Research Article

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Ingestion of bioactive collagen hydrolysates enhance facial skin moisture and elasticity and reduce facial ageing signs in a randomised double-blind placebo-controlled clinical study

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Abstract

BACKGROUND: Several human studies have demonstrated occurrence of two major collagen peptides, prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly), in human peripheral blood. Some *in vitro* studies have demonstrated that Pro-Hyp and Hyp-Gly exert chemotaxis on dermal fibroblasts and enhance cell proliferation. Additionally, Pro-Hyp enhances the production of hyaluronic acid by dermal fibroblasts. These findings suggest that the amounts of Pro-Hyp and Hyp-Gly in blood are important factors to show the efficacy of collagen hydrolysates on skin health.

RESULTS: We conducted a randomised double-blind placebo-controlled clinical trial of ingestion of two types of collagen hydrolysates, which are composed of different amounts of the bioactive dipeptides Pro-Hyp and Hyp-Gly, to investigate their effects on the improvement of skin conditions. Improvement in skin conditions, such as skin moisture, elasticity, wrinkles, and roughness, were compared with a placebo group at baseline, and 4 and 8 weeks after the start of the trial. In addition, the safety of dietary supplementation with these peptides was evaluated by blood test. Collagen hydrolysate with a higher content of bioactive collagen peptides (H-CP) showed significant and more improvement than the collagen hydrolysate with a lower content of bioactive collagen peptides (L-CP) and the placebo, in facial skin moisture, elasticity (R2), wrinkles and roughness, compared with the placebo group. In addition, there were no adverse events during the trial.

CONCLUSION: This study demonstrated that the use of the collagen hydrolysate with a higher content of Pro-Hyp and Hyp-Gly led to more improvement in facial skin conditions, including facial skin moisture, elasticity, wrinkles and roughness.

Keywords: clinical study; collagen hydrolysate; collagen peptide; skin health; bioactive peptides; skin roughness

INTRODUCTION

There are many products that have beneficial effects on skin health available in the current health food market. Collagen hydrolysates have been developed over the past two decades as supplements or cosmeceutical products for use worldwide. Although a number of studies have demonstrated the efficacy of collagen hydrolysates on skin conditions, little is known regarding what peptides derived from collagen hydrolysates function as bioactive peptides and have physiological effects, which is fundamental information for the maintenance of healthy facial skin.

Denatured collagen forms a substance called gelatin, which when treated by enzymatic hydrolysis results in what are called collagen hydrolysates. Collagen hydrolysates are soluble in water at ambient temperature due to low molecular weight, and possess no gelation ability. This high solubility of collagen hydrolysates allow for the development of products in drink- and jelly-stick-form.

Pharmacological bioavailability trials revealed that two types of collagen dipeptides, prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly), were available at high concentrations for several hours in the human blood stream after oral administration.[1–5] It has been demonstrated that ^{14}C-labelled Pro-Hyp reaches the skin and bone tissues rapidly after ingestion by mice.[6] Moreover, in a clinical study, Pro-Hyp was identified in urine after collagen hydrolysate intake.[7] These findings suggest that Pro-Hyp and Hyp-Gly are stable and relatively resistant to peptidases in the blood,[4,8] and are able to reach the skin tissues.

In addition, some *in vitro* studies demonstrated the physiological function of Pro-Hyp and Hyp-Gly in skin dermal fibroblasts. Pro-Hyp stimulated chemotaxis of dermal fibroblasts[9] and both Pro-Hyp and Hyp-Gly enhanced cell proliferation activity.[10,11] Additionally, it was observed that Pro-Hyp enhanced the production of hyaluronic acid in dermal fibroblasts.[11]

Pro-Hyp and Hyp-Gly involvement in such physiological roles may be important to improve the efficacy of collagen hydrolysates on the maintenance of skin health. The current study, a randomised double-blind placebo-controlled clinical trial, was carried out to evaluate the efficacy of two types of collagen hydrolysates with differing contents of the bioactive dipeptides Pro-Hyp and Hyp-Gly.

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MATERIALS AND METHODS

Investigational products

The placebo, maltodextrin TK-16, was purchased from Matsutani Chemical Industry Co., Ltd. (Itami, Japan). Two forms of collagen hydrolysates derived from fish gelatin, which were composed of different ratios of free-formed Pro-Hyp and Hyp-Gly, were used in this study. One form of collagen hydrolysate (L-CP) had a low ratio of dipeptide-to-product content, with about 0.1 g kg^{-1} of product. The other form of collagen hydrolysate (H-CP) had a high ratio of dipeptide-to-product content, with more than 2 g kg^{-1} of product. These products were provided by Nitta Gelatin Inc. (Osaka, Japan), and are commercially available under the Wellnex brand. Each 5 g test sample was packed in an aluminium sachet and could not be distinguished by the subjects or investigators.

Study design

This clinical study was conducted in the Shanghai Skin Disease Hospital (Shanghai, China), under the supervision of Dr Xuemin Wang, MD. Randomised administration of the products was carried out in 85 Chinese female subjects who were shown to have no medical issues by blood test performed prior to the study.

The randomised double-blind placebo-controlled study consisted of three groups: Placebo, L-CP and H-CP. Participants were randomly assigned to one of the three groups in a 1:1:1 ratio using a computer generated randomisation schedule. This study was conducted from February to April in 2012. At the start of the trial, each group contained 28 or 29 subjects. Five-gram samples were ingested orally in hot milk, coffee, or any other beverages, once a day after dinner for 8 weeks. Efficacy was assessed at baseline, week 4 and week 8. The amount of daily protein except for collagen peptides was not confined in the study but sustainable intake amount was continued through this trial.

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the ethics committee of Shanghai Skin Disease Hospital, and written informed consent was obtained from all subjects participating in the study. Selection criteria were: age between 35 to 55 years, subject conscious of their dry and rough skin, body mass index less than 30, not regularly using other supplements or health foods, no treatment with sex hormones over the prior 3 months, and not pregnant. The subjects were advised to avoid excessive eating, drinking, exercise, strong sunburn, change in lifestyle, and change cosmetics.

