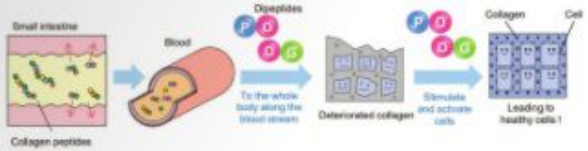
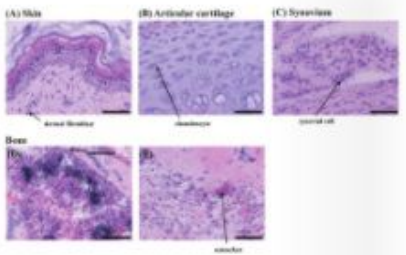
คอลลาเจนไดเปปไทด์มีขนาดโมเลกุลที่เล็กกว่าคอลลาเจนไตรเปปไทด์และคอลลาเจนตามธรรมชาติ อีกทั้งสามารถถูกดูดซึมได้ทั้งในรูปแบบกรดอะมิโนและไดเปปไทด์ [2, 3] ส่งผลให้คอลลาเจนไดเปปไทด์มีอัตราเร็วในการดูดซึมที่ลำไส้และชีวประสิทธิผล (Bioavailability) สูงกว่าคอลลาเจนชนิดอื่น [4]

ผลการศึกษาด้านเภสัชจลนศาสตร์จากงานวิจัยหลายฉบับบ่งชี้ว่า prolyl-hydroxyproline (Pro-Hyp หรือ PO) และ hydroxyprolyl-glycine (Hyp-Gly หรือ OG) สามารถถูกดูดซึมที่ลำไส้และอยู่ในกระแสเลือดได้หลายชั่วโมงหลังจากได้รับ คอลลาเจนไดเปปไทด์ 2 ชนิดดังกล่าวทางช่องปาก [3, 5]





รูปที่ 1 ภาพรังสีแสดงการกระจายของ [^{14}C] PO ในเนื้อเยื่อบริเวณต่างๆ ของหนูทดลองหลังจากได้รับ [^{14}C] PO ทางช่องปาก เป็นเวลา 30 นาที [6]

Figure 1 Autoradiographs of rat tissue sections obtained at 30min after oral administration of [^{14}C] PO. [6].

Kawaguchi Tomoaki และคณะ ได้ทำการศึกษาโดยให้อาหาร ^{14}C-labelled PO (PO ที่ถูกติดฉลากด้วย [^{14}C]) แก่หนูทดลองโดยการยัดลงกระเพาะอาหาร พบว่า ^{14}C-labelled PO สามารถกระจายไปยังเซลล์เป้าหมาย (Target cells) ได้แก่ dermal fibroblast ที่ผิวหนัง chondrocyte ที่กระดูกอ่อนผิวข้อ synovial cell ที่เยื่อบุข้อ osteoblast และ osteoclast ที่กระดูกได้ โดย ^{14}C-labelled PO มีลักษณะเป็นอนุภาค (particles) สีดำ ดังแสดงในรูปที่ 1 [6]

ไดเปปไทด์ คู่ PO และ OG สามารถกระตุ้นการเพิ่มจำนวนของเซลล์ไฟโบรบลาสต์ (Fibroblasts) ซึ่งมีบทบาทในการสังเคราะห์คอลลาเจน และ extracellular matrix ได้ นอกจากนี้พบว่าไดเปปไทด์ คู่ PO สามารถกระตุ้นการสังเคราะห์กรดไฮยารูโลนิก (Hyaluronic acid; HA) ซึ่งเป็น polyanionic natural polymer ที่ร่างกายสังเคราะห์ขึ้นได้เอง มีบทบาทในการช่วยหล่อลื่นและลดการยึดตึงบริเวณข้อต่อ [7] Kumar Suresh และคณะ พบว่าคอลลาเจน ที่มี PO และ OG สามารถช่วยบรรเทาอาการปวดข้อโดยลด WOMAC score (รูปที่ 2) ในผู้ป่วยข้อเข่าเสื่อมได้อย่างมีนัยสำคัญเมื่อเทียบกับยาหลอก [8]

มีรายงานการศึกษาผลของการรับประทานคอลลาเจน 2 ชนิดที่มีปริมาณ bioactive dipeptides ได้แก่ PO และ OG แตกต่างกันต่อคุณสมบัติของผิวหน้าในด้านความชุ่มชื้น ความยืดหยุ่น ริ้วรอย และความหยาบกร้าน โดยทำการศึกษาเปรียบเทียบกับยาหลอก (Maltodextrin) ในอาสาสมัคร 85 คน ที่รับประทานผลิตภัณฑ์ทดสอบแตกต่างกันเป็นระยะเวลา 8 สัปดาห์ พบว่าคอลลาเจนสามารถฟื้นฟูคุณสมบัติของผิวในด้านต่างๆ ได้แก่ ความชุ่มชื้นของผิวหน้า ความยืดหยุ่นของผิว (R2) ริ้วรอยและความหยาบกร้านของผิวได้ในลักษณะ dose-dependent manner โดยคอลลาเจนชนิดที่มีปริมาณ PO และ OG สูงกว่า สามารถฟื้นฟูคุณสมบัติของผิวได้ดีกว่าคอลลาเจนชนิดที่มีปริมาณ PO และ OG ต่ำกว่าและยาหลอก [9]

จากหลักฐานการศึกษาทั้งหมดที่กล่าวมาข้างต้นชี้ให้เห็นถึงความเป็นไปได้ที่ไดเปปไทด์ คู่ PO และ OG จะสามารถถูกดูดซึมและกระจายไปยังอวัยวะเป้าหมายเพื่อให้คุณประโยชน์ต่อร่างกาย โดยเฉพาะอย่างยิ่งประโยชน์ต่อสุขภาพผิวและข้อต่อ [8, 9] ดังนั้น การเลือกรับประทานผลิตภัณฑ์คอลลาเจนไดเปปไทด์ ที่มีองค์ประกอบของ PO และ OG ในปริมาณที่เหมาะสมต่อวันจึงเป็นอีกหนึ่งแนวทางในการฟื้นฟูสุขภาพผิวและข้อต่อ

ณัฐพร อมรนพรัตนกุล
Nattaporn Amornopparattanakul
Nutraceutical Ingredient Specialist
Pure Chemicals Co., Ltd.
Nattaporn@purechemicals.com

คอลลาเจนไดเปปไทด์ที่มี PO และ OG เป็นองค์ประกอบ: นวัตกรรมของคอลลาเจนเพื่อสุขภาพผิวและข้อ

คอลลาเจนไดเปปไทด์ (Collagen dipeptides) คือ คอลลาเจนที่มีองค์ประกอบของกรดอะมิโน 2 หน่วยเชื่อมกันด้วยพันธะเปปไทด์ มีความแตกต่างจากคอลลาเจนไตรเปปไทด์ (Collagen tripeptides) ซึ่งเกิดจากการต่อกันของกรดอะมิโนอย่างน้อย 3 หน่วย และต่างจากคอลลาเจนตามธรรมชาติ (Native collagen) ซึ่งมีโครงสร้างโมเลกุลขนาดใหญ่ทำให้ดูดซึมได้ยาก [1]

ข้อมูลเพิ่มเติม/Additional Information
ข้อมูลจากเอกสารแบรนด์ Wellnex™ collagen dipeptides ผลิตโดย Nitta Gelatin Inc. โดย Pure Chemicals Co., Ltd. เป็นผู้จัดจำหน่ายเพียงเจ้าเดียวในประเทศไทย
https://www.purechemicals.com

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COLLAGEN DIPEPTIDES CONTAINING PO AND OG: THE INNOVATIVE COLLAGEN FOR SKIN AND JOINT HEALTH

Collagen dipeptides are forms of collagen containing two units of amino acid linked together by a peptide bond. They differ from collagen tripeptides as the tripeptides consist of three units of amino acids linked by two peptide bonds. The collagen dipeptides also differ from native collagen, which has a large molecular structure, thus limiting intestinal absorption [1].

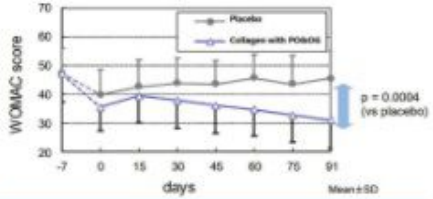
Collagen dipeptides have a smaller molecular size than collagen tripeptides and native collagen and can be absorbed in both single amino acid and dipeptide forms [2, 3]. These two factors result in higher intestinal absorption rates and bioavailability [4].

Several results from pharmacokinetics studies have indicated that prolyl-hydroxyproline (Pro-Hyp or PO) and hydroxyprolyl-glycine (Hyp-Gly or OG) can be absorbed by the intestine and remain in the bloodstream several hours after oral ingestion [3, 5].

Kawaguchi Tomoaki and his team conducted a study by feeding ^{14}C-labelled PO (PO radiolabeled with [^{14}C]) into experimental rats stomachs. They found that ^{14}C-labelled PO was distributed into target cells i.e. dermal fibroblasts in skin, chondrocytes in articular cartilage, synovial cells in synovium, and osteoblasts and osteoclasts in bone. ^{14}C-labelled PO presented as black particles as shown in Figure 1 [6].

PO and OG dipeptides have induced fibroblast proliferation which subsequently played a role in collagen and extracellular matrix synthesis. In addition, the dipeptide PO induced hyaluronic acid (HA) synthesis. HA is a polyanionic natural polymer which can be synthesized in the human body where it assists in joint lubrication, thus reducing joint stiffness [7]. Kumar Suresh and his team found that collagen containing PO and OG reduced joint pain by significantly reducing the WOMAC score (Figure 2) in knee osteoarthritis patients when compared to a placebo [8].

Two types of collagen, with different amounts of PO and OG bioactive dipeptide content, were studied in terms of their effect on facial skin hydration, elasticity, wrinkles, and roughness in comparison to a placebo (maltodextrin). Eighty-five volunteers were assigned to administer the test products for 8 weeks. Both of these collagens improved various skin properties, including facial moisture, skin elasticity (R2), wrinkles and roughness of the skin in a dose-dependent manner. The collagen with the higher PO and OG content improved these properties more than the collagen with lower PO and OG content and the placebo [9].



รูปที่ 2 คะแนน WOMAC ของอาสาสมัครกลุ่มที่ได้รับยาหลอก (เส้นสีเทา) และกลุ่มที่ได้รับคอลลาเจนที่มีองค์ประกอบของ PO และ OG (เส้นสีน้ำเงิน) ที่เวลาต่างๆ (คะแนน WOMAC ที่สูงกว่าคะแนนที่บ่งชี้ถึงความรุนแรงของอาการข้อเข่าเสื่อมที่มากกว่า) ผลการศึกษาบ่งชี้ว่าการรับประทานคอลลาเจนที่มีองค์ประกอบของ PO และ OG สามารถช่วยลดค่า WOMAC ลงได้อย่างมีนัยสำคัญ

Figure 2 The WOMAC scores of placebo (grey line) and collagen with PO and OG (blue line) at different time points. The higher WOMAC score indicated more severity of symptom in osteoarthritis. The results indicated that oral administration of collagen with PO and OG significantly reduced WOMAC score.

The above evidence supports the possibility that PO and OG dipeptides can be absorbed and distributed to target organs to provide health benefits, especially for skin and joint health [8, 9]. Thus, selection of products containing PO and OG collagen dipeptides for administration in appropriate daily amounts is worthy of consideration for the improvement of skin and joint problems.

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

Naoki Inoue,[a*] Fumihito Sugihara[a] and Xuemin Wang[b]

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.

