

# The Role of Hypoxic Mesenchymal Stem Cells Conditioned Medium in Increasing Vascular Endothelial Growth Factors (VEGF) Levels and Collagen Synthesis to Accelerate Wound Healing

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## Abstract

Full-thickness wound are areas damage of skin associated with loss of epidermis and dermis. The wound healing mechanism consists proliferation, migration and remodeling. Hypoxic conditional medium of mesenchymal stem cells (HMSCs-CM) contains lots of soluble molecules, such as protein growth factor and cytokine anti-inflammation. The soluble molecule of HMSCs-CM plays a critical role in wound healing by upregulation of VEGF and collagen synthesis. The objective of this study was to evaluate the effect of HMSCs-CM on VEGF and collagen concentrations in rats with incised wounds. The methods of this study were an experimental animal study with post-test only control group design was performed involving 24 Wistar rats. The rats were randomized into four groups consisting of sham, control and two treatment groups (gel of HMSCs-CM at doses of 200  $\mu$ L and 400  $\mu$ L). The VEGF levels and collagen density were analyses using ELISA assay and Masson-trichome specific staining, respectively. One-way ANOVA and Post Hoc LSD were used to analyses the data. The results of this study showed that a VEGF levels was significant increased on day 6 with doses-dependent manner. Interestingly, the VEGF levels gradual decrease on day 9. In addition, the decreased of VEGF levels on day 9 in this study in line with our findings in which we found there was a trend in the decreased of collagen density, it indicated the completion of remodeling phase and there has been an acceleration in wound healing. This study demonstrated that HMSCs-CM were able to regulate VEGF levels and collagen synthesis in accelerate wound healing. The role of HMSCs-CM stimulate cutaneous wound healing should be clarified further.

**Keywords:** *hypoxic conditional medium of mesenchymal stem cells (HMSCs-CM), vascular endothelial growth factor, collagen synthesis, paracrine factors*

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## INTRODUCTION

Wound healing process is mediated by multiple steps including cells proliferation, extracellular matrix deposition, angiogenesis, and remodeling (Eming, *et al.*, 2014). Enhancement production of collagen and angiogenesis is one of the crucial factors contributing to the optimum wound healing (Falanga, 2005; Wang, *et al.*, 2016). The transforming growth factor  $\beta$  (TGF- $\beta$ ) and platelet derived growth factor (PDGF) have a crucial role in activating fibroblast leading to enhancement collagen disposition. There was association between the increase of vascular endothelial growth factor (VEGF) with fibroblast proliferation and collagen synthesis (Lu, *et al.*, 2010). Recently, mesenchymal stem cells under hypoxic condition (HMSCs) have an ability to secrete several signaling molecules such as VEGF, TGF- $\beta$ , and PDGF to the medium culture known as HMSCs-conditional medium (HMSCs-CM) that associated with wound healing acceleration (Wu, *et al.*, 2007). However, the HMSCs application have limitation because of poor engraftment and survival of HMSCs <1% at the injured site (Eggenhofer, *et al.*, 2012; Lee, *et al.*, 2009; Song, *et al.*, 2012). Therefore, investigating the paracrine molecules released by HMSCs to increase VEGF level associated with collagen enhancement leading to optimum wound healing is a crucial to be explored.

HMSCs-CM has been shown enhance the quantities of paracrine factors leading to the optimum tissue healing improvement (Putra, *et al.*, 2019; Yustianingsih, *et al.*, 2019). The one mechanism healing of HMSCs-CM is to promotes the enhancement of angiogenesis and re-epithelization in injured tissues, that is in part mediated by several soluble factors such as VEGF (Muhar, *et al.*, 2019; Xia, *et al.*, 2018). Furthermore, a previous study has reported that HMSCs-CM also have a ability to induce chemotaxis and angiogenesis regarding accelerated tissue regeneration (Quade, *et al.*, 2020). The VEGF is one of the most important

proangiogenic mediators in addition fibroblast proliferation associated with collagen synthesis that is an essential to induce wound healing at any stage of healing process (Goodarzi, *et al.*, 2018; Johnson & Wilgus, 2014).

