ESTABLISHMENT OF AGED HUMAN DERMAL FIBROBLASTS BY ULTRAVIOLET IRRADIATION

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ABSTRACT

Introduction: Photoaging is a degenerative condition that leads to skin fragility, loss of function and cosmetic dissatisfaction. The senescence of human dermal fibroblasts (HDFs) plays vital roles in the pathogenesis of skin aging. Therefore, we aim to develop a senescent HDFs model with an optimized acute ultraviolet (UV) irradiation protocol for investigating mechanism and developing novel treatment intervention for photoaging. ***Materials and methods:*** HDFs were isolated from human abdominal skin and the expression of some fibroblast-specific markers was evaluated by flow cytometry. HDFs were then divided into three groups: normal (non-UV), UV1, and UV2. The irradiation dosages were UVB 780 mJ/cm^2 + UVA 480 mJ/cm^2 for 10 mins in group UV1, and UVB 1170 mJ/cm^2 + UVA 720 mJ/cm^2 for 15 mins in group UV2. The HDFs were then assessed with some markers for aging. The cell morphology and size were observed by microscopy. SA-β-galactosidase expression of cells was stained by SA-β-Gal kit and measured using Image J software. The differentiation potential of HDFs into chondrocytes, osteocytes, and adipocytes was checked by inducible medium. Cell proliferation was stained by Alamar blue assay and measured by spectrophotometer. Gene expression of collagen 1, collagen 3, MMP-3, p16, and p21 were quantified by real-time RT–PCR. ***Results:*** The results showed that isolated cells exposed spindle-shape morphology, and highly expressed with fibroblast markers such as CD 90 (63%), Vimentin (85.55%), and S100A4 (61.17%). After UV irradiation, in the UV-treated groups, the cell appearance became flattened and larger. Cell sizes were significantly increased in UV-irradiated HDFs ($p<0.05$). The integrated density of SA-β-gal signals was higher in the UV groups than in the normal group ($p<0.05$). The time for fibroblasts to differentiate into adipocytes and chondrocytes was longer in both UV groups compared to normal HDFs. The division ability of cells significantly decreased in both UV groups at 72 hours and was maintained until 8 days ($p<0.001$), with a lower proliferation rate in the UV2 group. Moreover, UV light increased the mRNA expression of MMP-3, p16, and p21 and decreased the expression of collagen 1 and 3 in the UV2 group ($p<0.05$). ***Conclusion:*** These results demonstrated that the single irradiation dose of UVB 1170 mJ/cm^2 + UVA 720 mJ/cm^2 for 15 mins has successfully established the HDF senescence model.

Keywords: *human dermal fibroblast, ultraviolet, senescence*

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I. BACKGROUND

Photoaging has been an emerging problem which has complex mechanism. The study of skin photo-damage pathogenesis on human has some limitations such as long-time follow-up, complicated and invasive procedure to investigate and ethical issues. In much research, senescent human dermal fibroblast (HDF) accumulation has been

shown as the feature of skin aging. Therefore, to elucidate the mechanism of cutaneous photo-damaged and develop treatment methods for photoaging, senescent HDF model has been developed.

Fibroblasts are principle cell type of connective tissue, which play vital roles in extracellular matrix production, wound healing and other diseases pathogenesis such as fibrosis or cancer. Fibroblasts have spindle shape and attach well on artificial surfaces in cell culture. Their Hayflick-limit is 70 to 80 times before they enter an irreversible state called senescence. Cellular senescence is defined as cells entering growth arrests permanently after several divisions. Senescent cells are irreversibly cell-cycle arrested via the p53–p21CIP1 or the p16INK4a–Rb axis, accumulate senescence-associated β-galactosidase activity (SA-β-gal) and display a typical morphology. In addition, cells can be premature senescent by stress-causing factors such as ultraviolet irradiation (UVR), copper sulphate (CuSO4) or hydrogen peroxide (H2O2)[4]. Among others, UV irradiation is the most common cause of skin aging in real life.