Physiological measurements of the skin

Instrumental measurements of skin condition were evaluated at three points: at baseline prior to regular ingestion (baseline), and after 4 weeks and 8 weeks of ingestion. The subjects washed off their make up by conventional methods, and were acclimatised for 30 min in the waiting lounge at a constant temperature of 20 ± 2 °C and humidity of 50 ± 5% before facial skin evaluation.

Skin moisture

The change of the dielectric constant measured by an electrical capacitance method was used as an estimate of the amount of skin moisture at the cheek and canthus using a Corneometer CM820 (Courage and Khazaka, Cologne, Germany). Three measurements were taken and averaged.

Skin elasticity

Skin elasticity was measured by the suction method using a Cutomater SEM575 (Courage and Khazaka). Decompression suction was carried out for 5 s with a pressure of 300 mbar and a mouth diameter of 2 mm. The return rate, R2 (skin elasticity: Ua1/Uf1), after expansion was assessed at the cheek and canthus.

Skin wrinkles and roughness

Analysis of the cutaneous surface of the area from the cheek to the canthus was conducted using a VisioFace SSA (Skin Surface Analysis; Courage and Khazaka) on the following items: number of wrinkles, wrinkle area, wrinkle depth, and roughness.

Table 1. Panel demographics

Group	Number of subjects: Baseline	Dropout	Week 8	Mean age at week 8*
Placebo	28	2	26	42.31 ± 4.80
L-CP	29	1	28	43.25 ± 4.06
H-CP	28	2	26	42.31 ± 4.92

*Data are expressed as mean ± SD.
L-CP, lower content of bioactive collagen peptides; H-CP, higher content of bioactive collagen peptides.

Statistical analysis

Comparison of skin moisture, elasticity, and VisioFace SSA data at different time points within a group were carried out with paired Student's *t*-test. Comparison between the two experimental (H-CP and L-CP) and placebo groups was performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for evaluation of significance. Comparison of skin moisture and elasticity between the groups was performed using the difference of these variables before ingestion and after 4 weeks (changing rate week 4) or 8 weeks (changing rate week 8) of ingestion.

Significance was defined as $P < 0.05$ using the data analysis software SPSS Ver. 13.0 (IBM Inc., Armonk, NY, USA). Each value was expressed as the mean ± standard deviation (SD).

RESULTS

Panel demographics

Five subjects dropped out over the course of the study, due mainly to difficulty in visiting the hospital. There was no significant difference in age between the groups (Table 1). None of the subjects involved in the study demonstrated any dietary problems.

Skin moisture

Skin moisture results are summarised in Table 2. Skin moisture at the cheek and canthus in both the L-CP and H-CP groups showed a significant increase between baseline and weeks 4 and 8 ($P < 0.05$), while the placebo group did not show such an increase. In the L-CP group, skin moisture at the canthus was significantly higher than the placebo group by week 8. On the other hand, both cheek and canthus skin moisture in the H-CP group was significantly higher by week 8 ($P < 0.05$) when compared to the placebo group.

The change of skin moisture from baseline, namely changing rate (%) week 4 and 8, at the cheek and canthus in both the L-CP and H-CP groups showed a significant increase compared to the placebo group ($P < 0.05$).

Moreover, the change in skin moisture from baseline in the H-CP group was significantly greater ($P < 0.05$) at the cheek by week 8 and at the canthus by weeks 4 and 8, when compared to the L-CP

Table 2. Facial skin moisture throughout treatment

Group	Baseline	Week 4	Changing rate (%), week 4	Week 8	Changing rate (%), week 8
Cheek					
Placebo	25.53 ± 14.31	26.68 ± 15.96	4.5 ± 14.92	25.42 ± 14.88	−0.43 ± 14.54
L-CP	23.25 ± 12.51	27.73 ± 12.88*	19.27 ± 15.14†	28.63 ± 12.57*	18.79 ± 12.85†
H-CP	23.14 ± 12.40	29.08 ± 12.05*	25.67 ± 23.55†	33.53 ± 12.52*†	30.99 ± 16.78†‡
Canthus					
Placebo	72.49 ± 11.58	71.76 ± 10.73	−1.01 ± 7.27	70.85 ± 10.20	−2.31 ± 6.87
L-CP	70.11 ± 11.25	75.56 ± 9.57*	7.77 ± 8.91†	78.42 ± 8.21*†	10.60 ± 11.55†
H-CP	65.50 ± 11.75†	75.87 ± 10.82*	13.67 ± 22.78†‡	82.78 ± 7.47*†	20.87 ± 10.75†‡

Data are expressed as mean ± SD, in arbitrary units.
*Intragroup comparison ($P < 0.05$, vs. baseline).
†Intergroup comparison ($P < 0.05$, vs. placebo group).
‡Intergroup comparison ($P < 0.05$, L-CP group vs. H-CP group).
'Changing rate (%)' shows the changing rate in % figures between baseline and the time after ingestion, baseline, which was calculated by the equation: (score after ingestion − score at baseline) × 100/score at baseline.

Table 3. Facial skin elasticity (R2) throughout treatment

Group	Baseline	Week 4	Changing rate (%), week 4	Week 8	Changing rate (%), week 8
Cheek					
Placebo	0.736 ± 0.060	0.750 ± 0.041	1.90 ± 6.59	0.738 ± 0.045	0.27 ± 7.34
L-CP	0.739 ± 0.058	0.745 ± 0.044	0.81 ± 7.71	0.749 ± 0.039	1.35 ± 7.44
H-CP	0.725 ± 0.058	0.751 ± 0.059*	3.59 ± 5.74	0.767 ± 0.058*†	5.79 ± 7.59†‡
Canthus					
Placebo	0.735 ± 0.121	0.681 ± 0.088*	−7.35 ± 13.61	0.697 ± 0.087*	−5.17 ± 10.48
L-CP	0.689 ± 0.138	0.673 ± 0.099	−2.32 ± 13.93	0.677 ± 0.105	−1.74 ± 17.13
H-CP	0.721 ± 0.124	0.737 ± 0.106	2.22 ± 10.26†	0.785 ± 0.097*†	8.88 ± 13.18†‡

Data are expressed as mean ± SD, in arbitrary units.
*Intragroup comparison ($P < 0.05$, vs. baseline).
†Intergroup comparison ($P < 0.05$, vs. placebo group).
‡Intergroup comparison ($P < 0.05$, L-CP group vs. H-CP group).
'Changing rate (%)' shows the changing rate in % figures between baseline and the time after ingestion, baseline, which was calculated by the equation: (score after ingestion − score at baseline) × 100/score at baseline.

group. Additionally, the changing rate of H-CP showed a two-fold increase in the L-CP group by week 8 in cheek moisture and by weeks 4 and 8 in canthus.