Recently, some studies showed VEGF could stimulate vascular permeability, adhesion molecule expression, and granulation tissue formation to strengthen the healed wound (Johnson & Wilgus, 2014; Putra, *et al.*, 2018). In regard to mediate a collagen synthesis, the VEGF used the PI3K/Akt pathway to activate fibroblast into myofibroblast (Lu, *et al.*, 2010). These facts suggest that there is a correlation between VEGF with the active fibroblast to produce collagen synthesis leading to the optimum wound healing. On the other hand, HMSCs-CM containing several signaling molecules particularly VEGF has been shown induce the wound healing acceleration (Fu, *et al.*, 2006; McFarlin, *et al.*, 2006; Wu, *et al.*, 2007). However, how the HMSCs-CM increase VEGF levels and collagen synthesis to accelerate the wound healing process remains unclear. Therefore, in this study we examined the effect of HMSCs-CM in increasing the VEGF levels and collagen synthesis associated with the optimum wound healing in rat full-thickness wound model.

## METHODS

### The Induction of Full-thickness Wound Model

Twenty-four wistar rat (four to six-month old, male, body weight 250-300 grams) were obtained from local farmer at the Java Rat Labs, Tembalang, Semarang, Indonesia. The full-thickness wound model was generated as described previously (Kuntardjo, *et al.*, 2019). In brief, after the hair removal from the dorsal surface and anaesthesiae 6- mm full- thickness skin wounds were created on one side of the midline. The animals were randomly divided into four groups: sham group (n=6) was not treated and the control (n=6) was under NaCl administration, whereas for

T1 (n=6) and T2 (n=6) groups treatment were received one topical gels administration at the same day of the full thickness skin wound induction with two different concentration of HMSCs-CM (200 $\mu$ L and 400 $\mu$ L) respectively, diluted in 0.1 gram of gels. All research activities were in accordance to 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/2019/Komisi Bioetik.

### MSCs Isolation and Characterization

MSCs were obtained from umbilical cord of 19 days pregnancy of female rats based on our previous study (Nugraha & Putra, 2018). The umbilical cord was minced into small pieces (2-5 mm) and cultured in DMEM (Sigma-Aldrich, St. Louis, MO, catalog #D6046-500ML) enriched with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, catalog #Gibco™ 26140079, 100 IU/mL penicillin/streptomycin (GIBCO, Invitrogen, catalog #Gibco™ 15140122) incubated at 37 °C with 5% CO<sub>2</sub>. The medium was refreshed one in 3 days. The characteristics of MSCs were analyzed by flowcytometric analysis at the fourth passage. The cells were subsequently incubated at room temperature and in the dark with fluorescein isothiocyanate (FITC)-conjugated, allophycocyanin (APC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies, Per-CP, including CD73, CD90, CD105, and lineage negative. The analysis used BD Pharmingen™ (BD Biosciences, Franklin Lakes, NJ, USA) at 4°C for 30 min. The cells were washed twice with 1% BSA/PBS, resuspended in 200  $\mu$ L 1% BSA/PBS and analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA).

### MSCs Differentiation Assay

The MSCs were grown in culture dishes at densities of 5x10<sup>3</sup> and 1x10<sup>4</sup> cells/well, to which was added osteogenic induction medium containing 10 mmol/L  $\beta$ - glycerophosphate, 10<sup>-7</sup> mol/L/0.1  $\mu$ M dexamethasone, 50 $\mu$ mol/L ascorbate-2-phosphate

(Sigma-Aldrich, Louis St, MO) and 10% FBS in DMEM. Osteogenic differentiation was observed by Alizarin Red staining after 21 days to examine calcium deposits. The cells were rinsed in PBS and fixed with cold 70% ethanol (v/v) for 1 hour at room temperature, then rinsed three times with twice-distilled water. A volume of 1 mL 2% Alizarin Red solution (w/v) (pH 4.1-4.3) was added and the cells incubated for 30 minutes at a room temperature, then rinsed four times in twice- distilled water (Putra, *et al.*, 2018).

### Collection and Analysis of HMSCs-CM

Once MSCs reached 70% confluence in 75 cm<sup>2</sup> flask containing complete medium, they were washed twice with 5 mL PBS and incubated in DMEM (Gibco, USA) and then placed in a hypoxic chamber (Anaerobic Environment; ThermoForma, Waltham, MA, USA) containing 15 mL DMEM for 24 h. The airtight humidified hypoxic chamber was maintained at 37°C and continuously supplied with 5% CO<sub>2</sub>, 10% H<sub>2</sub>, and 85% N<sub>2</sub>. The oxygen level in the chamber was ~0.5%. After incubation, HMSCs-CM was collected and centrifuged twice at 1,500 rpm for 3 min at 4 °C to eliminate debris and dead cells. Paracrine factors secreted-HMSCs was analyzed under flowcytometry including TGF- $\beta$  (BD Biosciences, catalog #560429), IL-6 (BD Biosciences, catalog #558276), IL-12 (BD Biosciences, catalog # 560154), and IL-10 (BD Biosciences, catalog #558274).

