Skipjack (Katsuwonus pelamis) elastin hydrolysate-derived peptides attenuate UVA irradiation-induced cell damage in human HaCaT keratinocytes

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Introduction

As the largest organ of the human body, the skin protects against various physical and environmental assaults. However, the function of the skin inevitably declines with age and the risk of skin diseases increases because of a wide variety of environmental factors (Yaar & Gilchrest, 2003). Of all the environmental factors, ultraviolet (UV) irradiation has been shown to be the most detrimental to the human skin (Lan, Hung, Fang, & Ching-Shuang, 2019). Sunlight is the main source of UV exposure for humans and can be classified into UVA (315-400 nm), UVB (280-315 nm), and UVC (200-280 nm) (De Grujil, 2000). Short-wavelength UVC is the most damaging type of UV radiation. However, it is mostly absorbed by the ozone layer, preventing it from reaching the earth’s surface (Diffey, 2002). In this regard, UVC exerts little or no physiological threat to humans. With a much stronger penetration capacity than UVB, UVA constitutes 95% of the solar ultraviolet light of significance to human health and ecosystems and it is known to cause serious skin damage by inducing the generation of reactive oxygen species (ROS) (Berneburg, Plettenberg, & Krutmann, 2000; Jiang et al., 2009). Excessive generation of ROS depletes the...
skin’s inherent antioxidant capacity, inducing cell damage, photoaging, and/or skin cancer (Wenk et al., 2001). The overproduction of ROS also drives the pro-apoptotic proteins, Bax and Bak, to translocate into the mitochondrial outer membrane, inducing the release of cytochrome C and subsequently activate caspases and trigger cell apoptosis program (Lopez & Tait, 2015; Tait & Green, 2010), ultimately leading to apoptosis in HaCaT cells (Tada-Oikawa, Oikawa, & Kawanishi, 1998). Therefore, through the generation of ROS, UVA irradiation has been shown to be a particularly potent inducer of DNA damage, serious oxidative stress and cell death.

Elastin is the core protein (>90%) of elastic fibers and gives elasticity to tissues, such as skin, aorta, ligaments, and lungs (Mitieux & Weiss, 2005). A small amount of elastin in the dermis allows for the elasticity and flexibility of the skin. However, breakdown and loss of elastic fiber function becomes prevalent with aging (Zhang & Duan, 2018). Food-derived peptides have been suggested to induce biological responses including enhanced synthesis of elastin, fibroblast growth, and the extracellular matrix, all of which are beneficial for the skin (Shigemura et al., 2012; Tajima, Wachi, Uemura, & Okamoto, 1997). Hydrophobic protein-derived peptides from natural materials including food have long attracted tremendous research attention due to their antioxidative properties (Hattori, Hiroto, Kumagai, Kumagai, Feng, & Takahashi, 1998). Food-derived elastin peptides such as Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, and Ala-Hyp-Gly (Shigemura et al., 2012) have been shown to be potentially valuable in skin care, but detail mechanism is largely lacking. In this regard, it is suggested that ingestion of elastin hydrolysate might improve the condition of the skin. Recent consumer preference for marine products, the availability and relatively low cost coupled with its demonstrated presence of elastin peptides and biological potency of skipjack (Shiratsuchi, Nakaba, Shigemura, Yamada, & Sato, 2013) makes it a good candidate for further exploring the mechanism of photoaging. We hypothesized that skipjack elastin hydrolysate small peptides have free radicals scavenging ability. Because UVA-induced cell damage is primarily ascribed to the deleterious effects of ROS, molecules with free radical scavenging properties are particularly promising as radio-protectors. In the present study, we focused on identifying small peptides from the skipjack elastin hydrolysate with various bioactivities, especially in free radicals scavenging and explored the mechanism for their photoprotective effects.

2 | MATERIALS AND METHODS

2.1 | Materials

Skipjack elastin hydrolysate was purchased from Hayashikane Sangyo Co., Ltd. (SHIMONOSEKI, Japan). Chemical synthesis of the crude peptides was done by China Peptide Co. Ltd. (Shanghai, China). Human HaCaT Keratinocytes cell line was obtained from the American Type Culture Collection (ATCC, Virginia, USA). Sephadex G-15 column and SB-C18 column were purchased from GE Life Science Co. (Pittsburgh, USA) and Agilent Technologies (Palo Alto, USA), respectively. The LC-MS/MS used in this study was a combination of BRUKER Daltonics (Billerica, Massachusetts, Germany) maXis Impact equipped with electrospray ionisation (ESI) source and Tandem LC-Agilent Technologies 1290 Infinity (Palo Alto, USA). ROS detection kit, MTT cell proliferation kit, cytotoxicity assay kit and cell mitochondrial membrane potential detection kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Annexin V/PI apoptosis Analysis kit was purchased from Thermo Fisher Scientific (Lafayette, Colorado, USA). Fetal bovine serum (FBS) and α-MEM were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). UV-int® 150 UVA radiometer and UVA lamps were products from Nanjing Huaqiang Co. (Nanjing, China). FC-500 Flow cytometer was obtained from Beckman (California, USA). A radiometer UV-365 was purchased from Hangzhou Youwei Technology Co. Ltd. (Hangzhou, China).

