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ORIGINAL ARTICLE

Evaluation of Potential Effect of Bone Marrow Mast Cells on Burn Wound Healing in Rat

Shima Mousazadeh[1], Amir Amniattalab ✉[1], Rahim Mohammadi[2]

[1] Department of Pathology, Faculty of Veterinary Medicine, Urmia Branch, Islamic Azad University, Urmia, Iran. [2] Department of Surgery and Diagnostic Imaging, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

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ABSTRACT

The objective of this work was to assess the potential effect of bone marrow mast cells (BMMCs) on burn wound healing in rats. We included 45 male rats into three groups of 15 animals each: CNTRL group: We treated the burn wounds with normal saline (0.1 ml). TRTMENT group: In this group, the burn wounds were treated with bone marrow mast cells (1×10^6/ml). SSD (Positive control) group: In this group, the wounds were treated with silver sulfadiazine 1% ointment. Wound area reduction measurements, histopathological studies, and biochemical assessments levels showed significant differences between rats in TRTMENT group in comparison with other groups ($p < 0.05$). Accelerated repair of the wounds in TRTMENT group showed that local application of BMMCs could be taken into consideration in burn wound healing.

Introduction

Burn injuries are under-appreciated injuries that are associated with substantial morbidity and mortality.[1] Burn injuries, particularly severe burns, are accompanied by an immune and inflammatory response, metabolic changes and distributive shock that can be challenging to manage and can lead to multiple organ failure. Of great importance is that the injury affects not only the physical health, but also the mental health and quality of life of the patient. Accordingly, patients with burn injury cannot be considered recovered when the wounds have healed; instead, burn injury leads to long-term profound alterations that must be addressed to optimize quality of life. Burn care providers are, therefore, faced with a plethora of challenges including acute and critical care management, long-term care and rehabilitation. The aim of this Primer is not only to give an overview and update about burn care, but also to raise awareness of the ongoing challenges and stigmata associated with burn injuries.[1] To accelerate burn wound healing, numerous medical managements have been developed and adopted.[2]

Burns are caused by several mechanisms including flame, scald, chemical, electrical, and ionizing and non-ionizing radiation. Recently, tissue engineering and regenerative medicine strategies have been developed to improve skin regeneration that can restore normal skin physiology and limit adverse outcomes, such as infection, delayed re-epithelialization, and scarring.[2]

Grafts have been used for burnt patients for three decades. Although associated with dramatic survival improvement,[3] they have two major drawbacks: Fragility and poor cosmetic quality of healed zones, mostly due to their lack of underlying dermis, which must be provided by prior skin allografts,[4] and by immaturity of the resulting dermal–epidermal junction.[5] Cellular-based therapies can be applied throughout the continuum of burn care by targeting the stages of wound healing: hemostasis, inflammation, cell

✉ Corresponding author. Email: amir.amniattalab@iau.ac.ir

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proliferation, and matrix remodeling.[2]

Cell therapy is therapeutic administration of living cells aimed at tissue regeneration, support for any defective function (such as wound healing) or modulation of pathophysiological processes (such as hyperinflammation or immune dysfunction). This definition extends to tissue engineering involving cell-containing biomaterials.[3]

Mast cells are fascinating, multifunctional, bone marrow-derived, tissue dwelling cells. They can be activated to degranulate in minutes, not only by IgE and antigen signaling via the high affinity receptor for IgE, but also by a diverse group of stimuli. These cells can release a wide variety of immune mediators, including an expanding list of cytokines, chemokines, and growth factors.[4] Mast cells have an armamentarium of inflammatory mediators interleukins such as IL-6 and IL-8, and growth factors, such as vascular endothelial growth factor, platelet derived growth factor and proteases that are released in degranulation.[6] As a result of extra cellular matrix degradation and changes in the microenvironment following initial mast cell secretion, the mast cell populations may change significantly in number, phenotype and function. There is, moreover, strong evidence that mast cells significantly influence angiogenesis.[7,8] These characteristics of the mast cells has encouraged us to conduct a study to assess local mast cell therapy in site of transection of sciatic nerve to observe whether the cells could be of benefit in peripheral nerve regeneration. The aim of the present preliminary study was to assess effect of a single local transplantation of bone marrow-derived mast cells on burn wound healing in rat.

