AHA-98: A Novel Tetrapeptide Against UV-Induced Skin Photoaging

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Abstract

Objective: A primary driver of premature skin aging is UV irradiation-induced photoaging, which results in reduced collagen production and deterioration of cellular processes. This study aimed to evaluate the protective effects of a novel tetrapeptide AHA-98 against UV-induced photoaging and investigate its potential applications in skincare products.

Methods: Extensive *in vitro* experiments examining AHA-98's protective effects on UVB-exposed human skin fibroblasts (HSF) and human keratinocytes cells (HaCat). These investigations included detailed analyses of cell viability rates using MTT assays, measurement of type I collagen expression and hyaluronic acid production using ELISA, assessment of cell migration capacity through standardized wound healing assays, detection of apoptosis rates using flow cytometry, measurement of relative changes in p-JNK content using immunofluorescence, and determination of cytochrome C content in the cytoplasm relative to mitochondria using ELISA. A rigorously designed 9-week randomized, single-blind, placebo-controlled clinical trial involving 30 healthy participants. Participants were randomly assigned to the AHA-98 group and placebo group, applying creams with or without AHA-98 twice daily to their facial skin. Skin elasticity parameters (R2, R7, and Q1) were evaluated at baseline and at 3-week intervals using Cutometer MPA580 instrument.

Results: *In vitro* studies showed that AHA-98 significantly enhanced the viability rate of UVB-exposed HSF cells compared to control group. AHA-98 increased type I collagen expression and enhanced hyaluronic acid production in UVB-irradiated cells. Wound healing assays demonstrated a improvement in cell migration rates in AHA-98-treated cells. AHA-98 reduced UVB-induced apoptosis in cells. The phosphorylation of JNK, a key regulator of apoptosis, was also attenuated by AHA-98. Furthermore, the release of cytochrome c from mitochondria to the cytosol was diminished in the presence of AHA-98 compared to which exposed to UVB. The clinical trial results were equally promising, demonstrating significant improvements in multiple skin elasticity parameters. Specifically, overall elasticity (R2) improved by 28%, the ratio of elastic recovery to total deformation (R7) increased by 33%, and elastic recovery (Q1) enhanced by 28% in the AHA-98 group compared to placebo group after 9 weeks of treatment.

Conclusion: AHA-98 demonstrates promising protective effects against UV-induced photoaging through multiple mechanisms and shows significant skin firming effects in clinical applications, suggesting its potential as an innovative ingredient for anti-photoaging skincare products.

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1. Introduction

The skin, as the largest organ of the human body, serves as a crucial barrier against external environmental factors. Ultraviolet (UV) radiation from sunlight is one of the most significant contributors to skin aging, a process known as photoaging[1]. Chronic UV exposure induces a cascade of biochemical and physiological changes in the skin, leading to the degradation of collagen and elastin fibers, the accumulation of abnormal pigmentation, and the generation of reactive oxygen species (ROS)[2]. These alterations not only compromise the skin's structural integrity but also impair its barrier function, resulting in the appearance of wrinkles, sagging, and age spots, which are hallmarks of photoaged skin.

Photaging, characterized by the gradual deterioration of skin quality due to chronic exposure to ultraviolet (UV) radiation, is a significant concern in dermatology and cosmetic science[3]. UVB irradiation, in particular, has been identified as a major contributor to this process. It induces a cascade of cellular and molecular changes that lead to the degradation of the extracellular matrix (ECM), a critical component of skin structure and function. The ECM, composed primarily of collagen type I and hyaluronic acid, provides essential support and elasticity to the skin[4][5]. However, UVB exposure can disrupt this structural integrity by triggering pathways that promote cellular damage and apoptosis[6].

In recent years, the mechanisms underlying UV-induced skin damage have been extensively investigated. UVB exposure activates the c-Jun N-terminal kinase (JNK) pathway, which phosphorylates JNK (p-JNK) and induces the release of mitochondrial cytochrome c[7]. This release of cytochrome c activates caspases, leading to apoptosis and further contributing to the aging process[8]. To counteract these detrimental effects, photoprotection strategies are essential. These may include the use of sunscreens, antioxidants, and other protective agents that inhibit the JNK pathway and stabilize the ECM. Understanding the molecular mechanisms underlying UVB-induced photoaging is crucial for developing effective interventions that can mitigate the impact of UV radiation on skin health.

