



Oral Administration of Glycine and Leucine Dipeptides Improves Skin Hydration and Elasticity in UVB-Irradiated Hairless Mice

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Abstract

Placenta is a special organ that contains many nutrients such as growth factors, minerals, and bioactive peptides. Dipeptides of glycine and leucine are major components of porcine placenta extracts (PPE) that has been used as an alternative of human placenta extracts. In this study, we investigated whether major peptides of PPE, Glycyl-L-Leucine (Gly-Leu), L-Leucyl-Glycine (Leu-Gly), and L-Leucyl-L-Leucine (Leu-Leu), affect skin hydration and elasticity *in vitro* and *in vivo*. We found that Gly-Leu and Leu-Gly dipeptides induced the expression of transglutaminase 1 in normal human epidermal keratinocytes (NHEKs) whereas Leu-Leu dipeptides did not. Treatment with Gly-Leu or Leu-Gly significantly increased hyaluronan (HA) synthesis in NHEKs and the upregulation of hyaluronan synthase 2 (HAS2) mRNA level was confirmed. In addition, elastase activity was inhibited in NHEKs treated with Gly-Leu or Leu-Gly dipeptides. Oral administration of Gly-Leu or Leu-Gly dipeptides increased skin hydration and elasticity in UVB-irradiated hairless mice. The significant upregulation of HA in UVB-irradiated hairless mice was observed in response to oral administration of Gly-Leu or Leu-Gly. These results suggest that the major dipeptides of porcine placenta, Gly-Leu and Leu-Gly, are potentially active ingredients for skin moisturization formulations.

Key Words: Gly-Leu, Leu-Gly, Hyaluronan, elasticity, Porcine placenta extract

INTRODUCTION

Human skin is the largest organ of the body and extends to approximately 2 m² in area (Tobin, 2006; Nichols and Katiyar, 2010). Skin protects the body against excessive water loss and dangerous external factors including pollutants, UV irradiation, and chemicals (Makrantonaki and Zouboulis, 2007; Bonte, 2011). Accumulated UV exposure leads to skin aging, which causes wrinkle formation, acute erythema, tanning, and loss of hydration and elasticity (Scharffetter-Kochanek *et al.*, 2000; Matsumura and Ananthaswamy, 2004; Kohl *et al.*, 2011).

There are several factors that control skin moisturization and elasticity. First, hyaluronan (HA) regulates moisture, elasticity, and architecture of tissue, repairing tissue, promoting cell motility, and scavenging free radicals (Hsu and Chiang, 2009; Wen *et al.*, 2010). HA, a nonsulfated glycosaminoglycan, is a component of the extracellular matrix (ECM) and is composed of repeating units of D-glucuronic and N-acetyl-D-glucosamine (Kogan *et al.*, 2007). HA is synthesized by three

isoform enzymes, hyaluronan synthase 1 (HAS1), hyaluronan synthase 2 (HAS2), and hyaluronan synthase 3 (HAS3) (Rilla *et al.*, 2013). A previous study has reported that UV irradiation induced loss of HA and down-regulation of HAS enzymes in skin (Dai *et al.*, 2007). Second, differentiation of keratinocytes prevented extensive water loss as well as microbial pathogens and other dangerous factors in the skin (Gschwandtner *et al.*, 2013). Last, concentration and organization of elastic fibers regulate skin tissue elasticity and resilience (Hahn *et al.*, 2006). Elastase leads to the degradation of elastin, inducing wrinkle formation (Suganuma *et al.*, 2010).

Placenta is a specialized organ of pregnancy and is very important for growth and development of the fetus (Gude *et al.*, 2004). Placenta has many components including growth factors, hormones, enzymes, bioactive peptides, vitamins, and minerals (Jash *et al.*, 2011). Placenta extract has been studied in many fields of science due to its many biological functions including anti-aging, suppression of acute liver injury and lipid peroxidation, and induction of mitogenesis and melanogenesis (Togashi *et al.*, 2000; Pal *et al.*, 2002; Wata-

