RESEARCH ARTICLE

Achatina fulica Mucus Ameliorates UVB-induced Human Dermal Fibroblast Photoaging via the TGF-β/Smad Pathway

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Abstract

BACKGROUND: Ultraviolet B (UVB) induces skin photoaging by reducing collagen deposition via impairment of the TGF-β/Smad signaling pathway. *Achatina fulica* mucus (AFM) is a native medicine acting as vehicle of anti-aging ingredients. The present investigation examined the effect of AFM on UVB-induced fibroblast photoaging by assessing TGF-β, Smad3, and Smad7 mRNA expressions.

METHODS: AFM was extracted from *A. fulica* using electrical shock and freeze-dried into a powder. Normal human dermal fibroblast (NHDF) cultures were irradiated with/without 100 mJ/cm^2 UVB and treated with/without 10% platelet-rich plasma or different concentrations of AFM: 3.9 µg/mL in AF3 group; 15.625 µg/mL in AF15 group, and 62.5 µg/mL in AF62 group. The mRNA expressions of TGF-β, Smad3, and Smad7 in NHDF were evaluated by quantitative polymerase chain reaction.

RESULTS: TGF-β mRNA expressions in the AF3 (0.85±0.01), AF15 (0.94±0.02) and AF62 (1.64±0.03) groups were significantly higher (p<0.05) compared with that in the UVB group (0.55±0.04). Moreover, Smad3 expressions in the AF3 (1.42±0.25), AF15 (1.89±0.13), and AF62 (2.50±0.31) groups were significantly higher (p<0.05) compared with that in the UVB group (0.57±0.08). Furthermore, Smad7 expressions in the AF3 (1.57±0.18), AF15 (0.87±0.03), and AF62 (0.25±0.09) groups were significantly lower (p<0.05) than that in the UVB group (2.57±0.06).

CONCLUSION: AFM ameliorates UVB-induced fibroblast photoaging by upregulating the TGF-β/Smad3 expressions and downregulating Smad7 expression.

KEYWORDS: *Achatina fulica*, TGF-β, Smad, collagen, UVB, fibroblast, photoaging

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Introduction

Photoaging is a process of skin aging caused by ultraviolet (UV) irradiation, especially UVB irradiation. UVB-induced skin aging is characterized by worsening of skin condition such as abnormal pigmentation, roughness, dryness, increased wrinkling, and deterioration of skin elasticity. The main histological appearance of skin photoaging are characterized by the transformation of the content of the extracellular matrix (ECM) and a creeping loss in collagen deposition.(1,2) Of all UV rays, UVC (200–280 nm) has

the most potent energy, but it cannot penetrate the Earth's ozone layer. UVB (280-320 nm) has a shorter wavelength than UVA (320-400 nm), resulting in greater energy that can penetrate the epidermis and superficial dermis.(3) Exposure to UVB can induce skin photoaging by activating several signaling proteins that causes various skin damages through different signaling pathways. Transforming growth factor-β (TGF-β)/Smad3 is one of the signaling pathways that is blocked by UVB irradiation, which has an impact on the production of collagen.(4,5)

TGF-β signaling is essential for preserving the integrity and structure of cutaneous connecting tissue. It is a key regulator of the synthesis of collagen and other ECM components. By inhibiting the expression of matrix metalloproteinase-1 (MMP-1), it serves to prevent the fragmentation of collagen.(5,6) Through the binding and activation of the receptor complex, TGF-β starts cellular activity. The activated receptor then phosphorylates and activates Smad3. Phosphorylated Smad3 is transferred to the nucleus, which in turn regulates procollagen transcription to promote collagen synthesis.(7) This process is inhibited by Smad7 as a Smad inhibitor. Smad7 is a TGF-β signaling pathway negative regulator that inhibits Smad3. It is normally expressed in a very low amount in normal keratinocytes but overexpressed in several pathological conditions such as photoaging and skin carcinogenesis.(8)

