PUSH
Collagen Dipeptide Concentrate

Wound Care Supplement

Pressure Injuries • Diabetic Wounds
Pre-Post Operative • Dry-Fragile Skin
Preventative Care
Not Your Average Collagen Powder

Clinical Studies Available www.globalhp.com

Collagen is a key component to healthy skin. PUSH Collagen Dipeptide works by improving skin structure, which leads to smoother skin texture and a higher level of moisture.

Features
- Patented blend of highly concentrated dipeptides
- Available in single serve packets & 72 serving cans
- Mixes easily with 2-3 oz of water
- Great tasting pineapple & mixed berry flavors
- Will not plug enteral feeding tubes
- Sugar & gluten free

Benefits
- Promotes wound healing¹
- Increases skin moisture, elasticity & texture²
- Found in human plasma for up to 4 hours

PUSH Collagen Dipeptide
GH15B: Pineapple 19.5 oz (1 Can)
GH15: Pineapple 19.5 oz (2 Cans)
GH16B: Pineapple 30 pckts/bx
GH16: Pineapple 30 pckts/bx - 6 bx/cs (180 packets)
GH17B: Mixed Berry 30 pckts/bx
GH17: Mixed Berry 30 pckts/bx - 6 bx/cs (180 packets)
GH18B: Mixed Berry 18.8 oz (1 Can)
GH18: Mixed Berry 18.8 oz (2 Cans)

INGREDIENTS: Collagen Dipeptide, Natural Flavors, Citric Acid, Sucralose.

INGREDIENTS: Collagen Dipeptide, Pineapple Juice Powder, Citric Acid, Pineapple Flavor, Sucralose.


Moisture

Texture

Nutrition Facts

Pineapple

Nutrition Facts

Mixed Berry

Serving size 1 Scoop/Packet (7.4g)
Amount per serving
Calories 20
% DV* 
Total Fat 0g 0%
Saturated Fat 0g 0%
Cholesterol 0mg 0%
Sodium 0mg 0%
Total Carbohydrate 2g 1%
Dietary Fiber 0g 0%
Total Sugars 0g 0%
Includes 0g Added Sugars 0%
Protein 5g 0%

Serving size 1 Scoop/Packet (7.7g)
Amount per serving
Calories 30
% DV* 
Total Fat 0g 0%
Saturated Fat 0g 0%
Cholesterol 0mg 0%
Sodium 0mg 0%
Total Carbohydrate 2g 1%
Dietary Fiber 0g 0%
Total Sugars 0g 0%
Includes 0g Added Sugars 0%
Protein 5g 0%

INGREDIENTS: Collagen Dipeptide, Pineapple Juice Powder, Citric Acid, Pineapple Flavor, Sucralose.
Molecular Structure Comparison

**PUSH Collagen Dipeptide**

Patented blend of powdered collagen dipeptides that helps reduce healing time. Highly concentrated Proline-Hydroxyproline (PO) and Hydroxyproline-Glycine (OG) team up to form a dipeptide that is not easily degraded.

These dipeptides send out signals to cells to energize the collagen peptide production by fibrocytes and chondrocytes, promoting growth of hyaluronic acid & aids in wound healing!

**Standard Collagen**

The longer and less stable molecular chains can make it more challenging for standard collagen to make it into the bloodstream.

Dipeptides are very stable and have a high absorption rate into the bloodstream. A dipeptide-rich collagen is shown to be more efficient than standard collagen at stimulating wound healing.

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**Supplement Protocol**

**Moderate & Severe Wounds**
Pressure Ulcer • Deep Tissue Wound
Unstageable 3 or 4 • Surgical Site • Skin Tear
Abrasions • Stasis Ulcers • Bruises

**Instructions**
• Mix serving, with 2-3 oz of cool water
• 1 serving BID, can be taken orally or via G-tube
• Take a minimum of 4 hours apart

Once all wounds are healed, continue taking once per day for a month

+ Can also be mixed with thickened liquids
+ Administer with water flushes for enteral patients with as little as 2 oz of water

*Servings may vary according to individual assessment by a health care professional.
Collagen Peptides

A natural protein derived from collagen. It is one of the major constituent elements that plays a foundational role in the make-up of the human body.

