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Secretome Hypoxia Mesenchymal Stem Cells Inhibited Ultraviolet Radiation by Inhibiting Interleukin-6 through Nuclear Factor-Kappa Beta Pathway in Hyperpigmentation Animal Models

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Abstract

BACKGROUND: Ultraviolet B (UVB) radiation is the main factor causing hyperpigmentation. Secretome hypoxic mesenchymal stem cells (S-HMSCs) contain bioactive soluble molecules such as growth factors and anti-inflammatory cytokines that can prevent melanin synthesis and induce collagen formation. However, the role of S-HMSCSs on interleukin-6 (IL-6), p50, and p65 gene expression in hyperpigmentation is still unclear.

AIM: This study aimed to determine the effect of administration of S-HMSCSs gel on the expression of IL-6, p50, and p65 in a hyperpigmented rat skin model induced by UVB light exposure.

METHODS: Twenty-five male Wistar rats of hyperpigmented were created as an animal model under exposed to UVB 6 times in 14 days at 302 nm with a MED of 390 mJ/cm2. The animal was randomly assigned into five groups consisting of two treatment groups (treated by S-HMSCs at a 100 µL as T1 and 200µL as T2 on bases gel) for 14 days, control groups (UVB-irradiation), sham (negative control), and base gel groups. On the 14th day, IL-6, p50, and p65 were terminated and analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Statistical analysis will perform using one-way ANOVA followed with post hoc least significant difference test.

RESULTS: Analysis of IL-6 (8.59 ± 3.32), p50 (4.35 ± 2.27), and p65 (4.09 ± 1.82) gene expression in the treatment group decreased along with the increase in the concentration of S-MSCs compared to the control group.

CONCLUSION: The administration of S-HMSCs gel is expected to affect the speed of decreasing the hyperpigmentation process significantly.

Introduction

Hyperpigmentation is a process leading the high expression of melanin pigments caused by several factors, including ultraviolet (UV) exposure and environment pollutants [1]. The excess accumulation of melanin and collagen depletion can cause esthetic problems and induces pigmentation disorders [2], [3]. At present, chemical compound such as hydroquinone, retinoic acid, and natural product with antioxidant activity is the primary preference for the skin whitening agent for preventing hyperpigmentation. However, these treatments have induced sight-to-severe irritation in human skin, skin damage leading to skin cancer [4], [5]. Exposure to UVB irradiation is associated with hyperpigmentation due to an oxidative stress response, resulting in the accumulation of reactive oxygen species (ROS) [6]. Excess ROS triggers signal transduction and activates nuclear transcription factor-kappa B (NF-kβ), an inflammatory mediator [7]. The inflammatory response mediated by the p50 and p65 subunit heterodimers of NF-kβ induces the release of proinflammatory cytokines such as IL-6, IL-1β, and IL-10 that cause skin inflammation [8], [9]. Release of IL-6 increases the degree of pathological damage to hyperpigmented skin tissue [10]. This response also causes protein and DNA oxidation, leading to oxidant damage and dermal carcinogenesis [11]. Medium-conditioned mesenchymal stem cells (MSCs) prevent inflammation by inhibiting the NF-k pathway through the release of anti-inflammatory cytokines [12]. The hypoxic state of MSCs increases the release of cytokines and growth factors in the secretome [13]. The effect of hypoxic secretome MSCs (S-HMSCs) on NF-kβ and IL-6 expression in hyperpigmentation remains unclear.

MSCs are derived from stromal cells with plastic-adherent and multipotent differentiation capability which can express various markers, including CD90 and CD29, and lack of other surface marker expression such as CD45 and CD31 [14], [15]. S-HMSCs are a secretome produced by MSC under hypoxic conditions containing tons of various anti-inflammatory cytokines and growth factors such as IL-10 and transforming growth factor-beta (TGF-β) that have beneficial therapeutic effects in the inhibition of skin pigmentation particularly through inhibit of IL-6 expression [1], [16], [17]. The previous studies reported that TGF-β acts as functional paracrine and autocrine factors which inhibit nuclear factor-kappa beta (NF-kB) pathway, leading to decrease the transcription of proinflammatory cytokine [18]. On the other hand, inflammatory factors play prominent role in its pathogenesis of hyperpigmentation. Several studies reveal that inhibit the expression of proinflammatory cytokine (IL-6) potentially inhibit skin pigmentation [19]. However, the beneficial effects of S-HMSCs in hyperpigmentation to control inflammation on hyperpigmentation remain unclear. Therefore, the effect of IL-10 and TGF-β released by S-HMSCs in the hyperpigmentation associate with anti-inflammatory effect on hyperpigmentation are needed more exploration. In this study, we investigated the role of S-HMSCs in controlling UVB-induced IL-6 and p50/p65 (NF-kB) gene expression in the hyperpigmentation animal model.