### HMSCs-CM Treatment to Full-thickness wound model

HMSCs-CM gel was made by supplementing the 0.1 gram of base gel (Filalite gelcreme fagron, catalog #3079670) with the HMSCs-CM in two several concentrations of 200 $\mu$ L and 400 $\mu$ L. To concise gels, briefly the HMSCs was stirred with 0.1 gram of base gels until homogeneous.

The full-thickness rats lubricated with different doses of HMSCs-CM in topical gel. The control groups were lubricated with basis of gel,

whereas the treatment groups (T1 and T2) received the gel of HMSCs-CM 200  $\mu$ L and 400  $\mu$ L, respectively (Kuntardjo, *et al.*, 2019). The gel was applied for control and treatment groups one doses on the day of induction of the full-thickness wound.

### VEGF ELISA assay

The VEGF levels in the blood serum was measured using enzyme-linked immunosorbent assay (ELISA) (Fine Test ELISA Kit, catalog #ER0069) according to the manufacture instructions. The ELISA plate was coated with capture antibody and incubated overnight at 4°C. The wells were washed, then blocked for one hour, after which they were incubated with rat VEGF standard solution and the treatment and control groups for two hours, then diluted to 1:100 so that the VEGF concentrations could be determined on the standard curves. After washing, the wells were incubated with detection antibody for one hour, then washed again several times. The wells were incubated with Avidin-HRP for 30 minutes, washed thoroughly, and incubated with substrate solution for fifth teen minutes, which was followed by the addition of the stop solution. The results were analysed at a wavelength of 450 Å using a microplate reader. The VEGF levels (pg/mL) were measured on days 3, 6 and 9 after the HMSCs-CM administration.

### Collagen Analysis

Skin tissue of full-thickness wound area was collected at day 9 and placed in 10% neutral-buffered formalin then embedded in paraffin or

Histogel (Thermo Scientific, Watham, MA, USA). Subsequently the tissue paraffin block was cut using a microtome to a thickness of 5-10  $\mu$ m then stained with Masson Trichrome (Bio optica, catalog #04-010802 and observed under the microscope (Leica DM IL LED). The percentage of collagen density was calculated from the area of collagenous tissue formed on each slide using ImageJ (Wise, *et al.*, 2018).

### Statistical Analysis

All the statistical data were analysed in SPSS 24.0 software and evaluated using one-way ANOVA to determine the mean difference of VEGF levels and collagen density with LSD comparison post hoc test preceded by normality test. Values of  $p < 0.05$  was considered as statistically significant.

## RESULTS

### MSCs Characterization and HMSCs-CM Profile

The MSCs were successfully isolated from the umbilical cord, based on their adherence to plastic. After reaching 80% confluence, the cells were harvested for fourth passages. The isolated cells showed peculiar fibroblast-like (spindle shape) morphology assessed the marker expression of MSC using flowcytometry (Figure 1). Flowcytometry analysis showed that the MSCs cells express an MSC-specific marker including positive expression of CD105 (95.9%), CD73 (99.2%), and CD90 (99.9%) and lack of Lin (2.0%). In line with the flowcytometry analysis, we also examine oste-

