Effect of Bone Marrow Derived Mesenchymal Stem Cells on Healing of Induced Full-Thickness Skin Wounds in Albino Rat

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Background and Objectives: Mesenchymal stem cells have delivered new approaches to the management of wound healing in severe skin injuries. This work was planned to evaluate the effect of bone marrow-derived mesenchymal stem cells (BMSCs) on healing of induced full thickness skin wounds in albino rats using topical & systemic injections.

Methods and Results: Forty adult male albino rats were classified into 2 groups after induction of full thickness skin wound; untreated group and stem cell-treated group. The latter was further subdivided into topically and systemically treated ones. BMSCs were isolated & labeled by PKH26 before injection. Healing of wounds was evaluated grossly. Skin biopsies were obtained one & three weeks after wound induction. Sections were stained with Hematoxylin & Eosin, Masson's trichrome and immunohistochemical stain for vascular endothelial growth factor (VEGF). Epidermal thicknesses and mean area percent of both collagen fibers & VEGF immunopositive cells were measured using image analyzer & results were subjected to statistical analysis. PKH26 fluorescent-labeled cells were found in the regenerated epidermis, hair follicles and dermis in BMSCs-treated groups. By the end of the third week, the wounds of BMSCs-treated groups showed full regeneration of epidermis, re-organization of collagen and decrease in VEGF immunopositive cells. Delayed wound healing was seen in 20% of systemically treated rats. Significant increase in the mean area percent of collagen fibers was detected in topically treated group.

Conclusions: Both methods of BMSCs injection were effective in healing of full thickness skin wound but topical method was more effective.

Keywords: BMSCs, PKH26, Skin, VEGF, Wound healing

Introduction

Skin wound persists as a health care crisis inspite of increased understanding of the cellular and molecular responses to injury. Contributing significantly to this crisis is the lack of reliable therapies for slowly-healed wounds such as chronic and deep wounds. Even under optimal healing conditions, normal wound repair is imperfect failing to regenerate skin structure and function (1).

The apparent safe and broad application of mesenchymal stem cells (MSCs) to treat various diseases has provided a compelling reason to investigate their potentiality in the treatment of problematic wound and promotion of regeneration rather than scar formation (2). However, the question of the ideal route of administration of MSCs remains one of the many unresolved issues facing efficient clinical application of MSCs therapy. Two main methods of stem cells injection are known; either through direct local injection into the wound or through indirect systemic injection. In the case of direct local injection, there is the advantage of easy technique and precision. However, if the wound is an extensive lesion, the locally injected stem cells may not function properly. On the other hand, in the systemic delivery method only a minor portion of the cells actually arrive at the specific target

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sites (3).

This study was carried out to evaluate the effect of bone marrow derived MSCs (BMSCs) on healing of induced full thickness rat skin wounds using two delivery methods of injection; local (topical) and systemic.

Materials and Methods

Isolation, culture and labeling of MSCs from rat bone marrow (4)

Bone marrow cells were obtained from the long bones of 8 weeks old male albino rat by aspiration. Bones were flushed with Dulbecco's Modified Eagle's medium (DMEM) (Sigma, USA, D5796) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA, F6178). Bone marrow was slowly layered over Ficoll- Hypaque (Sigma, USA, F8016) in a ratio of 2 : 1 in sterile conical tubes and was centrifuged (at 1,200 rpm for 30 minutes at room temperature). The opaque layer containing mononuclear cells was aspirated and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (Sigma, USA, P4333). Cells were incubated at 37°C in 5% humidified CO₂ for 14 days. Media were changed every 3~4 days. When large colonies developed (80~90% confluence), cultures were washed twice with phosphate buffer saline (PBS) (P5493, Sigma, USA) and cells were trypsinized with 0.25% trypsin (Sigma, USA, T1426) in 1 ml ethylene diamine tetra acetate (EDTA) (Sigma, USA, E6758) for 5 minutes at 37°C. After centrifugation (at 2,400 rpm for 20 minutes at room temperature), cell pellets were resuspended with serum-supplemented medium and incubated in 25 cm² culture flasks (Sigma, USA, C6356). The resulting cultures were referred to as first-passage cultures. MSCs in culture were characterized by their plastic adhesiveness and fusiform shape (5).