Materials and Methods

Pokeweed Mitogen-Stimulated Spleen Cell Conditioned Medium (PWM-SCM)

Spleen cells from a donor rat were cultured at a density of 2 × 10^6 cells/ml in RPMI 1640 medium containing 4 Mm L-gluta-mine, 5 ×10^5 M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nones-sential amino acids (complete RPMI1640) containing lectin (8 mg/ml) and placed in 75-cm^2 tissue culture flasks. The cells were incubated at 37 °C in a 5% CO2 humidified atmosphere. After 5–7 days, medium was collected, centrifuged for 15 min at 3200 g, filtered through a 0.22 mm millipore filter and used as PWM-SCM.

Preparation of the Bone Marrow Derived Mast Cells (BMMCs)

Bone marrow of a donor male rat was used to generate mast cells based on a method described by others.[9] Briefly, the animal was anesthetized, euthanized (see above) and intact femurs were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe. The suspension of bone marrow cells was centrifuged at 320 g for 10 min, and cultured at a concentration of 5 ×10^5 nucleated cells/ ml in RPMI 1640 with 10% FCS, 100 units/ml penicillin, 100 mg/ml streptomycin (Life technology), 10 mg/ml gentamycin, 2 mM L-glutamine and 0.1 mM nonessential amino acids (referred to as enriched medium). PWM-SCM 20% (v/v) was added to the enriched medium. Flasks were then incubated at 37 ° C in a 5% CO2 humidified atmosphere. Non-adherent cells were transferred to fresh medium at least once a week. After 3–4 weeks, mast cell purity of > 90% was achieved as assessed by flow cytometry.

Ethical Considerations

The procedures of this work were approved by the University Ethical Committee and filed at IR.IAU.URMIA.REC.1402.081 code. We followed instructions of National Academy of Sciences Publication with number of 85-23 that was revised in 1985.

Animals and Study Design

We included 45 male rats into three groups of 15 animals each: CNTRL group: We treated the burn wounds with normal saline (0.1 ml). TRTMENT group: In this group, the burn wounds were treated with bone marrow mast cells (1 × 10^6/ml). SSD (Positive control) group: In this group, the wounds were with silver sulfadiazine 1% ointment.

Animal housing was in standard conditions of temperature (22 ± 3 °C), humidity (60 ± 5%), and a 12-hour light/dark cycle. They were fed on standard pellet diet and tap water. On the final day of the sampling, the rats were euthanized through intraperitoneal administration of a ketamine-xylazine combination. Euthanasia was carried out using a dosage five times more than that of the anesthesia induction dose

The Procedures for Burn Wound Creation and local administration of the cells

General anesthesia was performed by intraperitoneal injection of ketamine 10% (60 mg/kg) and xylazine 2% (5 mg/kg). Afterward dorsal surface of the animals was shaved and prepared for burn creation. Burn wounding process was equal among all animals. They were placed on the plate of the device in ventral recumbence position and full thickness burn wound was created on dorsal lumbar surface 2 cm in diameter within a 10-second interval at 95 °C. The animals were under surveillance daily in the term of general health, appetite, weight, depression, and inflammation of the wound area. The cells were administered locally. One mL cell suspension was subcutaneously injected at four sites around the wound bed using a syringe, maintaining a distance of 3 mm from the rim. The injection was administered at a single dose immediately after wound creation (day 0).

Excision Wound Model and Planimetric Studies

Wound-healing property was evaluated by wound contraction percentage and wound closure time. Photographs were taken immediately after wounding and on days 7, 14, and 21 post operation by a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc., San Jose, CA, USA) and wound contraction percentage was calculated using the following formula:

Percentage of wound contraction = $(A0 - At)/A0 \times 100$

where *A0* is the original wound area and *A*t is the wound area at the time of imaging. All rats were closely observed for any infection and if they showed signs of infection were separated, excluded from the study, and replaced.

