Protective effects of simvastatin against ultraviolet B-induced photoaging of human dermal fibroblasts

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Received: 25 February 2021
Accepted: 09 May 2021

Background: Simvastatin is a beta-hydroxy-beta-methylglutaryl-CoA (HMG-CoA) inhibitor molecule with several pleiotropic (immunomodulatory, anti-inflammatory, and antioxidant) activities. In this study, we evaluated the protective effect of simvastatin on ultraviolet B (UVB)-induced photoaging of normal human dermal fibroblast cultures by assessing fibroblast proliferation, collagen deposition, and fibroblast morphology.

Methods: This study was an in vitro experiment using normal human skin fibroblast cell cultures. Fibroblasts were then cultured and observations were made of fibroblast proliferation, collagen deposition, and cell morphology using various concentrations of simvastatin (0 nM, 0.01 nM, 0.1 nM, 0.5 nM, 1 nM, and 5 nM) and UVB exposure (100 mJ/cm^2).

Results: After UVB exposure, a significant decrease in fibroblast proliferation and collagen deposition was observed. Cells appeared thinner, and fibroblasts were less organized and more pointed. Simvastatin with 0.01 nM, 0.1 nM, 0.5 nM, 1 nM, and 5 nM levels could significantly maintain cell proliferation and collagen deposition compared to UVB-irradiated cell groups without simvastatin. Interestingly, fibroblast proliferation and collagen deposition in the simvastatin group above 0.5 nM were not significantly different from the normal human dermal fibroblast group. An increased level of collagen deposition was also confirmed by observing the fibroblast morphology, which had more red-smeared cells on Sirius red staining. The antioxidant activity of simvastatin might play a role in fibroblast proliferation and collagen deposition, protecting against UVB by inhibiting reactive oxygen species. Simvastatin maintained fibroblast morphology, possibly by preventing DNA damage and maintaining membrane-bound collagen fiber deposition.

Conclusions: Our findings revealed that simvastatin pretreatment mitigated UVB-induced photoaging in human dermal fibroblast cells by maintaining fibroblast proliferation, collagen deposition, and fibroblast morphology.

Keywords: fibroblast, photoaging, simvastatin, UVB

Iran J Dermatol 2022; 25: 34-40

DOI: 10.22034/ijd.2021.270743.1330

INTRODUCTION

As the body's largest organ, the indispensable role of our skin is to protect the body from damaging exposures [1]. The most skin-damaging exposure is ultraviolet (UV) irradiation, which is found in sunlight [2]. This exposure causes premature aging to the skin, called photoaging, characterized by leathery, lax skin with coarse wrinkles, "broken"-appearing blood vessels, and poor pigmentation

of brown spots [3]. Photoaging is an important topic because most people are concerned about their physical appearance [4].

When fibroblasts are irradiated with UV radiation, whether UVA or UVB, reactive oxygen species (ROS) production is triggered, eliciting oxidative stress that leads to skin function damage [5,6]. Ultraviolet exposure also increases the production of proinflammatory cytokines by keratinocytes, thus triggering inflammation in the skin [7]. The immune response to the skin is also suppressed due to UV exposure, especially UVB, due to the release of immunosuppressive cytokines and migration of antigen-presenting cells to draining lymph nodes [8]. Since UVB possesses more energy than UVA, the degree of damage is higher [9].

Currently, the drug modality most commonly used for reducing photoaging is the retinoid class, which includes agents such as tretinoin [4,9]. Tretinoin increases tactile smoothness through several mechanisms such as thickening the epidermis (due to spongiosis), making the stratum corneum pattern more compact, stimulating collagen production, and preventing UV-induced collagenase activation. However, in some circumstances, adverse effects of hypervitaminosis A appear, such as erythema, peeling, and burning [4]. Although the efficacy and safety profile are good, further exploration is still needed for other molecules that can be used for the prevention of photoaging with higher efficacy and fewer side effects.