Benefits

• Promotes Wound Healing
• Increases Skin Moisture and Texture
• Improves Skin Elasticity
• Reduces Wrinkle Formation
• Encourages Healthy Bone Metabolism
• Helps Maintain Bone Density
• Reduces Joint Pain
• Reduces Bruising
• Radiation Treatment

What Makes PUSH Collagen Dipeptides Different?

A patented blend of powdered collagen dipeptides that helps reduce heal time. Highly concentrated Proline-Hydroxyproline (PO) and Hydroxyproline-Glycine (OG) team up to form a dipeptide formula that is not easily degraded. Studies show that PO and OG reach the cellular level in the skin, bones and joints. These dipeptides send out signals to cells to energize the collagen peptide production by fibrocytes and chondrocytes and promotes the growth of hyaluronic acid. This creates resilient tissue, stimulates cell division and aids in wound healing.
What is Bioavailability?

Bioavailability, also known as absorption, is the first element of efficacy. The human body is able to absorb di and tripeptides. These aren’t broken down into free amino acids during digestion process. Research has shown that the two dipeptides that have keys to bioavailability and efficacy are Proline-Hydroxyproline (PO) and Hydroxyproline-Glycine (OG).

PO & OG Bioavailability

PO & OG have a high resistance to degradation. In clinical trials, PO and OG were found in human plasma up to 4 hours after ingestion. This indicates that PO and OG are absorbed by humans, which then in turn stimulates the wound healing process.

PUSH Wound Healing Mechanism

Pressure Ulcers Heal in 3 Stages

1. **Inflammatory Stage** PO and OG stimulate the fundamental metabolism for wound healing

2. **Granulation Stage**

   PUSH stimulates dermal fibroblasts to migrate into the vacant tissue and enhance the components of the extracellular matrix - hyaluronic acid, elastin, and collagen in the re-making phase.

3. **Re-making Stage** PO can help re-epithelialization
Halt the Progression
of Early Wound Development & Pressure Ulcers

Practicing preventative measures such as implementing proper nutrition, can help eliminate enormous pain and suffering, save lives and reduce healthcare expenditures by millions of dollars.

Stage 1
$2,000

Stage 2
$3,000-$10,000

Stage 3
$5,900-$15,000

Stage 4
$19,000-$21,000

Average cost in treating a pressure ulcer is $10,700

Other Expenses That Increase Healthcare Cost
Nursing time for wound care & patient positioning • Wound care dressings & supplies
Specialty beds & overlays • Surgical debridement cost • Medications & ointments

Global Health Products • 1.800.638.2870 • www.globalhp.com
Collagen is denatured to gelatin by heating, and the gelatin hydrolysate (CH) formed by the hydrolysis of this gelatin in the presence of enzymes is used in foods and cosmetics. It has been found that, after oral ingestion of CH, not only amino acids but also di- and tripeptides were assimilated in human peripheral blood and that the peptides remained in the blood for a relatively long time (1–3). After the ingestion, Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp have been identified as CH-derived peptides in the blood (4). Of these peptides, Pro-Hyp has been reported to stimulate cell proliferation, cell growth, and hyaluronic acid synthesis in cultured dermal fibroblasts and synovium cells (5–7). Pro-Hyp also exerted chondroprotective effects in the articular cartilage in vitro and in vivo (8). On the other hand, Hyp-Gly was transported across the rat small intestinal tract into the blood using the in situ vascular perfusion techniques (9). The objective of the present study was to quantify Hyp-Gly in the blood of subjects by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after oral ingestion of CH.

