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Effects of Hair Follicle Stem Cells and Simvastatin on Deep Partial Thickness Wound Healing: Histological and Molecular Assay

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A B S T R A C T

Introduction: Restoration of burn wounds in a shorter time with fewer side effects is one of the main concerns of wound management. In this study, the effects of simultaneous use of hair follicle stem cells (HFSCs) and simvastatin (SMV) in partial-thickness burn wound healing and C-X-C motif chemokine ligand 12 (CXCL12) / C-X-C chemokine receptor type 4 (CXCR4) and vascular endothelial growth factor (VEGF) expression are investigated.

Materials & Methods: Animals (male Wistar rats) were categorized into five groups: 1) control (non-treated), 2) vehicle, 3) cell (HFSCs), 4) drug (SMV), and 5) cell-drug (HFSCs + SMV). The bulge of rat whisker was isolated and cultured in DMEM/F12 and transplanted to the burn-wound site. Daily dressing was performed with petrolatum gel containing 5 µM simvastatin. At the end of therapeutic intervention, histological assessment (H&E), molecular assay (p-value blot), and tensile strength assay were performed. Statistical analyses were performed using GraphPad Prism version 6.01 (two-way repeated-measures ANOVA, one-way ANOVA, and Tukey's post-hoc test). A p-value was considered statistically significant. Quantitative analysis of wound healing parameters was performed using ImageJ 1.46r software.

Results: The diameter of epidermis, dermis structural integrity, and expression of SDF-1/CXCR4 and VEGF in cell-drug groups were significantly improved compared with other groups ($P < 0.05$). No significant differences were observed between the vehicle and control groups.

Conclusion: Hair follicle stem cell transplantation simultaneously with simvastatin treatment accelerates the healing of the burn wounds and skin tensile strength by elevating the SDF-1 and VEGF expression levels in the injected region.

Keywords: HFSCs, Simvastatin, VEGF, SDF-1/CXCR4, Tensile strength, Partial-thickness burn wound

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Introduction

Burns are one of the most severe and common skin traumas. Therefore, burn wound healing is still considered a principal challenge in modern and emergency medicine (1, 2).

Wound healing is a complicated procedure that includes the interaction between different types of cells, growth factors, and extracellular matrix (3).

Most of the burn patients do not have enough skin to be used as a graft; thus, stem cell therapy is one of the effective, low-mortality, and high-quality treatments to cover the skin in burn wounds, which causes the restoration of skin excesses and reduces the risk of hypertrophic scars (4).

Hair follicle stem cells have important potential in wound healing (5). These cells can be more useful than other adult stem cells regarding their easier access and high differentiation potential. (6, 5) Hair follicle stem cells have no known risk of carcinogenesis and are capable of differentiating into distinct cell types. Thus, the first set of keratinocytes involved in wound healing comes from the bulge area of the hair follicle (7).

Chemokines and their receptors participate in stem-cell recruitment. SDF-1 is a natural chemokine that exhibits rapid expression alongside its receptor (CXCR4) in reaction to tissue damage, and by calling stem cells to the site of damage, it improves tissue repair and blood vessel formation (8, 9).

Various effects of statins have been reported in different phases of wound healing, especially inflammation and vascular repair (10). Topical use of simvastatin increases wound healing by improving angiogenesis and the proliferation of endothelial cells, increasing the synthesis of VEGF and its release at the wound site, having anti-inflammatory effects, and accumulating collagen bundles. Simvastatin also increases the expression levels of SDF-1 during burn wound healing (3, 9, 11). Some previous studies have examined the effect of simultaneous treatment with a type of stem cell and simvastatin on burn wound healing (3).

Considering the importance of hair follicle stem cells and the simvastatin drug in wound healing and angiogenesis, for the first time, the simultaneous effects of these two in burn wound healing and the expression of SDF-1/CXCR4 and VEGF proteins were studied.

Materials and methods

Study Design

30 male Wistar rats (200-250 gr) were acquired from the Animal Laboratory of Iran University of Medical Sciences. They were housed in the animal house for one week before wounding in accordance with the guideline and authorization of the Institution of Iran University of Medical Sciences. The rats were preserved in controlled environmental condition (light-dark cycle: 12h/12h, temperature 25±1 °C) (1,3), and received water and food. They were arranged randomly into five equal experimental groups (n=6 in each): (1) control or lesion group (burn wound without treatment), (2) vehicle (animals received petroleum cream alone), (3) HFSCs group (animals received 1×106 hair follicle stem cells by intradermal injection), (4) SMV group (animals with topical simvastatin administration), and (5) HFSCs+SMV (animals were treated with combination of HFSCs and simvastatin).

