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Topical Gel of Mesenchymal Stem Cell-Conditioned Medium-induced Serum Injury Accelerates Wound Healing in Skin Excision Tissue

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ABSTRACT

Background: Umbilical cord-derived mesenchymal stem cells (UC-MSCs) accelerate wound closure by increasing VEGF and PDGF levels leading to re-epithelialization, cell infiltration, and angiogenesis. It has been found that MSC-conditioned medium (MSC-CM) can enhance the migration of fibroblasts in scratch assays. However, the effect of MSC-CM-induced serum injury (MSC-CM-S) formulated in gel to accelerate wound healing remains unclear.
Objective: This study aims to evaluate the impact of several doses of topical gel of MSC-CM-S in accelerating wound healing.
Methods: The MSCs were cultured medium-supplemented serum injury of wounded rat (8:1) to get MSC-CM-S. The topical gel of MSC-CM-S was made by base gel supplemented with MSC-CM-S. Eighteen Wistar rats were randomly assigned into control (C) and treatment groups (T1, T2). Groups received serum-free medium gel (C), 25 µl MSC-CM-S in topical gel (T1), and 50 µl MSC-CM-S in topical gel (T2), twice daily for nine days. PDGF and VEGF level and fibroblast density were measured by ELISA and HE staining on days three and six, respectively.
Results: This study showed a significant increase in the level of VEGF, PDGF and fibroblast density on days three and six. The T2 led to optimum enhancement level of VEGF, PDGF and fibroblast density.
Conclusion: Topical gel of MSC-CM-S was effective to accelerate wound closure by enhancing PDGF and VEGF levels in full-thickness skin defect rats.

Keywords: skin excision tissue, PDGF, UC MSCs-CM, VEGF, wound healing

INTRODUCTION

Chronic non-healing wound has been a significant clinical problem worldwide, which is the conventional available treatment only achieves a 50% healing rate and quick result[1,2]. Recent study reported that MSCs transplantation may provide a novel approach in accelerating the wound healing process[3-5]. Exogenous populations of MSCs delivered either systemic or direct target may produce cytokines to stimulate endogenous stem cells for regeneration in injury area in addition to differentiation and trans-differentiation[6]. However, MSCs have several limitations such as the complexity requirements of cells culture and a small percentage of cells surviving in damaged skin tissues post cell transplantation[7]. Several studies reported that the regeneration mechanism of MSC particularly due to soluble molecule released by MSCs known as paracrine mechanism[5]. Therefore, new approach in tissue regeneration using soluble molecule released MSCs is needed.

Mesenchymal stromal cells (MSCs) are defined as a stromal multipotent cell that are mostly used in regenerating several tissue damages, particularly in wound healing. MSCs have three main criteria, they must easily adhere to plastic flask; express CD105, CD90, CD73, and have lack expression of CD45, CD14, CD34, CD11b, and CD31 and HLA-DR; and may differentiate into osteocytes, chondrocytes, and adipocytes under certain conditions[8]. They can be isolated from the umbilical cord, bone marrow, adipose, and other tissue sources. Thereto, umbilical cord-mesenchymal stem cells (UC-MSCs) represent an alternative source of MSCs, due to noninvasively collected and still have pluripotent characteristic[9].

Several studies reported that the problem of non-healing wounds is impairment in the production of soluble molecules produced by local inflammatory cells and fibroblasts[2]. Under inflammation conditions, MSCs may release a variety of soluble molecules, such as anti-inflammatory IL-10 and TGF-β or proliferation molecules, particularly VEGF and PDGF[10,11]. These facts suggest that the soluble molecules released MSCs into the culture medium, known as a conditioned medium (CM), might be one of the effective ways in wound healing treatment. The previous study has shown that MSCs-CM under TNF-α stimulation may secrete large quantities of soluble mediators and develop their immunosuppressive potential, however, the involvement of exogenous TNF-α may trigger allergic reactions. These are due to most exogenous TNF-α also released by mast cells and eosinophils related to allergic mechanism[12,13]. The injury serum reported contains several pro-inflammatory cytokines, particularly TNF-α.