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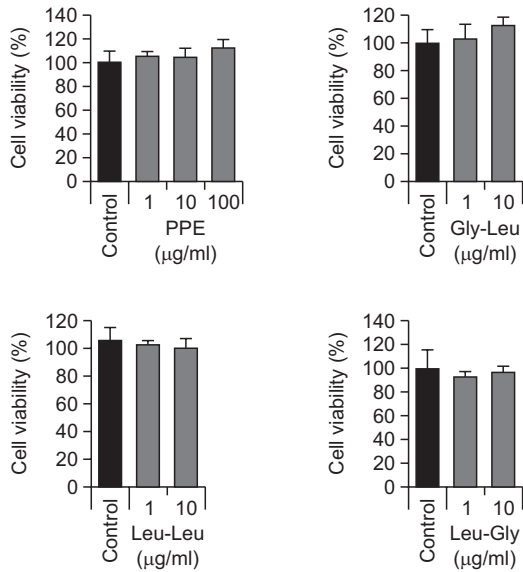


Fig. 1. Effects of PPE, Gly-Leu, Leu-Leu, and Leu-Gly on growth of NHEKs. Cell viability was determined using Ez-Cytox assay and measured at 450 nm. Values are presented as the mean \pm SD of three determinations (n=3).

nabe *et al.*, 2002). Porcine placental extract (PPE) has been recently developed as an oral supplement for use instead of human placenta extract (HPE) (Yoshikawa *et al.*, 2014). Previous studies have shown that PPE decreased shoulder stiffness, knee pain, and postmenopausal climacteric symptoms (Koike *et al.*, 2012, 2013a, 2013b). PPE has been shown to protect skin photoaging through decreased matrix metalloproteinase-2 (MMP-2) and MMP-9 mRNA expression in human skin fibroblasts and *in vivo* (Yoshikawa *et al.*, 2013; Hong *et al.*, 2015). PPE also improved the appearance of fine wrinkles below the eye (Yoshikawa *et al.*, 2014). However, the effects of PPE on skin moisturization and elasticity have not been reported, and the main peptides have not been identified.

In this study, we attempted to elucidate the effects of PPE, Glycyl-L-Leucine (Gly-Leu), L-Leucyl-Glycine (Leu-Gly), and L-Leucine-L-Leucine (Leu-Leu) dipeptides on skin moisturization and elasticity in normal human keratinocytes (NHEKs) and UVB-induced hairless mice.

MATERIAL AND METHODS

Experimental material

PPE was purchased from Biofac A/S (Kastrup, Denmark). Gly-Leu, Leu-Gly, and Leu-Leu were purchased from Bachem AG (Bubendorf, Switzerland).

Cell culture

NHEKs from neonatal origin were purchased from Invitrogen (Carlsbad, CA, USA). NHEKs were cultured in EpiLife® medium (Life Technologies, NY, USA) with 60 μ M CaCl₂, human keratinocyte growth supplement (Invitrogen), and 1% penicillin/streptomycin (Welgene, Gyeongsan, Korea). Cells were maintained at 37°C in a 5% CO₂ incubator.