Setting and Participants

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Sample Size

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Randomization

They were arranged randomly into five equal experimental groups (n=6 in each).

Measurements & Validity and Reliability

HFSCs Identification

Flow cytoplasmic analysis was used to determine the percentage of stem cell expressing the especial markers (CD34, Nestin and Kr15).

HFSCs Viability

MTT assay was used for measuring HFSCs proliferation and survival rate. HFSCs were exposed to 0-30 μM simvastatin. The optical density was determined by ELISA reader at 57o nm.

Histological Analysis

Tissue specimens with a surrounding rim of normal skin were removed on days 0, 3, 7, 14 and fixed in 10% formalin. Paraffin sections of 5 μm-thicks were stained by Hematoxylin and Eosin (H&E) (to evaluate epithelial thickness). All images were assessed by Image J version 1.46 r (NIM, Bethesda, MD).

Western Blot Analysis

In order to detect the expression level of CXCL12/CXCR4 and VEGF proteins, western blot analysis was performed. Tissue samples were lysed with RIPA buffer (CMG RIPA) and supernatants were collected and their protein concentration was measured and normalized by Bradford test and spectrophotometry (Labtron, UK). According to an equal concentration of samples (40 μg protein) SDS-PAGE electrophoresis was performed. After transferring the protein from the gel to the PVDF membrane, blotting was done using anti SDF-1α antibody (1:1000 Santa Cruz), anti CXCR4 (1:1000 Santa Cruz), and anti VEGF antibody (1:1000 Santa Cruz) as primary antibodies. Detection process was performed with horseradish peroxidase-conjugated goat anti-rabbit IgG (cell signaling 1:10000) as secondary antibody. The western blotting was replicated four times.

Tensiometry

To evaluate the biomechanical strength of the wounds on day 14 post-burn, wound breaking strength was measured using a calibrated tensiometer (Zwick 72.5, Germany). Skin strips measuring 12 cm in length, 5 cm in width, and 3.5 cm in thickness, containing a wound in the center, were stretched at a speed of 10 mm/min until rupture. Tissue stress/strain (wound breaking strength) was assessed (3,5)

Anesthetized with Ketamine (15 mg/kg) and Xylazine (2 m/kg) (1,3), the hairs on the back of rats were shaved and disinfected with 70% isopropyl alcohol and povidone-iodine burn induction was performed by a rectangular iron lid attached to a temperature-controlled super soldering Station (RX 711AS, Goot, Japan) with the tip 15×15 mm at optimized 120 °C temperature. The lid was steadily applied on the interscapular area for 10 seconds, in order to create a deep partial thickness burn (deep second-degree burn).

In simvastatin groups, topical simvastatin was provided as previously described(1, 3). For this purpose, 5 mg of simvastatin and 995 mg of petroleum cream (vehicle) were mixed. 10 mg of the mixture (containing 50 μg of simvastatin) was administered to the wound site. In HFSCs groups, the amount of 1×10^6 cells were administered intradermally into the four corners of the wound. In HFSCs+SMV groups, after injection (transplant) of

HFSCs, topical simvastatin was used to dress the wound. In vehicle groups, only petroleum gel (cream) was used. The control groups did not receive any treatment.

Ethical Considerations

This study was approved by the Ethics Committee of Iran University of Medical Sciences (Ethical code: No.26603).

Statistical and Data Analysis

Statistical analyses were performed using GraphPad Prism version 6.01. Data are expressed as mean ± SD. The normality of the data distribution was verified using the Shapiro-Wilk test. For parameters evaluated across multiple time points, including epidermal thickness (H&E staining) and the protein expression levels (SDF-1, CXCR4, and VEGF) obtained via Western blot, two-way analysis of variance (two-way repeated-measures ANOVA) was employed to assess the interaction between treatment groups and time. For parameters measured at a single time point or without time-dependent analysis, such as the skin biomechanical index (tensile strength), one-way analysis of variance (one-way ANOVA) was utilised. In all cases, Tukey's post-hoc test was applied for multiple comparisons between groups. A p-value was considered statistically significant. Quantitative analysis of wound healing parameters, including diameter and area measurements, was performed using ImageJ 1.46r software (National Institutes of Health, USA) and Measurement 3.0 Microstructure software.