* *Correspondence to: N Inoue, Peptide Division, Nitta Gelatin Inc., 2-22 Futamata, Yao, Osaka 581-0024, Japan. E-mail: na-inoue@nitta-gelatin.co.jp*

a *Peptide Division, Nitta Gelatin Inc., Osaka, Japan*

b *Shanghai Skin Disease Hospital, Shanghai, China*

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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Hydroxyproline-containing dipeptides and tripeptides quantified at high concentration in human blood after oral administration of gelatin hydrolysate

SATOMI ICHIKAWA[1], MASASHI MORIFUJI[1], HIROKI OHARA[1], HITOSHI MATSUMOTO[1], YASUO TAKEUCHI[1] & KENJI SATO[2]

[1]*Food and Health R&D Laboratories, Meiji Seika Kaisha, Ltd, Sakado-shi, Saitama, Japan, and,* [2]*Graduate School of Life and Environment Sciences, Kyoto Prefectural University, Kyoto, Japan*

Abstract

Several hydroxyproline (Hyp)-containing food-derived collagen peptides were identified in human blood after oral ingestion of gelatin hydrolysates. However, these types of peptides were not quantified in human plasma. In this report, a sensitive LC-MS/MS method was introduced for simultaneous quantitative analysis of Hyp-containing peptides. All peptide concentrations were determined accurately, with all coefficients of determination (r^2) >0.999. The method achieved detection and quantification limits of 0.01 pmol/ml and 12.5–1,000 pmol/ml in plasma, respectively. Concentrations were quantified for nine Hyp-containing peptides in human plasma by this method, identifying Pro-Hyp (C_{max} = 60.65 ± 5.74 nmol/ml) as the major constituent of food-derived collagen peptides, while the minor components were Ala-Hyp-Gly, Ser-Hyp-Gly, Ala-Hyp, Phe-Hyp, Leu-Hyp, Ile-Hyp, Gly-Pro-Hyp, and Pro-Hyp-Gly (C_{max} from 23.84 to 0.67 nmol/ml). Thus a total of nine Hyp-containing peptides in human plasma were successfully quantified by this approach. The concentration of Hyp-containing peptides is substantially higher than that following oral administration of other peptides.

Keywords: *Collagen, hydroxyproline, plasma, hydroxyproline-containing peptide, quantification*

Introduction

Collagen is a major constituent of connective tissues of animals, birds, and fish. Gelatin, a denatured form of collagen, is prepared on an industrial scale from these animals (Shrieber and Seybold 1993). Collagen has a unique triple helix configuration with a repeating sequence $(Gly\text{-}X\text{-}Y)_n$, with X and Y being mostly proline and hydroxyproline (Hyp) (Ramshaw and Shah 1998; Bos et al. 1999). Gelatin-based food derivatives obtained from animals, especially fish and pigs, have been attracting worldwide attention as health-food ingredients. Significant amounts of Hyp-containing peptides were found to be present in the peripheral blood of human volunteers after oral ingestion of porcine skin gelatin hydrolysates (Iwai et al. 2005). Recently, some Hyp-containing peptides were also detected in human blood after ingestion of hydrolysate from fish scales (Ohara et al. 2007a). The major constituents of Hyp-containing

Corespondence: Hiroki Ohara, PhD, Food and Health R&D Laboratories, Meiji Seika Kaisha, Ltd, 5-3-1 Chiyoda, Sakado-shi, Saitama 350-0289, Japan. Tel: 81 49 284 7569. Fax: 81 49 284 7598. E-mail: hiroki_ohara@meiji.co.jp

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peptides that remained in the blood were identified as Ala-Hyp, Pro-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Phe-Hyp, Pro-Hyp-Gly, Gly-Pro-Hyp, Ile-Hyp and Leu-Hyp. These collagen-based peptides represent functional peptides involved in various physiological activities. For example, Pro-Hyp and Gly-Pro-Hyp exert chemotactic effects on fibroblasts, peripheral blood neutrophils (Postlethwaite et al. 1978; Laskin et al. 1986) and monocytes (Postlethwaite and King 1976) in cell culture systems. Gly-Pro-Hyp is also suggested to be involved in platelet aggregation (Knight et al. 1999). Recently, Shigemura et al. (2009) indicated that Pro-Hyp enhanced mice fibroblast cell proliferation. Therefore, it could be assumed that food-derived collagen peptides in blood may be involved in some of the biological activities suggested by animal and human experiments.

However, no quantitative analysis of peptides has been reported in an earlier human absorption study. Previous methods to quantify these types of peptides involved subtraction of free Hyp and Hyp-containing peptide concentrations after determining the Hyp concentration in plasma using reverse-phase high-performance liquid chromatography (Iwai et al. 2005; Aito-Inoue et al. 2006; Ohara et al. 2007a). Thus, quantification of food-derived Hyp-containing peptides has been evaluated by semi-quantitative methods such as determining the recovery of Hyp in each peptide peak. Moreover, it has been difficult to detect and isolate small amounts of food-derived peptides that do not have any marker amino acids or modified amino acids from animal and human blood after oral ingestion.

To overcome this problem, a sensitive and convenient liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) method was introduced for simultaneous analysis of Hyp-containing peptides in human plasma after oral ingestion of fish-scale gelatin hydrolysate. Recently, digested mixtures of collagen type II and type I containing many specific peptides and common peptides were analyzed by MS/MS sequencing (Zhang et al. 2006). However, only tetrapeptides to nonapeptides were analyzed to define the collagen type, whereas specific dipeptides and tripeptides from human blood samples were not analyzed.

The goal of the present study was to quantify food-derived Hyp-containing peptides in a complex matrix such as human plasma.

Materials and methods

Gelatin hydrolysate

Enzymatic hydrolysate of fish-scale gelatin was a kind gift from Nitta Gelatin, Ltd (Osaka, Japan). This preparation was of food grade and it can be obtained commercially. The average molecular weight of peptides in this gelatin hydrolysate, which did not contain the free form of Hyp, was about 5,000 Da.

Chemicals

Acetonitrile (high-performance liquid chromatography grade), pentafluoropropionic acid, and trichloroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp, and Phe-Hyp were purchased from Kokusan Chemical (Tokyo, Japan), and Pro-Hyp and Gly-Pro-Hyp were purchased from Bachem (Bubendort, Germany).

Preparation of standard samples

Standards prepared for nine Hyp-containing peptides (Ala-Hyp, Pro-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Phe-Hyp, Pro-Hyp-Gly, Gly-Pro-Hyp, Ile-Hyp and Leu-Hyp) were dissolved in blank human plasma or water, mixed and diluted to 1 nmol/ml, 5 nmol/ml, 10 nmol/ml, 25 nmol/ml, 50 nmol/ml and 100 nmol/ml. They were then mixed with equal amounts of 5% (w/v) trichloroacetic acid. After filtration with a 4-mm, 0.22-μm PVDF filter (Millipore, Bedford, MA, USA), 5 μl of the resulting filtrate was injected into the LC-MS/MS system.

Human study design

The present study was performed according to the Helsinki Declaration and was approved by the Ethical Committee of Meiji Seika Kaisha, Ltd, Food and Health R&D Laboratories. Five healthy male volunteers with no incidence of gelatin allergy (33.0 ± 5.6 years old and 69.8 ± 7.4 kg body weight) participated in the study. Subjects did not consume any food or beverages except for water in the 12-h period prior to the experiment. On the morning of the experiment, the subjects were fasting and each subject orally ingested the fish-scale gelatin hydrolysate concentrate (0.385 g/kg body weight) in water (20% w/v). Three hours after ingestion of the gelatin hydrolysate preparation, the subjects were served a collagen-free lunch, consisting of only a rice ball with salt. Approximately 5 ml venous blood was collected from the cubital vein before (0 h) and 0.5 h, 1 h, 2 h, 4 h, and 7 h after ingesting the hydrolysate. Plasma was obtained after blood centrifugation at 880 x *g* for 10 min at 4°C and stored in tubes at −80°C until analysis was performed.

Pre-treatment of blood sample for LC-MS/MS

The plasma was de-proteinized by adding equal amounts of 5% (w/v) trichloroacetic acid. The supernatant was then centrifuged at 14,010 x *g* for 10 min at 4°C. After filtering through a 4-mm, 0.22-μm PVDF filter, 5 μl of the resulting filtrate was injected into the LC-MS/MS system.

LC-MS/MS analysis

Samples were analyzed by LC-MS/MS. The LC analysis was performed using an ACQUITY UPLC system (Waters, Milford, MA, USA). A particular Octa Decyl Sillica (ODS) column that retains polar compounds tightly was better adapted to this analysis than the conventional ODS column that was used previously. Therefore an ACQUITY UPLC HSS T3 column (2.1 x 50 mm, 1.7 μm; Waters) was used for the separation. Gradient elution was carried out with 0.05% (v/v) pentafluoropropionic acid and acetonitrile at a constant flow rate of 0.3 ml/min. The gradient profile with the following proportions (v/v) of acetonitrile was applied (*t* (min), % acetonitrile): (0 min, 0%), (4 min, 0%), (9 min, 25%), (9.01 min, 80%), (10 min, 80%) (3 min: time was required to reach initial conditions). The column temperature was maintained at 40°C. The Quattro Premier XE tandem quadrupole mass spectrometer was used in positive ion electrospray mode. The ion source was operated at 120°C with a capillary voltage of 3.5 kV. Nitrogen was employed for the desolvation gas at 400°C and 850 l/h. The mode of acquisition was multiple reaction monitoring (MRM) at an argon collision gas pressure of 5.0 x 10^{-3} mbar. The list of peptides and the MRM transitions, along with

Table I. MRM method parameters.

Peptide	Retention time (min)	MRM transition
Ala-Hyp	1.7	203.3 > 132.1
Pro-Hyp	2.7	229.2 > 70.2
Ala-Hyp-Gly	2.1	260.3 > 189.0
Ser-Hyp-Gly	1.9	276.3 > 189.1
Phe-Hyp	7.8	279.3 > 119.9
Pro-Hyp-Gly	4.0	286.3 > 189.0
Gly-Pro-Hyp	5.6	286.3 > 154.7
Ile-Hyp	7.0	245.3 > 131.9
Leu-Hyp	7.2	245.3 > 131.9

Cone voltage: Pro-Hyp, 25 V; others, 20 V. Collision energy, 15 eV.

the retention times, cone voltages, and collision energies for the method, are presented in Table I. The data were acquired using MassLynx Software version 4.1 (Waters) and were processed using the TargetLynx application manager.

Pharmacokinetic analysis

Analysis of blood concentration–time data was carried out with a non-compartment model using WinNonlin Professional (version 5.2.1; Pharsight Co., Mountain View, CA, USA). The total area under the concentration–time curve ($AUC_{0-7\ h}$) was calculated by the trapezoidal rule based on the plasma concentrations up to the time of final measurement using the WinNonlin Professional program.

Results

Analysis of standards

Figure 1 shows typical MRM chromatograms of the nine Hyp-containing peptide standards. The total run-time per sample was only 13 min. The sensitivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was defined as the concentration of the nine Hyp-containing peptides with a signal-to-noise ratio of 3, for the chromatographic peaks from 0.01 pmol/ml to 100 nmol/ml, stepwise. The LOQ was the lowest standard concentration with a signal-to-noise ratio of 10. The LOD and LOQ for a 5 μl injection, coefficients of determination and recovery for each of the nine Hyp-containing peptides in plasma are presented in Table II. The method achieved detection and quantification limits of 0.01 pmol/ml and 12.5–1,000 pmol/ml in plasma, respectively. The LOQ was as follows: Ala-Hyp, 225 pmol/ml; Ser-Hyp-Gly, 125 pmol/ml; Ala-Hyp-Gly, 200 pmol/ml; Pro-Hyp, 1000 pmol/ml; Pro-Hyp-Gly, 125 pmol/ml; Gly-Pro-Hyp, 75 pmol/ml; Ile-Hyp, 50 pmol/ml; Leu-Hyp, 12.5 nmol/ml; and Phe-Hyp; 150 pmol/ml.