2.2 | Purification and identification of the Skipjack elastin peptide

Prior to LC-MS/MS analysis, the Skipjack elastin hydrolysate was subjected to gel filtration purification using Sephadex G-15. Distilled water was used as eluent. Flow rate was 5 mL/min. Each peptide solution (5 mL/tube) was collected and detected by spectrophotometer at 214 nm. One peak was obtained and the fraction was concentrated and filtered for LC-MS/MS analysis. The LC-MS/MS detection was performed by a combination of BRUKER Daltonics Maxis Impact equipped with electrospray ionization (ESI) source and Tandem LC-Agilent Technologies 1290 Infinity SB-C18 column (2.1x50 nm, 1.8 μm). The mobile phase of the liquid chromatography was acetonitrile containing 0.1% trifluoroacetic acid. The flow rate was set at 200 μL/min. Molecular mass and peptide sequence of all spectra were determined through the Bruker Daltonics software Data Analysis 4.0 (Billerica, Massachusetts, Germany) and the MASCOT (Matrix Science, v2.204) search engine software against Swiss-Prot and NCBI databases. The search was implemented using no specific enzyme cleavage sites and an MS/MS mass tolerance of 0.6 Da. The peptides with average local confidence (ALC) over 75% were used for further analysis. The analysis was performed using two individual samples and only peptides, which were positively identified in both samples were accepted. Identified peptides were synthesized later by solid phase using Fmoc strategy (China Peptide Co. Ltd, Shanghai, China).

2.3 | Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC of the synthesized peptides was evaluated according to the procedure described by Prior R.L (Prior et al., 2003). Analyses were conducted by automated plate reader in phosphate buffer (PBS, pH 7.4) at 37 °C. The measurements were made in plates with 96 black flat-bottom wells. Trolox was used as the standard solution. Fluorescein (FL) was used as the fluorescent substrate. The reaction was started by thermal decomposition of 2,2-Azobis(2-Adamipinopropane) Hydrochloride (AAPH) (Sigma, St. Louis, MO, USA) in 75 mM phosphate buffer (pH
7.4). In each well, 200 μl of FL (78 nM) and 20 μl of sample, blank (PBS), and standard (Trolox) were placed, and then 20 μl of AAPH (119.4 mM) was added. The mixture was preheated at 37 °C for 15 minutes prior to the addition of AAPH. The reaction was activated by the addition of AAPH. Fluorescence was measured at 5-minute intervals for 180 minutes at an excitation of 485 nm and an emission of 538 nm. The measurements were taken in triplicate. The ORAC values were calculated by applying formula (1) and expressed as μmol Trolox equivalents/μmol peptide (μmol TE/μmol peptide).

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\text{ORAC} = \frac{C_{\text{Trolox}} (\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}) \cdot k}{C_{\text{peptide}} (\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}}) - \text{F}_0 \cdot \Sigma n \cdot \Delta t} 
\]

(1)

In formula (1), \(C_{\text{Trolox}}\) is the concentration (μM) of Trolox, \(k\) is the sample dilution factor, and AUC is the area below the fluorescence decay curve of the sample, blank or Trolox, respectively, which is calculated by applying formula (2) in a Microsoft Excel spreadsheet (Washington, USA).

\[
S = 0.5 \times [2 \times (F_0 + F_1 + \cdots F_{n-1} + F_n) - F_0 - F_n] \times \Delta t, 
\]

(2)

In formula (2), \(F_0\) is the initial fluorescence and \(F_n\) is the fluorescence at time \(n\).

### 2.4 | Cell culture

Human keratinocyte, HaCaT, cell line was maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO₂. The cells were cultured in minimum Eagle’s medium containing 10% heat-inactivated fetal bovine serum.