Results

Histological analysis was used to evaluate epidermal thickness (with hematoxylin & eosin) in all groups (Figures 1A and 1B).

Results of a three-day wound evaluation: The cell (HFSCs) and drug (simvastatin) groups showed a significant difference in the thickness of formed epidermis compared with the control group ($p < 0.05$). Furthermore, the greatest degree of dermis regeneration was observed in the cell-drug group, exhibiting a statistically significant difference in comparison to the remaining groups.

Results of seven-day wound evaluation: On the seventh day, the epidermis was reformed in all groups. There was a significant increase in the epidermal thickness in the drug-cell group in contrast to other groups ($p < 0.05$). The thickness of the dermis in the cell-drug, cell, and drug groups was increased compared to the control group.

Results of fourteen-day wound evaluation: The number of keratinized layers in the skin was increased. In the healed area of the wound, skin appendages, including blood vessels and hair follicles, were seen. On the 14th day, the thickness of the dermis in the cell-drug group was increased, and most of the collagen deposition in the created tissue (over 90%) was similar to the healthy skin. The healed area in the vehicle group was similar to the control group. There was no significant difference in the cell and drug groups compared to the control group.

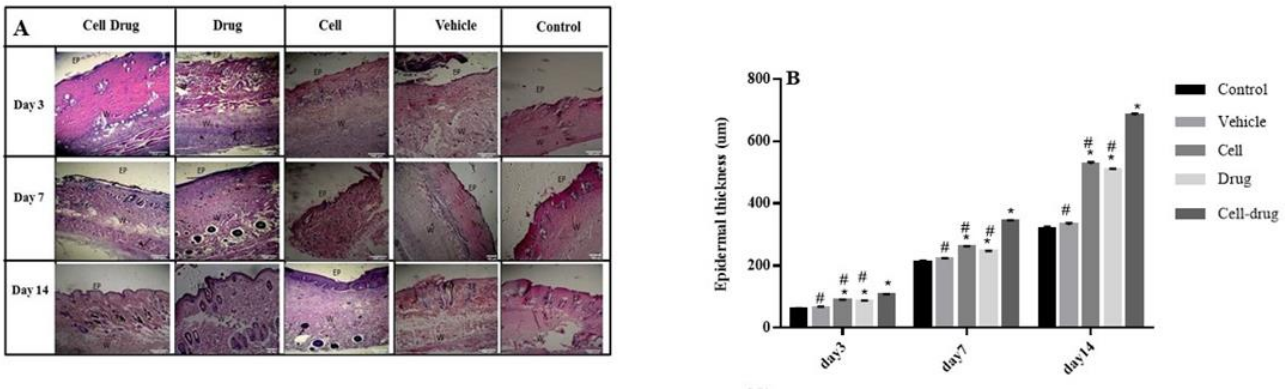

Figure 1. Tissue regeneration in histological analysis. (A) Images of histological sections from the wound site (H&E) on days 3, 7 and 14; (Scale bars = 100 μm) (B) Epidermal thickness in different groups on days 3, 7 and 14 after wounding (Mean±SD) (*P<0.001 significant increase compared to the control group, #P<0.001 significant difference compared to cell-drug group).

A Western blot test was used to assess the expression level of SDF-1, CXCR4, and VEGF proteins on days 3, 7, and 14 after wound formation in different groups (Figures 2A-C).