Collagen hydrolysate was prepared from porcine skin gelatin by enzymatic hydrolysis. Consists of peptides with the average molecular weight of 1200 Da, containing a small amount of Pro-Hyp and Hyp-Gly. The human study was carried out according to a protocol described by Iwai et al. (1). This study was performed according to the Helsinki Declaration and was approved by the Ethical Committee of Nitta Gelatin Inc. Five male volunteers (mean age, 39.8±7.9; mean body weight, 68.6±8.8 g) ingested 8 g of the CH dissolved in 100 ml of water, and the venous blood samples were collected before and 0.5, 1, 2, and 4 h after the ingestion. The blood samples were collected with the collaboration of Dr. Shigehiro Arita at Arita Clinic (Osaka, Japan). The plasma prepared from the samples was then deproteinized by adding three volumes of ethanol and ethanol-soluble fraction was stored at −80°C until analysis. The amounts of (a) free Hyp and (b) total Hyp in the plasma samples were measured for the CH at the measurement time, and the amount of Hyp-containing peptide (Hyppeptide) [(b)−(a)] was calculated. Measurements were carried out by high-performance liquid chromatography (HPLC; TSK-GELOD–80TSQA, Tosoh, Tokyo, Japan), and the phenylisothiocyanate (PITC; Wako Pure Chemical Industries, Osaka, Japan) labeling method was used for the N-terminus (4), and measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The measurement conditions were as follows: HPLC/NANOSPACSI-2 (Shiseido Co., Ltd., Tokyo, Japan); column/Hypersil GOLD PFP (2.1×150 mm, Thermo Fisher Scientific Inc., MA, USA); elution condition, (i) 0.2% formic acid and 2 mM aqueous solution of ammonium acetate and (ii) methanol in a gradient condition: injection volume, 5 μl; column temperature, 40°C.

A tandem mass spectrometer (TSQ Vantage, Thermo Fisher Scientific) was used. The ionization method was positive electrospray ionization (ESI) at 1500 V. The selectivity of the monitoring (SRM) conditions were as follows: Pro-Hyp plus charge ESI SRM ms/ms, 229.1170.3 at 41 eV; Hyp-Gly plus charge ESI SRM ms/ms, 189.1N85.9 at 16 eV. As shown in Fig. 1, the concentration of Hyp-peptide increased to maximum level (32.3 mmol/ml) 1 h after the CH ingestion. The concentration decreased half of the maximum 4 h after the ingestion. Hyp-Gly and Pro-Hyp were identified in all subjects by LC–MS/MS. The ratio of Hyp-Gly to Pro-Hyp in human plasma 1 h after the ingestion was distributed ranging from 6.3% to 22.1% (Table 1). As shown in Fig. 2, the concentration of Hyp-Gly increased to maximum level (4.2 mmol/ml) 1 h after the ingestion. The concentration decreased one-quarter of the maximum 4 h after the ingestion. In the present study, Hyp-Gly, which has not been detected in the previous studies (1–4), is quantified in the blood of all the five subjects by LC–MS/MS. Hyp-Gly can be detected by using the LC–MS/MS system, because CH used contained a small part of Hyp-Gly. Very recently, Shigemura et al. also identified Hyp-Gly as a novel food-derived collagen peptide in human peripheral blood by pre-column derivation with phenyl isothiocyanate. The ratio of Hyp-Gly to Pro-Hyp depended on subjects and ranged from 0.00 to 5.04 (10). As Hyp-Gly inhibited the differentiation of mouse osteoclasts in vitro and in vivo (Mano, H., Nakatani, S., Sekiguchi, Y., Shimizu, J., Sugihara, F., Haketa, Y., and Wada, M., Abstr. 27th Ann. Meet. Jpn. Soc. Bone Miner. Res., p. 227, 2009), Hyp-Gly may play active roles in the bone tissue.

REFERENCE
INTRODUCTION
In today’s super aging society, more and more elderly persons are seen to require long-term hospitalization or home healthcare due to illness or injury. When bedridden, such elderly persons have difficulty changing their posture by themselves, and are prone to pressure ulcers if not receiving sufficient nutrition.

The purpose of most conventional pressure ulcer treatments is to prevent the ulcers from becoming chronic by cleaning and protecting the affected areas, as well as maintaining the area moist. In addition, the common method of promoting the healing of pressure ulcers is to ensure proper nutritional management and feed the elderly with nutrition, which is partially composed of dietary supplements. For instance, the amount of essential amino acids is often discussed for protein support from the nutritional viewpoint.