#### 2.4.1 | UVA irradiation intensity and cell viability

HaCaT cells were seeded into 96-well plates at a density of 3 × 10^4 cells/well and maintained in culture medium for 24 hours. Before exposure to UVA irradiation, the media in each well was replaced by PBS to eliminate possible phototoxic reaction of some components in the culture medium. Cells were then treated with various intensities (2.8, 5.6, and 10 J/cm²) of UVA light after the confluence reached 80%-90%. To prevent the cells from overheating during irradiation, plates were kept on ice during the whole test. Cells without UVA irradiation were set as the negative control. UVA irradiation were supplied by two paralleled UVA lamps with a peak emission frequency of 365 nm at 8000 μW/cm² output (emission spectrum: 320-400 nm). Cells were irradiated at a distance of approximately 20 cm. The irradiation intensity was measured by a UVA radiometer, which was put at the same distance from the UVA source as the cells. It took about 3 hours to reach the highest UVA irradiation intensity of 10 J/cm². After UVA irradiation, cells were incubated with fresh complete medium at 37 °C for 24 hours.

The effect of the different UVA irradiation doses on cell viability was monitored using the MTT (3-(4,5-dimethylthiazol-2-11)-2, 5-diphenyl tetrazolium bromide) assay. MTT assay depends on the principle that mitochondrial dehydrogenase reduces tetrazolium salt in viable cells. In brief, 10 μL of 5 mg/mL MTT was added to each well to reach a final concentration of 0.5 mg/mL. After incubation at 37 °C for 4 hours in light-proof conditions, the supernatants were aspirated. Formazan crystals in each well was dissolved in Dimethyl sulfoxide (DMSO, 150 μL) and the plate was further analyzed by Tiangen microplate reader (Beijing, China) at a wavelength of 490 nm.

#### 2.4.2 | Cell viability of the peptides under UVA irradiation

The effect of the peptides on the viability of cells under UVA (2.8, 5.6, 10 J/cm²) treatment was monitored by MTT assay. HaCaT cells were seeded into 96-well plates at a density of 3×10^4 cells/well. When the confluence reached 80%-90%, cells were pretreated with culture medium containing predetermined (results not shown) doses (0.5 and 0.05 mM) of the peptides for 14 hours. Cells preincubated with peptides and without UVA irradiation were set as the control. Other cells were treated with 10 J/cm² UVA irradiation and then incubated for 24 hours at 37 °C. Finally, MTT assay was performed by the same protocol as mentioned in 2.4.1. Considering that 10 J/cm² is the actual daily UVA intensity to the human skin (10 J/cm² = 30 minutes of sun exposition, at 12 noon in Central Europe) (Mapelli, Calo, & Marabini, 2016), 10 J/cm² UVA irradiation was chosen and applied for the subsequent assays.

#### 2.5 | Measurement of intracellular ROS

Intracellular ROS was assessed after cells were pretreated with the synthesized peptides (0.5 and 0.05 mM) and then exposed to 10 J/cm² UVA irradiation. Production of intracellular ROS was quantified according to the method of Wang and Joseph using fluorometric assay with the cell-permeable, nonfluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) by performing a fluorometric assay (Wang & Joseph, 1999). This probe is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which could be trapped within cells and subsequently changes to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation by intracellular ROS. In brief, HaCaT cells in a 6-well plate at a concentration of 2.5×10⁵ cells/well were preincubated with or without peptides for 14 hours prior to UVA exposure. After 10 J/cm² UVA irradiation, the cells were immediately washed with medium without serum and subsequently incubated with 10 μM DCFH-DA in α-MEM without serum at 37 °C for 30 minutes, and then washed twice with fresh medium without serum. A Nikon fluorescence microscope (Tokyo, Japan) was used for the imaging. To detect the intensity of fluorescence, cells with the same treatment were digested using 0.25% trypsin and then harvested. Cells were then incubated with DCFH-DA for 30 minutes and washed twice with medium without serum. The level of intracellular ROS was determined using FC-500 flow cytometer (Beckman, California, USA).

In formula (3), \(C_{\text{peptide}}\) is the concentration (μM) of the peptides, \(k\) is the sample dilution factor, and AUC is the area below the fluorescence decay curve of the sample, blank or Trolox, respectively, which is calculated by applying formula (2) in a Microsoft Excel spreadsheet (Washington, USA).

\[
S = 0.5 \times [2 \times (F_0 + F_1 + \cdots F_{n-1} + F_n) - F_0 - F_n] \times \Delta t, 
\]

(3)
2.6 Measurement of mitochondrial membrane potential (MMP)

