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Increased serial levels of platelet-derived growth factor using hypoxic mesenchymal stem cell-conditioned medium to promote closure acceleration in a full-thickness wound

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ABSTRACT The healing process of a full-thickness wound involves a complex cascade of cellular responses to reverse skin integrity formation. These processes require growth factors, particularly platelet-derived growth factor (PDGF). Conversely, hypoxic mesenchymal stem-cell-conditioned medium (HMSC-CM)-contained growth factors notably contribute to acceleration of wound healing. This study aims to investigate the role of HMSC-CM in controlling the serial levels of PDGF associated with accelerated wound closure in full-thickness wounds. Twenty male Wistar rats with full-thickness wounds were developed as animal models. The animals were randomly assigned to four groups, comprising two treatment groups (treated using HMSC-CM at a high dose as P1 and at a low dose as P2), a control group (administration of base gel), and sham group (healthy group). PDGF levels were examined using an enzyme-linked immunosorbent assay. Using ImageJ software, wound closure percentages were determined photographically. The study showed that there was a significant increase in PDGF levels on days 3 and 6 after HMSC-CM treatment, followed by a decrease in PDGF levels on day 9. In line with these findings, wound closure percentage also increased significantly on days 6 and 9. In the rat model, HMSC-CM administration may promote acceleration of wound closure by increasing serial PDGF levels in the full-thickness wound.

KEYWORDS healing process; HMSC-CM; PDGF; wound closure; wound healing

1. Introduction

The full-thickness wound is the loss of the cutaneous layer, which extends to the subcutaneous layer caused by several factors such as mechanical, chemical, biological, and thermal injuries (Hu et al. 2018). The healing process of a full-thickness cutaneous involves a highly organized physiological cascade of cellular responses to restore skin integrity formation (Bartaula-Brevik 2017; Riis et al. 2017). The prolonged duration of wound healing may increase the risk of chronic wound development, which contributes to morbidity, particularly in developed countries (Okur et al. 2020). Furthermore, one of the crucial phases in wound healing is the induction of fibroblast activation into myofibroblast to produce collagen synthesis associated with wound closure (Herrera et al. 2018). These processes require paracrine factors essentially transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (Hu et al. 2014). A recent study revealed that mesenchymal stem cells (MSC) under hypoxic conditions can release a massive number of growth factors including TGF-β and PDGF into a culture medium known as the hypoxic conditioned medium of

MSC (HMSC) that highly contribute to an acceleration of wound healing (Chen et al. 2014). PDGF enhances wound closure through the PI3K/AKT/eNOS signaling pathway (Wu et al. 2019). Furthermore, PDGF can block fibroblast into myofibroblasts associated with decreasing scar formation in wound closure (Mulholland 2020). Additionally, TGF-β stimulates wound contraction through induction of smooth muscle alpha-actin expression and accelerates re-epithelialization thus contributing to wound closure (Ramirez et al. 2014). This indicated that HMSC potentially induces the acceleration of wound closure in the full-thickness wound through a release of PDGF; however, the association of PDGF levels to wound closure following HMSC administration needs further explanation (Putra et al. 2019).

MSCs are derived from stromal cells with plastic-adherent and multipotent differentiation capability that can express various markers, including CD90, CD105, CD73, CD44, and CD29, and lack of other surface marker expressions such as CD45, CD34, CD14, CD11b, CD79a, CD19, and human leukocyte antigen class II (Dominici et al. 2006; Lv et al. 2014). HMSC-CM is a conditioned medium produced by MSC under hypoxic conditions containing tons of various anti-inflammatory cytokines and growth factors such as interleukin 10 (IL-10), PDGF, and TGF-β that have beneficial therapeutic effects in the optimum wound healing (Burlacu et al. 2013; Li et al. 2017; Muhar et al. 2019). Previous studies reported that PDGF act as functional paracrine and autocrine factors, which accelerate the healing process by promoting myofibroblast activation to synthesize collagen associated with wound closure (Chen et al. 2008; Riis et al. 2017). Conversely, PDGF is also a potent growth factor produced by various cells including platelets, fibroblasts, macrophages, endothelial cells, and keratinocytes, which contribute to tissue repair (Park and Kim 2017). PDGF is a responsible molecule in initiating wound healing by regulating cell growth a division as well as angiogenesis (Dehkordi et al. 2019).