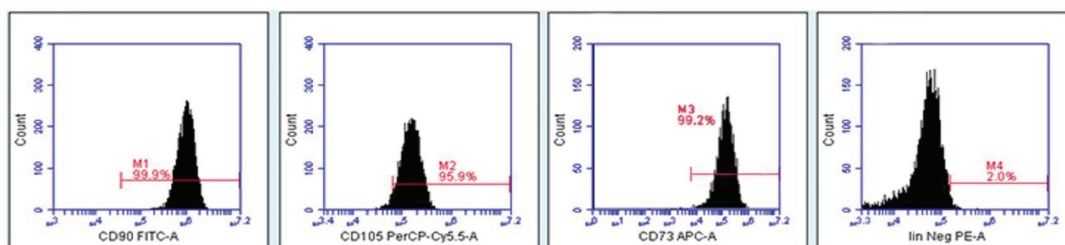
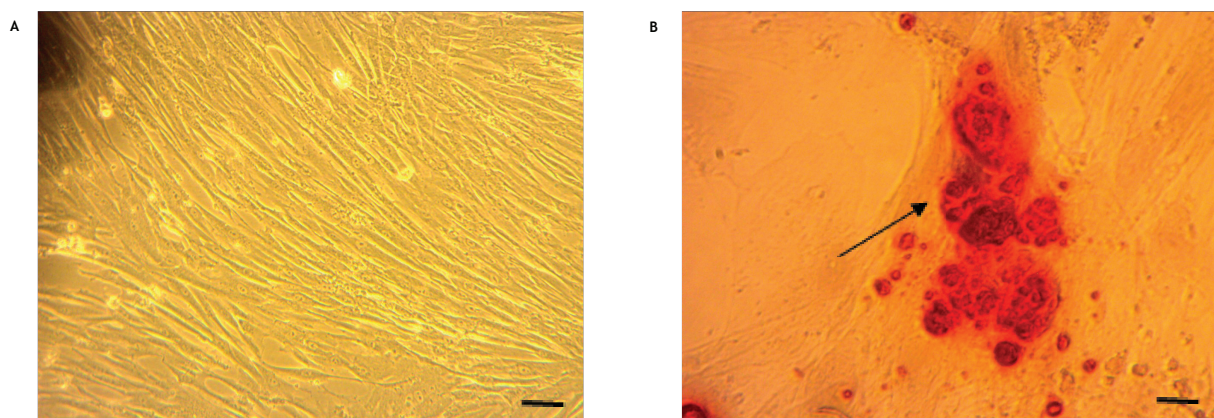


Figure 1. MSCs characterization. Graph displayed the expression MSC; positive markers (CD105, CD73, and CD90) and lack of the negative marker Lin (Lin) expression.





**Figure 2. The morphology and differentiation of MSCs.** (A) Umbilical cord MSC-like from *in vitro* culture showing fibroblast-like and polygonal cells, (B) Osteogenic differentiation, Alizarin Red staining appearing in MSC population. (magnification x10, scale bar 200  $\mu$ m).

ogenic capabilities of MSCs. In which we found that the differentiation of MSC into osteogenic was occurred that indicated by calcium deposits as red appearance using Alizarin red dye staining method (Figure 2). This is according to the International Society of Cellular Therapy (ISCT).

To induce HMSCs-CM, the MSCs was cultured under hypoxia condition with 5% O<sub>2</sub> for 24 h. Under flowcytometry analysis, we found the higher paracrine factors in HMSCs-CM compare to MSCs-CM, including VEGF, TGF- $\beta$ , IL-10, IL-6, and IL-12 (Figure 3). This clearly indicated that the HMSCs-CM significantly contains more of paracrine factor than MSCs-CM.

#### **The Increase of VEGF Levels Following HMSCs-CM Administration in Full-thickness wound**

VEGF is the main growth factors with angiogenesis capability particularly associated with the fibroblast proliferation due to its ability to increased collagen synthesis in wound healing process. To examine the VEGF levels following HMSCs-CM administration we used ELISA. In this study we found a significant increase ( $p < 0,05$ ) of VEGF levels on day 3 after topical gel at high doses

of HMSCs-CM 400  $\mu$ L administration and continuo to increase up to day 6 (24.78pg/mL $\pm$ 5.07) and 28.96pg/mL $\pm$ 11.46), respectively. However, there is no significant increase of VEGF levels at the low doses of HMSCs-CM 200  $\mu$ L on day 3. Interestingly, we also found the significant decrease ( $p < 0.05$ ) of VEGF levels in treatment groups of HMSCs-CM 200  $\mu$ L and 400  $\mu$ L on day 9 of (10.51pg/mL $\pm$ 0.91) and (9,37pg/mL $\pm$ 1,18), respectively (Figure 4). However, the control group gradually increase the VEGF levels compare day 6. This indicated that the proliferation phase was occurred on treated groups.

#### **The Increased of Collagen Density Following HMSCs-CM Administration in Full Thickness Wound**

The increase of collagen disposition indicated that the wound healing process come to the end of proliferation phase associated with wound improvement. To analyses the role of collagen in strengthening wound closure, the collagen density was stained by masson-tricome to calculate the percentage of collagen density (Figure 5A). In this study we found a significant increase of collagen density in HMSCs-CM 200 $\mu$ L and 400 $\mu$ L of 63.25% $\pm$ 9.71% and 69.75% $\pm$ 10.34%, respectively (Figure 5B).

