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Research Article

Intra-Myocardial Injection of Mesenchymal Stem Cells Improve Skin Wound Healing

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Abstract

Introduction: Bone Marrow Mesenchymal Stem Cells (BM-MSCs) have a significant effect on facilitating the regeneration of damaged cells during the homing process. Through their ability to induce host repair cells and secrete factors that promote skin wound healing, MSCs have demonstrated great attention. To understand the mechanism underlying this process, in this research, the skin wound healing effect of intra-myocardial injection of BM-MSCs labeled with BrdU (5-Bromo 2'deoxyuridine) was investigated on the BALB/c mice**.**

Materials and Methods: First, BM-MSCs were isolated from the femur of mice. The isolated MSCs were labeled with BrdU and injected (2000 cells/10μM) into the hearts of the mice containing skin wounds. The wound size was recorded in 3, 7, and 14 days after MSCs injection in both the control and MSCS-received groups. The skin biopsy of wounds was prepared to evaluate the level of two keratinocytes differentiated markers, CK8 and CK14, on 3, 7, and 14 days via DAB (3'3-diaminobenzidine) and DAPI staining.

Results: The mean±SD of wound sizes presented a reduction at a significant level on days 3, 7, and 14 after receiving the MSCs than control groups. Also, the injected MSCs accelerated the closure of wound edges. The apoptosis rate decreased significantly until 14 days after MSCs injection in groups without receiving MSCs ($P<0.0001$). Furthermore, the staining with DAB resulted in brown color, indicating the activation of peroxidase (an apoptotic cell marker). The color shifted from brown in the control mice to blue in the mice receiving MSCs indicating a reduction in the apoptotic cells. The evidence revealed an increase in BrdU-positive cells on day 14 compared to day 3, signifying the homing of injected MSCs to the wound site. Moreover, the differentiated keratinocytes were tracked through decreased CK8 and increased CK14 in the MSCs-received group at day 14 compared to the control mice.

Conclusion: The significant wound healing potential of BM-MSCs was proved by homing to the wound site, differentiating into keratinocytes, and reducing the apoptosis rate. These outcomes presented the role of BM-MSCs as a therapeutic intervention to promote wound healing. However, Ffurther clinical trials are necessary to explore the mechanisms and their effects on reducing scar formation in patients with skin wounds.

Keywords: BM-MSCs; Homing; Wound healing; BrdU; Keratinocytes

Introduction

Wound healing is a significant global challenge including different kinds of wounds such as acute, chronic, diabetic, burning, and trauma. Recognizing the importance of advancing wound care and promoting effective treatments, organizations like the Association for the Advancement of Wound Care (AAWC) and the World Health Organization (WHO) have dedicated their attention to this field [1-4]. The advanced wound care market in the United States reached a value of $9.8 billion in 2018, and it

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is predicted that there will be a Compound Annual Growth Rate (CAGR) of more than 5% by 2025 for these products in Asia-Pacific nations [5,6].

Over the course of history, wound care has gained remarkable advancements, evolving from primitive methods like cauterizing wounds with red-hot iron or egg yolk to the utilization of modern wound care technologies and treatments. Recently, wound healing procedures include a wide range of approaches, including autologous grafts of split-thickness as the gold standard, keratinocyte donations, cultured epithelial auto-grafting, specialized dressings with components like chitosan or hyaluronic acid, topical agents, negative pressure wound therapy, cell therapy, and even stem cell therapy. The primary objective of these interventions is to facilitate healing, prevent infections, and alleviate discomfort [7,8].

Recent studies have shed light on the advantages and limitations of both traditional and modern wound-healing techniques, prompting scientists to propose the use of cell therapy for repairing various types of wounds [9,10]. Stem cell therapy, in particular, has gained significant consideration in regenerative medicine because of its advantageous properties and biological functions [11]. Among the available sources for cell therapy, bone marrow-derived mesenchymal stem cells (BM-MSCs) are more identified due to their potential for differentiating to diverse cells, especially keratinocytes [12-15].

These cells possess unique qualities that make them suitable for wound healing. For instance, these cells are able to self-renewal [16] and exhibit multipotency by secreting cytokines, chemokines, growth hormones, vascular, and epidermal growth factors [17-20]. Apoptosis, an essential mechanism in the wound healing process, showed a vital function in removing dead cell debris from the wound site, thereby reducing scar formation [21]. Stem cells have also been found to reduce apoptosis rates through Wnt/β-catenin signaling [22]. Studies have suggested that MSCs can inhibit apoptosis which was induced by heat stress in HaCAT (aneuploid immortal keratinocyte cell) and DFL (Duodenal-type follicular lymphoma) cells [23]. The skin, the body's largest organ, consists of multiple tissue layers and appendage structures that collectively form the integumentary system, which serves a unique function [24,25]. Anatomically, the skin comprises three main parts: the outermost epidermis, middle dermis, and hypodermis. Although each layer has distinct functions, they work together in an integrated manner [24]. In this study, we evaluated the wound healing in mice model after injections of BM-MSCs [26].

