PAPER

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Oral intake of collagen peptide NS improves hydration, elasticity, desquamation, and wrinkling in human skin: a randomized, double-blinded, placebo-controlled study

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Collagen hydrolysate, which contains bioactive peptides, is used as a dietary supplement for the refinement of elasticity, hydration, desquamation, and wrinkling of aging human skin. Here, we conducted a double-blind, randomized, and placebo-controlled oral administration study on the effects of a collagen peptide (CPNS) containing dipeptides, including Gly-Pro and Pro-Hyp, on skin wrinkling, desquamation, elasticity, and hydration. Our results show that an intake of 1650 mg per day of CPNS for 12 weeks had beneficial effects on skin health in a cohort of women aged from 30 to 60 years ($n = 100$). Compared with the placebo group, skin desquamation, hydration, skin wrinkling, and elasticity were significantly improved after 4, 4, 12, and 12 weeks of administration, respectively. In a safety test of CPNS ingestion, none of the participants showed any side effects during the clinical study period. These results demonstrate that the low molecular weight bioactive peptides contained in CPNS, such as Gly-Pro and Pro-Hyp, exert positive effects on skin hydration, elasticity, desquamation, and wrinkling.

1. Introduction

Various environmental and endogenous factors influence skin damage and aging. The two main layers of human skin are the dermis and the epidermis, constituting connective tissue and stratified squamous epithelium, respectively. The extracellular matrix (ECM) elements of the dermis layer, such as hydroxyapatite, collagen, glycosaminoglycan (GAG), and enzymes are mostly generated by fibroblasts.[1–3] Wrinkling, coarse skin, dryness, telangiectasia, pigmental degradation, and decrease of tensile strength due to photoaging are visible because of a decrease in metabolic reactions and include various changes in dermal ceramide and collagen.[4,5] A recently emerging trend in the management of skin care is the intake of dietary supplements to enhance the arrangement and appearance of the skin "from the inside out".

Previous studies have demonstrated that oral intake of enzymatically hydrolyzed collagen peptides can have positive effects on skin health, such as dermal ECM generation, fibro-

blast growth, antioxidation, recovery, elasticity, and wrinkle reduction.[6–10] Particularly, gelatin- and collagen-oriented hydrolysates are widely applied these days and are produced in a variety of fields, such as functional foods, nutraceuticals, medical supplies, and the biotech industry.[11–13] The molecular weight of gelatin, a water-soluble polymer, is considerably higher than that of collagen hydrolysates, and it is produced by enzymatically hydrolyzing collagen that is normally used as a food ingredient and delivery carrier for the regulated distribution of bioactivities.[14–17] When the high molecular weight gelatin is hydrolyzed by treating with a particular fungal or bacterial protease, the outputs, small molecular weight peptides, are conveniently called collagen hydrolysates.[11,12,18] There is growing evidence indicating that collagen peptides improve not only human skin indicators associated with aging, such as hydration, wrinkles, and elasticity, but also the antihypertensive effect in spontaneously hypertensive rats (SHRs).[19] They also reduce skin cellulite[20] and osteoporosis and joint pain[21–23] and have a positive effect on hair follicles and cell proliferation.[24] The effectiveness of collagen hydrolysates in improving damaged skin has been demonstrated, yet the acknowledgment of the beneficial effects and functions of low molecular weight bioactive proteins and peptides from collagen hydrolysates is still low.[14,25] Enzymatically produced bioactive peptides are characterized as a combination of short sequences of amino acid monomers bound via peptide bonds, which invigorate functional effects and may control the

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homeostasis system.[26,27] After oral administration of collagen hydrolysate, the bioactive peptides hydroxypropyl-glycine (Hyp-Gly) and prolyl-hydroxyproline (Pro-Hyp) are distributed at high levels throughout the bloodstream, indicating that they play a crucial role in the improvement of skin status.[28,29] The Gly-Pro dipeptide is mainly produced as an end product of enzymatic hydrolysis of Gly-Pro, alongside other peptide products (Gly-Pro-X).[30] The Gly-Pro dipeptide is highly resistant to proteases of the intestine and hydrolysis by enterocytes, which result in part permeation into the human bloodstream.[31] However, particular receptors for Gly-Pro have not been identified, and the functional efficacy of Gly-Pro or Gly-Pro-containing collagen peptides has not been thoroughly studied so far.[32]

As previously mentioned, lots of research related to collagen hydrolysates and bioactive peptides is being actively conducted. However, in vivo testing has limitations, and the difference in physiological function between mice and humans is difficult to prove. More research is needed to verify the efficacy and safety of collagen hydrolysate intake by conducting controlled clinical trials.[4,33,34] In some clinical studies, the oral administration of collagen hydrolysates was ineffective against photoaging skin,[35,36] and the number of volunteers or the completeness of the design was insufficient.[37] Other clinical trials were also conducted with collagen hydrolysates extracted from pigs[24,38–40] or chickens,[41,42] not from fish. In addition, some clinical studies have proved that simply enzymatically hydrolyzed collagen products without revealing the bioactive peptide component exert beneficial effects on skin hydration.[36,38,43,44] Above all, most clinical studies conducted oral supplementation with physiologically active ingredients such as antioxidants, vitamins, and collagen hydrolysates rather than collagen hydrolysate intake alone.[37,45–48] To the best of our knowledge, this is the first study to estimate the photoaged skin improvement efficacy through a randomized, double-blinded, and placebo-controlled clinical study design using fish-derived collagen hydrolysates containing specific bioactive dipeptide forms, Gly-Pro, and Pro-Hyp, not combined with any antioxidants and vitamins.