The linearity of the method was investigated by spiking blank human plasma (obtained before collagen ingestion) with known concentrations of the nine Hyp-containing peptides at six concentration levels ranging from 1 to 100 nmol/ml. The linearity of measurement over the calibration curve range was good for all peptides measured, and all coefficients of determination (r^2) were >0.999. Furthermore, the recovery of standards added to blank human plasma (obtained before collagen ingestion) was investigated with 25 nmol/ml of the nine Hyp-containing peptides, and their recovery rates were 97–100%

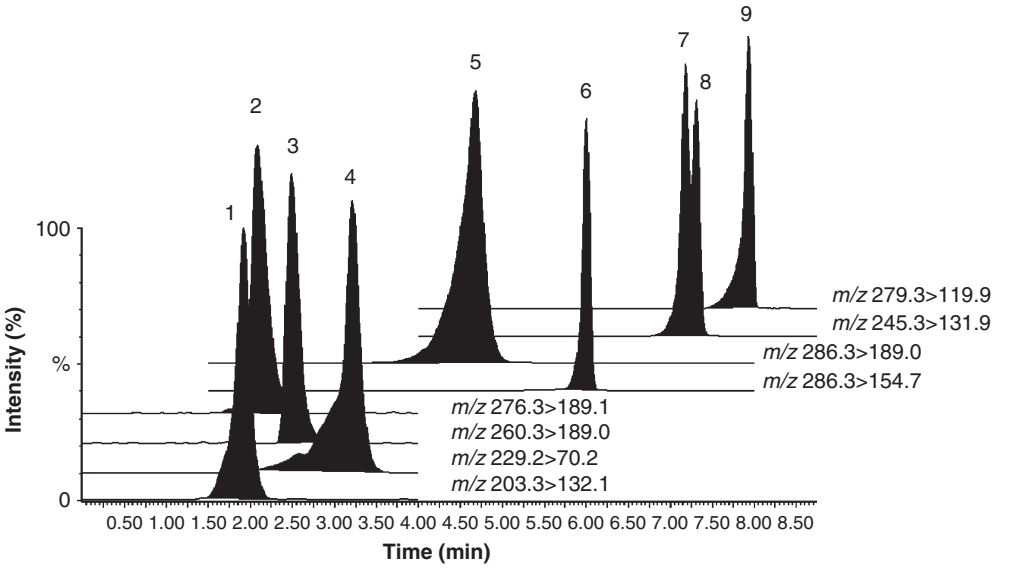

Figure 1. MRM chromatogram of nine Hyp-containing peptides. Peak 1, Ala-Hyp (*m/z* 203.3 > 132.1); peak 2, Ser-Hyp-Gly (*m/z* 276.3 > 189.1); peak 3, Ala-Hyp-Gly (*m/z* 260.3 > 189.0); peak 4, Pro-Hyp (*m/z* 229.2 > 70.2); peak 5, Pro-Hyp-Gly (*m/z* 286.3 > 189.0); peak 6, Gly-Pro-Hyp (*m/z* 286.3 > 154.7); peak 7, Ile-Hyp (*m/z* 245.3 > 131.9); peak 8, Leu-Hyp (*m/z* 245.3 > 131.9); peak 9, Phe-Hyp (*m/z* 279.3 > 119.9).

(Table II). In addition, other concentrations of the nine Hyp-containing peptides at 1 nmol/ml, 5 nmol/ml, 10 nmol/ml, 50 nmol/ml and 100 nmol/ml were investigated. Their recovery rates were 94–107% (data not shown). Therefore, this method is adequate to detect these nine Hyp-containing peptides.

Levels of nine Hyp-containing peptides in human plasma

Figure 2 shows the amounts of the nine Hyp-containing peptides in human plasma after oral ingestion of fish-scale gelatin hydrolysate. Only negligible amounts of each peptide were observed before the ingestion of fish-scale gelatin hydrolysate. In all subjects, the nine Hyp-containing peptides in the plasma increased after oral ingestion and reached a

Table II. Correlation coefficient, recovery, limit of quantification, and detection data obtained from LC-MS/MS analysis of nine Hyp-containing peptides in human plasma (n = 6).

Peptide	Correlation coefficient	Percentage recovery (% relative standard deviation)	LOQ (pmol/ml)	LOD (pmol/ml)
Ala-Hyp	0.999	100 (2)	225	0.01
Ser-Hyp-Gly	0.999	99 (1)	125	0.01
Ala-Hyp-Gly	0.999	99 (3)	200	0.01
Pro-Hyp	0.999	100 (5)	1,000	0.01
Pro-Hyp-Gly	0.999	98 (4)	125	0.01
Gly-Pro-Hyp	0.999	99 (2)	75	0.01
Ile-Hyp	0.999	97 (1)	50	0.01
Leu-Hyp	0.999	99 (1)	12.5	0.01
Phe-Hyp	0.999	99 (3)	150	0.01

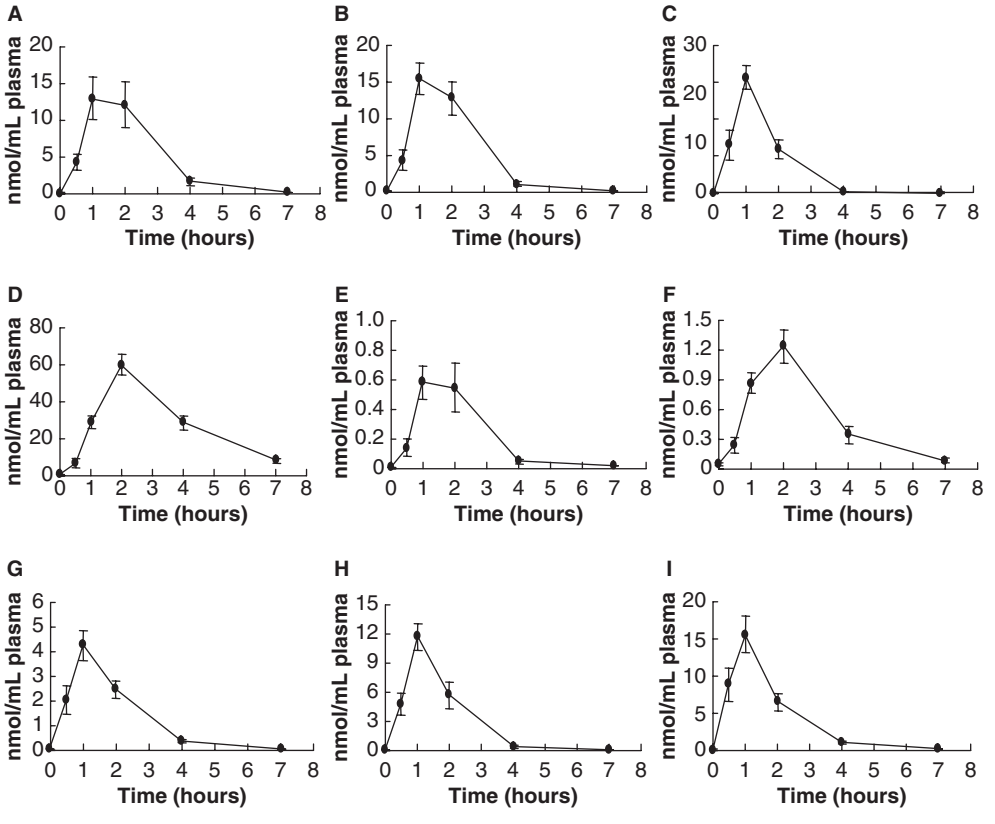

Figure 2. Plasma levels of nine Hyp-containing peptides after oral ingestion of fish-scale gelatin hydrolysate. (a) Ala-Hyp; (b) Ser-Hyp-Gly; (c) Ala-Hyp-Gly; (d) Pro-Hyp; (e) Pro-Hyp-Gly; (f) Gly-Pro-Hyp; (g) Ile-Hyp; (h) Leu-Hyp; (i) Phe-Hyp. Values presented as the mean ± standard error, $n = 5$ subjects.

maximum 1–2 h after ingestion. The T_{max} (h), C_{max} (nmol/ml), and AUC (h nmol/ml) of the nine Hyp-containing peptides are presented in Table III. The T_{max} values for Pro-Hyp and Gly-Pro-Hyp were reached 2 h after oral ingestion of fish-scale gelatin hydrolysate. On the other hand, the T_{max} values for the seventh through ninth Hyp-containing peptides were from 1 to 1.6 h after oral ingestion of the hydrolysate. The C_{max} in plasma was 60.65 ± 5.74 nmol/ml plasma, and the C_{max} of Pro-Hyp was higher than that of the other eight Hyp-containing peptides. The calculated $AUC_{0-7\,h}$ of each Hyp-containing peptide was as follows: Ala-Hyp, 34.55 ± 8.48 h nmol/ml; Ser-Hyp-Gly, 36.25 ± 5.26 h nmol/ml; Ala-Hyp-Gly, 37.72 ± 3.98 h nmol/ml; Pro-Hyp, 201.17 ± 18.78 h nmol/ml; Pro-Hyp-Gly, 1.49 ± 0.31 h nmol/ml; Gly-Pro-Hyp, 3.62 ± 0.57 h nmol/ml; Ile-Hyp, 9.06 ± 1.19 h nmol/ml; Leu-Hyp, 21.30 ± 3.36 h nmol/ml; and Phe-Hyp, 28.85 ± 4.50 h nmol/ml. This result indicated that Pro-Hyp was the major Hyp-containing peptide in plasma after oral ingestion of fish-scale gelatin hydrolysate, as reported earlier (Ohara et al. 2007a).

Discussion

Several Hyp-containing food-derived collagen peptides were identified in human blood after oral ingestion of gelatin hydrolysates. However, none of these peptides were

Table III. $AUC_{0-7\ h}$ of nine Hyp-containing peptides in human plasma after oral ingestion of fish-scale gelatin hydrolysate.

Peptide	T_{max} (h)	C_{max} (nmol/ml)	$AUC_{0-7\ h}$
Ala-Hyp	1.60 ± 0.24	13.70 ± 2.78	34.55 ± 8.48
Ser-Hyp-Gly	1.40 ± 0.24	16.58 ± 1.72	36.25 ± 5.26
Ala-Hyp-Gly	1.00 ± 0.00	23.84 ± 2.44	37.72 ± 3.98
Pro-Hyp	2.00 ± 0.00	60.65 ± 5.74	201.17 ± 18.78
Pro-Hyp-Gly	1.40 ± 0.24	0.67 ± 0.14	1.49 ± 0.31
Gly-Pro-Hyp	2.00 ± 0.00	1.24 ± 0.17	3.62 ± 0.57
Ile-Hyp	1.00 ± 0.00	4.26 ± 0.60	9.06 ± 1.19
Leu-Hyp	1.00 ± 0.00	11.71 ± 1.35	21.30 ± 3.36
Phe-Hyp	1.00 ± 0.00	15.61 ± 2.46	28.85 ± 4.50

Values presented as the mean ± standard error, $n = 5$ subjects.

quantified in human plasma. In this report, a LC-MS/MS method was introduced to quantify Hyp-containing peptides in human plasma after oral ingestion of fish-scale gelatin hydrolysate. The recovery of standards added to plasma was quantified, confirming that this method could be used to measure concentrations of Hyp-containing peptides without derivatization. In addition, the linearity of the measurements was evaluated, and results confirmed that it was accurate over the calibration curve range for all peptides. Previous approaches to measuring peptides containing Hyp were based on their derivatization with phenyl isothiocyanate (Iwai et al. 2005; Ohara et al. 2007a; Aito-Inoue et al. 2006).

The major constituent of food-derived collagen peptides remaining in blood was confirmed to be Pro-Hyp ($AUC_{0-7\ h}$ = 201.17 ± 18.78 h nmol/ml), while the minor components were Ala-Hyp-Gly, Ser-Hyp-Gly, Ala-Hyp, Phe-Hyp, Leu-Hyp, Ile-Hyp, Gly-Pro-Hyp, and Pro-Hyp-Gly ($AUC_{0-7\ h}$ from 37.72 to 1.49 h nmol/ml). This result indicated that Pro-Hyp was the major Hyp-containing peptide in plasma after oral ingestion of fish-scale gelatin hydrolysate, as reported earlier (Ohara et al. 2007a). In the present study, Pro-Hyp reached its maximum concentration in plasma 2 h after oral ingestion of fish-scale gelatin hydrolysate, while Ala-Hyp and Ala-Hyp-Gly reached their maximum concentrations 1 h after ingestion of the hydrolysate. Another study reported that more than 75% of Pro-Hyp remained 24 h after being added *in vitro* to human serum (Iwai et al. 2005). Therefore, Pro-Hyp can be considered indigestible by human blood.

It is well known that the abundance of the oligopeptide transporter (PEPT-1) in the brush-border membrane of the intestinal epithelium is the principal mechanism for regulation of transport of products of protein digestion (dipeptides and tripeptides). Gly-Pro-Hyp can be partially hydrolyzed by the brush-border membrane-bound aminopeptidase N to remove Gly, and the resulting Pro-Hyp may be transported into small intestinal epithelial cells via the H^+-coupled PEPT-1 (Aito-Inoue et al. 2007). It therefore may be possible for Hyp-containing dipeptides or tripeptides to be absorbed transcellularly, at least partly, via this peptide transporter (Adibi 2003).

