

## Evaluation of the *in vivo* skin moisturizing effects and underlying mechanisms of pomegranate concentrate solution and dried pomegranate concentrate powder

Su-Jin Kang<sup>1,2\*</sup>, Beom-Rak Choi<sup>3\*</sup>, Seung-Hee Kim<sup>3</sup>, Hae-Yeon Yi<sup>3</sup>, Hye-Rim Park<sup>3</sup>, Mi-Sun Sung<sup>3</sup>,  
Chang-Hyun Song<sup>1,4</sup>, Il-Je Cho<sup>1</sup>, Young-Joon Lee<sup>1,2\*\*</sup>, Sae-Kwang Ku<sup>1,4\*\*</sup>

<sup>1</sup>The Medical Research Center for Globalization of Herbal Medicine, Daegu Haany University, Gyeongsan

<sup>2</sup>Department of Preventive Medicine, College of Korean Medicine, Daegu Haany University, Gyeongsan

<sup>3</sup>Research Institute, Health-Love Co., Ltd., Anyang

<sup>4</sup>Department of Histology and Anatomy, College of Korean Medicine, Daegu Haany University, Gyeongsan

**Objectives:** The aim of this study was to confirm the dose-dependent skin moisturizing effects of dried pomegranate concentrate powder (PCP) and pomegranate concentrate solution (PCS) in ICR mice.

**Materials and methods:** To observe the *in vivo* skin moisturizing effects and possible underlying mechanisms of PCP and PCS, oral PCP (100, 200, and 400 mg/kg) and PCS (1, 2, and 4 mL/kg) were administered to normal ICR mice. Changes in body weight, skin water content, and skin type I collagen and hyaluronan contents were measured. Additionally, the mRNA expression levels of hyaluronan synthase (Has) 1, 2, and 3, and collagen type I alpha (COL1A) 1 and 2 were determined in the dorsal skin of mice by real-time reverse transcription polymerase chain reaction (RT-PCR).

**Results:** Significant and dose-dependent increases in dorsal skin water content and type I collagen and hyaluronan contents were seen in PCP and PCS-treated mice. Moreover, the mRNA levels of Has 1, 2, and 3, involved in hyaluronan synthesis, and of COL1A1 and COL1A2, involved in collagen synthesis, were significantly and dose-dependently upregulated in PCS- and PCP-treated mice.

**Conclusions:** In this study, PCP and PCS led to favorable skin moisturizing effects as indicated by increased skin water content and the upregulation of hyaluronan and collagen synthesis enzymes in mice treated with PCS (4 mL/kg) and PCP (200 mg/kg).

**Key Words** : Pomegranate concentrate solution, dried pomegranate concentrate powder, skin moisturizing effects, mouse, collagen, hyaluronan

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\* These authors contributed equally to this work.

• Correspondence to : Sae Kwang Ku

Department of Histology and Anatomy, College of Korean Medicine, Daegu Haany University  
1 Haanydaero, Gyeongsan, Gyeongsangbuk-Do, 38610, Republic of Korea  
Tel : +82-53-819-1549, Fax : +82-53-819-1576, E-mail : gucci200@hanmail.net

• Correspondence to : Young Joon Lee

Department of Preventive medicine, College of Korean Medicine, Daegu Haany University  
1 Haanydaero, Gyeongsan, Gyeongsangbuk-Do, 38610, Republic of Korea  
Tel : +82-53-819-1296, Fax : +82-53-819-1576, E-mail : gksxntk@dhu.ac.kr