Here, a collagen peptide product produced by Nongshim Co., Ltd, Seoul, Korea, CPNS, was used to demonstrate the physiochemical properties of collagen hydrolysates. The main source of CPNS is fish scales, and it is manufactured by employing specific fungal proteases. The composition of CPNS, with Pro-Hyp and Gly-Pro as its main components, distinguishes it from other commercial collagen peptides produced using collagenase. In an earlier in vivo study, hairless mice with photoaging induced by ultraviolet ray exposure for 12 weeks were orally administered with CPNS and showed improvements in wrinkle-related indicators, such as wrinkle formation and epidermal thickness, and moisturization-related indicators, such as moisture loss and moisture content, which surpassed the results observed in control animals.[10] In addition, in an in vitro test, the CPNS spurred hyaluronic acid (HA) production in human dermal fibroblasts (HDF) by stimulation of HAS2 transcription.[10] The purpose of this clinical study was thus to estimate the anti-photoaging efficacy of CPNS in the human dermis and determine its effect on the process of skin aging.

2. Materials and methods

2.1 Preparation of collagen hydrolysate and determination of the dose

The fish collagen hydrolysate CPNS was manufactured by the Research and Development (R&D) center of Nongshim Co., Ltd (Seoul, Korea). Concisely, CPNS was derived from tilapia (Oreochromis niloticus) fish scales that were enzymatically degraded with multiple fungal proteases (Amano Enzyme Inc., Nagoya, Japan). In a previous study, the main collagen peptides of CPNS were identified by utilizing a dual-piston pump preparative high-pressure liquid chromatography system (prep-HPLC, Gilson, Inc., Middleton, USA). Moreover, the low molecular weight peptide fractions below 500 Da were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS, Thermo Fisher Scientific, Waltham, USA) to identify the specific peptide sequences. Unlike other studied collagen hydrolysates,[12,49–51] CPNS contains 4% Gly-Pro as its representative peptide10, whereas the other short-chain peptides existed in minor quantities (data not shown). Based on the efficient dose determined in a previous study,[10] the titrated dose for humans was estimated utilizing the body surface area (BSA) normalization method.[52,53] Based on a dose of 300 or 500 mg per kg per day used in mice, 1650 mg per day for humans was determined as the titrated dose for clinical trials, adapted to 60 kg, the average body weight of an adult.

2.2 Clinical study design

This study was conducted to evaluate human skin hydration, desquamation, wrinkling, and elasticity after 12 weeks of daily intake of CPNS, following a randomized, double-blind, placebo-controlled supplementation design. All study procedures were in line with the applicable Good Clinical Practice guidelines and the standard operating procedures of COREDERM Inc, the skin research center (Seoul, Korea), and clinical research organizations in the bio-food sector.[54] The clinical protocol was recognized by the institutional review board of COREDERM Inc. Skin Research Center in Seoul, Korea. For reference, the approval number is CDE-1700-020. All participants were informed about the study details and provided written informed consent. Participants were assigned to two groups based on block randomization, the CPNS group and the placebo group. A 3300 mg test product (tablet) contained 1650 mg of CPNS along with vehicle ingredients. The placebo product (tablet) had the same cellulose, flavor, and excipients for tablet figuration as the test formulation, except for CPNS (Table 1).
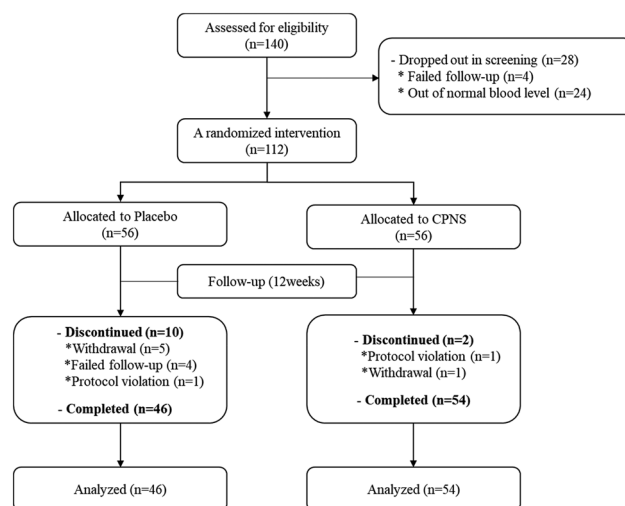
2.3 Study participants

We recruited a total of 140 women (aged from 30 to 60 years) who were mentally and physically healthy, had dry facial skin

Table 1 Ingredients of test and placebo tablet preparations

Ingredients	Test		Placebo	
	Content (mg)	Content (%)	Content (mg)	Content (%)
CPNS	1650	50	0	0
Cellulose	1485	45	3135	95
Excipients	132	4	132	4
Flavor	33	1	33	1
Total	3300	100	3300	100

Fig. 1 The clinical study flow diagram. Abbreviations: CPNS, collagen peptide NongShim.

(water content ≤ 49) and crow's feet, which refer to wrinkles around the eyes greater than grade 3, and satisfied all inclusion and exclusion criteria (Table 2). Ahead of the intervention, we confirmed that the participants were willing and able to visit the center on the prescribed days and follow the schedule and all regulations. During the screening phase, 28 volunteers were excluded because the levels of blood parameters were not in the normal range or because they withdrew consent. A total of 112 participants were administered either the test product (with CPNS included) or the placebo (without CPNS included), and among the 112 participants, 12 participants were withdrawn from the study later because of either being lost to follow-up or due to protocol violations. Finally, 100 participants completed the study in good condition (Fig. 1). From the aspect of safety, before and 12 weeks after administration, all of the participants took a blood test (WBC, RBC, hemoglobin, platelet, T Protein, albumin, T Bil, SGOT, SGPT, total cholesterol, triglyceride (TG), glucose, BUN, creatinine), and we surveyed concomitant medication and medical history (Table 3). Also, to verify that the diet was maintained as usual during the study period, we recorded that the subjects were required to bring a self-assessment of their usual diet to

the dietary recording sheet provided 3 days before (−3 days), 4, 8, and 12 weeks after the product intake. In addition, physical examination and body weight analysis and analysis of BMI at baseline (0 W) and 12 weeks after the product intake were performed. We confirmed that the results of the blood test were in normal ranges of all blood parameters in all the participants even though some parameters had a significant difference between baseline and 12 weeks after administration. And weight, BMI, and the diet of the participants were not a significant difference between the test and placebo group after administration (Table 4). Moreover, participants were advised not to alter their daily skincare routine, not to undergo dermatological procedures on the test areas of their face, and not to