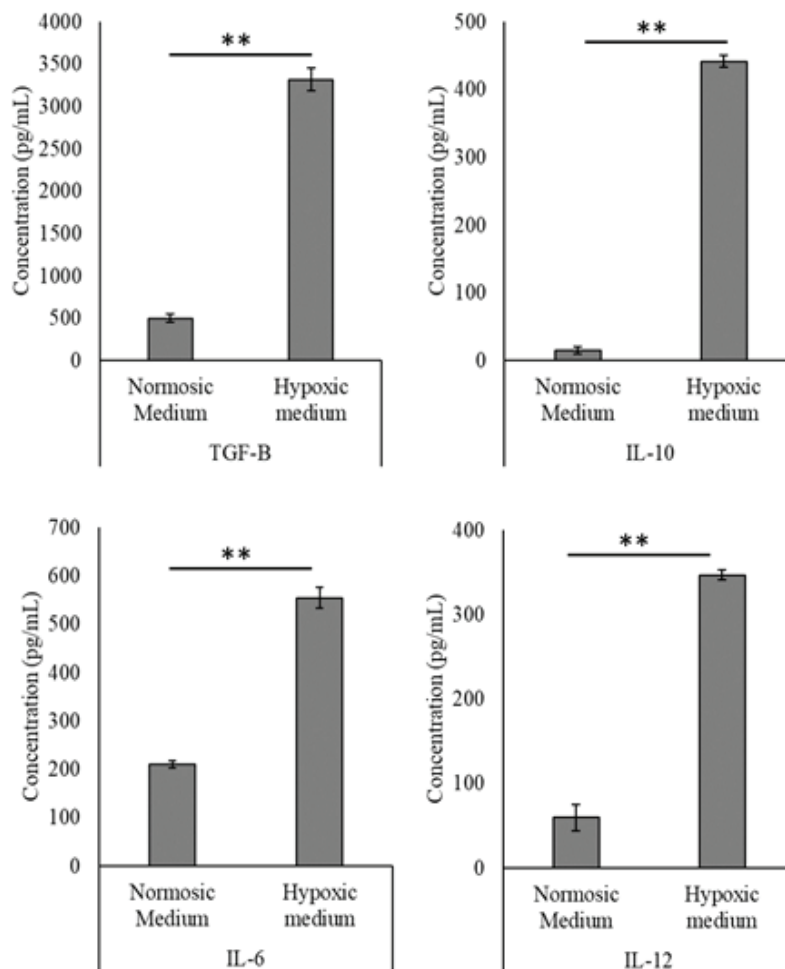
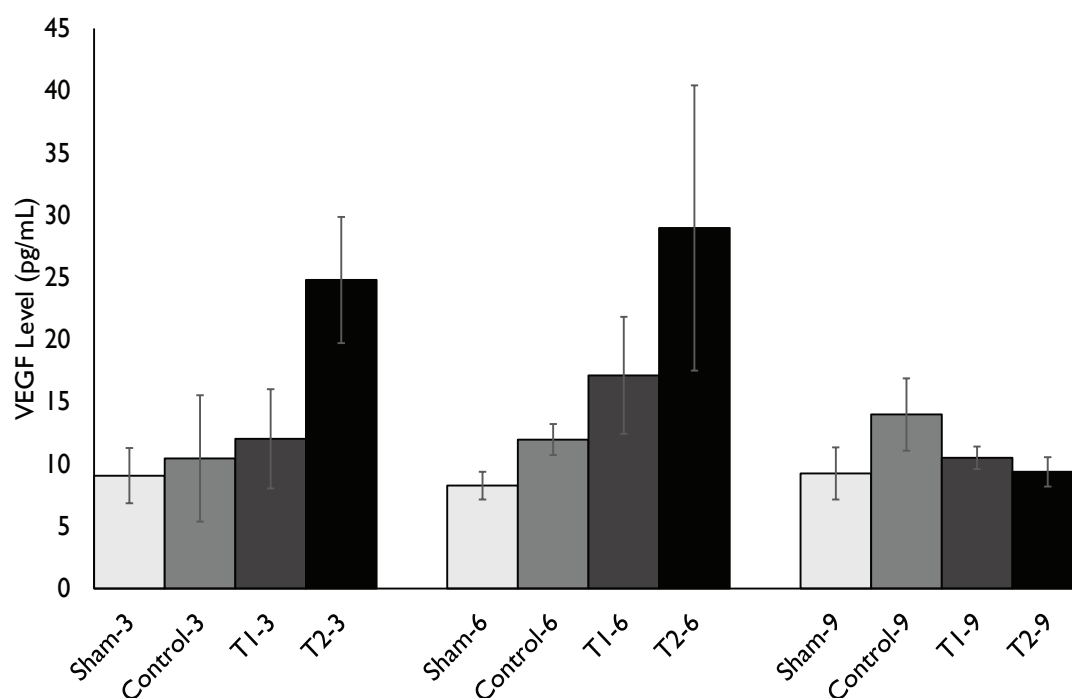