Currently, many studies are conducted in trying to find anti-aging drugs from natural sources. *Achatina fulica* is a pest that is difficult to eradicate in agricultural areas, but it turns out that *A. fulica* mucus (AFM) has a natural remedy for the anti-aging effect that was previously employed in ancient medicine but has not been scientifically proven. (9) Previous studies have found that AFM contains several bioactive ingredients such as achasin, a broad-spectrum antibiotic and anti-inflammatory agent, acharan sulfate, a type of glycosaminoglycan, and antioxidants all of which play a role in collagen synthesis.(10-14)

This experiment was conducted to investigate AFM which is abundant in antioxidants, anti-inflammatory agents and glycosaminoglycans. In this study, regenerative effect of AFM on UVB-induced photoaging in fibroblasts was investigated by assessing TGF-β, Smad3, and Smad7 mRNA expressions.

Methods

Preparation of AFM and Platelet-rich Plasma (PRP)

Mucus from *A. fulica* was collected by 5-10 V electrical shock that lasted 30-60 seconds.(10) The mucus was then put in sterile bottles and freeze-dried into a powder. The 10% PRP was provided by the Department of Dermatology and Venereology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada.

Cell Culture

Normal human dermal fibroblasts (NHDFs) in passage >4, which were taken from the foreskin of boys aged 11–13 years, were provided by the Department of Dermatology and Venereology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) was used to culture the cells. It was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were maintained at 37°C in a humidified environment with 5% CO_2. The trypsinization process was then used to subculture the cells in growth media to create a fresh culture. This protocol was approved by Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (No.: KE/FK/0682/CE/2018).

UVB Irradiation and AFM Treatment

NHDFs were plated in a 24-well tissue culture plate until reaching 80–90% confluency and were then washed with phosphate-buffer saline (PBS). NHDFs, which were placed in newly prepared PBS-filled wells were then exposed to UVB irradiation using a Spectrolinker XL-1500 UV crosslinker (Spectronics Corporation, Westbury, NY, USA) (range 280-320 nm, with a peak at 312 nm). Phototherapy radiometer (International Light Technologies, Newburyport, MA, USA) was used to measure the UVB radiation. Following 330 seconds of UVB radiation at a dosage of 100 mJ/cm^2, the cells were rinsed three times with PBS. AFM with concentrations of 3.9 µg/mL (AF3 group), 15.625 µg/mL (AF15 group), and 62.5 µg/mL (AF62 group) were applied to the cells. Afterwards, the cells were incubated at 37°C for 72 hours in a humidified environment with 5% CO_2.

Measurement of TGF-β, Smad3, and Smad7 mRNA Expressions

TGF-β, Smad3, and Smad7 mRNA expression levels were determined using quantitative real-time polymerase chain reaction (qRT-PCR). The level of GAPDH mRNA expression served as a housekeeping gene. miRCURY LNA™ Universal RT microRNA PCR Universal cDNA Synthesis Kit II, 8-64 rxns (Lot No. 629693, Exiqon, Vedbaek, Denmark) was used to synthesis cDNA after RNA was extracted from the

cells using the miRCURY™ RNA Isolation Kit – Cell & Plant (Lot No. 32213, Exiqon). A 7500 FAST Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and Thunderbird™ SYBR™ qPCR Mix (Catalog No. QPS-201, Toyobo, Osaka, Japan) were used for the qRT-PCR. qRT-PCR was conducted with primers as follows: TGF-β forward: 5'-GTACCTGAACCCGTGTTGCT-3' and reverse: 5'-CGGTAGTGAACCCGTTGATGT-3', Smad3 forward: 5'-GTGCTCCATCTCCTACTAC-3' and reverse: 5'-CCTCTTCCGATGTGTCTC-3', Smad7 forward: 5'-CGGATCTCAGGCATTCCTCG-3' and reverse: 5'-GGCACAGCATCTGGACAGTC-3', and GAPDH forward: 5'-GGAGCGAGATCCCTCCAAAAT-3' and reverse: 5'-GGCTGTTGTCATACTTCTCATGG-3'. For a total of 40 cycles, the thermocycling protocol was defined as: 2 minutes of initial denaturation at 95°C, 10 seconds of subsequent denaturation at 95°C, 15 seconds of annealing at 58°C, and 20 seconds of extension at 72°C. Cycle thresholds (CTs) of TGF-β, Smad3 and Smad7 mRNA were determined and used for calculation of $2^{-\Delta\Delta CT}$ formula. Three duplicates of each experiment were performed.