This research focuses on elucidating the specific mechanisms by which UV radiation induces skin aging and exploring novel strategies for effective skin protection. By employing a combination of in vitro and in vivo models, we aim to investigate the role of key signaling pathways and molecular targets in UV-induced skin damage. Furthermore, we will evaluate the efficacy of AHA-98 in modulating these pathways and promoting skin repair. This study is expected to provide valuable insights into the prevention and treatment of photoaging, contributing to the development of advanced skincare products and therapeutic interventions.

2. Materials and Methods

2.1. Cell line and culture conditions

The HaCaT cell and HSF cell were procured from Kunming wild animal cell bank，the Chinese Academy of Science. The cells were grown in DMEM (Dulbecco's modified eagle medium) (Gibco, MA, USA), which were supplemented with 10% FBS (Fetal bovine serum) (Sigma, MO, USA) and 1% antibiotics (penicillin 100 U/mL and streptomycin 100 U/mL)(Beyotime, SH, CN). The cells were incubated at 37 ℃ with 5% CO_2 and cells were passaged after they reached 70-80% confluency.

2.2. Cell viability assay

The HSF cells were resuspended in DMEM(Gibco, MA, USA) complete medium, seeded in 96-well plates and incubated for 24 h.The cells were treated with AHA-98 at concentrations of 10ppm,50 ppm, 100 ppm and 200ppm, while the control group received complete culture medium with an equivalent volume of solvent.The cells were cultured for an additional 48 hours. After incubation, the medium was removed and the MTT reagent (Beyotime, SH, CN) was added (50 μL of 5 mg/mL) and incubated in a CO_2 incubator for 4 h at 37°C. The formazan crystals were solubilised with 150 μL of DMSO (Dimethylsulfoxide). Finally, the plates were read at 490 nm using a micro plate reader (Molecular Devices, SH, CN).

2.3. UVB-damaged cell viability assay

The HSF cells were resuspended in DMEM(Gibco, MA, USA) complete medium, seeded in 96-well plates and incubated for 24 h.The cells were exposed to UVB irradiation for 15 minutes except the control group.After irradiation, the experimental groups were treated with AHA-98 at concentrations of 50 ppm, 100 ppm and 200ppm, while the control group received complete culture medium with an equivalent volume of solvent.The cells were cultured for an additional 24 hours. After incubation, the medium was removed and the MTT reagent (Beyotime, SH, CN) was added (50 μL of 5 mg/mL) and incubated in a CO_2 incubator for 4 h at 37℃. The formazan crystals were solubilised with 150 μL of DMSO (Dimethylsulfoxide). Finally, the plates were read at 490 nm using a micro plate reader (Molecular Devices, SH, CN).

2.4. Collagen I content assay

The HSF cells were resuspended in DMEM(Gibco, MA, USA) complete medium, seeded in 12-well plates and incubated for 24 h.The cells were exposed to UVB irradiation for 15 minutes except the control group.After irradiation, the experimental groups were treated with AHA-98 at concentrations of 50 ppm and 100 ppm, while the control group received complete culture medium with an equivalent volume of solvent.The cells were cultured for an additional 24 hours.The content of collagen I in the cell lysates were measured using commercially available Enzyme-linked immunosorbent assay(ELISA) kits(Eilab, WH,CN), following the manufacturer's instructions. Additionally, the protein content in the cell lysates was determined using a commercially available BSA kit(Beyotime, SH, CN).

2.5. Hyaluronic acid content assay

The HaCaT cells were resuspended in DMEM(Gibco, MA, USA) complete medium, seeded in 6-well plates and incubated for 24 h.The cells were exposed to UVB irradiation for 15 minutes except the control group.After irradiation, the experimental groups were treated with AHA-98 at concentrations of 50 ppm and 100 ppm, while the control group received complete culture medium with an equivalent volume of solvent.The cells were cultured for an additional 48 hours.The content of hyaluronic acid in the cell lysates were measured using commercially available Enzyme-linked immunosorbent assay(ELISA) kits(Eilab, WH,CN), following the manufacturer's instructions. Additionally, the protein content in the cell lysates was determined using a commercially available BSA kit(Beyotime, SH, CN).