Solar light consists of UVB (290–320 nm) and UVA (320–400 nm) can go through the epidermis and reach the dermis of human skin. These lights are able to be absorbed by endogenous chromophores and photosensitizers, which leads to the generation of reactive oxygen species (ROS) resulting in the damage of DNA, proteins and lipids. UVB can directly interact with DNA and generate dipyrimidine photoproducts such as cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone, consequence in carcinogenicity ability. UV radiations can activate several cell surface receptors causing multiple signal transduction pathways related to growth, differentiation, senescence and connective tissue degradation. These receptors include receptors for epidermal growth factor (EGF), tumor necrosis factor (TNF), interleukin-1 (IL-1) and keratinocyte growth factor (KGF). The UV responses consist of acute conditions (inflammation, sunburn, tanning) or chronic processes (photo-aging, immunosuppression, carcinogenesis)[3].

Most of previous studies have evaluated the effect of UVB on the senescence of dermal fibroblast, might be due to the fact that UVA is poorly absorbed by nucleotides, causing less direct DNA damage than UVB. However, UVA can still be absorbed by other cellular chromophores, generating free radicals, and consequently causing oxidative stress in cells[8]. Also, low dose of UVA and UVB irradiation on dermal photoaging has not been well characterized, with most of previous studies focused on the cytotoxic dose of UV to cause fibroblast damage or apoptosis. Because both UV types can cause simultaneously effect on skin cells, herein, we aimed to establish a senescent human dermal fibroblast *in vitro* model by acute sub-cytotoxic UVA-UVB irradiation.

II. MATERIALS AND METHODS

Fibroblast isolation and culture. Human dermal fibroblasts (HDFs) were isolated from human abdominal skin tissue of a healthy women (aged 44 years), discarded after abdominal plastic surgery. Informed consent was obtained from the patient. All procedures were approved by the Ethics Committee at the University of Medicine and Pharmacy Ho Chi Minh City (approval no. 56/HĐĐĐ-ĐHYD, date 28/01/2021). Briefly, the epidermal and fat layers of the skin tissue were removed, remaining dermis was cut into small pieces size 1 mm^2 by sharp blade and placed in the 125 cm^2 flask surface. The

cells started to grow out of the explant after 5 to 7 days cultured by Dulbecco's modified Eagle's medium F12 (DMEM/F12; Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA) and 1% penicillin–streptomycin (Sigma, St. Louis, USA) in a humidified incubator with 5% CO2 at 37°C. Cells were passaged at 70%–80% confluence, and used for the experiments at the 4th passage to avoid *in vitro* replicative-induced senescence. The cells were cryopreserved by Cryosave I (Regenmedlab, Viet Nam) for further experiments.

Fibroblast characterization. Fibroblasts were defined as mesenchymal stem cell-related, therefore they possessed the differentiation potential into osteocytes, chondrocytes and adipocytes. To investigate the differentiation ability, 4th-passage HDFs were cultivated in specific adipogenic, osteogenic, and chondrogenic differentiation medium (Regenmedlab, Vietnam) for one to three weeks, then stained with Oil Red O (Sigma Aldrich, USA), Alizarin Red S (Sigma Aldrich, USA) and Safranin O (Sigma Aldrich, USA). Flow cytometry was used for analysis of specific markers expression for fibroblast such as Vimentin and S100A4, and some markers of mesenchymal stem cells such as CD14, CD34, HLA-DR, CD44, CD90, and CD105. Stained cells were analyzed using flow cytometry (BD FACSCaliburTM, BD Biosciences, USA).

UV exposure to fibroblast. For establishing senescent HDFs, the lamp using was Reptile UVB 100 compact 25W PT2187 (Exo Terra, USA). The distance from the lamp to the surface of the plate was consistently 10 cm. Before every exposure, the UV lamp was warmed up for 10 mins to set stable condition for irradiation and UV dose was measured with two UV-AB meters included Extech UV-AB light meter, model UV505 (Wilsonville, USA) and Santacary XAR-UV UVB light meter (Guangdong, China). HDFs were divided into three groups: normal; UV1 and UV2 single dose, equivalent as UVB 780 mJ/cm^2 + UVA 480 mJ/cm^2 for 10 mins, and UVB 1170 mJ/cm^2 + UVA 720 mJ/cm^2 for 15 mins, respectively. In preliminary experiments, we tested five different doses of UVA and UVB. Upon irradiation with doses in 10 mins and 15 mins, most of cells were viable by observing under microscope. Therefore, we chose these doses to investigate non-cytotoxic effects of UV irradiation on HDFs. Cells were seeded in a 6-well disk. Medium culture was removed, and cells were washed with phosphate buffer saline (PBS) twice before irradiated under UV-AB radiation. All UV irradiations were performed under a thin layer of 100 µl 1x PBS to prevent cells from drying up. After UV-AB irradiation, cells were rinsed again twice with PBS and incubated immediately in fresh culture media with serum for 24 h, 48 h, 72 h and 96 h.