Table 2 Inclusion and exclusion criteria for recruitment of volunteers

Inclusion criteria

• Female or male subjects aged between 25 and 60 with eye wrinkles and pigmentation on the face that began or had already been conducted (visual assessment grade 3 or higher).
• Healthy subjects who are free from acute and chronic diseases including skin ailment.
• Those who were briefed in detail by the investigator about the purpose and contents of the test and possible occurrences of adverse reactions and who willingly signed the informed consent form.
• Those who can do a follow-up observation during the testing period.

Exclusion criteria
• Those who are pregnant and feed or have a pregnancy plan within 24 weeks.
• Those who take oral contraceptives, hormones, and diuretics.
• Those who have received treatment for oral steroids or retinoids, topical steroids ointment within 24 weeks before the start of the study.
• Those who have participated in a similar test within 24 weeks before the start of the study.
• Those who have skin matters on the test site like moles, pimples, red spots, scalds (burns), or scars.
• Those who have used or taken similar effective cosmetic and topical products or supplements on the test site within 12 weeks before starting this study.
• Those who got a treatment of surgery (skin dermabrasion, Botox, laser, skincare, *etc.*) on test site within 24 weeks.
• Those who have a chronic or wasting disease (asthma, diabetes, high blood pressure, *etc.*)
• Those who smoke or quit smoking for less than a year.
• Those who have a psychiatric disease and an infectious skin disease.
• Those who have a specific allergic reaction to foods that are related to the test product.
• Those who have experience in drug-sensitive and allergic reactions.
• Those who have cardiovascular, endocrine, digestive, and urinary diseases.
• Those who are employed in this center.
• Those who are considered as a nonqualified person by the judge of the investigator and ineligible for the blood test.

Table 3 Baseline characteristics of the participants

Parameter	Mean ± S.D		
	Placebo group ($n = 46$)	Test group ($n = 54$)	P-Value
Age	44.9 ± 5.2	45.7 ± 7.5	0.530
Weight (kg)	59.98 ± 1.47	57.60 ± 1.07	0.185
BMI (kg m^{-2})	23.04 ± 0.56	22.46 ± 0.42	0.400
Exposure hours to UV (daily/%)			
Less than 1h	32.61	14.81	0.115
1–3 h	60.87	77.78	
More than 3 h	6.52	7.41	
Sleeping hours (daily/%)			
Less than 5 h	8.70	5.56	0.367
5–8 h	76.09	87.04	
More than 8 h	15.22	7.41	
Hydration (A.U)			
Cheek	38.80 ± 0.87	36.29 ± 1.05	0.075
Volar forearm	32.23 ± 1.01	32.01 ± 0.97	0.871
TEWL (g m^{-2} h^{-1})			
Cheek	13.92 ± 0.61	13.08 ± 0.51	0.290
Volar forearm	7.27 ± 0.30	7.69 ± 0.23	0.265
Desquamation (%)			
Cheek	14.69 ± 0.50	16.04 ± 0.48	0.056
Volar forearm	18.81 ± 0.64	20.10 ± 0.58	0.139
Wrinkling (μm)			
R_a	12.85 ± 0.33	13.05 ± 0.32	0.663
R_{max}	80.46 ± 2.13	82.71 ± 1.97	0.440
R_z	59.65 ± 1.50	61.08 ± 1.45	0.499
R_p	40.04 ± 0.99	41.15 ± 1.03	0.447
R_v	45.31 ± 1.38	46.42 ± 1.23	0.548
Elasticity			
K (mm)	1.140 ± 0.007	1.139 ± 0.005	0.833
Indent (mm)	0.557 ± 0.015	0.563 ± 0.013	0.767
Alpha (ratio)	0.027 ± 0.001	0.027 ± 0.001	0.823
CoR (ratio)	0.682 ± 0.010	0.686 ± 0.009	0.744
Area (mm^2)	71.319 ± 3.293	71.854 ± 2.942	0.891

Student's t-test for continuous variables and chi-square or Fisher's exact test for categorical variables were used to compare the difference between the groups.

make changes to their lifestyle and dietary habits, daily skin-care products, or dietary supplements. They were also told not to be exposed to excessive sunlight (ex: outdoor activities such as outdoor swimming, skiing, hiking, and long-term travel) during the study periods (Table 3).

2.4 Study schedule

All participants were asked to ingest four tablets containing 1650 mg of CPNS once a day. Moreover, they agreed to only use cosmetics provided by the center for a wash-out period of 2 weeks and the intervention period to ensure a normal skin status. All participants visited the center five times in total during the clinical trial period to estimate the measuring parameters: before administration formulation at baseline (0 week), at 4, 8, and 12 weeks after the start of the administration of the prepared formulation for the assessment of effects, and 2 days after the end of the ingestion period at 12 weeks for a safety test. Participants were told not to wear make-up for 12 h before visiting the assessment center. At each visit, participants washed their entire face with a foam cleanser named 'Facial Care Smart Whip' of Welcos Co., Ltd, which has no functional ingredients that affect the skin and is available on the market,

and waited for 20 min at a controlled temperature (22 ± 2 °C) and humidity (50 ± 5%) before the evaluations.