Histopathological Preparation and Quantitative Morphometric Studies

After wound creation, on days 7, 14, and 21 a tissue sample from edge of the wound as well as normal skin were taken and fixated in an appropriate fixative (10% buffered formalin). The samples underwent routine tissue processing of dehydration and paraffin wax embedding. 5 µm, sections were stained with hematoxylin and eosin (H&E) stains. Using light microscopy, photomicrographs of various stages of the healing processes were scrutinized. Mononuclear cells, polymorphonuclear cells, and fibroblastic aggregation were quantitatively analyzed. Image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics) was adopted to score morphological results. Using a five-step scoring system, the histological parameters were classified based on the intensity of occurrence (−, absence; +, discrete; ++, moderate; +++, intense; and ++++, very intense).[10]

Biochemical Investigations

We followed the methods of others for biochemical investigations.[11] To do this, we took the specimens of wound tissue assessed their enzyme activities. We used a morter to grind the samples. We treated one half gram of each sample with 4.5 mL of an appropriate buffer. Ultra-Turrax homogenizer (IKA Werke) mixture was used to homogenize the samples. Following filtration of the homogenates with use of a refrigerator centrifuge the samples were centrifuged at 4 °C. For assessments of the enzymatic activities at room temperature supernatants were used. The activities of the following biochemical parameters in cluding, total antioxidant activity (TAC), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GPx) were assessed.

Statistical Analysis

We did Kruskal-Wallis variance analysis to analyse differences in groups. Multiple comparison checks were done to get the differences in case the *P* value (from the Kruskal-Wallis test statistics) was statistically significant. To make comparison among days, we did Mann-Whitney U checks. We did the Bonferroni correction to retrieving multiple comparisons, if any. We used SPSS version 11.5 (SPSS Inc) and set *p* value at <0.05 as significant level.

Results

Findings of mast cell staining Bone marrow stromal cells of the mice were successfully harvested and cultured. In the first culture of the cells both adherent and confluent cells were observed that were appeared as heterogeneous cells. Within the first week the adherent cells were observed as confluent cells. In contrary to other common culture media, the confluent cells could live longer. In the second passage, because of limited space in the smaller flasks (T25), the confluent cells were appeared densely and on days 18 and 19 the first culture cells were appeared more homogenous. A few dividing cells were also observed. Following 3 to 4 passages and change of the culture media on day 21, the cells were homogenous enough to be harvested. The harvested cells were counted and their viability was assessed using trypan blue with Neubauer method. From each flask 12,000,000 cells with viability rate of 90% were harvested. After centrifugation, the supernatant was discarded and the pellet was resuspended in a 1 ml culture media and spread on slides. The slides were air dried at room temperature. They were fixed using carnoy and stained using toluidine blue, alcin blue and gimsa stains. The granules of mast cells were purple to red where stained with toluidine blue. These cells were matachromatic. The granules were blue and the nuclei were red where stained with alcin blue and violet where stained with gimsa (Figure 1).

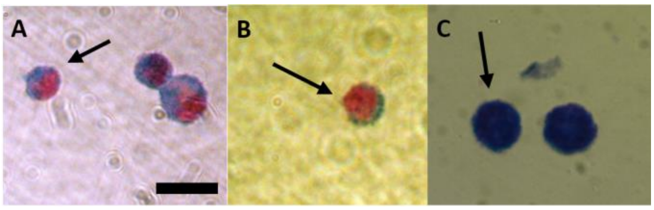

Figure 1. BMMCs (arrows) from rat were cultured in the medium during the third week of culturing bone marrow cells. (A) Gimsa staining, (B) Alcian blue and (C) Toluidine blue. Scale bar: 10 µm.

Reduction in Wound Area

Table 1 shows reduction in wound are during the study period. The rate of healing of wounds in BMMCs treated animals was significantly accelerated in comparison with rate of healing of others ($p < 0.05$).

Biochemical Results

Topical usage of BMMCs ended up significant increase in the mean values of TAC and SOD in TRTMNT group in comparison with other groups ($p < 0.05$). BMMCs significantly diminished MDA level in TRTMNT group in comparison with others ($p < 0.05$). Levels of GPx in TRTMNT animals were significantly augmented in compared to others ($p < 0.05$) (Table 2).

Histology and Morphometric Results

Between TRTMNT and other experimental groups significant differences were found regarding re-epithelialization, new vessel formation, acute hemorrhage, cellular infiltration, congestion, production of collagen and edema. In this work, we observed significantly higher scores in re-epithelialization and new vessel formation in TRTMNT group in comparison with others ($p < 0.05$). Table 3 and Figures 2 to 4 show that mean values for cell count and the qualitative study mean rank of wound score in TRTMNT animals were significantly higher than those of others ($p < 0.05$).

Table 1. Impact of BMMCs on circular excision wound contraction area (mm^2). Values are given as mean ± SEM.