Immunophenotyping of separated cells: (a) 100 ml of the cell suspension was used for immunophenotyping using flow cytometry (Accuri, USA). The MSCs are positive for CD29 (Sigma, USA, SAB 4501582) and negative for CD45 (Sigma, USA, OX-1 84112004) (6).

(b) Gating: Using side scatter (to detect the cellular granularity) and forward scatter (to detect the cell size), the target cell population was determined.

(c) Reading: The percentage of cells positive for CD29 was counted out of the CD45 negative population.

To track the migration and homing of MSCs at the site of wound healing, cultured cells were labeled with fluorescent cell tracer PKH26 (Sigma, USA, MINI26) according to manufacturer's (Sigma) instructions. Labeled Cells appeared as red fluorescence that can be detected with flu-

orescent microscope (7).

Experimental design

Forty adult male albino rats (150~180 g) were housed in Kasr ElAiny Animal House, Faculty of Medicine, Cairo University according to the guide for the care and use of laboratory animals.

1-Wound creation (8): The animals were anaesthetized using chloroform inhalation (Sigma-Aldrich, 611778). Skin was disinfected by absolute alcohol followed by epilation of hair. By using a clean transparency sheet template and a permanent marker, the vertices of the experimental wounds (1×1 cm dimensions) were outlined on the right dorsal side of all groups. Full-thickness skin specimen including subcutaneous tissue was excised with a #11 BP blade in each animal (Fig. 1). Haemorrhage, if any, was controlled by dapplying pressure with sterile cotton gauze. The rats were then divided into two groups:

Group I (non treated group): twenty wounded rats that received 0.5 ml phosphate buffered saline (PBS) (P5493, Sigma, USA) injections only without MSCs. Half of them received topical injections and the other half received systemic injections.

Group II (MSCs-treated group): including twenty wounded rats that were subdivided according to the method of MSCs injection into two groups ten rats each: Topically treated rats: each rat received a single intradermal injection of MSCs (2.0×10^6) diluted in 0.5 ml of PBS that was injected into 8 sites in the wound margins & systemically treated rats: each rat received one systemic injection (through the caudal vein) of MSCs (2.0×10^6) diluted in 0.5 ml of PBS.

Fig. 1. Wound creation (the dimensions of the wound measured 1×1 cm). Wound expands to variable extents after surgery due to the loose nature of rats' skin.

Penicillin (5 mg/kg intramuscular) and anti-inflammatory Meloxicam (0.2 mg/kg intramuscular) were administered for three consecutive days after wound induction. The exposed skin was covered by Tegaderm (3M™ Tegaderm™ Non-Adherent Contact Layer 5642, USA) that was maintained for more than 24 hours in order to preserve tissue fluid. The animals were housed individually to avoid damage to the wounds. The wounds were photographed at days 0, 3, 7, 14, and 21 with the rats in a standard prone position by using a 4 mega pixels digital camera (Samsung).

Gross evaluation of wound healing

Evaluation of wound healing was done by using the following parameters:

Wound size: Wound contraction was calculated on 3rd, 7th, 14th and 21st postoperative days as a percentage reduction in wound area. Progressive decrease in the wound area was monitored periodically by tracing the wound margin on a tracing paper. The tracing is then placed onto graph paper and the number of squares counted. The total open wound area at each tracing was subtracted from that of the initial tracing to determine the area of contraction and re-epithelization during the period since wounding (9). The percentage of wound closure was calculated as: (area of original wound-area of actual wound)/area of original wound $\times 100$ (10).

Granulation tissue formation: Granulation tissue evaluation was performed daily up to day 7 and then on days 14 and 21 after surgery and categorized as the following score:

1. No granulation tissue seen.
2. Granulation tissue depressed below the skin edge.
3. Granulation tissue proliferated to the level of skin edges.
4. Granulation tissue elevated above skin edges.
5. Granulation tissue elevated above skin edges, projecting over the advancing border of epithelium.

A granulation score of 3 was considered better than 4 or 2, which were considered better than 5 or 1 (8).