A previous study reported that HMSC-CM contain substantial growth factors particularly PDGF that improve refractory wound healing by regulating fibroblast (Chen et al. 2014; Saheli et al. 2020). The beneficial effects of HMSC-CM in wound healing acceleration are designed by an ability of IL-10 contained in HMSC-CM to control inflammation; additionally, contained growth factors, particularly PDGF, are also stimulating cell proliferation to accelerate wound closure (Muhar et al. 2019; Noronha et al. 2019). Another study also reports that the HMSC-CM can enhance wound healing through an increase in vascular endothelial growth factor (VEGF) and macrophage recruitment to wound sites triggering wound closure acceleration (Chen et al. 2014). Conversely, PDGF is also a potent growth factor produced by various cells including platelets and macrophages, which contributes as chemoattracting molecules for leucocytes migration to initiate inflammation (Park and Kim 2017). These facts are supported by another study that reports that the PDGF is one of the responsible agents in initiating early inflammation besides wound healing acceleration that depends on its release time (Landén et al. 2016). Thus, the serial analyses of PDGF released by HMSC-CM in the healing phase associated with wound closure require more exploration. In this study, we investigated the role of HMSC-CM in controlling the serial level of PDGF associated with wound closure in the full-thickness wound animal model.

2. Materials and Methods

2.1. Rat umbilical cord MSC isolation and characterization

The procedures in this study were approved by the Institutional Ethics Review Board of UNISSULA University, Semarang. The isolation of MSC from an umbilical cord (UC-MSC) of 19 days pregnancy of female rat was performed using a previously described method with modification (Hamra et al. 2021). Briefly, the umbilical cord was mechanically dissected and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) contained 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) under normoxic condition. The cultured cells at passage 5 (P5) and under 80% confluence were applied for the next experiment.

The cultured cells were subjected to flow cytometry analysis to confirm MSC phenotypic characteristics. Briefly, the cultured cells were labeled with rat MSC antigens CD44, CD90, CD105, and their isotype controls (BD Bioscience, USA) and analyzed using BD accuri C6 plus. Furthermore, UC-MSC were cultured in the DMEM (GIBCO, USA) supplemented with 0.05 µM ascorbate-2-phosphate, 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 10 mM b-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 1% antibiotic/antimycotic, and 10% fetal bovine serum (Gibco, USA) to validate the differentiation capacity of a cultured cell into an osteocyte. The osteogenic medium was replaced at a 3-day interval for 21 days. Prior to Alizarin red staining, cells were washed in phosphate-buffered saline and fixed in 95% methanol for 10 min. Alizarin Red S solution 2% was added for 5 min and then rinsed with water and imaged under an epifluorescence microscope to visualize the Ca^{2+} deposits. Calcium deposition of the differentiated cell was visualized as red bright color after Alizarin red staining.

2.2. Hypoxic preconditioned medium MSC preparation

UC-MSC cultured in serum-free complete medium were incubated under hypoxia condition (5% O_2) for 24 h. The hypoxia-preconditioned medium was centrifuged at 2000 rpm at 8 °C temperature for 20 min and passed through a 0.22 µm filter membrane (Corning, USA) to remove the remaining cell debris. The cytokine profile of IL-17A, INF-γ, and IL-6 in HMSC-CM was measured us-

ing a cytometric-based assay (BD Biosciences, USA). The concentrations of IL-10, TGF-β, and PDGF in HMSC-CM were also measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA). Colorimetric absorbance was recorded at a wavelength of 450 nm. The HMSC-CM was kept at −80 °C temperature until the treatment.