Simvastatin is a beta-hydroxy-beta-methylglutaryl-CoA (HMG-CoA) inhibitor that is commonly prescribed to reduce cholesterol levels. Many studies show that simvastatin has pleiotropic properties, including immunomodulatory [10], anti-inflammatory [11], and antioxidant [12] effects. As a member of the statin group, simvastatin is considered the best choice in terms of cost and safety compared to other statin members [13]. Skin photoaging is closely related to the alteration of immune cells and inflammation of the skin [14,15]. To the best of our knowledge, there is no study relating simvastatin to photoaging prevention. Therefore, we aimed to evaluate the ability of simvastatin to mitigate skin photoaging in human dermal fibroblasts.

MATERIALS AND METHODS

Ethical Approval. This study was performed in accordance with the Declaration of Helsinki, and the procedures were approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia, with reference number: KE/FK/0167/EC.

Simvastatin preparation. Simvastatin (molecular weight 418.6 g/mol) was taken from 10 mg tablets, which were pressed into powder form. Simvastatin was mixed with 2 mL of complete Dulbecco Minimal Essential Medium (DMEM) solution. Then, gradual dilution was done to obtain 2 mL of consecutive concentrations of 0.01 nM, 0.1 nM, 0.5 nM, 1 nM, and 5 nM.

Cell isolation and culture. Fibroblasts were isolated from healthy skin with a mixture of DMEM, penstrep, gentamicin sulfate, and amphotericin medium, then stored and transferred using an icebox. Fibroblasts were cultured using a complete medium consisting of DMEM, serum bovine fetal, amphotericin B, penstrep, and ceftriaxone until reaching 60% confluency. Passage III subculture was used in this experiment and placed into 96 wells that contained 15-20 x 10^5 cells per well in serum-free media, 37 ºC, and 5% CO_2. Simvastatin was added to each well in the different concentrations, then incubated for 48 hours.

Group allocations. The cells were divided into seven groups: 1: control group: no treatment; 2: UVB group: UVB irradiation without simvastatin; 3, 4, 5, 6, and 7: UVB + simvastatin pretreatment (0.01 nM, 0.1 nM, 0.5 nM, 1 nM, and 5 nM respectively).

UVB irradiation. Before being given UVB irradiation, the cells were washed twice using 200 µL of phosphate-buffered saline (PBS). Then, PBS was removed until 100 µL of PBS was left in each well. Cells were irradiated with 100 mJ/cm^2 of UVB. The UVB dosage was adopted from a previous study (5). Simultaneously with this process, other cell groups were stored in a dark room with the same conditions as irradiated cells. UVB irradiations originated from a 365-nm UV illumination lamp (35 Å, 20 cm). A 4 mm-thick glass was placed 20 mm above the lamp to eliminate short waves. A plastic Petri dish was placed on the glass before irradiation was done from the bottom surface.

Proliferation of fibroblasts. Measurement of fibroblast proliferation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay began by removing all the

remaining media in the well, then adding 50 µL of MTT and 200 µL of the new medium. The microplate was then wrapped in an aluminum foil sheet and incubated for 4 hours. MTT and medium were discarded. As much as 200 µL of DMSO and 25 µL of glycine buffer were added to each well, then fibroblast proliferation was assessed using a 570-nm wavelength spectrophotometer. The obtained absorbance data were converted into proliferation percentage using the formula: (absorbance of each group/absorbance of the control group) ×100%.

Collagen deposition measurement. Measurement of non-soluble collagen was done by removing the medium in each well containing fibroblast cells before washing each well with 200 µL of PBS. The fibroblast suspension was fixed with Bouin solution for 1 hour and then washed with running water until the yellow color disappeared. As much as 200 µL of Sirius red was added to each well, then incubated for 1 hour. Sirius red was discarded, and each well was washed with 0.1 N of hydrochloric acid until Sirius red disappeared from the supernatant and walls of each well. As much as 200 µL of 0.5 N sodium hydroxide was then added and left for 30 minutes. The measurement was done using a 570 nm wavelength spectrophotometer. The obtained absorbance data were converted into collagen deposition percentage using the formula: (absorbance of each group/absorbance of the control group) ×100%.