Skin elasticity (R2)

Skin elasticity (R2) results are summarised in Table 3. The placebo group showed elasticity of the canthus decreased significantly between baseline and weeks 4 and 8. The L-CP group showed no significant improvement in facial skin elasticity between baseline and weeks 4 and 8, and no significant differences between the placebo group at weeks 4 and 8. On the other hand, in the H-CP group, elasticity of the cheek increased significantly between baseline and weeks 4 and 8, as well as the elasticity of the canthus by week 8. Skin elasticity of both the cheek and canthus in the H-CP group was significantly higher ($P < 0.05$) than in the placebo group by week 8. Moreover, improvement of elasticity from baseline in the H-CP group was significantly higher ($P < 0.05$) than the placebo group by week 4 at the canthus and by week 8 at both the cheek and canthus. Furthermore, there was a significant difference in change rate of elasticity improvement at both the cheek and canthus between the L-CP and H-CP groups by week 8 ($P < 0.05$).

Skin surface analysis by VisioFace SSA

Skin surface analysis results by VisioFace SSA are summarised in Table 4. In the L-CP group, wrinkle area by weeks 4 and 8 was reduced significantly ($P < 0.05$), and roughness also improved significantly ($P < 0.05$) by week 8, when compared to baseline.

On the other hand, the H-CP group showed significant improvement compared to baseline in many categories, including the number of wrinkles by week 8, and wrinkle area, wrinkle depth, and roughness by weeks 4 and 8. Moreover, comparison between the H-CP and placebo groups showed significant differences ($P < 0.05$) in the number of wrinkles by week 8, and both wrinkle depth and roughness by weeks 4 and 8.

Additionally, there were significant differences ($P < 0.05$) between the H-CP and L-CP groups, including the number of wrinkles and wrinkle depth by week 8, and roughness by weeks 4 and 8.

Blood test

Blood test analysis results are shown in Table 5. Each value at baseline and after 8 weeks of ingestion was within the limits of standard values. Furthermore, no adverse effects were observed during the clinical trial.

Table 4. Facial skin wrinkles and roughness throughout treatment

Group	Baseline	Week 4	Week 8
Number of wrinkles			
Placebo	0.021 ± 0.004	0.020 ± 0.003	0.021 ± 0.004
L-CP	0.021 ± 0.005	0.021 ± 0.005	0.020 ± 0.006
H-CP	0.021 ± 0.004	0.021 ± 0.004	0.017 ± 0.005*†‡
Wrinkle area			
Placebo	0.73 ± 0.24	0.73 ± 0.21	0.73 ± 0.22
L-CP	0.69 ± 0.15	0.68 ± 0.14*	0.67 ± 0.15*
H-CP	0.71 ± 0.15	0.68 ± 0.12*	0.65 ± 0.11*
Wrinkle depth			
Placebo	56.79 ± 3.60	56.91 ± 3.06	56.52 ± 2.29
L-CP	56.60 ± 4.48	56.21 ± 4.70	55.93 ± 5.03
H-CP	55.86 ± 2.49	54.08 ± 3.02*†	51.78 ± 3.26*†‡
Roughness			
Placebo	23.69 ± 1.74	23.58 ± 1.60	23.42 ± 1.60
L-CP	23.32 ± 1.42	22.93 ± 1.49	22.32 ± 1.63*†
H-CP	23.15 ± 2.26	21.65 ± 2.23*†‡	20.27 ± 2.18*†‡

Data are expressed as mean ± SD, in arbitrary units.
*Intragroup comparison ($P < 0.05$, vs. baseline).
†Intergroup comparison ($P < 0.05$, vs. placebo group).
‡Intergroup comparison ($P < 0.05$, L-CP group vs. H-CP group).

DISCUSSION

The present study demonstrated that ingestion of H-CP, which contains a higher content of the free-formed bioactive peptides Pro-Hyp and Hyp-Gly, resulted in significantly better improvements in facial skin conditions compared to ingestion of L-CP, which has a lower content of these bioactive peptides. These results suggest that, despite using the same raw material, it may be possible to control the effects of collagen hydrolysates on facial skin conditions by modifying the manufacturing process and thus the dipeptide content. Previous reports have demonstrated the effects of Pro-Hyp and Hyp-Gly on skin dermal fibroblasts as signal transducers, which can stimulate metabolism, migration, proliferation, and production of hyaluronic acid.[9–11] In addition, these dipeptides are absorbed into the blood by peptide transporters of the small intestinal epithelial cells in the human digestive and absorption process.[12] Taking into account the bioavailability of these oligopeptides, we hypothesise that it may be possible to enhance uptake of bioactive peptides like Pro-Hyp and Hyp-Gly by increasing the concentration of free-formed bioactive peptides in collagen hydrolysate products. Another type of collagen hydrolysate product, which we have previously reported on, contains more than 3 g kg^{-1} of Pro-Hyp and Hyp-Gly and may have similar or improved effectiveness in enhancing facial skin moisture, elasticity (R2) and roughness, with as little as half the ingested dose (2.5 g) utilised in the present study.[13] On the other hand, we need to consider an effect of beverage co-ingested with collagen hydrolysate for better absorption of collagen bioactive peptides. In the presence study, we reflected the actual use of powder type of collagen hydrolysate by ingestion with tea, coffee, juice, milk, a kind of hot soup like miso soup, etc. Further studies are needed to better understand the optimum combinations with drink type and general food to enhance the functional effects of collagen hydrolysate.