The mitochondrial membrane potential of cells was assessed using JC-1 staining. The JC-1 fluorescent probe, which enters the mitochondria and changes from red to green as the membrane potential declines, was used to estimate mitochondrial polarity. In brief, cells were seeded at 2.5 x 10⁵ cells/well in a 6-well plate and incubated at 37 °C for 12 hours. After growing adhesively, the cells were, respectively, treated with peptides A and B (0.5 and 0.05 mM) for 14 hours before being exposed to 10 J/cm² UVA irradiation. After irradiation, JC-1 (10 μM) was added to the cells and incubated at 37 °C for 20 minutes, following the manufacturer’s instructions. After that, cells were washed with PBS twice and images were visualized by fluorescence microscope. Besides, cells under the same treatment were collected by flow cytometry. For each tube, 500 μL JC-1 staining buffer was added and then cells were incubated at 37 °C for 30 minutes. Cells were then washed once by adding 1.5 mL of PBS to each tube and resuspended in a final volume of 500 μL PBS for flow cytometry. Flow cytometry was performed using 488 nm excitation, green and red filters for detection. The ratio of the intensity of green fluorescent monomers to the intensity of JC-1 aggregates reflects the mitochondrial potential. Therefore, mitochondrial membrane potential was expressed as the ratio of red/green fluorescence and the fraction of damaged cells.

2.7 Apoptosis analysis by Annexin V-Fluorescein Isothiocyanate (FITC) staining

Staining was performed according to the Annexin V-FITC apoptosis detection kit protocol to determine whether the synthesized peptides had protective effects on UVA-exposed HaCaT cells by reducing apoptosis. Phosphatidylserine (PS) is translocated from the inner to the outer layer of the plasma membrane in apoptotic cells. Therefore, Annexin V, which is a Ca²⁺-dependent phospholipid-binding protein with high affinity for PS, can be used for identifying apoptotic cells (Mapelli et al., 2016). Briefly, cells were seeded at 2.5 x 10⁵ cells/well in a 6-well plate and incubated at 37 °C for 12 hours. After growing adhesively, the cells were respectively treated with peptides A or B (0.5 and 0.05 mM) for 14 hours before exposure to 10 J/cm² UVA irradiation. Both floating and attached cells which received UVA irradiation were harvested with 0.25% EDTA-trypsin. Then, the cells were washed twice with PBS and incubated with Annexin V-FITC for 20 minutes at room temperature. Thereafter, propidium iodide was added and then incubated for 10 minutes. Stained cells were examined by flow cytometry using 488 nm excitation and 525 nm emission. The rate of apoptosis was analyzed by the Flowjo software. Besides, nuclear morphology of cells with the same UVA irradiation treatment was assessed using Hoechst 33258 staining. After UVA irradiation, cells were washed twice with PBS and fixed for 10 minutes in 4% paraformaldehyde followed by staining with 10 μg/mL Hoechst 33258 for 20 minutes. The cells were washed with PBS twice and observed under Nikon fluorescence microscope (Tokyo, Japan).

2.8 Statistical analysis

All measurements were performed in triplicates. All statistical analysis was carried out using the Origin software version 8.0 (OriginLab Co, USA). Data are presented as means ± SD. Group comparison was analyzed using paired t-tests. Results were classified as significant (p < 0.05), highly significant (p < 0.01), or extremely significant (p < 0.001).

3 RESULTS

3.1 Purification and identification of peptides

Sample purification was done by column equipped with Sephadex G-15. As shown in Figure 1a, the sample was concentrated into one peak. The peptide peak (5 mL, tube 23) was collected repeatedly and concentrated for ESI-MS/MS analysis. The concentrated peak fraction was subjected to sequence identification by ESI-MS/MS. The acquired MS/MS data was first searched with software Mascot MS/MS Ions Search against amino acid sequences. Peptide tolerance was set to 0.6 Da and no specific cleavage sites were arranged. Two peptides TGVLTVM (peptide A) and NHIINGW (peptide B) matching the criteria of a cross-correlation possibility (p-values < 0.01) were identified. The acquired MS/MS data was also searched using analysis software Data analysis 4.1 and the MS/MS spectra of these two peptides were further confirmed in the database. In the MS/MS spectrum of the two peptides (Figure 1b), parent ions were labeled and b & y ions were marked. The peptides A and B with molecular structure as shown in Figure 1c were confirmed to be the main constituents of the peak sample by both database search and ion comparison.

3.2 In vitro antioxidant activity of peptides

The in vitro antioxidant activity, Oxygen Radical Absorbance Capacity (ORAC), of the two peptides was determined by ORAC assay. The results (Figure 1d) showed that the ORAC value of peptide A (1.86 ± 0.22 μmol TE/μmol peptide) with molecular weight of 719.71 Da (98.66% purity) was significantly higher (p < 0.05) than that of peptide B (1.51 ± 0.10 μmol TE/μmol peptide) with molecular weight of 852.95 Da (99.31% purity).

