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Role of Exosomes Derived from Secretome Human Umbilical Vein Endothelial Cells (Exo-HUVEC) as Anti-Apoptotic, Anti-Oxidant, and Increasing Fibroblast Migration in Photoaging Skin Models

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Abstract

BACKGROUND: Prolonged skin exposure to ultraviolet light rays leads to photoaging, which is characterized molecularly by an increase in reactive oxygen species (ROS), cell apoptosis, and a decrease in collagen. Photoaging therapy has been a challenge until recently. Fibroblasts exposed to ultraviolet B (UVB) light proved to be a good model for photoaging skin. They are also the primary dermal cells that stimulate collagen production and extracellular matrix (ECM), which contribute to skin aging. Exo-HUVEC is rich in growth factors, cytokines, and miRNAs, and they all play a key role in cell-to-cell communication. The migration of fibroblasts is crucial for the development, repair, and regeneration of skin tissue during the repair of skin aging.

AIM: An *in vitro* experimental study was conducted to analyze the effect of Exo-HUVEC on oxidative stress levels, cell apoptosis, and fibroblast migration rate after UVB ray exposure on fibroblasts.

METHODS: The fibroblast cultures were divided into five groups, including one without UVB exposure, one with UVB exposure, and one with UVB+Exo-HUVEC exposure at 0.1%, 0.5%, and 1%, respectively. Oxidative stress levels were measured using the ELISA test for malondialdehyde (MDA). Furthermore, flow cytometry was used to measure apoptosis using PI/Annexin markers, while a scratch assay examination was used to measure fibroblast migration rate using imaging readings.

RESULTS: There were significant differences in the levels of MDA, PI/Annexin, and the rate of fibroblast migration between the UVB-irradiated control group and the Exo-HUVEC treatment group ($p < 0.001$).

CONCLUSION: Exo-HUVEC is a marker of photoaging improvement, which has anti-apoptotic effects and reduces oxidative stress, as well as increases fibroblast migration rate.

Introduction

A photoaging image appears when the skin experiences chronic exposure to ultraviolet light. Fibroblasts are the primary cells in the skin's dermis layer that stimulate the production of collagen and extracellular matrix (ECM), which are responsible for aging [1], [2], [3]. UV light exposure triggers the formation of reactive oxygen species (ROS) and cell apoptosis through intrinsic and extrinsic pathways [4], [5], [6]. Consequently, reactive oxygen species stimulate apoptosis both extrinsically by multimerizing CD95 and intrinsically by increasing the release of cytochrome c. These two processes activate the caspase cascade and caspase-3, resulting in cell apoptosis [7], [8]. Flow cytometry is employed to detect apoptosis using Annexin V and propidium iodide (PI) [9], [10].

Malondialdehyde (MDA) is a biomarker for increased oxidative stress in the body, produced by a lipid peroxidation chain reaction [11]. Reactive oxygen species trigger the activation of mitogen-activated protein kinase (MAPK), nuclear factor-kB (NF-kB), and activator protein-1 (AP-1), which play vital roles in regulating Matrix Metalloproteinase (MMP-1, MMP-3, and MMP-9) transcription. Furthermore, increased expression of these genes triggers dermal collagen fragmentation [7], [11]. These molecular changes reduce fibroblast cell migration, a physiological process that is coordinated and crucial for tissue development, repair, and regeneration. Cell migration is the movement

of individual cells, groups, or clusters of cells from one location to another, leading to increased cell activity. The increase in fibroblast migration can be detected using a scratch test in μm. Furthermore, collagen is a vital component of the extracellular matrix (MES) in human skin. A decrease in its level in the dermis will be clinically manifested as skin wrinkles [1].

Human umbilical vein endothelial cells (HUVEC) are endothelial cells that play a vital role in the inflammatory process and neurovascularization-angiogenesis through paracrine and endocrine effects [12]. HUVEC has never been used as a skin-antiaging treatment. However, secretomes taken from HUVEC culture conditioned media are rich in components of growth factors and cytokines, such as Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), and Transforming Growth Factor-β (TGF-β) [13]. Exosomes derived from Secretome Human Umbilical Vein Endothelial Cells (Exo-HUVEC) are 30–100 nm microvesicles belonging to cell metabolic products that function as carriers of bioactive messenger RNA (mRNA) and micro RNA (miRNA) proteins for intercellular communication and protein transfer to desired target cells. Exo-HUVEC is a cell-free therapy with a low rejection rate and no carcinogenesis, making it safe to use, and it is expected to reduce MDA levels and inhibit apoptosis, increasing the rate of fibroblast migration [12], [14].