Statistical Analysis

Data was presented as mean and standard deviation (SD) and analyzed with SPSS version 26 (IBM, Armonk, NY, USA). To assess significant group differences, analysis of variances ANOVA and the least significant difference (LSD) *post hoc* test were employed. The criterion for statistical significance was considered to be $p<0.05$.

Results

AFM Upregulated TGF-β mRNA Expression

TGF-β mRNA was amplified, characterized by an increase in the relative fluorescence unit (RFU) value along with an increase in the template amplification reaction as shown in the amplification curve (Figure 1A). The absence of amplicon in no template control (NTC) showed that there was no contamination during the preparation and qRT-PCR process. The melt curve (Figure 1B) and melt peak curve (Figure 1C) showed that the qRT-PCR process for TGF-β mRNA took place specifically according to the PCR primer used. This was indicated by the formation of one peak on the melt peak curve. The optimum melting temperature for TGF-β and GAPDH were 81°C and 82.5°C, respectively.

The UVB group's TGF-β mRNA expression (0.55±0.04) was significantly lower ($p<0.001$) in comparison to the normal control (NC) group's. The PRP group showed significantly lower TGF-β mRNA expression (0.92±0.02) than the NC group ($p<0.01$). However, TGF-β mRNA expression in the PRP group was significantly higher than that in the UVB group ($p<0.001$). The mRNA expressions of TGF-β in the AF3 (0.85±0.01), AF15 (0.94±0.02) and AF62 (1.64±0.03) groups were significantly higher ($p<0.001$) compared with that in the UVB group. With substantial differences ($p<0.001$) among the AF62 group and the AF15 and AF3 groups, the AF62 group exhibited the highest level of TGF-β mRNA expression. Additionally, a significant difference ($p<0.05$) was observed regarding TGF-β mRNA expression levels between the AF3 and AF15 groups (Figure 1D).

AFM Upregulated Smad3 mRNA Expression

Smad3 mRNA was amplified, indicated by an increase in the RFU value along with an increase in the template amplification reaction as seen in the amplification curve (Figure 2A). The absence of amplicon in NTC indicated no contamination occurred during the preparation and qRT-PCR process. The melt curve (Figure 2B) and melt peak curve (Figure 2C) showed that the qRT-PCR process for Smad3 mRNA took place specifically according to the PCR primers used. This was shown by the formation of one peak on the melt peak curve. The optimum melting temperature for Smad3 and GAPDH were 86°C and 82.5°C, respectively.

In comparison to the NC group, the UVB group's Smad3 mRNA expression level (0.57±0.08) was considerably lower ($p<0.001$). The PRP group had a considerably higher ($p<0.001$) expression of Smad3 mRNA (1.85±0.19) in comparison to the NC and UVB groups. Compared to the NC and UVB groups, the mRNA expression levels of Smad3 in the AF3 (1.42±0.25), AF15 (1.89±0.13), and AF62 (2.50±0.31) groups were considerably greater ($p<0.001$). Smad3 mRNA expression in the AF3 group was significantly lower ($p<0.001$) compared with the one in the PRP group, but it was higher in the AF15 group ($p>0.05$) and in the AF62 group ($p<0.001$). Expression of Smad3 mRNA increased along with the increase in the AFM concentration. The highest Smad3 mRNA expression was found in the AF62 group, followed by the AF15 and AF3 groups with significant differences ($p<0.001$) between groups (Figure 2D).