2.5 Measurement of skin hydration

Skin moisture was measured on the face and the volar forearm, 5 cm distal to the elbow, using a Corneometer®CM 825 (C+K, Germany). This device is well known for being able to estimate the moisture content in the stratum corneum (SC).[55] All electrical phenomena are caused by electric charges, with capacitance indicating the ability to store these charges, and when the anode plate of a probe is charged, it creates an electric field in between. The principle of the storing of generated charges can thus be used to measure the moisture content in the SC of the epidermis that has high resistance to electricity. Since the measured capacitance is relatable to the water content of the SC, the higher the reading, the higher the moisture content; moreover, the more hydrated the SC is, and the more intensive the electronic conductivity. The measured output levels are represented in arbitrary units (au).

2.6 Measurement of trans-epidermal water loss (TEWL)

TEWL is one of the well-known factors for the estimation of the moisture barrier capacity of the skin.[56] It was measured on the participant's face and their volar forearm using a Tewameter® TM 300 (C+K, Germany). The device's "open chamber" principle represents the most common method for the estimation of TEWL. The Tewameter® probe gauges the density gradient of moisture vaporization from the specific skin area indirectly with the double sensors (relative humidity and temperature) inside the hollow cylinder. This is the only method that allows for assessing the TEWL continuously without influencing its microenvironment. The measured values express the evaporation rate in g m^{-2} h^{-1}.

2.7 Measurement of skin desquamation

Desquamation on the face and the volar forearm was assessed with a Standard D-Squame® disc (Heiland electronic GmbH, Wetzlar, Germany). When assessing the desquamation of the skin, a D-Squame pressure instrument (D500; Heiland electronic GmbH) was used to apply a constant pressure of 225 g cm^{-2}. The obtained skin desquamation was analyzed using a SquameScan® 850 (Heiland electronic GmbH) which measures the total protein (%) of corneocytes on disc images to assess desquamation on the disc. The skin desquamation on the disc was assessed with an optical microscope with a 10-fold magnification lens (SOPTOP MDX320, China).

2.8 Measurement of skin wrinkling

Crow's feet were evaluated using two different methods, visual assessments, and instrumental measurements. For the visual assessments, a dermatologist and an investigator, who has over 10 years of experience in the skin clinical research field, assessed the participant's skin, following a double-blinded method, and recorded grades according to the wrinkle scoring criteria of the MFDS (Ministry of Food and Drug Safety, KOREA) guidelines. Instrumental measurements were con-

Table 4 The variation of diet, weight, BMI, and blood test of the participants

Parameter		Mean±S.D (P-Value[1])				P-Value[2]
		Placebo group ($n = 46$)		Test group ($n = 54$)		
Diet	−3 days	2.01 ± 0.05	—	2.00 ± 0.04	—	—
	4 weeks	2.01 ± 0.06	(0.205)	1.99 ± 0.09	(0.628)	0.594R
	8 weeks	2.00 ± 0.14	(0.556)	1.99 ± 0.07	(0.315)	0.863R
	12 weeks	2.00 ± 0.05	(0.265)	2.00 ± 0.06	(0.354)	0.879R
Weight	0 week	59.98 ± 9.98	—	57.60 ± 7.87	—	—
	12 weeks	60.41 ± 9.76	(0.024*)	58.04 ± 8.07	(0.016*)	0.968
BMI (kg m^{-2})	0 weeks	23.04 ± 3.80	—	22.46 ± 3.06	—	—
	12 weeks	23.20 ± 3.64	(0.025*)	22.64 ± 3.19	(0.015*)	0.891
Blood test						
WBC	0 week	6.01 ± 1.41	—	5.73 ± 1.33	—	—
	12 weeks	6.26 ± 1.49	(0.195)	6.02 ± 1.46	(0.113)	0.863
RBC	0 week	4.34 ± 0.29	—	4.34 ± 0.35	—	—
	12 weeks	4.38 ± 0.26	(0.140)	4.37 ± 0.31	(0.322)	0.745
Hemoglobin	0 week	12.87 ± 0.96	—	12.74 ± 1.19	—	—
	12 weeks	13.07 ± 0.84	(0.006*)	12.88 ± 0.96	(0.049*)	0.571
Platelet	0 week	269.35 ± 46.58	—	277.80 ± 55.81	—	—
	12 weeks	271.50 ± 50.23	(0.615)	280.04 ± 62.26	(0.622)	0.989
T Protein	0 week	7.34 ± 0.35	—	7.35 ± 0.37	—	—
	12 weeks	7.36 ± 0.38	(0.684)	7.35 ± 0.33	(0.972)	0.735
Albumin	0 week	4.63 ± 0.21	—	4.63 ± 0.23	—	—
	12 weeks	4.64 ± 0.25	(0.942	4.63 ± 0.22	(0.907)	0.896
T Bil	0 week	0.65 ± 0.21	—	0.61 ± 0.23	—	—
	12 weeks	0.57 ± 0.24	(0.016*)	0.54 ± 0.22	(0.030*)	0.737
SGOT	0 week	18.74 ± 4.29	—	19.96 ± 5.21	—	—
	12 weeks	20.39 ± 6.76	(0.102)	20.44 ± 5.67	(0.496)	0.327
SGPT	0 week	13.24 ± 5.23	—	15.06 ± 6.48	—	—
	12 weeks	16.09 ± 7.40	(0.023*)	15.52 ± 6.14	(0.144)	0.112
Total cholesterol	0 week	186.67 ± 29.97	—	186.85 ± 28.93	—	—
	12 weeks	184.89 ± 32.56	(0.564)	192.72 ± 29.72	(0.021*)	0.052
Triglyceride	0 week	89.30 ± 40.05	—	85.37 ± 35.50	—	—
	12 weeks	92.61 ± 40.15	(0.475)	94.20 ± 55.34	(0.188)	0.509
Glucose	0 week	89.67 ± 6.18	—	90.22 ± 8.55	—	—
	12 weeks	91.17 ± 8.25	(0.177)	91.39 ± 7.45	(0.330)	0.839
BUN	0 week	11.41 ± 2.75	—	11.56 ± 2.86	—	—
	12 weeks	12.27 ± 2.74	(0.035*)	13.27 ± 10.20	(0.246)	0.602
Creatinine	0 week	0.67 ± 0.09	—	0.67 ± 0.08	—	—
	12 weeks	0.65 ± 0.09	(0.067)	0.65 ± 0.07	(0.004*)	0.843

