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The Comparison of Normoxic and Hypoxic Mesenchymal Stem Cells in Regulating Platelet-derived Growth Factors and Collagen Serial Levels in Skin Excision Animal Models

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Abstract

BACKGROUND: The healing process of a skin excisions involves a complex cascade of cellular responses to reverse skin integrity formation. These processes require growth factors particularly platelet-derived growth factors (PDGF). On the other hand, hypoxia- preconditioned mesenchymal stem cells (MSCs) could secrete growth factors that notably contribute to wound healing acceleration, characterized by the enhancement of collagen density.

AIM: This study was aimed to investigate the role of hypoxia-preconditioned MSCs in regulating the serial levels of PDGF associated with the enhancement of collagen density in the skin excision animal models.

METHODS: Twenty-seven male Wistar rats of skin excision were created as animal models. The animals were randomly assigned into four groups consisting of two treatment groups (treated by normoxia-preconditioned MSCs as T1 and hypoxia-preconditioned MSCs as T2), positive control (treated with phosphate-buffered saline) and sham (non-treated and healthy rats). PDGF levels were examined by ELISA. The collagen density was determined using Masson's trichrome staining.

RESULTS: This study showed that there was a significant increase in PDGF levels on days 3 and 6 after hypoxia- preconditioned MSCs treatment. In line with these findings, the collagen density was also increased significantly after hypoxia-preconditioned MSCs treatment on days 3, 6, and 9.

CONCLUSION: Hypoxia-preconditioned MSCs could regulate the serial PDGF levels that lead to the enhancement of collagen density in the skin excision rat's model.

Introduction

The persistent non-healing wound has been a major medical problem worldwide and the standard available treatment only achieves a 50% of healing rate and a short-term effect [1], [2]. Recent studies stated that mesenchymal stem cells (MSCs) transplantation may additionally provide a singular approach in accelerating the wound recovery processes [3], [4], [5]. Exogenous populations of MSCs, added either systemically or directly, may additionally produce diverse cytokines to stimulate endogenous stem cells for regenerating the harmed area [6], [7]. However, MSCs have a number of limitations, such as the complexity requirements of the range of available and a small percentage of cells surviving in injured skin tissues after transplantation [5], [7]. Several studies stated that the regeneration mechanism of MSCs particularly because of soluble molecules released through MSCs called the paracrine mechanism [8], [9], [10]. Therefore, a new technique for tissue regeneration employing MSCs released by soluble molecules is required. On the other hand, the current studies found that platelet-derived growth factors (PDGF) are one of the primary factors that have a pivotal impact on the wound microenvironment, main to speedy increases in cell migration, proliferation, and differentiation [11], [12]. The PDGF acts as a regulator in cell growth, cell division, and angiogenesis for facilitating chemotaxis, fibroblast proliferation, and collagen deposition leading to accelerated wound closure [13], [14]. Excluding, one individual of successful wound restore is fibroblast proliferation-stimulated by way of PDGF which lays down extracellular matrix (ECM) proteins (hyaluronan, fibronectins, and proteoglycans) and subsequently produces collagen

and fibronectin [11], [12], [13]. However, the exact mechanisms of MSCs in regulating PDGF signaling and collagen deposition are nevertheless uncertain. Therefore, this study was aimed to investigate the comparison of normoxia MSCs (N-MSCs) and hypoxic MSCs (H-MSCs) in regulating the serial levels of PDGF associated with the enhancement of collagen density inside the skin excision animal models.

MSCs are a population of pleiotropic cells with self-renewing ability that have the potential to distinguish into various canonical mesenchymal cells specifically adipocytes, chondrocytes, and osteocytes [15], [16], [17]. MSCs exhibit some of markers, together with CD29, CD44, CD73, CD90, CD105, CD166, and lack of CD14, CD34, CD45, or CD11b, CD79a or CD19, and HLA elegance II expression [6], [11], [18]. A current observation reported that hypoxia offers advantages to MSCs to grow their functionality of self-renewal, proliferation, and survival genes modulation [16]. H-MSCs can mimic a physiological niche within the bone marrow that probably offers advantages of a wound healing method [9], [19], [20]. This is consistent with the previous reports which stated that one of the therapeutic problems of administering MSCs is the ability of negative engraftment and occasional cell survival during the transplantation method in the hypoxia condition [5]. This fact confirmed that the competence of MSCs to live on underneath hypoxia circumstances in inhibiting scar formation turned into vital to be explored [21]. Furthermore, H-MSCs turned into extra strong cells to manipulate inflammation and boost up wound recuperation than N-MSCs thru growing molecules and increase aspect, together with IL-10 and PDGF [9], [11], [22]. These conditions may want to boost up the wound closure characterized through the increase of ECM deposition, mainly collagen [4].

