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Hypoxia Mesenchymal Stem Cells Accelerate Wound Closure Improvement by Controlling α-smooth Muscle actin Expression in the Full-thickness Animal Model

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Abstract

BACKGROUND: The active myofibroblast producing extracellular matrix deposition regarding wound closure is characterized by alpha-smooth muscle actin (α-SMA) expression. However, the persistence of α-SMA expression due to prolonged inflammation may trigger scar formation. A new strategy to control α-SMA expression in line with wound closure improvement uses hypoxic mesenchymal stem cells (HMSCs) due to their ability to firmly control inflammation for early initiating cell proliferation, including the regulation of α-SMA expression associated with wound closure acceleration.

AIM: This study aimed to explore the role of HMSCs in accelerating the optimum wound closure percentages through controlling the α-SMA expression.

MATERIALS AND METHODS: Twenty-four full-thickness rats wound model were randomly divided into four groups: Sham (Sh), Control (C) by NaCl administration only, and two treatment groups by HMSCs at doses of 1.5×10^6 cells (T1) and HMSCs at doses of 3×10^6 cells (T2). HMSCs were incubated under hypoxic conditions. The α-SMA expression was analyzed under immunohistochemistry staining assay, and the wound closure percentage was analyzed by ImageJ software.

RESULTS: This study showed a significant increase in wound closure percentage in all treatment groups that gradually initiated on days 6 and 9 ($p < 0.05$). In line with the increase of wound closure percentages on day 9, there was also a significant decrease in α-SMA expression in all treatment groups ($p < 0.05$), indicating the optimum wound healing has preceded.

CONCLUSION: HMSCs have a robust ability to accelerated wound closure improvement to the optimum wound healing by controlling α-SMA expression depending on wound healing phases.

Introduction

Full-thickness wounds are a loss of skin integrity associated with damaged tissue of the epidermis and dermis [1]. The challenge of a full-thickness wound repair is the exposed wound structures and impaired cell proliferation and migration due to prolonged inflammatory phase [2], [3]. In wound healing processes, the fibroblasts have a dominant role in promoting a wound contraction acceleration leading to wound closure [4]. These occurred through releasing several growth factors such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) to induce fibroblast differentiation into myofibroblast characterized by α-smooth muscle actin (α-SMA) expression [5]. However, the prolonged inflammatory phase causes impaired fibroblast differentiation that leads to delayed wound contraction, potentially resulting in scar formation [6], [7]. Recent studies have shown that hypoxic mesenchymal stem cells (HMSCs) enhance wound healing acceleration in several damaged tissues by increasing growth factors such as vascular endothelial growth factor, TGF-β, and PDGF to activate fibroblast differentiation into myofibroblasts [8], [9], [10], [11]. However, there are no reports on how days the HMSCs increase wound closure to the optimum wound healing by controlling α-SMA expression released by myofibroblast.

HMSCs are known as hypoxic multipotent stromal progenitor cells grown in hypoxic culture conditions to strengthen their multiple differentiation capabilities and paracrine pathways in releasing the tons of growth factor and anti-inflammatory

cytokines associated with tissue damage amelioration [12], [13], [14]. The clones of HMSCs can be isolated from the umbilical cord (UC), cord blood, placenta, bone marrow, mobilized peripheral blood, adipose tissue, and dental pulp [13], [15]. Several studies also reported that HMSCs could suppress the excessive inflammatory responses to accelerate the shifting of inflammation to the proliferation phases by releasing several anti-inflammatory cytokines, including interleukin-10 (IL-10), TGF-β, hepatocyte growth factor, and prostaglandin E2 [9], [11], [16]. HMSCs express the surface markers CD105, CD90, and CD73, and a lack of hemopoietic markers such as CD11b, CD14, CD19 or CD79a, CD45, CD34, or human leucocyte antigen (HLA) class II expression [11], [17].