2.6. *In vitro* wound healing assay

The HaCaT cells were resuspended in DMEM(Gibco, MA, USA) complete medium, seeded in 12-well plates and incubated for 24 h.The medium was then replaced, and two vertical lines

were scratched in the center of each well using a p200 micropipette tip. The cells were washed once with PBS, and images were taken at positions marked by the lines.The experimental groups were treated with AHA-98 at concentrations of 50 ppm and 100 ppm, while the control group received complete culture medium with an equivalent volume of solvent.The cells were then cultured for an additional 24 hours. Finally, images of the same positions were captured again under an inverted microscope after 24 hours of culture.The percentage of wound closure was calculated using Image J software.

2.7. Apoptosis assay

The HSF cells were resuspended in DMEM complete medium, cultured in 6-well plates and incubated for 24 h. The cells were exposed to UVB irradiation for 8 minutes except the control group. After irradiation, the experimental groups were treated with AHA-98 at concentrations of 50 ppm and 100 ppm, while the control group received complete culture medium only with an equivalent volume of solvent. The cells were cultured for an additional 16 hours. Subsequently, the cells were digested with trypsin-EDTA-free digestion solution (0.25% trypsin), collected, and stained with Annexin V-FITC and propidium iodide (PI) according to the apoptosis detection kit(Beyotime, SH, CN) instructions. Apoptosis was assessed using flow cytometry.

2.8. p-JNK Immunofluorescence assay

The HSF cells were resuspended in DMEM complete medium, cultured in 12-well plates and incubated for 24 h. The cells were exposed to UVB irradiation for 8 minutes except the control group. After irradiation, the experimental groups were treated with AHA-98 at concentrations of 50 ppm and 100 ppm, while the control group received complete culture medium only with an equivalent volume of solvent. The cells were cultured for an additional 24 hours.The cells were fixed with fixative solution for 15 minutes at room temperature, permeabilized with permeabilization solution for 10 minutes, and blocked with blocking solution for 1 hour at room temperature. The cells were incubated with primary antibody anti-p-JNK(Beyotime, SH, CN) overnight at 4 ℃, followed by secondary antibody(Beyotime, SH, CN) for 1 hour at room temperature in the dark. The nuclei were stained with Hoechst 33342(Beyotime, SH, CN) for 10 minutes at room temperature in the dark. After washing with wash solution, the cells were observed and imaged under a fluorescence microscope using a fluorescence exciter and filter.

2.9. Cytochrome c content assay

The HSF cells were resuspended in DMEM complete medium, cultured in 10cm petri dishs and incubated for 24 h. The cells were exposed to UVB irradiation for 8 minutes except the control group. After irradiation, the experimental groups were treated with AHA-98 at concentrations of 50 ppm and 100 ppm, while the control group received complete culture medium only with an equivalent volume of solvent. The cells were cultured for an additional 6 hours.Mitochondrial separation was performed using a mitochondrial isolation kit(Beyotime, SH, CN) to obtain cytosolic and mitochondrial fractions.The content of cytochrome c in the cytosol and mitochondria was measured using commercially available Enzyme-linked immunosorbent assay(ELISA) kits(Eilab, WH,CN), following the manufacturer's instructions. Additionally, the protein content in the cytosol and mitochondria was determined using a commercially available BSA kit(Beyotime, SH, CN).

2.10. Clinical trial

A one-sided blind study was designed with a placebo control. Thirty healthy human volunteers, aged 30 to 62 years, were selected after signing informed consent forms. All measurements were conducted under controlled conditions of 22–24℃ and 40–60% relative humidity. The experiments were performed on the cheeks of the volunteers. Each volunteer was provided with two creams: one was the base cream, and the other contained the active ingredients. Volunteers were instructed to apply the creams twice daily on their cheeks for a period of 9 weeks and to return for skin measurements at weeks 3, 6, and 9.The skin elasticity parameter R2,R7 and Q1 were measured using the Cutometer MPA 580(Courage+Khazaka,CG,DE). The changing rate for the individual values of different factors, taken every week, of volunteers were calculated by the following formula:Changing rate(%) = [(A-B) / B] × 100. Where A = individual value of any factor of 3rd, 6th, 9th week and B(baseline) = zero hour value of that factor.

