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Transdermal Delivery and Biological Activity of Arginine Oligomer Conjugation of Palmitoyl Tripeptide-1

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Abstract

GHK is a promising cosmeceutical peptide that stimulates the synthesis of ECM and modulates the activity of Matrix Metallo Proteinases (MMPs). However, due to the hydrophilic properties of GHK, skin permeability is a challenge. To overcome this limitation, we considered two peptide modifications, namely palmitoyl attachment and arginine oligomer conjugation. First, we evaluated the optimal modification of GHK for skin penetration using the Franz diffusion cell assay with 3D human reconstructed skin. We found that skin permeability was slightly increased by attaching palmitoyl at the N-terminus of GHK, while it was significantly increased by conjugating arginine oligomers at the C-terminus of GHK. In the comparison between R4, R6, and R8, R4 showed the highest penetrating potential, and the permeability of Pal-GHK-R4 was about 7-fold higher than that of GHK. Based on the skin permeability results, the optimal peptide, Pal-GHK-R4, was synthesized using SPPS, after which its biological activities were verified. Evaluating MMP-2 activity by gelatin zymography and collagen production by Sircol collagen assay showed that Pal-GHK-R4 had approximately100-fold lower active concentrations than GHK. In other words, we established the novel peptide Pal-GHK-R4 as a modification that significantly increases both the biological activities and skin permeability of GHK.

Keywords: GHK, Pal-GHK-R4, Arginine oligomer, Skin permeability, MMP-2 activity, Collagen production.

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1. Introduction

Tripeptide-1 (Glycyl–Histidyl–Lysine, GHK) is a synthetic peptide containing glycine, histidine, and lysine; it occurs naturally in human plasma and declines with age. Pickart first discovered that GHK causes liver tissue from older humans to synthesize proteins with the same characteristics as younger tissue, and this peptide has thus been described as a growth factor for a variety of cells [1, 2]. Since the discovery of GHK, its diverse biological activities have been established. In

particular, several studies have shown that GHK stimulates skin regeneration by synthesizing extracellular matrix (ECM) such as collagen and glycosaminoglycans (GAGs) and also modulates the activity of matrix metalloproteinases (MMPs); it has, therefore, become known as a promising cosmeceutical peptide [3-5].

However, the challenge is that the skin permeability of GHK is low because of its hydrophilic properties [6]. The conjugation of cosmeceutical peptides with other molecules is an effective way to obtain better physicochemical properties [7]. A common modification that can enhance permeability is to attach fatty acids, which are lipophilic molecules, to the bioactive peptides [8]. Lintner and Peschard described how the transdermal delivery of peptides with palmitoyl attached at the N-terminus was enhanced compared to that of non-palmitoylated peptides, but the active concentration of palmitoyl tripeptide-1 (Pal-GHK) was not far from GHK [9].

Another modification is to conjugate bioactive peptides with cell-penetrating peptides (CPPs) [10, 11]. CPPs have been demonstrated to mediate the intracellular penetration of cargos and can be applied to skin penetration based on the structural similarity of the lipid bilayer between the cell membranes and intercellular lipids [12, 13]. The trans-activating transcriptional activator (TAT) peptide of HIV-1 is a representative cationic CPP, and its penetrating potential strongly depends on having lysine (K) or arginine (R) among its amino acid sequence. Enabling lysine and arginine oligomers to act as CPPs, Brooks et al. [14], Rudolph et al. [15], and Lim et al. [16] conjugated K9 to the N-terminus of GHK (K9-GHK) and found that its transdermal delivery was enhanced compared to GHK, although bioactivities were not significantly improved [16].

Previous studies comparing the penetrating potential of lysine and arginine have shown that arginine is considerably more effective than lysine [17, 18]. However, the optimal amount of arginine for efficient translocation varies depending on the drugs. In this paper, to obtain the desired efficacy of GHK as a cosmeceutical peptide, we aimed to establish the optimal amount of arginine for the efficient skin penetration of GHK and Pal-GHK. Based on the *in vitro* skin permeability results, the optimal peptide was synthesized, after which its *in vitro* biological activities were verified.