The current research additionally confirmed that MSCs comprise numerous molecules especially IL-10 that can set off regenerative tissue repair through regulating an anti- inflammatory pathway to promote skin excision wound restoration [14], [18]. The previous research additionally found out that hypoxia-precondition can increase the synthesis of hypoxia-inducible factor (HIF-1a) thereby activating several genes involved in angiogenesis and wound healing, including PDGF and TGF-β which activate fibroblasts into myofibroblasts which are characterized by the collagen production [19], [20]. Furthermore, PDGF acts as a prime suppressor of the anti-inflammatory reaction in accelerating the shift from anti-inflammatory to proliferation phase through down-regulating the expression of the seasoned-anti-inflammatory cytokines [21], [22]. PDGF additionally has been said might induce macrophage polarization from the pro-inflammatory M1 phenotype into anti-inflammatory M2 phenotype, mainly related to a boom of TGF-β expression triggering the fibroblast activation related to the wound recovery acceleration without scarring [23], [24]. Ultimately, those statements provide direct evidence that PDGF and collagen are potential biomarkers in resolving skin tissue excision wounds. Hence, in this study, we aimed to investigate the contrast between H-MSC and N-MSC in regulating PDGF and collagen serial levels in pores and skin excision animal models.

Methods

Research design

This post-test-only control group design was carried out in the Stem Cell and Cancer Research (SCCR) Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung (Unissula), Semarang from October to December 2020. The study was approved by the experimental animal's ethics committee of the Medical Faculty of Sultan Agung Islamic University, Semarang, Indonesia.

Skin excision animal model

Thirty-six male Wistar rats weighed 200g were caged at a 22 ± 2°C and 60% relative humidity, with a 12:12-h light-dark cycle. To establish the animal model of skin excision, rats were anesthetized by isofluorane inhalation then the dorsal skin was shaved and cleaned with a tincture of iodine. One skin excision circular 6 mm biopsy punch excision was done for each rat. The animals were randomly divided into four groups; T1 treatment (n = 9) were treated by Normoxic-MSCs (N-MSCs) topically, and T2 treatment (n = 9) H-MSCs topically, while the control group (n = 9) was treated with phosphate-buffered saline (PBS) and combination of placenta extract 10% and neomycin sulfate 0.5% (Bioplacenton®) and Sham group or untreated group, Sham (n = 9) [25].

MSCs isolation culture and H-MSCs induction

MSCs were isolated from the umbilical cord (UC) obtained from pregnant single Wistar-albino rats and expanded as described previously [8]. Briefly, UCs were chopped into smaller pieces and transferred into a T25 culture flask (Corning, Tewksbury, MA, USA) containing DMEM (Gibco™ Invitrogen, NY, USA) which supplemented with 10% FBS (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL) and 0.25% streptomycin (100 μg/mL) (Gibco™ Invitrogen, NY, USA) and incubated at 37°C, 5% CO_2 and ≥95% humidity. The medium was replaced every 3 days and harvested after reaching 80% confluence (14 days). The 4–6th passage MSCs-like were employed for the experiments.

To induce H-MSCs, MSCs derived from the fourth 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 for the following experiment [26].

H-MSCs characterization by flowcytometry

H-MSCs surface markers at the 4–6th passage were analyzed by flow cytometry analysis according to company protocols. Briefly, the cells were subsequently incubated in the darkroom 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. H-MSC cells were stained with MSC-specific antibody for 30 min at 4°C, then examined and analyzed with a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA) [27], [28].