AFM Downregulated Smad7 mRNA Expression

Smad7 mRNA was amplified, characterized by an increase in the RFU value along with an increase in the template amplification reaction as shown in the amplification curve (Figure 3A). The absence of amplicon in NTC showed that

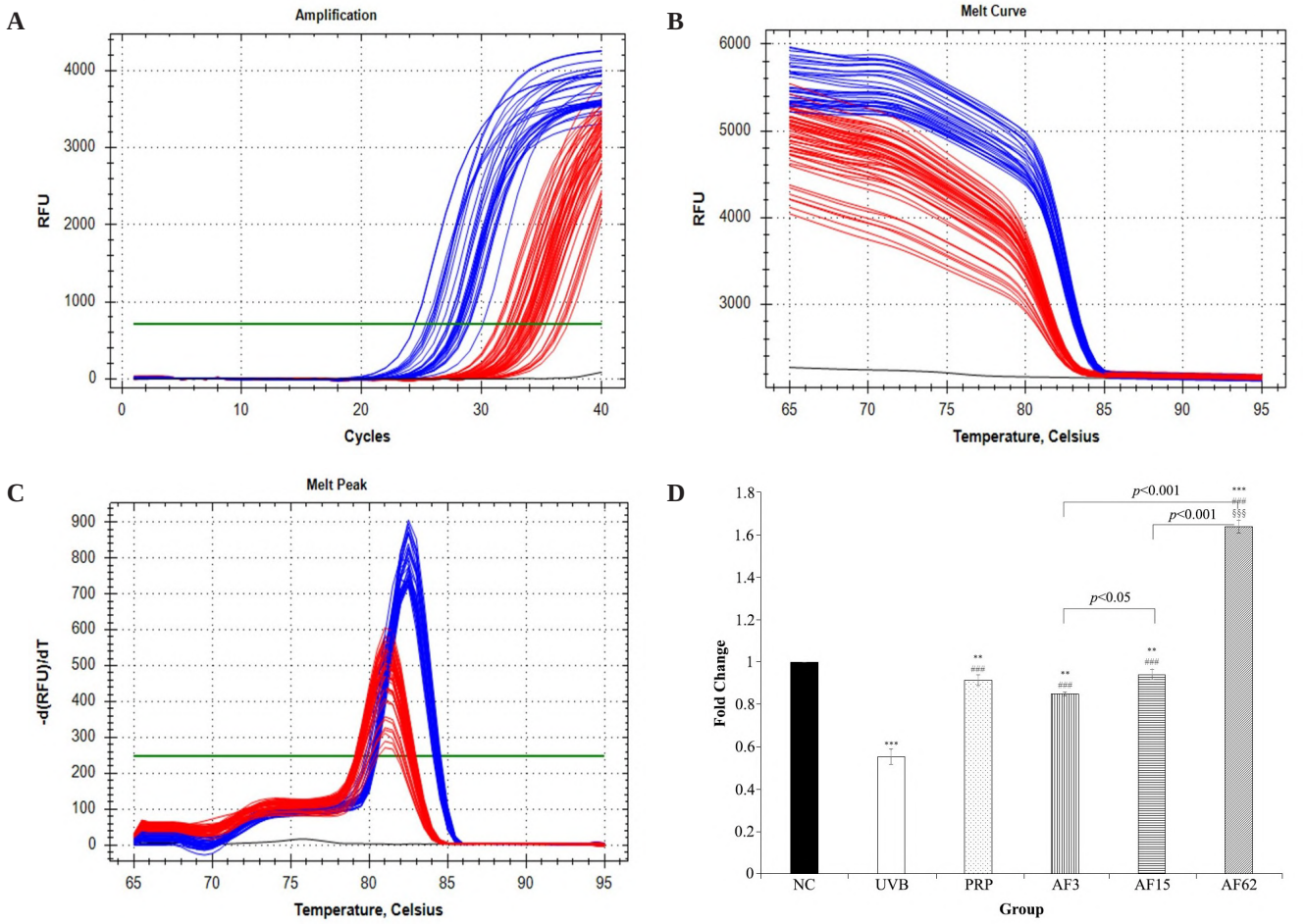

Figure 1. qRT-PCR analysis of TGF-β mRNA expression. NHDFs were irradiated with 100 mJ/cm² UVB and treated with 10% PRP or different concentrations of AFM. The results were presented as mean±SD. A: Amplification curve; B: Melt curve; C: Melt peak curve; D: TGF-β mRNA expression in all groups. Red lines: TGF-β; blue lines: GAPDH; black line: NTC. **p*<0.05 *vs* NC, ***p*<0.01 *vs* NC, ****p*<0.001 *vs* NC. #*p*<0.05 *vs* UVB, ##*p*<0.01 *vs* UVB, ###*p*<0.001 *vs* UVB. §*p*<0.05 *vs* PRP, §§*p*<0.01 *vs* PRP, §§§*p*<0.001 *vs* PRP.

there was no contamination during the preparation and qRT-PCR process. The melt curve (Figure 3B) and melt peak curve (Figure 3C) showed that the qRT-PCR process for Smad7 mRNA took place specifically according to the PCR primer used. This was indicated by the formation of one peak on the melt peak curve. The optimum melting temperature value for Smad7 and GAPDH were 84°C and 82.5°C, respectively.