Skin moisture and elasticity depends on the condition of the extracellular matrix, which consists of primarily collagen, hyaluronic acid, and elastin. In an *in vitro* study using human dermal fibroblast cells, Ohara *et al.*[11] reported that Pro-Hyp enhanced cell proliferation and hyaluronic acid synthesis with up-regulated hyaluronic synthase 2 (HAS2) mRNA levels. In addition, they demonstrated that Pro-Hyp stimulates phosphorylation of signal transducer and activator of transcription 3 (STAT3), which is a fundamental intracellular signaling factor.[11] Recently, we have reported the daily oral administration of Pro-Hyp + Hyp-Gly improved skin barrier dysfunction and moisture in HR-1 hairless mice.[14] These reports suggest that Pro-Hyp and Hyp-Gly have a crucial effect in improving the barrier function to enhance skin moisture. We hypothesise that Pro-Hyp and Hyp-Gly stimulated production of hyaluronic acid in the dermis. Hyaluronic acid has been shown to play crucial roles in skin moisture and elasticity.[15] Additionally, several animal studies demonstrated that oral intake of collagen hydrolysates stimulated the synthesis of type I collagen and other extracellular matrix molecules.[16–18]

Regarding the degree of the efficacy between the cheek and the canthus, moisture and elasticity were slightly better in the canthus. In general, the elasticity and thickness of human skin depends on age and measurement site.[19]

In the present study, the H-CP group showed improvement in the number of wrinkles and depth of wrinkles by VisioFace SSA.

Proksch *et al.*[20] have shown that the synthesis of procollagen Type I and elastin, components of the dermal extracellular matrix, led to a pronounced, statistically significant reduction in eye wrinkle volume in a double blind clinical trial. Their data support the idea that a decline in the number of eye wrinkles and wrinkle depth around the eye area effectively improves eye wrinkles, which was similar to the results of the present study (any data not shown)[21].

Regarding the effect of collagen hydrolysate on facial spots, we have previously reported in a clinical study that collagen hydrolysates help reduce ultra-violet spots after 4 weeks of ingestion.[22] Gu *et al.*[23] reported that hyaluronan plays a beneficial role by interacting with fibroblasts to enhance epidermal morphogenesis in a co-culture system. Okawa *et al.*[24] suggested that induced hyaluronic acid in dermal fibroblasts followed by oral administration of collagen hydrolysate may provide beneficial effects on maintaining epidermal and dermal homeostasis in mice. Additionally, Le Vu *et al.*[25] demonstrated that Pro-Hyp induced an increase in expression of Krtap and Krt genes in keratinocytes in co-culture with fibroblasts. These findings suggest that Pro-Hyp may affect signalling to change the phenotype of keratinocytes through the regulation of dermal cells.

Further studies are needed to better understand the mechanisms of the bioactive peptides, Pro-Hyp and Hyp-Gly, which may be associated with their bioavailability. The findings would contribute not only to a better understanding of collagen hydrolysate but also to further the understanding of fundamental mechanisms in anti-ageing.

CONCLUSIONS

The present study demonstrates that both L-CP and H-CP are effective supplements for the improvement in skin moisture and roughness in women who were conscious of their dry and rough skin. Fortified collagen hydrolysate, H-CP, demonstrated a greater improvement in skin elasticity and reducing wrinkles on facial skin.

The present study is the first of its kind to demonstrate that there is a significant difference between conventional collagen hydrolysate and new types of collagen hydrolysate with higher

Table 5. Blood test of subjects in the clinical study

Item	Unit	Placebo group ($n = 26$)		L-CP group ($n = 28$)		H-CP group ($n = 26$)	
		Baseline	Week 8	Baseline	Week 8	Baseline	Week 8
Total protein	g L^{-1}	75 ± 4	72 ± 4	76 ± 4	74 ± 3	77 ± 5	74 ± 4
Albumin	g L^{-1}	43 ± 2	42 ± 2	45 ± 2	42 ± 2	44 ± 2	42 ± 2
Albumin/globulin	Ratio	2 ± 3	3 ± 9	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.4 ± 9
GPT	IU L^{-1}	20 ± 13	14 ± 6	17 ± 10	18 ± 8	17 ± 10	18 ± 6
ALP	IU L^{-1}	109 ± 36	92 ± 27	106 ± 28	101 ± 27	102 ± 29	100 ± 27
γ-GTP	IU L^{-1}	19 ± 11	18 ± 8	23 ± 11	23 ± 13	20 ± 8	21 ± 8
GOT	IU L^{-1}	22 ± 8	18 ± 4	22 ± 7	19 ± 5	21 ± 7	19 ± 4
LDH	IU L^{-1}	190 ± 24	189 ± 22	195 ± 27	188 ± 26	186 ± 25	202 ± 22
Total bilirubin	μmol L^{-1}	8 ± 4	9 ± 3	7 ± 3	9 ± 4	8 ± 4	10 ± 3
BUN	mmol L^{-1}	5 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 2	5 ± 1
Creatinine	μmol L^{-1}	56 ± 8	60 ± 7	54 ± 8	59 ± 8	56 ± 9	57 ± 7
UA	μmol L^{-1}	256 ± 74	253 ± 34	236 ± 47	251 ± 56	252 ± 60	260 ± 34
CPK	U L^{-1}	88 ± 26	82 ± 24	83 ± 32	77 ± 27	82 ± 31	90 ± 24

Data are expressed as mean ± SD.
GPT, glutamic pyruvate transaminase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyltransferase, GOT, glutamic oxaloacetic transaminase; LDH, *lactate dehydrogenase*; BUN, blood urea nitrogen, UA, urinary acid; CPK, creatinine phosphokinase.

contents of specific bioactive dipeptides such as Pro-Hyp and Hyp-Gly for improvement of human skin conditions.

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Effects of Collagen Peptide Ingestion on UV-B-Induced Skin Damage

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Note

Effects of Collagen Peptide Ingestion on UV-B-Induced Skin Damage

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The effect of daily ingestion of collagen peptide on the skin damage induced by repeated UV-B irradiation was examined. Ingestion of collagen peptide (0.2 g/kg/d) suppressed UV-B-induced decreases in skin hydration, hyperplasia of the epidermis, and decreases in soluble type I collagen. These results suggest that collagen peptide is beneficial as a dietary supplement to suppress UV-B-induced skin damage and photoaging.

