Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Dec 15; 10(A):1-7.
https://doi.org/10.3889/oamjms.2022.10348
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pathophysiology*

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Effect of Secretome-Hypoxia Mesenchymal Stem Cells on Regulating SOD and MMP-1 mRNA Expressions in Skin Hyperpigmentation Rats

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Edited by: Ksenija Bogoeva-Kostovska
Citation: Zukhiroh Z, Putra A, Chodidjah C, Sumarawati T, Subchan P, Trisnadi S, Hidayah N, Amalina ND. Effect of Secretome-Hypoxia Mesenchymal Stem Cells on Regulating SOD and MMP-1 mRNA Expressions in Skin Hyperpigmentation Rats. Open Access Maced J Med Sci. 2022 Dec 15; 10(A):1-7. https://doi.org/10.3889/oamjms.2022.10348
Keywords: Secretome; Superoxide dismutase; Matrix metalloproteinases-1; Hyperpigmentation; qRT-PCR
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Received: 07-Jun-2022
Revised: 07-Aug-2022
Accepted: 05-Dec-2022

Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Ultraviolet B (UVB) radiation is the main factor causing hyperpigmentation. MSC secretome contains bioactive soluble molecules such as cytokines and growth factors that can accelerate skin regeneration. However, the molecular role of the secretome in hyperpigmentation is still unclear.

AIM: This study aimed to determine the effect of secretome hypoxia mesenchymal stem cells (S-HMSC) gel on the expression of superoxide dismutase (SOD) and matrix metalloproteinases (MMP-1) genes in skin tissue of hyperpigmented rats induced by UVB light exposure.

MATERIALS AND METHODS: Experimental research with post-test only control group. The control, base gel, T1 and T2 groups were UVB irradiated 6 times in 14 days at 302 nm with an minimal erythema dose of 390 mJ/cm^2, respectively, while sham group did not receive UVB exposure. T1 was given 100 uL of S-HMSC gel and T2 was given 200 uL of S-HMSC gel every day for 14 days, while base gel received base gel. On day 15, skin tissue was isolated and analyzed for SOD and MMP-1 expression using qRT-PCR.

RESULTS: The relative expression of the SOD gene in the treatment group (P1 = 0.47 ± 0.20, P2 = 1.22 ± 0.47) increased with increasing dose compared to the control group (UVB = 0.05 ± 0.01, Base gel = 0.05 ± 0.02). The relative expression of the MMP-1 gene in the treatment group (T1 = 5.82 ± 1.16, T2 = 2.86 ± 1.57) decreased with increasing dose compared to the control group (Control = 10.10 ± 2.31, and Base gel = 9.55 ± 1.29).

CONCLUSION: Administration of S-HMSC gel can increase SOD gene expression and decrease MMP-1 gene expression in skin tissue of hyperpigmented rats model induced by UVB light.

Introduction

Ultraviolet B (UVB) radiation is the main factor causing hyperpigmentation and the cessation of collagen synthesis which has an impact on the formation of melanin pigment and wrinkles on the skin [1], [2]. UVB induction induces an increase in reactive oxygen species (ROS) levels thereby accelerating the skin aging process, increasing melanocyte proliferation, and melanin synthesis [3]. ROS overexpression causes abnormalities of activator-protein 1 [AP-1] which inhibits collagen synthesis through the release of matrix metalloproteinase enzymes (MMPs) including MMP-1, thereby inhibiting collagen synthesis [3], [4]. The antioxidant enzyme extracellular superoxide dismutase (SOD) has a central role in suppressing abnormal ROS levels. SOD is also one of the main factors that reduce melanin production due to UVB irradiation [1], [5].

Repeated exposure to UVB is known to cause adverse effects on human skin, including causing cancer, immunosuppression, erythema, and hyperpigmentation due to mutations in oncogenes and tumor suppressor genes [4], [6]. In 2015, 4.2% of 142 positive subjects experienced hyperpigmentation after exposure to three times minimal erythema dose UVB [7]. UVB exposure causes 8% incidence of squamous carcinoma known as melanoma skin cancer with high metastatic potential [8]. The incidence of melanoma increased in 2020 with ~100,350 new cases and 6,850 deaths. Primary melanoma cell types that are cultured 40–60% show an increase in ROS expression. Increased ROS induces the release of MMPs including MMP-1, MMP-2, and MMP-9 to break down extracellular matrix [ECM] proteins such as collagen, fibronectin, elastin, proteoglycans that play a role in photoaging [9].