Materials and Methods

Experimental Wound Model (In vivo)

The wound mice model was approved by the Ethics Committee of Qazvin University of Medical Sciences (No: 14003425). 12 male BALB/c mice (5-6 weeks of age and ~ 200 g weighing), were obtained from the Razi Vaccine and Serum Research Institute (Karaj, Iran). The three mice were assigned to the control group (without any treatment) and nine to the MSCs receiving group. The isolation of BM-MSCs was done using three mice. The maintenance of laboratory animals adhered to international ethical standards, ensuring appropriate environmental conditions such as a regulated light/dark cycle, feeding with pellet and water, room temperature of 18-20 ºC, and regular cage cleaning and disinfection.

BM-MSCs Isolation and Culture

The BM-MSCs isolation began by euthanizing the mice through cervical dislocation, followed by the sterilization of their incisions using 70% ethanol. Subsequently, the side appendices consisting of muscles and tendons were detached from the distal limbs (specifically tibias and femurs) using sterile surgical instruments. After washing the side appendices with PBS (phosphate-buffered saline), the clean bones were placed on a cell culture Petri dish containing DMEM (BioIDEA). The tibia and femur were used to extract the bone marrow using an insulin syringe containing a 33-gauge needle. The MBCs were transferred to a T-25 flask containing a complete culture medium and conducted to incubation for five days at 37 °C with 95% humidity and 5% CO2. Cells with adherent and spindle-shaped were observed after three days, with a cell density of 70-80%. The PBS was used to wash the cells and after trypsinization cells were counted. Sub-culturing was performed every 4-5 days to maintain an 80% or higher confluency of MSCs and promote cell survival [27].

Labeling of MSCs with BrdU for Tracking of Homing Cell

BM-MSCs proliferation and their homing in wounds were tracked after labeling with thymidine analog, BrdU (5-Bromo 2'deoxyuridine). Briefly, MSCs (~ 2000 cells/mL) were incubated at 37 °C with 5% CO2 for 24h in a complete medium containing 10 µM of BrdU powder.

Creating Wounds on Mouse Models and Injecting MSCs-BrdU

For the creation of wounds on the mouse models and the injection of MSCs-BrdU, the mice were anesthetized using a combination of ketamine and xylazine. The dorsal hairs were shaved and sterilized with 70% isopropyl alcohol. Circular incisions were made on the dorsal skin using forceps and a scalpel. The solution containing MSCs-BrdU (2000 cells/10µM) was injected intramuscularly into the hearts of the mice [28,29]. Three mice were considered as the control group (without MSCs-BrdU injection) and experimental groups that received MSCs-BrdU-labeled (n=9). Changes in wound size were measured in both groups on days 0, 3, 7, and 14. Labeled cells visualized by an anti-BrdU antibody during immunohistochemistry analyses.

Immunohistological (IHC) Assay

IHC assay was done after euthanizing the mice on 3, 7, and 14 days. Tissue samples from the wound area were embedded in paraffin and subjected to hematoxylin-eosin (H&E) staining using the Abcam protocol.

DAB (3'3-diaminobenzidine) Staining

Deparaffinization of samples was done using xylene and dehydrated with graded alcohol. Next, they pretreated 20-minute in the microwave in TBS 1X (pH 7.0) at 750W. Subsequently, the samples were left in the solution (15 min) and washed with PBS. To prevent nonspecific antibody binding, the samples were exposed to a mixed solution of H_2O_2: methanol (1:9) and primary antibodies (anti-CK14 and anti-CK8 from Sigma-Aldrich) for 24h. Following PBS washing, the samples were treated with a linker, polymer, and 100 λ DAB (chromogen) for 5 min. Finally, the samples were stained using H&S before being mounted for observation under a dark field microscope.

DAPI Staining

The protocol used for staining tissue sections in this study included washing with PBS, antigen retrieval with HCL for 30 min, sodium borate buffer for acid neutralization, triton 0.3% to make the membrane permeable, and blocking the secondary antigen with goat serum 10%. The first step in sample preparation for primary and secondary antibody conjugated with PI interaction with tissue samples contained diluted primary antibodies (anti-CK14 and anti-CK8 from sigma-Aldrich) (1:100) (overnight, 2-8) and incubated tissues (37°C) with diluted secondary antibody (1:150). Finally, DAPI was added, and the samples were put in the dark room. The CK14 and CK8 were traced under an Olympus fluorescence microscope.