Collagen peptide (CP) discussed in this chapter was developed in hope that it would contribute to improving pressure ulcers as a functional ingredient, rather than to feed amino acids. Sections 2 to 4 describe the latest information on the functional material CP, focusing on its absorption kinetics after oral intake, effects on pressure ulcers, and its mechanism. Section 5 introduces applications to enteral feeding products based on ideal amino acid scores.

CP Absorption
It has already been reported that CP derived dipeptides and tripeptides such as hydroxyproline (Hyp) are detected in human blood after CP is orally ingested[1]). The pig hide-derived CP is characterized by an average molecular weight of 1300 and containing dipeptides such as prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly). To elucidate the kinetics of peptides entering into the blood after CP intake, absorption tests were conducted in 5 male volunteers. After having them intake 8 g of CP, their blood was sampled from the vein at 0, 0.5, 1, 2, and 4 h to measure Pro-Hyp and Hyp-Gly, peptides usually found in the collagen alignment, and prolyl-hydroxyprolyl-glycine (Pro-Hyp-Gly) and glycyl-prolyl-hydroxyproline (Gly-Pro-Hyp), both of which are tripeptides. The results found that Pro-Hyp transferred to the blood the most, followed by Hyp-Gly3). As for the absorption behavior of Pro-Hyp, the maximum blood concentration was reached at about 1 h, after which the concentration declined gradually (Figure 1).

Furthermore, a rat experiment revealed that MMP-9 production was expressed broadly defined as its effects on healing wounds. The following paragraphs discuss the effects of Collapep PU on a rat model with skin wounds.

Using model rats with skin wounds, Horiuchi et al. compared the wound healing promotion effects between Collapep PU and milk casein (control food, casein group), administered at 1 g daily[5]). In the initial stage of wound healing, the production of matrix metalloproteinase (MMP), which breaks down unwanted substrates due to the injury, increases. At day 0 of recovery, MMP-9 production of the Collapep PU group was significantly increased compared to the milk casein group. The analyses of localization in the skin tissue revealed that MMP-9 production was expressed locally near the basement membrane at the boundary between the epidermis and dermis of the wound area in the Collapep PU group, while in the casein group, it was found dispersed all over the skin. On day 4 of recovery, MMP-9 production had stopped in both groups.

The percentage of nascent collagen (NaCl-soluble collagen) in the skin tissue during the recovery process was also measured.

In this way, Pro-Hyp and Hyp-Gly absorbed after CP intake are believed to repair damaged skin tissues and promote remodeling.

Oral administration of CP is also expected to demonstrate strong effects on the treatment of pressure ulcers, discussed in the following section, through the above absorption and transfer processes after CP intake.

Effects of Collapep PU intake on Pressure Ulcers
This section discusses the results of clinical studies investigating the efficacy of Collapep PU in pressure ulcer patients. One study found the promotion of wound healing by combining CP and amino acids mixtures[6], suggesting the efficacy of CP intake for pressure ulcer patients. However, the efficacy of the single CP intake has never been reviewed, and therefore, this section aims to validate the effects of single CP use.

Collapep PU was used as the study food and maltodextrin as the placebo (TK-16, Matsutani Chemical Industry Co., Ltd.). Subjects were 81 Indian male and female patients with Stage-2 and -3 pressure ulcers. They were randomized into treatments with Collapep PU (CP group, 40 subjects) and placebo (placebo group, 41 subjects) in a double-blind study. The patient age ranged from 18 to 70 years, and their BMI was ±18.5 but <35.0. People who were diabetic, pregnant, or breastfeeding were excluded from registration.