2.3. Animal model and treatment

Twenty healthy adult male Wistar rats weighing 200–250 g were applied in this study. All the procedures and protocols were approved by the Animal Experiment Ethics Committee of the UNISSULA, Indonesia. The full-thickness cutaneous wound rat model was developed as described in a previous study (Hamra et al. 2021). Briefly, the rats were anesthetized via isoflurane inhalation, and the dorsal hairs were shaved off and sterilized using 10% of povidone–iodine solution. The full-thickness wound was created using a 6 mm sterile biopsy punch on the dorsal skin. They were randomly divided into four groups, namely, sham group (control untreated), control group (treated by bases gel), and two treatment groups (treated by 200 µL of HMSC-CM gel as a high dose (P1) and by 400 µL of HMSC-CM gel as a low dose (P2). The HMSC-CM gel was produced by mixing 100 mg of sterilized water-based gels with HMSC-CM.

2.4. PDGF level analysis

Blood samples were collected from the orbital sinus vein using a hematocrit tube on days 3, 6, and 9 after treatment and centrifuged at 2000 rpm for 10 min. The serum was collected and stored at −80 °C temperature. The blood PDGF concentration was analyzed using ELISA on the basis of manufacture protocol (FineTest, Wuhan, China) at a 450 nm wavelength.

2.5. Statistical analysis

Quantitative data are shown as the mean ± the standard error of the mean for at least three independent experiments. Statistical differences were determined using a one-way analysis of variance followed by the Duncan post hoc test. Significant differences were considered at $p < 0.05$.

3. Results and Discussion

3.1. MSC isolation and characteristic

The MSC showed plastic adherence capacity and fibroblast or spindle-like shape (Figure 1a). Moreover, MSC exhibits differentiation capacity into osteocyte clones presented as a bright color on Alizarin red staining (Figure 1b). The immunophenotypical analysis of surface markers MSC showed that they expressed CD44, CD90, and CD105 (Figure 1c). To induce HMSC-CM, the MSC was cultured under hypoxia condition with 5% O_2 for 24 h. Under flow cytometry and ELISA analysis, we found several paracrine factors contained in HMSC-CM, including IL-17A, TNF-α, IL-6, IL-10, TGF-β, and PDGF (Figure 2).