The active fibroblast post the TGF-β stimulation characterized by α-SMA expression has a primary role producing matrix extracellular associated with wound healing acceleration [18], [19]. This fact suggested that α-SMA expression is the crucial molecule indicating that the wound closure process is well preceding [20]. However, the prolonged α-SMA expression can also induce pathological healing features, particularly in the abnormal collagen synthesis that potentially induces scar formation [21], [22]. Hence, controlling α-SMA expression during wound healing processes is critical to ensure that the wound healing process is well-running or tends toward pathological conditions [23], [24], [25]. Recent studies reported that HMSCs might increase wound closure processes by increasing α-SMA expression to reach optimum wound healing [26]. On the other hand, HMSCs have a central role in improving fibrosis diseases by reducing prolonged α-SMA expression [27]. These facts suggested that HMSCs have dual roles in controlling healing phase-related α-SMA expression, whether by increasing or decreasing those molecules leading to the optimum wound healing; however, in what healing phases of the α-SMA expressed by HMSCs-regulated myofibroblast regarding wound closure acceleration is still unclear. Therefore, exploring the role of HMSCs in controlling α-SMA expression regarding wound closure is a crucial point to be investigated. In this study, we investigated the effect of HMSCs in accelerating the optimum wound closure percentages through controlling the α-SMA expression in the full-thickness animal models.

Materials and Methods

MSCs isolation and HMSCs induction

Rat MSCs were isolated from a 19-day pregnant female rat. Briefly, donor rats were anesthetized, and the abdomens were dissected out. Under sterile conditions, the UC was collected and washed in phosphate-buffered saline (PBS). The UC artery and vein were removed, then the UC was cut into lengths of 2–5 mm using a sterile scalpel. The sections were then distributed evenly in T25 flasks using Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% PBS, 100 IU/ml penicillin/streptomycin (GIBCO, Invitrogen), then incubated at 37 C with 5% CO_2. The medium was renewed every 3 days, and the cells were passaged after reaching 80% confluence. UC-MSCs at passages 4–6 were employed for the following experiments.

To induce H-MSCs, MSCs derived from the 4th passage were incubated under 5% O_2 condition in a hypoxia incubation chamber (STEMCELL Technologies, Biopolis, Singapore) for 24 h at 37°C and 5% CO_2, then collected by harvesting technique the HMSCs in the sake of the following experiment.

MSCs in-vitro osteogenic differentiation assay

The MSCs were grown in a 24 well plate (1.5×10^4 cells/well) with a standard medium containing DMEM (Sigma-Aldrich, Louis St, MO), enriched with 10% fetal bovine serum (Gibco™ Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL) (Gibco™ Invitrogen, NY, USA) at 37°C, 5% CO_2 and ≥95% humidity. After 95% confluent, the standard medium was aspirated and replaced with an osteogenic differentiation medium containing Human MesenCult™ Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), augmented with 20% Human MesenCult™ Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore), and 1% L-Glutamine (Gibco™ Invitrogen, NY, USA). The differentiation medium was renewed every 3 days. After bone matrix formation occurred, the osteogenic differentiation was visualized by staining with 1 ml of 2% alizarin red solution.

Characterization of MSCs

MSC surface antigens were analyzed by flow cytometry analysis at the fourth passage according to company protocols. Briefly, the cells were incubated in the dark with allophycocyanin mouse anti-human CD73, fluorescein isothiocyanate mouse anti-human CD90, perCP-Cy5.5.1 mouse anti-human CD105, and phycoerythrin mouse anti-human Lin negative (CD45/CD34/CD11b/CD19/HLA-DR) antibodies. The analysis was performed using BD Stemflow™ (BD Biosciences, San Jose, CA, USA). MSCs were stained with a specific antibody for 30 min at 4°C, examined with a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA), and analyzed with BD Accuri C6 Plus software (BD Biosciences, San Jose, CA, USA).

Full-thickness wound skin animal model and HMSCs administration

Twenty-four male Wistar rats weighing 200 g were caged at 24 ±2°C and 60% relative humidity, with a 12:12-h light-dark cycle. To establish the animal model of a full-thickness skin defect, rats were anesthetized by isoflurane inhalation; then, the dorsal skin was shaved and cleaned with the tincture of iodine. One circular 6 mm biopsy punch excision was done for each rat. The animals were randomly divided into four groups: T1 (n = 6) and T2 treatment (n = 6) were treated subcutaneous injection with two different concentrations of HMSCs at doses of 1.5×10^6 and 3×10^6 cells respectively, while the sham group (n = 6) was not treated and the control (n = 6) was under NaCl administration.

Wound closure percentages measurement

The wound measurement was taken over days 3 and 6. The wound closure percentages were photographically analyzed by ImageJ software. The percentages of wound closure were calculated using: $\frac{A0-At}{A0}\times100\%$ where A_0 is the original wound area after creating a wound and A_t is the area of a wound at the time of measurement in days 3, 6, and 9.