Methods

This experimental study employed a post-test control group design. Fibroblast culture from the prepuce was used because it was free of UV exposure and did not photo-age. The HUVEC was extracted from the baby's umbilical cord after a cesarean section. This study was conducted at Dermama Biotechnology Laboratory of Surakarta Indonesia and Clinical Pathology Gajah Mada University Indonesia and was approved by the Health Research Ethics Committee of Dr Moewardi Hospital/Faculty of Medicine at Sebelas Maret University, Surakarta, Central Java, Indonesia (785/VIII/HREC/2021).

Fibroblast culture

Primary fibroblast cultures were collected from the prepuce of the post-circumcised child after the parents agreed and provided informed consent. Fibroblasts were cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco, New York, NY, USA) supplemented with 10% fetal bovine serum (Gibco, New York, NY, USA), 1% penicillin-streptomycin (Gibco, New York, NY, USA), and 1% fungizone (Gibco, New York, NY, USA). Subsequently, sub-culture was performed until passage 4, at which point cells were harvested and placed into 24 wells, each containing 0.05 × 106 cells/μL.

HUVEC secretome isolation

The isolated HUVEC secretome was obtained from the proximal umbilicus after it was cut from the proximal part and cleaned of blood by venipuncture. Subsequently, the umbilicus was examined immediately for a day. It was cannulated and cleaned from the left blood using 0.2% buffer and collagenase before being incubated at 37°C for 15 min. The endothelial and subendothelial layers were subjected to centrifugation and washed 3 times with phosphate-buffered saline (PBS). The culture was placed on a disc and allowed to grow for 3 days. Cell subculture was also performed using the warm trypsin method after fibroblastoid formation.

Exo-HUVEC isolation

The isolation of Exo-HUVEC was achieved by transferring the HUVEC secretome into a sterile tube and adding Invitrogen® reagent. Samples were centrifuged after being incubated overnight at temperatures ranging from 2 to 80°C. The supernatant was then discarded, leaving the pellets at the bottom of the tube. They were suspended with PBS and purified using the affinity method. Finally, the Exo-HUVEC was isolated for a week at temperatures ranging from 2 to 80°C.

Treatment model

The study was divided into five groups, each of which was conducted in threes. The groups included one which contained fibroblasts treated with PBS without UVB exposure, one with UVB exposure, and one with UVB + Exo-HUVEC exposure at 0.1%, 0.5%, and 1%, respectively. The large dose was based on the results of the preliminary study. UVB exposure was selected as a model of skin aging because of its convenience, speed, and the extrinsic factors causing aging are primarily from UV exposure. Waldmann NB-UVB type 109 was used to achieve UVB irradiation, exposure to UVB is carried out once with a dose of 600 mJ/cm^2 and was applied to all wells for 80 s, for the other three groups, after exposure to UVB were given exposure to Exo-HUVEC. Previously, cells were incubated using 5% CO_2 for 24 h at 37°C. An ELISA test (Bio-Rad Lab) was used to determine MDA levels, while a flow cytometry test was used to detect cell apoptosis using Annexin V/PI (BD FACS Canto II). The fibroblast migration rate was calculated in percentage using the imaging reading method with a scratch assay described by Yarrow *et al*.

Statistical analysis

SPSS for Windows was used to analyze the data. The average distribution of the data was analyzed using one-way ANOVA with a Post Hoc test. However, the Kruskal–Wallis test was used after the Mann–Whitney test for analysis in cases where the data were not normally distributed. $p < 0.05$ was considered statistically significant.

Results

The highest MDA value after treatment in the control group with UV exposure had an average of 402.733±49.395, while the lowest MDA value in the control group without UV exposure had an average of 57.433 ± 0.651 (Figure 1). This study also evaluated the Exo-HUVEC group by measuring MDA levels after treatment. The 0.1% Exo-HUVEC group had the highest MDA levels, with an average of 245.100±1.700, while the 1% Exo-HUVEC group had the lowest MDA levels, with an average of 79.600 ± 3.132. However, the MDA levels in the 1% Exo-HUVEC group were not significantly different from the no-exposure group (Figure 1).