2.11. Statistical Analysis

Data are expressed as mean±SD of at least three experiments performed in duplicate. Data were analyzed by ImageJ and SPSS 17.0 software. Figures were drawn by GraphPad Prism 8.0.*$P < 0.05$ was considered as a mark of statistical significance.

3. Results

3.1. AHA-98 protects HSF cells against UVB damage

To evaluate the cytotoxicity of AHA-98, the cell viability was assessed using the MTT assay after treating HSF cells with various doses of AHA-98 for 48 hours. The results showed that AHA-98 exhibited no cytotoxic effects at concentrations ranging from 10 to 200 ppm (Figure 1A). To investigate the photoprotective effects of AHA-98 on HSF cells, the MTT assay was used to assess cell viability. The results show that UVB irradiation significantly reduces cell viability, indicating that UVB causes substantial cell damage. Different concentrations of AHA-98 were administered to cells post-UVB irradiation in order to evaluate the photoprotective effects of AHA-98. The results indicate that AHA-98 significantly enhances cell viability (Figure 1B). Furthermore, in cells that were not subjected to UVB irradiation, treatment with varying concentrations of AHA-98 had no significant impact on cell viability. This indicates that AHA-98 selectively targets UVB-induced cell damage.
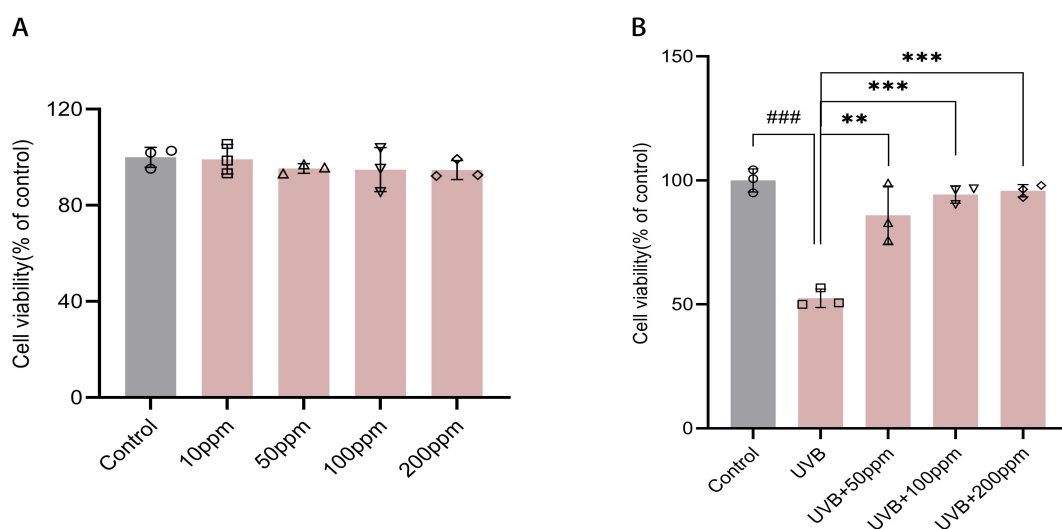
Figure 1. Effect of AHA-98 on HSF Cells and UVB-treated HSF Cells. (A) HSF cells (n=3) were treated with different doses of AHA-98 for 48 h and cytotoxicities were analyzed by MTT assays; (B) UVB-treated HSF Cells(n=3) were treated with different doses of AHA-98 for 24 h and cell viability was analyzed by MTT assays.$^{\#\#\#}P<0.001$ compared to the untreated control group.$^{**}P<0.01,^{***}P<0.001$ compared to the UVB-treated group.