Time of complete healing: Time of complete healing was recorded as the day on which wound healed completely. Healing was considered complete when hairy skin covered the entire wound and the area of the remaining granulation tissue was zero. The duration required for complete wound healing was recorded in each animal and the mean duration needed to complete healing was calculated for all studied groups (8).

Microscopic evaluation of wound healing

Animals were re-anaesthetized using chloroform in-

halation and full-thickness skin specimens, including about 0.5 cm around the healing wound (served as control skin for its group) were taken one week (from one half of the animals of each group) and three weeks (from the other half) after wound induction. After collections of specimens, wounds were repaired by suturing and, these animals were excluded from the study.

The skin specimens were flattened, fixed in 10% buffered formalin solution for 24~48 hours, dehydrated in ascending grades of ethanol and embedded in paraffin. Serial sections of 7 μ m thickness were cut and subjected to the followings:

- Fluorescence detection (to detect PKH26-labeled MSCs) by fluorescent microscope in unstained paraffin sections (11).
- Hematoxylin & eosin (H&E) stain for histological evaluation (12).
- Masson's trichrome stain for collagen fibres (13).
- Immunohistochemical staining for vascular endothelial growth factor (VEGF) (Lab Vision, USA, RB-9031-R7) (14).

Morphometric measurements: Data were obtained using "Leica Qwin 500 C" image analyzer computer system Ltd. (Cambridge, England). The epidermal thickness, mean area percent of collagen fibers and mean area percent of VEGF immunopositive cells were detected for the healing skin as well as the control skin. All measurements were detected in 10 randomly chosen non overlapping fields for each section.

Statistical methods: Data were tabulated and statistically analyzed to evaluate the difference between the groups under study as regards the various parameters. The mean, standard deviation and analysis of variance (ANOVA) were calculated using EXCEL and SPSS 9.0 Software. Results were considered statistically significant when p was < 0.05 (15).

Results

Characteristics of MSCs in culture

MSCs in culture had fibroblast-like morphology and they adhered to the tissue culture substrate within 24~48 h. They reached confluence within 7~14 days (Fig. 2).

The present work demonstrated no gross or microscopical differences between topically and systemically injected BPS wounds in non-treated group. So, their results were expressed together as the results of non-treated group.

Gross evaluation of wound healing

Postoperatively, the animals appeared comfortable as

evidenced by normal appetite and behaviour. All rats showed no infection or mortality throughout the experimental period.

Wound size: In all groups, wounds had an area that exceeded 1.5 cm² by the 3rd day. In non treated group, the wound area decreased slowly from 7th day onwards, however complete closure couldn't be detected in any rat till the end of experiment. In topically treated rats, the wound area decreased from seventh day onwards in all rats with the most rapid rate of wound healing (40.2% reduction after 14 days and 100% reduction after 21 days). By the end of the third week, the wounds were completely closed in all rats. In Systemically treated rats, the wound area decreased from seventh day onwards in all rats with a relatively rapid rate compared to non- treated group. By the end of the third week, the wounds were completely closed in 4 out of 5 rats (Table 1).

Granulation tissue formation: In non treated group, by the 3rd day, the wound was slightly covered at the edges by scab with no granulation tissue (score 1). By the 7th day, the wound was almost completely covered by scab while granulation tissue couldn't be detected grossly (score 1). By the 14th day, dark slightly dry granulation tissue

covered the wound edges and extended toward the centre (score 3). By day 21st the peripheral part of wound was covered by scar tissue with no hair. In topically & systemically treated rats, the wound was completely covered by thick scab by the 3rd day. By the 5th day, healthy granulation tissue in the form of granular red wet and soft tissue covered the wound edges and extended toward the centre, its level was below the skin edges (score 2). On the 7th day, the healthy granulation tissue proliferated to the same level of skin edges; covered the wound completely (score 3). Sero-sanguinous discharge could be detected at and around the wound. From the 7th day onwards, skin started to cover the granulation tissue (Fig. 3).

Time of complete healing: None of the wounds healed completely by the 14th day of surgery in all experimental groups. By the 21st day after wounding, complete healing couldn't be achieved in any rat in non treated group. Complete healing with complete regaining of hair was achieved in all animals of topically treated rats & in 4 out of 5 rats of systemically treated group.