After peptide ingestion, dipeptides were detected in human blood, but their concentrations were quite low. Matsui et al. (2002) reported that the dipeptide Val-Tyr was observed in plasma 2 h after oral peptide administration. The maximal Val-Tyr concentration in plasma was 2,041 ± 148 fmol/ml. Morifuji et al. (2009) reported the plasma levels of Val-Leu, Ile-Leu and Leu-Leu after ingestion of soy and whey protein hydrolysate. The maximal Val-Leu, Ile-Leu and Leu-Leu concentrations in

plasma were 25 nmol/l, 40 nmol/l and 6 nmol/l, respectively. In the present study, Pro-Hyp was the major Hyp-containing peptide in plasma after oral ingestion of fish-scale gelatin hydrolysate, and the maximal level in plasma was 60.65 ± 5.74 nmol/ml plasma. The C_{max} of Pro-Hyp was higher than that of Val-Tyr. Stimulation of human fibroblast proliferation and hyaluronan synthesis by Pro-Hyp has been achieved at a concentration of 100 nmol/ml (Ohara et al. 2007b). The amount of Pro-Hyp in plasma 2 h after oral ingestion of fish-scale gelatin hydrolysate is approximately 60 nmol/ml plasma. Therefore, the total Pro-Hyp content in plasma or skin is estimated to reach approximately 100 nmol/ml. This suggests that oral ingestion of collagen can result in biological activities that depend on food-derived Hyp-containing peptides.

Conclusions

Concentrations of nine Hyp-containing peptides were determined in human plasma after oral ingestion of fish-scale gelatin hydrolysate. Pro-Hyp was the major constituent of food-derived collagen peptides, while the minor components were Ala-Hyp-Gly, Ser-Hyp-Gly, Ala-Hyp, Phe-Hyp, Leu-Hyp, Ile-Hyp, Gly-Pro-Hyp, and Pro-Hyp-Gly. The concentration of Hyp-containing peptides is substantially higher than that following oral ingestion of other peptides.

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ORIGINAL ARTICLE

Collagen-derived dipeptide, proline-hydroxyproline, stimulates cell proliferation and hyaluronic acid synthesis in cultured human dermal fibroblasts

Hiroki OHARA,[1] Satomi ICHIKAWA,[1] Hitoshi MATSUMOTO,[1] Minoru AKIYAMA,[2] Norihiro FUJIMOTO,[2] Takashi KOBAYASHI,[2] Shingo TAJIMA[2]

[1]*Food and Health R&D Laboratories, Meiji Seika Kaisha, and* [2]*Department of Dermatology, National Defense Medical College, Saitama, Japan*

ABSTRACT

Orally ingested collagen undergoes degradation to small di- or tripeptides, which are detected in circulating blood 2 h after ingestion. The influence of collagen-derived peptides on dermal extracellular matrix components and cell proliferation was studied using cultured human dermal fibroblasts. Of the various collagenous peptides tested here, the dipeptide proline-hydroxyproline (Pro-Hyp) enhanced cell proliferation (1.5-fold) and hyaluronic acid synthesis (3.8-fold) at a dose of 200 nmol/mL. This was concomitant with a 2.3-fold elevation of hyaluronan synthase 2 (*HAS2*) mRNA levels. Small interfering RNA (siRNA)-mediated knockdown of the *HAS2* gene in human dermal fibroblasts inhibited Pro-Hyp-induced *HAS2* mRNA transcription and cell mitotic activity. Addition of genistein or H7, a protein kinase inhibitor, abolished the Pro-Hyp-induced *HAS2* mRNA stimulation. Pro-Hyp elevated phosphorylation of signal transducer and activator of transcription 3 (STAT3) within a short time period (60 min). These results suggest that Pro-Hyp stimulates both cell mitotic activity and hyaluronic acid synthesis, which is mediated by activation of *HAS2* transcription.

Key words: collagen peptide, fibroblast, hyaluronan, hyaluronan synthases 2, signal transducer and activator of transcription.

INTRODUCTION

Collagen is a major constituent of connective tissues of animals, birds and fish. Gelatin, a denatured form of collagen, prepared on an industrial scale from these materials,[1] is used as a folk medicine to improve joint condition by reducing pain.[2] Some animal experiments and preclinical human trials have also suggested that oral ingestion of gelatin hydrolysate might have beneficial effects. We previously reported that daily ingestion of a type I collagen hydrolysate mixture, including 5 g of fish type I collagen hydrolysate, improved skin moisture content and elasticity.[3] Therefore, we hypothesized that supplementation with collagen hydrolysate potentially changes extracellular matrix (ECM) metabolism in the skin.

It is difficult to understand how collagen, a high molecular weight protein, could be absorbed in the intestine and transported to the dermis. Osser *et al.*[4] determined the bioavailability of collagen hydrolysate after p.o. administration in mice using ^{14}C-labeled hydrolysate. The distribution of labeled amino acids in skin was confirmed and 58% of the peak value of labeled amino acids remained 192 h after administration. Iwai *et al.*[5] identified a small dipeptide, proline-hydroxyproline (Pro-Hyp), in the blood of healthy human volunteers who had ingested porcine skin collagen hydrolysate. Although there are no previous reports describing absorption of collagen hydrolysate

Correspondence: Hiroki Ohara, Ph.D., Food Functionality Research Center, Food and Health R & D Laboratories, 5-3-1 Chiyoda, Sakado-shi, Saitama 350-0289, Japan. Email: hiroki_ohara@meiji.co.jp
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through the intestinal epithelium, it was found that some small peptides, such as carnosine (beta-Ala-L-His), were absorbed from the small intestine.[6] It is well known that the abundance of the oligopeptide transporter (PEPT-1) in the brush-border membranes of intestinal epithelium is the principal regulatory mechanism for transport of protein digestion products (dipeptides and tripeptides).[7] It therefore may be possible for Hyp-containing di- or tripeptides to be absorbed transcellularly, at least partly, through this peptide transporter. We have previously reported that several collagen-derived peptides were detected in human blood 2 h after oral ingestion of fish collagen hydrolysate.[8] The major constituent of collagen-derived peptides that remained in blood was identified as Pro-Hyp (39% of total Hyp-containing peptides), while the minor components were Ala-Hyp (15%), Ala-Hyp-Gly (16%), Pro-Hyp-Gly (5%), Leu-Hyp (2%), Ile-Hyp (2%) and Phe-Hyp (3%).[5,8]

In this report, we studied eight collagen-derived Hyp-containing peptides on cell motility and modulation of ECM proteins using *in vitro* cultured dermal fibroblasts, which are the major ECM-producing cells in skin.

METHODS

Reagents

Hydroxyproline (Hyp) and proline (Pro) were purchased from Nacalai Tesque (Kyoto, Japan). Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp and Phe-Hyp were purchased from Kokusan Chemical (Tokyo, Japan), and Pro-Hyp was from Bachem (Bubendort, Germany). Genistein, sodium orthovanadate and basic fibroblast growth factor (bFGF) were purchased from Wako Pure Chemical Industries (Osaka, Japan). H7 dihydrochloride (H7) was from Sigma (St Louis, MO, USA). (6-^{3}H) Glucosamine/HCl (1,3 TBq/mmol) was purchased from Amersham Bioscience (Piscataway, NJ, USA).

Cell culture

Human dermal fibroblast cells were explanted from a skin biopsy sample from a normal individual. Cells were grown at 37°C in 35-mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) in a humidified atmosphere of 5% CO_2/95% air and were subcultured every 7 days. Cells in passage 5–7 were used.

Cell growth kinetics

Cells were plated at a density of 3.6×10^4 in each well of a 6-well plate and grown for 1 day in 10% FBS-DMEM. On day 1, culture medium was replaced with 1% FBS-DMEM with collagen-derived Hyp-containing peptides (200 nmol/mL). Cells were incubated for a further 6 days. On day 7, cells were trypsinized (0.25%) and cell numbers were counted with a hemocytometer. For a dose-dependent analysis of the Pro-Hyp peptide on cell proliferation, fibroblasts were again plated at a density of 3.6×10^4 in 6-well plates and incubated for 1 day in 10% FBS-DMEM. After a further incubation of 6 days in 1% FBS-DMEM containing Pro-Hyp (0, 50, 100, 200 and 400 nmol/mL), cells were trypsinized and cell numbers were counted. bFGF (50 ng/mL) treatment was used as a positive control.

Glycosaminoglycan synthesis

Cells were grown to confluency in 10% FBS-DMEM and then placed in serum-free DMEM for 24 h. Cells were treated with Pro-Hyp (200 nmol/mL) and (6-^{3}H) glucosamine/HCl for the last 24 h of treatment. Glycosaminoglycans were isolated from the medium and cell layer separately as previously described.[9] Cells were trypsinized and an aliquot was taken for cell counting. (^{3}H)-labeled hyaluronate was electrophoretically resolved on a cellulose acetate membrane. For quantitative determinations, each spot was cut out and dissolved in 1 mL dioxane. Radioactivity was determined with a Beckman LS 9000 liquid scintillation system (Beckman Instruments, Fullerton, CA, USA).

Total RNA isolation, cDNA synthesis and quantitative real-time reverse transcription polymerase chain reaction (RT–PCR) analysis

Total RNA was isolated from human dermal fibroblasts using the RNeasy mini-kit (QIAGEN, Hilden, Germany). Extracted RNA was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at a wavelength of 260 nm. RT was performed using a cDNA synthesis kit (Fermentas, Burlington, ON, Canada). The cDNA was stored at −80°C until subsequent analysis. Real-time PCR was performed using the Applied Biosystems 7900 HT

Real-Time PCR system according to the supplier's recommendations. Primers and probes (TaqMan Assays-on-Demand Gene Expression Products) of the genes for collagen type I (*COL1A1*), type III (*COL3A1*), type IV (*COL4A1*), elastin (*ELN*), hyaluronan synthases 1, 2 and 3 (*HAS1*, *HAS2*, *HAS3*), and β-glucuronidase (*GUS*) were designed by Applied Biosystems from gene sequences obtained from GenBank (accession no.: NM_00008.3 for *COL1A1*, NM_00009.3 for *COL3A1*, NM_001845.3 for *COL4A1*, NM_000501.1 for *ELN*, NM_001523.1 for *HAS1*, NM_005328.1 for *HAS2*, NM_005329.2 for *HAS3* and NM_000181.3 for *GUS*). Relative mRNA amounts of selected genes were calculated using the standard curve method. mRNA quantity was normalized to the amount of *GUS* mRNA in each cDNA sample. TaqMan quantitative RT–PCR results are presented as the mean ± standard error of the mean (SEM).

Small interfering RNA (siRNA)-mediated silencing of *HAS2* expression

Four siRNA duplexes, designed with symmetric 3′TT overhangs to target different nucleotide sequences (no. 1, cat no. SI00075810; no. 2, cat no. SI00075817; no. 3, cat no. SI00075824; no. 4, cat no. SI00075831) of the human *HAS2* gene, and ''allstars'' negative control siRNA were obtained from QIAGEN. Human dermal fibroblast cells were grown to subconfluency in 10% FBS-DMEM (6-well plates). Subconfluent cells were placed in serum-free DMEM and transfected separately with one of four siRNA duplexes or with control siRNA at a final concentration of 5 nmol/L using HiPerFect Transfection Reagent (QIAGEN) according to the manufacturer's instructions. Furthermore, cells were treated with Pro-Hyp (200 nmol/mL) in some of the transfection experiments. The suspension efficiency of each siRNA duplex was examined by measuring *HAS2* mRNA expression levels using real-time RT–PCR 24 h after transfection.

Effect of cell growth kinetics by *HAS2* siRNA transfection

Cells were plated at a density of 3.6×10^4 in each well of a 6-well plate and grown for 1 day in 10% FBS-DMEM. On day 1, cells were transfected with control siRNA or *HAS2* siRNA (no. 4) as described above. Differences in the cell growth kinetics between *HAS2* siRNA and control siRNA transfected cells were examined in the absence or presence of Pro-Hyp (200 nmol/mL) by counting cell numbers on day 7. Cell numbers were determined using the cell counting kit-8 (Dojindo, Kumamoto, Japan).