3.2. AHA-98 upregulates the extracellular matrix synthesis

Collagen I expression in HSF cells was suppressed following UVB irradiation, suggesting that the extracellular matrix was disrupted. The UVB-damaged group exhibited a significant reduction in collagen I protein expression compared to the control group. However, treatment with 50 ppm of AHA-98 restored collagen I protein expression (Figure 2A). These findings suggest that AHA-98 effectively mitigates UVB-induced damage in HSF cells by enhancing collagen I protein expression, thereby offering protection against photoaging. Hyaluronic acid is a key factor in skin hydration and anti-photoaging, and its preservation is vital for maintaining skin health. AHA-98 significantly enhances the hyaluronic acid (HA) content in HaCaT cells following UVB irradiation. UVB exposure notably reduces the HA content in cells, which is crucial for maintaining skin hydration and elasticity. However, treatment with varying concentrations of AHA-98 post-UVB irradiation restores the HA content (Figure 2B).
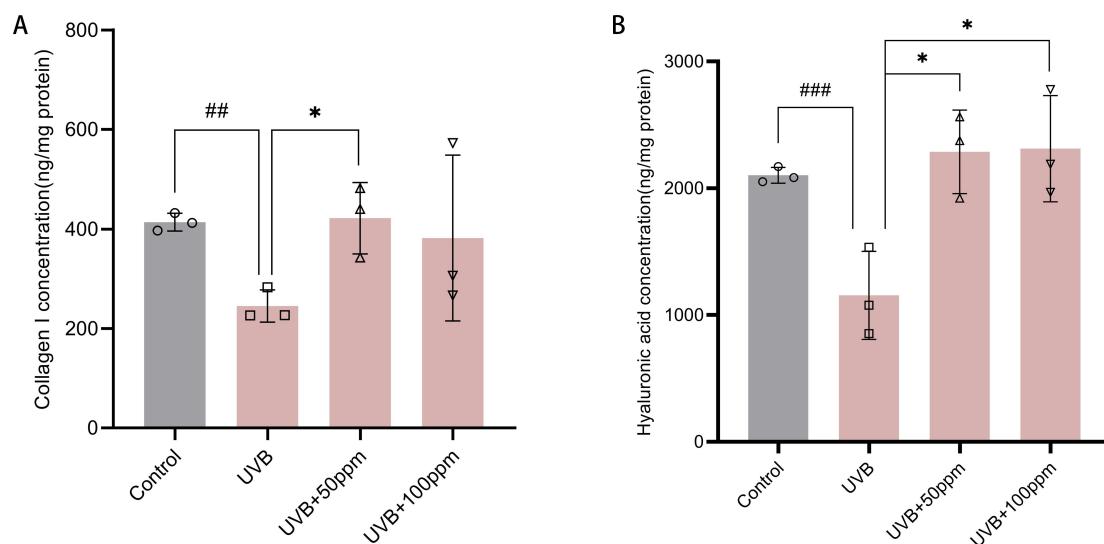