Cell morphology and size change. Cells were inspected with an inverted microscope to observe normal growth and appearance. To measure the size of cells, HDFs were detached from culture plates surface using Trypsin-EDTA 0.25% (Gibco, USA), then centrifugated at 300 x g to remove supernatant and re-suspended with fresh culture media. Suspended fibroblast images were captured under inverted microscopic and cells size were measured by AxioVision 4.8 software.

SA-β-Galactosidase expression. SA-β-Gal staining was used to investigate the senescence of HDFs. Cells were plated on 48-well plates with the culture medium and

incubated at 37°C and 5% CO2 for 24 hours. After UV-exposure, the cells were fixed with 0.125 mL Fixative Solution (ab102534, Abcam, UK) at room temperature for 15 mins and subsequently stained by the Staining Solution Mix in the SA-β-Gal staining kit (ab102534, Abcam, UK) following the instructions of the manufacturer. Positive senescent cells stained in blue were observed using an inverted microscope. Three images per well were taken, and the corresponding quantification of the SA-β-Gal staining was analyzed by ImageJ (National Institutes of Health, USA).

Cell proliferation. Cell proliferation was determined using Alamar blue assay (Resazurin sodium salt, Sigma-aldrich, USA) following the instructions of the manufacturer. HDFs were allocated into normal (non-UV), UV1 or UV2 groups. Each group of cells had three technical replicates seeded in 96-well plates (1×10^3 cells/well) and cultured in 100 µL of DMEM/F12 with 10% FBS. Subsequently, at 0, 24, 48 and 72h after UV exposure, the Alamar blue solution was then added to each well (10 µL per well) and cells were incubated at 37°C for 1 h. The absorbance at 595 nm was measured using a microplate reader (DTX 880 multimode detector, Beckman coulter, USA).

Gene Expression Analysis. Total RNA was extracted from HDFs ($1x10^6$ cells) using the phenol-chloroform method with the easy-BLUE Total RNA Extraction Kit (Boca Scientific Inc., Dedham, MA, USA). Real-time quantitative RT-PCR was performed using the Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) with primer sequences listed in Table I, following the protocol of the manufacturer. The final concentration of each primer was 10 µM. The standard PCR conditions were 55˚C for 10 minutes, followed by 95˚C for 1 minute, and then 40 cycles of 95˚C for 10 seconds and 60˚C for 45 seconds. Results were determined as the relative quantity value of the target group and calculated using the 2-ΔΔCq method[7].

Table 1. ***Human primer sequences for real-time quantitative polymerase chain reaction***

Target gene	Sequences (5'-3')	Seq. Len. (mer)	Ref
hCOL1A1	F: CCCTGGAAAGAATGGAGATGAT R: ACTGAAACCTCTGTGTCCCTTCA	22	NM_000088.4
hCOL3A1	F: TATCGAACACGCAAGGCTGT R: AAAAGCAAACAGGGCCAACG	22	NM_000090.4
hMMP3	F: ATGGACAAAGGATACAACAGGGA R: TGTGAGTGAGTGATAGAGTGGG	22	NM_002422.5
hGAPDH	F: AATGGGCAGCCGTTAGGAAA R: GCGCCCAATACGACCAAATC	20	NM_001256799.3
P16	F: CATAGATGCCGCGGAAGGT R: CTAAGTTTCCCGAGGTTTCTCAGA	19	AH005371.3
P21	F: CTTCGACCTTTGTCACCGAGA R: AGGTCCACATGGTCTTCCTC	21	BC000312.2
F, forward; R, reverse; MMP, matrix metalloproteinase			

Data analysis. Experimental values were analyzed using GraphPad Prism 10 (GraphPad, California, USA). All data were expressed as mean ± standard deviation (SD) or mean ± standard error (SE). Comparison among two groups was performed by t-test or Mann-Whitney test. Differences between more than two groups were analyzed by one-way or two-way ANOVA with Tukey's multiple comparison post-hoc test. $p < 0.05$ was considered significant.