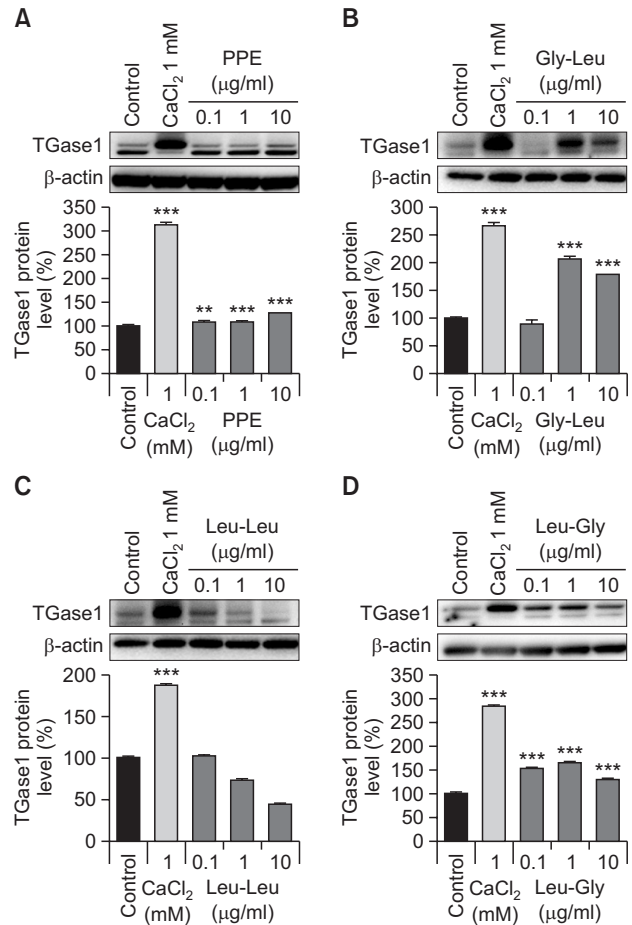


Fig. 2. Effects of PPE, Gly-Leu, Leu-Leu, and Leu-Gly on the protein level of TGase1 in NHEKs. The protein expression of TGase1 was measured in NHEKs treated with (A) PPE for 48 h and (B) Gly-Leu, (C) Leu-Leu, and (D) Leu-Gly for 24 h by western blotting. Treatment of CaCl₂ was used as a positive control. Values are the mean value of expression normalized to that of β -actin from three independent experiments \pm SD. ** p <0.01, *** p <0.001 compared with control.

Cell viability assay

NHEKs were seeded into 96-well culture plates at 1×10^4 cells/well. After 24 h at 37°C, the media was replaced with EpiLife® media containing PPE, Gly-Leu, Leu-Gly, and Leu-Leu diluted to the appropriate concentrations for 24 h. Then cells were washed with DPBS, EZ-Cytox reagents (Daeil Lab Service, Seoul, Korea) were added, and the cells were incubated at 37°C for 1 h. The absorbance was measured using a microplate reader (Tecan, Mannedorf, Switzerland) at a wavelength of 450 nm.

Western blot analysis

Cells were lysed in extraction buffer (0.1 M Tris-HCl, pH 7.2, 1% TritonX-100, 200 mM NaCl, protease inhibitor cocktail) at 4°C for 30 min. The lysates were subjected to centrifugation at 13,000 rpm for 20 min, and the supernatant was obtained. Blots were incubated with antibodies against anti-TGase1 (Santa Cruz Biotechnology, CA, USA) and β -actin (Santa Cruz Biotechnology). After incubation, membranes were rinsed

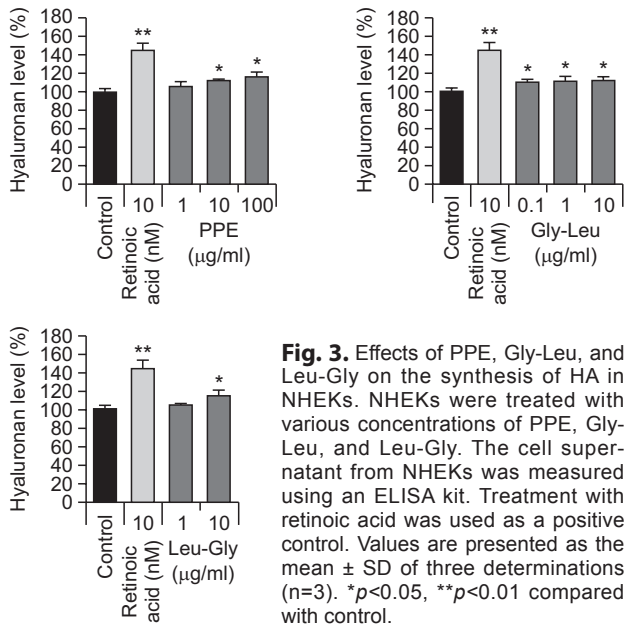


Fig. 3. Effects of PPE, Gly-Leu, and Leu-Gly on the synthesis of HA in NHEKs. NHEKs were treated with various concentrations of PPE, Gly-Leu, and Leu-Gly. The cell supernatant from NHEKs was measured using an ELISA kit. Treatment with retinoic acid was used as a positive control. Values are presented as the mean ± SD of three determinations (n=3). **p*<0.05, ***p*<0.01 compared with control.

three times with TBS-T and were incubated with donkey anti-rabbit IgG antibody (Bethyl Laboratories, Montgomery, TX, USA) and goat anti-mouse IgG antibody (Bio-Rad, CA, USA) for 1 h at room temperature. Binding antibodies were detected using a WEST-ZOL® Plus Western Blot Detection System (INTRON Biotechnology, Sungnam, Korea) and visualized with ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).