3.3 Peptides A and B increases the viability of UVA-induced HaCaT cells

In order to evaluate the cytotoxicity of UVA irradiation, the viability of HaCaT cells exposed to UVA irradiations of 2.8, 5.6, and 10 J/cm² were measured by MTT assay. After UVA irradiation, obvious changes in cell morphology were observed under microscope (Figure 2a). Compared with the control group, cells in the UVA (model) group exposed to UVA irradiation detached from the wall, shrank, became round, and
apoptotic bodies formed. The results showed that 3 hours of UV irradiation decreased cell viability in a dose-dependent manner (Figure 2b). At 2.8, 5.6 and 10 J/cm² UVA irradiation, the viability of HaCaT Keratinocytes decreased to 78.33% ± 5.48%, 35.97% ± 3.54%, and 18.09% ± 0.68%, respectively. These indicated that a UVA-irradiated HaCaT cell model was successfully established. As shown in Figure 2c, at concentrations of 0.5 and 0.05 mM, peptides A and B exhibited no cytotoxic effects on HaCaT cells after 24 hours treatment. In fact, peptide B treatment stimulated cell proliferation by 6.33% ± 4.59% (0.5 mM) and 6.88% ± 4.65% (0.05 mM) (p < 0.05), while treatment with peptide A showed no significant changes in cell viability (Figure 2c). The attenuation of UVA-induced cytotoxicity by peptides A and B in HaCaT cells was then evaluated. The survival rate of cells without peptide treatment but exposed to UVA radiation of 2.8, 5.6, and 10 J/cm² (UVA groups, Figures 2d-f) were normalized as 100% and used as the control in each case. Compared to the normalized control (UVA), the relative cell viability of cells in each group revealed the protective effect of the two peptides. As shown in Figures 2d-f, relative cell viability of cells treated with peptide A increased by 27.55% ± 12.27% (0.05 mM) under 2.8 J/cm² UVA, and 23.20% ± 10.46% (0.5 mM) and 30.96% ± 41.37% (0.05 mM) under 5.6 J/cm² UVA irradiation. However, at peptide concentrations of 0.5 mM (2.8 J/cm² UVA intensity) and 0.5 and 0.05 mM (10 J/cm² UVA intensity), the trend of increase in cell viability did not reach significant levels. Treatment with peptide B significantly increased the viability of HaCaT cells (p < 0.05) at all UVA intensities applied except at 2.8 J/cm² (0.05 mM). It was observed that, peptide B (0.5 mM) increased cell viability by 12.32% ± 7.30% (p < 0.05), 22.92% ± 5.19% (p < 0.001), and 73.33% ± 5.30% (p < 0.01) under 2.8, 5.6, and 10 J/cm² UVA, respectively, compared with the normalized UVA group (Figures 2d-f). The results showed that, peptides A and B exhibited no apparent cytotoxicity on HaCaT cells, making them good candidates for further exploration.

3.4 ROS scavenging capacity of peptides A and B in UVA-induced HaCaT cells

MTT was performed to analyze the capacity of peptides A and B to scavenge ROS induced by UVA irradiation in HaCaT cells. 10 J/cm² UVA irradiation was used because that is the actual daily UVA intensity to the human skin (10 J/cm² ≈ 30 minutes of sun exposition, at 12 noon. in Central Europe) (Mapelli et al., 2016). A cell-permeable, nonfluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was used to perform a fluorometric assay. This probe is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which could be trapped within cells and subsequently change to highly fluorescent 2′,7′-dichlorofluorescein upon oxidation by intracellular ROS. Therefore, the fluorescence intensity is proportional to the amount of cellular ROS generation (Chen, Zhong, Xu, Chen, & Wang, 2010). As shown in Figure 3a, an increase in fluorescence...
FIGURE 2. Effect of peptides A and B on cell viability. Cell viability was determined by MTT assay and measured by microplate reader. (a) Morphology of HaCaT cells before (control group) and after UVA irradiation (UVA group). (b) Cell viability of HaCaT cells treated with 0, 2.8, 5.6, and 10 J/cm² UVA irradiation, respectively. (c)–(f) Cell viability of HaCaT cells pretreated (14 hours) with peptides A and B under 0, 2.8, 5.6, and 10 J/cm² UVA irradiation. *p < .05; **p < .01; ***p < .001 compared with the UVA group.

Intensity was observed in cells irradiated with UVA (10 J/cm²), which was reduced in the presence of peptides A and B. Quantitative analysis of the fluorescence intensity showed that UV irradiation significantly increased (p < .05) intracellular levels of ROS. However, pretreatment with peptides A and B remarkably inhibited the generation of ROS (p < .05) (Figure 3b). As shown in Figure 3c, the mean fluorescence intensity in cells treated with 0.5 and 0.05 mM of peptide A, reduced by 56.57% ± 12.62% and 64.86% ± 9.52%, respectively, whereas peptide B treatment (0.5 mM) showed a 40.68% ± 15.67% reduction in mean fluorescence intensity. However, the change induced by peptide B at 0.05 mM did not reach significant levels. Thus peptides A and B effectively reduced intracellular ROS generation and/or accumulation.