WBC: white blood cell, RBC: red blood cell, T Protein: total protein, T bil: total bilirubin, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic-pyruvic transaminase, BUN: blood urea nitrogen, (1) paired t-test or Wilcoxon signed-rank test, *$P < 0.05$ it means that there is a significant difference when compared to the baseline (0 W) (2) independent t-test, R RM-ANOVA, *$P < 0.05$ it means that there is a significant difference between the groups.

ducted using a PRIMOS® lite (Canfield, USA). Based on digital micromirror devices (DMDs), the principle of this method is to measure the digital stripe projection. The device can take a photo and transform it into a 3D image. The analyzed value of the same region was determined by comparing two images (before and after measurements) using the matching and overlay system, and the result gave the following parameters: R_a represents the arithmetic average value of profile peaks within the total length; R_{max} is the maximum of all peak-to-valley values; R_z is the average maximum height of the profile; R_p is the maximum profile peak height; and R_v is the maximum profile valley height.[9,57]

2.9 Measurement of skin elasticity

The elasticity of the dermis was estimated by assessing the participant's cheek with a Ballistometer BLS780 (Dia-Stron Ltd, United Kingdom). The measuring principle is based on the tra-

ditional ballistometric method of impacting force on the skin surface.[58] When placed on the surface of the skin and applying vibrational energy, the waveform of the vibration (reduction) is analyzed and expressed as the value of each parameter and the bounce profile. We obtained the following four parameters: K represents skin firmness or softness; indent indicates skin softness; alpha is the rate of energy breaking; CoR is the coefficient of restoration; and the area is the area between the zero datum and under the curve of the bounce profile.

2.10 Statistical analysis

The statistical analysis was conducted using SPSS 12.0. (IBM, USA) and SAS 9.4 (SAS Institute, USA). All variables of measurement at the baseline were assessed for normal distributions using the Shapiro–Wilk test and kurtosis & skewness measurements. All continuous data were analyzed using paired and independent t-tests if normality was satisfied and the

Wilcoxon signed-rank test and the Mann–Whitney U test if distributions were not normal. Categorical data were analyzed using chi-square tests or Fisher's exact tests. All data are presented as the mean ± standard error (S.E.), and $P < 0.05$ was considered to indicate statistical significance.

3. Results and discussion

3.1 Participant baseline characteristics

A total of 100 participants (CPNS group = 54; placebo group = 46) completed the clinical study, and their data were entered into the analyses. Homogeneity tests between groups did not reveal significant differences in age, body weight, TEWL, desquamation, wrinkling, or elasticity at the baseline (Table 3).

3.2 Effect of CPNS on skin hydration and TEWL

Skin hydration on the face was significantly ameliorated at 4 weeks ($P = 0.000$), 8 weeks ($P = 0.000$), and 12 weeks ($P = 0.000$) after the start of CPNS administration, compared to the baseline, whereas no variation from the baseline was observed in the placebo group after 4, 8, and 12 weeks of administration ($P = 0.370$, $P = 0.861$, and $P = 0.169$, respectively). When comparing the two groups directly, the skin was significantly more hydrated in the CPNS group than in the placebo group at 4, 8, and 12 weeks (Table 5 and Fig. 2) ($P = 0.000$, respectively). Improvements in skin hydration were also greater in the CPNS group than in the placebo group (5.19-fold at 4 weeks, 52.00-fold at 8 weeks, and 6.63-fold at 12 weeks). Skin hydration on the forearm did not show a significant difference between the groups (data not shown). Furthermore, TEWL and the forearm skin tended to show greater improvements in the CPNS group than in the placebo group; however, the difference between the groups was not statistically significant (data not shown).

3.3 Effect of CPNS on skin desquamation

Forearm desquamation was significantly ameliorated in the CPNS group at 4 weeks ($P = 0.000$), 8 weeks ($P = 0.001$), and 12 weeks ($P = 0.000$) compared to the baseline, while no significant difference was observed in the placebo group at 4, 8, and 12 weeks ($P = 0.708$, $P = 0.892$, and $P = 0.474$). The comparison between groups showed that the desquamation level was significantly lower in the CPNS group than in the placebo group at 4, 8, and 12 weeks ($P = 0.017$, $P = 0.039$, and $P = 0.016$) (Fig. 3 and 4). The CPNS group showed an 8.74-fold improve-
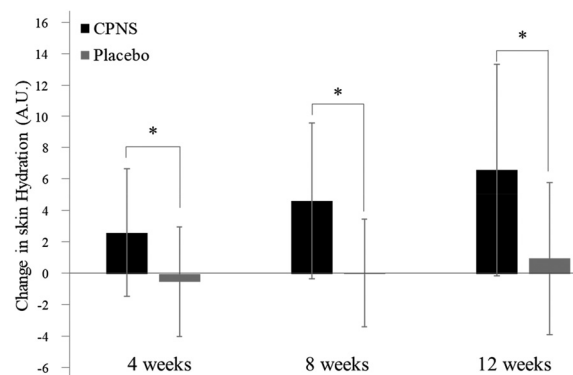