H-MSCs osteogenic differentiation assay

The H-MSCs were cultured in 24-well plate (1.5 × 104 cells/well density) with standard medium containing DMEM (Sigma-Aldrich, Louis St, MO), supplemented with 10% FBS (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL), and 0.25% streptomycin (100 μg/mL) (Gibco™ Invitrogen, NY, USA) at 37°C, 5% CO_2, and ≥95% humidity. After reaching 95% confluent, the standard medium was aspirated and replaced with osteogenic differentiation medium containing Human MesenCult™ Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), augmented with 20% Human MesenCult™ Osteogenic Differentiation 5× Supplement (Stem Cell Technologies, Singapore) and 1% L-Glutamine (Gibco™ Invitrogen, NY, USA). The differentiation medium was replaced every 3 days. The bone matrix was formed after 15 days and can be visualized by 2% of Alizarin red solution staining [29].

N-MSCs and H-MSCs administration

After the skin excision was made, the rats were treated once by 3 × 10^6 N-MSCs as T1 group and 3 × 10^6 H-MSCs as T2 group, and the control group received PBS. The interventions were ip administrated twice a day until the animal was sacrificed.

ELISA assay

On days 3, 6, and 9 after the treatment, ELISA is used to measure the level of PDGF released in rat's peripheral blood of treatment and control group. The analysis is done according to the manufacturer's instructions (Fine Test, Wuhan, China) using a standard curve and performed in five replications. The colorimetric absorbance wavelength used is 450 nm.

Macroscopic analysis

The induced skin isolated was euthanized using CO2 inhalation on the 3rd, 6th, and 9th day after the treatment. The tissue is taken by cutting the skin around the wound area with a size of 2 × 2 cm. Tissue samples were fixed by immersing in 10% formalin for 24 h and embedded in paraffin. The tissue paraffin block was cut using a microtome to a thickness of 5μm then stained with Masson Trichrome (Bio Optica, catalog #04010802) and observed under the light microscope (Olympus CX21, Tokyo, Japan). The percentage of collagen density was calculated from the area of collagenous tissue formed on each slide using Image J (30).

Statistical analysis

The statistical analysis was performed using the SPSS ver.23 (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed as mean ± standard deviation. For intergroup analysis, the Kruskal–Wallis variance analysis was used to analyze significant differences among the groups. $p < 0.05$ was considered statistically significant.

Ethical clearance

The study was approved by the Health Research Ethical Committee of the medical faculty of Sultan Agung Islamic University, Semarang, Indonesia, under No. 388/XII/2020/Komisi Bioetik.

Results

H-MSCs characterization

The results of this study showed that the isolated cells used in this study were differentiated into osteocytes marked in red after being stained using Alizarin Red dye (Figure 1a) and able to differentiate into adipocyte cells marked by the presence of a fat dot (yellow arrow) on Oil Red O staining (Figure 1b). These results indicated the isolated cells have MSC characteristics because they are multipotent. Moreover, the results of cell phenotype analysis using a flow cytometer showed that the cells used in this study expressed CD 105, CD90, and CD73 as shown in Figure 1c. These results are following the characteristics of the MSC determined by the International Society for Stem Cell Research (ISSCR).

PDGF levels

Based on the results of the study, administration of normoxic and H-MSCs significantly increased the PDGF concentration on the 3rd and 6th days ($p < 0.05$).