Microscopical results

Fluorescence detection: Examination of unstained paraffin sections using fluorescent microscope was performed to detect and track PKH26-labeled MSCs. In non-treated group, fluorescence could not be detected in any section. In both topically & systemically treated groups, PKH26-labeled MSCs appeared as red fluorescent cells after one week within the proliferated granulation tissue and after three weeks in regenerated epidermis, hair follicles and dermis (Fig. 4).

Hematoxylin and eosin stained skin sections: Histological examination of skin samples in non treated group with H&E staining revealed ulceration with failure of crust formation, few blood vessels, mild inflammatory cell infiltrate and failure of re-epithelialization after one week. After three weeks, the wound site was almost completely covered by thick regenerated epidermis, however, skin appendages failed to regenerate completely. The underlying healed dermis showed persistence of inflammation, numerous blood vessels, and many fibroblasts. Examination

Fig. 2. Confluent rat MSCs isolated after 14 days showing fibroblastic morphology in the form of spindle shaped cells (inverted microscope $\times 400$).

Table 1. Mean values of wound area (cm²) and percent of reduction (in parentheses) in excisional wounds of rats

Group	Wound area in post wounding days			
	3rd day	7th day	14th day	21st day
Non treated group	2.44 $\pm$ 0.28 (–144.2%)	1.47 $\pm$ 0.37 (–47.3%)	0.88 $\pm$ 0.18 (12.1%)	0.5 $\pm$ 0.01 (50%)
Topically treated rats	1.8 $\pm$ 0.40 (–80.4%)	1 $\pm$ 0.46 (–0.46%)	0.6 $\pm$ 0.23 (40.2%)	0 $\pm$ 0.00 (100%)
Systemically treated rats	1.9 $\pm$ 0.27 (–90.2%)	1.2 $\pm$ 0.16 (–20.1%)	0.7 $\pm$ 0.18 (30.1%)	0.04 $\pm$ 0.13 (96%)

Fig. 3. Photographs showing the wound area in all groups (non-treated, topically treated & systemically treated) at 3rd, 7th, 14th, 21st days. A rat from non-treated group at 3rd day shows expanded wound area that is partially covered at the edges by scab. At 7th day, scab covers most of it & granulation tissue cannot be detected (score 1). At 14th day; the central part is covered by remnants of scab while peripheral areas are covered by dark, slightly dry granulation tissue (score 3). At 21st day, the wound area is decreased, the central part is covered by granulation tissue and the peripheral part is covered by scar tissue with no hair. In topically & systemically treated group at 3rd day, the wound is completely covered by thick scab. At 7th day, red wet granulation tissue covers the wound edges and extends toward the centre proliferating to the level of skin edges (score 3) with sero-sanguinous discharge. At 14th day; the wound area is greatly decreased, the small central part is still covered by remnants of granulation tissue and the peripheral part is covered by skin. At 21st day, complete wound healing with hairy skin can be detected. Delayed healing is seen in small parts of the wound (arrows) in systemically treated group.

of skin samples in both topically & systemically treated groups, after one week revealed early development of crust, evident inflammatory infiltrate, wide highly cellular granulation tissue, evident neovascularisation and early growth of the epidermis over the edges of the ulcer. After three weeks, the epidermis regained its normal thickness and showed mature differentiation with well recovered skin appendages. The regenerated dermis showed early resolution of inflammation and few blood vessels (Fig. 5). Delayed healed wound with inflammatory cell infiltrate, few blood vessels and absent hair follicles and sebaceous gland were seen only in 20% of systemically treated group.

Masson's trichrome stained skin sections: Non treated group showed scanty and irregularly arranged collagen bundles deposition after one week. After 3 weeks, deposi-

tion of homogenous amorphous intercellular substance and few collagen fibers aligned horizontally in one direction i.e. failure of collagen remodeling was noticed. Examination of skin samples in both topically & systemically treated groups, after one week revealed early deposition of newly formed thin collagen fibers. Dense arrangement (remodeling) of thick collagen fibers in different directions in a network like manner was noticed after 3 weeks in both groups (Fig. 6). Delayed healed wound with dense but thinner collagen bundles was seen only in 20% of systemically treated group (Fig. 7).