Effect of protein kinase inhibitors on Pro-Hyp-induced *HAS2* mRNA expression

Confluent cultures of human dermal fibroblasts were washed twice with phosphate buffered saline and placed in serum-free DMEM. Twenty-four hours later, the cells were pre-incubated for 2 h with or without the following inhibitors at optimal concentrations: tyrosine kinase inhibitor (50 μg/mL genistein),[10] serine/threonine kinase inhibitor (80 μmol/L H7)[11,12] and tyrosine phosphatase inhibitor (100 μmol/L sodium orthovanadate).[10] Sodium orthovanadate was used as a control. Cells were then incubated for a further 24 h with or without 200 nmol/mL Pro-Hyp.

Direct monitoring of signal transducer and activator of transcription 3 (STAT3) phosphorylation

Human dermal fibroblasts were plated at a density of 2.0×10^4 in each well of a 96-well plate and grown for 1 day in 10% FBS-DMEM. On day 1, the culture medium was replaced by serum-free DMEM. Twenty-four hours later, cells were treated with 200 nmol/mL Pro-Hyp for 0, 30 or 60 min. The amount of activated (phosphorylated) STAT3 protein was analyzed using the cellular activation of signaling enzyme-linked immunosorbent assay (ELISA) kit for STAT3 Y705 (CASE; SuperArray Bioscience, Frederick, MD, USA) according to the manufacturer's instructions.[13]

Statistics

Statistical analyses were conducted using SPSS ver. 10.02 for Windows with data expressed as mean ± SEM. Data were analyzed by Dunnett's multiple comparison test or an unpaired Student's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

Effects of collagen-derived Hyp containing peptides on cultured human dermal fibroblast proliferation

Human dermal fibroblasts were cultured in the presence of collagen-derived Hyp containing peptides

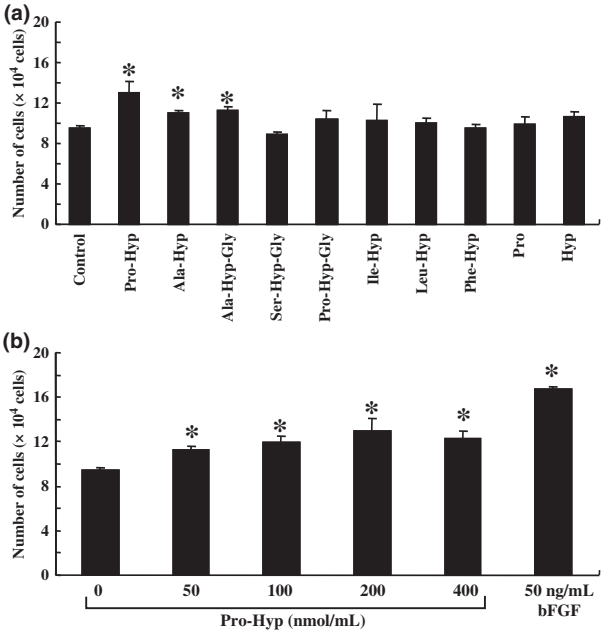

Figure 1. Effects of hydroxyproline (Hyp)-containing peptides on fibroblast proliferation. (a) Fibroblasts were treated with 1% fetal bovine serum Dulbecco's modified Eagle's medium (FBS-DMEM) containing various species of collagen-derived Hyp containing peptides (200 nmol/mL) for 6 days. On day 7, cells were harvested and the number of cells in four different fields was counted with a hemocytometer. Values are mean ± standard error of the mean (SEM) obtained from three independent experiments. *Statistical significance at $P < 0.05$. (b) Fibroblasts were treated with 1% FBS-DMEM containing proline-hydroxyproline (Pro-Hyp) (0, 50, 100, 200 and 400 nmol/mL). On day 7, cell numbers were counted. Values are mean ± SEM from three independent assays. Basic fibroblast growth factor (bFGF) (50 ng/mL) was used as a positive control. *Statistical significance at $P < 0.05$.

(Pro-Hyp, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp, Phe-Hyp, Pro and Hyp) for 6 days, each at a concentration of 200 nmol/mL, and cell numbers were counted (Fig. 1a). The peptides, Pro-Hyp, Ala-Hyp and Ala-Hyp-Gly, stimulated fibroblast proliferation slightly but significantly ($P < 0.05$). Maximal stimulation of cell proliferation (1.5-fold) was observed by Pro-Hyp treatment. On the basis of these cell kinetic results, further studies were focused on the peptide Pro-Hyp. Cell numbers were increased dose-dependently by Pro-Hyp at concentrations ranging 0–200 nmol/mL, but declined at a dose of 400 nmol/mL. A positive control using 50 ng/mL bFGF stimulated cell proliferation 1.8-fold as previously described (Fig. 1b).[14]

Effects of collagen-derived Hyp containing peptides on hyaluronan synthase (*HAS*) mRNA levels

No significant changes in *COL1A1, COL3A1, COL4A1, ELN*, *HAS1* and *HAS3* mRNA levels were

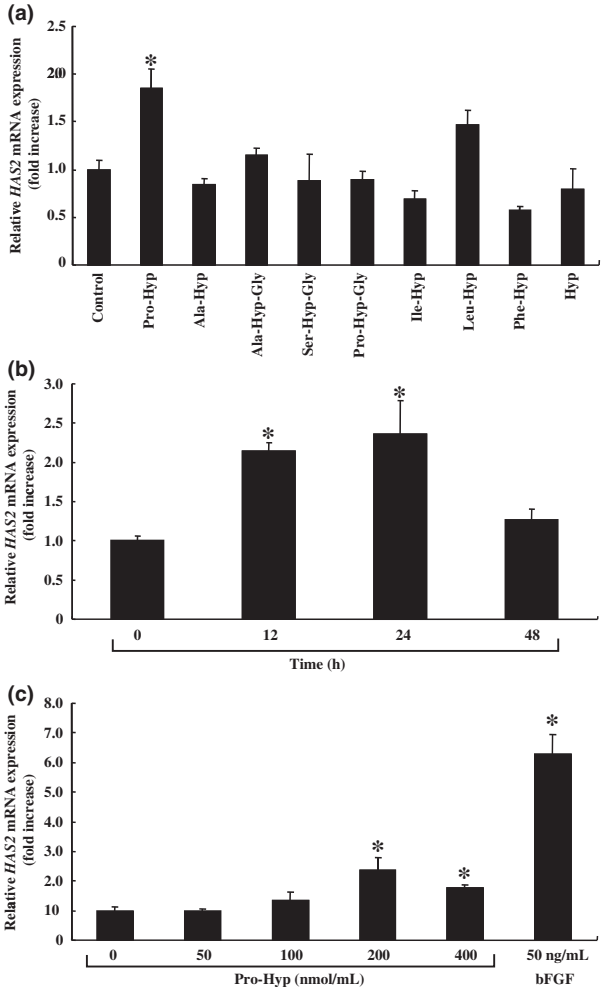

Figure 2. Hyaluronan synthase 2 (*HAS2*) mRNA levels in cultured fibroblasts treated with various hydroxyproline (Hyp)-containing peptides. (a) Fibroblasts were treated with various species of collagen-derived Hyp containing peptides (200 nmol/mL) for 24 h in serum-free Dulbecco's modified Eagle's medium (DMEM). (b) Fibroblasts were treated with proline hydroxyproline (Pro-Hyp) (200 nmol/mL) for 0, 12, 24 and 48 h in serum-free DMEM. (c) Fibroblasts were treated with Pro-Hyp for 24 h at a dose of 0, 50, 100, 200 and 400 nmol/mL in serum-free DMEM. RNA was extracted from the cells and *HAS2* mRNA levels were determined by real-time polymerase chain reaction. Cells treated with basic fibroblast growth factor (bFGF) (50 ng/mL) for 24 h were used as a positive control. Values are mean ± standard error of the mean from triplicate assays. *Statistical significance at $P < 0.05$.

observed by any of the collagen-derived Hyp containing peptides examined here as measured by real-time PCR (data not shown). Relative *HAS2* mRNA content was significantly increased by Pro-Hyp treatment ($P < 0.05$), but was unchanged by other collagen-derived Hyp containing peptides (Fig. 2a). The effect of Pro-Hyp on mRNA expression of *HAS2* was determined by real-time PCR. To accomplish this, 0, 12, 24 and 48 h after addition of Pro-Hyp, cells were harvested, total mRNA isolated and quantitative real-time PCR for *HAS2* was performed. Maximum mRNA levels were reached 24 h after addition of Pro-Hyp (Fig. 2b). The average induction of *HAS2* mRNA at 24 h was 2.3-fold above controls. Based on these results, in subsequent experiments, we determined *HAS2* mRNA levels at 24 h. These results led us to study the effects of Pro-Hyp in detail using various doses of Pro-Hyp. The increase of *HAS2* mRNA levels by Pro-Hyp was dose-dependent up to a concentration of 200 nmol/mL, but declined at 400 nmol/mL (Fig. 2c).

Effects of collagen-derived Hyp containing peptides on hyaluronic acid synthesis

Treatment of cultured human dermal fibroblasts with 200 nmol/mL Pro-Hyp for 24 h increased total hyaluronic acid synthesis (medium + cell) approximately 3.8-fold (Fig. 3), which is comparable to the increase of *HAS2* mRNA levels (Fig. 2c). Treatment with bFGF (50 ng/mL) also increased *HAS2* mRNA levels by approximately 4-fold as previously reported.[15]

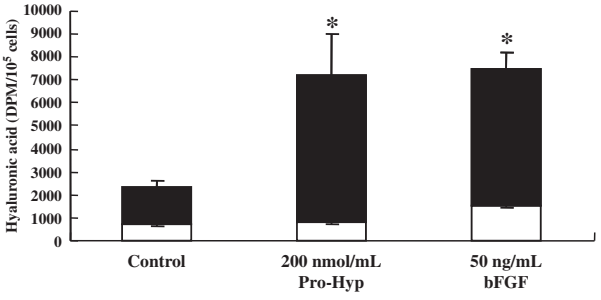

Figure 3. Hyaluronic acid synthesis is increased by proline-hydroxyproline (Pro-Hyp). Fibroblasts were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) containing Pro-Hyp (200 nmol/mL) and (^{3}H) glucosamine for 24 h. Glycosaminoglycans were isolated from medium (closed) and cells (open column) as described in Methods. Total hyaluronic acid synthesis was calculated from the culture medium and cells. Cells treated with basic fibroblast growth factor (bFGF) (50 ng/mL) for 24 h were used as a positive control. Values are mean ± standard error of the mean from five independent experiments. *Statistical significance at $P < 0.05$.

Effect of *HAS2* mRNA expression and cell growth kinetics by *HAS2* siRNA transfection

To further examine the role of *HAS2* in Pro-Hyp-induced cell proliferation, siRNA experiments were performed. We investigated the consequences of suppression of the *HAS2* gene by designing specific *HAS2* siRNA duplexes. Transfection of cells with 5 nmol/L of siRNA duplexes targeted to four different sites of *HAS2* cDNA led to 0.1–0.3-fold *HAS2* transcript suppression irrespective of the species of

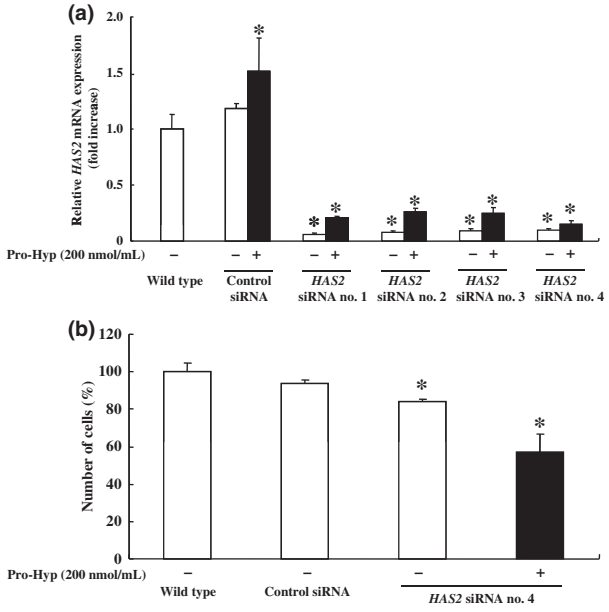

Figure 4. Effects of small interfering RNA (siRNA)-mediated knockdown of hyaluronan synthase 2 (*HAS2*) on the expression level of *HAS2* mRNA and cell growth kinetics. (a) Fibroblasts were transfected with 5 nmol/L of control siRNA or four different *HAS2* siRNA duplexes (nos. 1, 2, 3 or 4) in the absence or presence of proline-hydroxyproline (Pro-Hyp) (200 nmol/mL). The expression levels of *HAS2* mRNA were quantified by real-time reverse transcription polymerase chain reaction 24 h after transfection in serum-free Dulbecco's modified Eagle's medium. Values are mean ± standard error of the mean (SEM) from triplicate assays. *Statistical significance at $P \leq 0.05$. (b) Fibroblasts were transfected with 5 nmol/L of control siRNA or *HAS2* siRNA (no. 4) in the absence or presence of Pro-Hyp (200 nmol/mL). On day 7, cell numbers were counted. Values are mean ± SEM from triplicate assays. *Statistical significance at $P \leq 0.05$.