2. Materials and Methods

2.1. Franz Diffusion Cell Assay

The skin permeability measurements were undertaken using a Franz diffusion cell system (PermeGear, USA) with a diffusion area of 1.33 cm^2 and a receptor compartment capacity of 8.5 mL. The receptor compartment was filled with phosphate-buffered saline (PBS; pH 7.4) and maintained a temperature of 37.0 ± 0.5°C and a stirring speed of 500 rpm. 3D human reconstructed skin (Neoderm®-E, Tegoscience, Korea) was placed between the donor and receptor compartments, followed by 30 min of stabilization, and then donor samples were added to the skin. After 4, 8, 12, 16, 18, and 24 h, 0.5 mL of the receptor samples were collected, and the receptor phase was immediately replenished with the same volume of PBS. The quantification of peptides in receptor samples was carried out using high-performance liquid chromatography (HPLC).

2.2. HPLC Analysis

The quantification of peptides in receptor samples was carried out by an Agilent 1100 series high-performance liquid chromatography (HPLC; Agilent Technologies, USA) using Capcell Pak C18 reverse-phase column (5 mm, 4.6 × 250 mm, Shiseido Co., Ltd., Japan) at a temperature of 30°C. The mobile phases consisted of water containing 0.1% trifluoroacetic acid (TFA) and acetonitrile containing 0.1% TFA under gradient elution conditions at a flow rate of 1 mL/min. 5 µL of samples were injected, and the run time of each sample was 50 min. The detection using a diode array detector (DAD) was carried out at a wavelength of 220 nm.

2.3. Peptide Synthesis

Pal-GHK-R4 was synthesized using standard Fmoc solid-phase peptide synthesis (SPPS) with 2-chlorotrityl chloride (CC) resin (1% divinylbenzene (DVB) cross-linked, 100~200 mesh, 1.41 mM/g, BeadTech, Korea). The procedure consisted of three major steps: (1) synthesis of H-Arg(Pbf)Arg(Pbf)Arg(Pbf)Arg(Pbf)-CTL resin; (2) synthesis of H-GlyHis(Trt)Lys(Boc)-Arg(Pbf)Arg(Pbf)Arg(Pbf)Arg(Pbf)-CTL resin; (3) synthesis of Pal-GlyHisLys-ArgArgArgArg-OH (Pal-GHK-R4). Finally, the crude Pal-GHK-R4 was purified with a preparative HPLC (Prep-LC; 5 cm inner diameter column) and then lyophilized.

2.4. Cell Culture

Human dermal fibroblasts (Hs68) were purchased from the American Type Culture Collection (ATCC; Manassas, Virginia, USA) and cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. The cells were sub-cultured by trypsinization when their density reached optimal confluence and then used for experiments.

2.5. MTT Assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Hs68 cells were seeded with a density of 1×10^5 cells/well in 6-well plates and grown for 24 h, followed by ultraviolet (UVB) irradiation with an intensity of 200 mJ/cm^2 at 312 nm (VL-215-LM, Vilber Lourmat, Eberhardzell, Germany). After UVB irradiation, the cells were treated with serial diluted GHK and Pal-GHK in a medium containing 1% serum for 24 h and then incubated with MTT after suctioning the medium. Finally, the MTT formazan was extracted using dimethyl sulfoxide (DMSO), and its absorbance was measured at 570 nm using a microplate reader (Sunrise, Tecan, Switzerland).

2.6. Gelatin Zymography

MMP-2 secreted into the medium was detected by gelatin zymography. The supernatants were mixed with a non-reducing sample buffer and loaded without heat denaturation. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 8% polyacrylamide gels containing 0.1% gelatin at a voltage of 90 V for 2 h. After electrophoresis, the gels were washed twice using 2.5% Triton X-100 at room temperature for 1 h to remove the SDS. The gels were incubated in development buffer (50 mM Tris-HCl, pH 7.5; 10 mM $CaCl_2$, 150 mM NaCl) at 37°C overnight and stained by Coomassie brilliant blue R-250. After destaining with a methanol/acetic acid/water mixture, clear zones on the blue background were measured.

2.7. Sircol Collagen Assay

Total collagen content in the medium was quantified using the Sircol soluble collagen assay (Biocolor, Belfast, UK). Sircol dye reagent was added to the supernatants and then gently mixed at room temperature for 30 min to allow collagen-dye complex. After centrifugation, ice-cold acid-salt wash reagent was gently layered onto the pellet to remove unbound dye, followed by adding alkali reagent to dissolving collagen-bound dye, and the absorbance at 570 nm was measured by a microplate reader. The levels of collagen were calculated based on a standard calibration curve obtained by bovine type I collagen supplied with the kit.