III. RESULTS

Fibroblast isolation and cell culturing

After 5 days of primary culture, cells began to appear from the skin tissue (Fig. 1A) and proliferated well at day 10 (Fig. 1B) and were nearly full confluence at day 14 (Fig. 1C). After 1st, 2nd and 4th subculture, cells showed fibroblast-like spindle-shape morphology and attached well to the plate surface, without signs of replicative senescence (Fig 1. D-F).

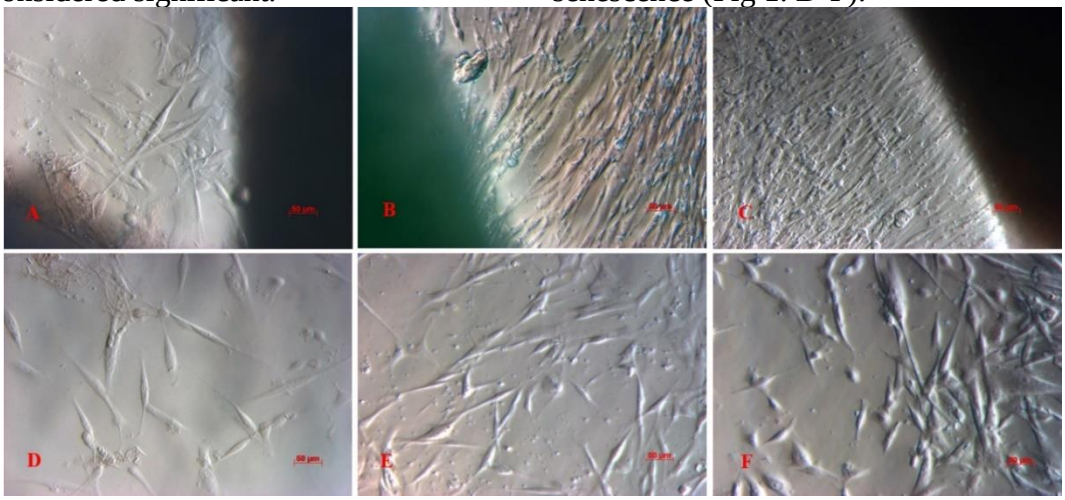

Fig 1. ***Fibroblast morphology and proliferation after isolation.*** *Primary culture of cells for* ***(A)*** *5 days,* ***(B)*** *10 days,* ***(C)*** *14 days and subculture* ***(D)*** *passage 1,* ***(E)*** *passage 2,* ***(F)*** *passage 4 (magnification 20X).*

Fibroblast characterization

Cells appeared as elongated spindle shape (Fig. 2A) and were able to differentiate into osteocytes (Fig. 2B), chondrocytes (Fig. 2C) and adipocytes (Fig. 2D). Flow cytometry analysis of cells showed that they were positive for some MSC-related markers such as CD44 (99,89%), CD90 (63%), and CD105 (65,68%), and negative for CD14 (2%), CD34 (0,68%) and HLA-DR (0.31%). Two specific fibroblast markers as Vimentin (85.55%) and S100A4 (61.17%) were positive. These results indicated that isolated cells had characteristics of fibroblast.

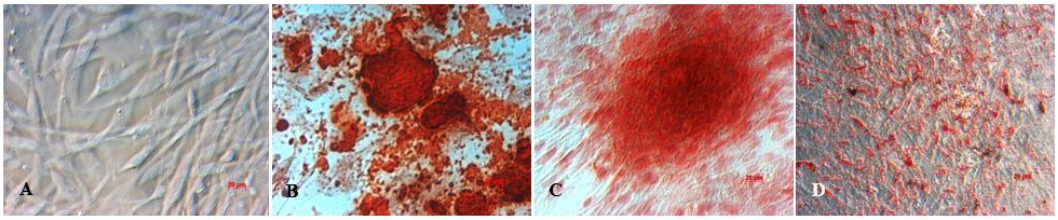

Fig 2. ***Characterization of fibroblast.*** *Representative image of fibroblast morphology under microscopic* ***(A)****. Osteogenic, chondrogenic and lipogenic differentiation of cells showed calcium accumulation staining by Alizarin Red S* ***(B)****, chondrogenic proteins staining by Safranin O* ***(C)****, and lipid droplets staining by Oil Red O* ***(D)****. Scale bar: 20 µm.*