Observation of cell morphology. Observation of fibroblast cell culture was conducted after staining with Sirius red. Cells were visualized using a camera on a microscope (total magnification: 100×).

Statistical analysis

Data were expressed as mean ± standard deviation (SD). For multiple comparisons, one-way analysis of variance (ANOVA) was used followed by Dunnett's post hoc test. All analyses were performed using SPSS v. 23 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Proliferation of fibroblasts. From the results of the fibroblast proliferation assay, UVB irradiation decreased the number of viable fibroblast cells by up to 50% compared with the control group (Figure 1). Single UVB irradiation directly damaged cells due to the single layer of fibroblast cells [13]. Significant inhibition of fibroblast proliferation was also observed at the 100 mJ/cm^2 irradiation dose.

Figure 1 demonstrates the ability of simvastatin to significantly maintain cell proliferation after UVB irradiation in all concentration series compared with the UVB group. Interestingly, cell proliferation in the simvastatin group was able to exceed the cell proliferation in the control group at simvastatin concentrations of 0.5, 1, and 5 nM, with the amount of cell proliferation exceeding 100% compared with the control.

Collagen deposition. Collagen deposition in fibroblast cells in the UVB group decreased by more than 50%, as shown in Figure 2. Simvastatin administration significantly increased collagen

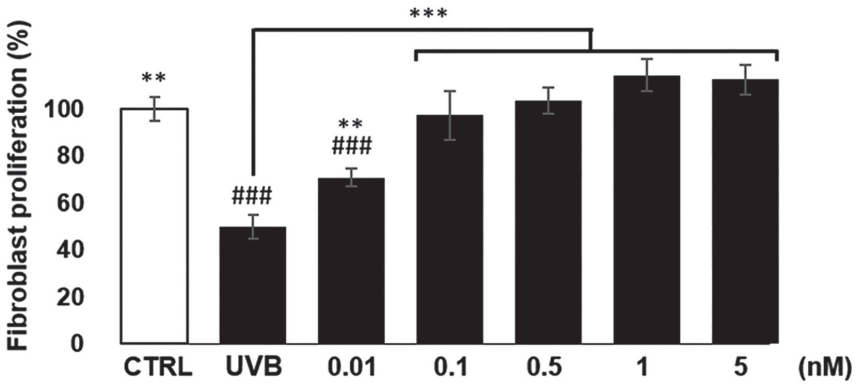

Figure 1. Simvastatin maintained fibroblast proliferation after UVB irradiation. Groups of simvastatin concentrations at 0.1 – 5 nM were not significantly different from the control group (CTRL). Cells were irradiated with UVB (100 mJ/cm^2) and incubated with or without simvastatin for 48 hours. Data in mean ± SD. Statistically significant differences: ** $P < 0.01$, *** $P < 0.001$ compared to UVB group. ### $P < 0.001$ compared to control group.

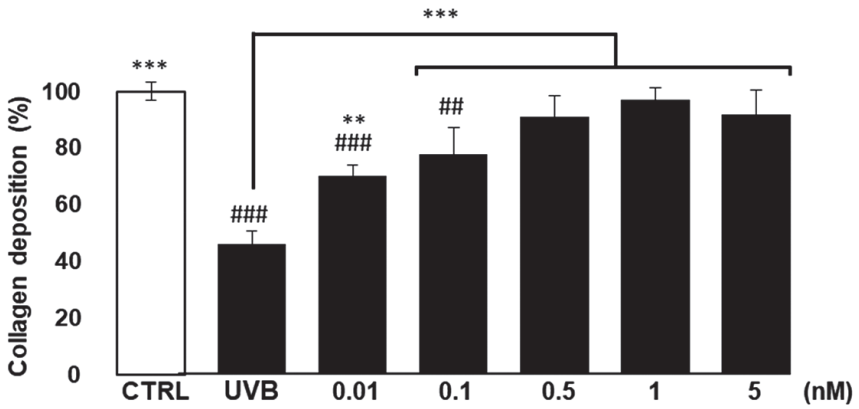

Figure 2. Simvastatin maintained collagen deposition after UVB irradiation. Groups of simvastatin concentrations at 0.5 – 5 nM were not significantly different from the control group (CTRL). Cells were irradiated with UVB (100 mJ/cm^2) and incubated with or without simvastatin for 48 hours. Data in mean ± SD. Statistically significant differences: ** $P < 0.01$, *** $P < 0.001$ compared to UVB group. ## $P < 0.01$, ### P < 0.001 compared to control group.