2.8. Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) (version 23.0, IBM, USA). The significance of the differences between groups was evaluated using an independent-sample T-test. The values of $p < .05$ were considered statistically significant.

3. Results and Discussion

3.1. In Vitro Skin Permeability of GHK Depending on Arginine Oligomer Mixture and Conjugation

The optimal number of arginine for the skin penetration of GHK was established through Franz diffusion cell assay using the 3D human reconstructed skin after conjugating GHK with R4 (GHK-R4), R6 (GHK-R6), and R8 (GHK-R8). Values were calculated as the cumulative permeated amount for each hour in comparison with the donor amount of peptide. Figure 1A shows that the permeability of GHK (1.08% at 4 h and 2.53% at 24 h) was very low, while the permeability was increased by conjugation of arginine oligomer in order of GHK-R4 (5.57% at 4 h and 12.58% at 24 h) > GHK-R6 (3.65% at 4 h and 9.30% at 24 h) > GHK-R8 (3.07% at 4 h and 8.35% at 24 h). In other words, the optimal arginine oligomer for the skin penetration of GHK is R4. As shown in Figure 1B, comparing this result with the permeability of mixing GHK with R4 (GHK + R4) and R6 (GHK + R6), the permeability was in the order of GHK-R8 > GHK + R4 > GHK + R6. Simply put, the arginine oligomer mixture shows lower skin permeability than conjugation, but the same trend is observed: R4 showed higher penetrating potential than R6.

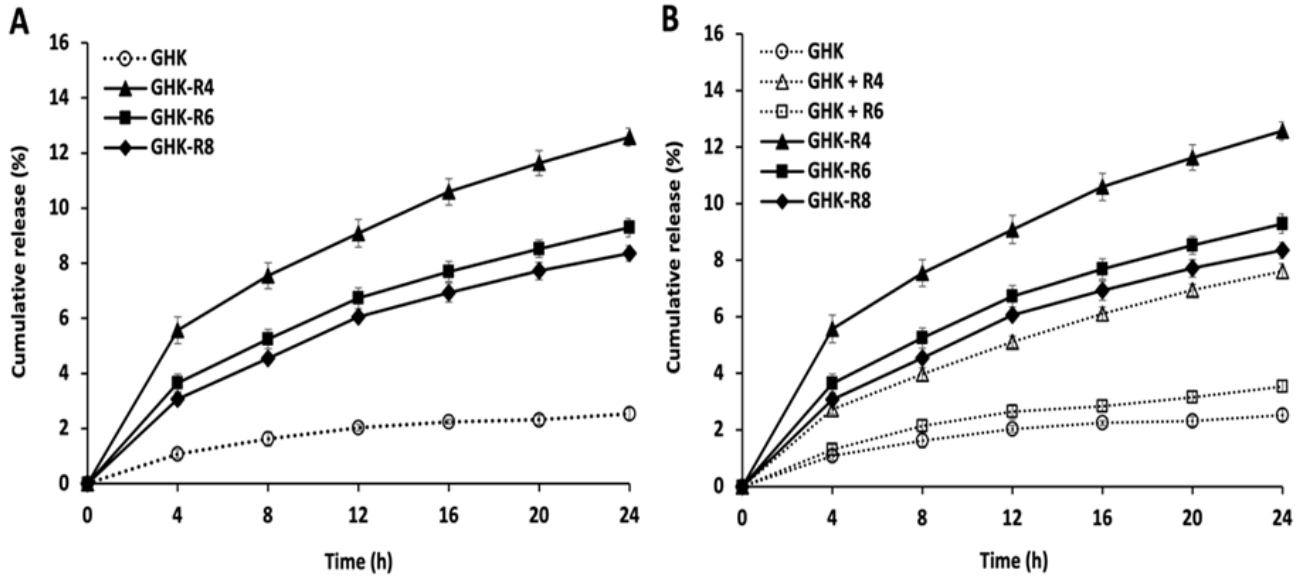

Figure 1.
In vitro skin permeability of GHK depending on arginine oligomer mixture (GHK + R4 and GHK + R6) and conjugation (GHK-R4, GHK-R6, and GHK-R8). (A) Skin permeability of GHK and arginine oligomer conjugation. (B) Skin permeability of GHK and arginine oligomer mixture compared with conjugation. Skin permeability was measured through Franz diffusion cell assay as described in Materials and Methods. Values are expressed as cumulative release compared with donor amount and presented as mean ± standard deviation (n = 3).