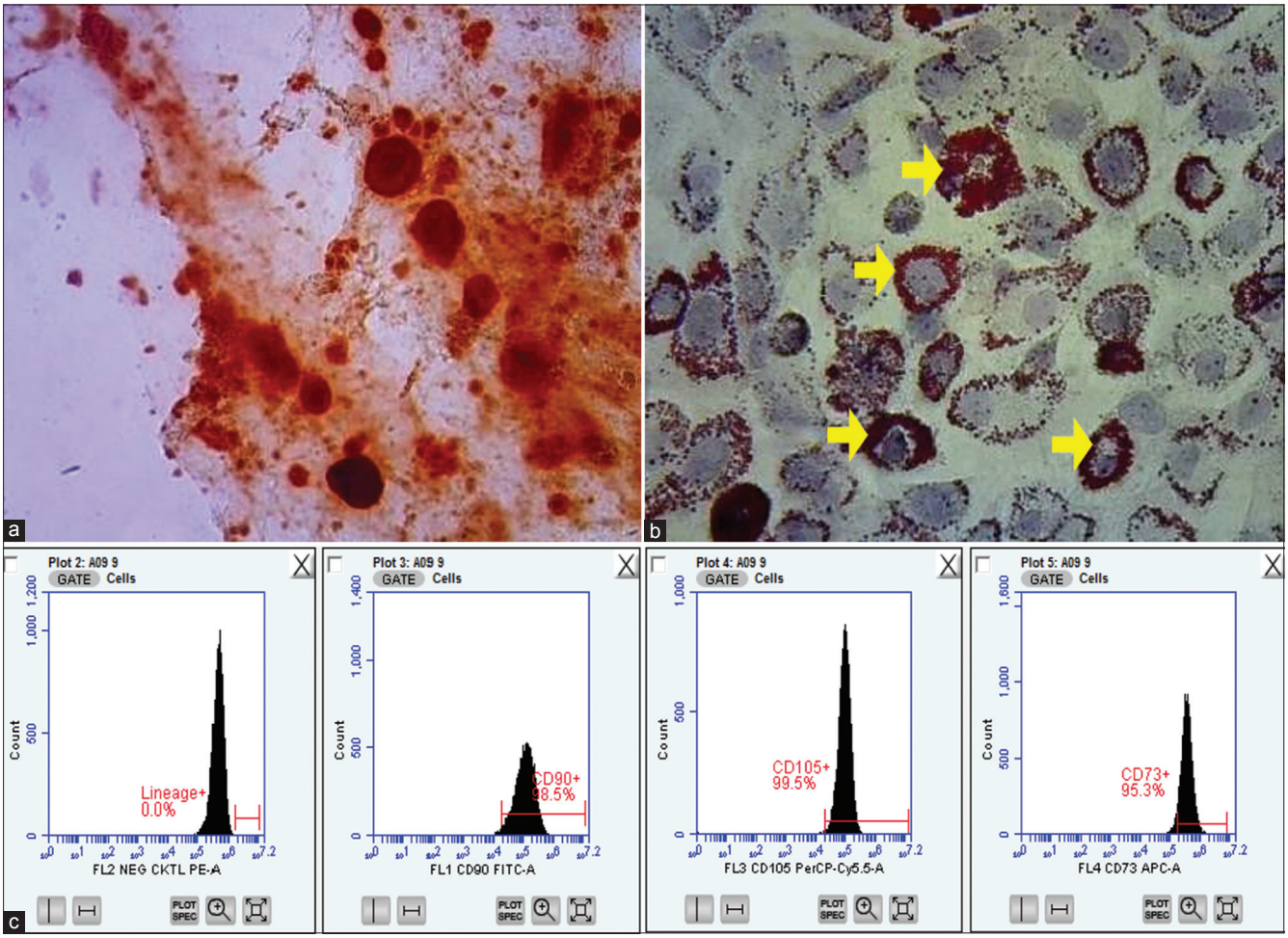

Figure 1: Validation of the differentiation ability of H-MSCs. (a). H-MSCs differentiate into osteocytes marked in red on Alizarin Red staining, (b). H-MSCs differentiate into adipocyte cells marked by the presence of a fat dot (yellow arrow) on Oil Red O staining, (c). Flowcytometric analysis of H-MSCs with the marker expression of CD73, CD90, and CD105

Although both MSCs provided a significant increase in PDGF, H-MSCs tended to provide higher PDGF concentrations (D3: 99.2 ± 10.5; D6: 90.5 ± 10.3) compared to N-MSCs (D3: 86.3 ± 7.0; D6: 88.9 ±) ($p > 0.05$). On the 9th day after treatment, the N-MSC group (87.1 ± 10.3) still showed a significant increase ($p < 0.05$). However, the PDGF levels in the H-MSC group (71.9 ± 6.9) showed a decrease in concentration that approached the Sham group ($p > 0.05$) (Figure 2).