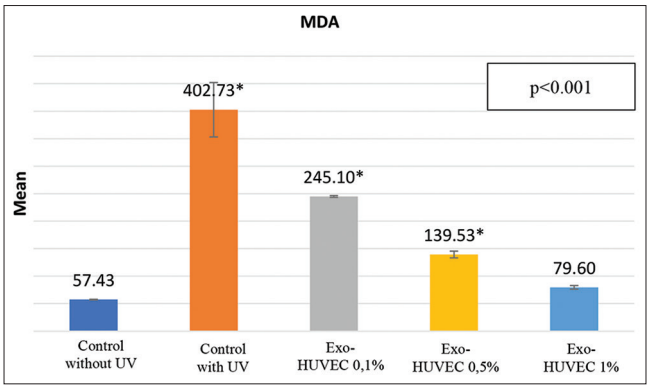

Figure 1: MDA value bar diagram based on treatment concentration, MDA is biomarker for oxidative stress: The dark blue bar chart shows the MDA value of the control group, the orange bar shows the MDA value of the UV group, the gray bar shows the MDA value of the 0.1% Exo-HUVEC group*, the yellow bar shows the MDA value of the 0,5% Exo-HUVEC group*, and light blue bars indicate the MDA value of the 1% Exo-HUVEC group (*p < 0.001 compared to control group, significant)*

The control group with UV exposure had the highest overall PI/Annexin levels after treatment, with an average of 13.07 ± 0.31 and the control group without UV exposure had the lowest PI/Annexin levels, with an average of 3.20 ± 0.10 (Figure 2). The greatest decrease in PI/Annexin levels occurred after the administration of 1% Exo-HUVEC compared to the other Exo-HUVEC groups (0.1% and 0.5%), with an average level of 5.53 ± 0.15 (Figure 2).

Fibroblast migration was assessed as part of the evaluation of the dermal repair process caused by exposure to UVB rays. This study employed a scratch assay, which discovered that there were significant differences in fibroblast migration between various preparations treated with Exo-HUVEC ($p < 0.001$) at 0, −6, −12, −24, −48, and −72 h (Figure 3).

The migration rate of fibroblasts with 1% Exo-HUVEC produced the best results from 6 h, 12 h, 24 h, 48 h, and 72 h with rate value similar to control group although it still shows a significant difference ($p < 0,001$), which were comparable to the results of the control group without UV, while the 0.1% Exo-HUVEC produced the slowest results, which were comparable to the control group with UV (Figure 3 and Table 1).

Discussion

Fibroblast culture is recognized as the standard material in tissue engineering [9], [10]. The culture irradiated with UVB as a model of skin photoaging showed changes in molecular composition that could be used by the level of malondialdehyde (MDA) to determine ROS and PI/Annexin to determine apoptosis [9], [15], [16]. Exo-HUVEC UVB-exposed human dermal fibroblasts (HDF) were administered to inhibit cell apoptosis and ROS-induced extrinsic aging caused by UV exposure, as well as to evaluate increased HDF migration as a marker of aging repair.

Oxidative stress is widely considered to be a key mechanism behind the detrimental effects of acute and chronic UV radiation exposure on human skin. According to this study, the oxidative stress levels in HDF were described as a whole. Based on result in Figure 1, previous studies also corroborate the results above that acute exposure to UV light results in the formation of free MDA and MDA-derived epitopes. MDA levels can increase up to 20-fold in human skin when exposed to UV light [17]. The comparison results show that the higher levels of Exo-HUVEC administration resulted in a significant decrease in MDA levels. There was a significant decrease in MDA levels followed by a significant increase in antioxidant levels in the study that evaluated the effectiveness of klotho protein as an antiaging protein in HUVEC combined with 33.3 mmol/L glucose in photoaging skin conditions [18].

Direct exposure to UV radiation causes apoptosis in fibroblasts and keratinocytes. Fibroblasts are useful in maintaining structure and strength of skin tissue through extracellular matrix (ECM), adhesive