HA assay

HA content was measured from culture media of the NHEK cultures with a Hyaluronan DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA). NHEKs were seeded into 96-well culture plates at 1×10⁴ cells/well. After 24 h, the cells were washed with DPBS, and serum-free media was added. After starvation for 24 h, NHEK cells were cultured with various concentrations of PPE, Gly-Leu, and Leu-Gly. After 24 h, the HA concentration in the culture supernatant was measured.

Elastase inhibition assay

The activity of porcine pancreatic elastase (Sigma, St. Louis, MO, USA) was examined using N-succinyl-(L-Ala)₃-p-nitroanilide as the substrate. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 1 U/mL elastase, and 0.5 mg/ml N-succinyl-(L-Ala)₃-p-nitroanilide. The reaction mixture was pre-incubated for 30 min at 25°C before adding the substrate. The release of p-nitroaniline was measured at 410 nm using a 96-well reader. The percent inhibition of elastase was calculated as follows:

$$\text{Inhibition activity (\%)} = [1 - (S - B) / C] \times 100$$

where S is enzyme activity in the presence of porcine pancreatic elastase, B is the activity without elastase, and C is the activity without sample.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from NHEK cells and mouse skin tissue with the Trizol reagent (Takara, Otsu, Japan). The quality

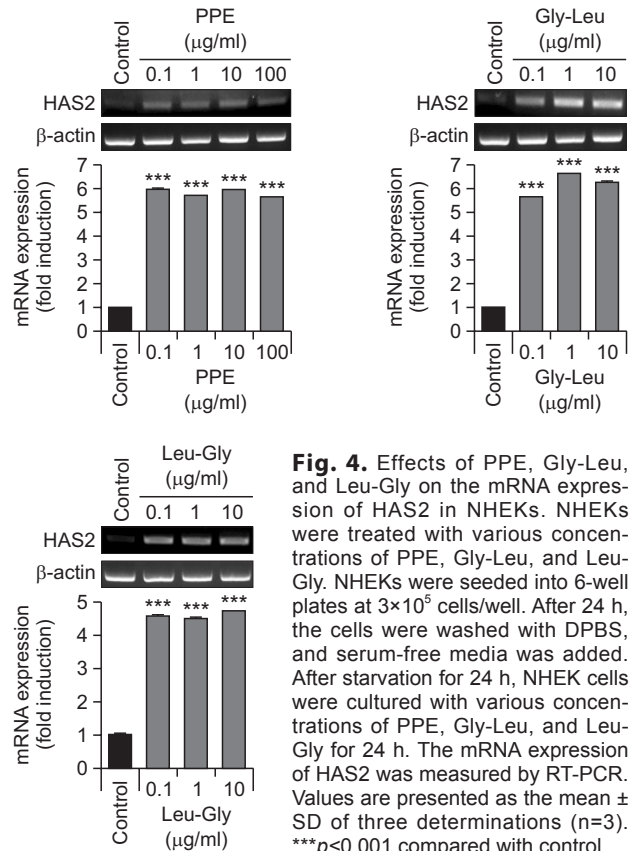


Fig. 4. Effects of PPE, Gly-Leu, and Leu-Gly on the mRNA expression of HAS2 in NHEKs. NHEKs were treated with various concentrations of PPE, Gly-Leu, and Leu-Gly. NHEKs were seeded into 6-well plates at 3×10⁵ cells/well. After 24 h, the cells were washed with DPBS, and serum-free media was added. After starvation for 24 h, NHEK cells were cultured with various concentrations of PPE, Gly-Leu, and Leu-Gly for 24 h. The mRNA expression of HAS2 was measured by RT-PCR. Values are presented as the mean ± SD of three determinations (n=3). ****p*<0.001 compared with control.