BrdU-CK8/CK14 Detection in Skin Samples

Dual staining was performed to detect BrdU and CK8/CK14 in skin samples after 30 minutes adjacent to HCL (1 mol/L) at 37°C.

Statistical Analysis

The Mean±SD (standard deviation) was calculated and the comparison between groups (ANOVA) was analyzed with Graph Pad Prism and the significant level was p<0.05.

Results

The morphology of cultured MSCs is illustrated in Figure 1. The mean±SD of wound sizes on the dorsal skin of both the control group and MSCs received are presented in Figure 2. In the MSCs-receiving mice, a significant decrease in the wound size was found (3, 7, and 14 days). The closure of wound edges occurred at a faster rate in mice injected with BrdU-labeled MSCs than in control mice.

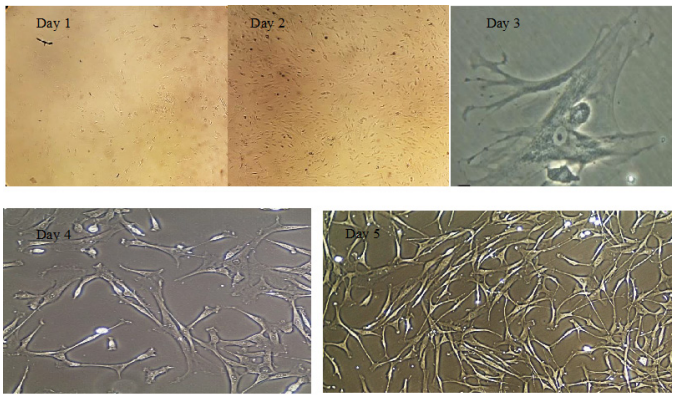
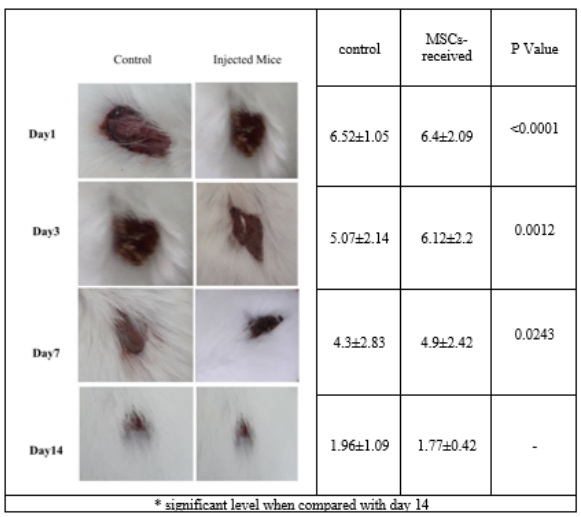


Figure 1: Morphological of MSCs from day 1 to day 5.



	control	MSCs-received	P Value
Day1	6.52±1.05	6.4±2.09	<0.0001
Day3	5.07±2.14	6.12±2.2	0.0012
Day7	4.3±2.83	4.9±2.42	0.0243
Day14	1.96±1.09	1.77±0.42	-

* significant level when compared with day 14

Figure 2: Wound area (mm) of control and MSCs-receivers' groups.

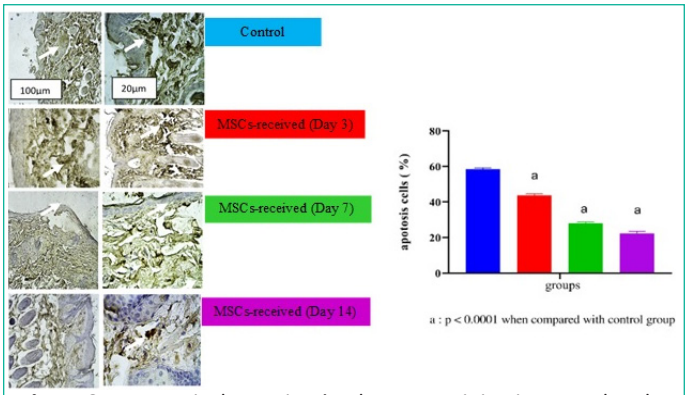


Figure 3: Apoptosis determination by DAB staining in control and MSCs-received groups. Brown color shows apoptotic cells which is caused by peroxidase reaction with DAB.