	Wound area (mm^2)		
Groups	**Day 7**	**Day 14**	**Day 21**
CNRTL	261.76 ± 12.97	77.25 ± 8.24	16.14 ± 5.27
SSD	188.71 ± 18.66*	65.65 ± 7.19*	33.18 ± 5.85*
TRTMNT	145.73 ± 11.81**	47.11±5.57**	92 ± 4.36**

The treated groups are compared by Student t test with other groups. .*,**$p < 0.05$ versus other groups.

Table 2. Comparison of the activities of TAC, SOD, MDA and GPx in the animals of the all experimental groups. Data are expressed as Mean ± SD.

Indices	CNTRL	SSD	TRTMNT
TAC	0.57±0.15	0.86±0.09**	1.88±0.21*
SOD (U/mg protein)	0.22±0.07	0.42±0.07**	0.72±0.092*
MDA (nmol/mg protein)	0. 97±0.02	0.52±0.26**	0.34±0.13*
GPx (U/mg protein)	0.14±0.09	0.27±0.04**	0.49±0.08*

The BMMCs treated group is compared by Student t test to other groups. *, **p < 0.05 *vs.* other groups. TAC: Total antioxidant Capacity, SOD: Super oxide dismutase, MDA: Malondialdehyde and GPx: Glutathion peroxidase.

Table 3. The histological parameters classified based on the intensity of occurrence in 5 levels described by others.[10]

Groups	CNTRL			SSD			TRTMNT		
Days	**7**	**14**	**21**	**7**	**14**	**21**	**7**	**14**	**21**
Acute Haemorrhage	+++	++	-	+++	+	+	+*	-	-
Congestion	+++	+	-	+++	+	+	+*	-	-
Epithelialization	-	+	++	+	++	+++	++*	+++*	++++*
Collagen	+	++	++	+	++	+++	++*	+++*	++++*

Classification of histological parameters according to the intensity of occurrence: - absence; + discrete ;++ moderate; +++ intense; ++++ very intense. Histopatological damages were assessed as explained under material and methods on days, 7, 14 and 21 of lesion. *$p < 0.05$ *vs.* SSD and TRTMNT groups.

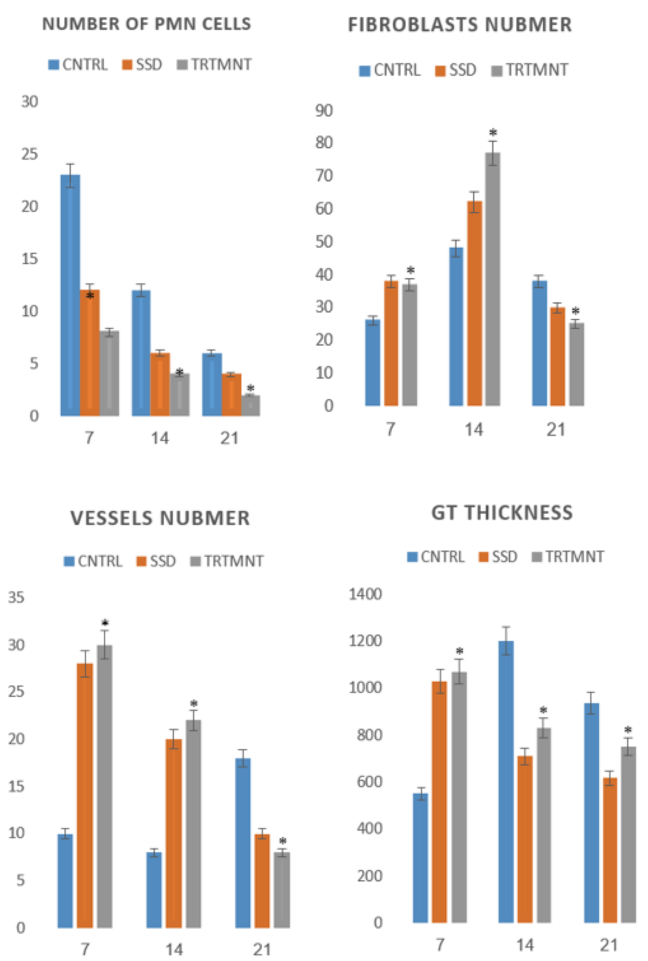

Figure 2. Line graphs show the findings of number of PMN, fibroblasts, vessel numbers and granulation tissue (GT) thickness in studied groups on days 7, 14 and 21 post wounding. Data are presented as mean ± SD. * $p < 0.05$ among studied groups.