3.2. In Vitro Skin Permeability of Pal-GHK Depending on Arginine Oligomer Mixture and Conjugation

Palmitoyl attached GHK is a modification that enhances permeability. To further increase the permeability of GHK, we aimed to establish the optimal number of arginine for the skin penetration of Pal-GHK after conjugating Pal-GHK with R4 (Pal-GHK-R4), R6 (Pal-GHK-R6), and R8 (Pal-GHK-R8). Figure 2A illustrates that the permeability of Pal-GHK (2.48% at

4 h and 4.61% at 24 h) was slightly increased compared to that of GHK (1.08% at 4 h and 2.53% at 24 h). However, the permeability was significantly increased by conjugation of arginine oligomer in order of Pal-GHK-R4 (7.95% at 4 h and 17.75% at 24 h) > Pal-GHK-R6 (5.65% at 4 h and 12.01% at 24 h) > Pal-GHK-R8 (4.96% at 4 h and 10.62% at 24 h). In a similar trend to the case of GHK described above, the optimal arginine oligomer for the skin penetration of Pal-GHK is R4. As shown in Figure 2B, when comparing the permeability of mixing Pal-GHK with R4 (Pal-GHK + R4) and R6 (Pal-GHK + R6), the permeability was in the order of Pal-GHK-R8 > Pal-GHK + R4 > Pal-GHK + R6. Briefly, the arginine oligomer mixture shows lower skin permeability than conjugation but the same trend: R4 had higher penetrating potential than R6.

Taken together, the skin permeability of Pal-GHK-R4 was about 7-fold higher than that of GHK. However, in previous studies, the enhancement of skin permeability did not necessarily lead to a decrease in the active concentration, that is, an increase in bioactivity. So, we aimed to attach palmitoyl to the N-terminus and conjugated R4 to the C-terminus (Pal-GHK-R4) and then verify the biological activities of Pal-GHK-R4 as compared with GHK.

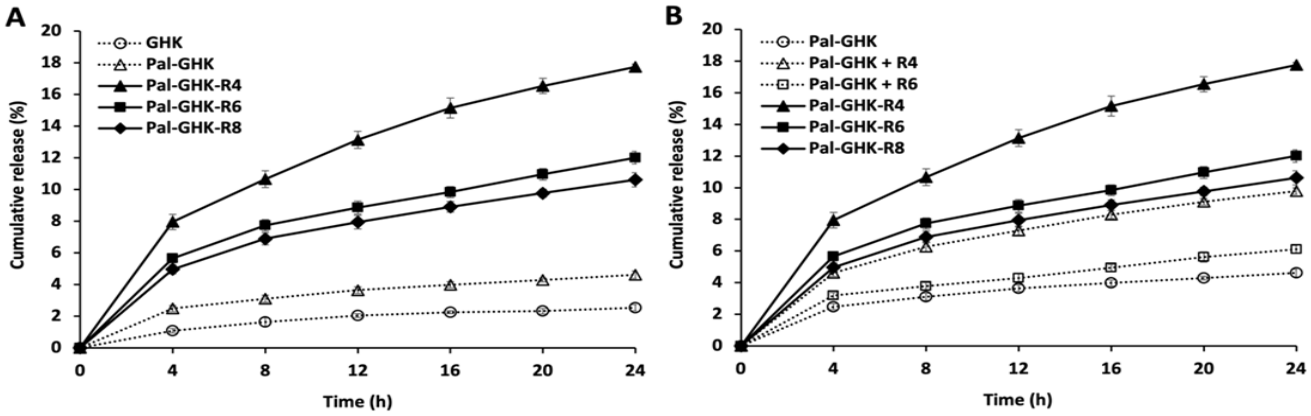

Figure 2.
In vitro skin permeability of Pal-GHK depending on arginine oligomer mixture (Pal-GHK + R4 and Pal-GHK + R6) and conjugation (Pal-GHK-R4, Pal-GHK-R6, and Pal-GHK-R8). (A) Skin permeability of Pal-GHK and arginine oligomer conjugation. (B) Skin permeability of Pal-GHK and arginine oligomer mixture compared with conjugation. Skin permeability was measured through Franz diffusion cell assay as described in Materials and Methods. Values are expressed as cumulative release compared with donor amount and presented as mean ± standard deviation (n = 3).