3.5 Peptides A and B attenuate mitochondria membrane potential (MMP) decline in UVA-induced HaCaT cells

Changes in MMP caused by peptides A and B in UVA irradiated HaCaT cells were examined by both fluorescence microscope and flow cytometry using the lipophilic fluorochrome JC-1 staining. JC-1 is an indicator of mitochondrial health and polarization, and its accumulation in the mitochondria leads to the formation of JC-1 aggregates detectable in the red channel by flow cytometry (Qian, Jung, & Kim, 2008). Fluorescence images taken by fluorescence microscope were shown in Figure 4a. According to the fluorescence images, MMP changes can be visually distinguished by color change from red to green. Loss of MMP was observed in cells irradiated by 10 J/cm² UVA. However, treatment with peptides A and B (0.05 and 0.5 mM) resulted in significant protection against the loss of MMP and reversed mitochondrial dysfunction in UVA-irradiated HaCaT cells as seen in the reduction in green fluorescence (Figure 4a). When irradiated by UVA (10 J/cm²), MMP of the HaCaT cells (UVA group) decreased by 12% compared to the control group. However, MMP significantly increased in the peptide-treated groups (Figure 4b). Furthermore, a much higher ratio of red/green fluorescence (p < 0.001) in the UVA group compared with the control group implied a dramatic decline in MMP of the UVA-induced cells while cells preincubated with peptides showed trends of recovered ratio of red/green fluorescence (Figure 4c). Figure 4d shows a much better fraction of UVA-induced damage cells in the UVA group compared to the control group (p < 0.001). However, preincubation with peptides A and B reduced the fraction of UVA-induced damage cells (p < 0.05) (Figure 4d). UVA irradiation caused severe cell damage (25.20% ± 2.01%); however, by treatment with peptides, the damage was recovered as reflected by the fraction of damaged cells decreasing by 12.40% ± 0.28% (0.5 mM) and 13.13% ± 3.96% (0.05 mM) in peptide A, while that of peptide B decreased by 10.80% ± 0.72% and 12.90% ± 3.35% for 0.5 and 0.05 mM, respectively, compared with the UVA group. Accordingly, the results suggest that peptides A and B may reverse MMP decline.
3.6 | Peptides A and B ameliorate UVA-induced apoptosis rate in HaCaT cells

To observe the apoptosis rate of UVA-irradiated HaCaT cells, nuclear morphology was evaluated by fluorescence microscopy using Hoechst 33258 staining. As shown in Figure 5a, the control group showed clear images indicating very rare apoptotic cells. However, in the UVA group (10 J/cm² UVA), shrunk, bright nuclei, and condensed chromatic cells typical of cell apoptosis were observed. Interestingly, the number of apoptotic nuclei in cells treated with peptides A and B decreased, revealing the protective effect of the two peptides. Phosphatidylserine (PS) is translocated from the inner to the outer layer of the plasma membrane in apoptotic cells. Therefore, Annexin V, which is a Ca²⁺-dependent phospholipid-binding protein with high affinity for PS, was also used for identifying apoptosis cells (Mapelli et al., 2016). As shown in Figures 5b-c, the apoptosis rate in the UVA group was 13.08% ± 0.36%. However, this was significantly reduced by 3.43% ± 0.99% (0.5 mM) and 6.44% ± 0.50% (0.05 mM) for peptide A, and 8.52% ± 0.30% (0.5 mM) and 7.05% ± 0.70% (0.05 mM) for peptide B. Taking together, peptides A and B effectively ameliorates the rate of UVA-induced apoptosis in HaCaT cells.