## Introduction

Skin aging results in clinical signs including irregular dryness, dark or light pigmentation, sallowness, deep furrows or severe atrophy, dehydrogenation, telangiectasia, premalignant lesions, laxity, and a leathery appearance<sup>1</sup>. Various complex cellular and extracellular components are involved in the skin-aging phenomenon; several studies highlight the involvement of reactive oxygen species (ROS)<sup>2</sup>, the degradation of the extracellular matrix (ECM)<sup>3</sup> and melanin pigmentation<sup>4</sup>, and a decrease in moisture of the skin keratin layers<sup>5,6</sup>. Functional anti-aging skin protective agents have focused on antioxidant, anti-wrinkle, whitening, and skin moisturizing effects as their underlying mechanisms of action. Various antioxidants that reduce ROS and scavenge free radicals, such as ascorbic acid,  $\alpha$ -tocopherol, carotenoid, flavonoid, butylated hydroxy toluene, butylated hydroxy anisole, and propylene glycol, have been used as ingredients in anti-aging protective agents<sup>7-9</sup>. The antioxidant efficacy of such agents has been tested via free radical scavenging or effects on the activity of antioxidant enzymes that contribute to enzymatic defense mechanisms, including superoxide dismutase (SOD)<sup>10</sup>. Their anti-wrinkle and skin regeneration effects have been assessed via the activities of various dermal enzymes involved in the formation of skin wrinkles and the degradation of the ECM, including hyaluronidase, elastase, collagenase, and matrix metalloproteinase (MMP-1)<sup>3,11</sup>. Moreover, the whitening effects of such agents have been measured using skin melanin content as well as tyrosinase activity, an enzyme involved in melanin formation<sup>4,9,12,13</sup>, while their skin moisturizing effects have been detected by measuring skin water content in animal and human pilot studies<sup>9,14,15</sup>.

Pomegranate is a rich source of crude fiber, pectin, sugars, and several tannins<sup>16</sup>. In addition, pomegranate seed oil and juice contain flavonoid and

anthocyanidin species, which exert an antioxidant activity that is three-fold more potent than that of red wine or green tea extract<sup>17,18</sup>. Furthermore, the chemopreventive and adjuvant therapeutic applications of pomegranate to human breast cancer have been recently<sup>19</sup>. Owing to these significant biological activities, the popularity of pomegranate juice has risen worldwide. We have previously reported the favorable *in vitro* and *in vivo* skin protective effects of a pomegranate concentrate solution (PCS), including its possible skin moisturizing effects *in vitro*<sup>20,21</sup>. However, detailed studies investigating the underlying mechanisms of action and demonstrating the skin-moisturizing effects of PCS and pomegranate concentrate powder (PCP) *in vivo* are warranted.

The objective of this study was to confirm the dose-dependent skin moisturizing effects of PCP and PCS in normal ICR mice, as well as to determine their possible underlying mechanisms of action, focusing on hyaluronan and type I collagen synthesis. To do so, changes in body weight, and skin water, type I collagen, and hyaluronan contents, as well as mRNA expression of enzymes involved in hyaluronan synthesis, including hyaluronan synthase (Has) 1, 2, and 3, and collagen synthesis, including collagen type I alpha (COL1A) 1 and 2, were measured in the dorsal skin of mice.

## Materials and Methods

### 1. Animals

Healthy 6-week old male SPF/VAF outbred CrJOri:CD1 (ICR) mice (OrientBio, Seungnam, Korea) were used after acclimatization for 7 days. Five mice were allocated per polycarbonate cage in a temperature (20-25°C) and humidity (45-55%) controlled room with a 12 hour light-dark cycle. Mice had *ad libitum* access to chow (Samyang, Seoul, Korea) and water. All laboratory animals were treated according to the national regulations of the

usage and welfare of laboratory animals, and approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) prior to animal experiment.