3.3. Synthesis of Pal-GHK-R4

Using standard Fmoc SPPS, Pal-GHK-R4 in the form of white powder was obtained with a purity of 99.5% by an HPLC and a yield of 28.2%, and the molecular weight was measured as 1203.80 (theoretical molecular weight: 1202.82) using HPLC-mass spectrometry (HPLC-MS).

3.4. Effect of Pal-GHK-R4 on Cell Viability of UVB Irradiated Hs68 Cells Compared with GHK

To evaluate the cytotoxicity of the novel peptide Pal-GHK-R4 compared with GHK, the UVB-irradiated Hs68 cells were treated with various concentrations of Pal-GHK-R4 (5.0, 6.5, and 8.5 μM) and GHK (29.7, 45.1, and 59.5 mM) for 24 h. The results of the MTT assay revealed that the IC30 value of Pal-GHK-R4 was about 8.8 μM (see Figure 3B) and that of GHK was about 45.3 mM (see Figure 3A), and the cell viabilities were in a concentration-dependent manner.

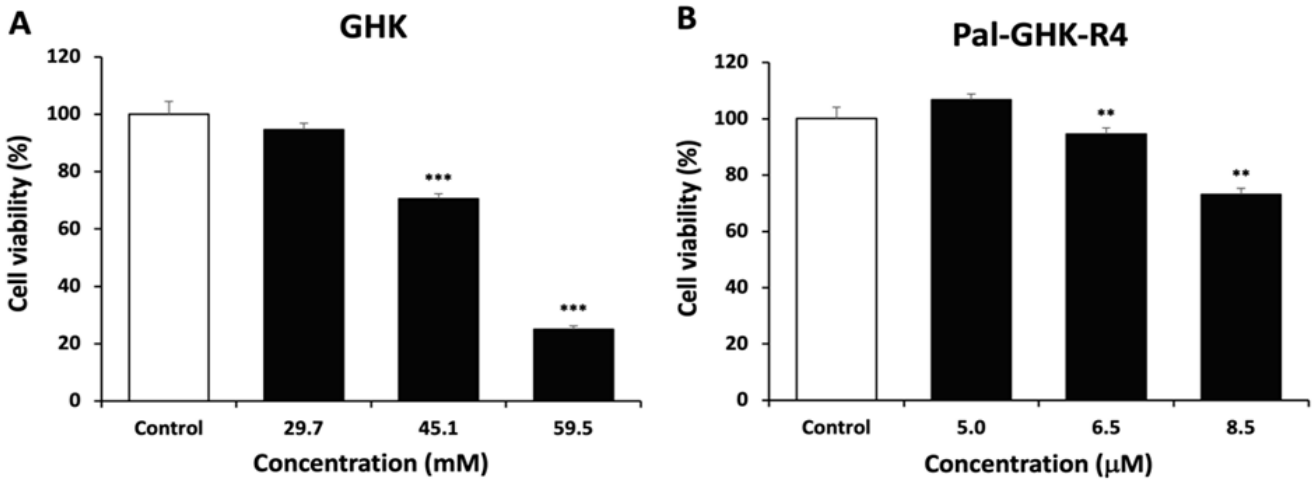

Figure 3.
Effect of GHK and Pal-GHK-R4 treatment on cell viability of Hs68 cells under UVB irradiation. (A) Effect of GHK on cell viability of UVB irradiated Hs68 cells. $^{***}p < .001$ versus control. (B) Effect of Pal-GHK-R4 on cell viability of UVB irradiated Hs68 cells. $^{**}p < .01$ versus control. Cell viability was measured by MTT assay as described in Materials and Methods. Values are expressed as a percentage of control and presented as mean ± standard deviation (n = 3).