Anti-VEGF stained skin sections: Non treated group showed mild immunoreactivity in perivascular mononuclear cells, fibroblastic cells and endothelial lining of blood vessels after 1 week. After three weeks, moderate im-

Fig. 4. Photomicrograph of a section in the skin defect area of (A) topically treated rat & (C) systemically treated rat - 1 week duration, showing PKH26-labeled MSCs which appears as red fluorescent cells "thin arrows" within the proliferated granulation tissue and new blood vessels "V" in the ulcer defect. (B) Topically treated rat & (D) systemically treated rat - 3 weeks duration showing red fluorescent cells "blue arrows" within the regenerated epidermis "E", dermis, blood vessels and hair follicles "thick arrow" (PKH26, $\times 100$).

munoreactivity in the endothelial lining of blood vessels within the regenerating scar was noticed (Fig. 8). Examination of skin samples in both topically & systemically treated groups, after one week revealed widespread dense immunoreactivity at the endothelial lining of blood vessels all through the dermis and was marked mostly in the upper part close to resolved crust, perivascular mononuclear cells and fibroblastic cells also showed dense positive immunoreactivity. After 3 weeks mild localized immunoreactivity at the endothelial lining of blood vessels and fibroblastic cells within the regenerated dermis was noticed in both groups (Fig. 9).

Morphometric results

Epidermal thickness: The present study demonstrated

statistically non significant differences in epidermal thickness between non treated, topically treated and systemically treated groups after 1& 3 weeks ($p > 0.05$) (Fig. 10).

Mean area percent of collagen fibers: After 1 week, there was statistically significant difference between (topically treated and systemically treated rats) and non treated group ($p < 0.05$). However the difference between topically treated and systemically treated was statistically non significant ($p > 0.05$). After 3 weeks, the topically treated group showed significant increase ($p < 0.05$) in the area percent of collagen fibers as compared with non treated and systemically treated groups (Fig. 11).

Mean area percent of VEGF immunopositive cells: After 1 week, there was statistically significant difference between systemically treated and non treated ($p < 0.05$).

Fig. 5. Photomicrographs of a skin section showing the junction between the normal skin & the wound area (arrow). Apparently normal epidermis "E" and dermis "D" with many hair follicles "F" and sebaceous gland "S" are seen in the adjacent control skin. (A) Non treated rat - 1 week duration. The wound is showing complete absence of the epidermis, skin appendages, papillary dermis and most of reticular dermis. After 3 weeks (B), the wound is covered by thick fully regenerated epidermis. The regenerated dermis shows numerous congested blood vessels "V" with absence of hair follicles and sebaceous glands. (C) Topically treated rat & (E) systemically treated rat - 1 week duration, the wound is filled with highly cellular granulation tissue "GT" with numerous congested blood vessels. The adjacent epidermis is encroaching at the edge of the wound under the formed crust. (D) Topically treated rat & (F) systemically treated rat - 3 weeks duration, The wound area is hardly demarcated from the adjacent control skin. The wound area shows complete regeneration of the epidermis & dermis. Many hair follicles and sebaceous glands are seen mainly within the hypodermis "H". Panniculus muscle "M" is seen in the lower part (H&E, $\times 40$).

However the difference between topically treated and systemically treated was statistically non significant ($p > 0.05$). Also the difference between non treated, topically treated was statistically non significant. After 3 weeks, topically treated group showed significant decrease in immunopositive cells ($p < 0.05$) as compared with non treated group (Fig. 12).

Discussion

In this study, an attempt was done to find out the histo-

logical effects of BMSCs on healing of induced full thickness skin wound in adult rat using the main delivery methods of injection; topical and systemic.