## 2. Treatments

PCS (ASYA Meyve Suyu ve Gıda San A.S., Ankara, Turkey), a deep red-colored viscous solution, contains 2.31 mg/g of the active ingredient ellagic acid and is composed of 58.86% carbohydrate, 1.21% protein, 0.49% fat, 27.97% water, 1.47% ash, and 28.03 mg/100 g sodium. PCP (ASYA Meyve Suyu ve Gıda San A.S., Ankara, Turkey), a pink-colored powder, contains 1.15 mg/g of the active ingredient ellagic acid. Both PCS and PCP were prepared and supplied by Health Love Ltd. (Anyang, Korea). A PCP dosage of 200 mg/kg was selected based on previous clinical dosages used in humans (mouse dosage ~12-fold the human dosage;  $(1,000 \text{ mg}/60 \text{ kg}) \times 12 = 200 \text{ mg}/\text{kg}$ ), and dosages of 400 and 100 mg/kg were selected as the highest and lowest dosages, respectively, using a ratio of 2. Similarly, a PCS dosage of 2 mL/kg was selected based on previous clinical dosages used in humans (mouse dosage ~12-fold the human dosage;  $(10 \text{ mL}/60 \text{ kg}) \times 12 = 2 \text{ mL}/\text{kg}$ ), and dosages of 4 and 1 mL/kg were selected as the highest and lowest dosages, respectively, using a ratio of 2. PCP was dissolved in distilled water to obtain concentrations of 40, 20, and 10 mg/mL, and a volume of 10 mL/kg of body weight was orally administered, resulting in administration of 400, 200, and 100 mg/kg of body weight, respectively. PCS was diluted in distilled water in 2:3, 1:4, and 1:9 ratios (v/v) and a volume of 10 mL/kg was orally administered, resulting in administration of 4, 2, and 1 mg/kg of body weight, respectively. The different dosages of PCP and PCS were administered in six different groups of mice once per day for 56 days, and equal volumes of vehicle (distilled water) were orally

administered in a separate group of intact control mice.

## 3. Body weight

Changes in body weight were measured once a week, from one day prior to the beginning of treatment to sacrifice, including the second and the last (56<sup>th</sup>) treatment day, using an automatic electronic balance (Precisa Instrument, Dietikon, Switzerland). Animals were overnight fasted prior to the first treatment day and termination to reduce differences in feeding. Body weight gain during the 8-week treatment period was calculated as follows: body weight gain (g) = (body weight at sacrifice) – (body weight on the first treatment day).

## 4. Skin water content

Twenty-four hours following the final treatment, 6 mm-diameter dorsal skin samples were collected from all mice. Skin water content (%) was measured using an automated moisture analyzer balance (Ohaus, Pine Brook, NJ, USA) as described in our previous report<sup>9</sup>.

## 5. Collagen type I content in skin tissue

Dorsal skin type I collagen content was measured according to a previously established method<sup>10</sup>. Briefly, dorsal skin tissue samples collected 24 h following the final treatment administration were homogenized and prepared using a bead beater (GeneResearch Biotechnology Corp., Taichung, Taiwan), an ultrasonic cell disruptor (Madell Technology Corp., Ontario, CA, USA) and radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louise, MO, USA). The supernatant was separated by centrifugation at 15,000 rpm at 4 °C, and the amount of pro-collagen in each sample was measured using a pro-collagen type I C peptide assay kit (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. Absorbance was measured at 450 nm

using a microplate reader (Tecan, Männedorf, Switzerland).

## 6. Hyaluronan content in skin tissue

Hyaluronan content of the prepared supernatant from dorsal skin tissue homogenate samples was measured according to a previously established method<sup>22,23</sup>. Briefly, dorsal skin samples were defatted using acetone, dried, boiled in 50 mM Tris/HCl (pH 7.8) buffer for 20 min, and subjected to proteolytic digestion with 1% *w/v* actinase E (Sigma-Aldrich, St. Louise, MO, USA) for a week at 40 °C. Trichloroacetic acid (Sigma-Aldrich, St. Louise, MO, USA) was added to the samples at a final concentration of 10% *w/v* for deproteinization before centrifugation at 3,000 rpm at 4 °C for 20 min. The resulting supernatant was neutralized with 10 N NaOH. Hyaluronan levels were measured using the OnE hyaluronic acid enzyme-linked immunosorbent assay (ELISA) kit (Biotech Trading Partners, Encinitas, CA, USA) according to the manufacturer's instructions. Properly diluted samples and hyaluronan reference solutions were incubated in hyaluronic acid binding protein (HABP)-coated microwells. After washing to remove unbound molecules, HABP conjugated with horseradish peroxidase was added to

the microwells to form complexes with hyaluronan. The samples were subsequently washed and tetramethylbenzidine, a chromogenic compound, and hydrogen peroxide were added, resulting in a colored reaction. Color intensity was measured in optical density units using a spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea) at 450 nm. Hyaluronan levels in the test and control samples were determined against a reference curve prepared from a reagent blank (0 ng/mL) as well as the hyaluronan reference solutions provided in the kit (50, 100, 200, 500, and 800 ng/mL).