Therefore, activating MSCs in producing soluble molecules autogenously using injury serum may be more tolerated. However, the effect of MSC-CM-S with autologous serum injury in accelerating wound healing remains unclear. In this study, we investigated the therapeutic effects of several doses of topical MSC-CM-S with serum injured in accelerating wound healing by measuring VEGF and PDGF levels as well as fibroblast density on days three and six.

METHODS

Experimental animals

This post-test-only control group design study was conducted in the Stem Cell and Cancer Research Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung (Unissula). This study used four- to six-month-old Wistar rats that weighed 250-300 g, which were healthy and certified by the Agricultural and Fishery Service (Dinas Pertanian dan Perikanan) of Salatiga City under no. 524.3/0211/421. The rats were housed in the standardized cage at 23-26°C, with 50-60% humidity and 12 hours light-dark cycle. The rats were also adapted for seven days and observed for health and behavior changes before treatment. Eighteen rats were randomly divided into three groups, 50 µL serum-free medium gel (C), 25 µl MSC-CM-S in topical gel (T1), 50 µl MSC-CM-S in topical gel (T2) groups. All dying animals in the experimental interventions during the study were dropped out. The excision model rats were topically applied with the MSC-CM-S twice daily for six days. All research activities were followed and approved by the Commission on Test Animal Ethics (Komisi Etik Hewan Uji), Faculty of Medicine, Universitas Islam Sultan Agung, Semarang, under No. 204/VI/2017/Komisi Bioetik.

Surgical procedure

Rats were anesthetized by isoflurane inhalation, then the dorsal skin was shaved and cleaned with a tincture of iodine. One full-thickness circular 6 mm biopsy punch excision was made. Animals in the control group were topically applied with 50 µL serum-free medium gel in the wound area. On the other hand, rats on the T1 and T2 topically received 25 µl and 50 µl MSC-CM-S in a topical gel, respectively.

MSCs isolation, characterization and differentiation analysis

The umbilical cords of a female rat at 19 days of pregnancy were washed in PBS. The umbilical blood vessels were removed, then the umbilicus was cut into 2-5 mm in length using a sterile scalpel, and the sections were distributed evenly on T25 flasks. The medium used was 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 ^{o}C with 5% CO_2. The medium was refreshed every 3 days.

MSCs at the fourth passage were analyzed by flow cytometry. fluorescein allophycocyanin (APC)-, isothiocyanate (FITC)-, phycoerythrin (PE) and peridinin-chlorophyll-protein (perCP)-Cy5.5.1- conjugated anti-rat CD90.1, CD29, CD31, and CD45 antibodies (BD Bioscience, San Jose, CA, USA), respectively. Analysis was performed using BD Pharmingen™ (BD Bioscience, Franklin Lakes, NJ, USA) at 4°C for 30 min. The cells were washed and resuspended with PBS and analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA).

The MSCs were cultured in a standard medium containing DMEM with 10% FBS, 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) and 0.25% amphotericin B at 37°C and 5% CO_2 until reaching 95% confluency. Then the standard medium was replaced using an osteogenic differentiation medium containing Rat MesenCult™ Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore) with 20% Rat MesenCult™ Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore), 1% L-glutamine (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.25% amphotericin B. On the other side, for inducing adipogenic differentiation, the UC-MSCs-like with 95% confluency was cultured using an adipogenic differentiation medium made of Rat MesenCult™ MSC Basal Medium (Stem Cell Technologies), Rat MesenCult™ Adipogenic Differentiation Supplement (Stem Cell Technologies), 1% L-Glutamine (Gibco™ Invitrogen), 1% penicillin/streptomycin (100 U/mL; respectively) (Gibco™ Invitrogen) and 0,25% amphotericin B (62.5 µg/mL) (Gibco™ Invitrogen). The medium was changed every three days. After 21 days of incubation, alizarin red and oil red o solution were added and the cells to analyze the calcium and lipid deposition. The cells were incubated for 30 minutes at room temperature, then rinsed four times with distilled water.

Preparation of Conditional Medium (CM) and gel conditional medium

MSCs ($1x10^4$ cells/well) were cultured in a medium that was supplemented with an injured serum of wounded rats (8:1) (Sigma-Aldrich, Louis St, MO), then incubated for 24 hours at 37 ^{o}C with 5% CO_2. MSCs-CM was then collected after 48-hour incubation. The collected MSCs-CM was centrifuged at 2000 rpm for five minutes to remove cell debris. In addition, the DMEM serum-free medium was similarly processed for use as a control. Then, the gel conditional medium was made by supplementing the base gel with the conditional medium.