HAS siRNA (numbers 1–4) compared to wild type (Fig. 4a). We chose *HAS2* siRNA (no. 4) for further experiments examining the effects of *HAS2* siRNA transfection on cell growth. The results showed that *HAS2* siRNA (no. 4) transfection, both in the absence or presence of Pro-Hyp, significantly inhibited cell proliferation but the effect was greater in the presence of Pro-Hyp (Fig. 4b).

HAS2 mRNA induction by Pro-Hyp is dependent on protein kinases

To identify the signaling pathway induced by Pro-Hyp, we examined the effects of inhibitors of kinases on Pro-Hyp-induced *HAS2* mRNA expression in human dermal fibroblasts. Both genistein, a tyrosine kinase inhibitor, and H7, a serine/threonine kinase inhibitor, reduced basal *HAS2* mRNA levels. Threshold cycle (Ct) values of *GUS*, used as housekeeping gene, in genistein (23.97 ± 0.08) and H7 groups (24.01 ± 0.05) were not significantly different than that of the control group (24.03 ± 0.03). This result indicated that cell cytotoxicity is not affected by each inhibitor. These compounds were found to be effective antagonists of Pro-Hyp-induced stimulation. A control using sodium orthovanadate, a tyrosine phosphatase inhibitor, increased basal *HAS2* mRNA levels by approximately 2.4-fold. However, this showed no inhibiting effects on Pro-Hyp-stimulated *HAS2* mRNA expression (Table 1). On the other hand, inhibition of the *HAS2* mRNA expression by genistein and H7 does not depend on restraint of the transcription activity in this experiment and does not confirm it whether it is the thing by reduction of mRNA stability. However, we think that both these are possible.

Table 1. Effects of various kinase inhibitors on proline-hydroxyproline (Pro-Hyp)-induced hyaluronan synthase 2 (*HAS2*) mRNA expression

	HAS2 mRNA level	
Inhibitor	Pro-Hyp$^-$	Pro-Hyp$^+$
None	1 ± 0.05	2.36 ± 0.43*
Genistein	0.32 ± 0.01*	0.33 ± 0.01*
H7	0.02 ± 0.01*	0.02 ± 0.01*
Sodium orthovanadate	2.48 ± 0.78*	6.04 ± 2.14*†

Cells were pre-incubated for 2 h with genistein (50 μg/mL), H7 (80 μmol/L) or sodium orthovanadate (100 μmol/L), then incubated a further 24 h with Pro-Hyp (200 nmol/mL) in the presence or absence of these inhibitors. RNA was extracted from the cells and *HAS2* mRNA levels were determined by real-time polymerase chain reaction. Values are mean ± standard error of the mean from triplicate assays. *Significantly different from control levels (minus inhibitors, minus Pro-Hyp; $P < 0.05$). †Values are significantly different from cells treated with Pro-Hyp alone ($P < 0.05$).

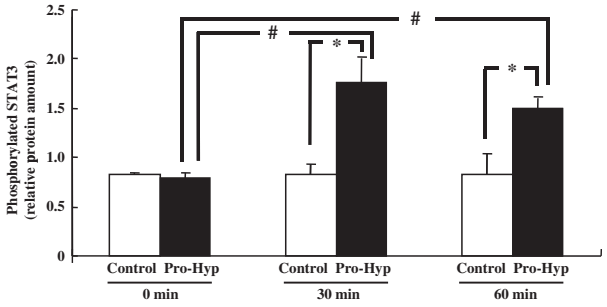

Figure 5. Effect of proline-hydroxyproline (Pro-Hyp) on the levels of phosphorylated signal transducer and activator of transcription 3 (STAT3) in human dermal fibroblasts. Cells were treated with 200 nmol/mL Pro-Hyp for 0, 30 or 60 min. The amount of phosphorylated STAT3 was determined by enzyme-linked immunosorbent assay. Values are mean ± standard error of the mean from triplicate assays. *Significant increase as compared with untreated cells ($P < 0.05$). #Significant increase as compared with 0 min ($P < 0.05$).

Effect of Pro-Hyp on phosphorylated STAT3 levels in human dermal fibroblasts

Recent reports indicate that phosphorylated STAT3 enhanced *HAS2* transcription.[16] Therefore, we examined phosphorylation of STAT3. Pro-Hyp (200 nmol/mL) markedly induced phosphorylation of STAT3 after 30 and 60 min of incubation, while phosphorylation of STAT3 was constant in control (untreated) cells (Fig. 5).

DISCUSSION

All species of collagen-derived Hyp containing peptides, except Pro-Hyp, did not change the expression of matrix-related genes including *COL1A1*, *COL3A1*, *COL4A1*, *ELN* and *HAS1–3* in cultured dermal fibroblasts. Pro-Hyp alone stimulated both cell proliferation and *HAS2* mRNA levels. In *in vitro* studies using cell culture systems, Pro-Hyp has been found to possess chemotactic activity for fibroblasts, peripheral blood neutrophils[17,18] and monocytes,[19] and is involved in collagen-platelet and collagen-cytokine interactions.[20,21] In addition, the presence of Pro-Hyp in human plasma for a relatively

longer period than other collagen peptides after ingestion of collagen hydrolysate[8] suggests its important physiological role in normal and pathological conditions such as wound healing and inflammation.

Maximal stimulation of cell proliferation and hyaluronan synthesis by Pro-Hyp in this experiment was achieved at doses of 200 nmol/mL, which is similar to physiological concentrations because the amount of collagen-derived Hyp containing peptides in plasma 2 h after oral ingestion of fish scale gelatin hydrolysate is reported to be approximately 140 nmol/mL plasma.[8]

The relationship between cell proliferation and hyaluronic acid is intriguing. Pro-Hyp-induced cell proliferation may be associated with Pro-Hyp-induced hyaluronic acid stimulation. Although there is no evidence that hyaluronic acid acts directly on fibroblast mitogenic activity, it has been shown that high levels of hyaluronic acid are present during cell mitosis and that inhibition of hyaluronic acid synthesis leads to prevention of cell mitosis and proliferation.[22,23] These previous studies suggest that hyaluronic acid does not produce mitogenic activity but instead promotes hydration of the extracellular space that aids cell proliferation.[24] In this study, we found that *HAS2* siRNA inhibited both Pro-Hyp-induced *HAS2* mRNA expression and Pro-Hyp-induced cell proliferation in human dermal fibroblasts. Increased fibroblast proliferation by Pro-Hyp in this study may be related to increased hyaluronic acid synthesis.

Hyaluronan, a linear glycosaminoglycan, consists of alternating D-glucosamine residues. Hyaluronan is found in almost all connective tissues and is thought to participate in many biological processes and hyaluronan levels are markedly elevated during embryogenesis, cell migration, wound healing, malignant transformation and tissue turnover.[25] Molecules of hyaluronan are generally of very high molecular mass, ranging approximately 10^5–10^7 Da, depending upon the tissue. Hyaluronan exhibits unusual physicochemical properties in concentrated solutions because of its capacity to interact with water molecules. A molecule of hyaluronan, therefore, has a large hydrodynamic volume and forms solutions with high viscosity and elasticity that provide space filling, lubricating and filtering functions.[26] In fact, hyaluronan injection has now proven to be the most safe and minimally complicated procedure in many injectable fillers for reconstructive and cosmetic medicine.[27] Hyaluronan synthesis is regulated by hyaluronan synthase genes (*HAS1*, *2* and *3*). *HAS1–3* are regulated independently. For instance HAS3 produces lower molecular mass hyaluronan than *HAS2*. HAS2, but not HAS1 or HAS3, is responsible for hyaluronan synthesis in cultured skin fibroblasts.[28] These unique properties of *HAS1–3* support our results that *HAS1* and *3* mRNA were unchanged by all species of collagen-derived Hyp containing peptides examined here, but *HAS2* mRNA alone was increased by Pro-Hyp. This was accompanied by a comparable increase of hyaluronan synthesis in cultured dermal fibroblasts. Our results also suggest that oral ingestion of collagen hydrolysate may lead to more viscous and elastic skin resulting in improvement of skin appearance.

A previous report investigated the effect of bFGF, insulin-like growth factor (IGF)-1 and transforming growth factor (TGF)-β1 on the expression of *HAS2* mRNA in dermal fibroblast cells.[15,29] Greenwel *et al.*[12] indicated that tyrosine dephosphorylation of nuclear proteins mimics the stimulation of *COL1A2* transcription by the TGF-β1-activated signaling pathway using genistein and H7. In the case of genistein, *COL1A2* mRNA expression did not change, while treatment with H7 increased *COL1A2* mRNA expression. It was suggested that TGF-β1 used a different signaling pathway than Pro-Hyp.

Recent reports have provided evidence for enhanced *HAS2* transcriptional activation by STAT3.[16] Epidermal growth factor (EGF) initiates a signaling pathway where activated EGF receptors (EGFR) induce phosphorylation of STAT proteins 1, 3 and 5 through the tyrosine kinase Src[30,31] and phosphorylated STAT leads to increased transcriptional initiation of *HAS2* gene expression.[32] Therefore, Pro-Hyp-induced *HAS2* mRNA upregulation may be mediated by the Src kinase pathway and phosphorylation of STAT3. In this study, we found that a tyrosine kinase inhibitor and a serine/threonine kinase inhibitor reduced Pro-Hyp-induced *HAS2* mRNA upregulation, but a tyrosine phosphatase inhibitor showed no such effects on Pro-Hyp-stimulated *HAS2* mRNA. The results were, therefore, consistent with the notion that preventing phosphorylation specifically and negatively affects *HAS2* transcription. Conversely, blocking kinase activity downregulates production of *HAS2* transcripts. Furthermore, this counteracts the

stimulation of *HAS2* by Pro-Hyp. Thus, like EGF signaling, treatment with Pro-Hyp significantly elevated phosphorylation of STAT3, while kinase inhibition was able to counteract *HAS2* upregulation by Pro-Hyp. Although more detailed studies are necessary, this evidence suggests that phosphorylation of STAT3 proteins by activated kinase will be involved, at least in part, in the Pro-Hyp signaling pathway. The possibility of the presence of a specific receptor for Pro-Hyp still remains. A recent report indicated that Pro-Hyp may be transported into small intestinal epithelial cells through the H^+-coupled transporter, PEPT-1.[33] Moreover, Pro-Hyp may be transported into osteogenic cells by PEPT-1, where Pro-Hyp then becomes a direct signal. Therefore, in the case of fibroblast cells, Pro-Hyp may be transported into the cells where it is able to signal directly.

In conclusion, we found that collagen-derived Hyp-containing peptides stimulated cell proliferation, *HAS2* mRNA expression and hyaluronan production in human dermal fibroblasts. These results suggest that cells and ECM in the skin are modulated by oral ingestion of collagen hydrolysate. The underlying mechanism and the effects of various collagen-derived Hyp-containing peptides on cell motility and modulation of ECM proteins using *in vitro* cultured epidermal keratinocytes require further investigation.