α-*SMA expression analysis*

The expression of α-SMA in wound tissue was evaluated using the immunohistochemistry technique with α-SMA-positive markers. The wound tissues of each animal on day 6 were fixed in 10%-buffered formaldehyde, embedded with paraffin, sectioned, and deparaffinized. After rehydration, tissues were incubated with the anti-α-SMA as a primary monoclonal antibody (LSBio, Seattle, USA) and the biotinylated secondary antibodies (Biocare Medical, Pacheco, CA, USA) according to the manufacturer's protocol. We then observed the tissues under the microscope (Olympus).

Statistical analysis

Data are presented as the means ± standard deviation. All calculations were carried out using IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA). The statistical significance of the difference group's differences was assessed using one way-ANOVA and continued with Fisher's least significant difference *post hoc* analysis. The differences between independent variables were analyzed using two way-ANOVA and continued with Duncan *post hoc* analysis. p values: *, $p < 0.05$.

Results

HMSCs characterization and differentiation

The H-MSCs incubated under hypoxic conditions showed homogeneously dense fibroblast with spindle shape appearance that meets the standard *in-vitro* characteristics of MSCs (Figure 1a). Osteogenic differentiation assay, the HMSCs exhibited the calcium deposition under visualized Alizarin red staining that indicated the multipotency of HMSCs was well-maintained as yet (Figure 1b). The flow cytometric analysis confirmed that HMSCs expressed high levels of CD90 (99.9%), CD105 (95.9%), and CD73 (99.2%), and a lack of Lin (2%), according to the International Society of Cellular Therapy as an immunophenotype characteristic of MSCs (Figure 1c).

Wound closure

After HMSCs treatment was given to each group, the length of the wound site was measured in each group on days 3, 6, and 9 (Figure 2). There was a significant increase ($p < 0.05$) in wound closure percentages on days 6 and 9 after HMSCs treatment, compared to the control groups. Comparing the wound closure rate in the treated groups, the T2 group showed a markedly highest wound closure on day 9 (80.71 ± 2.44%) (Figure 3). Furthermore, there was an inconsiderable difference between the rate of wound closure in the sham with control groups (52.19 ± 1.30% and 55.48 ± 3.44%, respectively).

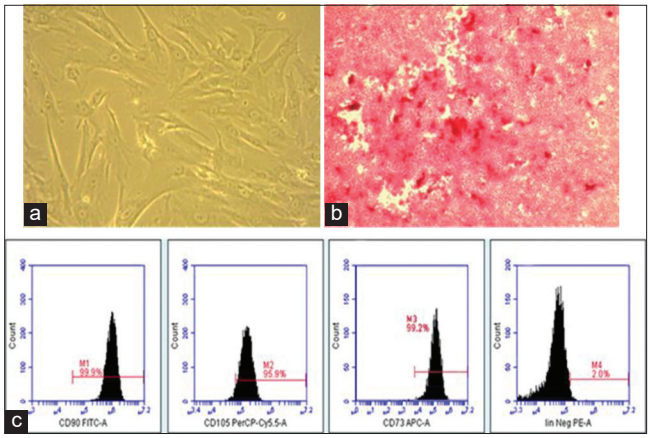

Figure 1: (a) Hypoxic mesenchymal stem cell (H-MSC) showed fibroblast and spindle-like shaped characteristic (scale bar 100 μm); (b) Osteogenic differentiation of HMSCs was visualized as bright red colors (scale bar 50 μm) (magnification ×10); (c) immunophenotyping analysis of H-MSCs expressed CD90 (99.9%), CD105 (95.9%), CD73 (99.2%), and lacked of Lin expression (2.0%)