4 | DISCUSSION

Numerous reports suggest that natural sources of peptides exert significant benefits on human health. As such the search (generation, separation, purification, and identification) for novel peptides from different protein sources have attracted enormous attention over the years. Elastin peptides in particular have long been known to be potent photoprotective agents (Zhang et al., 2017). Herein two novel peptides, TGVLTVM (peptide A) and NHIINGW (peptide B), with significantly different antioxidant capacities as measured by ORAC were identified. Peptide structure has been linked to its antioxidant capacity. Specifically, molecular size, hydrophobicity and electron transferring ability of the amino acid residues in the sequence are key determinants of ORAC and that the lower the peptide molecular weight, the higher the chance to cross the intestinal barrier and exert a biological effect (Qian et al., 2008; Suetsuna & CHEN, 2002). From the results, peptide A had a lower molecular weight (719.71 Da, 98.66% purity) compared to Peptide B (852.95 Da, 99.31% purity). Furthermore, peptides with hydrophobic N-terminal positions (CH₃), as in peptide A, are reported to have highly potent inhibitory activity. Besides, antioxidant activity is also influenced by the presence of aromatic amino acids (Kim,
FIGURE 4  Effects of peptide treatments on mitochondria membrane potential (MMP) in UVA-induced HaCaT cells. HaCaT cells were pretreated with or without peptides A and B (0.5, 0.05 mM) for 14 hours and then irradiated with UVA (10 J/cm²) for 3 hours. Mitochondrial membrane potential (MMP) was analyzed using JC-1 staining. (a) Fluorescent images of intracellular JC-1 taken by fluorescent microscope. The JC-1 aggregates were dyed in red and monomers in green. (b) Quantitative analysis of ratio of red/green fluorescence detected by flow cytometry. (c) Scatter grams of cells dyed with JC-1 detected by flow cytometry, the percentage numbers showed the proportion of damaged cells with declining MMP. (d) Quantitative analysis of the fraction of damaged cells with MMP decline detected by flow cytometry; ### $p < .001$, compared with control group. * $p < .05$, ** $p < .01$, compared with UVA group. MMP: mitochondrial membrane potential

Ahn, Moon, & Je, 2018; Vilcacundo, Miralles, Carrillo, & Hernández-Ledesma, 2018), of which only peptide B (NHIIINGW), contains the aromatic tryptophan (W) with the capacity to directly supply hydrogen ions. These may account for the differences in ORAC values between the two peptides.

ORAC is a widely used method for assessing antioxidant capacity in biological samples and foods (Zou, He, Li, Tang, & Xia, 2016). Previous studies have reported of ORAC values of hydrolysates and peptide fractions of glutelin from cocoa (0.07 ± 0.01 – 0.28 ± 0.02) (Tovar-Pérez, Guerrero-Becerra, & Lugo-Cervantes, 2017), egg white hydrolysates-derived peptides (0.96 ± 0.07 – 1.30 ± 0.01 μmol/TE/mg) (Nimalaratne, Bandara, & Wu, 2015), and oat protein-derived peptides (0.14–0.67 μM TE/μM) (Du, Esfandi, Willmore, & Tsopmo, 2016). Compared to these results, it could be said that peptides A and B have good antioxidant capacities. Antioxidant activity is predicted by factors including the peptide chain length as well as the constituent and position of amino acids. For example, amino acids with aromatic residue such as tyrosine (Y), tryptophan (W), phenylalanine (F), and the imidazole group of histidine (H) can quench free radicals by direct electron transfer. Furthermore, the presence of proline (P), valine (V), alanine (A), leucine (L), and sulfur containing amino acids methionine (M) and cysteine (C) and single hydrogen atom-containing amino acid like glycine (G) significantly contributes to the antioxidant ability of the resulting peptides (Huang, Ou, & Prior, 2005; Li, Jiang, Zhang, Mu, & Liu, 2008). In the two peptides studied, both had G. In addition, peptide A had 3 (V, L, and M), whiles peptide B contained 2 (W and H) known highly antioxidant amino acids. This higher number of constituent antioxidant amino acids in peptides A B relative to the others may have accounted for the relatively higher ORAC values of the two peptides.

HaCaT cells are immortalized human keratinocytes and have been widely used to study the molecular mechanism and pathophysiology associated with UVA irradiation of the skin which is usually characterized by significant reduction in cell viability with obvious nuclear morphological changes (Choi & Jeon, 2018; Gao, Guo, Chen, Du, & Wang, 2007; Hseu et al., 2018). The results showed that a successful UVA-irradiated HaCaT cell model was established, evident in reduced cell viability and increased formation of apoptosis cell bodies.

Exposure to UVA leads to increases in fibroblast damage through different photoaging pathways (Wang et al., 2014). Peptides A and B substantially ameliorated UVA-induced cytoxicity without exhibiting apparent self-toxicity. It thus suggests that peptides A and B may interfere with UVA-induced cellular photoaging pathways and holds great potential in reversing the decline in cell proliferation that is characteristic of UVA irradiations.
Peptides A and B inhibited UVA-induced apoptosis in HaCaT cells. Cells were pretreated with or without peptides A and B (0.5 and 0.05 mM) for 14 hours and then irradiated with UVA (10 J/cm²). After UVA irradiation, cells were stained with Hoechst 33258 and Annexin V/FITC. (a) Representative morphology visualized under a fluorescence microscope. Cells with brightly fluorescent and fragmented nuclei were apoptotic. (b) Proportion of early (Q3), late (Q2) apoptosis cells, dead cells (Q1), and normal cells (Q4) under 10 J/cm² UVA irradiation, which was detected by flow cytometry. (c) Quantitative analysis of apoptosis rate (%) of cells detected by flow cytometry. ### p < .001 compared with control group. *p < .05, **p < .01, ***p < .001 compared with UVA group.

