the applied BM-SCs.

In metformin-treated diabetic rats, significant difference was found when compared to the control group. The wound healing effect of metformin may be related to the formation of new blood capillaries as confirmed by H&E and CD31 immunohistochemistry. This explanation was confirmed by other researchers [9] who suggested that metformin promoted angiogenesis via improving the angiogenic functions of endothelial progenitor cells (EPCs) in diabetic mice. In addition, other authors [38] clarified that the EPCs decreased in number and functions in diabetic patients with subsequent vascular complications and impaired wound healing. Moreover, another mechanism was postulated to explain the wound healing effect of metformin. The insulin tissue resistance had been found to interfere with the healing of excisional skin wounds by delaying wound contraction and surface re-epithelialization, as shown by other investigators [6]. Metformin is a true insulin sensitizer affecting insulin action in peripheral tissues [5], which contributed to the indirect effect of metformin on wound healing.

Interestingly, in another animal model opposite results were reported [39]. The authors reported reduction in the proliferation of HaCaT keratinocytes in culture media in presence of metformin. This was due to an alteration of the cell cycle without induction

of apoptosis, as proven by means of flow cytometry. Furthermore, in a physiological setting, the authors revealed reduced healing process and wound closure progress in metformin-treated ulcers in diabetic patients. They explained their findings by suggestion that metformin interfered with surface re-epithelialization of the wound area by reducing cell proliferation of keratinocytes [39]. The discrepancy between our and their results could be attributed to the different experimental design, methods and dosage of metformin.

The angiogenic response is essential for wound healing. Formation of new blood vessels is necessary to sustain the newly formed granulation tissue and the survival of keratinocytes [40]. In agreement with this notion, the congested blood vessels scattered among the granulation tissue, in the current study, particularly in the combined therapy group confirmed the importance of neovascularization in maintaining the vitality of the newly formed granulation tissue.

Finally, the different mechanisms of wound healing encountered in each group of the single treatment modality can be summarized to synergistically promote better wound healing in the group of combined treatment modality.

In summary, the results of the current study indicated a synergistic effect of both combined BM-MSCs and metformin on skin healing in this animal wound model of STZ-induced diabetes. This combination accelerated wound healing and increased wound closure progress by stimulation of surface re-epithelialization, differentiation, and promoting angiogenesis. The combined approach could be considered as a novel treatment modality for the management of patients with diabetic foot ulcers to enhance wound healing and to decrease the risk of progression of gangrene.

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Conflict of interest

The authors have no conflicts of interest to declare.

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