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〈速報〉

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

Fumihito SUGIHARA, Naoki INOUE

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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Fumihito SUGIHARA , Naoki INOUE
Nitta Gelatin Inc., Peptide Division
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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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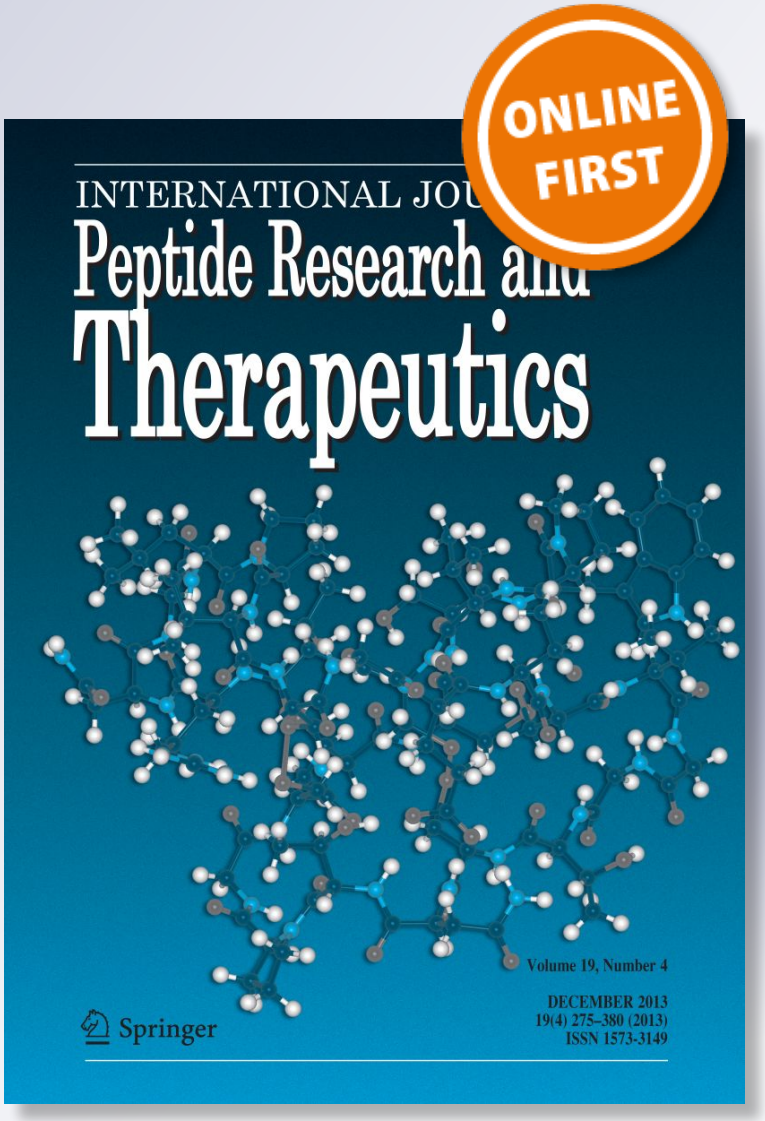

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Effects of Dietary Supplementation with Fish Scales-Derived Collagen Peptides on Skin Parameters and Condition: A Randomized, Placebo-Controlled, Double-Blind Study

Seiko Koizumi[1] · Naoki Inoue[1] · Maiko Shimizu[1] · Chang-ju Kwon[2] · Hwa-young Kim[2] · Kyoung Sik Park[3]

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Abstract Fish scales-derived collagen peptides (CPs) are characterized by their specific amino acid composition with a high concentration of glycine, proline and hydroxyproline. These amino acids have been known to exert beneficial effects on human skin. The aim of the present study was to examine the effects of collagen peptides obtained from fish scales on changes in periorbital wrinkles, facial skin hydration, and skin elasticity in healthy women aged 30–60 years. In the present randomized, placebo-controlled, double-blind trial, 71 subjects consumed a 20 mL beverage containing 3000 mg of CPs or placebo once per day over 12 weeks. Significant decreases in periorbital wrinkles ($p < 0.05$) were observed in the treatment group after 12 weeks of CPs ingestion compared to the control group. This study also demonstrated a consistent trend of enhanced facial skin moisture ($p < 0.001$) and skin elasticity ($p < 0.001$) by dietary intake of CPs without any side effects or adverse events. These findings indicate that fish-derived CPs hold great promise as a natural supplement with cutaneous anti-aging properties.

Keywords Collagen hydrolysate · Periorbital wrinkle · Skin hydration · Skin elasticity

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CPs	Collagen peptides
γ-GTP	γ-Glutamyltransferase
Gly	Glycine
Hyp	Hydroxyproline
IRB	Institutional review board
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
PP	Per-protocol
Pro	Proline
RBC	Red blood cell
TEWL	Transepidermal water loss
WBC	White blood cell

Introduction

Collagen is the main structural protein in the various connective tissues such as skin, tendons, cartilage and bone. It makes up from 25 to 30% of the whole-body protein content in mammals (Squire and Parry 2017). Commercial collagen is extracted from animal sources including porcine skin, bovine bone and fish scales (Blanco et al. 2017). Collagen peptides (CPs) are produced from the process of collagen hydrolysis involving breaking down the molecular bonds between individual collagen strands and peptides using combinations of physical, chemical and biological means (Liu et al. 2015). CPs are widely used as a dietary supplement with intend to aid joint mobility or enhance skin health.

In particular, fish scales-derived CPs have been reported to have specific amino acid composition with a high concentration of hydroxyproline (Hyp), glycine (Gly), and proline

✉ Kyoung Sik Park
parkks@cju.ac.kr

[1] Nitta Gelatin Inc., Yao, Osaka, Japan

[2] Ju Yeong NS Co. LTD., Seoul, Republic of Korea

[3] Department of Biomedical Science, College of Natural Science and Engineering, Cheongju University, Cheongju, 298 Daesung-ro, Chungwon-gu, Cheongju-si, Chungbuk 28503, Republic of Korea

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(Pro) in absorption level (Hu et al. 2017; Kim et al. 2012). Some Hyp-containing peptides including Pro-Hyp and Hyp–Gly are not completely digested into free amino acids and detected in human blood after ingestion of fish-derived CPs (Ohara et al. 2007). Notably, Prolyl-hydroxyproline (Pro-Hyp) stimulates cell proliferation and hyaluronic acid synthesis in dermis (Ohara et al. 2010). Recently, Shin et al. demonstrated that the dietary intake of fish scales-derived CPs significantly improves the wrinkle formation, thickness of the skin, acute erythema and skin hydration (Shin et al. 2016). From our previous studies, we observed a significantly increased viscoelasticity of human skin by dietary supplementation with 2.5–5 g of fish scales-derived CPs (Inoue et al. 2016; Sugihara and Inoue 2012; Sugihara 2015).

This present study was designed to evaluate the effects of fish scales-derived CPs on periorbital wrinkles, facial skin hydration and skin elasticity in healthy women of South Korea.

Materials and Methods

Study Design

This was a randomized, placebo-controlled, double-blind study examining the effects of CPs ingestion over 12 weeks. The protocol documents were submitted to Ellead Institutional Review Board (IRB), and the study was conducted after approval in January 2015 (IRB Number: EL-150113018A002) in compliance with the ethical principles of the Declaration of Helsinki. The research supervisor or researcher explained this study to the subjects signed the informed consent forms. Informed consent was obtained from all individual participants included in this study.

Subjects were randomly assigned to one of the two treatment regimens: CPs or placebo daily. The subjects consumed CPs or placebo product once a day for 12 weeks at the same time points from February to May in 2015. Efficacy assessments were conducted at baseline, and after 6 weeks and 12 weeks of ingestion.

Subjects

Eighty healthy women were enrolled as participants in this study. All participants were aged 30–60 years and confirmed to be "healthy" by blood test. Participants were tested negative for severe acute kidney disease, heart disease and other chronic diseases. They had no allergies or skin sensitivities to cosmetics, medicines, healthy functional foods, or ordinary exposure to sunlight. Selection criteria also included the presence of periorbital wrinkles corresponding to a grade of 2–6 defined by the global photodamage score as diagnosed by a dermatologist. Participants were randomly assigned to one of the two groups in a 1:1 ratio using a computer generated randomization schedule. The subjects were prohibited from taking dietary supplement including vitamins and minerals except for the test product. The subjects were prohibited from using any cosmetics except for the given cosmetics in the test area during the test. Skincare products or activities such as facial masks, massage, skin decortication and laser surgery which may affect the test were prohibited. Excessive exposure to UV was also prohibited during the test.

Study Beverage

The active products contained 3000 mg of CPs (average molecular weight: 3000 Da) derived from the scales of Tilapia fish (*Oreochromis mosambicus*) in the form of a 20 mL beverage. This product was manufactured by Nitta Gelatin Inc. (India), and provided by Ju Yeong NS Co. LTD. (Seoul, South Korea). The placebo product excluded CPs in 20 mL beverage. Color and smell of the placebo product were modified by fragrance and coloring agents to mimic the active products.

Dermatological Measurements

Instrumental measurements on the facial skin were conducted at baseline, and after 6 weeks and 12 weeks of ingestion. The subjects washed out their makeup and then were acclimatized for 30 min in the waiting lounge at a constant temperature of 20–24 °C and humidity of 40–60% RH before facial skin evaluation.

Periorbital Wrinkle

A replica of the periobital wrinkle was made using a silicone kit. To produce the replica, an adhesive plastic frame was pasted around the periorbital wrinkle. Silicon was applied and an impression was made which hardened into a replica of the skin. This replica was analyzed using a visiometer (Skin-Visiometer SV700, Courage & Khazaka, Germany). A visiometer assesses wrinkles by analyzing the intensity of light passing through the replica. Light is measured with a CMOS sensor and analyzed using image analyzer software and calculated by Lambert & Beer's Law. Replicas were analyzed for five criteria: R1: skin roughness; R2: maximum roughness; R3: average roughness; R4: smoothness roughness; R5: arithmetic average roughness. Photographs were also taken of each subject at baseline, after 6 weeks, and after 12 weeks of ingestion.

Skin Moisture Content

The skin moisture content on the cheek was assessed using Corneometer CM825 (Courage & Khazaka, Germany) at baseline, 6 weeks and 12 weeks after taking the product. The test method is based on a capacitance measurement of dielectric constant between electrodes separated on the skin surface. The average values were calculated from three measurements of skin moisture content.

Skin Elasticity

The skin elasticity on the cheek was assessed using Cutometer MPA580 (Courage & Khazaka, Germany) at the baseline, 6, and 12 weeks after taking the product. The test method draws skin with suction into the aperture of the probe and after a defined time releases it again. The method is non-invasive using a 2 mm of probe pressed into the skin. The results were calculated from three measurements with Mode 1, 450 mbar of constant suction for 2 s of suction time and 2 s of relaxation time. R2, R5 and R7 are parameters related to the skin elasticity.

Blood Tests

Blood tests were conducted following items: total protein, albumin, AST (aspartate aminotransferase), ALT (alanine aminotransferase), bilirubin, creatinine, total cholesterol, glucose, hemoglobin, hematocrit, WBC (white blood cell), RBC (red blood cell), platelet, MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), and γ-GTP (γ-glutamyltransferase).

Statistical Analysis

The study results were analyzed from PP (per-protocol) datasets. PP analysis refers to inclusion in the analysis of only those patients who strictly adhered to the protocol. Statistical comparisons (paired or unpaired *t* tests, Mann–Whitney rank sum tests) were performed by using SIGMASTAT 4.0 (SPSS, Chicago, IL, USA). A p value of less than 0.05 was considered to indicate statistical significance.

Results

Panelist Summary

Three subjects were excluded from participation among 80 subjects because they requested to withdraw. The remaining 77 subjects took either the CPs or placebo (39 subjects in CPs group, 38 subjects in placebo group). Six more subjects dropped out of the study after taking the products because of personal reasons (2 subjects in CPs group, 4 subjects in placebo group). The final 71 subjects (37 subjects in CPs group, 34 subjects in placebo group) completed this study (Fig. 1). Baseline characteristics for the placebo and CPs groups are shown in Table 1. No significant differences were detected between the two groups.

Blood Tests

At baseline and after 12 weeks of treatment, hematological and hemato-biochemical analyses were conducted. The results from the blood tests in the both CPs and placebo groups showed statistically significant differences between before and after taking in total protein, AST, ALT, creatinine, glucose, hematocrit, MCV, MCH and MCHC (Table 1). Furthermore, there were significant differences in platelet and MCV between the CPs and placebo groups after 12 weeks of treatment. However, all of the differences may have no significant meanings due to the changes within the clinically normal ranges.

Adverse Effects

There were no reports of subjects who experienced adverse events. Also, there were no premature terminations caused by a serious adverse event, adverse drug reaction or other adverse event.