ROS is known to induce oxidative stress and premature skin aging under UVA irradiation (Kammeyer & Luiten, 2015; Rajagopalan et al., 2018). It depletes the skin’s inherent antioxidant capacity, results in serious oxidative stress, and induces oxidative damage in membranes, DNA, proteins, and other skin tissues. Wang et al. (2014) reported that 100 μmol/L N-terminal 5-mer peptide analog P165 reduced UVA-induced intracellular concentration of ROS by 25.38%. Our results demonstrated the superior capacity of peptides A and B, as free radical scavengers in reducing ROS accumulation in UVA-irradiated HaCaT cells compared to N-terminal 5-mer peptide analog P165. Similar to Korean Propolis (Kim & Yoo, 2016), peptides A and B dose-dependently inhibited intracellular ROS generation. UVA-induced cell damage is primarily ascribed to the deleterious effects of ROS and as such molecules with free radical scavenging properties are particularly promising as radio-protectors. Peptide A with a higher ORAC value was expected to have a higher photoprotection effect; however, the results showed otherwise. This might be attributable to a number of factors. The specific antioxidant amino acid constituents might play a greater role compared to the overall ORAC capacity. For example, Trp (W), is known to have the highest antioxidant activity in biological systems among free amino acids. The presence of W in the structure of peptide B and absent in peptide A could be the most significant driving force of the antioxidant activity (Nimalaratne et al., 2015; Shen, Chahal, Majumder, You, & Wu, 2010). Interestingly, the Food and Drug Administration has stated that ORAC values on their own may not realistically reflect the in vivo situation (Prior, 2015). Furthermore, ORAC is best used to quantify and reflect peroxyl radical scavenging capacity and may not be able to adequately quantify an antioxidant’s reducing capacity. As such ORAC values on their own may not be comprehensive enough in determining all aspects of the antioxidation of a substance (Huang et al., 2005).

MMP is a key parameter in assessing cellular energy metabolism. Research findings suggest that the over production of ROS and Ca²⁺ overload are major activators of membrane permeability transition pore (MPTP) and mitochondrial membrane potential depolarization. Consequently, it has been proven that attenuating oxidative-stress-induced mitochondrial Ca²⁺ overload can reduce the loss of mitochondrial membrane potential (MMP) (Wang et al., 2018). Accordingly, the results suggested that peptides A and B may reverse the decline in
energy metabolism ostensibly by preventing mitochondrial dysfunction and oxidative stress (Liang & Sundberg, 2011) caused by UVA irradiation in HaCaT cells.

Evidence also indicates that the excessive production of UVA irradiation-induced ROS initiates cell damage leading to decreased MMP, the release of cytochrome C, and eventual cell death (Svobodova, Walterova, & Vostalova, 2006). It was reported (Kim & Yoo, 2016) that propolis reduced UVA-induced mitochondria damage, apoptosis, and cell death through its ROS-scavenging capacity. It is therefore reasonable to infer that the inhibition of cellular ROS overproduction and accumulation by peptides A and B may be responsible for the observed attenuation of mitochondrial damage, reversed MMP decline and consequently reduced cell death and apoptosis.

5 CONCLUSION

In conclusion, two novel elastin peptides, TGVLTVM (peptide A) and NHIIINGW (peptide B), were identified and purified from the skipjack and confirmed to exert protective effects against UVA irradiation-induced skin damage. The two peptides were capable of scavenging intracellular ROS, reversed the loss of MMP, and reduced apoptosis in UVA irradiated HaCaT cell model, confirming that Skipjack hydrolysate contains small peptides with free radicals scavenging ability, and through the attenuation of oxidative stress and mitochondrial damage exert photoprotective effect on UVA irradiation-induced cell damage. Consequently, TGVLTVM and NHIIINGW might be useful as promising ingredients in sunscreens or antiaging cosmetic products.

ACKNOWLEDGMENT

This was supported by the National Key R&D Program of China (2018YFD0901101) and the Research and Development Program in Key Areas of Guangdong Province (2019B020210002). The funders had no role in the design, collection, and analysis of data, writing, and the decision to submit the article for publication.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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How to cite this article: Amakye, W. K., Yang, L., Yao, M., Yuan, E., Ren, R., & Ren, J. (2021). Skipjack (Katsuwonus pelamis) elastin hydrolysate-derived peptides attenuate UVA irradiation-induced cell damage in Human HaCaT Keratinocytes. Food Frontiers, 2, 184–194. https://doi.org/10.1002/fft2.74