Fig. 2 Variance value in skin hydration in individual's intake CPNS formula or placebo. Skin hydration was measured with a Corneometer® CM 825, and changes from the baseline values are shown, with arbitrary units (A.U.). Data are expressed as the mean ± S.D. *Indicate significant differences between the CPNS group and the placebo group ($P < 0.05$; t-test).
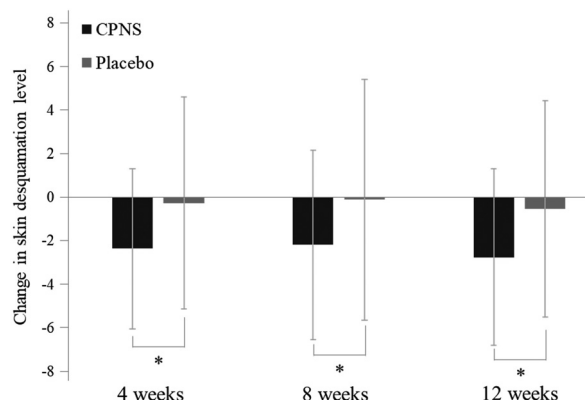

Fig. 3 Variance value in skin desquamation in individual's intake CPNS formula or placebo. Skin desquamation was measured with a D-squame® disc, and changes from the baseline values are shown. Data are expressed as the mean ± S.D. *Indicate significant differences between the CPNS group and the placebo group ($P < 0.05$; t-test).

ment compared to the placebo group at 4 weeks, a 19.82-fold improvement at 8 weeks, and a 5.19-fold improvement at 12 weeks. An improvement in skin desquamation on the face was observed at 12 weeks in the CPNS group. However, there was

Table 5 Skin hydration values on face measurement with a Corneometer® CM 825

| Time | CPNS Group ($n = 54$) | | | Placebo Group ($n = 46$) | | |
	Mean (S.D.)	P-Value		Mean (S.D.)	P-Value	CPNS/Placebo P-Value
0 week	36.29 (7.75)			38.80 (5.92)		
4 weeks	38.93 (7.84)	0.000*		38.33 (5.22)	0.370	0.000*
8 weeks	40.97 (8.17)	0.000*		38.89 (5.74)	0.861	0.000*
12 weeks	42.92 (7.66)	0.000*		39.80 (6.74)	0.169	0.000*

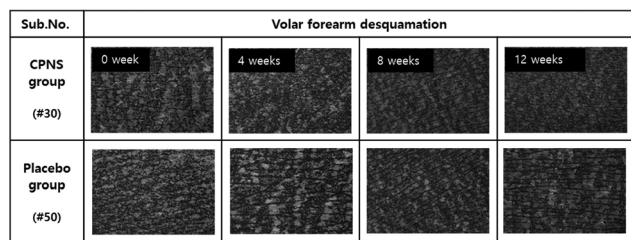P-Values for test comparison with baseline values; *indicates $P < 0.05$.

Fig. 4 Effect of CPNS on volar forearm desquamation in the CPNS group.

no significant difference between the two groups (data not shown).

3.4 Effect of CPNS on skin

According to the visual grading of the participants' crow's feet wrinkles, the CPNS group showed significant improvements compared to the placebo group at 12 weeks ($P = 0.039$) (Table 6 and Fig. 5), with the CPNS group's appearance level being 4.67-fold higher than that of the placebo group. The assessment of instrumental measurements showed significant improvements in the CPNS group compared to the placebo group in all wrinkle parameters (R_a, R_{max}, R_z, R_p, and R_v) after 4 weeks ($P = 0.006$, $P = 0.006$, $P = 0.004$, $P = 0.003$, and $P = 0.028$, respectively) and 12 weeks of administration ($P = 0.028$, $P = 0.015$, $P = 0.011$, $P = 0.020$, and $P = 0.044$) (Table 7). Moreover, the differences in variance values (baseline–after administration) of wrinkle indicators (R_a, R_{max}, R_z, R_p, and R_v) showed 3.26-fold, 5.04-fold, 4.07-fold, 7.73-fold, and 4.11-fold improvements compared to the placebo group at 4 weeks and 2.68-fold, 4.10-fold, 3.71-fold, 3.58-fold, and 3.83-fold improvements at 12 weeks of administration. None of the parameters showed significant improvements at 8 weeks.

3.5 Effect of CPNS on skin elasticity

Some measured parameters of elasticity were significantly ameliorated in the CPNS group after 8 weeks of oral intake compared to the baseline, and some (K, alpha, CoR, and area) also after 12 weeks of administration ($P < 0.05$ in all cases). However, the placebo group did not show any improvements in skin elasticity from baseline values during the entire study period, except for the parameter K at 8 and 12 weeks after the start of placebo administration. In comparison with the placebo group, the CPNS group showed significant improvements in most skin elasticity parameters (K, alpha, and CoR)
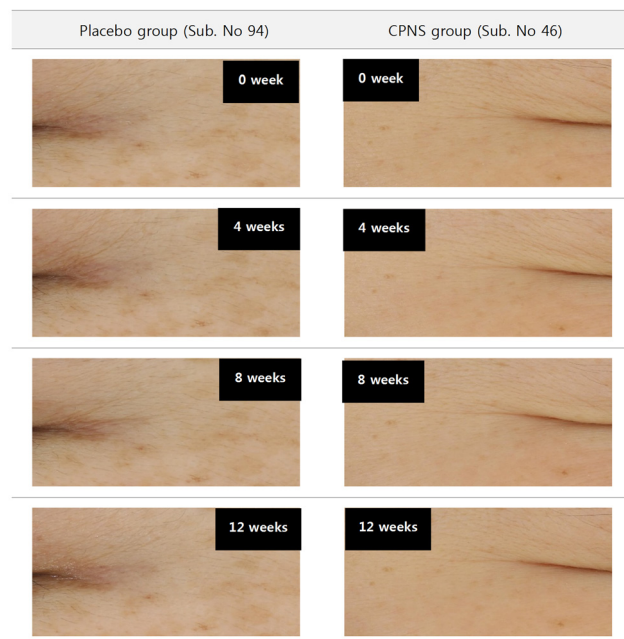