Table 1: Comparison test for fibroblast migration variables

Group	Fibroblast migration (μm)					
	0 h[a]	6 h[b]	12 h[a]	24 h[a]	48 h[b]	72 h[b]
Control without UV	58.31 ± 0.89	54.56 ± 1.01	52.17 ± 1.30	47.11 ± 2.94	14.70 ± 1.71	9.14 ± 1.82
Control with UV	58.48 ± 0.90	58.45 ± 0.77*	57.78 ± 1.53*	54.69 ± 1.25*	28.36 ± 0.97*	25.85 ± 0.83*
Exo-HUVEC 0.1%	58.78 ± 0.78	58.32 ± 0.88*	57.11 ± 0.54*	52.59 ± 0.97*	28.19 ± 1.58*	24.35 ± 1.14*
Exo-HUVEC 0.5%	57.35 ± 1.83	56.30 ± 1.66*	53.98 ± 1.06*	50.13 ± 4.68	26.04 ± 1.68*	15.08 ± 1.42*
Exo-HUVEC 1%	57.76 ± 1.25	56.25 ± 1.34*	53.19 ± 2.01*	50.09 ± 1.90*	20.81 ± 3.44*	11.78 ± 1.25*

[a]Kruskal–Wallis Test, [b]Anova test; (*p < 0.001 compared to control group, significant).

proteins, and ground substances also play role in wound healing, angiogenesis, cancer progression, inflammation, as well as in paracrine and autocrine signaling processes through cytokines and growth factors [19]. Meanwhile, keratinocytes have a key role in skin repair process, keratinocytes are the executor of the re-epithelialization process, in which keratinocytes will migrate, proliferate, and differentiate to restore the function of the epidermal barrier, thus programmed death will inflict role of these two cells decreasing or even vanishing [20]. Flow cytometry was employed in this study to visualize apoptotic conditions in fibroblasts using Annexin V and propidium iodide (PI). Propidium iodide (PI) is commonly used along with Annexin V to determine whether cells are viable, apoptotic, or necrotic by examining differences in plasma membrane integrity and permeability. The PI/Annexin protocol is a prevalent method for studying apoptotic cells [8]. Phosphatidylserine (PS), normally found in the cytoplasmic membrane, is located on the membrane's extracellular surface when cells die. This location serves as the binding site for Annexin V. Furthermore, the addition of propidium iodide (PI) distinguishes (labels) the next stage of apoptosis and cell death [9], [10]. PI/Annexin levels in this study increased by 66.4% compared to the control group in a previous study used to assess apoptosis in UV-exposed HDF [21]. Results in this study are consistent with a Turkish study which state the administration of exosomes from wheat can cause a decrease in apoptosis, which is measured by PI/Annexin levels [16]. The decrease in the apoptotic process affects the migration of fibroblasts in the extracellular matrix, which increases collagen deposition in the photoaged skin. Collagen produced by fibroblasts controls cell adhesion and migration of fibroblast cells during the skin regeneration process [22].

Liang *et al*., who assessed the proliferation of fibroblast cells, showed that administration of mesenchymal stem cells with higher concentrations resulted in a gradual increase in the number of cell migrations, indicating the effect of fibroblast cell migration that could trigger the process of re-epithelialization, collagen deposition, and angiogenesis [15]. The irradiation of fibroblasts can significantly inhibit collagen deposition and cell migration ($p<0.05$) [23]. The reduction in direct type I collagen deposition is caused by collagen breakdown and indirectly by inhibiting collagen synthesis from the procollagen promoter [24]. Exo-HUVEC is rich in growth factors and cytokine components, such as Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor-β (TGF-β) [25].