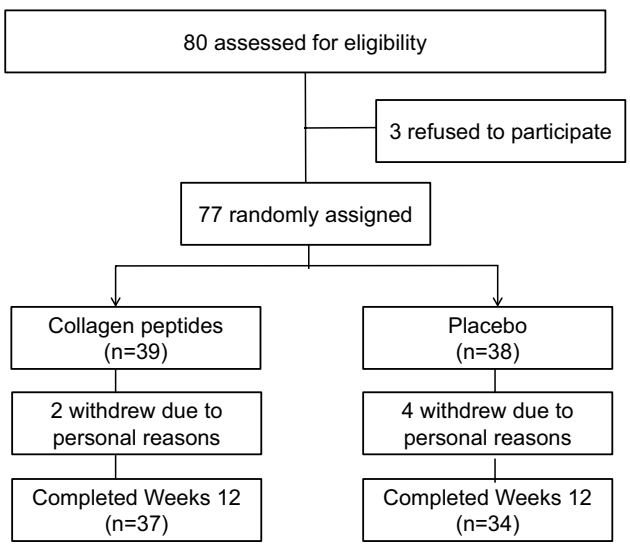

Fig. 1 Trial profile and design

Table 1 Baseline characteristics and changes in hematological and hemato-biochemical parameters after drinking either a CPs beverage or a placebo beverage for 12 weeks

	Placebo (n = 34)		CPs (n = 37)	
	Baseline	Week 12	Baseline	Week 12
Age (years)	47.37 ± 4.36	–	46.49 ± 5.74	–
Body weight (kg)	57.08 ± 6.35	–	56.19 ± 7.16	–
Systolic BP (mmHg)	113.21 ± 9.41	–	115.51 ± 10.24	–
Diastolic BP (mmHg)	66.44 ± 9.01	–	68.23 ± 8.84	–
Total protein (g/dL)	7.58 ± 0.41	7.26 ± 0.39***	7.50 ± 0.34	7.13 ± 0.28***
Albumin (g/dL)	4.57 ± 0.19	4.51 ± 0.22	4.63 ± 0.18	4.56 ± 0.21
AST (U/L)	23.63 ± 4.53	17.78 ± 4.40***	21.38 ± 3.79	17.07 ± 3.93***
ALT (U/L)	19.89 ± 8.54	14.07 ± 6.66***	16.38 ± 5.27	13.41 ± 5.85**
γ-GTP (U/L)	13.39 ± 6.17	14.63 ± 5.77	14.20 ± 9.70	14.56 ± 8.59
BUN(mg/dL)	13.38 ± 3.19	13.11 ± 3.17	12.09 ± 2.73	12.07 ± 2.84
Creatinine (mg/dL)	0.54 ± 0.08	0.65 ± 0.09***	0.56 ± 0.08	0.65 ± 0.08***
Total cholesterol (mg/dL)	186.71 ± 24.92	189.34 ± 24.83	183.46 ± 23.68	182.64 ± 26.10
Glucose (mg/dL)	89.07 ± 6.49	86.86 ± 7.45*	89.10 ± 9.70	85.38 ± 7.44**
Hemoglobin (g/dL)	12.81 ± 0.81	12.85 ± 1.09	13.06 ± 0.99	12.92 ± 1.05
Hematocrit (%)	38.27 ± 2.19	40.25 ± 2.91***	39.06 ± 3.10	40.64 ± 3.13***
WBC (Thous/μL)	5.92 ± 1.30	5.45 ± 1.22**	5.49 ± 1.52	5.51 ± 1.22
RBC (Mil/μL)	4.21 ± 0.23	4.32 ± 0.27**	4.27 ± 0.35	4.30 ± 0.31
Platelet (Thous/μL)	233.76 ± 50.46	233.07 ± 44.25	233.46 ± 54.17	252.51 ± 46.54**,##
MCV (fL)	90.96 ± 4.32	93.02 ± 4.51***	91.49 ± 4.32	94.46 ± 4.62***,#
MCH (pg)	30.47 ± 1.85	29.67 ± 1.73***	30.61 ± 1.72	30.04 ± 1.71**
MCHC (%)	33.48 ± 0.69	31.90 ± 1.01***	33.45 ± 0.63	31.79 ± 0.85***

Data are expressed as mean ± SD

BP blood pressure, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase; *BUN* bilirubin, *WBC* white blood cell, *RBC* red blood cell, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *MCHC* mean corpuscular hemoglobin concentration, *γ-GTP* gamma-glutamyltransferase

*p < 0.05; **p < 0.01; ***p < 0.001: significantly different from baseline

#p < 0.05; ##p < 0.01: significantly different between CPs group and placebo group

Wrinkle

The wrinkle and roughness parameters, R1 (skin roughness), R2 (maximum roughness), R3 (average roughness), R4 (smoothness roughness) and R5 (arithmetic average roughness) were calculated and statistically analyzed. The magnitude of % decreases at Week 12 in R1, R3, R4 and R5 was significantly greater ($p < 0.05$) in the CPs group than in the placebo group (Table 2). The representative photos showing the effect of CPs treatment on periorbital wrinkles were displayed in Fig. 2.

Facial Skin Moisture Content

The results of the skin moisture content assessment on the cheek from PP analysis showed a significant increase of facial skin hydration in the CPs group over time ($p < 0.05$, Table 2). Also, significant treatment-by-time interactions were found in facial skin moisture content ($p < 0.05$).

Skin Elasticity

The skin elasticity parameters, R2 (gross elasticity), R5 (net elasticity) and R7 (biological elasticity) were calculated and statistically analyzed. Table 2 represents the changes in skin elasticity by treatment for 12 weeks. A statistically significant increase was found in all of the skin elasticity parameters (R2, R5 and R7) in the CPs group after 12 weeks of ingestion compared to baseline ($p < 0.05$). Furthermore, CPs treatment induced a greater increase with a statistical significance at Week 12 in R, R5 and R7 compared to the placebo group ($p < 0.001$).

Discussion

The present study demonstrated that the supplementation with fish scales-derived CPs 3000 mg for 12 weeks was significantly effective in improving periorbital wrinkle, skin moisture content and skin elasticity. These findings confirmed the results of the previous clinical studies showing

Table 2 % Changes in skin parameters after drinking either a CPs beverage or a placebo beverage for 12 weeks

	Placebo (n = 34)			CPs (n = 37)		
		% Change from baseline			% Change from baseline	
	Baseline	Week 6	Week 12	Baseline	Week 6	Week 12
Periorbital wrinkles						
R1 (skin roughness)	0.144 ± 0.004	2.009 ± 1.491	3.698 ± 1.398	0.139 ± 0.003	1.171 ± 1.285	−4.094 ± 1.390$^{\#\#\#}$
R2 (maximum roughness)	0.110 ± 0.002	−1.841 ± 1.158	−0.988 ± 1.275	0.106 ± 0.002	0.102 ± 1.445	−3.022 ± 1.345
R3 (average roughness)	0.083 ± 0.001	−1.597 ± 0.900	−1.496 ± 1.328	0.082 ± 0.001	−2.256 ± 1.492	−5.116 ± 1.286$^{\#}$
R4 (smoothness roughness)	0.063 ± 0.002	6.568 ± 3.052	8.715 ± 3.385	0.062 ± 0.002	0.578 ± 2.779	−4.198 ± 2.649$^{\#\#}$
R5 (arithmetic average roughness)	0.018 ± 0.001	5.882 ± 4.919	11.274 ± 5.528	0.018 ± 0.001	−1.351 ± 3.055	−0.450 ± 5.793$^{\#}$
Skin moisture content						
Skin moisture content	49.585 ± 1.106	1.856 ± 1.047	4.017 ± 1.307	49.812 ± 1.224	6.646 ± 1.446$^{\#}$	10.891 ± 1.315**,$^{\#\#\#}$
Skin elasticity						
R2 (gross elasticity)	0.736 ± 0.008	−0.264 ± 0.458	0.938 ± 0.677	0.730 ± 0.007	2.272 ± 0.493$^{\#\#\#}$	4.320 ± 0.534**,$^{\#\#\#}$
R5 (net elasticity)	0.491 ± 0.011	0.315 ± 0.908	0.574 ± 0.837	0.486 ± 0.010	4.857 ± 0.777$^{\#\#\#}$	6.543 ± 1.089*,$^{\#\#\#}$
R7 (biological elasticity)	0.348 ± 0.008	1.744 ± 0.932	2.034 ± 1.389	0.344 ± 0.007	4.807 ± 0.779$^{\#\#}$	6.814 ± 1.049*,$^{\#\#\#}$

Data are expressed as mean ± SEM

*$p < 0.05$; **$p < 0.01$: significantly different from baseline

$^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$: significantly different between CPs group and placebo group

the daily intake of CPs 3–10 g has beneficial effects on skin health (Inoue et al. 2016; Sugihara and Inoue 2012; Sugihara 2015; Choi et al. 2014; Proksch et al. 2014a, b). There is a strong body of evidences for beneficial effects of fish scales-derived CPs on skin health in several trials. The ingestion of fish scales-derived CPs 3000 mg/day for 4 weeks significantly improved skin moisture content (Choi et al. 2014). In addition, Proksch et al. showed that ingestion of CPs obtained from porcine 2500 mg/day for 8 weeks improved skin wrinkle (Proksch et al. 2014b). Also, they demonstrated that the dietary intake of porcine-derived CPs 2500 and 5000 mg/day for 8 weeks improved skin elasticity without affecting skin moisture content and transepidermal water loss (TEWL) (Proksch et al. 2014a). While each of previous investigations only partially evaluated the beneficial effects of fish scales and porcine-derived CPs on skin parameters including skin moisture content, wrinkles and skin elasticity, this study demonstrated the improvement of all the parameters above by ingestion of them. Furthermore, most of previous human studies have examined the short-term effects of CPs within 8 weeks. To make up the restrictions of intake period in earlier trials, the present study expanded the treatment period up to 12 weeks.

In terms of the underlying mechanism, several human studies have demonstrated high absorption of two major CPs, prolyl-hydroxyproline (Pro–Hyp) and hydroxyprolyl-glycine (Hyp–Gly), into human blood (Kim et al. 2012; Sugihara et al. 2012). Some in vitro studies indicated that Pro–Hyp enhances chemotaxis on dermal fibroblasts (Postlethwaite et al. 1978) and Pro-Hyp and Hyp–Gly promote cell proliferation (Ohara et al. 2010). Additionally, Pro–Hyp enhances the production of hyaluronic acid in dermal fibroblasts (Ohara et al. 2010). Taking into account the bioavailability of these dipeptides, the improvement of skin condition after the intake of fish scales-derived CPs characterized by a high concentration of Hyp, Gly and Pro may be attributed to the high absorption of these bioactive peptides.

Conclusions

This study was conducted to evaluate the clinical efficacy of CPs to improve periorbital wrinkles, skin moisture content, and skin elasticity over 12 weeks. A daily intake of fish scales-derived CPs 3000 mg for 12 weeks was effective in improving periorbital wrinkle, skin moisture content

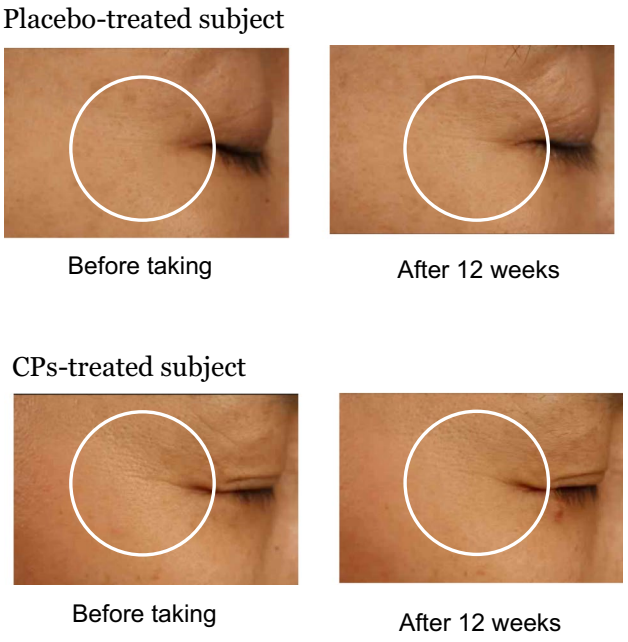

Fig. 2 Representative photographs indicating visual reduction of periorbital wrinkle formation at baseline and after 12 weeks of ingestion of placebo or collagen peptides

and skin elasticity. These beneficial effects of fish scales-derived CPs on skin health may result from their specific amino acid composition with a high concentration of Hyp, Gly and Pro in absorption level.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (IRB Number: EL-150113018A002) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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