and quantity of the RNA were determined by NanoDrop2000 (Thermo Scientific, Waltham, MA, USA). To synthesize cDNA, 1 µg quantities of total RNA were mixed with 100 pmol quantities of oligo (dT) (ELPIS, Daejeon, Korea), followed by denaturation at 65°C for 5 min and chilling on ice for 5 min. The annealed samples were then incubated with reverse transcriptase and 2 mM dNTPs (Fermentas, Waltham, MA, USA) for 1 h at 42°C. Reverse transcription was terminated by heating for 10 min at 70°C. For amplification, the cDNA was mixed with HiPi PCR Mix (ELPIS) and each of the following primer sets: HAS2: Forward: 5'-CAGAATCCAAACAGACAGTTC-3', Reverse: 5'-TAAGGTGTTGTGTGTGACTG-3'; β-actin: Forward: 5'-GTGGGCTGCCCGAGCACCA-3', Reverse: 5'-CTCCTTAATGTCACGCACGATTTC-3'. The resulting PCR products were visualized by electrophoretic separation on 3% agarose gels and staining with RedSafe™ Nucleic Acid Staining Solution (ELPIS). Specific primers for β-actin were added as a control.

Experimental animals

Six-week-old female albino hairless mice (SKH-1) were purchased from Orient Bio (Seongnam, Korea). The hairless mice were acclimated for 1 week before starting the experiments and then divided into 6 groups of 10 mice each. The feeding environment was maintained under controlled temperature (24 ± 2°C) and humidity (55 ± 10%) and automatic lighting (12 h light and dark cycle). Feed was provided (Feed Lab Korea, Guri, Korea) to the hairless mice. Laboratory animal breeding management was based on the "Guide for the Care and Use

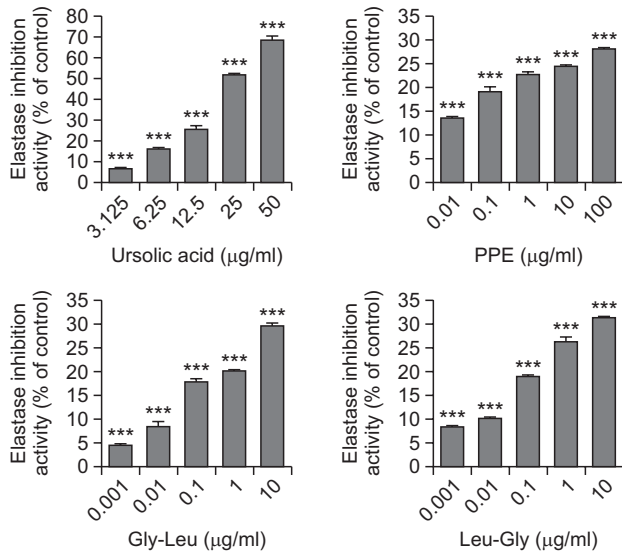


Fig. 5. Effects of PPE, Gly-Leu, and Leu-Gly on elastase inhibition. Treatment with ursolic acid was used as a positive control. Values are presented as the mean \pm SD of three determinations (n=3). *** p <0.001 compared with control.

of Laboratory Animals,” and all experiments were approved by the Institutional Animal Care and Use Committees of Gyeonggi Institute of Science & Technology (Suwon, Korea).

UVB irradiation

The UVB source was six fluorescent lamps (TL 20W/12RS SLV, wave length 290 to 390 nm, peak emission 315 nm; (Philips, Amsterdam, Netherlands), and the UVB irradiation intensity was measured with a UV meter (VARIOCONTROL, Waldmann ver.2.03, Villingen-Schwenningen, Germany). The mice were exposed to UVB irradiation three times per week. The starting dose of UVB irradiation was 75 mJ/cm² during the first week and then increased weekly by 1 minimal erythema dose (MED) until reaching 3.3 MED, which was maintained until 8 weeks.

Skin hydration and elasticity evaluation

Skin hydration content and elasticity were measured on the dorsal skin of the mice using Corneometer (CK Electronics GmbH, Cologne, Germany) and Cutometer (CK Electronics).

Histopathologic analysis

After the end of the experiment, the dorsal skins of all animals were biopsied and placed in 10% formalin. The dorsal skin tissues were stained with haematoxylin and eosin (H&E) and Alcian blue. The stained tissues were photographed using a Nikon ECLIPSE Ti-E inverted fluorescent microscope (Nikon, Tokyo, Japan) and analyzed using NIS-Element BR 3.0 software (Nikon, Tokyo, Japan).