Key words: photoaging; type I collagen; skin hydration

Collagen is the most abundant protein in the vertebrate body, comprising about one-third of total protein. Collagen extracted with hot water from animal bone, hide, or fish scales is called gelatin, and its hydrolysate is often called collagen peptide (CP) when used as a supplement. Ingestion of gelatin or CP affects various functions of the body, including bone,[1)] the Achilles tendon,[2)] and skin[3)] and skin appendages.[4)] One of the outer insults that damage the skin is ultraviolet irradiation. Ultraviolet is divided into three categories according to wavelength: UV-A (400–315 nm), UV-B (315–280 nm) and UV-C (<280 nm). Repeated skin exposure to UV-B results in an aged skin phenotype (photoaging), including wrinkle formation. Although ingestion of CP has various beneficial effects on the body including the skin,[3)] it remains unknown whether UV-B-induced skin injury is affected by ingestion of CP. In this study, we administered CP prepared from fish scale to hairless mice repeatedly exposed to UV-B irradiation for 6 weeks, and UV-B-induced skin damage was examined.

All animal experiments were approved by the Ethics Committee of Tokyo University of Agriculture and Technology. Six-week-old male Hos:HR-1 hairless mice (SLC Japan, Tokyo) were housed in collective cages at $20 \pm 2\,^{\circ}C$ on a 12-h light/12-h dark cycle, with free access to water and Labo MR Stock diet (Nosan, Tokyo, Japan). After 5 d of acclimation, mice were divided into three groups (seven mice per group) such that the body weight and hydration of the stratum corneum did not differ significantly among groups. CP (FCP-A, Nippi, Tokyo; derived from scales of *Tilapia zillii*) was dissolved in distilled water at 0.025 g/ml and administered p.o. at 0.2 g/kg, body weight daily. The mice were housed in a stainless steel cage ($5 \times 9 \times 4$ cm) and subjected to UV-B irradiation ($0.3\,mW/cm^2$) emitted from a UV-B lamp (GL20SE; Sakyo Denki, Tokyo). UV-B was irradiated 3 times per week 1 min each time, in the first week. The exposure time was then increased to 2 min × 3 times per week in the 2nd week, 3 min × 3 times in the 3rd week, and 4 min × 2 times in the 4th week, and was finally maintained at 3 min × 7 times in the 5th and 6th weeks (total energy, 0.846 J/mouse).

Hydration of the stratum corneum of the lumbar skin was measured once a week with a Corneometer CM 825 (Courage+Khazaka Electronic, Köln, Germany) after being kept at $20 \pm 2\,^{\circ}C$ and $50 \pm 5\%$ humidity for 2 h. After the 6-week experimental period, the mice were sacrificed by cervical dislocation, and seven skin samples from each group were assembled and subjected to extraction of protein.[1)] The extracted type I collagen was visualized by western blot using rabbit antiserum raised against porcine skin-derived type I collagen[1)] as the first antibody, and horseradish peroxidase-labeled mouse monoclonal IgG anti-rabbit IgG was employed as the second antibody.[1)] Skin samples were also obtained after the 6-week experimental period and were prepared for histological examination. Four sites were randomly selected in sections from each mouse, and the thickness of the epidermis was measured under the microscope using Axio Vision software (version 4.5, Zeiss, München, Germany). The mean of four values for each mouse was used to calculate the mean and SD for each experimental group. Differences in the mean for each group were analyzed by the Tukey-Kramer method using Prism 4 software (MDF, Tokyo).

Throughout the experimental period, body weight did not differ significantly among the non-irradiated mice [UVB(−)], the UV-B-irradiated mice (UVB), and the UV-B-irradiated mice fed CP (UVB+collagen) (data not shown). After 3 weeks, hydration of the stratum corneum in the UVB group was significantly lower than that in the UVB(−) group. However, the hydration of the stratum corneum did not decrease significantly in the UV-B-irradiated mice fed CP (UVB+collagen) compared to UVB(−) (data not shown). After 5 weeks (Fig. 1) or 6 weeks (data not shown), skin hydration in the UVB+collagen group was significantly higher than in the UVB group. The results, in Fig. 1 suggest that ingestion of CP suppresses UV-B-induced change in the outermost region of the skin, the stratum corneum of the epidermis. Therefore, we examined the effects of CP ingestion on the thickness of the epidermis. The thick-

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Abbreviations: CP, collagen peptide; TBS, Tris-buffered saline; PB, phosphate buffer; Pro-Hyp, prolyl-hydroxyproline

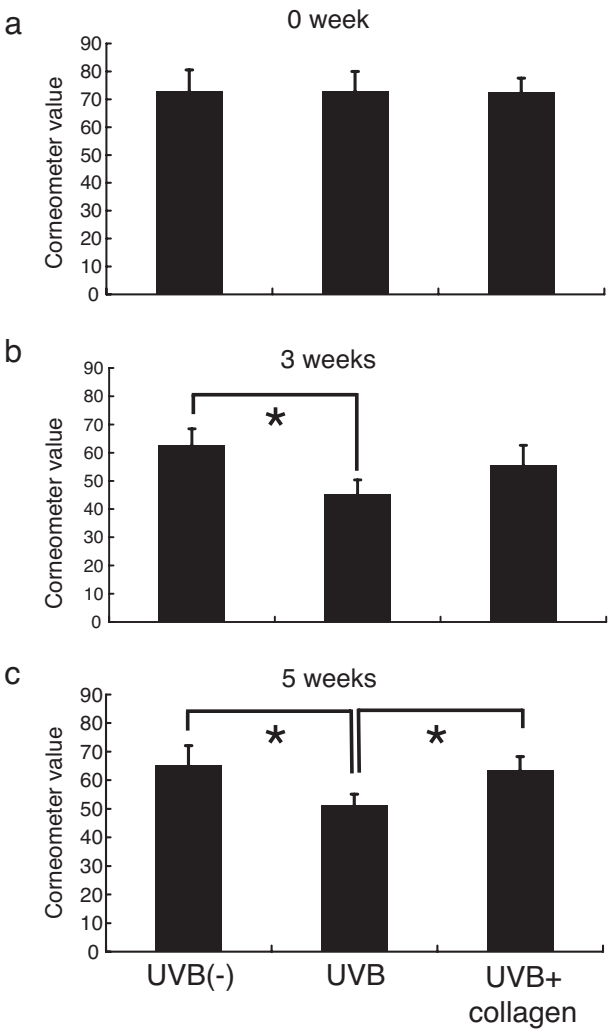

Fig. 1. Effects of UV-B Irradiation and CP Ingestion on Hydration of the Stratum Corneum.