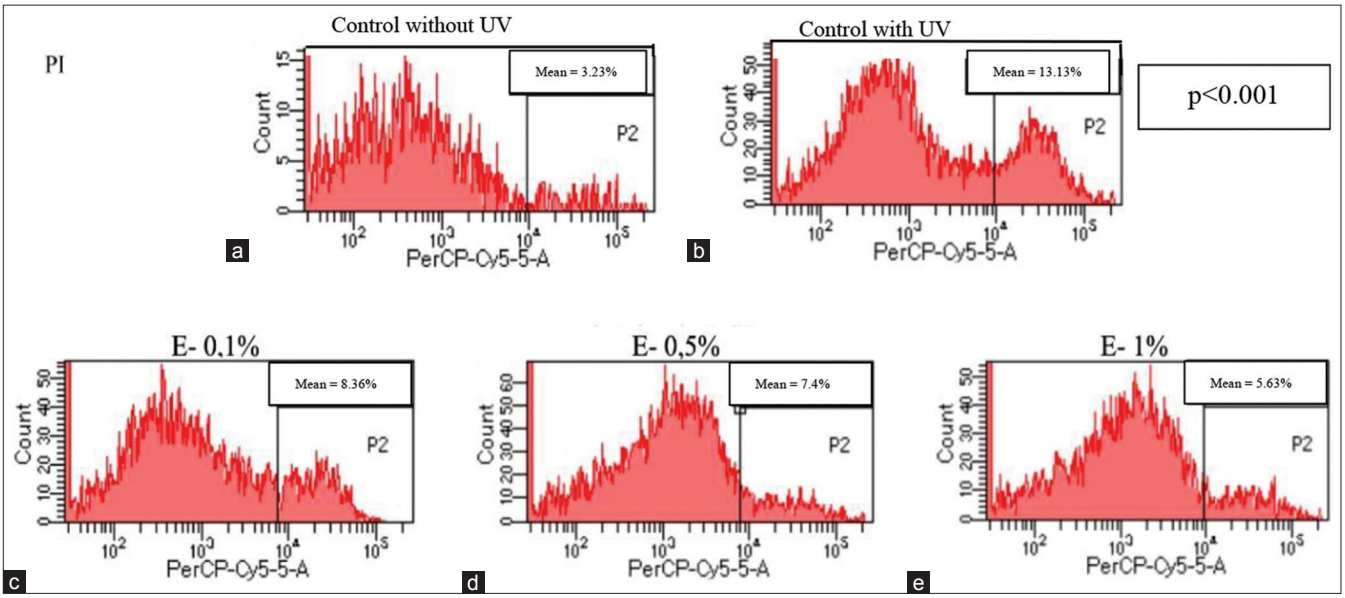

Figure 2: PI/Annexin flowcytometry diagram based on treatment concentration, PI/Annexin flowcytometry is a very sensitive method for detecting apoptosis: a. PI/Annexin flowcytometry for the control group (mean = 3.23%); b. PI/Annexin flowcytometry for UV group (mean = 13.13%); c. PI/Annexin flowcytometry for the 0.1% Exo HUVEC group (mean = 8.36%); d. PI/Annexin flowcytometry for the 0.5% Exo-HUVEC group (mean = 7.4%); e. PI/Annexin flowcytometry for the 1% Exo-HUVEC group (mean = 5.63%)

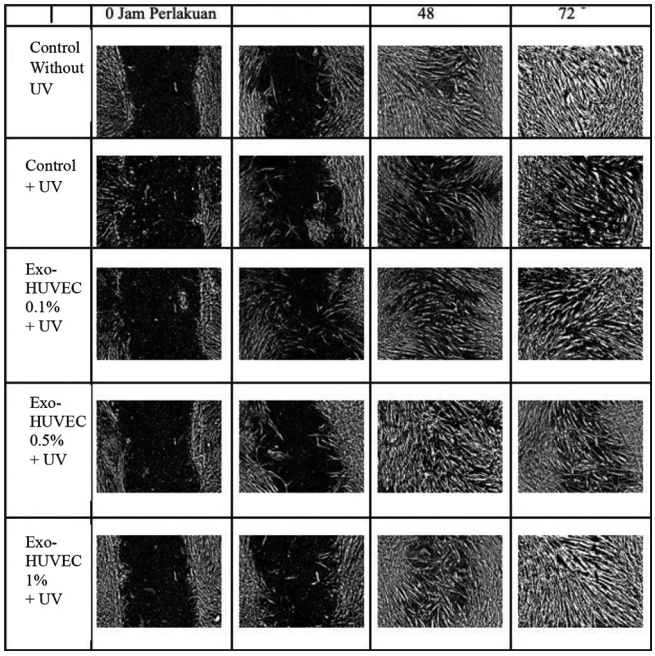

Figure 3: Fibroblast migration on 0, −24, −48, and −72 h after treatment: 1st row shows fibroblast migration in the control group 9.14 μm after 72 h; 2nd row shows UV group with fibroblast migration 25.85 μm after 72 h; 3rd row, shows the 0.1% Exo-HUVEC group with 24.35 μm fibroblast migration after 72 h; 4th row shows the 0.5% Exo-HUVEC group with 15.08 μm fibroblast migration after 72 h; 5th row shows the 1% Exo-HUVEC group with 11.78 μm fibroblast migration after 72 h (white bars represent fibroblasts)