Statistical analysis

All of the data are expressed as mean \pm SD. Statistical significance was determined by independent *t*-test. A value of p <0.05 (*), p <0.01 (**), or p <0.001 (***) was considered statistically significant.

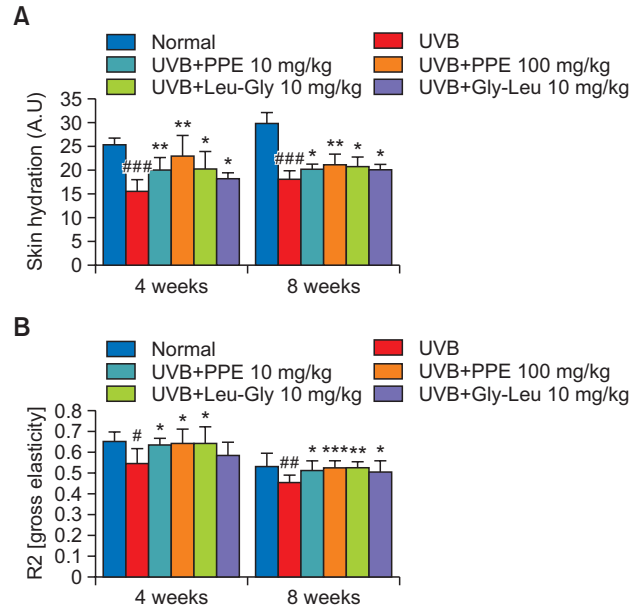


Fig. 6. Effects of PPE, Gly-Leu, and Leu-Gly intake on skin hydration and elasticity in UVB-induced hairless mice. (A) Skin hydration (B) elasticity. * p <0.05, ** p <0.01, *** p <0.001 compared with UVB-induced mice; # p <0.05, ## p <0.01, ### p <0.001 compared with non UVB-induced mice. Values are presented as the mean \pm SD of three determinations (n=10).

RESULTS

Effects of PPE, Gly-Leu, Leu-Leu, and Leu-Gly on NHEK viability

To determine the cytotoxic effects of PPE, Gly-Leu, Leu-Leu, and Leu-Gly, we applied these compounds at different concentrations to NHEKs. Treatment with PPE, Gly-Leu, Leu-Leu, and Leu-Gly showed no cytotoxicity at concentrations up to 100 μ g/ml and 10 μ g/ml (Fig. 1).

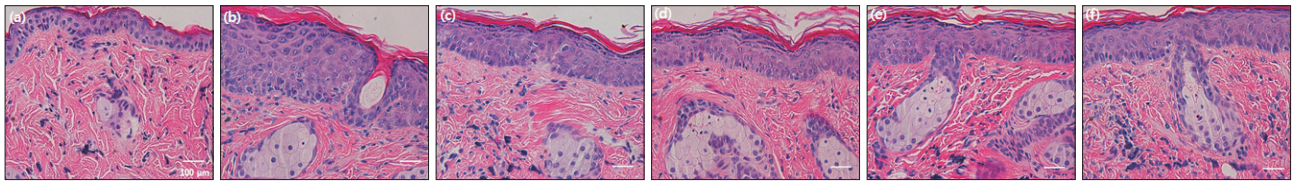
PPE, Gly-Leu, and Leu-Gly increased the expression of TGase 1 in NHEKs

To identify the effects of PPE, Gly-Leu, Leu-Leu, and Leu-Gly on keratinocyte differentiation, we measured TGase 1 protein expression level in NHEKs treated with PPE, Gly-Leu, Leu-Leu, and Leu-Gly. TGase 1 protein level was significantly increased with PPE in a dose-dependent manner (Fig. 2A). Gly-Leu and Leu-Gly treatment also increased TGase 1 protein level (Fig. 2B, 2D), but Leu-Leu treatment did not change TGase 1 protein level (Fig. 2C).