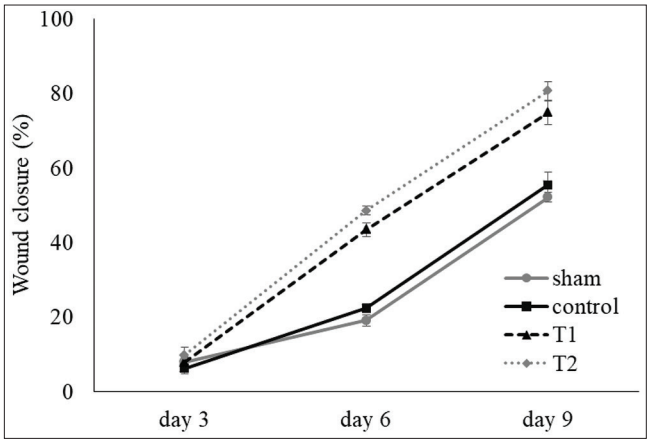

Figure 2: Comparison of wound closure rate in the four groups of full-thickness wound model, Wound closure rate was higher in T2 hypoxic mesenchymal stem cells-treated groups compared with control groups ($p < 0.05$). Each point represents the mean areas of wound closure in any of all groups

α-*SMA expression level*

In wound healing, the persistence of α-SMA expression indicated that the early fibrosis processes are occurring. Therefore the α-SMA expression is a good marker of fibrosis formation during wound healing [18]. In this study, the presence of α-SMA in all group studies was assessed by immunofluorescence. Nine-day after treatment, the percentages of α-SMA expression showed a significant decrease in T1 and T2 of the treated groups up to 3.40 ± 0.84% and 1.40 ± 0.37%, respectively ($p < 0.05$). However, in the sham and the control groups, the high α-SMA expression in the wound bed remained, which indicated the myofibroblast still produce those molecules (Figure 4).

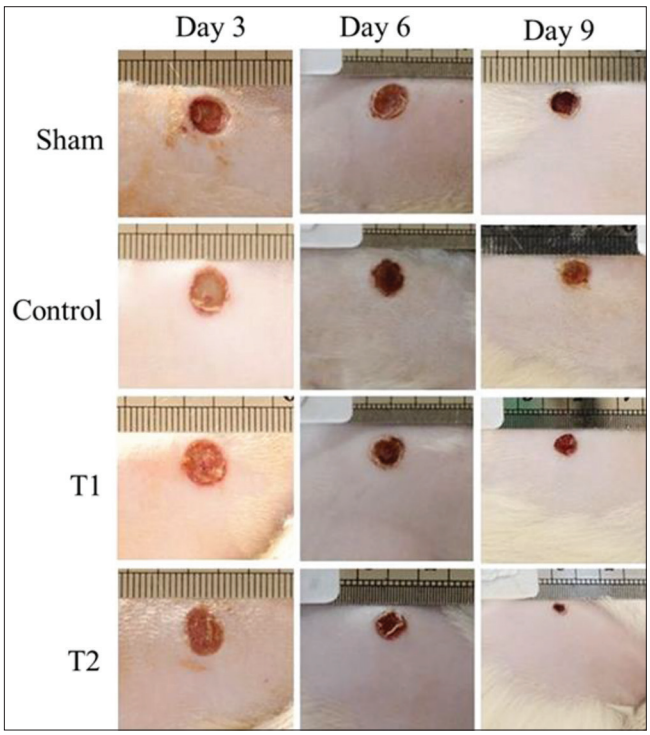

Figure 3: Effect of hypoxic mesenchymal stem cells (HMSCs) administration on wound closure in rat skin. Representative images of skin wounds in male rat skin at different healing stages following treatment with HMSCs. Mice were randomly assigned to one of four groups: Sham, control (NaCl administration), (T1) (HMSCs 1.5 × 106 cells administration), and (T2) (HMSCs 3 × 106 cells administration)

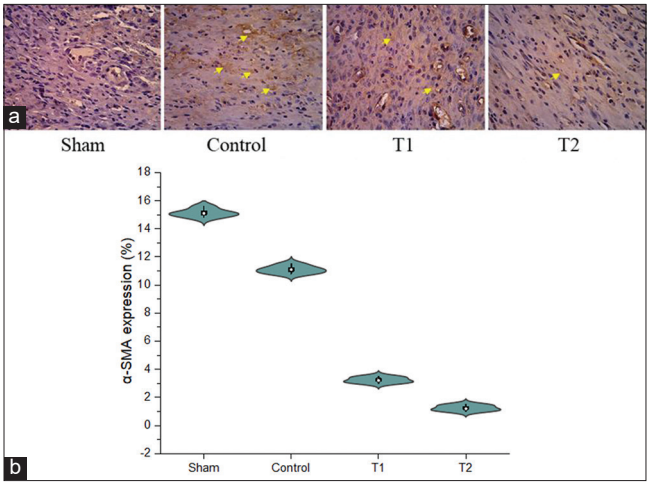

*Figure 4: (a) Immunohistochemistry staining for the alpha-smooth muscle actin (α-SMA) (magenta) to detect myofibroblast in full-thickness wound healing experiment (×100 magnification, 100 μm scale bar) (b) percentages of α-SMA expression during wound healing processes under hypoxic mesenchymal stem cells treatment at day 9. Data in the diagram are shown as the mean ± SD of biological replicates, *$p < 0.05$*