deposition compared with the UVB group. The highest collagen deposition was found in cell groups given 1 nM simvastatin. Moreover, collagen deposition in the simvastatin group was not significantly different from the control group at simvastatin concentrations of 0.5, 1, and 5 nM, with the amount of collagen deposition reaching approximately 90% compared with the control.

Fibroblast morphology. After UVB irradiation, morphological changes occurred in the UVB group (Figure 3). Cells appeared thinner, and fibroblasts were less organized and more pointed. The results also showed that the red color from collagen deposition increased along with the increases in simvastatin concentration in the cell culture.

DISCUSSION

As human life expectancy increases, there is an increase in cumulative damaging exposure to the skin. Chronic exposure to UVB irradiation results in clinical and histological changes that are consistent with aging such as wrinkles, abnormal pigmentation, loss of elasticity, and repair of disturbed cells. The effects of chronic exposure include decreased collagen production by fibroblasts and increased matrix metalloproteinase (MMP) expression [17]. Therefore, materials containing bioactive compounds with anti-photoaging activity are important for medical and cosmetic purposes [17].

From our findings, UVB irradiation inhibits fibroblast proliferation and collagen deposition and induces apoptosis. Increasing ROS production after UVB irradiation may trigger collagen degradation by MMP activation via the MAPK/AP-1 signaling pathway [16]. Besides, a single UVB irradiation can cause cell damage due to the monolayer properties of cultured fibroblast cells [16]. This *in vitro* model is different from *in vivo* models that have many cellular layers; hence, chronic exposure is needed to produce visible effects. Significant inhibition of fibroblast proliferation was also observed in UVB irradiation up to 100 mJ/cm^2 [5].

Damaged fibroblast morphology can be explained by the direct effect of UVB on fibroblast DNA. UVB irradiation activates the death receptors on the cell membrane that can activate the caspase cascade and apoptosis [18]. UVB irradiation increases cyclobutane pyrimidine dimers (CPD) in human dermal fibroblasts, triggering apoptosis. However, the apoptosis process induced by UVB is also thought to be beneficial for preventing tumor growth in the skin [19].

The results of our study showed that simvastatin maintained a higher proliferation of fibroblasts compared with the UVB group. The antioxidant activity possessed by simvastatin is thought to be related to the decrease in ROS in fibroblasts, which in turn increases fibroblast viability and proliferation. However, ROS is able to stimulate the growth of fibroblasts at a low concentration [20]. Besides, the prevention of direct DNA damage by UV has a role in increasing fibroblast proliferation. In UVB-induced DNA damage, the production of pyrimidine dimers and 6-4 photoproducts are increased. At low UV doses, DNA replication is inhibited, and