UV irradiation dose

HDFs were irradiated with five UV doses. Normal cells were 70 per cent confluent with spindle shape (Fig. 3A). In 5 mins UV-AB dose, cells maintained normal shape and stretched well on the cultured flask surface (Fig. 3B). In 10- and 15-mins doses, cells slightly shrank, while retaining adhesion (Fig. 3C and D). In 20- and 30-mins doses, cells clustered and peeled off the culture surface (Fig. 3E and F). Therefore, two doses in 10 and 15 mins were chosen for further experiments as UV1 and UV2 groups.

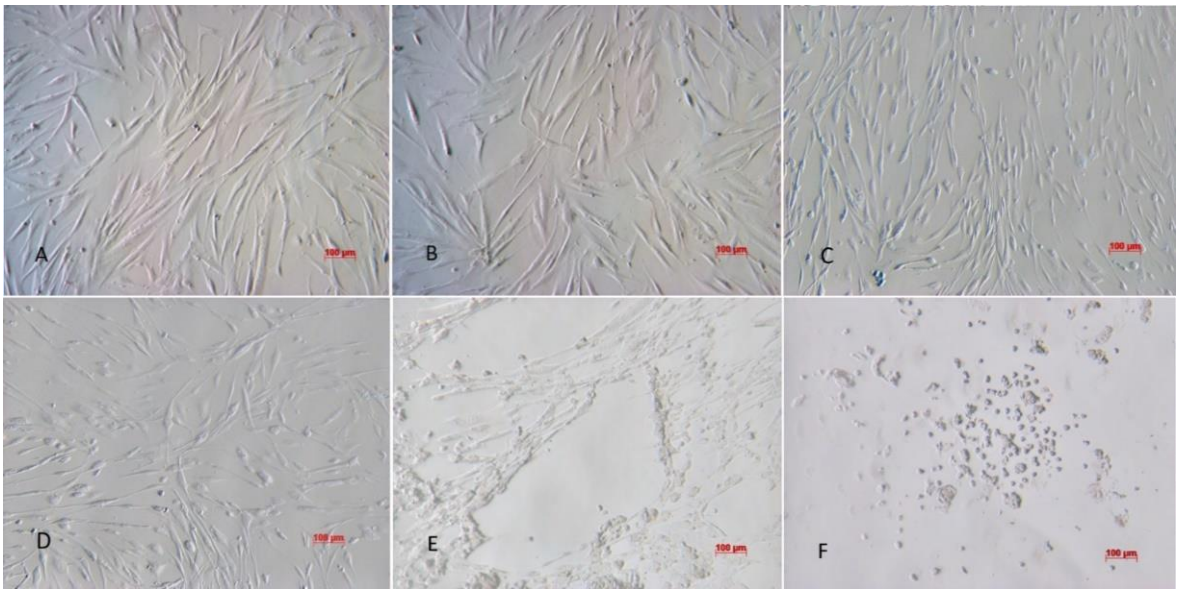

Fig 3. ***Cell reactions under various UV-AB irradiation doses***. ***(A)*** *normal cells without UV irradiation. Cells with different UV doses* ***(B)*** *UVB 390 mJ/cm^2 +UVA 240 mJ/cm^2 (5 mins),* ***(C)*** *UVB 780 mJ/cm^2 +UVA 480 mJ/cm^2 (10 mins),* ***(D)*** *UVB 1170 mJ/cm^2 +UVA 720 mJ/cm^2 (15 mins),* ***(E)*** *UVB 1560 mJ/cm^2 +UVA 960 mJ/cm^2 (20 mins),* ***(F)*** *UVB 2340 mJ/cm^2 +UVA 1440 mJ/cm^2 (30 mins).*