Materials and Methods

MSCs isolation and characterization

The procedure in this study was approved by the Ethical Committee of Medical Faculty Sultan Agung Islamic University Semarang. The isolation of MSCs from an umbilical cord of 19 days pregnancy of female rat was performed using a previously described method with modification [20]. Briefly, the umbilical cord was mechanically dissected and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) contained 10% Fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) under normoxic condition. The cultured cells at passages 5 (P5) and under 80% confluence were employed for the next experiment.

The MSC surface markers were determined as previously described [21]. Briefly, the cells at 4th passage were detached and stained with anti-rat monoclonal antibodies including APC-conjugated CD73, FITC-conjugated CD90, PerCP-conjugated CD105, and PE-conjugated hematopoietic stem cell lineage Lin for 30 min at 4°C. The labeled cells were analyzed using flow cytometry BD Accuri C6 PLUS; BD Biosciences, San Jose, CA, USA. The MSCs differentiation capacity was determined using osteogenic differentiation assay. Briefly, the cells were plated on 4 × 10^4 cells in 3.5 cm culture dishes under osteogenic medium that composed of DMEM high glucose supplemented with 10% FBS, 1% Penstrep, 1 × 10^{-2} M sodium β-glycerophosphate, 1 × 10^{-4} M dexamethasone, and 5 × 10^{-5} M ascorbic acid. The medium was replaced every 3 days for 15 days. The calcium deposition showed a red bright color after Alizarin Red staining Zigma [22], [23], [24], [25], [26].

Secretome hypoxic mesenchymal stem cell preparation

MSCs cultured in serum-free complete medium were incubated under hypoxia condition in the hypoxic chamber maintaining a gas mixture composed of 5% O_2, 5% CO_2, and balanced N_2 at 37°C for 24 h. After 24 h incubation, 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, NY, USA) to remove the remaining cells debris. The S-HMSCs isolation, especially for 10–50 kDa molecules, containing IL-10 (18 kDa) and TGF (25 kDa) using tangential flow filtration. The S-HMSCs were keet on 2–8°C temperature until the treatment [20], [27].

Secretome mesenchymal stem cells gel preparation

S-HMSCs 100 uL and 200 uL were dissolved in 200 mg of water-based gel as P1 and P2, respectively. The sample was applied topically to the dorsal skin one a day at day 14 until day 28 after UVB-irradiation [28].

Hyperpigmentation rat model and H-MSCs administration

The 25 male healthy Wistar mice weighing about 250 ± 25 g CV = 10% were fed ad libitum and reared under 28°C temperature and 12 h photoperiod. After 1 week of acclimatization, the mice were randomly divided into the following five groups: sham, UVB-irradiated control, UVB-irradiated and UVG water-based gel (Base gel), UVB-irradiated and 100 μL H-MSCs added in 0.2 g water-based gel (P1), and UVB-irradiated and 200 μL H-MSCs added in 0.2 g water-based gel (P2). Each group consisted of five mice. This study used UVB light (broadband with peak emission at 302 nm CL-100M, UVP, USA). The mice were exposed to 390 mJ/cm^2 of UVB irradiation 3 times a week on day 1, 3, and 5) for 2 weeks according to the previous study with slight modification [19]. Vehicle-treated UVB-irradiated mice were topically administered 100 μL and 200 μL H-MSCs on the dorsal skin daily until day 28. Control mice did not receive any treatment.

Melanin content assay

Amount of total melanin present in dorsal skin were used as an index of melanogenesis. The mice were sacrificed after the final treatment and biopsies were obtained from the dorsal skin, which were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Section, approximately 5 μm thick, was stained with Fontana-Masson staining solution (Bio optica). The stained slides were examined under a light microscope (Olympus CX21, Tokyo, Japan) [2].

P50/P65 and Interleukin-6 gene expression

Total RNA from skin healed tissue was extracted with Trizol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, the first-strand cDNA was synthesized with 1 μg of total RNA using Super-Script II (Invitrogen). SYBR Green I dye was used for reverse transcription in an ABI 7500 fluorescence quantitative PCR instrument, and the mRNA levels of p50/p65, GAPDH, and IL-6 were measured using the respective primers. The thermocycler conditions were as follow: initial step at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s, and 60°C for 1 min. Expression levels were re-corded as cycle threshold (Ct). Data were acquired using the 7500 Software (Applied Biosystems Life Technologies, Foster City, CA, USA). All reactions were performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta}$Ct method.