Fig. 5 Effect of CPNS on skin wrinkles in CPNS and placebo groups, wrinkle grade: 0: no wrinkles and skin condition is fine, 1: some signs of fine wrinkles, 2: some fine wrinkles, 3: a lot of fine wrinkles and some are progressing into slightly deeper wrinkles, 4: some slightly deeper wrinkles, 5: slightly deeper wrinkles have become more defined; however, there are no signs of deep wrinkles, 6: signs of slightly deeper wrinkles turning into deep wrinkles, 7: some deep wrinkles, 8: wrinkles are deep and numerous, 9: wrinkles are very deep and numerous.

after 12 weeks ($P = 0.031$, $P = 0.012$, and $P = 0.013$,), while the area parameter was improved at 8 and 12 weeks ($P = 0.005$, and $P = 0.000$). K, alpha, and CoR showed 1.89-fold, 2.00-fold, and 1.60-fold improvements in the CPNS group compared to the placebo group at 12 weeks, and the area parameter showed a 13.09-fold improvement at 8 weeks and a 36.56-fold improvement at 12 weeks, compared to the placebo group (Table 8).

4. Discussion

Dryness leads to skin aging due to skin barrier dysfunction and skin-related disease development, such as winter xerosis, atopic dermatitis, and eczema.[59–61] Dry skin becomes more notable with age and is related to a reduction in ceramide contents and natural moisturizing factors,[62] and skin moisture

Table 6 Visual assessment of skin wrinkles on crow's feet by investigators

Time (week)	CPNS group ($n = 54$)		Placebo group ($n = 46$)		CPNS/placebo P-Value
	Mean (S.D.)	P-Value	Mean (S.D.)	P-Value	
0	4.48 (1.09)		4.33 (1.07)		
4	4.48 (1.09)	1.000*	4.30 (1.05)	0.323	0.323
8	4.39 (1.06)	0.006*	4.30 (1.05)	0.323	0.074
12	4.34 (1.08)	0.003*	4.29 (1.04)	0.183	0.039*

Table 7 Variance value in skin wrinkle parameters between CPNS and placebo groups

| Parameter | Time (week) | Variance value | | CPNS/placebo *P*-value |
		CPNS group (*n* = 54)	Placebo group (*n* = 46)	
R_a	4	−1.337 ± 1.668	−0.410 ± 1.631	0.006*
	8	−0.646 ± 1.480	−0.314 ± 1.571	0.281
	12	−1.173 ± 1.442	−0.437 ± 1.853	0.028*
R_{max}	4	−7.993 ± 11.594	−1.585 ± 10.875	0.006*
	8	−3.741 ± 10.069	−0.944 ± 9.920	0.166
	12	−7.029 ± 9.652	−1.713 ± 11.761	0.015*
R_z	4	−6.019 ± 7.711	−1.478 ± 7.615	0.004*
	8	−3.317 ± 7.182	−1.147 ± 6.873	0.128
	12	−5.443 ± 6.964	−1.469 ± 8.399	0.011*
R_p	4	−3.974 ± 5.890	−0.514 ± 5.419	0.003*
	8	−2.428 ± 6.221	−0.823 ± 4.701	0.155
	12	−3.738 ± 5.852	−1.043 ± 5.516	0.020*
R_v	4	−4.529 ± 7.887	−1.102 ± 7.364	0.028*
	8	−1.626 ± 6.154	−0.507 ± 7.360	0.409
	12	−3.780 ± 5.987	−0.987 ± 7.724	0.044*

Skin-wrinkling parameters: R_1, skin roughness; R_2, maximum roughness; R_3, average roughness; R_4, smoothness depth; R_5, arithmetic average roughness. *P*-Values for independent *t*-test and Mann–Whitney *U* test comparisons between values in the CPNS group and the placebo group; *indicates $P < 0.05$.

Table 8 Variance value in skin elasticity parameters between CPNS and placebo groups

| Parameter | Time (weeks) | Variance value | | CPNS/placebo *P*-value |
		CPNS group (*n* = 54)	Placebo group (*n* = 46)	
K	4	−0.007 ± 0.034	−0.005 ± 0.027	0.761
	8	0.026 ± 0.041	0.019 ± 0.040	0.239
	12	0.034 ± 0.039	0.018 ± 0.034	0.031*
Indent	4	0.005 ± 0.043	0.003 ± 0.047	0.820
	8	0.027 ± 0.042	0.008 ± 0.045	0.038*
	12	0.039 ± 0.044	0.013 ± 0.054	0.012*
Alpha	4	0.000 ± 0.004	0.000 ± 0.003	0.731
	8	−0.001 ± 0.004	0.000 ± 0.005	0.260
	12	−0.002 ± 0.004	0.001 ± 0.005	0.012*
CoR	4	0.003 ± 0.020	0.002 ± 0.021	0.726
	8	0.004 ± 0.021	−0.004 ± 0.023	0.060
	12	0.008 ± 0.027	−0.005 ± 0.026	0.013*
Area	4	0.535 ± 7.466	−1.236 ± 7.933	0.253
	8	4.280 ± 7.816	−0.327 ± 8.103	0.005*
	12	6.325± 7.871	−0.173 ± 8.431	0.000*

Skin-elasticity parameters: K, start height; indent, indentation depth; alpha, rate of exponential decay; CoR, coefficient of restitution; area, area under the curve. *P*-Values for independent *t*-test comparisons between values in the CPNS group and the placebo group; *indicates $P < 0.05$.

content is influenced by water attached to HA in the epidermis and the dermis.[63] Koyama *et al.*[64] reported that in Japanese women, the ingestion of hydrolyzed collagen peptides enhanced the absorption rate of water content in the SC. Moreover, the intake of bioactive peptides improves skin conditions such as moisture, sebum, and gloss.[65,66]

Collagen, which is an abundant protein and a major element of connective and structural tissue in mammals, is found in cartilage, bones, and skin, as well as joints, hair, cornea, and blood vessels.[67] Collagen is widely known to help prevent aging and improve bone, ligament, and cartilage health when taken orally.[25,68,69] Due to its excellent moisture retention capability, it has been widely used as a cosmetic material for enhancing skin elasticity and moisture. However,

because collagen is a large-size protein with a high molecular weight of about 300 kDa, there is an ongoing controversy over its absorption rate; the latest trend in related research has thus been to develop functional materials with collagen peptides whose bioavailability has been enhanced by reducing their molecular weight using enzymatic hydrolysis. For the investigation of physicochemical properties of collagen hydrolysates, the CPNS used here was prepared from fish scales, and manufactured by hydrolyzing the fish scale collagen by treatment with fungal proteases.