Discussion

Depending on the mast cell phenotype and stimulus, mast cells initiate the transcription, translation and secretion of a varied array of cytokines including PDGF, VEGF. It has already been shown that PDGF, VEGF bear beneficial effects on peripheral nerve regeneration.[12-15] Mast cells have been proposed as angiogenesis promoters and the mast cell count appears to be a reliable prognostic marker in some tumors.[16] Mast cells cause

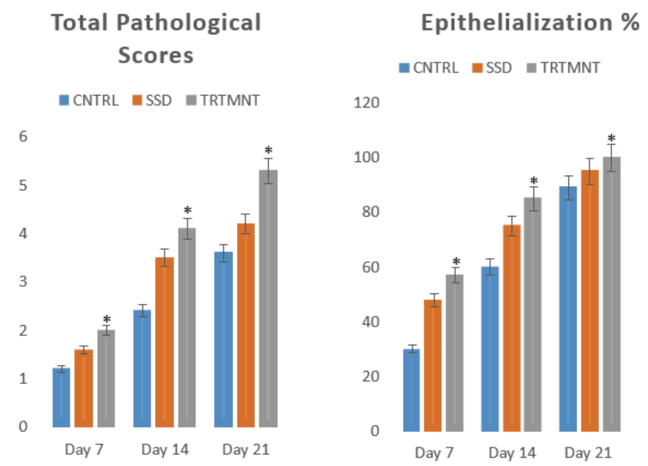

Figure 3. Line graphs show the quantitative results of epithelialization and total pathological scores in experimental groups. Data are presented as mean ± SD. * $p < 0.05$ among experimental groups.

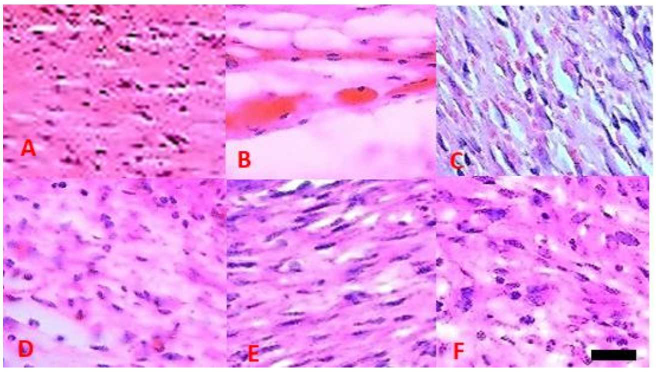

Figure 4. Histological features of the skin on days 7 (A-C) and 14 (D-F) after wound creation in experimental groups. Wounds with surrounding skin were prepared for histological microscopic evaluation by H&E staining. CNTRL (A, D), SSD (B, E), TRTMNT (C, F) groups. Scale bar: 60 µm.

neovascularization by producing angiogenic factors, such as VEGF, or substances with angiogenic properties, such as tryptase, FGF, TNF, interleukin (IL)-8, histamine and heparin. Angiogenesis is a complex process governed by many different variables. Growth factors, including VEGF, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), play important roles.[7] Mast cells play an important role in fibroblast proliferation and wound contraction. Its secretion of tryptase and histamine are keys to fibroblast production of collagen and αSMA contraction. They maintain angiogenesis to supply vital nutrients to repairing cells. Without the functioning of mast cells, wound healing is impaired in animal models.[7]

In this work, effect of bone marrow mast cells (BMMCs) was recorded to be more than silver sulfadiazine. Early epithelialization in BMMCs group prohibited wound from infection and infiltration of microorganisms to the healing tissue. In TRTMENT group, on day 21, the maximum stages of wound contraction were noticed in comparison with other animals representing promising effect of BMMCs in burn wound contraction and closure.

Within the inflammatory stage, numerous radicals are produced as a result of damage.[22] Radicals are frequently connected to oxidative stress, that ends up to peroxidation of lipid and compromised wound repair.[22] Diminished oxidative stress augments the inflammatory reaction, and our results revealed that BMMCs could be able to remove radicals.[23] Therefore, In the present study the biochemical indices were meaningfully improved in BMMCs treated animals in comparison with those of other experimental groups.