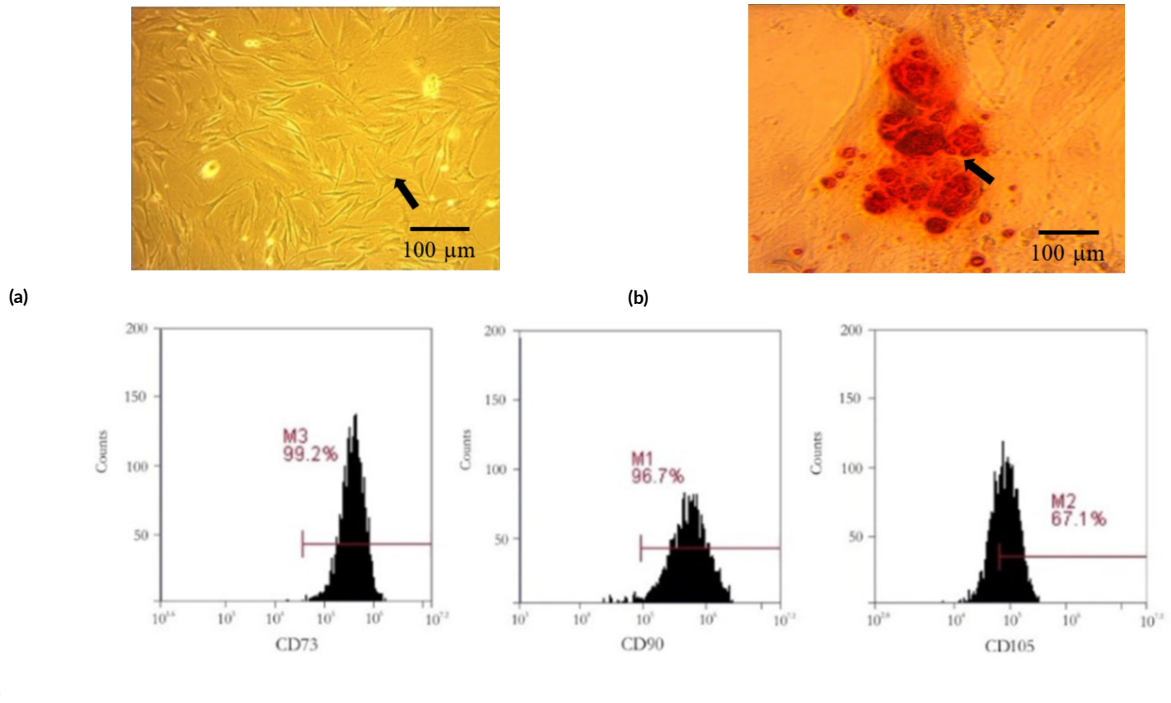

FIGURE 1 (a) MSC characterization and differentiation. The black arrow presented the fibroblast-like cells (magnification 100×, scale bar 100 µm). (b) MSC differentiation. The red bright color was marked by the black arrow in a response to the calcium deposition in osteocyte-differentiated MSC via staining by Alizarin red (magnification 40×, scale bar 50 µm). (c) The marker of MSC. Clones MSC positively expressed CD73 (99.2%), CD90(96.7%), and CD105 (67,1%).

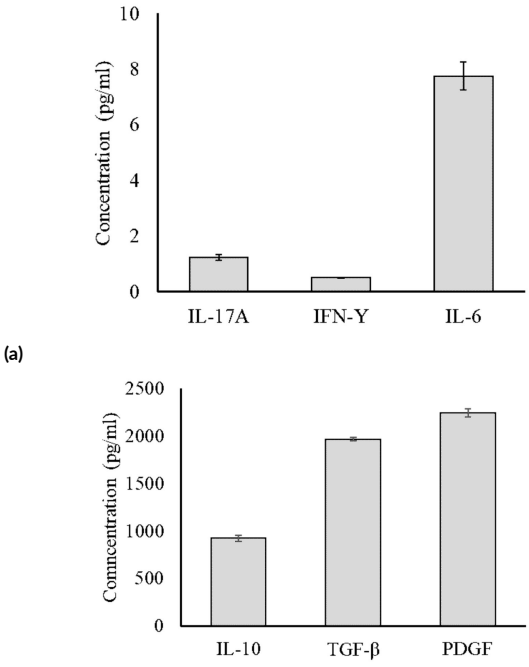

FIGURE 2 Levels of cytokine in HMSC-CM. The cytokine concentrations were determined in 24 h culture supernatants under (a) flow cytometry and (d) ELISA. Data are presented as mean ± SD. n = 3 for each group.

3.2. *HMSC-CM enhance wound healing by increasing PDGF concentration*

To investigate the roles of HMSC-CM in wound healing, two different doses of HMSC-CM were administered into the full-thickness cutaneous wound rats model 1 h post skin excision and the wound closure improvement was assessed on days 3, 6, and 9 posttreatment. Our study showed that the rat treated by HMSC-CM 400 µL showed significantly increased PDGF levels on days 3 and 6 in both groups (P1: 251.8 ± 20.4 pg/mL; P2: 291.4 ± 28.21 pg/mL; and P1: 302.2 ± 15.93 pg/mL, P2: 307.6 ± 32.56 pg/mL, respectively), followed by a significant decrease of PDGF levels on day 9 after HMSC-CM treatment (P1: 282.5 ± 20.83 pg/mL; P2: 239.8 ± 23.14 pg/mL). ($p < 0.05$; Figure 3).

3.3. *HMSC-CM improved wound closure*

To examine the wound closure improvement, we measure the wound diameter on days 3, 6, and 9 after the HMSC administration. Our study showed that rats treated by HMSC-CM promote wound closure acceleration on days 6 and 9. There was a decrease in the area of the wound on days 6 and 9 after HMSC-CM administration. (P1: 2613.25 ± 203.2 mm^2; P2: 2367.25 ± 164 mm^2; and P1: 831.75 ± 83.1 mm^2; P2: 643.5 ± 95.4 mm^2, respectively) (Figures 4 and 5).