The hydration of the stratum corneum in non-irradiated (UVB(−)) mice, UV-B-irradiated (UVB) mice, and UV-B-irradiated, and CP-administered (UVB+collagen) mice (7 mice per group) was measured with a Corneometer at 1-week intervals. Values before the experiment (a) and at 3 weeks (b) and 5 weeks (c) are shown. *Significant difference between the UVB(−) and UVB or between the UVB and UVB + collagen groups ($p < 0.05$).

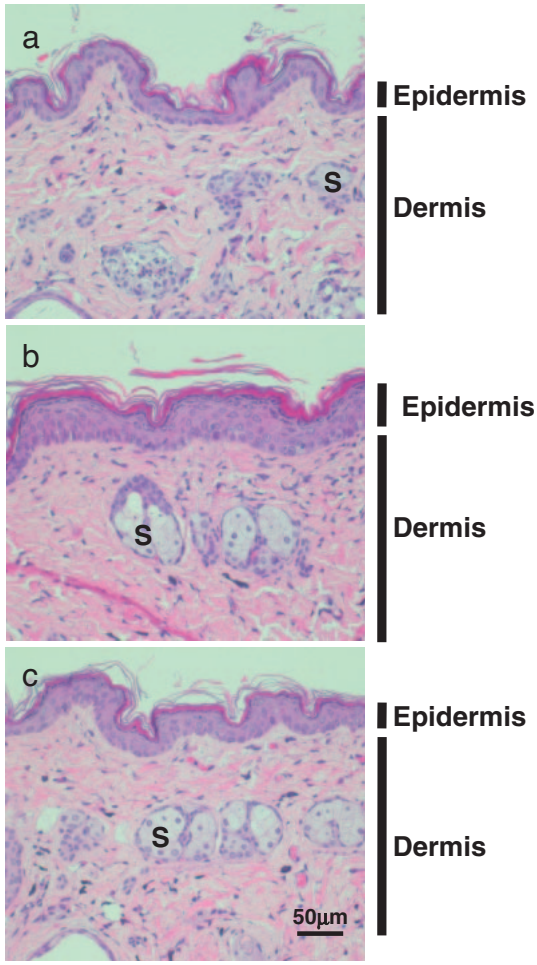

Fig. 2. Histology of the Skin.

Skin samples from each mouse were taken after the 6-week experimental period, and thin sections were stained with Hematoxylin and Eosin. a, the UVB(−) group; b, the UVB group; c, the UVB+collagen group. S, sebaceous gland. Bar, 50 μm.

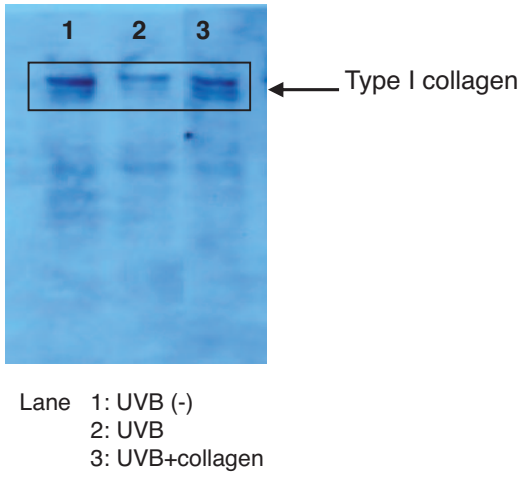

Fig. 3. Western Blot Analysis of Soluble Type I Collagen in the Skin.

Soluble type I collagen was detected using anti-porcine type I collagen antibody. Lane 1, the UVB(−) group; lane 2, the UVB group; lane 3, the UVB+collagen group.

ness of the epidermis in the UVB (Fig. 2b, 37.8 ± 9.5 μm) was significantly larger than that in the UVB(−) group (Fig. 2a, 27.1 ± 5.9 μm) at 6 weeks. In contrast, this increase in epidermal thickness was suppressed by CP ingestion (Fig. 2c, 31.1 ± 5.6 μm), and no significant difference was seen between the UVB(−) group and the UVB+collagen group. The effect of UV-B on the dermis was examined by Western blot analysis of soluble type I collagen.[5] The soluble type I collagen decreased markedly under repeated UV-B irradiation for 6 weeks (Fig. 3, the lanes 1, 2); the relative amount of type I collagen in the UVB group was 47, and the UVB(−) group had a relative value of 100. However, the decrease in soluble type I collagen was evidently suppressed by CP ingestion (relative amount, 117; Fig. 3, lane 3). These results suggest that the skin change in either the epidermis or the dermis is suppressed by daily ingestion of CP. Thus the present study indicates that ingestion of collagen peptide can suppress UVB-induced damage to the skin.

Chronic exposure to UV-B irradiation is known to damage skin structure and function, and induces photoaging, characterized by wrinkles, laxity, roughness, and irregular pigmentation. UV-B irradiation induces the

production of reactive oxygen species (ROS) that damage the anti-oxidative defense mechanisms of the skin, which results in immune suppression, cancer formation, and premature skin aging through the oxidation of cellular and non-cellular components. The mechanism of photoaging has been reviewed by Yaar and Gilchrest.[6] ROS activate cell surface receptors such as epidermal growth factor receptor (EGFR) and lead to intracellular signaling. Expression of nuclear factor AP-1 is induced by activated kinases, UV-B-induced cysteine-rich 61 protein (CYR61), or ROS themselves. Increased AP-1 transcription and its activity interfere with the synthesis of collagen and up-regulate the matrix degrading enzymes MMP-1 and MMP-3. It also blocks the effect of transforming growth factor-β (TGF-β), suppressing collagen gene expression and activating keratinocyte proliferation. Elevated epidermal proliferation (hyperplasia) and decreased collagen production are partly induced by UV-B-induced SMAD7 expression too. On the other hand, UV-B irradiation activates another nuclear factor, NF-κB, which results in the expression of proinflammatory cytokines and MMPs. Thus UV-B irradiation results in epidermal hyperplasia and decreased collagen in the dermis through altered signal transduction and the transcription of relevant genes.