The choice of MSCs was attributed to its reported advantages. MSCs are easily isolated from a variety of tissues, they expand rapidly in cell culture, they are multipotent, they seem to be largely immunologically inert, paving the way for allogeneic transplantations, they form supportive stroma for hematopoiesis and support hematopoietic stem cell engraftment and lastly, they secrete numerous trophic factors which modulate inflammation, re-

Fig. 6. Photomicrographs of a skin section showing the junction between the normal skin & the wound area (arrow). Apparently normal epidermis "E" and dermis "D" with many hair follicles "F" and sebaceous gland "S" are seen in the adjacent control skin. (A) Non treated rat - 1 week duration, the wound shows scanty and irregularly arranged blue collagen bundles in the regenerated reticular dermis. After 3 weeks (B), the wound is filled with densely arranged blue stained collagen fibers aligned horizontally in one direction. (C) Topically treated rat & (E) systemically treated rat - 1 week duration, the wound shows prominent deposition of thin light blue collagen fibers within the regenerated granulation tissues. (D) Topically treated rat & (F) systemically treated rat - 3 weeks duration, The wound area shows dense thick collagen bundles arranged in different directions in a network like manner. Hypodermis "H". Panculus muscle "M" is seen in the lower part (Masson's trichrome, $\times 40$).

modeling, and apoptosis (16).

PKH26 was chosen to label MSCs according to previous study (17). The choice of PKH26 in this study was attributed to its reported advantages as PKH26 is rapid, simple labeling procedure, achieves bright staining without altering protein and cell function and is physiologically stable for up to 4 months in vivo (18-20).

In this study, a full-thickness skin wound was created by using blade, transparency sheet template and a permanent marker to create fixed wound size of 1×1 cm dimensions on right dorsal side of all groups. This specific wounding technique as described by Borena et al. (8) was chosen as it was accurate and easy to perform.

In the present work, there were no apparent gross or histological differences between rats injected topically or systemically with PBS in non treated group, so they were considered and discussed as if they are the same. This was decided during the course of the study.

The present work demonstrated better and faster healing of BMSCs-treated wound compared with non treated group. These findings could be attributed to the injected BMSCs. This explanation was further supported by detection of red fluorescent PKH26 labeled MSCs within the regenerated wound of treated groups while no fluorescence could be detected in any section of non treated group.

In topically treated group, injection of BMSCs in

wound might have caused some trauma and inflammation leading to release of cytokines, which may affect wound healing. However, the injection of BMSCs was made slowly using a fine hypodermic needle to minimize the trauma. Furthermore, the injection induced trauma was considered negligible compared with the surgical trauma inflicted in creation of wound and thus may be of little significance (8).

In the present study, gross evaluation of BMSCs-treated wounds revealed early and fast wound contraction and

Fig. 7. Photomicrograph of a skin section from systemically treated rat, 3 weeks duration at junction between healed wound and delayed healing wound area (red arrow). Delayed healing wound shows dense collagen bundles formed of thin fibres; most of them are arranged in parallel direction with marked increase in the dermal thickness (D). The upper surface is covered with normal epidermis (E). Many hair follicles (F) and sebaceous glands (S) are seen at the adjacent healed wound (Masson's trichrome, $\times 40$).

early closure of wound, early crusting, and early deposition of healthy granulation tissue consequently resulting in complete healing and covering of wound with regenerated hairy skin by the end of the third week. On the other hand, delayed crusting, deposition of unhealthy granulation tissue and delayed closure of the wound with failure of hair regaining were detected in non treated wounds. The better and faster wound healing can be explained by the capacity of BMSCs to secrete growth factors and cytokines and to their ability to trans-differentiate into the healing tissue components (2, 21).

In the present work, better wound contraction might be attributed to the possible action of BMSCs that could differentiate into myofibroblasts and fibroblasts. It was also suggested that BMSCs could recruit more fibroblasts from the surrounding tissues through chemotaxis (22-24).

In this study, in addition to gross evaluation of wound healing, biopsies were collected for light microscope examination at two stages of wound healing on 7th and 21st post-operative days. In addition, skin around the induced wound was used as control skin to avoid variability in skin thickness between different rats. This could provide more precise evaluation of the healing procedure.