## 7. Real-Time RT-PCR

Total RNA in the dorsal skin samples collected 24 h following the final treatment was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to a previously established method<sup>23,24</sup>. RNA concentration and quality were determined using the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). To remove contaminating DNA, samples were treated with recombinant DNase I (Ambion, Austin, TX, USA). RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

**Table 1.** Oligonucleotides for *Realtime* RT-PCR Used in This Study

Target	5' - 3'	Sequence	NCBI accession No. (Molecular weights)
Has 1	Sense	GCATGGGCTATGCTACCAAGTAT	NM_008215 (2107 bp)
	Antisense	AGGAGGGCGTCTCCGAGTA	
Has 2	Sense	GACCCTATGGTTGGAGGTGTG	NM_008216 (4262 bp)
	Antisense	ACGCTGCTGAGGAAGGAGATC	
Has 3	Sense	AGACCGAGCTAGCCTTCCTAGT	NM_008217 (5912 bp)
	Antisense	TAATGGCCAGATACAGCATGAG	
COL1A1	Sense	GCGGTAACGATGGTGCTGTT	NM_007742 (5946 bp)
	Antisense	CTTCACCCTTAGCACCAAC	
COL1A2	Sense	ATTGTCGCCAGTGAG CTGGTCCTGCTGGT	NM_007743 (5222 bp)
	Antisense		
$\beta$ -actin	Sense	AGTGCGTTTTACACCCTTT	NM_007393 (1935 bp)
	Antisense	AAGCCATGCCAATGTTGCT	

NCBI = National Center for Biotechnology Information; RT-PCR = reverse transcription polymerase chain reaction; COL1A = Collagen, type I, alpha; Has = Hyaluronan synthase.

Briefly, a cDNA strand was synthesized from the total RNA and the cDNA was amplified by PCR under the following conditions: 58 °C for 30 min, 94 °C for 2 min, 35 cycles at 94 °C for 15 sec, 60 °C for 30 sec, 68 °C for 1 min, and 72 °C for 5 min. Analysis was performed using the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) and expression levels were calculated relative to vehicle controls. The expression level of  $\beta$ -actin mRNA was used as a control for tissue integrity in all samples. The oligonucleotide primer sequences used are listed in Table 1.

### 8. STATISTICAL ANALYSIS

All values are expressed as the mean  $\pm$  standard deviation (SD) of ten samples. The obtained data were analyzed by one-way analysis of variance (ANOVA), followed by the least-significant differences (LSD) multi-comparison test to determine significant differences. Statistical analyses were conducted using SPSS ver. 14.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant at a value of  $P < 0.05$ .

## Results

### 1. Body weight and body weight gain

No significant changes in body weight were observed in PCP- and PCS-treated mice compared with vehicle-treated control mice throughout the experimental period (Table 2, Fig 1). As compared with control mice, the percent body weight gain of PCS-treated mice during the 56-day treatment period was 6.50%, 0.60%, and 1.81% in mice administered 4, 2, and 1 mL/kg of PCS, respectively, while that of PCP-treated mice was 1.81%, -0.15%, and 3.17% in mice administered 400, 200, and 100 mg/kg of PCP, respectively.

### 2. Changes in skin water content

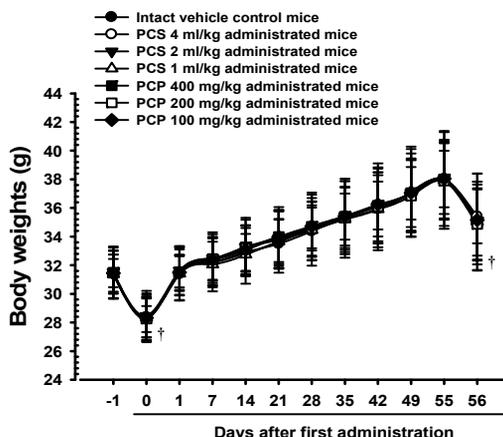


Fig. 1. Body Weight Changes. No significant changes on the body weights were demonstrated in all PCP and PCS treated mice as compared with intact vehicle control mice, throughout all experimental periods in this experiment. Values are expressed mean  $\pm$  S.D. of 10 mice, g. PCP = Dried pomegranate concentrate powder; PCS = Pomegranate concentrated solution. Day -1 means 1 day before first administration, Day 0 means the day of first administration, Day 56 means at 24 hrs after last 56th administration. All animals were overnight fasted before first administration and sacrifice, respectively ( $\dagger$ ).