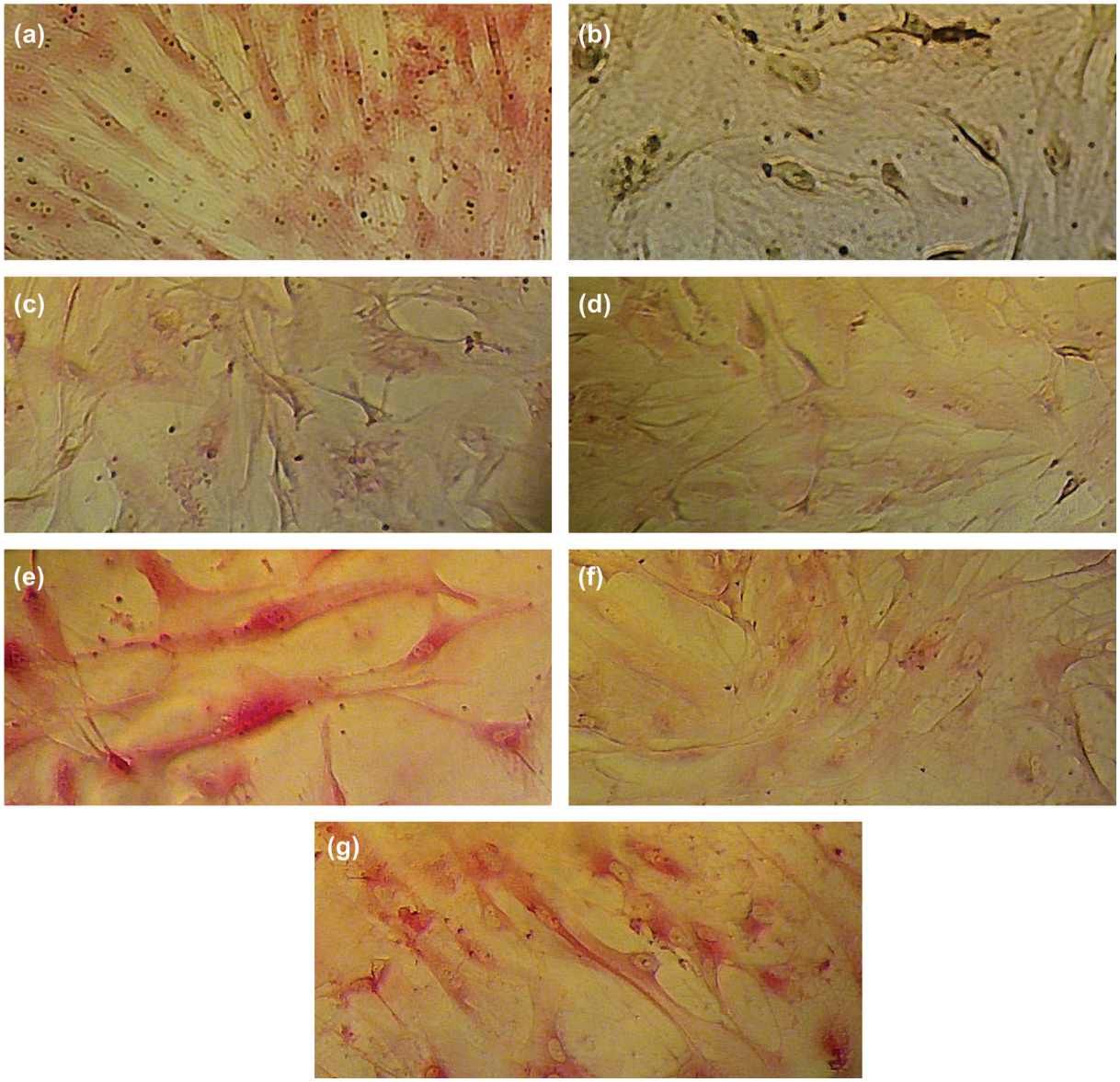

Figure 3. Morphological changes of human dermal fibroblast cultures after UVB irradiation, observed under light microscope (100×): (a) control group (did not receive UVB irradiation); (b) cells only treated with UVB (100 mJ/cm^2); (c-g) cells treated with UVB (100 mJ/cm^2) plus simvastatin (c) 0.01 nM, (d) 0.1 nM, (e) 0.5 nM, (f) 1 nM, and (g) 5 nM.

cells undergo arrest, followed by apoptosis [19]. Research reporting the effects of simvastatin on dermal fibroblasts is limited to the animal wound model by Khoshneviszadeh *et al.*, which showed that simvastatin improves wound healing by inducing epithelialization and proliferation of Sprague Dawley rats' fibroblasts from stereological and histopathological analysis [21].