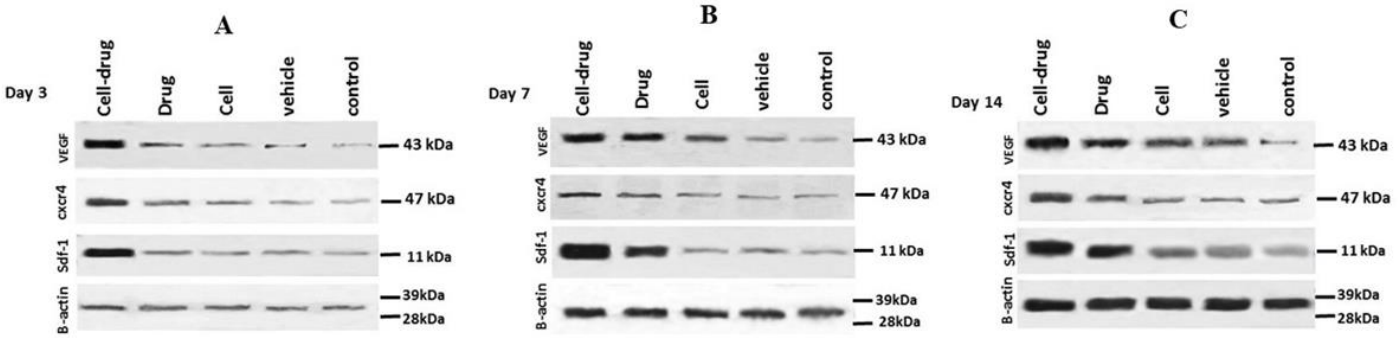

Figure 2. The level of VEGF; SDF-1; CXCR4 proteins expression in the wound site according to the β actin reference protein on days (A) 3, (B) 7 and (C) 14 after the wound in different groups.

On days 3, 7, and 14, the expression of SDF-1, CXCR4, and VEGF proteins increased the most in the cell-drug group in comparison to other groups, reaching its maximum level on day 14 (Figure 3A-C).

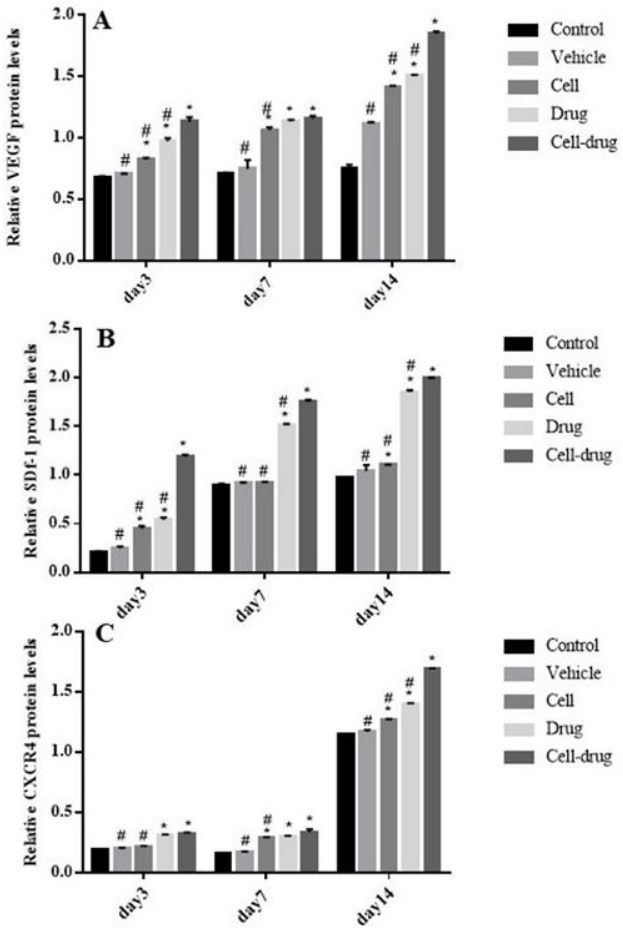

Figure 3. VEGF(A), SDF-1(B), CXCR4(C) proteins expression level in the granulation tissue of the wound site according to β actin reference protein on days 3, 7 and 14 after the wound induction in different groups (Mean±SD) (*P<0.001 significant increase compared to the control group, #P<0.001 significant difference compared to cell-drug group).

The finding of this experiment showed that the maximum stress value in the drug-cell group was significantly elevated relative to the control group ($p < 0.05$). However, the vehicle group exhibited a significant difference compared with the cell-drug group ($p < 0.05$). A lower stress value was also observed in the cell and drug groups, which demonstrated a difference compared with the drug cell group. (Figure 4).

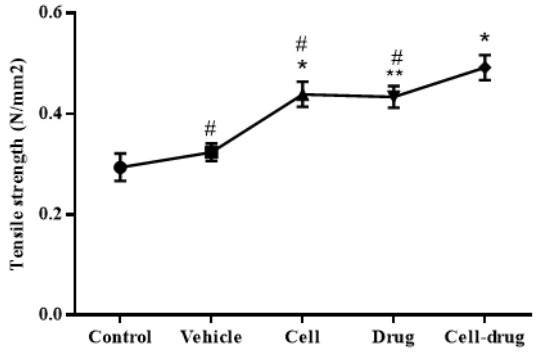

Figure 4. Biomechanical measures of maximum stress in the studied groups and 14 days after treatment (Mean±SD) (*P<0.05, **P<0.01 significant increase compared with the control group, #P<0.001 significant difference compared to cell-drug group).