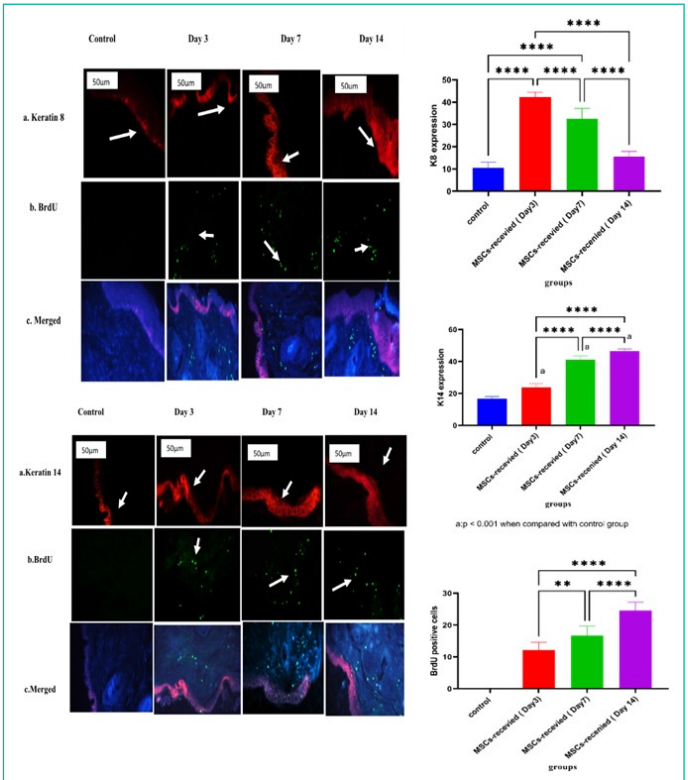


Figure 4: Detection of increased K14 and decreased K 8 level in control and MSCs-received groups. (**P<0.01, **** P<0.00001).

DAB (3'3-diaminobenzidine) Staining to Assess Apoptosis

Figure 3 displays the percentage of apoptotic cells in two groups after MSCs injection. The apoptosis rate decreased significantly until 14 days after MSCs injection. In addition, the staining process using HRP and DAB resulted in a brown color, indicating the activation of peroxidase, which serves as an apoptotic cell marker. Notably, the color change from brown in the control mice to blue in the MSCs-received mice indicated a reduction in apoptotic cells.

Detection of CK14 and CK8 levels after DAPI Staining

Apoptotic cells were seen as blue fluorescent color, the green color showed the BrdU incorporation into the DNA of new cells (Figure 4). The evidence revealed an increase in BrdU-positive cells on day 14 compared to day 3, signifying the homing process in which injected MSCs moved to the wound site. Moreover, the differentiated MSCs into keratinocytes could be tracked through the expression of Cytokeratin-8 (CK8) and Cytokeratin-14 (CK14) using monoclonal antibodies. The level of CK8 decreased in the MSCs-received mice at day 14 than the control mice, while the level of CK14 presented a significant increase from day 3 to day 14.

Discussion

The effective healing of skin wounds is one of the main challenges. Conventional treatment methods do not always yield optimal outcomes in wound healing. As a result, novel approaches like cell therapy have emerged as potential avenues for enhancing wound treatment [30]. We investigated the wound healing potentials of systemic injection of BM-MSCs. For this purpose, BrdU-labeled MSCs tracked at injury sites in male BALB/c mice after differentiation into keratinocytes by their specific markers CK8 and CK14.The differentiation of keratinocytes into the cornified layer of skin is crucial and necessary for re-epithelialization.

In a previous study, we assessed the wound healing effect of placenta-derived human amniotic epithelial cells (hAECs) after pressure ulcers on days 3, 7, 14, and 21. Cell therapy with hAECs can result in an increase in wound closure by promoting re-epithelialization, decreasing scar formation by affecting collagen I and III expression, decreasing TGF-β1/ TGF-β3 level, and angiogenesis inducing [31].

Alain et al. examined the impact of keratinocytes on the re-epithelialization process. Through tissue engineering, they demonstrated that keratinocytes migrate to the wound bed during re-epithelialization, leading to increased cell migration and enhanced cellular connection to the dermal layer due to up-regulation of laminin 5 level [32].

Previous studies showed that MSCs were activated following injury, resulting in the emergence of new progenitors within the wound healing process. Immunofluorescence staining of CK14 and BrdU in the wound area indicated that stem cells could move to the wound site and produce keratinocytes within two days of injury [33]. Another study highlighted that MSCs express CK14 and differentiate into different skin cell types [34].