Fibroblast senescence-induced by UV irradiation

At the beginning, HDFs revealed spindle shape in normal, UV1 and UV2 groups (Fig. 4A1, B1, and C1). After UV-induced, in the UV1 (Fig. 4B3, B4 and B5) and UV2 groups (Fig. 4C3, C4 and C5), HDFs morphologies became flattened and larger at 48 h, 72 h and 96 h. Spindle-shape was replaced by polygonal-appearance and cells proliferation decreased compared to that of the normal group (Fig. 4A3, A4 and A5). Cell sizes significantly increased in UV-irradiated HDFs compared to the normal group at 24 h, 48 h, 72 h and 96 h ($p<0.05$) (Fig. 4D, E and F). Moreover, differentiation potential into adipocytes and chondrocytes of fibroblasts in the UV1 (Fig. 4H5 and H6) and UV2 groups (Fig. 4I5 and I6) also decreased compared to those in the normal group (Fig. 4H4 and I4). The division ability of cells significantly decreased in both UV groups at 72 h and was maintained until day 6, with a lower proliferation rate in the UV2 group compared to the UV1 group (Fig. 4J). In cells staining with SA-β-gal, there was no blue expression at 0 h (Fig. 4K1, L1 and M1), however, at 24 h, 48 h, 72 h and 96 h, blue-stained HDFs

were observed in the UV1 (Fig. 4L2, L3, L4 and L5) and UV2 groups (Fig. 4M2, M3, M4 and M5). The integrated density of SA-β-gal signals was significant higher in the UV groups than in the normal group at 96 h ($p<0.05$) (Fig. 4N). Furthermore, mRNA expressions of collagen 1 and collagen 3 were significant downregulated ($p<0.05$) and mRNA expression of MMP-3, p16 and p21 were upregulated in the UV2 group compared to that of the normal group, with significant differences between two group in the p16 gene (Fig. 4O).