Figure 2. (A) Effect of AHA-98 on the expression of collagen I in HSF Cells. UVB-treated HSF Cells(n=3) were treated with different doses of AHA-98 for 24 h.(B) Effect of AHA-98 on the expression of hyaluronic acid in HaCaT cells. UVB-treated HaCaT Cells(n=3) were treated with different doses of AHA-98 for 48 h.$^{\#\#}P<0.01,^{\#\#\#}P<0.001$ compared to the untreated control group.$^{*}P<0.05$ compared to the UVB-treated group.

3.3. AHA-98 promotes wound healing in HaCaT cells

To explore whether treatment with AHA-98 enhances cell migration associated with wound healing, scratch assays were performed in HaCaT cells. Notably, cells treated with AHA-98 (100ppm) exhibited a significant increase in the area of scratch closure compared to the control group (Figure 3). This finding indicates that AHA-98 positively influences cell migration, which may have implications for wound healing processes.
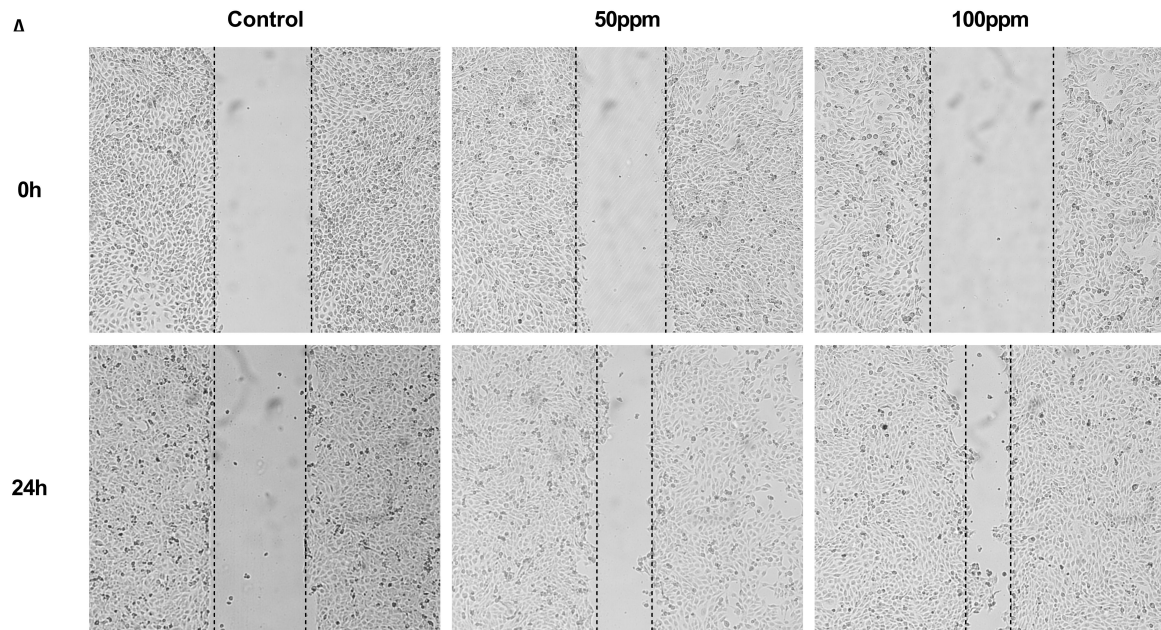
Figure 3. After the indicated times, the cells migration were photographed under a phase contrast microscope.