Discussion

When burn wound healing, it appears that unique treatments are necessary. Cell therapy is a revolutionary method in the treatment of burns. In the present investigation, hair follicle stem cells were employed as primary cells for rat burn wound healing. In addition to their great multipotential power, these cells are more accessible and may be obtained by biopsy. Hair follicle stem cells do not express MHC class I, hence there is no need to suppress the immune system if they are employed for treatment (6, 14).

Conversely, hair follicle stem cells and skin keratinocytes have a same embryonic origin, and hence, the use of these cells for wound healing is more efficient than other stem cells (11, 14-16).

In this research, for the first time, the combined use of hair follicle stem cells and Simvastatin in burn wound healing has been employed. Simvastatin co-treatment of hair follicle stem cells showed improvement of wound healing factors in comparison to the monotherapy. The efficiency of stem cell therapy and medication in the treatment of burn wounds has also been described in earlier research (3, 17).

Histological parameters analysis revealed that the application of hair follicle stem cells and simvastatin at the wound site led to accelerated burn wound closure, increased thickness of the formed epidermis, improved dermal tissue remodeling, restoration of skin appendages such as hair follicles and sebaceous glands, and formation of organized, thick granulation tissue with mature blood vessels. Previous investigations have identified hair follicle stem cells and simvastatin as potential options for burn wound therapy (3, 5, 6, 9, 18, 19).

It appears that the transplanted stem cells may contribute to repair other than by maturing into repairing cells and enhancing collagen formation. These include paracrine effects and increased production of angiogenesis promoting growth factors such as VEGF (3, 20).

The increase in density of newly created blood vessels in the treatment groups reveals the angiogenic activity of hair follicle stem cells and simvastatin and presumably the endothelial cells along with new blood vessels derived from hair follicle stem cells.

Hair follicle stem cells may influence angiogenesis by direct differentiation or by secreting growth factors (14, 21, 22).

In the present work, enhanced expression of SDF-1 in the hair follicle bulge stem cells were shown to summon stem cells to the injury site. In the past investigations, SDF-1 expression was observed in the basal layer of epidermis and in the outer sheath of hair roots (18, 23, 24).

Tensile strength is a highly essential aspect in burn wound healing which verifies the deposition of collagen fibers and regeneration of skin structures (25-28). The highest firmness and elasticity in the tissue samples of the drug-cell therapy groups in comparison to other groups was due to enhanced synthesis and deposition of collagen. These results showed that the appropriate dose of simvastatin and the use of hair follicle stem cells injected at the border of the wound have positive effects on the improvement of the biomechanical, molecular and histological parameters of the wound which are similar to the previous studies (3, 5, 6, 9). The findings of the present research suggested that the simultaneous transplantation of hair follicle stem cells with topical simvastatin might be regarded as a potential method for burn wound healing.

Limitations and Strengths

Repair of in burn wounds with a larger area and over a longer period of time should be studied in humans. For the first time, simultaneous use of autologous hair follicle stem cells and topical simvastatin was used to repair burn wounds.

Conclusion

The results of this study demonstrated that the transplantation of hair follicle stem cells, simultaneously with topical simvastatin, can enhance the healing of deep second-degree burns in rats. This synergistic application holds promise for improving therapeutic strategies in the management of burn injuries in clinical settings.

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CRediT authorship contribution statement

Conceptualization, Methodology, Validation, Formal Analysis: MN, PH, Investigation, Resources, Software: SK, KS, Data Curation, Writing–Original Draft Preparation, Writing–Review & Editing, Visualization, Supervision, Project Administration: MN, AB.

Declaration of Generative AI and AI-assisted technologies in the writing process

No AI writing assistance was utilized in the production of this manuscript.

Conflict of interest

All authors declare that they have no conflict of interest.

Ethical considerations

Ethical concerns included ensuring integrity in library collection and data reporting, getting signed informed permission from all participants in accordance with the Declaration of Helsinki, and adhering to principles of human intervention.

Data availability statement

Data available on request from the authors.

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