ELISA assay

The PDGF and VEGF were analyzed using ELISA kits (Fine test, China). The assay was performed at room temperature according to the manufacturer's instructions. The ELISA plate was coated with capture antibody and incubated overnight at 4 ^{o}C. The wells were washed and then blocked for an hour. They have incubated with rat PDGF and VEGF standard solution and the intervention and control rat sera for two hours, then diluted to 1:100 so that the PDGF and VEGF concentrations could be determined on the standard curves. After washing, the wells were incubated with a detection antibody for one hour, then rewashed several times. The wells were incubated with Avidin-HRP for 30 minutes, washed thoroughly, and incubated with substrate solution for 15 minutes, which was followed by the

addition of the stop solution. The PDGF and VEGF levels were analyzed at a wavelength of 450 nm using a microplate reader on days three and six.

Histologic evaluation of fibroblasts

Wound tissues were fixed in formalin and blocked by paraffin. Horizontal sections were taken from each paraffin block. Haematoxylin and eosin (HE) staining was performed after dissolving the paraffin substances by processing xylene for 1 min. Fibroblasts were evaluated in each group by the pathologist.

Statistical analysis

The amount of PDGF and VEGF levels, as well as Fibroblast density, were presented descriptively. Data processing was performed using SPSS 23.0 for Windows. The test of normality used the Shapiro-Wilk test and the test of homogeneity used Levene's statistical test. Subsequently, a parametric difference test was performed followed by one-way ANOVA and LSD posthoc test.

RESULTS

MSCs Differentiation and Characterization

MSCs was cultured based on the plastic adherent capacity under standard condition. The cells showed spindle shape and fibroblast-like characteristics (Figure 1 (a)). To characterize the multi-potency of MSCs, we performed a differentiation assay into osteogenic for 27 days under the differentiation medium. The differentiation assay was identified as the red color of calcium (Figure 1(b)) and lipid deposits (Figure 1(c)) by Alizarin Red staining. From flow cytometry analysis, the MSCs positively expressed CD90.1 (99.5%), CD29 (96.1%) and lacked the expression of CD45 (1.3%) and CD31 (6.6%) (figure 1(d)). From this result, we may conclude successful culturing MSCs and ready to be used.

Topical gel of MSC-CM-S enhances the PDGF and VEGF level in serum of rats with skin excision

The PDGF and VEGF levels were measured from the blood sample of rats on days three and six by ELISA. On day three, the level of PDGF in the T2 group was significantly increased compared to the C group. The T2 concentration was noted at 361.49±19.24 pg/ml. There was also a significant increase in PDGF levels on day six in all treatment groups compared C group. The concentration of T1 and T2 were noted at 409.38±17.94 and 408.90±28.65 pg/ml, respectively (Figure. 2A). On the other hand, we also measured VEGF level by ELISA on days three and six. On day three, the level of VEGF in all treatment groups was significantly increased compared to the C group. The VEGF level of T1 and T2 were noted at 78.20±1.30 and 95.60±1.95 pg/ml, respectively. The VEGF level on day six was also significantly increased in all treatment groups compared to C group. VEGF levels of T1 and T2 were noted at 102.60±2.30 and 408.90±28.65 pg/ml, respectively (Figure. 2B).

Topical gel of MSC-CM-S enhances fibroblast density in wound area of rats with skin excision

The fibroblast density on days three and six were evaluated from the wounded area of rats. On day three, the fibroblast density of all treatment groups was significantly increased compared to the C group ($p<0.05$). The fibroblast density of T1 and T2 groups were noted at 16.2±0.84 and 18.2±0.75, respectively. On day six, the fibroblast density of T2 group was also significantly increased compared to C group. The fibroblast density of T2 groups was noted at 22.2±0.83 (Figure 3).