The UVB group's mRNA expression of Smad7 (2.57±0.06) was significantly higher (p<0.001) compared with the NC group's. The PRP group's Smad7 mRNA expression (0.83±0.16) was significantly lower than the UVB and NC groups' (p<0.001). In comparison to the UVB group, the mRNA expressions of Smad7 were significantly lower (p<0.001) in the AF3 (1.57±0.18), AF15 (0.87±0.03), and AF62 (0.25±0.09) groups. The AFM-treated groups indicated a significant difference (p<0.001) in Smad7 mRNA expression, with the AF62 group exhibiting the lowest level, followed by the AF15 and AF3 groups (Figure 3D).

Discussion

This study highlighted the role of AFM in attenuating the development of photoaging through increasing TGF-β and Smad3, and decreasing Smad7. Photoaging is primarily caused by exposure to UV irradiation, especially UVB irradiation, which is able to penetrate the skin layers reaching the superficial dermis and disrupting collagen homeostasis. (15) Collagen homeostasis is regulated by TGF-β as the main stimulator of collagen synthesis.(7,16)

UVB exposure induces photoaging in two ways, namely through increasing reactive oxygen species (ROS) levels and DNA damage.(1,17) UVB irradiation increases ROS production, which in turn triggers the activation of mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1), and inhibition of TGF-β production. (5,18,19) The finding of this study is appropriate with the previous study that UVB inhibited TGF-β expression. As