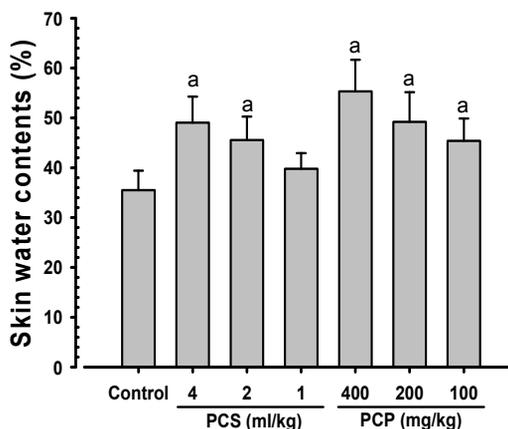
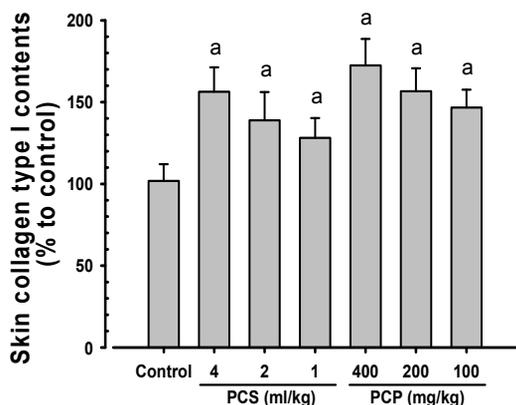


Fig. 2. Skin Water Contents, Note that significant increases of skin water contents were observed in PCS 4 and 2 ml/kg, PCP 400, 200 and 100 mg/kg treated mice as compared to those of intact vehicle control mice, at 24 h after end of last 56th administration, respectively. Values are expressed mean  $\pm$  S.D. of 10 mice, %/6 mm-diameter dorsal back skin. PCP = Dried pomegranate concentrate powder; PCS = Pomegranate concentrated solution,  $^{\#}P < 0.01$  as compared with intact vehicle control.

**Table 2.** Body Weight Gains

Groups	Periods	Body weights at			Body weight gains [B-A]
	First treatment [A]*	Last 56th treatment	Sacrifice [B]*		
<b>Controls</b>					
Intact vehicle		28.33±1.70	37.94±2.74	34.95±2.89	6.62±1.46
<b>PCS</b>					
4 ml/kg		28.36±1.39	38.02±1.98	35.41±1.88	7.05±1.12
2 ml/kg		28.45±1.76	38.01±2.75	35.11±2.71	6.66±1.32
1 ml/kg		28.35±1.56	37.96±3.42	35.04±3.38	6.69±2.15
<b>PCP</b>					
400 mg/kg		28.27±1.51	38.02±3.27	35.01±3.38	6.74±2.04
200 mg/kg		28.24±0.91	37.88±2.70	34.85±2.53	6.61±1.87
100 mg/kg		28.32±1.36	38.05±2.47	35.15±2.48	6.83±1.64

Values are expressed mean ± SD of 10 mice, g. PCP = Dried pomegranate concentrate powder; PCS = Pomegranate concentrated solution. \* All animals were overnight fasted



**Fig. 3.** Skin Collagen Type I Contents, Significant increases of skin collagen type I contents were observed in PCS 4, 2 and 1 ml/kg, PCP 400, 200 and 100 mg/kg treated mice as compared to those of intact vehicle control mice, at 24 h after end of last 56th administration, in the current experiment. Values are expressed mean ± S.D. of 10 mice, relative to intact mice %. PCP = Dried pomegranate concentrate powder; PCS = Pomegranate concentrated solution, <sup>a</sup>*p*<0,01 as compared with intact vehicle control.