DISCUSSION

Several studies reported that one of the mechanisms of MSCs in promoting wound healing by the release of soluble molecules, known as paracrine action[14-18]. MSCs must be activated by several conditions, such as TNF-α stimulation, to produce several soluble molecules. However, exogenous TNF-α may induce allergic reactions. Therefore, it is crucial to develop a new approach to creating autologous soluble molecules using injury serum that possibly contains TNF-α. This study performed a punch biopsy to make skin excision animal models according to the previous studies[6,14]. We used a topical gel formulation containing 25 µL and 50 µL MSC-CM-S induced serum injury and base gel.

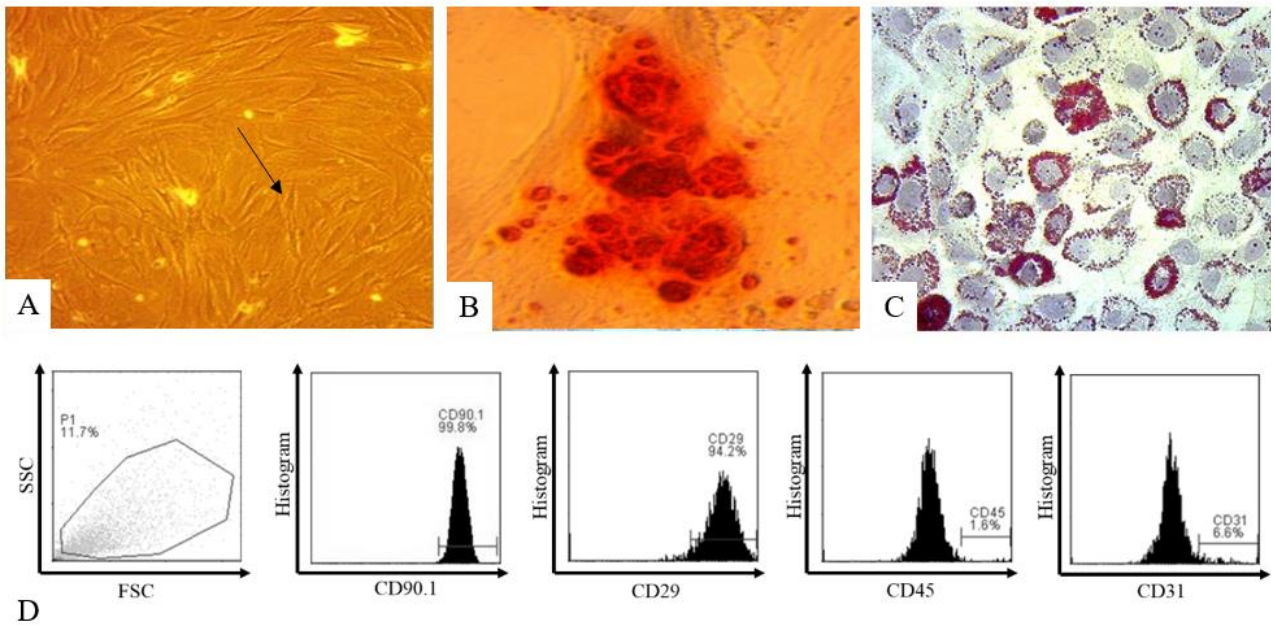

Figure 1. The characteristics of UC-MSCs. UC-MSC-like from the in vitro culture showed fibroblast-like cells and polygonal shape, with 100x magnification showed in arrow (A); the osteogenic (B) and adipogenic (C) differentiation test with Alizarin Red and Oil Red O Staining appears red color in MSC population. The characteristic of UC-MSCs expressed CD90.1 and CD29, and lacked the expression of CD45 and CD31 (D).