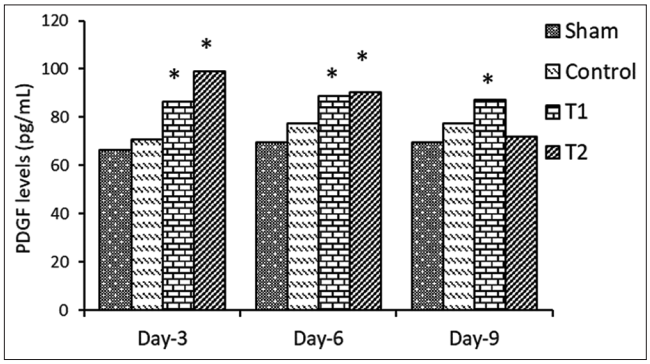

Figure 2: PDGF concentration on days 3, 6, and 9 after the treatment. PDGF: Platelet-derived growth factor, T1: 3 × 10^6 N-MSC, T2: 3 × 10^6 H-MSC, and the control group received PBS, Sham: untreated group

Collagen expression

The results also showed that the collagen density in wound tissue was significantly increased in the administration of N-MSC and H-MSC on days 3, 6, and 9 ($p < 0.05$; Figure 3) [24]. Although the administration of both N-MSC and H-MSC gave an increase in collagen density on days 3 (N-MSC: 21.4 ± 2.8; H-MSC: 35.4 ± 4.7) and 6 (N-MSC: 40.7 ± 5.7; H-MSC: 59.2 ± 6.8). Besides, the administration of N-MSC gave a linear increase until day 9 (55.6 ± 5.3), whereas the H-MSC group gave an increase in collagen density on day 6 (59.2 ± 6.8), and decreased on day 9 (39.2 ± 4.6) after administration.

Discussion

Wound healing is a complex dynamic process of replacing devitalized and missing cellular structures and tissue layers that consisting four-phase progression, namely, hemostasis, inflammation, proliferation, and remodeling [1], [12]. The transition

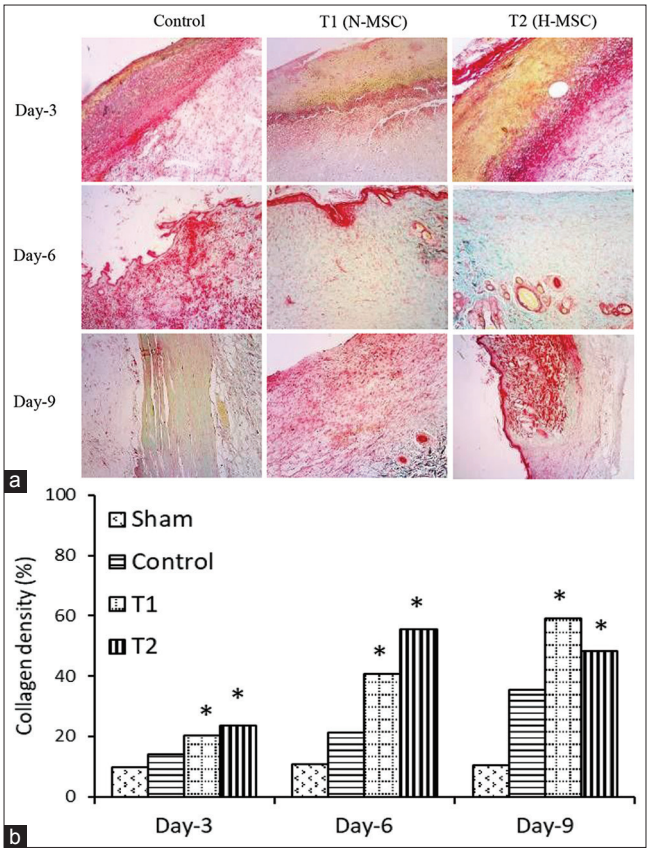

Figure 3: Collagen density on days 3, 6, and 9 after the treatment. PDGF: Platelet-derived growth factor, T1: 3 × 10^6 N-MSC, T2: 3 × 10^6 H-MSC, and the control group received PBS, Sham: untreated group