This clinical study found that oral administration of CPNS significantly improved skin hydration parameters after 4 weeks of intake, while no changes from baseline were observed in the placebo group at 4, 8, and 12 weeks. However, we did not

observe a significant difference in TEWL between the test and placebo groups. In our *in vitro* study, we aimed to estimate the beneficial effects of CPNS on the generation of HA as well as the generation of related proteins and genes associated with HA synthesis using human adult low-calcium high-temperature (HaCaT) keratinocytes.[70] The results of real-time PCR expression tests demonstrated that CPNS dose- and time-dependently induces the up-regulation of the HAS2 gene, which plays a crucial role in the down-regulation of the HYAL1 gene, related to HA degradation as well as HA synthesis. Moreover, western blotting results showed that the CPNS treatment led to improvements in parameters related to HAS2. These results demonstrate that in HaCaT cells, CPNS accelerates the synthesis of HA *via* the down-regulation of HYAL1 and the up-regulation of HAS2, which results in the effects of CPNS supplementation on skin moisture contents.[70]

Excessive UV irradiation causes skin photoaging and is responsible for the generation of MMP-1 followed by the disassembly of type 1 and 3 collagen in the skin.[71–74] The effect of CPNS on the concentration of MMP-1 induction in the supernatants of HDF cell cultures under ultraviolet B (UVB)-irradiation has been estimated, and it has been found that excessive UVB irradiation causes MMP-1 synthesis while CPNS considerably reduces the concentration of MMP-1 in a dose-dependent manner. In our skin wrinkling evaluation, we also verified if the response to the administration of CPNS were in line with the findings of an earlier *in vivo* study where oral ingestion of CPNS in UVB-irradiated mice inhibited MMP-1 and resulted in improvements in skin moisture contents and wrinkle formation.[10]

Type 1 collagen is the core component in the skin dermis, the massive fibers generally known as collagen.[75] Other previous clinical studies showed that collagen hydrolysate intake for 4 to 12 weeks affects skin elasticity.[4,66] In this study, CPNS enhanced skin elasticity and wrinkles after 12 weeks of administration. These distinct time points of efficacy might be explained by various factors, such as the collagen hydrolysate constituents of the peptide sequences, physical elements, environmental variables, and the plan of study. Our previous studies have shown that long-term administration of collagen hydrolysate is related to beneficial effects on skin health. Moreover, other collagen-related research has shown that nutraceutical supplements should be used to acquire notable efficacy, given that their bioactive ingredients can be fortified and thereby control the transduction of several cell signals in marked tissues.[65] The CPNS product administered here is suitable for increasing absorption rates and functional properties caused by the specific bioactive peptide Gly-Pro. After peroral administering CPNS to Sprague-Dawley rats, the plasma concentrations of dipeptide forms, Gly-Pro and Pro-Hyp, increased dramatically. Particularly, the Gly-Pro dipeptide is absorbed considerably faster than other CPNS-originated bioactive peptides such as Gly-Pro-Hyp tripeptide and Pro-Hyp dipeptide. According to Yamamoto *et al.*, Gly-Pro detected a notably faster Tmax than the other bioactive peptides of collagen hydrolysate, such as Pro-Ala, Pro-Hyp, Gly-Pro-Ala, and Gly-Pro-

Hyp with intraperitoneal administration.[71] Moreover, the absorption of bioactive dipeptides from the collagen hydrolysate into the skin may activate the movement and synthesis of fibroblasts.[7,76] These findings indicate that the rapid absorption of the Gly-Pro dipeptide occurs in the bloodstream and that it is promptly distributed to the organs because of the much smaller size of Gly-Pro compared to other peptides. Moreover, the Gly-Pro dipeptide has been found to increase the generation of type 1 procollagen as well as to strongly inhibit MMP-1 production in HDF cells.[10] Collectively, the high concentrations of both Gly-Pro and Pro-Hyp in the bloodstream after oral intake of CPNS could protect against skin photoaging. Our earlier *in vitro* study showed that CPNS treatment restricted MMP-1 production and procollagen synthesis, and the current clinical study shows that CPNS administration improves skin moisture, desquamation, elasticity, and wrinkle formation in women.[10] Skin hydration significantly influences skin desquamation and elasticity, because the structural alterations in elastin formation only occur in the water-bound protein form.[77] The improved skin hydration parameters that we investigated following the administration of CPNS may therefore have affected the decrease in skin desquamation and increase in skin elasticity that we observed. No side effects of CPNS administration were observed, and the vital signs in the CPNS group were in the normal range.

5. Conclusions

Oral ingestion of CPNS, 1650 mg per day for 12 weeks, significantly ameliorated several skin parameters in women aged 30–60 years. We observed a significant increase in skin hydration and a reduction in skin desquamation after 4 weeks of administration and reduced wrinkling after 12 weeks, as well as improved elasticity after 8 weeks. Since this clinical study only included women between the ages of 30 and 60 years, further studies are required for males of different ages. The cosmetic functional food market has been expanding rapidly, and CPNS may be used as a safe, economical, and efficient substance for nutraceutical foods, to ameliorate skin wrinkling, hydration, desquamation, and elasticity. Synthetically, these results suggest that Gly-Pro- and Pro-Hyp-containing CPNS may be an alternative supplement for anti-photoaging in the nutraceutical or nutricosmetic industry.

Author contributions

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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