Previous studies have reported that interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF) can suppress ROS levels through the

nuclear factor kappa-β pathway [10], [11]. Secretome hypoxia mesenchymal stem cells (S-HMSC) contain many cytokines such as IL-10, interleukin-6 (IL-6), inteleukin-1 and growth factors such as transforming growth factor-β (TGF-β), VEGF, platelet-derived growth factor, hepatocyte growth factor (HGF) which can prevent UVB hyperpigmentation by suppressing the amount of melanin and increasing collagen levels [12], [13], [14]. Soluble molecules S-HMSC including IL-10 and TGF-β have been shown to be able to control skin damage caused by UVB radiation, reduce ROS levels, inhibit melanin formation and reduce inflammation [3], [15], [16]. The advantages of giving S-HMSC compared to other compounds include S-HMSC is an active bio molecule that does not have immunogenicity, does not cause side effects and its small molecular size is very easy to be absorbed through the skin barrier to the dermis [17], [18], [19].

There is potential for S-HMSC as an alternative agent to prevent hyperpigmentation of the skin due to exposure to UVB rays. Therefore, in this study, we will investigate the effect of S-HMSC in gel preparations at concentrations of 100 μL and 200 μL on the expression of SOD and MMP-1 in hyperpigmented rat skin model induced by UVB irradiation *in vivo*.

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 with ethical clearance number: No. 53/II/2022/Komisi Bioetik. The isolation of MSCs from an umbilical cord (UC-MSC) 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% FBS (Sigma-Aldrich, St. Louis, MO), 100 IU/mL penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO) under normoxia 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 hemopoietic 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, Shenzhen, *China*).

S-HMSCs 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 was keep on 2–8°C temperature until the treatment.

S-HMSCs 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.

Hyperpigmentation rat model and Gel S-HMSCs administration

The twenty-five males healthy Wistar rats 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 rats were randomly divided into the following five groups: Sham, UVB-irradiated Control, UVB-irradiated and water-based gel (Base gel), UVB-irradiated and 100 μL S-hMSCs added in 0,2 g water-based gel (T1), and UVB-irradiated and 200 μL S-HMSCs added in 0.2 g water-based gel (T2). Each group consisted of five rats. This study used UVB light (broadband with peak emission at 302 nm CL-100M, UVP, USA). The rats 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 previous study with slight modification [22]. Vehicle-treated UVB-irradiated rats were topically administered 100 μL and 200 μL S-HMSCs on the dorsal skin daily until day 28. Control rats did not receive any treatment (Figure 1).

Melanin content assay

Amount of total melanin present in dorsal skin were used as an index of melanogenesis. The rats

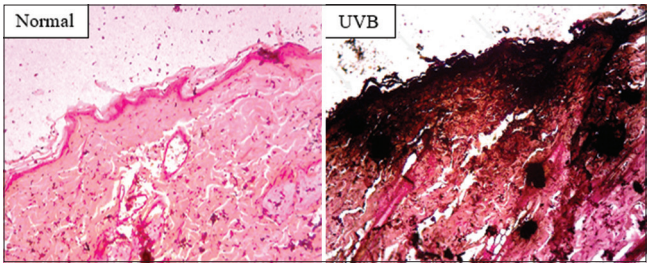

Figure 1: Validation of hyperpigmented animals with melanin content

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, San Faustino, Italy). The figure stained slides were examined under a light microscope (Figure 1) (Olympus CX21, Tokyo, Japan) [23].

SOD and MMP-1 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, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse transcription in the PCR max Eco 48 real time PCR instrument, and the mRNA levels of SOD, MMP-1, and GAPDH were measured using the respective primers (Table 1). 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 Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta}$Ct method.

Table 1: Primer sequences

Gene	Forward sequences
GAPDH	5'-TGACAACTTTGGCATCGTGG-3'
	5'-GGGCCATCCACAGTCTTCTG-3'
SOD	5'-AATGTGTCCATTGAAGATCGTGTGA-3'
	5'-GCTTCCAGCATTTCCAGTCTTTGTA-3'
MMP-1	5'-CCACTAACATTCGAAAGGGTTT-3'
	5'- GGTCCATCAAATGGGTTATTG -3'

Statistical analysis

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

Results

MSCs characterization and differentiation

MSC secretome were collected from the umbilical-cord MSC medium of pregnant rats that had reached the 4th passage. The results of the validation of the morphology of the MSC culture were obtained an image of cells attached to the bottom of the flask with spindle-like cell morphology under microscopic observation, while the results of the validation of osteogenic differentiation showed that MSCs could differentiate into osteocytes as indicated by the red calcium deposits in the MSC population. Using Alizarin Red staining (Figure 2a and b). In line with the osteogenic ability of MSCs, the results of isolated MSC cells were validated using flow cytometry to show that MSCs were able to express several MSC surface markers. The validation results showed that MSCs were able to express CD90 (99.80%), CD29 (94.20%) and did not express CD45 (1.60%) and CD31 (6.60%) (Figure 2c).