In the present work, healthy granulation tissue appeared faster in BMSCs-treated wounds than in non treated wounds. This gross observation was further supported by the histopathological findings of early deposition of relatively wider granulation tissue with more cellularity in BMSCs-treated wounds after 1 week compared with non treated wounds. These results were in agreement with earlier studies (21, 25, 26) and might be related to the role of BMSCs in the acceleration of granulation tissue formation that is extremely resistant to infection, provides a surface over which epithelium is able to migrate and contains fibroblasts that produce collagen for wound healing (24).

Fig. 8. Photomicrograph of a skin section from (A) non treated group - 1 week duration showing moderate localized immunoreactivity in the perivascular mononuclear cells (arrowheads), fibroblastic cells (arrows) and endothelial cells of blood vessels "V". After 3 weeks (B), moderate localized immunoreactivity is seen in some endothelial cells of dermal vessels (immunohistochemical stain for VEGF, $\times 400$).

Fig. 9. Photomicrograph of a skin section from (A) topically treated rat & (C) systemically treated rat - 1 week duration, showing widespread dense immunoreactivity in perivascular mononuclear cells (arrowheads), fibroblastic cells (arrows) and endothelial cells of blood vessels. (B) Topically treated rat & (D) systemically treated rat - 3 weeks duration, showing localized mild immunoreactivity in fibroblastic cells and endothelial cells of blood vessels "V" (immunohistochemical stain for VEGF, $\times 400$).

In this study, histopathological evaluation of wound from BMSCs- treated groups after 1 week revealed early development of crust, evident inflammatory infiltrate, wider and more cellular granulation tissue, evident neo-vascularisation, earlier deposition of newly formed collagen and enchoachment of adjacent epidermis over wound edges than in non treated wounds. These findings progressed after 3 weeks, so that in BMSCs-treated wounds, the epidermis regained its normal thickness and showed mature differentiation with well recovered skin appendages. The regenerated dermis showed earlier resolution of inflammation, few blood vessels, few fibroblasts, deposition of denser, thicker and well organized collagen fibres indicating successful skin regeneration.

On the other hand, in non treated wounds, the epidermis succeeded to regain its thickness while skin appen-

dages failed to develop. The healed dermis showed persistence of inflammation, numerous blood vessels, many fibroblasts, thick parallel poorly organized collagen fibres indicating healing with scar formation. The previous findings were in agreement with other studies (3, 21, 26).

In the present study early disappearance of inflammation in BMSCs-treated wounds was observed. This might facilitate earlier progress to the next phases of wound healing as reduced inflammation promotes regeneration rather than scarring (22). This notion might explain wound regeneration in BMSCs-treated groups and scar formation in non treated group.

In the present work, evaluation of wound from BMSCs-treated groups after one week revealed numerous blood vessels in H&E stained sections. This finding was further supported by the higher area percent of immunopositive

Fig. 10. The mean values of epidermal thickness (in micrometers) in the experimental animal groups. *Significant (S) ($p < 0.05$) as compared to 1 week-duration of its group.

Fig. 11. The mean values of area percent of collagen fibers in the experimental groups. *Significant (S) ($p < 0.05$) as compared to control skin. **Significant (S) ($p < 0.05$) as compared non treated group (1 week-duration). ***Significant (S) ($p < 0.05$) as compared to non treated group (3 weeks duration) and systemically treated group (3 weeks duration).

VEGF in early phases of wound healing (after 1 week) that decreased later on after three weeks. These findings might be attributed to the role of BMSCs in neovascularisation to sustain the newly formed granulation tissue (8, 24, 26).

BMSCs could differentiate into endothelial cells and pericytes (27). In the present work, this assumption might be supported by detection of PKH26 labeled MSCs within & around the regenerated blood vessels in the regenerated dermis of MSCs- treated groups and negative detection of fluorescence in any section of non treated group. Similar

Fig. 12. The mean values of area percent of immunopositive cells in the experimental groups. *Significant (S) ($p < 0.05$) as compared to control skin. **Significant (S) ($p < 0.05$) as compared to control skin and non treated group (1 week-duration). ***Significant (S) ($p < 0.05$) as compared to non treated group (3 weeks duration).

findings were reported by other investigators (28, 29) in their work on mouse skin wounds. Another mechanism by which BMSCs could enhance neovascularisation is through the release of pro-angiogenic factors via paracrine mechanism to recruit endothelial lineage cells into wound area (30-32).