from the inflammatory phase to the proliferative phase is a primary step during wound healing processes [31]. The inflammatory phase is essential for leading to hemostasis and recruitment of the innate immune system, which protects the body from invading pathogens and helps remove dead tissue [4], [32]. Prolonged inflammation can hinder the normal stage of wound healing and is also associated with excessive scarring [9], [32]. Furthermore, recent studies have reported that MSCs have an immunoregulatory role so that they can play a role in regulating immune cells in a wound healing process [10], [16], [33]. The injected MSCs are attracted by cytokines and growth factors secreted by resident inflammatory cells during the homeostasis process and migrate on the wound area [1], [34], [35]. Once in the wound area, the MSCs will secrete various paracrine molecules to regulate cellular responses and stimulate the secretion of various growth factors such as hepatocyte growth factor, fibroblast growth factor, and PDGF which can induce angiogenesis and tissue repair [4], [10], [36]. Therefore, this study provides treatment in the form of injection of hypoxic and normoxic MSC around the wound area to accelerate wound healing by shortening the transition period from the inflammatory phase to the proliferative phase marked by an increase in PDGF concentration.

Based on the results of the study, there was a significant increase in the concentration of PDGF due to the administration of normoxic and H-MSCs on days 6 and 9. The increase in this growth factor could indicate a shift in the inflammatory phase to the proliferative phase. Platelet-derived growth factor is a growth factor that can be secreted by several cells, one of which is macrophage Type 2 (M2). The increased PDGF level is indicated due to the polarization of Type 1 (M1) M2 that are known to secrete regenerative growth factors particularly PDGF. The previous studies reported that MSCs were able to trigger pro-inflammatory M1 polarization to anti-inflammatory M2 by secreting various cytokines such as IL-10 and TGF-β [5], [35], [37]. In addition to showing an increase in PDGF levels due to MSC administration, our data showed that H-MSCs provided a higher PDGF level compared to N-MSCs on the 6th day after the treatment. This phenomenon might be due to the increased survival rate of H-MSCs after being injected around the wound area. According to the previous studies, MSCs preconditioned in a hypoxic environment had a higher survival rate in hypoxic wound areas than MSCs cultured in normoxic conditions. The hypoxic precondition promotes the stabilization of HIF-1α, which decreases the level of reactive oxygen species in the MSC mitochondria, activating nuclear factor-kappa B [5], [19]. HIF-1α also stimulates normal cellular prion protein synthesis which promotes anti-apoptotic protein expression, repair growth factors, and antioxidant enzymes [38], [39].

PDGF is a growth factor that plays an important role in the wound closure process because it can initiate collagen formation by activating fibroblasts into myofibroblasts [18]. Collagen acts as a scaffold for the attachment of fibroblast cells in the wound area so that the wound can be closed [40]. Increased levels of PDGF can increase the activation of fibroblast cells increasing collagen [11]. This is consistent with the results of this study which found an increase in collagen density due to H-MSCs administration on the 6th day after treatment. The increase in the collagen density on the 6th day after the administration of H-MSCs indicated a faster wound closure process than other treatments. Although administration of H-MSCs and N-MSCs increased PDGF levels on day 6, there was a decrease in PDGF levels and collagen density on day 9 after H-MSC administration. The previous study has reported that fibroblast cell activation will stop when the wound closure process is complete [4]. Furthermore, the previous studies reported that IL-10 secreted by MSCs also has a role in inactivating fibroblast cells after the wound tissue has recovered [1], [30]. On the other side, the PDGF and collagen concentrations that are still increasing in the administration of N-MSC are indicated due to the ongoing proliferation phase and the wound closure process is not completed. Therefore, the present study indicated that the administration of H-MSCs can accelerate the closure of the excision wound by accelerating the proliferation phase which is marked by an increase in PDGF concentration and collagen density on the 6th day after the treatment and decreased PDGF

concentration and collagen density on the 9th day after the treatment. However, the limitation of this study is that there was no data related to IL-10 that can explain the process of changing the inflammatory phase to the proliferative phase. Hypoxic condition of MSC culture induced highest secretion level of cytokine IL-10.

Conclusion

H-MSCs were able to increase PDGF levels and collagen density compared to N-MSCs after treatment, which indicates an acceleration of the wound healing process.

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Author Contributions

All author made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspect of the work.

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