Statistical analysis

Statistical analysis was performed by SPSS 22.0. All data (from at least 3 separate experiments) are presented as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA and a least significant difference comparison *post hoc* test $p < 0.05$ indicated statistical significance.

Results

MSC isolation and characterization

MSCs were isolated and cultured from umbilical-cord pregnant mice aged 21 days based on their plastic adherent capability under standard culture condition. In this analysis, MSCs cell morphology exhibited characteristic spindle-shaped and fibroblast-like cells with plastic adherent capability (Figure 1a). In this study also examined the differentiation capacity in the end of fifth passage expansion, the MSC-like osteogenic differentiation assay was conducted by administering the standard and osteogenic medium for 21 days. Calcium deposition was visualized in red appearance using the alizarin red dye staining (Figure 1b). The MSCs were also successfully differentiated into adipogenic lineages under oil red-O staining(Figure 1c). Moreover, the immunophenotypically of MSCs were analyzed using flow cytometry. In this study, we found a high level of CD90 (99.50 ± 2.10%) and CD29 (96.10 ± 0.76%) and low level of CD45 (1.30 ± 0.05%) and CD31 (6.60 ± 0.34%) (Figure 1d).

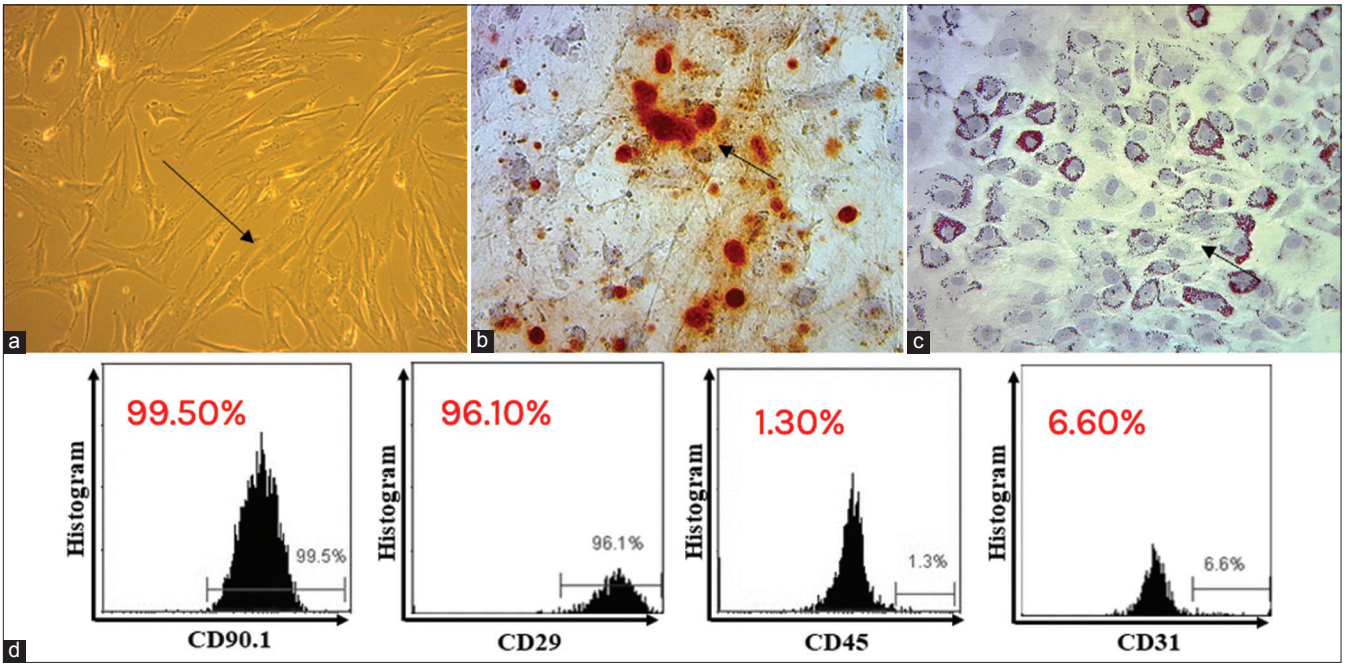

Figure 1: (a) MSC characterization and differentiation. The black arrow presented the fibroblast-like cells (magnification10x, scale bar 200 μm). (b) MSC differentiation. A red bright color marked by the black arrow in a response to the calcium deposition in osteocyte-differentiated MSCs through staining by Alizarin red (magnification ×40, scale bar 50 μm). (c) The red color marked by the black arrow in a response to the lipid deposition under Oil-red O staining. (d) The surface marker analysis of the expression of CD90, CD29, CD45, and CD31