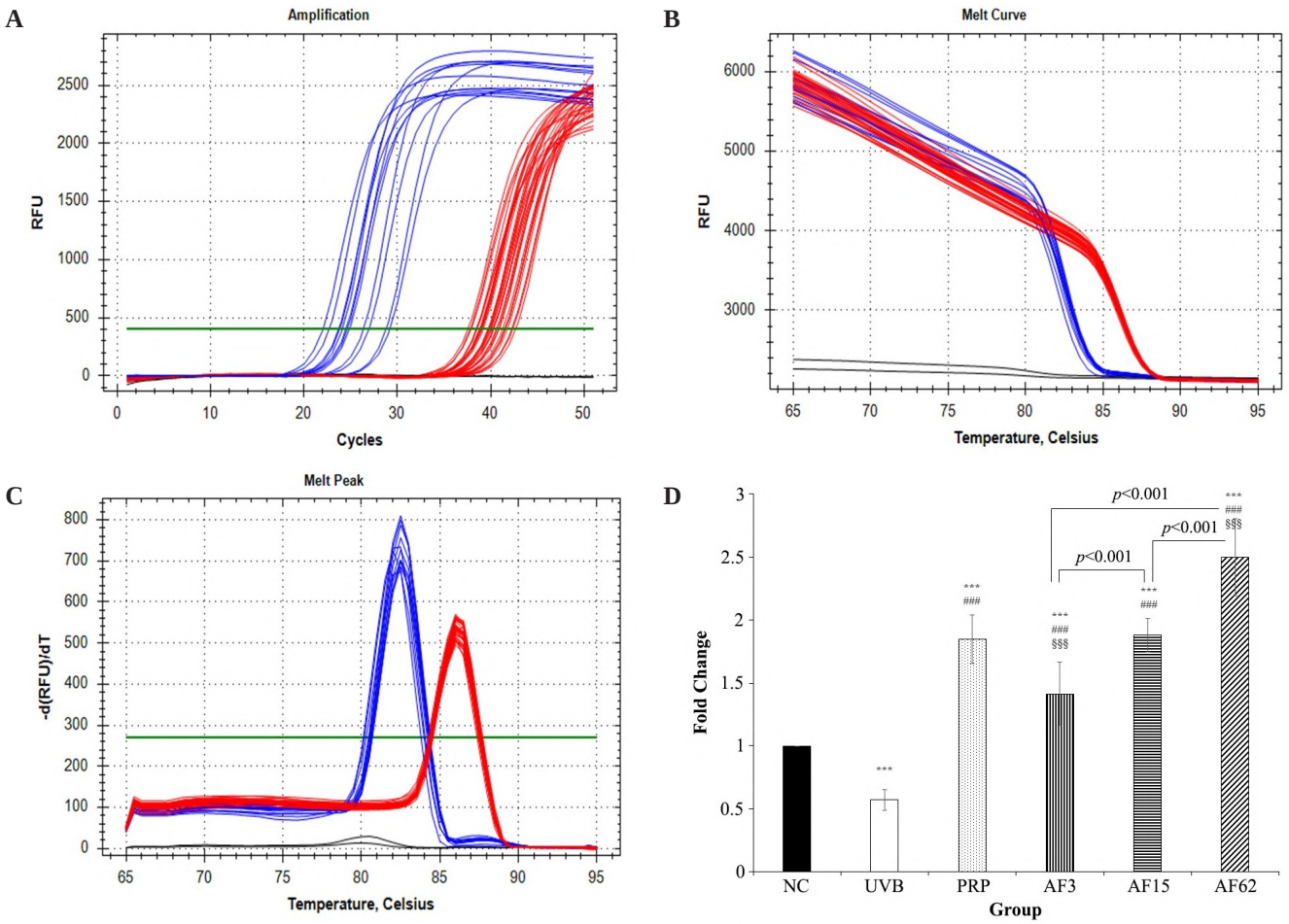

Figure 2. qRT-PCR analysis of Smad3 mRNA expression. NHDFs were irradiated with 100 mJ/cm² UVB and treated with 10% PRP or different concentrations of AFM. The results were presented as mean±SD. A: Amplification curve; B: Melt curve; C: Melt peak curve; D: Smad3 mRNA expression in all groups. Red lines: Smad3; blue lines: GAPDH; black line: NTC. **p*<0.05 *vs* NC, ***p*<0.01 *vs* NC, ****p*<0.001 *vs* NC. #*p*<0.05 *vs* UVB, ##*p*<0.01 *vs* UVB, ###*p*<0.001 *vs* UVB. §*p*<0.05 *vs* PRP, §§*p*<0.01 *vs* PRP, §§§*p*<0.001 *vs* PRP.

a regulator in the collagen synthesis process of the human skin, TGF-β is crucial for cell division, proliferation, and apoptosis.(20) Reduction in the production of ROS increases TGF-β activity via decreasing the activation of MAPK and nuclear factor kappa-B (NF-κB).(21,22) By selectively binding to its receptors, TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII), TGF-β starts cellular processes by phosphorylating Smad3. In order to increase collagen production, phosphorylated Smad3 can bind to Smad4 and proceed to the nucleus, where it modulates procollagen transcription.(6,23) AFM upregulated TGF-β mRNA expression in this study. The TGF-β-inducing activity of AFM may be associated with the presence of several bioactive ingredients, such as achasin, acharan sulfate, and antioxidants.(10-14)