3.4. AHA-98 regulates the JNK signaling pathway to reduces the apoptosis of HSF cells

AHA-98 significantly mitigates UVB-induced apoptosis in HSF cells. Exposure to UVB notably elevates the apoptosis rate in HSF cells, a critical factor in photoaging. However, post-UVB treatment with AHA-98 (100 ppm) effectively reduces apoptosis (4B). The result demonstrate that AHA-98 can robustly counteract the UVB-induced increase in apoptosis, thereby contributing to its anti-photoaging effects. To further investigate the mechanism by which AHA-98 protects against UVB-induced damage, we examined the activation of JNK, a critical kinase involved in stress responses and apoptosis. UVB-irradiated HSF cells exhibited a marked increase in phosphorylated JNK (p-JNK) levels, indicating the activation of the JNK signaling pathway. Immunofluorescence staining confirmed these findings, with a reduction in p-JNK staining intensity in AHA-98-treated cells compared to UVB-irradiated cells (Figure 4A and C). UVB irradiation significantly increased the ratio of cytosolic to mitochondrial cytochrome c compared to the control group, indicating enhanced apoptosis. However, treatment with AHA-98 (100 ppm) post-UVB irradiation reversed this trend, decreasing the cytosolic cytochrome c levels and restoring the mitochondrial cytochrome c content, suggesting a protective role against UVB-induced apoptosis (Figure 4D). These results suggest that AHA-98 may regulate the JNK signaling pathway to mitigate UVB-induced apoptosis in HSF cells, contributing to its potential anti-photoaging properties.
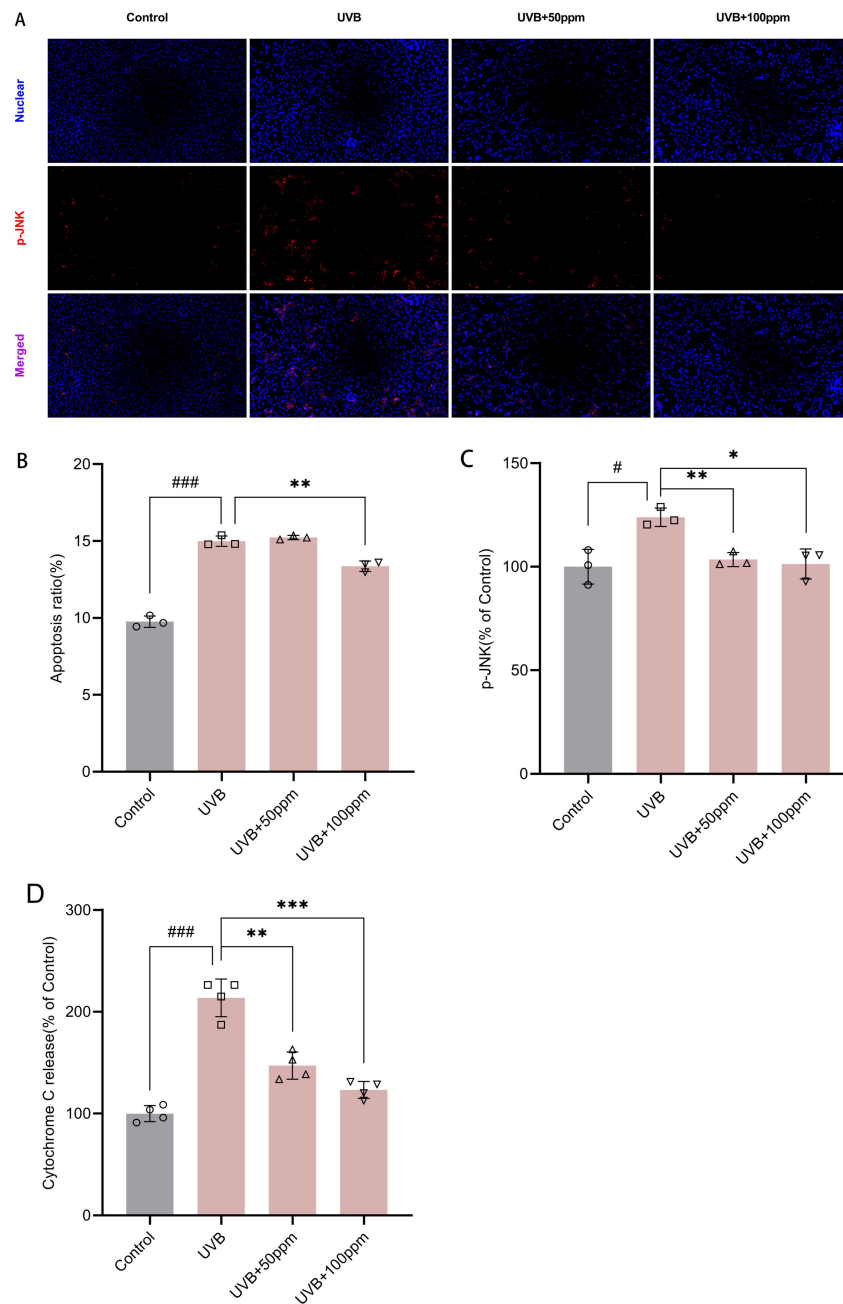
Figure 4. (A) Signals of p-JNK were visualized and digital images were obtained by using a confocal microscope. ImageJ was used to calculate the fluorescence intensity of the image. (B) Effect of AHA-98 treatment on apoptotic changes associated with UVB exposure. UVB-treated HSF cells(n=3) were treated with different doses of AHA-98 for 16 h. (C) Effect of AHA-98 on the expression of p-JNK in HSF cells.UVB-treated HSF cells(n=3) were treated with different doses of AHA-98 for 24 h. (D) Effect of AHA-98 on cytochrome c release in HSF cells. UVB-treated HSF cells (n=3) were treated with different doses of AHA-98 for 6 h.$^{\#}P<0.05$,$^{\#\#\#}P<0.001$ compared to the untreated control group.$^{*}P<0.05$,$^{**}P<0.01$,$^{***}P<0.001$compared to the UVB-treated group.