Angiogenesis, new blood vessels expansion, is one of primacies in process of healing of wound that could rise blood expansion via biochemical and pharmacological mechanisms and subsequently end up accelerative healing rate.[24]

According to results of the present study, maximum angiogenesis was observed in TRTMENT group. Angiogenesis provide a framework for formation of connective tissue in initial days of the healing. Others represented that by stablishing adequate blood circulation in granulation tissue, endothelial cells immigration and proliferation diminishes and apoptosis of redundant blood vessels ensues.[19]

Acceleration in blood vessel reduction of the groups treated by mast cells showed positive effect of BMMCs in the healing. Fibroblast numbers is considered as is a renowned index for assessment of quality of healing in connective tissue. Occurrence and initial production of fibroblasts in TRTMNT group showed that BMMCs could induce growth of fibroblasts. Formation of extracellular matrix and deposition of collagen by fibroblasts play crucial role in wound healing phases.[25] Production of granulation tissue on initial days of the healing phase is considered as one of vital factors in acceleration of the healing process.[26] The more fibroblast and blood vessel forms, the more granulation tissue develops. In the present study, maximum thickness of granulation tissue of SSD and TRTMNT group were observed on day 7 and Control group on day 14. In TRTMNT group application of BMMCs, granulation tissue was matured and its thickness was decreased on day 7 and the minimum thickness was achieved on day 21. When the study was over, thickness of granulation tissue in CONTROL animals was maximum and compared to BMMCs treated animals significant difference was recorded. This finding was in consistence with the findings of angiogenesis and proliferation of fibroblasts in BMMCs treated animals that were in charge of maturation of new blood vessels and maturation of fibroblast, and subsequent enhancement in healing in TRTMENT group. The first step of wound repair initiates with inflammatory phase that is characterized by emergence of inflammatory cells. Because of association of his phase with cellular events, contraction and closure wound, it is very critical phase in the course of wound healing.[27]

In TRTMENT group, on day 7, lower PMN cell count and higher blood vessels and fibroblast counts demonstrated that the healing process was accelerated using BMMCs. Newly molded epithelium, as a barrier between wound and environment, is characterized by higher number of cells and layers in comparison with the normal one.[28] When the surface of wound is covered by newly produced epidermal cells, differentiation starts, shape of the cells converts to normal and, reorganization and decrease in cell layer take place.[29]

Our findings showed that in BMMCs treated animals, epithelialization was almost complete on day 14 and this was occurred a week sooner than other animals that took place on the last day of the study. This finding indicated that epithelialization was induced via BMMCs treatment. Burn wounds are sterile when they are newly formed, however, because of presence of vascular necrotic tissue and breakage in integrity of epithelial layer, the wound is predisposed to infection and subsequently failure in healing and mortality in complicated cases ensue. Neutrophils in pathological sections are indicative of infection in wound.[30]

Because of dynamic feature of wound healing process, findings of inflammatory, proliferative and remodeling phases are interdependent and for proper conclusion, they should be analyzed altogether. Therefore, histomorphometrical findings of our work were scored from one to five grade. Greater grades were indicative of improved repair. As a result, the greatest grade demonstrated the most appropriate healing activity through enhancing the healing phases. Histopathologic scoring is a tool by which semi-quantitative data can be obtained from tissues. Scoring tissue lesions can be a useful tool for evaluating research tissues and corroborating morphologic findings. Following key principles can guide the pathologist to develop useful and valid scoring system that is both repeatable and meaningful for the project.[31] Findings of our work showed higher percentage in contraction of wound and pathological scores along with minor or loss of infection Treatment group.

The results of histomorphometery in the present study for dispersion of vessels were consistent with these findings. BMMCs treated animals showed significantly higher new vessel formation in comparison with SSD group. Enhanced new vessel formation after wound creation indicated that BMMCs could promote the healing process due to enhancement in cellular infiltration.

Although in the present study the outcomes were promising, however, extensive molecular, biochemical and histochemical assessments are required to evaluate mechanism of action of BMMCs in increasing TAC and other promising effects that remained unknown. These could be regarded as limitations of our study.

In conclusion, according to findings of our work, it could be concluded that BMMCs showed potential advantages in burn wound healing acceleration and improvement via antioxidant properties, induction of angiogenesis, proliferation of fibroblast, formation of granulation tissue in early days of healing phases, speeding up burn wound healing associated with earlier wound contraction and stability of damaged area by reorganization of granulation tissue and improved collagen deposition.

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Conflict of Interest

There were no conflict of interests to declare. No funding was received for this study. It is authors' own work.

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