S-HMSC upregulated SOD expression in hyperpigmentation rats

Exposure to UVB rays with moderate to high intensity has been shown to induce oxidative stress that leads to skin hyperpigmentation. Cytokines expressed by melanocytes such as IL-10 inhibit the accumulation of ROS through activation of the SOD enzyme. The results of this study showed that the S-HMSC gel was able to increase the expression of SOD along with the addition of the administered dose. Based on the results of the study, administration of S-HMSC 200 μL significantly induced SOD expression up to 1.22 times higher than in the entire treatment group. The administration of S-HMSC 100 μL also significantly increased the expression of SOD 0.47 times when compared to the other groups. Although both doses were able to increase SOD expression, treatment P2 had a significant effect compared to treatment P1 (Figure 3a).

S-HMSC downregulated MMP-1 expression in hyperpigmentation rats

Hyperpigmentation is a skin pathological condition whose repair and prevention involves tissue regeneration reactions including the prevention of melanogenesis and collagen reconstruction. Exposure to UVB rays has been shown to induce collagen degradation by increasing the enzyme MMPs. Cytokines expressed by S-HMSCs such as IL-1, IL-6, and TGF-β are able to inhibit MMPs enzyme synthesis and prevent collagen degradation by suppressing the

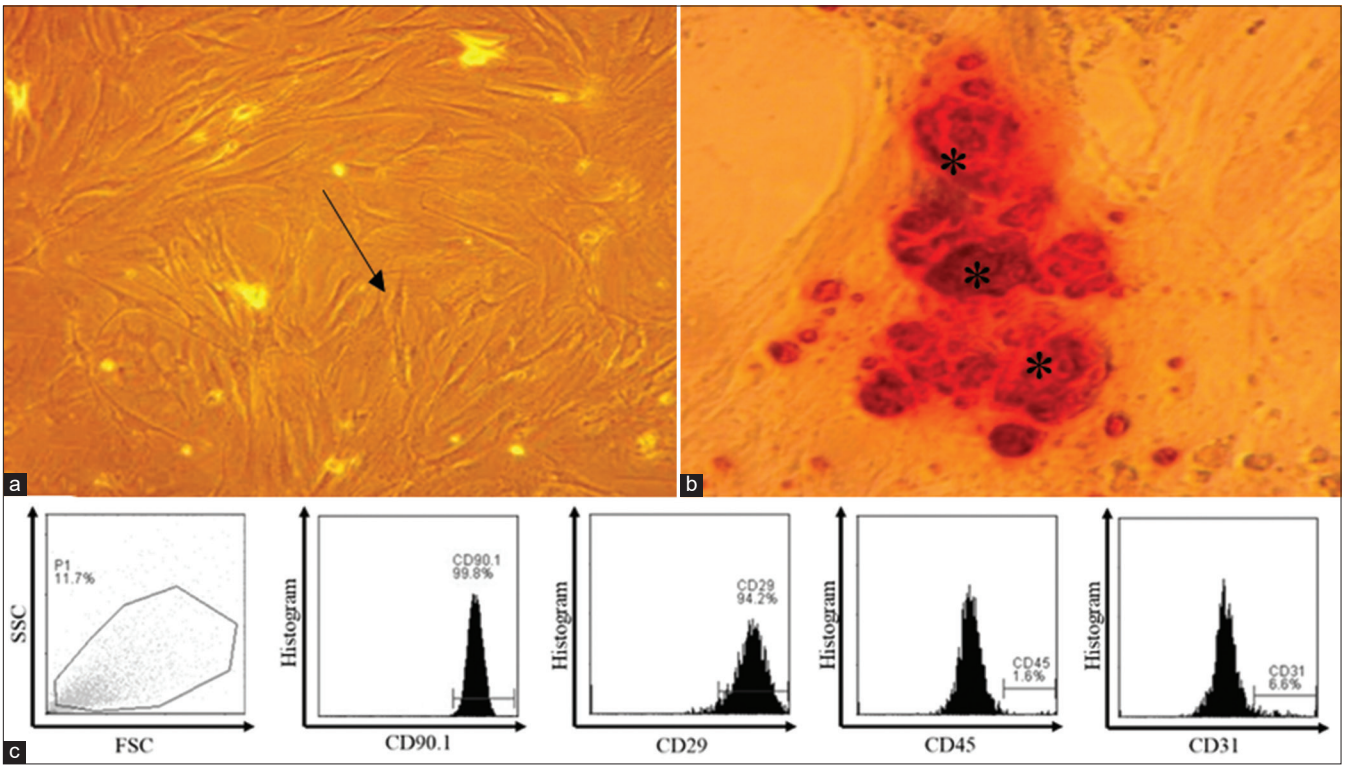

Figure 2: MSC Validation. (a) Isolated MSCs with 80% confluent showed spindle-like cells (pointed by arrows) at ×100 magnification. (b) Osteogenic differentiation using Alizarin Red staining appears in the MSC population at ×100 magnification. (c) Flow cytometry analysis of the expression of CD90, CD29, CD45, and CD31