The study food and placebo were administered daily at a dose of 5 g morning and evening (10 g/day) for 16 weeks, and evaluated using the three following international assessment scales:

1) PUSH (Pressure Ulcer Scale for Healing) score (0-17 points: the worse the symptom is, the higher the score is)
2) PSST (Pressure Score Status Tool) score (13-65 points: the worse the symptom is, the higher the score is)
3) Wound area on photo (cm2)

The difference in the above scores between baseline and 16 weeks was evaluated. The subjects were placed on standard treatment while being given the study food as an additional treatment. To confirm their nutritional state, chemical industry, and milk casein (control food, casein group), administered at 1 g daily[5]). In the initial stage of wound healing, the production of matrix metalloproteinase (MMP), which breaks down unwanted substrates due to the injury, increases. At day 0 of recovery, MMP-9 production of the Collapep PU group was significantly increased compared to the milk casein group. The analyses of localization in the skin tissue revealed that MMP-9 production was expressed locally near the basement membrane at the boundary between the epidermis and dermis of the wound area in the Collapep PU group, while in the casein group, it was found dispersed all over the skin. On day 4 of recovery, MMP-9 production had stopped in both groups.

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July 2012

PUSH Was Found to Aid in Healing Pressure Ulcers
Collapep PU was administered daily at a dose of 5 g morning and evening (10 g/day) for 16 weeks

(1) The PUSH score as -5.89 ± 1.97 in the CP group and -2.67 ± 1.26 in the placebo group, with a statistically significant intergroup difference (p<0.0001) (Table 1).
(2) The PSST score was -10.49 ± 3.79 in the CP group and -6.41 ± 3.63 in the placebo group, with a statistically significant intergroup difference (p<0.0001) (Table 1).
(3) The wound area was -10.04 ± 8.64 in the CP group and -6.41 ± 7.63 in the placebo group, with a statistically significant intergroup difference (p<0.0001) (Table 2).

Blood chemistry analyses showed that both blood protein and serum albumin levels were within the normal range in all subjects during the study period, suggesting their good nutritional condition (Table 3).

There was also no statistically significant difference between the two groups in terms of BMI, gender, and age. Compared to the placebo group, significant improvement was found in the CP group in scores on the three evaluation scales, suggesting that the addition of Collapep PU intake to the standard treatment promotes healing pressure ulcers.

Mechanism Promoting Wound Healing by Collapep PU
Mechanism Promoting Wound Healing by Oral Administration of Collapep PU.
The efficacy of oral administration of CP for the treatment of pressure ulcers is broadly defined as its effects on healing wounds. The following paragraphs discuss the effects of Collapep PU on a rat model with skin wounds.
A significant increase in nascent collagen was found in the Collapep PU group compared to the casein group from at day 1 of recovery. The results suggest that oral administration of Collapep PU promotes the efficient breakdown of unwanted substrates by locally promoting MMP-9 production near the wound, thereby producing nascent collagen. Finally, it was concluded that the oral administration of Collapep PU promotes the remodeling and re-epithelialization of skin tissue.

**Mechanism of Pressure Ulcer Healing Actions of Pro-Hyp**

What signals do specific CP-derived peptides, such as Pro-Hyp, send to fibroblasts? In what mechanism are such peptides involved in the promotion of wound healing?

We focused on Pro-Hyp found in Collapep PU, and investigated in vitro its effects on the genetic expression of cells. Three-dimensional culture with normal human dermal fibroblasts (NHDF [NB]) (Kurabo Industries Ltd.) embedded in floating collagen gel was used for analysis. The cells were embedded in the gel at a concentration of 5 × 105 cells/ml × 500 µl, and Pro-Hyp was added to the culture media to bring the final concentration to 5 mM. Twenty-four hours after cultivation, the collagen gel was broken down using collagenase, and the cells were sampled. Total RNA samples were collected from the cells, and the genetic expression level was quantified using the real-time polymerase chain reaction (PCR) method.

The results suggested that Pro-Hyp increases the mRNA expression of hyaluronic synthase 2 (HAS2), signal transducer and activator of transcription 3 (STAT3), hyaluronic acid receptor (CD44), MMP-9 (gelatinase B), and MMP-1 (interstitial collagenase), whereas it inhibits the mRNA expression of receptor for hyaluronic acid mediated mobility (RHAMM) and type I collagen (col1a1).