Significant increases in skin water content were observed in mice administered 4 and 2 mL/kg of PCS and 400, 200, and 100 mg/kg of PCP at the end of the treatment period compared with control mice. Further, mice administered 1 mL/kg of PCS showed a marked, albeit non-significant, increase in

skin water content compared with control mice (Fig 2). The percent skin water content changes in PCS-treated mice were 38.12%, 28.35%, and 12.08% in mice administered 4, 2, and 1 mL/kg of PCS, respectively, compared with control mice. Moreover, those of PCP-treated mice were 55.87%, 38.58%, and 27.86% in mice administered 400, 200, and 100 mg/kg of PCP, respectively, compared with control mice.

### 3. Changes in skin collagen type I content

At the end of the treatment period, significant increases in skin type I collagen content were observed in mice administered all doses of PCS (4, 2, and 1 mL/kg) and PCP (400, 200, and 100 mg/kg) compared with control mice (Fig 3). The percent type 1 collagen content changes in PCS-treated mice were 53.54%, 36.44%, and 25.83% in mice administered 4, 2, and 1 mL/kg of PCS, respectively, while those of PCP-treated mice were 69.35%, 53.83%, and 44.11% in mice administered 400, 200, and 100 mg/kg of PCP, respectively, compared with control mice.

### 4. Changes in skin hyaluronan content

Skin hyaluronan content was significantly increased in all groups treated with PCS (4, 2, and 1 mL/kg)

**Table 3.** Skin Hyaluronan and Collagen Type I Synthetic Gene – Has 1, Has 2, Has 3, COL1A1 and COL1A2 mRNA Expressions

Groups	Periods		mRNA expressions		
	Has 1	Has 2	Has 3	COL1A1	COL1A2
<b>Controls</b>					
Intact vehicle	1.02±0.15	1.00±0.10	0.99±0.14	0.98±0.08	0.98±0.17
<b>PCS</b>					
4 ml/kg	1.57±0.17 <sup>a</sup>	1.45±0.14 <sup>a</sup>	1.58±0.16 <sup>a</sup>	1.49±0.22 <sup>a</sup>	1.50±0.21 <sup>a</sup>
2 ml/kg	1.46±0.13 <sup>a</sup>	1.31±0.15 <sup>a</sup>	1.42±0.16 <sup>a</sup>	1.40±0.25 <sup>a</sup>	1.41±0.21 <sup>a</sup>
1 ml/kg	1.37±0.17 <sup>a</sup>	1.26±0.12 <sup>a</sup>	1.29±0.10 <sup>a</sup>	1.29±0.13 <sup>a</sup>	1.31±0.09 <sup>a</sup>
<b>PCP</b>					
400 mg/kg	1.68±0.22 <sup>a</sup>	1.56±0.10 <sup>a</sup>	1.79±0.19 <sup>a</sup>	1.71±0.22 <sup>a</sup>	1.71±0.24 <sup>a</sup>
200 mg/kg	1.58±0.20 <sup>a</sup>	1.47±0.11 <sup>a</sup>	1.59±0.14 <sup>a</sup>	1.54±0.16 <sup>a</sup>	1.52±0.14 <sup>a</sup>
100 mg/kg	1.46±0.14 <sup>a</sup>	1.36±0.11 <sup>a</sup>	1.45±0.15 <sup>a</sup>	1.43±0.19 <sup>a</sup>	1.43±0.17 <sup>a</sup>

Values are expressed mean ± SD of 10 mice, relative to control/ $\beta$ -actin. PCP = Dried pomegranate concentrate powder; PCS = Pomegranate concentrated solution; COL1A = Collagen, type I, alpha; Has = Hyaluronan synthase. \*All animals were overnight fasted. <sup>a</sup> $p < 0.01$  as compared with intact vehicle control.

and PCP (400, 200, and 100 mg/kg) compared with control mice at the end of the treatment period (Fig 4). The percent hyaluronan content changes in PCS-treated mice were 44.79%, 32.94%, and 25.24% in mice administered 4, 2, and 1 mL/kg of PCS, respectively. Moreover, those of PCP-treated mice were 55.54%, 44.22%, and 43.98% in mice administered 400, 200, and 100 mg/kg of PCP, respectively.