3.5. Effect of Pal-GHK-R4 on MMP-2 Activity of UVB Irradiated Hs68 Cells Compared with GHK

MMP-2 is a member of the MMP family, which is involved in the degradation of the ECM and can be induced by UVB. Figure 4 shows that UVB irradiation induced the activation of MMP-2 in Hs68 cells. The UVB-irradiated Hs68 cells were treated with Pal-GHK-R4 (2, 4, and 6 μM) and GHK (200, 300, and 400 μM), and the cell culture medium was collected to evaluate the MMP-2 inhibitory effect of Pal-GHK-R4 compared with GHK. The results of gelatin zymography revealed that the UVB-induced MMP-2 expression was significantly reduced by approximately 31.30% with the treatment of 2 μM Pal-GHK-R4 (see Figure 4B) and significantly reduced by 55.26% with the treatment of 200 μM GHK (see Figure 4A). In other words, the results suggest that approximately 100-fold lower concentrations of Pal-GHK-R4 are needed to inhibit MMP-2 activity compared to GHK.

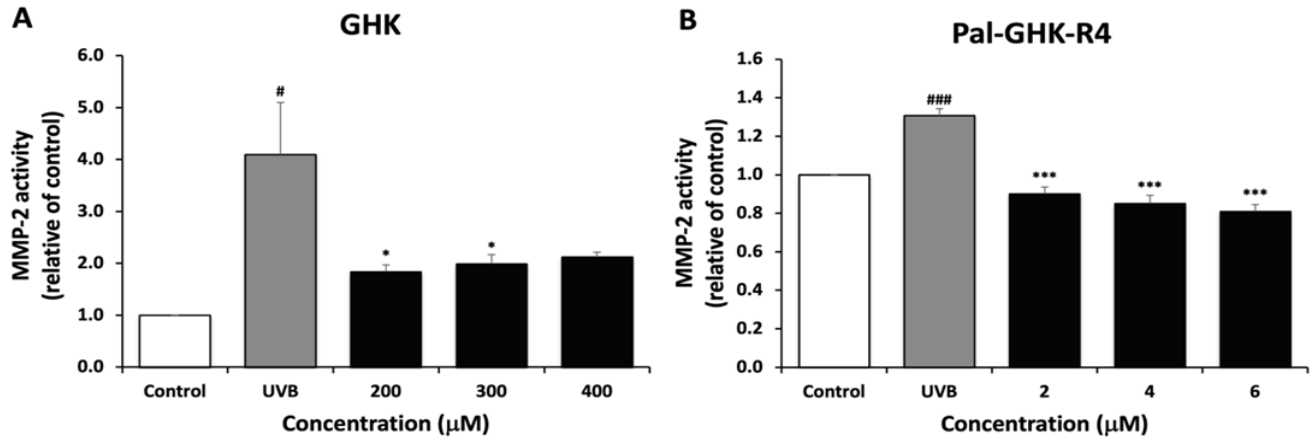

Figure 4.
Effect of GHK and Pal-GHK-R4 treatment on MMP-2 activity of Hs68 cells under UVB irradiation. (A) Effect of GHK on MMP-2 activity of UVB irradiated Hs68 cells. $^{\#}p < .05$ versus non-irradiation control. $^{*}p < .05$ versus UVB irradiation control. (B) Effect of Pal-GHK-R4 on MMP-2 activity of UVB irradiated Hs68 cells. $^{\#\#\#}p < .001$ versus non-irradiation control. $^{***}p < .001$ versus UVB irradiation control. MMP-2 activity was measured using gelatin zymography as described in Materials and Methods. Values are expressed as relative to control and presented as mean ± standard deviation (n = 3).

3.6. Effect of Pal-GHK-R4 on Collagen Production of UVB Irradiated Hs68 Cells Compared with GHK

Collagen is the main protein in the ECM and plays an important role in skin structure and health. To evaluate the effect of Pal-GHK-R4 on collagen production compared with GHK, the Hs68 cells were treated with Pal-GHK-R4 (2, 4, and 6 μM) and GHK (200, 300, and 400 μM) under UVB irradiation, and then the supernatants were used for this experiment. The results of the Sircol collagen assay show that the production of soluble collagen was significantly increased by approximately 51.35% with the treatment of 4 μM Pal-GHK-R4 (see Figure 5B) and significantly increased by approximately 70.54% with the treatment of 400 μM GHK (see Figure 5A), in comparison with UVB-induced collagen degradation in Hs68 cells. As with the inhibition of Matrix Metallo Proteinases (MMP)-2 activity, approximately 100-fold lower concentrations of Pal-GHK-R4 are needed for the stimulation of collagen production than GHK.