CK8 is the initial keratins expressed during embryogenesis. The expression of CK8 was investigated in different cell lines and found its association with increased cell migration and reduced doubling time. CK8 expression has also been associated with decreased re-epithelialization at wound sites [35]. In the current study, CK8 expression decreased while CK14 expression increased during wound edge re-epithelialization from day 3 to day 14. These outcomes are in line with previous reports, which demonstrated that GFP-labeled BM-MSCs can differentiate into keratinocytes and accelerate re-epithelialization when administered to the site of wounds [29].

Besides MSCs, Adipose Stem Cells (ASCs) are used in cell therapy and open new doors in improving wound healing. it was found that after injection of GFP-positive ADSCs into a wound site, these cells were differentiated into CK14-positive cells during 14 days of injury, and re-epithelialization was enhanced at the wound edges [36]. Acellular derivatives of MSCs also contribute to accelerated wound healing. These cell derivatives, comprising growth factors, cytokines, and chemokines, can facilitate the differentiation of skin stem cells into keratinocytes. MSCs exhibit low immunogenicity because of a lack the MHC-II [37]. Consequently, MSCs represent practicable candidates for injection at wound sites, with the challenge being achieving scar-free wound healing. Several studies have demonstrated that MSCs can reduce scar formation. Treatment of burn wounds with MSCs derived from burn wound debridement is able to speed up wound healing and minimize the formation of scars [38]. Intradermal injection of human MSCs into full-thickness incision wounds in rabbits has been found to enhance tensile strength and reduce scar formation on consecutive days (3, 7, and 14) post-wounding [39]. In line with these results, in our study, wound closure occurred more rapidly in MSCs-receiving mice compared to the control mice. It was presented that both systemic and local injections of PKH26-labeled MSCs accelerated skin regeneration in albino rats with full-thickness skin wounds, although a great proportion of collagen fibers was displayed in the topically injected rats [40]. A higher rate of wound healing potential of Umbilical Cord Blood-Derived Mesenchymal Stem Cells (UC-MSCs) was presented via the graft method [41]. A previous investigation demonstrated that allogeneic MSCs can expedite wound healing in burn patients, resulting in increased collagen fibers and dermal thickness. The use of autologous MSCs for wound healing presents challenges such as treatment delays for cell isolation and culture, as well as limitations in cell quantity [42]. Therefore, allogeneic MSCs were used in the current study. In the first phase, cells were cultured for several weeks, and in the second phase, MSCs were injected into mice. Also, the use of local applications of MSCs has limited beneficial effects. In another study, the impact of both systemic and local MC-MSCs injections on wound healing was reported in which both types of injection accelerated wound healing in old rats [43]. Another assessment was conducted to show the local and remote MSCs injection effect on wound healing and the results confirmed the effective promotion of skin regeneration in both injection methods [44]. Furthermore, the present study demonstrates that systemic injections also contribute to wound healing. In this particular investigation, MSCs were labeled with BrdU and incorporated into DNA. To track BrdU-labeled cells using an anti-BrdU antibody, denaturing treatments are necessary. It was found that during the denaturing treatments, the traceability of BrdU-labeled differentiated MSCs was changed due to the loss of antigenicity [45], but we successfully demonstrated the differentiation of BrdU-labeled cells into keratinocytes. In addition, we indicated a reduction in the apoptosis rate from day 3 to day 14 following MB-MSCs injection at the wound site. This aligns with a previous study that highlighted how MSCs inhibit cell apoptosis through exosome secretion [46] and expedite wound healing by suppressing apoptosis of skin cells through the modulation of signaling pathways [23]. Keratinocytes could secret the various microvesicles and microRNA to regulate the gene expression involved in the wound healing process [47,48]. Here, the differentiation of BM-MSCs into keratinocytes was seen at the wound site, along with the reduction of apoptosis rate from day 3 to day 14, suggesting that keratinocytes derived from BMSCs may have important activity in mitigating apoptosis at the wound site.

Conclusions

The current study provides evidence that BM-MSCs harbor a significant effect on wound healing acceleration by homing to the wound site, differentiating into keratinocytes, and reducing the rate of apoptosis. Our results highlighted the MSCs potential as an effective therapeutic intervention for improving wound healing outcomes. The study also emphasizes the importance of understanding the differentiation patterns of MSCs and their association with keratinocytes in the healing of wounds. A more comprehensive investigation is necessary to explore the mechanisms of their effects in reducing scar formation as well. Overall, this study contributes valuable insights into wound healing and promises novel treatment options for managing skin diseases.

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