These findings suggest that Pro-Hyp increases the mRNA expression of HAS2 via the upstream gene of STAT3 (Figure 2). Given that the mRNA expression of hyaluronic acid receptor CD44 increases as well, a more optimum microenvironment are expected to be created for fibroblasts. Furthermore, an increased mRNA expression of MMP-8,-1 matches the transient increase in MMP-9 production at the protein level in the initial stage of wound healing, described in the previous section.

These findings suggest that Pro-Hyp functions as a signal on cells in each process of wound healing and plays the role of adjusting genetic expression related to the remodeling of damaged skin tissue.

**Applications of Collapep PU to Enteral Feeding Products**

Protein contained in enteral feeding products need to have high nutritional value. However, Collapep PU contains a very small amount of essential amino acids, and no tryptophan in particular. In the preparation or designing of collagen peptide-based enteral feeding products, it is hence ideal to combine them with protein with high nutritional value. The following paragraph discusses the combination of casein sodium, a protein often used in enteral feeding products, from the viewpoint of amino acid score.

Table 4 shows the amino acid composition of different types of protein. If Collapep PU and casein sodium are combined, compositions were converted into relative figures per 1 g of nitrogen as the nitrogen-to-protein conversion factor differs between the two products. The amino acid pattern indicates the ideal essential amino acid levels in adults. If these values are achieved, it means that the amino acid score is calculated as 100. To maintain 70 mg of tryptophan, if Collapep PU and casein sodium are combined at a ratio of 1:4, the amino acid score of 100 can be established. The recommended protein level of 60 g/day for males over 70 years of age in the Japanese food intake standards is premised on the intake of protein with high nutritional value such as casein sodium. If 12 g out of the 60 g is replaced by Collapep PU, the amino acid score of 100 can be maintained. This means that consuming 12 g/day fulfills the required level of 10 g/day for providing the physiological functions of Collapep PU, discussed in section 3, enabling the design of an enteral feeding product meeting both objectives of promoting pressure ulcer healing and amino acid supply.

**CONCLUSION**

The findings of this study suggest that CP-derived peptide molecules such as Pro-Hyp with physiological functions act directly on skin cells and genetically adjust the remodeling of damaged skin tissues during the wound healing process. In addition, the intake of Collapep PU containing these molecules was found to demonstrate healing promotion effects even in patients with pressure ulcer and other symptoms for which the efficacy of the original treatment is reduced.

As an application for, if combined with substances with high nutritional value such as casein sodium, Collapep PU has been demonstrated to be used as an enteral feeding product while properly supporting originally intended nutrients. In the future, further study will be necessary to elucidate the mechanism of CP promoting pressure ulcer healing, and their applications will be required for the development of various CP-based food products for the elderly.

**REFERENCE**

10. 堺内 恵美子ら : アミノ酸研究., 42(2) ; 197, 2010.
Collagen Hydrolysates Enhance Facial Skin Moisture & Elasticity

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 condition, 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 administration1-4. It has been demonstrated that 13C-labelled Pro-Hyp reaches the skin and bone tissues rapidly after ingestion by mice5. Moreover, in a clinical study, Pro-Hyp was identified in the urine after collagen hydrolysate intake6. These findings suggest that Pro-Hyp and Hyp-Gly are stable and relatively resistant to peptidases in the blood7,8 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 fibroblasts9 and both Pro-Hyp and Hyp-Gly enhanced cell proliferation activity10,11. Additionally, it was observed that Pro-Hyp enhanced the production of hyaluronic acid in dermal fibroblasts12.

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 randomized 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.

METHODS & MATERIALS

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 (LCP) 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.

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. Randomized 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 randomized 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 randomization 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 diner for 8 weeks.

Results

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 ethic 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 three months, and non-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 makeup by conventional methods, and were acclimatized for 30 minutes 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, Germany). Three measurements were taken and averaged.