Vascular Endothelial Growth Factor (VEGF) stimulates fibroblast proliferation and migration because it is a key factor in the angiogenesis process [26], [27]. Furthermore, direct UV light exposure to fibroblast cells causes an increase in ROS and induces apoptosis, resulting in significant cell damage. Increased fibroblast migration could be a sign of reduced oxidative stress and cell apoptosis, which define as a coordinated physiological process and essential for the development, repair and regeneration of tissues. Fibroblast migration is the movement of individual fibroblast, groups or clusters of fibroblast from one location to another. The migration of fibroblasts increases the amount of collagen – ECM, resulting in a reduction in skin aging, particularly photoaging. A previous study showed that mesenchymal stem cells present during *in vivo* tests can promote the proliferation and migration activity of keratinocytes and fibroblasts, which are two important effector cells for skin regeneration. They also accelerate the re-epithelialization process, increase collagen maturity, and improve angiogenesis [14]. Exo-HUVEC contains a set of miRNAs that are widely available in HUVEC, with miR-126 being the most abundant. It is capable of increasing cell proliferation and migration, the number of cell colonies, as well as inhibiting apoptosis [25], [27].

Mesenchymal stem cell (MSCs) can reduce UV-induced skin damage by secreting paracrine factors including Keratinocyte Growth Factor (KGF), Fibroblast Growth Factor -1 (FGF-1), and Vascular Endothelial Growth Factor (VEGF), thereby promoting the production of collagen and fibronectin from skin fibroblasts [28]. In damaged tissue, Umbilical Cord-Mesenchymal Stem Cell (UC-MSCs) attract stem cells/progenitor cells through paracrine activity involving Stromal cell-Derived Factor-1/C-X-C chemokine receptor type 4 (SDF-1/CXCR4) and Monocyte Chemoattractant Protein-1/CC Chemokine Receptor 2 (MCP-1/CCR2) interactions. The role of Hepatocyte Growth Factor (HGF) in the HUVEC secretome is to increase cell proliferation. Paracrine chemo attractants and potent angiogenic factors influence the microenvironment by acting on different cell types, which function in tissue repair and angiogenesis [29]. Research by Kim *et al*. (2017) reported that MSCs secrete cytokines and various growth factors such as Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF), which are important for skin rejuvenation and wound healing by synthesizing collagen HDFs. The paracrine effect was evaluated as one of the main mechanisms of action in MSCs that are beneficial in growth factors for skin rejuvenation [30].

According to Feng *et al*., (2010) exosomes derived from stem cells in nano-sized conditioned media and can make a higher regeneration effect through penetration into the skin epidermis. Meanwhile, HUVECs are often the model system of choice for the bio-pharmacological industry and preclinical trials because they have several advantages [31]. Bodega reported that HUVEC-derived microvesicles (MV) contain active antioxidant molecules and have antioxidant roles, maintain blood redox status and prevent cardiovascular disease [32]. This study supports the research that has been carried out by Joo *et al*. (2015) with *in vitro* and *in vivo* methods reporting that HUVEC secretomes can increase angiogenic potential through upregulation of the Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) signaling pathway derived from tissue. UCB-MSC released a higher content of wound healing factors, compared to other MSCs [30].

Elevated levels of paracrine factors secreted from UC-MSCs in injured tissues may promote the recruitment of circulating mesenchymal and progenitor cells to injured tissues. Shen *et al*. (2015) reported that UC-MSC secretes various cytokines and chemokines, including increased amounts of Stromal cell-Derived Factor-1 (SDF-1), Monocyte Chemoattractant Protein-1 (MCP-1), HGF, IL-8, Insulin-like Growth Factor-1 (IGF-1) and VEGF [29]. Umbilical Cord Blood-derived Mesenchymal Stem Cell (UCB-MSCs) promote the migration, proliferation, and synthesis of fibroblast collagen. In addition, UCB-MSC conditioned media also promoted wound closure and epithelialization through subcutaneously injected animals. Because these benefits come from the use of UCB-MSCs, the development of cosmetic products using stem cells is an interesting research [30].

This study can provide an overview of the work of exosomes from HUVEC secretomes in the process

to inhibit or improve photoaging. The working effect starts from HUVEC which is an endothelial cell that has growth factors so that it can affect the cell regeneration process. The ability of exosomes from HUVEC secretomes in reducing MDA levels can reduce the apoptotic process (decreased caspase and Annexin/PI levels), as well as markers of aging improvement seen from increased migration.

Conclusion

The administration of Exo-HUVEC in this study resulted in a decrease in MDA levels, a decrease in Annexin/PI, and an increase in fibroblast migration rate in skin exposed to UVB as a photoaging model.

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