In the present work, after one week in BMSCs- treated groups, VEGF immunopositive reaction was found in perivascular mononuclear cells, fibroblastic cells and endothelial lining of blood vessels that were consistent with Sködl et al. (33). The VEGF positive reaction could be related to increased VEGF secretion by these cells during wound healing (34). Another explanation might be attributed to activated platelets that release VEGF, particularly after thrombin stimulation (35).

In the present work, neovascularization and the area percent of VEGF decreased after three weeks in BMSCs-treated groups which run in accordance with other investigators (24, 36). The increased vascularity and area percent of VEGF could be explained by up regulation of tissue expression of VEGF and its receptors in response to ischemia and hypoxia produced by tissue damage. VEGF in turn promotes capillary growth and differentiation during the proliferative phase of repair occurring approximately 3 to 7 days post-wounding. The influence of VEGF diminishes when inducers of VEGF like hypoxia decrease. Accordingly, VEGF and blood vessels decline again after wound healing (after 3 weeks) (35, 37).

Regarding the non treated group, the current study

showed persistent increase in neovascularization and in the area percent of VEGF after three weeks. The same findings were also detected in delayed healing wound of systemically treated group. These findings might be explained by persistence of VEGF inducers due to delayed healing (35, 37).

In the present study, more fibroblasts were detected until the end of the 1st week after wound creation then they decreased later on. Better collagen formation throughout the observation period was recorded in the BMSCs-treated groups compared with the non-treated group. These findings were in agreement with Fossum et al. (38) who found that BMSCs are able to transcribe both collagen types I and III.

In the present work, after one week in BMSCs- treated wound, the newly deposited collagen was relatively thin and oriented parallel to the skin surface. After three weeks, it became thicker and was arranged in different directions in a network like manner that mimic control skin. These findings might be attributed to the initial deposition of thin collagen fibrils (type III) that were later resorbed and replaced with thicker fibrils (type I) aligned with stress lines indicating maturity of wound (22, 25, 26). Eventually, in delayed healing of wounds in systemically treated group after three weeks, collagen fibres were still relatively thin and oriented parallel to the skin indicating delayed healing.

Re-epithelialization is widely accepted to be one of the major processes in wound healing that ensures successful repair. In the present work, after 1 week in BMSCs- treated groups, keratinocytes at the wound edges migrated across the wound gap over the newly formed granulation tissue but failed to do the same in non treated group. After 3 weeks in BMSCs- treated groups, the epidermis regained its normal thickness and showed full differentiation with well recovered skin appendages. On the other hand, in non treated group, thick epidermis succeeded to regenerate while skin appendages failed. These findings might be attributed to the role of BMSCs in promotion of re-epithelialization and hair regeneration (28).

BMSCs may trans-differentiate into epidermal keratinocytes and fully differentiated skin with hair (28). This assumption has gained evidence in the present work by detection of PKH26 labeled cells within the regenerated epidermis and hair follicles of MSCs-treated groups while no fluorescence was detected in any section of non treated group. In addition, BMSCs might enhance proliferation of resident epidermal cells in the presence of epidermal growth factor, possibly produced by bone marrow cells through a paracrine mechanism (39).

On comparing the histological effects of both topical and systemic delivery methods of BMSCs on healing of induced full thickness rat skin wound in the present study, both methods were found to enhance healing. However topical method was more effective as delayed healing was detected in 20% of systemically treated rats at the end of the third week. Similar results were observed by other researchers (3) using human cord blood derived mesenchymal stem cells for healing wounds of diabetic mouse model using topical & systemic injection.

The systemic delivery method mimics the route of endogenous MSCs via the circulatory system with final homing to target sites. During vascular transit, MSCs carry risk of being taken out of circulation, in organs such as the lungs, spleen, and liver. This may either delay their transit or reduce the numbers of cells that finally reach the specific target which might explain the efficiency of local injection compared with the systemic one (3, 40). In conclusion, both methods of BMSCs injection were effective in healing of full thickness skin wound but topical method was statistically more effective.

Potential conflict of interest

The authors have no conflicting financial interest.

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