Skin elasticity

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

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 LCP) 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 ratetwo) or 8 weeks (changing rateweek 8) of ingestion. Significance was defined as p<0.05 using the data analysis software SPSS Ver.13.0. 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 summarized 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 group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of subjects</th>
<th>Mean ± Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>L-CP</td>
<td>29</td>
<td>1</td>
</tr>
<tr>
<td>H-CP</td>
<td>28</td>
<td>2</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD

LCP lower content of bioactive collagen peptides, H-CP higher content of bioactive collagen peptides.

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.

Summary

The effects of collagen hydrolysates on skin condition, 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.
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 summarized 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 eight weeks of ingestion was within the limits of standard values. Furthermore, no adverse effects were observed during the clinical trial.

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 might 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.13 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.14 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.15 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.16 reported that Pro-Hyp 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 data on oral administration of Pro-Hyp+Hyp-Gly improved skin barrier dysfunction and moisture in HR-1 hairless mice.17 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 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 understanding of fundamental mechanisms in anti-ageing.

### Table 1: Facial skin wrinkles and roughness throughout treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
</tr>
<tr>
<td>L-CP</td>
<td>-0.01</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
</tr>
<tr>
<td>H-CP</td>
<td>-0.02</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
<td>-0.00</td>
</tr>
<tr>
<td>Wrist-dep</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Wrinkle-dep</td>
<td>0.05</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Roughness</td>
<td>0.07</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

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

### REFERENCES

Table 5. Blood test of subjects in the clinical study

<table>
<thead>
<tr>
<th>Item</th>
<th>Placebo group (n = 26)</th>
<th>L-CP group (n = 28)</th>
<th>H-CP group (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 8</td>
<td>Baseline</td>
</tr>
<tr>
<td>Total protein g L⁻¹</td>
<td>75 ± 4</td>
<td>72 ± 4</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>Albumin g L⁻¹</td>
<td>43 ± 2</td>
<td>42 ± 2</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>Albumin/globulin Ratio</td>
<td>2 ± 3</td>
<td>3 ± 9</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>GPT IU L⁻¹</td>
<td>20 ± 13</td>
<td>14 ± 6</td>
<td>17 ± 10</td>
</tr>
<tr>
<td>ALP IU L⁻¹</td>
<td>109 ± 36</td>
<td>92 ± 27</td>
<td>106 ± 28</td>
</tr>
<tr>
<td>γ-GTP IU L⁻¹</td>
<td>19 ± 11</td>
<td>18 ± 8</td>
<td>23 ± 11</td>
</tr>
<tr>
<td>GOT IU L⁻¹</td>
<td>22 ± 8</td>
<td>18 ± 4</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>LDH IU L⁻¹</td>
<td>190 ± 24</td>
<td>189 ± 22</td>
<td>195 ± 27</td>
</tr>
<tr>
<td>Total bilirubin μmol L⁻¹</td>
<td>8 ± 4</td>
<td>9 ± 3</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>BUN mmol L⁻¹</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Creatinine μmol L⁻¹</td>
<td>56 ± 8</td>
<td>60 ± 7</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>UA μmol L⁻¹</td>
<td>256 ± 74</td>
<td>253 ± 34</td>
<td>236 ± 47</td>
</tr>
<tr>
<td>CPK U L⁻¹</td>
<td>88 ± 26</td>
<td>82 ± 24</td>
<td>83 ± 32</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.

GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transferase; GOT, glutamic oxaloacetic transaminase; LDH, lactate dehydrogenase; BUN, blood urea nitrogen; UA, uric acid; CPK, creatinine phosphokinase.

INTRODUCTION

A pressure ulcer is defined as an area of localized damage to the skin and/or underlying tissue (usually over a bony prominence) caused by pressure, or pressure in combination with shearing stress. Pressure ulcers develop when continuous pressure affects cellular metabolism and impedes or obstructs capillary blood flow to the skin and underlying tissue, resulting in tissue ischemia. Malnutrition is an independent risk factor for the development of pressure ulcers. Appropriate nutrition is imperative for preventing and treating such wounds. Reddy M et al. reviewed that protein supplementation of long-term care residents improved wound healing compared with a placebo (it brought about an improvement in the Pressure Ulcer Scale for Healing (PUSH) score). For undernourished patients, supplementation with high amounts of energy and protein is recommended. In addition, various vitamins, zinc, and arginine and collagen hydrolysates should be supplied.