3.4. *Discussion*

Wound healing processes require a well-integrated of numerous molecular and physiological cascades that are regulated by cytokines, chemokines, and growth factors (Ridiandries et al. 2018). The one crucial phase in wound healing is the collagen synthesis produced by activated fibroblast that is associated with the wound closure acceleration (Desjardins-Park et al. 2018). Under controlled inflam-

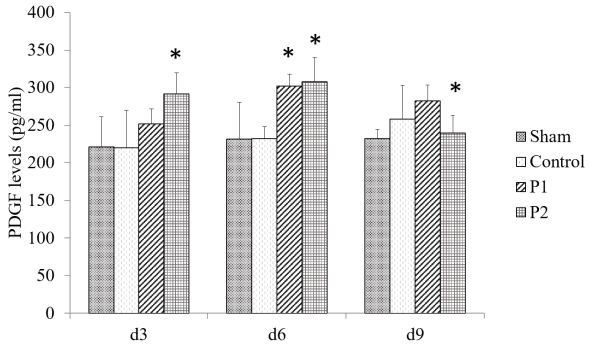

FIGURE 3 The increase in PDGF level on days 3 and 6 followed the decrease in PDGF level on day 9. Significant differences ($p < 0.05$) are marked with asterisks.

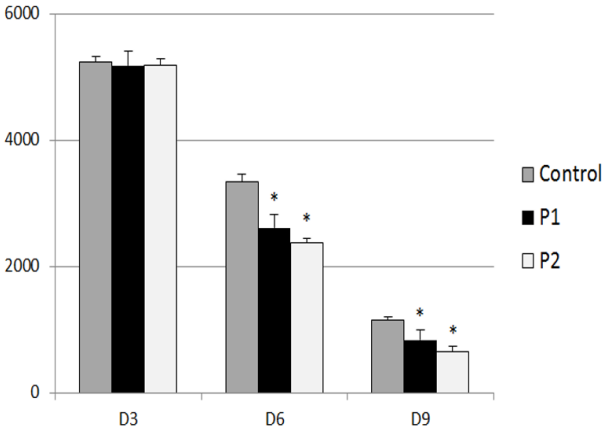

FIGURE 4 Wound closure improvement on days 6 and 9. Significant differences ($p < 0.05$) are marked with asterisks.

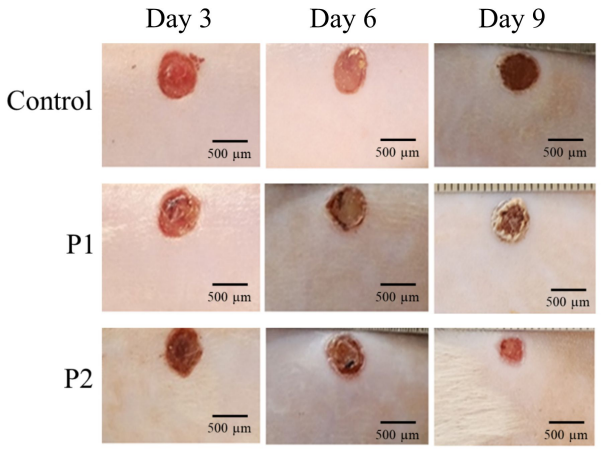

FIGURE 5 Photographically recorded wound closure improvement on days 3, 6, and 9. (a) There was no wound closure improvement in all groups. (b) The wounds in P1 and P2 at day 6 were slightly smaller than in control. (c) P1 and P2 showed better wound closure improvement than control.

mation, the macrophage-secreted PDGF actively stimulates fibroblast to produce collagen leading to wound closure (Zhang et al. 2010). Previous studies reported that MSC-CM-contained growth factors including PDGF significantly enhanced cell proliferation and regulate inflammatory niece, which affect the quality of skin regeneration (Mehanna et al. 2015; Desjardins-Park et al. 2018; Darlan et al. 2021). Conversely, PDGF released by platelets also has a role in attracting leucocyte migration to initiate inflammatory processes (Phipps et al. 2012; Xiang et al. 2019). Thus, investigating the role of PDGF regulated using HMSC-CM in accelerating wound closure on the basis of the time-dependent serial of wound healing phases is a crucial point in wound healing processes. We used the full-thickness animal model to explore the serial level of PDGF in inflammation to the proliferation phase as described by a previous study (Mehanna et al. 2015).