mitogen activated protein kinase (MAPK) pathway. In this study, the researchers found that the S-HMSC gel was able to reduce the expression of MMP-1 depending on the dose. Based on the results of the study, it was found that the administration of S-HMSC 200 μL significantly reduced the expression of MMP-1 up to 7.2 times compared to the UVB group. Administration of S-HMSC 100 μL significantly suppressed MMP-1 expression 4.27 times when compared to the UVB group. Although both doses were able to reduce MMP-1 expression, P2 treatment had a significant effect compared to P1 treatment. However, the application of base gel was not significant when compared to the UVB group (Figure 3b).

Discussion

Exposure to UVB irradiation is a major risk factor for skin photoaging contributed to hyperpigmented diseases and characterized by inflammatory conditions, accumulation of melanin, and decreased of collagen synthesis [6], [24]. UVB irradiation induces DNA damage through the formation of oxidative ROS, which results in activation of several melanin synthesis pathways, inactivation of the collagen synthesis pathway, inflammatory processes, and resulting in the formation of skin cancer [8], [25]. Current therapies do not all provide maximum results,

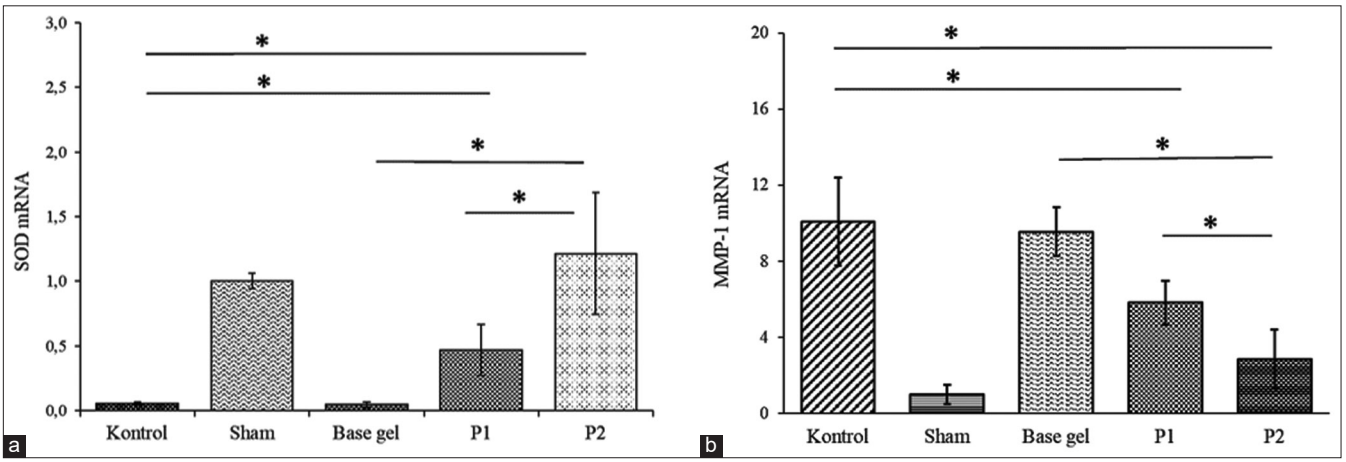

*Figure 3: The results of the RT-PCR test of the expression level of superoxide dismutase matrix metalloproteinases-1 mRNA in the skin tissue of each group *p < 0.05*