Secretome hypoxic mesenchymal stem cells gel-inhibited proinflammatory cytokine IL-6 gene expression in hyperpigmentation mice model

UVB exposure has been shown to induce the release of inflammatory cytokines associated with skin hyperpigmentation. Cytokines expressed by melanocytes such as IL-6 to inducing melanogenesis by overexpressing the NF-kB pathway. In this study, we found that the S-HMSCs gel was able to decrease the IL-6 gene expression in doses-dependent manner. Our study showed that the S-HMSCs 200 μL significantly decrease of IL-6 gene expression (3.49 ± 2.23) up to 5 times lower than in the whole treatment group (control group). The S-HMSCs 100 μL decreased IL-6 gene expression (8.59 ± 3.32) up to 2.5 times lower than control group (18.08 ± 5.96). The vehicle group has no significant different of IL-6 expression, its means that the vehicle has not effect on the cytokine expression (Figure 2).

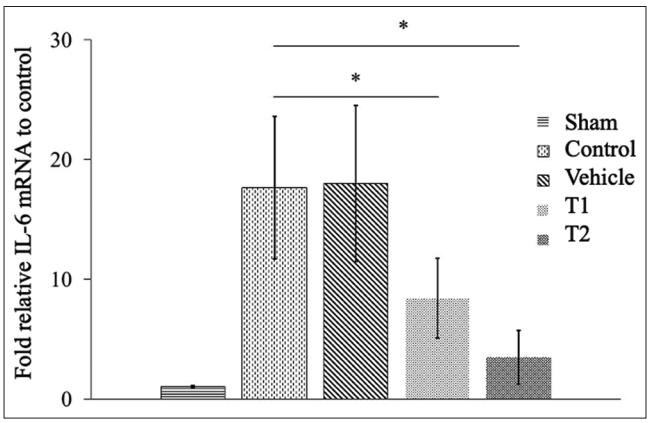

*Figure 2: The effect of S-HMSCs on IL-6 gene expression on UVB irradiation-induced hyperpigmentation rat models. RNA was extracted from the mice skin and analyzed for mRNA expression by qRT-PCR (n=5 ±SE). Data are presented as fold change in gene expression relative to UVB unexposed group. *Differences were considered statistically significant at p < 0.05. ns, which were considered statistically non-significant different at p > 0.05. S-HMSCs: Secretome hypoxic mesenchymal stem cells, qRT-PCR: Quantitative real-time polymerase chain reaction, IL-6: Interleukin-6*

Secretome hypoxic mesenchymal stem cells gel p50/p65 gene expression in hyperpigmentation mice model

In the present study, we also found that S-HMSCs gels significantly decreased p50/p65 gene expression in doses-dependent manner (Figure 3). In control group, p50/p65 gene expression was increased until 5.72 ± 2.50 and 12.96 ± 3.69-fold greater than sham group. Interestingly, the H-MSCs groups significantly decreased the p65 gene expression were T1 4.09 ± 1.82 and T2 3.15 ± 1.69-fold greater than control group.

Discussion

UVB radiation is the leading cause of skin photoaging, which results in hyperpigmentation characterized by excessive inflammatory conditions [29], [30]. The UVB-induced inflammatory response and photoaging of the skin are caused by the activation of the NF-kB signaling pathway in the epidermis that impacts the secretion of various proinflammatory cytokines, such as IL-6 [31], [32]. The previous studies have reported that NF-kB plays an important role in the maintenance and expansion of the skin photoaging process [33], [34]. This suggests that the NF-kB signaling pathway is expected to suppress UVB irradiation-induced inflammatory and photoaging responses. This study aimed to determine the effect of S-HMSCs gel on expressing IL-6, p50, and p65 mRNA in hyperpigmented rats. The anti-inflammatory ability of S-HMSCs gel was analyzed using a photoaging model of male Wistar rat skin, because it is a mammal with a skin structure similar to humans [35]. Mice were induced using UVB light with a wavelength of 302 nm and an energy intensity of 390 mJ/cm^2 3 times a week for 2 weeks [36].