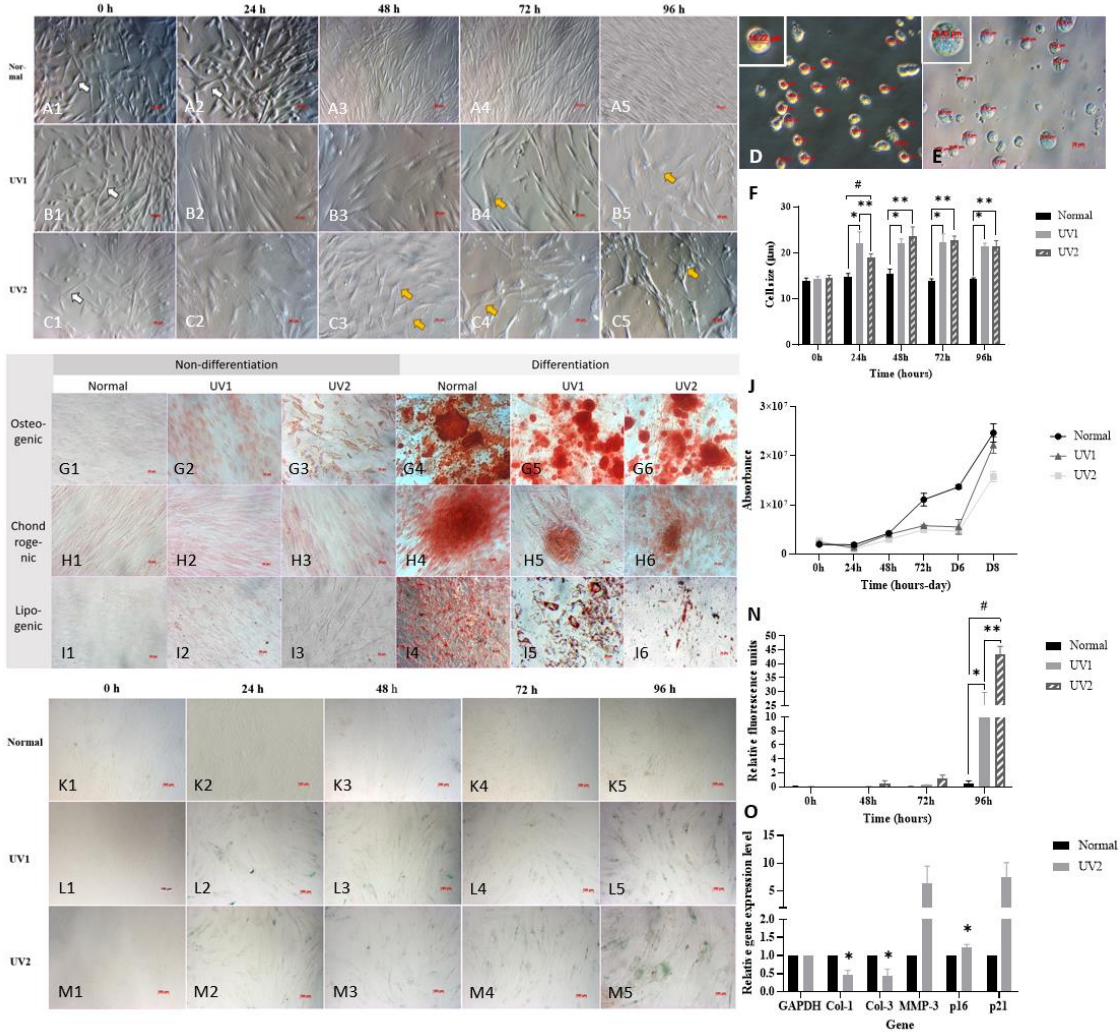

Fig 4. ***Senescent features of fibroblast induced by UV irradiation.*** *Fibroblasts morphology alteration after UV irradiation in normal* ***(A1-A5)****, UV1* ***(B1-B5)****, and UV2 group* ***(C1-C5)****; white arrows: normal cells, yellow arrows: senescent cells; scale bar = 50 μm. Cell sizes before* ***(D)*** *and after* ***(E)*** *UV irradiation (40X magnification). Cell size alteration bar chart between different groups at various time points; *p<0.05, normal vs UV1; **p < 0.05, UV1 vs UV2; #p <0.05, normal vs UV2, n=3* ***(F).*** *Undifferentiated cells were grown only in DMEM/F12 and showed only background staining for Alizarin Red S* ***(G1-G3)****, Safranin O* ***(H1-H3)****, and Oil Red O* ***(I1-I3).*** *On differentiation-induced groups, UV1 and UV2-exposed*

groups showed positive staining for Alizarin Red S ***(G5 and G6),*** *similar to normal group* ***(G4),*** *while revealed lower positive for Safranin O* ***(H5 and H6)****, and Oil Red O* ***(I5 and I6),*** *compared to normal group* ***(H4 and I4);*** *scale bar = 20 µm. Representative images of two replicates that were conducted independently. Alamar blue stained-cell proliferation rate was evaluated at different time points, data represent one experiment and error bars are calculated from three technical replicates* ***(J)****. Senescent fibroblast appeared blue with SA-β-galactosidase staining at 24 h, 48 h, 72 h and 96 h in UV1* ***(L2, L3, L4 and L5)*** *and UV2 group* ***(M2, M3, M4 and M5),*** *scale bar = 100 µm****.*** *Bar chart showed SA-β-galactosidase expression at different time points (n=2)* ***(N).*** *mRNA expression of collagen 1, collagen 3, MMP-3, p16 and p21 were displayed at 72 h after UV irradiation between normal and UV2 group (n=3; *p<0.05)* ***(O)****.*

IV. DISCUSSION

The senescence of human dermal fibroblasts (HDFs) plays vital roles in the pathogenesis of skin photoaging. Therefore, we aimed to develop aged HDFs with an optimized acute ultraviolet A and B irradiation protocol to investigate mechanism of photoaging and develop methods for ameliorating photoaging via cellular senescence attenuation.

Fibroblasts are principle cell type of connective tissue, which have spindle shape and attach well on artificial surfaces in cell. In this study, HDFs was isolated from human abdominal skin as discarded after cosmetic surgery, which was an available and immoral tissue source. After 14 days of primary culture, the cells were sub-cultured to passage 4 to prepare for other experiments. Then HDFs were identified by specific spindle-shape morphology and highly positive with Vimentin and S100A4 markers, as well as proved ability to differentiate into functional cells such as osteocyte, chondrocyte and adipocyte. As previous study, HDFs were shown to possess multilineage potential, giving rise to fat-, cartilage- and bone-like cells.