Ingested collagen is digested and absorbed in the digestive tract and appears in the blood partly in a peptide form.[7] Possible explanations for the effect of collagen peptide are as follows: first, antioxidative activity, and second, other biological activity of the CP-derived peptide.

The effects of ingested CP on UV-B-induced skin damage observed in this study may be due to the antioxidative activity of this CP-derived peptide, as CP was found to be antioxidative *in vitro* though it should be elucidated whether the concentration of this CP-derived peptide in the skin is high enough to exhibit antioxidative activity.[8] On the other hand, it was reported that the peptide-form CP in the blood contains oligopeptides such as prolyl-hydroxyproline (Pro-Hyp).[9] It had been reported that Pro-Hyp has a chemotactic activity for cultured fibroblasts.[10] Although it is still unclear whether Pro-Hyp mediates the function of ingested collagen, it is tempting to speculate that Pro-Hyp affects the signal transduction pathway of epidermal keratinocyte and/or dermal fibroblasts and antagonizes the effect of UV-B irradiation. In that event, Pro-Hyp might directly affect the function of epidermal cells. It is also possible that Pro-Hyp affects the function of dermal cells and consequently alters the phenotype of keratinocyte, because the function of the keratinocyte can be regulated by dermal cells.[11] In any case, *in vivo* distribution of the oligopeptide and its biological activities calls for further study.

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〈速報〉

Clinical effects of collagen hydrolysates ingestion on UV-induced pigmented spots of human skin: A preliminary study

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ABSTRACT

To examine the effects of ingesting collagen hydrolysates (CHs) from fish scale (fish CH) and swine skin (swine CH) on UV-induced pigmented spots (UV spots) of human skin, a placebo controlled randomized double blind study was conducted. Thirty-nine females ingested 5 g each of control food, fish CH or swine CH daily for 8 weeks. A within-group comparison showed that both fish and swine CHs significantly decreased the area of UV spots between before and 8 weeks after ingestion. In particular, swine CH significantly decreased the area from an early period of 4 weeks after ingestion.

1. INTRODUCTION

Heat-denatured collagen is gelatin, and the collagen hydrolysate (CH), which is formed by the hydrolysis of gelatin by an enzyme is utilized for food products and cosmetics. It has been demonstrated that following oral ingestion of CH, not only amino acids but also di- and tripeptides enter human bloodstream[1),2)]. In particular, it was reported that large amounts of peptides containing collagen-specific hydroxyproline (Hyp) enter the bloodstream and remain there for a relatively long time[3-5)]. Zague V. reviewed the effects of CH ingestion on skin properties from a pre–clinical point of view, and pointed out that controlled clinical trials are needed in addition to the previous pre-clinical and bioavailability assays[6)]. A clinical study of the effects of oral ingestion of CH on the skin characteristics showed that ingestion of 10 g of a swine-skin-derived CH (swine CH) for 60 days improved the epidermal water absorption capacity as compared with placebo ingestion[7)]. It was also reported that a four-week ingestion of 5 or 10 g of fish-scale-derived CH (fish CH) increased significantly water content in the horny cell layer[8)]. Other researchers observed a significantly increased viscoelasticity of human skin after an eight-week ingestion of food containing 4 g of swine CH[9)]. To examine the effects of ingesting fish and swine CHs on UV-induced pigmented spots (UV spots) of human skin, a double-blind parallel-group study was conducted.

2. MATERIALS AND METHODS

2.1. Test Food. The following three kinds of test food were used: fish CH[2)] (Nitta Gelatin Inc., Osaka, Japan), swine CH[2)] (Nitta Gelatin Inc.), and

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maltodextrin (as control food: placebo, Pinedex TK-16, Matsutani Chemical Industry Co., Ltd., Itami, Hyogo, Japan).

2.2 Study Design and Skin Measurement. A randomized double-blind method was employed with daily ingestion of 5 g of test food for 8 weeks from February to April 2009. This study was performed according to the Helsinki Declaration and was approved by Ethics Committee at Nishi Clinic (Fujiidera, Osaka, Japan) on February 13th 2009. The possible risks of the experiments were explained to all subjects, and informed consent was obtained prior to entry in the study. The subjects were healthy Japanese females aged 35 to 50 years with a subjective symptom of skin roughness or dry skin. Thirty-nine out of 60 subjects who participated in this study were selected on the basis of their medical history, skin condition, and responses to interview questions. They were assigned to the following groups by the randomized double-blind study method. The subjects' mean ages are shown as follows: Fish-CH-fed group: 13 subjects with the mean age of 42.8 ± 3.3 years; Swine-CH-fed group: 13 subjects with the mean age of 42.2 ± 3.9 years; Placebo-fed (maltodextrin) group[8]: 13 subjects with the mean age of 41.8 ± 4.6 years. They cleansed their face as they normally do to remove their makeup, and became acclimated in a room with constant temperature and humidity (temperature, 20℃ ± 2℃; humidity, 50% ± 5%) for 20 minutes. Then, melanin, pores, porphyrin, hemoglobin, speckles, UV spots, wrinkles and redness were examined using VISIA II (Canfield Imaging Systems, Fairfield, NJ, USA). These measurements were performed prior to the ingestion (0 w), 4 weeks (4 w) and 8 weeks (8 w) after the ingestion. For the purpose of evaluating safety and harmful factors, blood samples were collected from the subjects 0 w and 8 w. The samples were then put to hematological and biochemical tests.