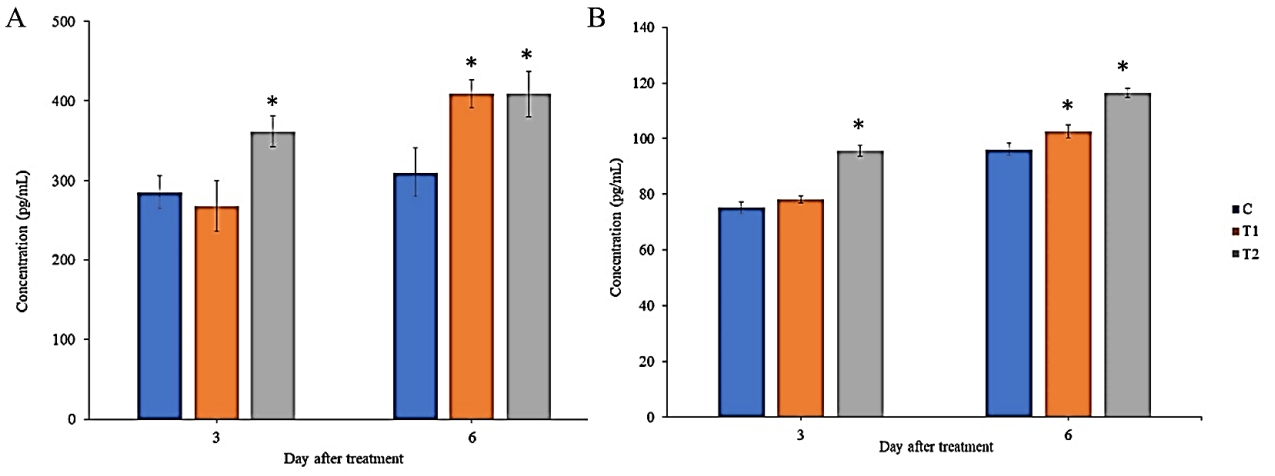

Figure 2. Quantitative measurement of PDGF and VEGF level on serum of skin excision model treated with MSC-CM-S. The PDGF (a) and VEGF (b) level of T1 and T2 groups on days three and six were analyzed by ELISA (* $p<0.05$).

Our result showed a significant increase in PDGF levels in T2 and VEGF levels T1 and T2 on day three compared to the C group. These suggest that there was a proliferation phase on day three post-MSC-CM-S treatment due to soluble factors such as IL-10 in MSC-CM-S may accelerate the shift of inflammation to the proliferation phase. The under-controlled inflammation may induce the activation of several cells to regenerate injured areas, particularly fibroblasts. The activated fibroblast may secrete PDGF and VEGF to accelerate wound healing. This is under the previous study reported that MSC activated by TNF-α may produce IL-10 and TGF-β as anti-inflammatory cytokines[19]. The increase of PDGF and VEGF is time-dependent and parallel with the acceleration of wound healing. These are in line with our study that we also found a significant increase in PDGF and VEGF levels on day six. Theoretically, PDGF may activate fibroblast to produce collagen in tissue regeneration, through the activation of phosphoinositide 3-kinase (PI3K) and p38 mitogen-activated protein kinase (MAPK) pathways[12]. On the other side, VEGF plays a crucial role as a mitogen in regulating endothelial cell survival and proliferation through PI3K/Akt pathway activation. This mechanism could enhance the expression of endothelial nitric oxide synthase (eNOS) and promote angiogenesis[12,20,21]. The increased level of PDGF and VEGF indicated that the MSC-CM-S could lead the shift of milieu from inflammation to the proliferation phase rapidly.

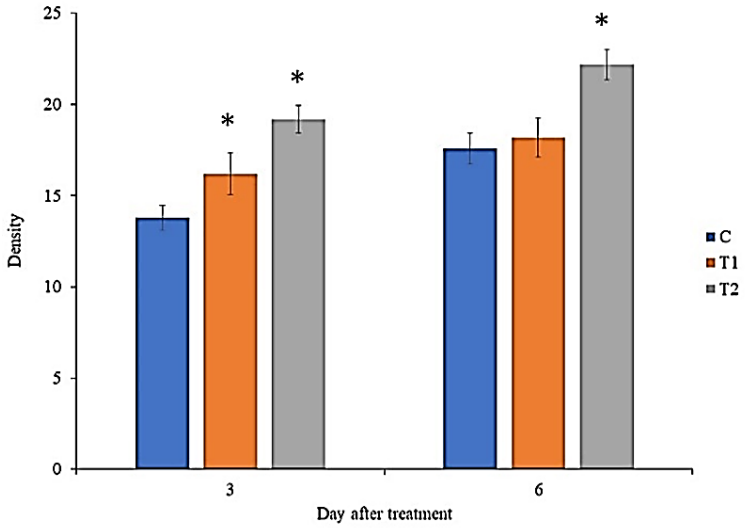

Figure 3. The analysis of fibroblast density analysis on skin excision tissue treated with MSC-CM-S. Fibroblast density of T1 and T2 groups on days three and six were analyzed using Haematoxylin-Eosin staining (*p<0.05).