especially in preventing skin hyperpigmentation, some agents cause adverse side effects such as genotoxicity and skin irritation [26], [27]. Recent studies have shown that hypoxic secretory Mesenchymal stem cells contain bioactive soluble molecules such as growth factors and anti-inflammatory cytokines that can regenerate and induce skin regeneration [17], [28], [29]. This study aims to determine the effect of giving variations in the dosage of gel S-HMSC on the formation of melanin and skin collagen in hyperpigmented male Wistar rats by molecular biomarker mainly MMP-1 and SOD.

Several previous studies have reported that SOD1 is significantly suppressed by repeating exposure to UVB in response to chronic photo-oxidative stress [5], [30], [31]. SOD, an enzyme that alternately catalyzes the dismutation of superoxide [O_{-2}] radicals into molecular oxygen [O_2] and hydrogen peroxide [H_2O_2], is an important antioxidant defense in all living cells exposed to oxygen radicals. Increased SOD expression in this study after S-HMSC administration may suggest that cytokines in S-HMSC containing IL-10, IL-12, HGF, G-CSF, INF-γ, and TNF-α were ably inducing the synthesis pathway and the activation of SOD enzymes by involving the transcription factor NF-κB [32], [33]. It has been reported that SOD is one of the key factors reducing melanin production caused by UV irradiation [8], [10], [30]. Another study has also shown that an increase in SOD may suppress the process of melanogenesis through the Tyrosinase pathway [34]. The previous studies have shown that the cytokines IL-10, IF-γ, and TNF - with their cell receptors will phosphorylate NADPH in a program that results in activation of the transcription factor NF-κB pathway through the mediation of phosphoinositide-3-kinase, MAPK, ERK1/2 [16], [34], [35]. Activated NF-κB will migrate into the nucleus and initiate the translation and transcription of various antioxidant gene expressions, including SOD [8].

In mammalian skin tissue, the ECM degradation is mediated by the endopeptidase enzyme activity known as MMPs and their inhibitors, called Tissue Inhibitor MMP 2 [36], [37]. The result showed that MMP-1 expression decreased after S-HMSC administration. We assume that S-HMSC may increase MMP-1 through TGF-β1, IL-1, and IL-6 which are inhibitors of the MMP-1 enzyme synthesis pathway. Previous studies have demonstrated that increased collagen production by fibroblasts may lead to increased deposition into the ECM regulated by the cytokine TGF-β1 [37], [38], [39]. Interestingly, other previous studies have shown that TGF-β1 not only increases collagen production but also inhibits MMPs in the collagen degradation pathway through MMP-3 and MMP-9 [38], [39]. TGF-βs bind to two serine/threonine kinase receptors consisting of TGF-βRI and TGF-βRII [40]. When the ligand binds, TGF-βRII phosphorylates TGF-βRI and activates intracellular signaling pathways such as Smad3, MAPK, including cellular signaling-associated protein kinase (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase [16], [41]. In skin fibroblasts, p38 MAPK and Smad3 unite in regulating TGF-β-induced MMP-13 expression, whereas ERK1/2 cooperates with Smad3 in controlling connective tissue growth factor expression [38], [42], [43].

The limitation of this study was that it did not explore the effect of administering S-HMSC gel on intracellular ROS levels which are strongly suspected as one of the factors provoking tissue damage due to exposure to UVB rays. This study also did not analyze the expression of TIMP as an MMP inhibitor in the collagen degradation pathway. On the other hand, this study also did not analyze collagen density as a parameter of tissue regeneration and repair of hyperpigmentation. Therefore, to further confirm and validate the research results, more challenging trials are needed in the future.

Conclusion

Administration of S-HMSC gel can increase SOD gene expression and decrease MMP-1 gene expression in skin tissue of hyperpigmented rats model induced by UVB light.

Acknowledgments

The authors thank to Stem Cell and Cancer Research, Laboratory at the Sultan Agung Islamic University's Faculty of Medicine in Semarang.

Author Contributions

ZZ, AP, and CC were responsible for experimental design and developed methodology. ZZ and NH carried out the experiments. ZZ, AP, and TS interpreted the results, performed data analysis and prepared the figures and tables. AP and NH wrote, reviewed, and revised the manuscript. Provided administrative, technical, or material support. PS and ST supervised the study.

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