2.3. Statistical Analyses. The parameters assessed 8 w after the ingestion of CHs were compared with those for the control to calculate *p*-values. Within-group changes 0 w, 4 w and 8 w after the ingestion were compared using the paired t-test to calculate *p*-values. The significance of differences was determined using JMP8.0.1 (SAS Institute Inc., Cary, NC, USA).

3. RESULTS AND DISCUSSION

VISIA II is a method that detects melanin, pores, porphyrin, hemoglobin, speckles, UV spots, wrinkles and redness on the facial surface by means of image analysis, and evaluates the dimension ratios in proportion to the total measured dimensions as an absolute score. Because initial values for these items differed markedly, it was difficult to carry out a simple comparison between groups 8 w after the ingestion. We hence made comparisons between the changes from 0 w to 4 w and from 0 w to 8 w in each group. For the ingestion of the placebo, no changes were observed in any items assessed. As shown in Table 1 and 2, the area of UV spots decreased significantly between 0 w and 8 w after the ingestion in the fish-CH- and swine-CH-fed groups ($p = 0.034$ and $p = 0.002$, respectively). In particular, the swine-CH-fed group significantly decreased the area from an early period of 4 w after the ingestion ($p = 0.016$) (Table 1 and 2). No abnormalities in blood test results were observed in association with their participation in this study (data not shown), thus substantiating the safety of ingesting the used fish and swine CHs.

In this clinical study, a within-group comparison shows that both fish and swine CHs significantly decrease the area of UV spots 8 w after the ingestion. However, this study has the following study limitation: these three groups each consisting of n = 13, were already different groups statistically. Therefore, further study is needed.

An orally-ingested CH is more likely to be

Table 1. Changes in scores of parameters assessed using VISIA Ⅱ

	Fish CH		
	0 w	4 w	8 w
Melanin	9.01 ± 1.49	8.71 ± 1.47	8.38 ± 1.46
Pores	1.59 ± 1.00	1.52 ± 1.07	1.41 ± 0.87
Porphyrin	0.44 ± 0.85	0.62 ± 1.28	0.27 ± 0.54
Hemoglobin	1.03 ± 0.46	0.99 ± 0.50	1.00 ± 0.48
Speckles	1.89 ± 0.75	1.96 ± 0.82	1.87 ± 0.81
UV spots	4.20 ± 1.93	4.07 ± 1.98	3.71 ± 1.89*
Wrinkles	0.86 ± 0.78	1.04 ± 0.97	0.77 ± 0.77
Redness	1.33 ± 0.90	1.22 ± 0.88	1.12 ± 0.74
	Swine CH		
	0 w	4 w	8 w
Melanin	8.43 ± 2.27	8.27 ± 2.49	8.38 ± 2.13
Pores	1.74 ± 1.12	1.68 ± 1.03	1.53 ± 0.89
Porphyrin	0.30 ± 0.33	0.32 ± 0.37	0.29 ± 0.36
Hemoglobin	1.44 ± 1.02	1.35 ± 0.92	1.57 ± 1.04
Speckles	2.47 ± 1.82	2.55 ± 1.88	2.44 ± 1.82
UV spots	4.39 ± 2.87	3.91 ± 2.60*	3.44 ± 2.38**
Wrinkles	0.58 ± 0.34	0.94 ± 0.53	0.59 ± 0.45
Redness	1.41 ± 0.88	1.58 ± 0.77	1.18 ± 0.74
	Control food		
	0 w	4 w	8 w
Melanin	9.32 ± 1.60	9.23 ± 1.44	9.06 ± 1.57
Pores	1.19 ± 0.56	1.18 ± 0.33	1.19 ± 0.34
Porphyrin	0.23 ± 0.32	0.17 ± 0.21	0.19 ± 0.22
Hemoglobin	1.17 ± 0.63	1.03 ± 0.59	1.11 ± 0.67
Speckles	2.52 ± 1.44	2.11 ± 0.91	2.08 ± 0.94
UV spots	4.95 ± 3.06	4.79 ± 2.42	3.99 ± 2.42
Wrinkles	0.79 ± 0.73	1.11 ± 1.16	0.66 ± 0.51
Redness	1.01 ± 0.53	1.08 ± 0.51	0.94 ± 0.33

Unit is % area. The data are shown as the mean ± SD, n=13 in each goup.
Each within-group comparison between before (0 w) and after (4 w and 8 w) the ingestion using paired t-test * $p<0.05$, ** $p<0.01$

transported to human dermal and epidermal tissues via the peripheral blood vessels in the form of di- and tripeptides after being absorbed into the blood. Proly-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly) are two major components of them[1-5]. Pro-Hyp was reported to stimulate cell proliferation, cell growth and hyaluronic acid synthesis in cultured dermal fibroblasts[10), 11)]. Hyp-Gly also enhanced the cell growth of mouse primary fibroblasts in a higher extent than Pro-Hyp[4)]. These

Table 2. Respective p values according to the corresponding t tests

	Fish CH		Swine CH		Control food	
	0 w vs 4 w	0 w vs 8 w	0 w vs 4 w	0 w vs 8 w	0 w vs 4 w	0 w vs 8 w
Melanin	0.444	0.139	0.447	0.855	0.870	0.669
Pores	0.555	0.124	0.525	0.086	0.914	0.971
Porphyrin	0.438	0.179	0.734	0.794	0.446	0.603
Hemoglobin	0.666	0.755	0.398	0.200	0.459	0.338
Speckles	0.583	0.902	0.213	0.747	0.233	0.241
UV spots	0.538	0.034*	0.016*	0.002**	0.826	0.208
Wrinkles	0.457	0.762	0.070	0.958	0.215	0.453
Redness	0.304	0.092	0.151	0.081	0.525	0.525

In each group, the corresponding t-test was used to compare with before the ingestion; * $p<0.05$, ** $p<0.01$.

peptides are suggested to modulate cells and the extracellular matrix proteins of human skin. We therefore believe that these peptides from the CHs used in this study decrease the area of UV spots of human skin via modulating the dermis and probably epidermis. The mechanisms underlying the effects after ingesting used CHs remain to be elucidated.

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