This study also showed that simvastatin helped fibroblasts to maintain collagen deposition. In several studies, antioxidants have been used as protective agents against UVB-induced photoaging. For example, *Coffea arabica* extract had a reducing activity and, as a free radical scavenger, had a UVB photoprotection effect. Simvastatin is also known to have antioxidant-like effects [12]. The intrinsic antioxidant activity of simvastatin to scavenge free radicals is the most effective compared to other statin members such as fluvastatin, atorvastatin, and pravastatin [22]. Another study

showed simvastatin lowers ROS by activating Nrf2 through the PI3K/Akt pathway in primary mouse embryonic fibroblasts [23]. This antioxidant activity might explain the protective effect of simvastatin against UVB.

The ability of simvastatin to maintain collagen deposition has implications for the fibroblast morphology that can be maintained after UVB exposure, resembling the condition of fibroblasts without UVB exposure. The 'spindle-shaped' or fusiform fibroblasts originate from membrane-bound collagen fibers [24]. The increased collagen deposition induced by simvastatin is also supported by several other studies. An *in vivo* study showed an increase in collagen bundles on stereological observation after administration of 2% simvastatin gel to rat skin [21]. Another study using simvastatin in the dosage form of nanoparticles showed an increase in mature collagen fibers in the wound healing process after 11 days of simvastatin gel treatment [25]. However, several studies have shown inhibition of collagen deposition in fibroblasts by simvastatin. When statins were administered to patients with coronary heart disease and atrial fibrillation, increased collagen degradation and decreased inflammation were seen [26]. Administration of simvastatin to human intestinal fibroblasts *in vitro* was found to inhibit the activation of TGF-β_1 intestinal fibroblasts by inhibiting Smad-3 phosphorylation. This mechanism explains the antifibrotic effect of simvastatin [27]. Moreover, in TGFβ_1-stimulated primary human airway fibroblast cultures, 1-15 µM of simvastatin dose-dependently inhibited collagen type I protein abundance [28]. It is important to note that these studies [26-28] did not involve UVB-induced photoaging and did not use human dermal fibroblasts.

The different roles of simvastatin in human dermal fibroblasts and fibroblasts from other organs can be explained by the different locations of fibroblasts, which determine the fibroblasts' intrinsic properties. Differences in fibroblast gene expression according to the surrounding also explain this diverse intrinsic fibroblast property [29].

Simvastatin treatment with concentrations of 5 µM and 10 µM in systemic sclerosis fibroblasts and normal human dermal fibroblast cultures for 3 or 4 days caused a decrease in collagen and mRNA type I collagen gene expression [30]. Besides, Louneva *et al.* found that mevalonate and geranylgeranyl pyrophosphate (GGPP), but not farnesyl pyrophosphate (FPP), were able to restore the expression of collagen genes that were previously inhibited by simvastatin [30]. These findings are methodologically similar to our study since both use simvastatin and normal human dermal fibroblasts. However, this study differs in UVB exposure and simvastatin concentration. The concentration of simvastatin used in the study of Louneva *et al.* was about a thousand times more than ours. This difference might explain how the effect of simvastatin that occurs in normal human dermal fibroblasts is also different.

Further investigations are needed concerning the molecular mechanisms through which simvastatin inhibits UVB-induced photoaging, such as MMP-1, MMP-3, MMP-8 enzyme activity, ROS levels, and gene expression related to collagen synthesis and further degradation pathways after simvastatin and UVB exposure.

CONLUSIONS

This study revealed that simvastatin pretreatment could mitigate UVB-induced photoaging in human dermal fibroblast cells by maintaining fibroblast proliferation, collagen deposition, and fibroblast morphology. Thus, simvastatin may be used as a novel anti-photoaging molecular treatment. Future research should explore the molecular mechanisms behind these anti-photoaging activities.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interest: The authors declare that no competing interests exist.

FUNDING STATEMENT

This work was supported by a research grant from the Indonesian Ministry of Higher Education. The funders had no role in study design, data collection and analysis, decision to publish, or manuscript preparation.

Acknowledgment

The authors would like to thank Zulti Lavita and Lintang Unggul Rini for contributing to this study.

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