Our study presented the fact that HMSC-CM can accelerate wound closure by increasing serial levels of PDGF, starting from days 3 to 6 after HMSC-CM treatment. However, the increase of PDGF level at day 3 is not yet correlated with the wound closure acceleration. We supposed that the increase of PDGF on day 3 in this case is not able yet to promote the shift of inflammation to the proliferation stage that indicated by there was no improvement in the wound closure diameter. The increase of PDGF was suggested as an attractant molecule for other inflammatory cells to initiate the inflammation process (Park and Kim 2017; Putra et al. 2019). Conversely, the increase of PDGF level was also stimulated by under controlled inflammation macrophages as reported in the previous study that HMSC could release massive anti-inflammatory molecule particularly IL-10 to accelerate the shift of inflammation to proliferation phase that induce macrophage polarization into macrophage type-2 (M2) with growth factor production capability leading to wound healing acceleration (Muhar et al. 2019). This study is in line with our finding on day 6 in which there was an increase of PDGF level along with wound closure diameter improvement.

Interestingly, in this study, we found that there was a decrease of PDGF level at day 9 following HMSC-CM administrations indicating the remodeling phase of the treatment group has occurred. However, in control groups as a normal wound healing process, the PDGF level is higher than the treated group that indicated that the normal healing process is under the proliferation phase. We suggest that HMSC-CM accelerate the wound healing processes by increasing PDGF levels in the early phase of wound healing. The mechanism of PDGF in accelerating wound closure through the binding of PDGF to PDGFR results in the activation of intracellular signaling pathways including Ras/Rac, MAPK, PI3K, and STA Src, which subsequently promote cell proliferation to support new tissue formation (Kardas et al. 2020). This finding was also in line with a previous study, which revealed that in this remodeling phase, the active inflammatory cells reverse to the inactive sites leading to the decrease of most growth factors particularly PDGF (De Oliveira et al. 2016). Theoretically, PDGF activates fibroblasts to produce collagen to support wound closure improvement (Zhang et al. 2010). Under hypoxic culture conditions, MSC released a massive amount of growth factors, particularly PDGF into medium (Bartaula-Brevik 2017; Putra et al. 2019). Additionally, the TGF-β induces the secretion and autocrine regulation of PDGF by the upregulation of PDGF-A and both PDGF receptors (Fischer et al. 2007). Thus, HMSC-CM contains tons of growth factors to induce tissue repair and regeneration through paracrine mechanism particularly, by activating fibroblast to collagen-produced myofibroblast leading to wound closure acceleration (Putra et al. 2019).

As mentioned above, the acceleration of cutaneous wound healing involves several factors such as platelet to produce PDGF as an attractant molecule in the early inflammation phase, macrophage type 2 (M2) to release PDGF as a growth factor for cell proliferation, which is associated with wound closure. As the limitation of this study, we do not analyze the role of platelet and M2 as the PDGF producer in each phase, including IL-10 as an anti-inflammatory marker. Thus, the role of HMSC-CM in regulating PDGF released by platelet and M2 associated with wound closure acceleration remains unclear. We also did not analyze IL-10 as a factor inducing polarization macrophage into M2, which contributes to producing PDGF.

4. Conclusions

We conclude that TGF-β in HMSC-CM accelerates wound closure by increasing PDGF production in the full-thickness wound rat model and HMSC-CM may become the new modality to increase wound treatment.

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Authors' contributions

AP conceived and designed the study. PD, NDA and BTD conducted the experiments and analyzed the data. TN and YWP wrote the paper. All authors contributed to manuscript revisions. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests

The authors declare no conflicts of interest.

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