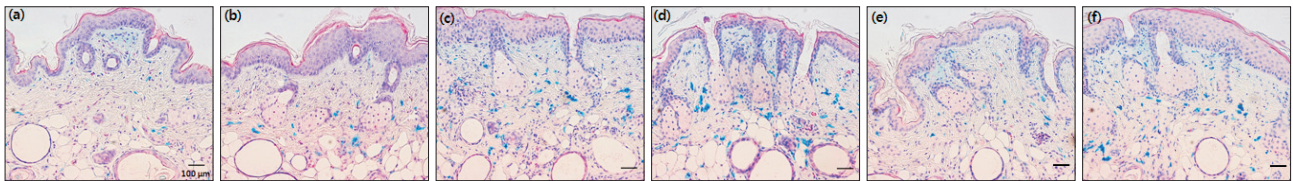
PPE, Gly-Leu, and Leu-Gly increased synthesis of HA and HAS2 mRNA levels in NHEKs

To investigate the effects of PPE, Gly-Leu, and Leu-Gly on synthesis of HA, HA was measured in NHEKs. Treatment of PPE increased HA level in a dose-dependent manner compared to the control (Fig. 3). Treatment with Gly-Leu and Leu-Gly also increased HA level at 10 μ g/ml concentration. It was reported that increased mRNA level of HAS2 induced HA production in human keratinocytes (Kim *et al.*, 2004). Since treatment with PPE, Gly-Leu, and Leu-Gly increased HA level, we examined the HAS2 mRNA expression level in NHEKs.

A



B



C

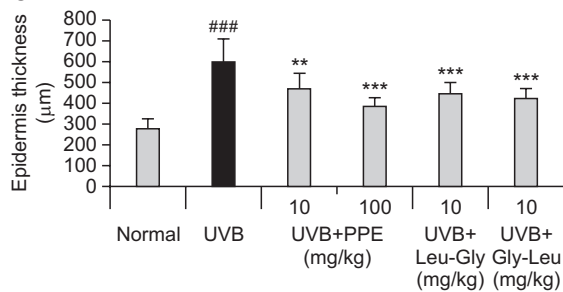


Fig. 7. Effect of PPE, Gly-Leu, and Leu-Gly intake on skin histopathological change and HA level in UVB-induced hairless mice. The dorsal skin tissues were stained with (A) H&E and (B) Alcian blue. (a) Normal, (b) UVB, (c) UVB+PPE 10 mg/kg, (d) UVB+PPE 100 mg/kg, (e) UVB+Leu-Gly 10 mg/kg, (f) UVB+Gly-Leu 10 mg/kg groups. (C) Epidermis thickness was measured with NIS-Elements BR 3.0 Software (Nikon). Values are presented as the mean \pm SD of three determinations (n=10). ** p <0.01, *** p <0.001 compared with UVB-induced mice; ### p <0.001 compared with non UVB-induced mice.

Treatment with PPE, Gly-Leu, and Leu-Gly significantly increased HAS2 mRNA expression level at all tested concentrations compared to the control (Fig. 4). These results suggest that treatment with PPE, Gly-Leu, and Leu-Gly increases HA synthesis through increased mRNA level of HAS2.

PPE, Gly-Leu, and Leu-Gly inhibited elastase activity in NHEKs

To determine the effects of PPE, Gly-Leu, and Leu-Gly on elastase activity, we measured elastase activity as described in the Methods section. PPE, Gly-Leu, and Leu-Gly treatment significantly reduced elastase activity in a dose-dependent manner (Fig. 5).

Oral administration of PPE, Gly-Leu, and Leu-Gly increased skin hydration and elasticity in hairless mice

To analyze the effects of PPE, Gly-Leu, and Leu-Gly on skin hydration and elasticity *in vivo*, we orally administered PPE, Gly-Leu, and Leu-Gly to UVB-exposed hairless mice for 8 weeks. Skin hydration and elasticity were significantly increased in the PPE intake group in a dose-dependent manner and also increased in the Gly-Leu and Leu-Gly 10 mg/kg intake groups compared with UVB-induced group (Fig. 6).