3.5. AHA-98 upregulates skin elasticity parameters R2, R7, and Q1

AHA-98, when applied in the form of an emulsion to the facial skin of volunteers, increased the skin elasticity parameters R2, R7, and Q1 compared to the baseline at weeks 3, 6, and 9 (Figure 5A, C and E).These increases were statistically significant compared to the placebo group at each time point (Figure 5B, D and F). The enhancement of these parameters indicates that AHA-98 effectively improves skin elasticity, which is a key factor in maintaining youthful skin appearance and reducing the signs of photoaging.
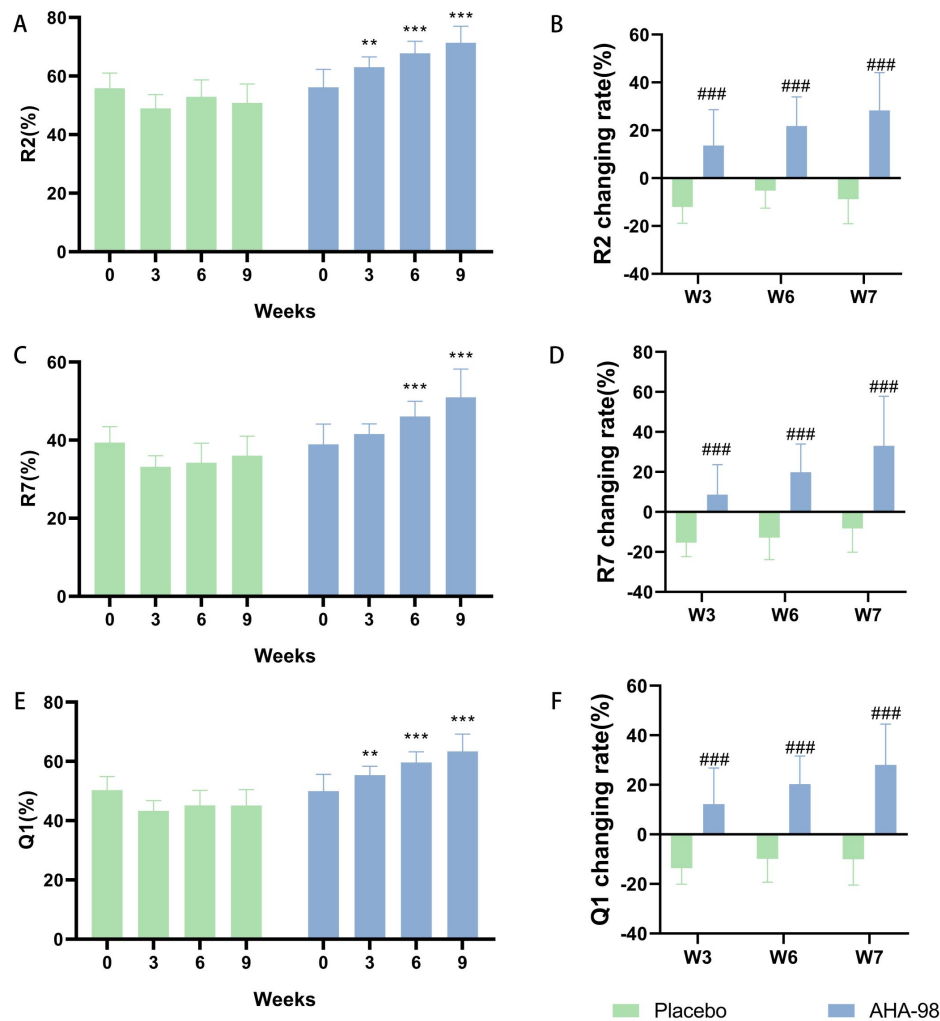

Figure 5. Effect of AHA-98 on skin elasticity parameters R2, R7, and Q1.Healthy human volunteers(n=15) were treated with base cream or cream contained AHA-98.Measurement of skin elasticity parameters R2(A), R7(C), and Q1(E) was conducted at weeks 3, 6, and 9.The calculation of the relative changes in R2(B), R7(D), and Q1(E) from the baseline was performed at each time point.**$P<0.01$,***$P<0.001$ compared to the baseline.###$P<0.001$ compared to the placebo group.

4. Discussion

This study explored the anti-photaging effects and underlying mechanisms of AHA-98. The results of this study suggest that the upregulation of p-JNK, which induces the release of mitochondrial cytochrome C into the cytoplasm and subsequently triggers apoptosis, is one of the mechanisms underlying UVB-induced skin aging. AHA-98 has been shown to counteract

this process by inhibiting JNK phosphorylation and reducing cytochrome C release, thereby mitigating UVB-induced cellular damage. Additionally, AHA-98 has demonstrated the ability to alleviate other types of damage caused by UVB exposure. This study provides comprehensive evidence of AHA-98's anti-photaging effects and elucidates its underlying mechanisms.

5. Conclusion

AHA-98 shows strong photoprotective effects by inhibiting JNK phosphorylation to reduce cytochrome C release and prevent apoptosis. It also boosts cell viability, collagen I and hyaluronic acid levels, and cell migration in photodamaged cells. Human trials confirm its ability to improve skin elasticity, highlighting its potential as an anti-photoaging agent for skincare products.

Acknowledgments

Conflict of Interest Statement

The authors declare no conflict of interest.

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