Discussion

In wound healing, myofibroblast contractile phenotype was acquired during proliferation phases to induce wound closure processes, which is characterized by an emergence of the α-SMA expression in response to the myofibroblast differentiation [28]. However, myofibroblast persistence during wound healing could induce fibroproliferative scar formation, characterized by an excessive amount of collagen deposition and, [29], [30] these processes are a relatively common complication of prolonged inflammatory reaction [31]. Therefore, the one primary strategy to accelerate the optimum wound healing without scar formation is by effectively controlling the α-SMA expression using HMSCs treatment. The HMSCs are the most promising therapeutic to control several fibrosis diseases, including scar formation, due to their ability to firmly control the prolonged inflammation for early initiating cell proliferation leading to the optimum wound healing acceleration [23], [32].

In this study, the decrease of α-SMA expression in all treatment groups on day 9 indicated that the healing process in these cases began reaching completed healing and come into the initiation phases of remodeling. On the other hand, the normal healing processes were still proceeding, marked by increasing α-SMA expression in control groups. We suggested that HMSCs accelerate the wound healing process by promoting the

shift of inflammation to proliferation phases triggering the remodeling phases early occurs. This is supported by a previous study that reported the decrease of α-SMA following HMSCs administration is associated with the optimum wound healing [33], [34], [35]. The lower expression of the α-SMA in wound healing processes indicated the myofibroblast has been under inactive states following the HMSCs administration, due to IL-10 produced by the HMSCs inhibit an active macrophages-produced TGF-β the myofibroblast induce [36], [37]. The HMSCs-secreted IL-10 also suppressed the myofibroblast pro-collagen gene in response to the under controlled inflammation [38].

In line with the decrease of α-SMA, there was also a significant increase in wound closure percentages on day 9 that indicated the wound healing in HMSCs-treated groups early occurred, in contrast, the α-SMA still being expressed in the control groups suggested that those group's wound healing processes still proceeded. The expression of α-SMA determines the direction of the wound healing fate due to the early and transient expressed of those is needed to accelerate the wound healing. However, the persistent a-SMA expression indicated excessively producing and contracting collagenous extracellular matrix by the myofibroblast leading to the stiff scar tissue formation [39], [40]. In this study, the increase of wound closure in HMSCs-treated groups was initiated after days 3 and gradually increased on day 6, indicated by a significant intensify in wound closure and continuously increased up to day 9. These findings suggested that HMSCs accelerate the shift of inflammation to proliferation phases than normally wound healing which greatly impacts on the initiation of cell proliferation early, particularly myofibroblast leading to wound closure acceleration [41]. This was supported by other findings, in which there was not an increase in HMSCs-treated groups regarding wound closure percentages on day 3 indicated the inflammation still occurred in those days, particularly in the previous days.

Controlling the inflammation could accelerate wound healing processes, and the HMSCs are the one robust agent to control the time-dependent inflammation. Under controlled inflammation, the proliferation phases may accomplish re-epithelialization, fibroplasia, and revascularization leading to the wound closure initiation, characterized by α-SMA expression [42]. Therefore, the expression of α-SMA controlled by HMSCs is not solely a marker for a myofibroblast activation to produce collagen associated wound closure, however also the driver of cell function and fate [43], [44]. The limit of this study is we did not analyze the TGF-β as the main activator for myofibroblast to express α-SMA leading to wound closure in addition to the IL-10, the potent anti-inflammatory cytokines. Therefore, we have no clear observation regarding the role of IL10 released by HMSCs in controlling the TGF-β associating with α-SMA expression and wound closure.

Conclusion

HMSCs have a robust ability to accelerate wound closure improvement in the proliferation phases by controlling the α-SMA expression in the myofibroblasts on a full-thickness animal model. Thus, HMSCs administration may be considered as a biological agent to promote optimal wound healing acceleration.

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