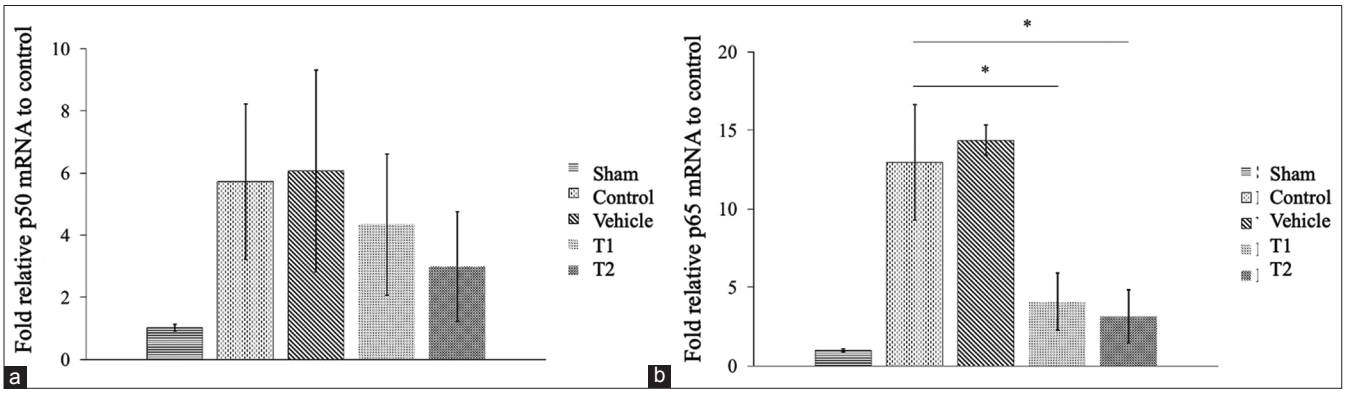

*Figure 3: The effect of S-HMSCs on (a) p50 and (b) p65 gene expression on UVB irradiation-induced hyperpigmentation rat models. RNA was extracted from the mice skin and analyzed for mRNA expression by qRT-PCR (n=5 ±SE). Data are presented as fold change in gene expression relative to UVB unexposed group. *Differences were considered statistically significant at p < 0.05. ns which were considered statistically non-significant different at p > 0.05. S-HMSCs: Secretome hypoxic mesenchymal stem cells, qRT-PCR: Quantitative real-time polymerase chain reaction*

This study analyzed the mRNA expression of IL-6, a proinflammatory cytokine that plays a role in the induction of systemic inflammation [28], [37]. The results showed a significant decrease in IL-6 mRNA expression in all treatment groups compared to the control group, where the optimum decline occurred in the S-HMSCs gel with a dose of 200 μL. This is thought to be due to the IL-10 content in the S-HMSCs gel, which can increase the inflammatory process in hyperpigmentation, thereby reducing IL-6 expression [25], [38], [39].

The suppression of the NF-kB pathway is supported by this study, where administration of S-HMSCs gel was able to significantly reduce p65 mRNA expression compared to controls in hyperpigmented mouse models. The most optimal decrease in p65 mRNA expression was produced by S-HMSCs gel at a dose of 200 μL to 3.15 ± 1.69. On the other hand, administration of S-HMSC gel also provided a downward trend in p50 mRNA expression up to 2.99 ± 1.76 in hyperpigmented mouse models. The decrease in p50 and p65 mRNA expression on gifts suggests that S-HMSCs were able to inhibit the inflammatory process in hyperpigmented mice [37], [40], [41], [42]. The T1 and T2 treatment groups reduced the p50 and p65 gene expression and significantly differed from the gel base administration.

The p50 (NF-kB1) and p65 (RelA) proteins belong to the NF-kB family and are the most common form of NF-kB protein that can form heterodimer [43]. The previous studies that the p50 and p65 heterodimer complexes are initially in the inactive phase have reported a protein inhibitor, inhibitory kappa B (IkB) [44]. Various stimuli, such as proinflammatory cytokines, UV, and free radicals formed in hyperpigmentation, activate the IkB kinase complex, which, further, phosphorylates IkB protein and has an impact on polyubiquitination and molecular proteolysis [36], [45]. The released p50 and p65 protein complexes then translocate to the nucleus, interacting with specific DNA sequences in the promoter regions of target genes and mediating transcription (Collett and Campbell, 2006; Giridharan and Srinivasan, 2018) [46]. The p50 subunit plays an important role in binding NF-kB to various DNA regions, and the p65 subunit regulates the transcriptional activity of the DNA complex [43].

Conclusion

Taken together, the results of this study indicate that gel S-HMSCs have anti-inflammatory activity in hyperpigmented mouse models through suppression of IL-6, p50, and p65 mRNA expression. This shows that S-HMSCs have the potential to be developed as targeted therapy in suppressing the inflammatory process, especially in skin hyperpigmentation.

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