In this study, we also found a significant increase in fibroblast density in T1 and T2 on day three, and in T2 on day six compared to the C group. These are in line with our previous findings concerning the increased PDGF and VEGF levels on days three and six. These suggest that MSC-CM-S could promote the release of PDGF and VEGF in injury areas that serve the proliferation induction of fibroblasts and release collagen for wound healing besides as chemotaxis of neutrophils, monocytes and fibroblasts. This is in line with our previous studies that reported that MSC-CM from TNF-α activation could accelerate wound closure of skin defect[22,23]. The MSC-CM from serum injury performed more acceptable inflammatory conditions to MSCs compared to the TNF-α precondition. Numerous inflammatory molecules contained in serum injury could provide more excessive inflammatory milieu to MSCs leading to the abundant release of anti-inflammatory molecules and growth factors[24]. These imply that the presence of MSC-CM-S may accelerate wound closure by promoting dermal fibroblasts by activating various stromal cells, including fibroblasts and endothelial cells in addition to re-epithelialization (Figure 4). Despite the positive potency observed, there are several limitations in this study. The MSC-CM-S molecules, epithelization and collagen level were not observed. The collagen fraction would give us a better understanding of wound healing.

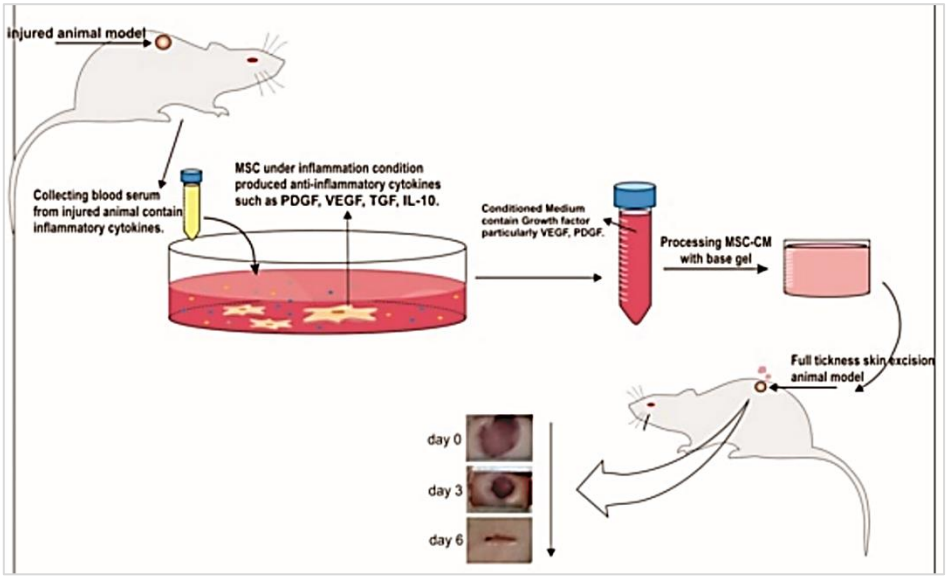

Figure 4. Schematic mechanism of topical gel of MSC-CM pre-conditioned with serum from an injured animal model.
The serum that contains proinflammatory cytokines could polarize MSCs from type-1 proinflammatory into type-2 anti-inflammatory. The type-2 MSCs could release several anti-inflammatory molecules and growth factors to the medium, called MSC-CM-S. The abundant concentration of these molecules was collected and processed into the gel. The gel contained MSC-CM-S could activate the fibroblast cells, induce collagen formation and accelerate wound healing.

CONCLUSION

The MSC-CM-S have successfully accelerated wound healing in the skin excision tissue rat model. The MSC-CM-S could enhance PDGF and VEGF levels as well as fibroblast density in the wound healing process. The MSC-CM-S displayed promising therapeutic potential for healing acceleration of skin excision tissue. Further researches are necessary to further explore therapeutic applications of MSCs in patients with skin excision.

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AUTHOR CONTRIBUTION

I.A. conceived and designed the experiment and drafted the manuscript, and P.S. performed critical revision of the manuscript for important intellectual content. M.A.B. and S.A.H. experimented with these studies. F.H. and H.A.S. analyzed the data.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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