Subsequently, we established senescent HDFs by UVA coordinating with UVB (UV-AB) irradiation. In our study, HDFs were inspected with different UV-AB doses to find the most suitable one which can cause cell senescence without affecting cell viability. Two doses were chosen as UVB 0.78 J/cm^2 + UVA 0.48 J/cm^2 (10 mins), and UVB 1.17 J/cm^2 + UVA 0.72 J/cm^2 (15 mins). This finding was similar to another study, which also indicated that UVB dose over 2.2 J/cm^2 can cause cellular damage immediately[5].

For cell assessment after UV irradiation, senescence induction was confirmed by well-recognized senescence hallmarks such as the activity of SA-β-galactosidase, cell metabolic activity, and the expression of senescence-related gene p16, p21 and fibroblast biological function-related gene as collagen 1, 3 and MMP-3.

As the results, acutely single UV2 dose in short time (UVB 1.17 J/cm^2 + UVA 0.72 J/cm^2 for 15 mins) showed the clearest effects in terms of changing cell morphology, inducing SA-β-galactosidase activity, and of reducing cellular metabolic activity. The reduction of cell proliferation via metabolic activity alteration was markedly recorded at 72 h and still maintained significant decrease at day 6 after irradiation, compared to the normal group. However, this activity was improved at day 8, might due to the fact that

only S- or G1- phase cells was affected by UV irradiation[12], therefore the remained unaffected cells might grow normally. The UV2 exposure dose also downregulated collagen 1 and 3, and upregulated MMP-3, p16 and p21 gene expression at 72 h, demonstrating the deterioration of fibroblast function as a consequence of senescence.

This finding was quite different from some previous studies which attributed aged-fibroblasts by using a very low dose of UVB (5 mJ/cm^2) in long period (48 hours)[1], or a series of 10 sub-cytotoxic (non-proapoptotic) exposures to UVB[2] at 250 mJ/cm^2, as well as involving a series of 8 sub-cytotoxic (non-proapoptotic) exposures to UVB at 3250 J/m^2. Previous study suggested that a low dose of UVB irradiation markedly increases MMP-1 expression in keratinocytes through BLT2-ROS-ERK-linked signaling pathway[6]. Another study also used low dose of UVA (1.0 mW/cm^2) for 1 hour every day, total 10 consecutive days (total dose: 36.0 J/cm^2) to develop senescent fibroblast model[11]. Our study used the combination of both UVA and UVB to provide more dynamic detrimental impact, therefore, we could create the senescence of cells by just single irradiation in 15 mins, which was more advantageous and timesaving. In addition, the effective UVA dose in the present study (0.72 J/cm^2) was also much lower than some previous studies [10] (20 J/cm^2) due to the same reason, as low dose can prevent cells from cytotoxic. To the best of our knowledge, there was a study conducted using a single dose (100 mJ/cm^2) of UVB to create photo-aged fibroblast[9], however, that study only confirmed of senescence by cell morphology and β-galactosidase activity. In the meanwhile, with the dominant criteria for assessing cell senescence, we were successfully established UV-induced aged-fibroblast rather than UV-induced damaged cells, which was then appropriate to study about photoaging *in vitro.*

V. CONCLUSIONS

Taken together, the current study demonstrated a method to induce photo-aged dermal fibroblast *in vitro* by single UV-AB radiation in short time, attempting to contribute to the understanding of mechanism and develop intervention for photoaging.

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AUTHOR CONTRIBUTIONS

All author contributed to design the experiment, Huynh Bach Cuc collected data, and draft the article; Vu Bich Ngoc provided some resources and supervised the investigation; Huynh Bach Cuc performed the experiments and analyzed the data. All authors have read, edited, and Van The Trung and Pham Van Phuc approved the final version of the manuscript.

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INFORMED CONSENT STATEMENT

The human subjects involved in this research received an informed consent.

CONFLICTS OF INTEREST

The authors declare no competing interests, financial or otherwise, associated with this publication.

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