Increased oxidative stress and ROS due to UVB exposure reduces Smad3 complex formation by inducing c-Jun through the MAPK pathway.(24) Functionally, c-Jun associates with the Smad protein to inhibit TGF-β-induced cellular responses and reduce TGF-β-induced TGF-β/Smad3 complex formation, so that TGF-β is unable to stimulate intracellular Smad3 transcription factor phosphorylation. (25,26) Consequently, Smad3 does not translocate into the nucleus and interact with Smad binding element (SBE) in the TGF-β target gene promoter region, which includes collagen and other ECM genes. Inhibition of Smad3 activation significantly decreases TGF-β-induced type I procollagen. Smad3 is an essential molecule that regulates the TGF-β stimulation of type I procollagen synthesis and suppresses MMP-1 synthesis through the Smad3-dependent pathway.(27) The finding of this study showed that AFM upregulated Smad3 mRNA expression, which might be due to its antioxidant activity. Several studies reveal that UVB radiation increases ROS in the skin, which decreases the levels of glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD). These enzymatic antioxidants

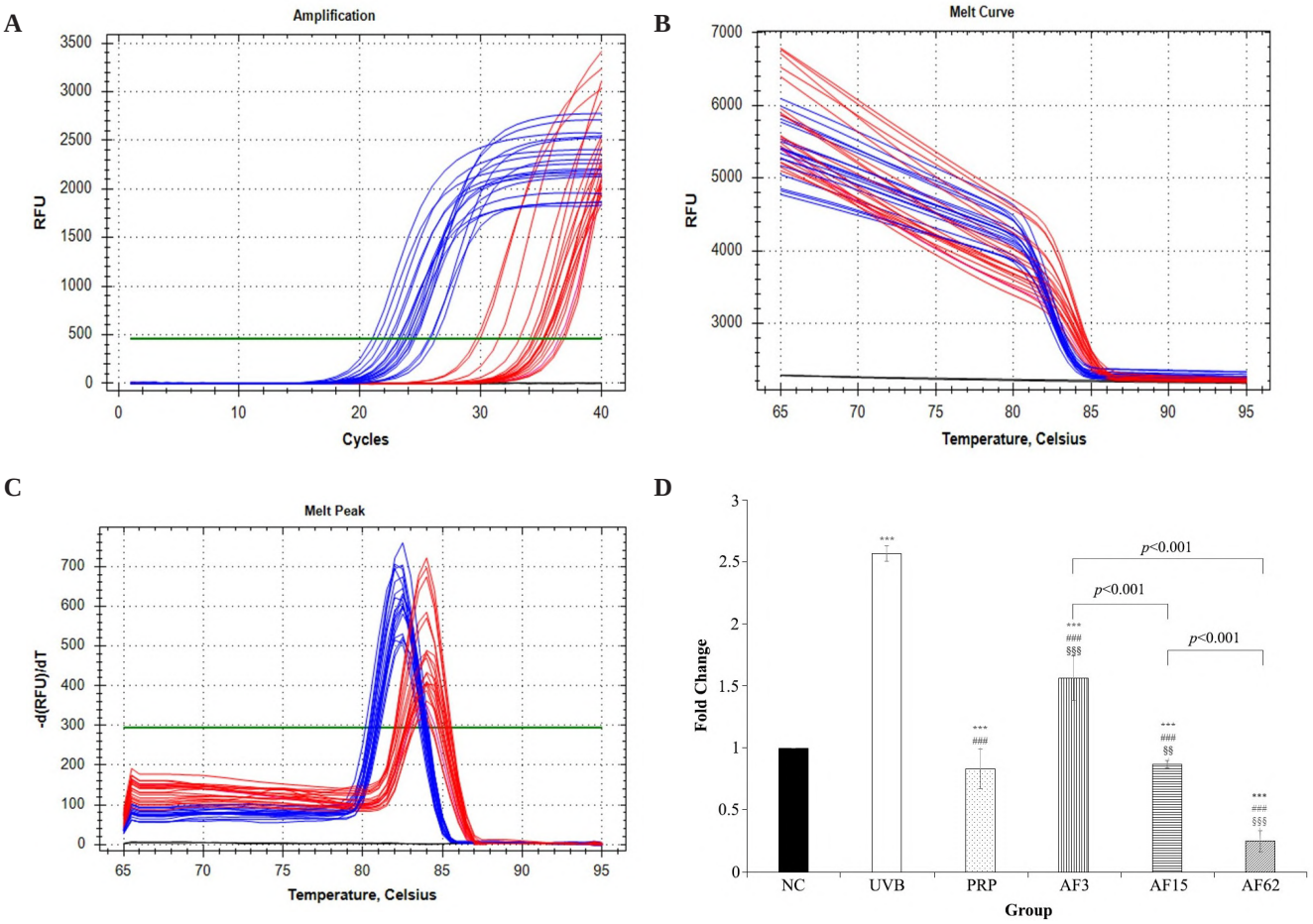

Figure 3. qRT-PCR analysis of Smad7 mRNA expression. NHDFs were irradiated with 100 mJ/cm^2 UVB and treated with 10% PRP or different concentrations of AFM. The results were presented as mean±SD. A: Amplification curve; B: Melt curve; C: Melt peak curve; D: Smad7 mRNA expression in all groups. Red lines: Smad7; blue lines: GAPDH; black line: NTC. *p<0.05 *vs* NC, **p<0.01 *vs* NC, ***p<0.001 *vs* NC. $^{\#}p$<0.05 *vs* UVB, $^{\#\#}p$<0.01 *vs* UVB, $^{\#\#\#}p$<0.001 *vs* UVB. $^{\S}p$<0.05 *vs* PRP, $^{\S\S}p$<0.01 *vs* PRP, $^{\S\S\S}p$<0.001 *vs* PRP.

play a role in neutralizing free radicals. Previously, it has been proven that AFM had enzymatic antioxidant activity such as SOD, CAT, and GPX beyond other active ingredients that have been discovered.(28,29)