##### 5. Effects on skin hyaluronan synthase genes

Significant increases in skin Has 1, Has 2, and Has 3 mRNA expression were observed in mice treated with all doses of PCS (4, 2, and 1 mL/kg) and PCP (400, 200, and 100 mg/kg) compared with control mice at the end of the treatment period (Table 3). The percent increases in skin Has 1 mRNA expression levels in PCS-treated mice were 54.52%, 43.22%, and 34.09% in mice administered 4, 2, and 1 mL/kg of PCS, respectively, while those of PCP-treated mice were 65.03%, 55.11%, and 43.71% in mice administered 400, 200, and 100 mg/kg of PCP, respectively. Has 2 mRNA expression levels increased 45.40%, 31.40%, and 25.60% in PCS-treated mice at doses of 4, 2, and 1 mL/kg,

respectively, and 56.10%, 46.50%, and 35.70% in PCP-treated mice at doses of 400, 200, and 100 mg/kg, respectively, compared with control mice. Lastly, the skin mRNA levels of Has 3 increased 58.65%, 43.16%, and 30.08% in mice treated with 4, 2, and 1 mL/kg of PCS, respectively, and 79.98%, 59.46%, and 45.88% in mice treated with 400, 200, and 100 mg/kg of PCP, respectively, compared with control mice.

##### 6. Effects on skin collagen type I synthetic genes

Significant increases in skin COL1A1 and COL1A2 mRNA expression were observed in mice treated with all doses of PCS (4, 2, and 1 mL/kg) and PCP (400, 200, and 100 mg/kg) compared with control mice at the end of the treatment administration period (Table 3). The percent increase in skin COL1A1 mRNA expression levels in PCS-treated mice was 52.30%, 43.50%, and 31.83% in mice administered 4, 2, and 1 mL/kg of PCS, respectively, while that of PCP-treated mice was 75.33%, 57.83%, and 46.06% in mice administered 400, 200, and 100 mg/kg of PCP, respectively, compared with control mice. Skin COL1A2 mRNA expression levels increased 53.84%, 44.32%, and

33.67% in PCS-treated mice at doses of 4, 2, and 1 mL/kg, respectively, and 75.33%, 55.99%, and 46.06% in PCP-treated mice at doses of 400, 200, and 100 mg/kg, respectively, compared with control mice.

## Discussion

Functional skin protective agents contain specific ingredients that have potent pharmacological actions, which can be divided into anti-aging, whitening, anti-wrinkle, moisturizing, and skin protective effects<sup>8)</sup>. Specifically, their anti-aging effects have been demonstrated by testing their antioxidant and free radical scavenging actions as well as their effects on the activity of antioxidant enzymes that contribute to enzymatic defense mechanisms<sup>10)</sup>. The skin regeneration and anti-wrinkle effects of these protective agents have been demonstrated by assessing the activities of various dermal enzymes involved in the formation of skin wrinkles<sup>3,11)</sup>. In addition, their whitening effects have been measured based on skin melanin content and the activity of tyrosinase, an enzyme involved in melanin formation<sup>4,12,13)</sup>, while their skin-moisturizing effects have been detected by measuring skin water content in animals and in human pilot studies<sup>5,6,9,14,15)</sup>. Therefore, various skin protective products have been developed for anti-aging, whitening, anti-wrinkle, moisturizing, and skin protective purposes<sup>4-6,9,14,15)</sup>. However, currently available anti-aging skin protective agents have a number of limitations, including high costs and pharmacological and side effects that are not fully understood<sup>25)</sup>. Due to these factors, various efforts have been made to find an affordable and functional skin-protective ingredient with few side effects, focusing on natural antioxidants<sup>2,8,9,21,26)</sup>.