Figure 3. Levels of paracrine factors in HMSCs-CM. Total paracrine factors from the medium of HMSCs and MSCs were analyzed by flowcytometry in which all of paracrine factor of HMSCs-CM higher than MSCs-CM. TGF- $\beta$ , transforming growth factor- $\beta$ ; IL-10, interleukin-10; IL-6, interleukin-6; IL-12, interleukin-12.

## DISCUSSION

HMSCs have gain considerable attention regarding tissue repair and regeneration due to the growth factor contained HMSCs may good be superior to cell-based therapy due to its safety and perceptuality. It was reported that the HMSCs could accelerate the wound healing through enhancement of angiogenesis and collagen synthesis mediated VEGF. To answer the ability of HMSCs-CM to increase VEGF levels associated with collagen

synthesis, the full-thickness wound animal model was inducted as described previous study. In this study, the VEGF levels significantly increased on day 3 and 6 in all groups treated by HMSCs-CM in time dependent-manner. These findings indicated that the paracrine factor contained in HMSCs-CM such as VEGF, TGF- $\beta$ , and PDGF have an effect in promoting the phases shiftiness acceleration of healing process from inflammation to proliferation phases marked by the increase of collagen density associated with wound closure. However,



**Figure 4.** The effect of HMSCs-CM on VEGF levels in blood serum. VEGF levels were measured by ELISA. sham= a group without intervention; control= bases topical gel; T1= 200 $\mu$ L HMSCs-CM topical gel; T2= 400 $\mu$ L HM-SCs-CM topical gel. \*\* $p < 0,05$ .

there is no significant increase of VEGF levels at day 3 following HMSCs-CM administration at low doses, we assumed that the lack of growth factor affecting the VEGF expression. This is supported by previous study that reveal the stem cell conditioned medium significantly increased the expression level of VEGF on radiation-induced skin injury animal model by increasing angiogenesis and fibroblast proliferation (Rong, *et al.*, 2019). Other studies have reported that in normal wound healing the VEGF levels start to increase on the fourth day after injury, however in this study the VEGF levels shows an increase in day 3 (Liang, *et al.*, 2006). Indicating that the secretion of VEGF by HMSCs involvement in the accelerate the transition from inflammatory phase into the proliferative phases through polarization of proinflammatory macrophage type M1 to proliferative macrophage type M2 (Bernardo and Fibbe, 2013). Interestingly,

previous study also revealed that VEGF induces fibroblast proliferation and collagen synthesis through activation of PI3K/Akt/HIF-1 $\alpha$  signalling pathway (Lu, *et al.*, 2010). The PI3K/Akt pathway was activated upon exposure to stress, such as injury and oxidative stress (Uranga, *et al.*, 2013).

These findings supported by our study that the increased of VEGF levels in this study in line with our findings in which we found there was a trend in the increase of collagen density at day 9 under HMSCs-CM treatment. The increase of collagen along with the decrease of VEGF indicates the completion of the remodelling phase which is marked by the end of activated fibroblast into myofibroblast following HMSCs-MC administration. Active myofibroblast produces collagen matrix, this finding was confirmed by previous study stated that active myofibroblasts characterized by the excessive production of ECM proteins such as