In normal cells, there is an equilibrium between free radicals such as ROS and the body's endogenous antioxidant defence mechanisms. If this balance is disturbed, as in photoaging, oxidative stress will occur. In photoaging caused by UVB exposure, there is ROS level elevation in skin fibroblasts.(30) UVB exposure induces the activation of Smad7, the most influential components in the skin development process.(31) Smad7 is usually expressed in low quantities in normal keratinocytes and in excessive amounts in pathological conditions, such as premature skin aging due to UVB exposure and skin carcinogenesis. Additionally, Smad7 inhibits Smad3 phosphorylation via TβRI, thereby blocking TGF-β signaling and inhibiting collagen production by skin fibroblasts.(6,32) The results of this study indicated that AFM downregulated Smad7 mRNA expression. It occurred because AFM might have a photoprotective effect, as demonstrated in the previous research related to the active compounds in AFM.(28)

This study revealed that AFM could inhibit UVB-induced photoaging via the upregulation of TGF-β and Smad3 mRNA expression levels. Additionally, AFM downregulated Smad7 mRNA expression, an inhibitor of Smad3 as a negative regulator in the TGF-β/Smad pathway. These outcomes demonstrated that AFM ameliorated the UVB-induced photoaging in fibroblasts by regulating TGF-β, Smad3, and Smad7 mRNA expressions.

Conclusion

This study shows that AFM ameliorates UVB-induced fibroblast photoaging by upregulating TGF-β/Smad3 expressions and downregulating Smad7 expression.

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Authors Contribution

CTN contributed to the research idea, research design, statistical analysis, manuscript preparation, article editing, and revision. SMH, YWW, and NA oversaw manuscript preparation, article review, and data analysis. TPA designed literature searches, research figures, data collection, and data analysis.

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