In this study, significant and dose-dependent increases in skin water, type I collagen, and hyaluronan contents were demonstrated in mice treated with various doses of PCP and PCS,

compared with vehicle-treated control mice. Moreover, the mRNA expression of Has 1, Has 2, and Has 3, involved in hyaluronan synthesis and of COL1A1 and COL1A2, involved in collagen synthesis, were significantly and dose-dependently increased in dorsal skin samples of PCP and PCS-treated mice. Additionally, treatment with PCP at a dose of 200 mg/kg of body weight showed similar *in vivo* skin moisturizing effects to treatment with PCS at a dose of 4 mL/kg of body weight. Thus, treatment with both PCP and PCS resulted in favorable skin moisturizing effects in normal mice through the upregulation of mRNA expression of genes involved in hyaluronan and collagen synthesis.

Keratin layers in the human skin maintain skin hydration; they are composed of 10-20% water. However, decreases in keratin layer water content accelerates the aging process, wrinkle formation, and itching<sup>6)</sup>. To maintain healthy elastic skin, keratin layers must retain adequate moisture, even under dry conditions<sup>27)</sup>. Skin moisturizing effects have previously been measured in animal and human studies by assessing the skin's water content<sup>9,14,15)</sup>. In this study, significant and dose-dependent increases in dorsal skin water content were demonstrated in all PCS- and PCP-treated mice compared with vehicle-treated control mice. Thus, both PCS and PCP exert favorable skin-moisturizing effects, which could be used in a functional skin protective product.

Type I collagen is the major structural protein in the skin. Collagen destruction is the main underlying mechanism thought to contribute to the appearance of aged skin due to chronic sun exposure<sup>28)</sup>. Skin wrinkling is a complex but inevitable process that involves the age-dependent decline of skin cell function, causing harmful proteolytic degradation of the ECM<sup>3)</sup>. Several scientific studies investigating skin wrinkling have highlighted that ECM degradation is significantly associated with increased dermal activity of enzymes including hyaluronidase, collagenase, elastase, and MMP-1 to form wrinkles<sup>3,11)</sup>. Ingredients

that promote type I collagen synthesis or inhibit the activity of enzymes involved in ECM degradation in aged skin have the potential for use in anti-wrinkle and moisturizing skin-care products<sup>1,10</sup>. In our previous *in vitro* experiments<sup>20</sup>, fibroblast (HDF-N cell) treatment with PCS influenced type I collagen synthesis, and favorably inhibited hyaluronidase and MMP-1 activities. In this study, PCP and PCS treatment resulted in dose-dependent increases in skin collagen type I content, with upregulation of the collagen synthetic genes COL1A1 and COL1A2; comparable results were obtained with a PCS dose of 4 mL/kg and a PCP dose of 200 mg/kg. Both the COL1A1 and COL1A2 genes are involved in type I collagen synthesis by producing the pro-alpha1(I) and pro-alpha2(I) chains that form a type I procollagen molecule<sup>29</sup>. Thus, PCS and PCP may exert their favorable skin moisturizing actions, in part, by increasing collagen synthesis.

Hyaluronan is a major ECM component of the skin that plays important roles in skin physiology, including maintaining water homeostasis<sup>20,23,30</sup>. Hyaluronan is synthesized by three proteins, Has 1, Has 2, and Has 3<sup>23,31,32</sup>. Similar to our previous *in vitro* study<sup>20</sup>, in which PCS treatment favorably increased hyaluronan synthesis in HaCaT cells, significant and dose-dependent increases in skin hyaluronan content as well as Has 1, Has 2, and Has 3 gene upregulation were demonstrated in all PCS- and PCP-treated mice. These results indicate that the skin-moisturizing effects of PCS and PCP are exerted, in part, by an increase in hyaluronan synthesis.

This study demonstrates the beneficial skin-moisturizing effects of PCP and PCS in normal mice, and highlights potential underlying mechanisms, including upregulation of mRNA expression of genes involved in hyaluronan and collagen synthesis. Similar results were obtained in mice administered PCP at a dose of 200 mg/kg and in mice administered PCS at a dose of 4 mL/kg. Thus, PCP and PCS exert promising

potent anti-aging effects that may be beneficial in functional skin protective agents.

### Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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