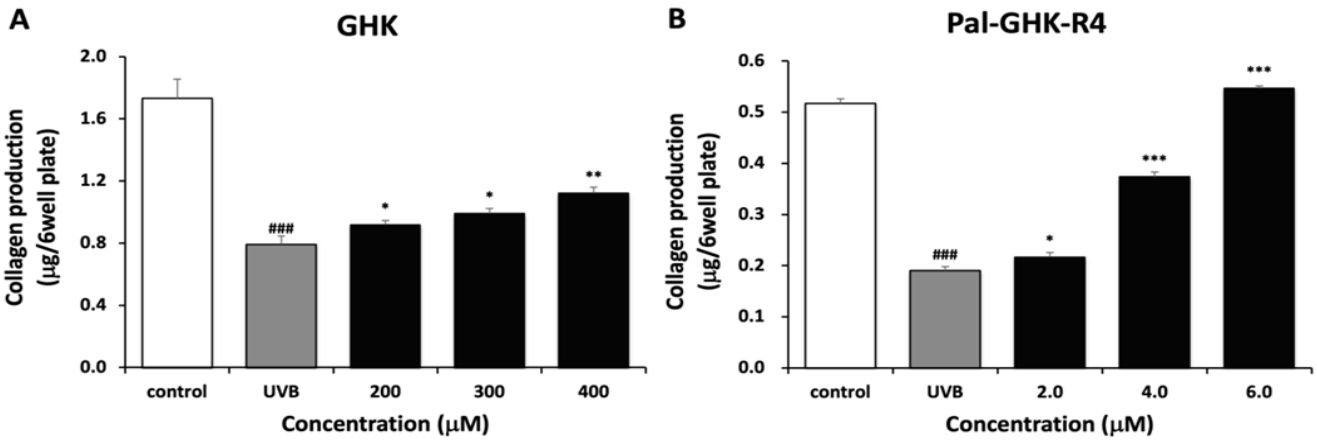

Figure 5.
Effect of GHK and Pal-GHK-R4 treatment on collagen production of Hs68 cells under UVB irradiation. (A) Effect of GHK on collagen production of UVB irradiated Hs68 cells. $^{\#\#\#}p < .001$ versus non-irradiation control. $^{*}p < .05$ and $^{**}p < .01$ versus UVB irradiation control. (B) Effect of Pal-GHK-R4 on collagen production of UVB irradiated Hs68 cells. $^{\#\#\#}p < .001$ versus non-irradiation control. $^{*}p < .05$ and $^{***}p < .001$ versus UVB irradiation control. Collagen production was measured by Sircol collagen assay as described in Materials and Methods. Values are expressed as mg/6well plate and presented as mean ± standard deviation (n = 3).

4. Conclusion

This study aimed to maximize the efficiency of GHK, which is well known as an anti-aging cosmeceutical peptide due to its activities of synthesizing ECM and modulating MMPs. First, we established the optimal amount of arginine for the efficient skin penetration of GHK and Pal-GHK. (1) By attaching palmitoyl to the N-terminus, the permeability (2.48% at 4 h and 4.61% at 24 h) was slightly increased compared to that of GHK (1.08% at 4 h and 2.53% at 24 h), which is approximately a 2-fold increase over GHK. (2) By conjugating R4 to the C-terminus, the permeability was significantly increased compared to that of GHK. (3) In the comparison between R4, R6, and R8, R4 showed the highest penetrating potential (7.95% at 4 h and 17.75% at 24 h), which is approximately a 7-fold increase over the permeability of GHK. (4) In the comparison between the arginine oligomer mixture and conjugation, the trend that R4 showed a higher penetrating potential than R6 remained the same, and conjugation had a permeability about 2 times higher than the mixture. Next, based on the skin permeability results, the optimal peptide, Pal-GHK-R4, was synthesized, and its biological activities were then verified. (1) To inhibit UVB-induced MMP-2 activity, approximately 100-fold lower concentrations of Pal-GHK-R4 are needed than GHK. (2) To stimulate collagen production under UVB irradiation, about 100-fold lower concentrations of Pal-GHK-R4 are needed than GHK. Therefore, this study established that the novel peptide Pal-GHK-R4 significantly increases both the biological activities and skin permeability of GHK.

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