Oral administration of PPE, Gly-Leu, and Leu-Gly increased HA level and HAS2 mRNA level in hairless mice skin

Epidermal thickness was increased and HA was decreased in UVB-induced hairless mouse skin. Oral supplement with PPE, Gly-Leu, and Leu-Gly significantly decreased epidermal thickness and increased the level of HA compared to those in the UVB-induced group (Fig. 7). We examined HAS2 mRNA level in dorsal skin using RT-PCR. HAS2 mRNA level was increased in the PPE intake group in a dose-dependent man-

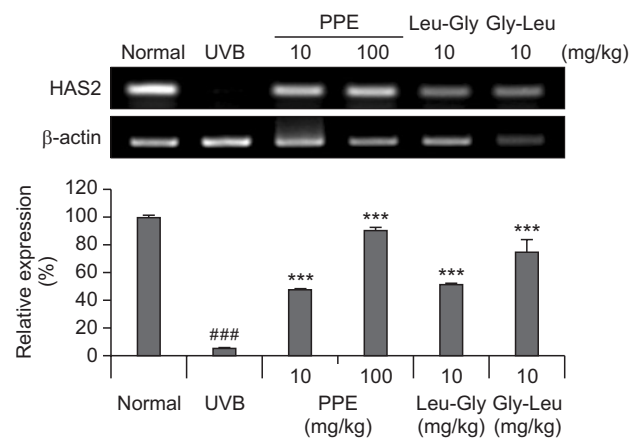


Fig. 8. Effects of PPE, Gly-Leu, and Leu-Gly intake on HAS2 mRNA expression level in UVB-induced hairless mice. Values are presented as the mean \pm SD of three determinations (n=6). *** p <0.001 compared with UVB-induced mice; ### p <0.001 compared with non UVB-induced mice.

ner compared to the UVB-induced group. In addition, HAS2 mRNA level was increased in the Gly-Leu and Leu-Gly 10 mg/kg intake groups (Fig. 8).

DISCUSSION

Gly-Leu, Leu-Gly, and Leu-Leu dipeptides were included in PPE. When we analyzed PPE, large quantities of Gly-Leu, Leu-Gly, and Leu-Leu were contained, in amounts of

1,200~2,400 mg/kg, 140~600 mg/kg, and 250~450 mg/kg, respectively. Glycine has been reported to increase collagen synthesis in rats (Chyun and Griminger, 1984). Based on this information, we tested whether PPE and glycine-containing peptides in PPE were effective for skin moisturization and elasticity.

We showed that treatment with PPE, Gly-Leu, and Leu-Gly increased keratinocyte differentiation (Fig. 2). In addition, synthesis of HA was increased by treatment with PPE, Gly-Leu, and Leu-Gly (Fig. 3). It was previously reported that synthesis of HA was inhibited by down-regulation of HAS2 expression (Rock *et al.*, 2011). We found that treatment with PPE, Gly-Leu, and Leu-Gly increased HAS2 mRNA expression in NHEKs, which resulted in HA synthesis (Fig. 4). We also found that PPE, Gly-Leu, and Leu-Gly decreased elastase activity (Fig. 5). Therefore, Gly-Leu and Leu-Gly could be functional peptides in PPE. Conversely, Leu-Leu showed no activity (Fig. 2C).

It has been reported that inhibition of HA synthesis decreased skin hydration and viscoelasticity by down-regulation of HAS2 in hairless mice (Rock *et al.*, 2015). We investigated the effects of PPE, Gly-Leu, and Leu-Gly in hairless mice *in vivo*. We found that oral administration of PPE, Gly-Leu, and Leu-Gly dipeptides increased skin hydration and elasticity in UVB-induced mice (Fig. 6). Oral administration of PPE, Gly-Leu, and Leu-Gly dipeptides also increased HA synthesis (Fig. 7) and HAS2 mRNA expression (Fig. 8).

Gly-Leu and Leu-Gly dipeptides of fermented porcine placenta extract have been reported to have a reducing effect on fatigue (Nam *et al.*, 2016). However, no studies have analyzed the effects of dipeptides on skin. In this study, we report the effects of PPE and its major peptides, Gly-Leu and Leu-Gly, on skin moisturization and elasticity for the first time.

In summary, oral supplementation with PPE, Gly-Leu, and Leu-Gly could protect skin from UV-damage by restoring the synthesis of HA and reducing the inhibition of elastase. Also, Gly-Leu and Leu-gly peptides were shown to be functional ingredients of PPE. Therefore, we suggest that PPE and its major peptides, Gly-Leu and Leu-Gly, could be potential candidate materials for skin moisturization and elasticity as dietary supplements.

ACKNOWLEDGMENTS

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