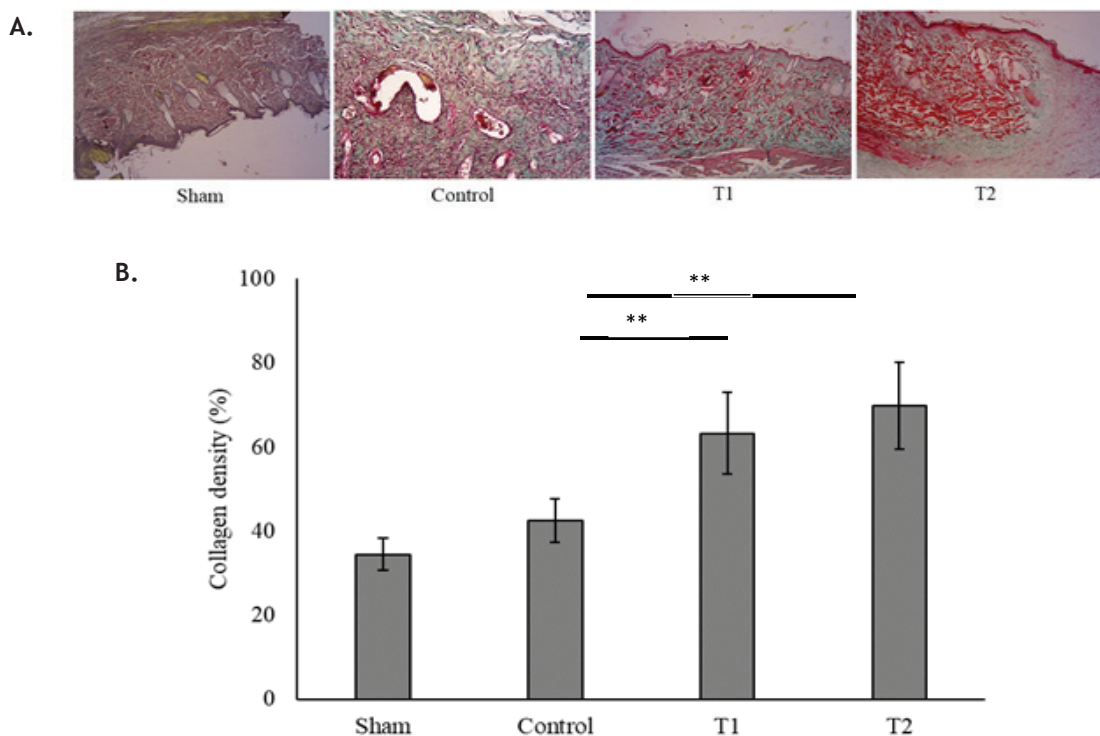


Figure 5. (A) Wound tissue was isolated at day 9 post-injury and analyzed histologically. Arrowheads indicate collagen formation (magnification  $\times 10$ , scale bar  $100 \mu\text{m}$ ). (B) Quantification of collagen density. sham= a group without intervention; control= bases topical gel; T1=  $200 \mu\text{L}$  HMSCs-CM topical gel; T2=  $400 \mu\text{L}$  HMSCs-CM topical gel.  $**p < 0,05$ .

collagen (Gibb, *et al.*, 2020). VEGF also significantly increased interleukin (IL)-10 expression, leading to myofibroblast differentiation and stimulate of excessive collagen synthesis (Al-Qattan, *et al.*, 2015; Wise, *et al.*, 2018). In addition, previous study also reported that one of VEGF mediator, nitric oxide (NO), enhances collagen deposition in diabetic wound via fibroblasts activation (Bao, *et al.*, 2009). Consistent with its mechanism, VEGF may promote healing on multiple levels. Although PDGF and TGF- $\beta$  is efficacious in cutaneous wound, VEGF may stimulate additional component of wound healing independently of PDGF and TGF- $\beta$  (Johnson and Wilgus, 2014; Smiell, *et al.*, 1999).

In summary, the administration of HMSCs-CM involves in increasing VEGF level and collagen synthesis that correlated with acceleration

in the optimum wound healing process. Unfortunately, the limitation of this study is we did not determine the concentration of other potential growth factor mediator of fibroblast activation in collagen synthesis, such as PDGF and TGF- $\beta$ . We also did not measure the inflammatory marker such as IL-10 that are a marker of the end of the inflammatory phase. So, this warrant furthers investigation in future studies. This study has the potential to becomes in the future one of the solutions to the problem of accelerating optimum wound healing.

## CONCLUSION

In conclusion, we demonstrated that HMSCs-CM administration accelerated wound healing by paracrine mechanism through increasing VEGF



level and collagen synthesis in full-thickness wound animal model. More important, HMSCs-CM has potential to be developed as a new therapeutic strategy in the future to accelerating optimum wound healing.

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