Collagen hydrolysate (CH), which is also referred to as collagen peptides, is widely utilized as a nutritional supplement. It is a mixture of peptides of different molecular weights derived from gelatin, a form of heat-denatured collagen, via enzymatic hydrolysis. Orally ingested CH is absorbed as both free amino acids and oligopeptides, such as prolylhydroxyproline (Pro-Hyp) and hydroxyproline (Hyp-Gly), and these oligopeptides are considered to be the major factors responsible for the physiological activity of CH.

Concerning the effects of CH on the skin, we reported that orally ingested CH containing Pro-Hyp and Hyp-Gly improved the water content, elasticity, and roughness of the skin in healthy women. As for the effect of CH on pressure ulcers, Lee SK et al. reported that the combined oral administration of CH, an amino acid mixture, and the standard treatment resulted in an improvement in the PUSH score after 8 weeks’ treatment. The objective of the present study was to assess the clinical effectiveness of CH supplementation in terms of its ability to induce remission in subjects with stage II or III pressure ulcers and the safety of this approach.

The first CH (CH-a) had a low dipeptide content (<0.01 g dipeptides per kg of product). The other CH (CH-b) had a high dipeptide content (>1 g dipeptides per kg of product). The mean molecular weights of CH-a and CH-b were 5,000 and 1,200, respectively. The characteristics of the test samples are shown in Table 1. Each 5-g test sample was packed in an aluminum sachet.

Inclusion Criteria

The inclusion criteria were as follows: inpatients or outpatients of either sex who were aged between 18 and 70 years; had been diagnosed with stage II or III pressure ulcers, as defined by the National Pressure Advisory Panel of the USA (2007); had a body mass index of 18.5 to 34.9 kg m⁻²; exhibited a pressure ulcer surface area of <80 cm² (multiplication of the major and minor diameters of the wound surface); were suffering from a stage II or III pressure ulcer (regardless of its location) with a PUSH (version 3.0) score of ≥6 that was likely to heal during the 6-month study period; and demonstrated moderate exudate production and a Braden score of ≥6.

Exclusion Criteria

The exclusion criteria were as follows: women who were pregnant or lactating; women of childbearing potential who were not taking adequate contraceptive measures; patients with stage IV pressure ulcers; patients that were being fed via a tube; patients with diabetic foot ulcers; patients who received immunotherapy or cytotoxic chemotherapy within the 60 days before enrollment; patients who had taken systemic steroids within the 30 days prior to enrollment; patients that had received topical therapy other than steroidial therapy during the 7 days prior to enrollment; patients who were human immunodeficiency virus-, hepatitis B virus-, or hepatitis C virus-positive; patients with pre-existing demyelinating disorders; patients with hepatic, renal, or metabolic disease that was likely to interfere with their participation in or completion of the study; patients with arterial or venous disorders that had the potential to cause ulcerated wounds; patients with a history of established diabetes mellitus and a fasting blood glucose level of >200 mg dl⁻¹; patients with any condition that would interfere with wound healing (e.g., a connective tissue disorder, immunological disorder, or clinical obesity); patients who were malnourished; patients with wounds caused by malignancy; patients with burns or scalds; patients who had used any form of complimentary alternative medicine in the preceding 2 months; patients that were known to exhibit hypersensitivity reactions to protein products; patients with any dermatological condition or disorder that might interfere with the appropriate assessment or treatment of ulcers; current smokers; patients who had participated in any other clinical study during the 3 months prior to this trial; patients who were unwilling or unable to comply with the study procedures; patients who were considered to be unsuitable candidates by the investigator for any reason.

Study Design

This was a 16-week double-blind, multi-centric, placebo-controlled, randomized clinical study that aimed to determine the effectiveness, safety, and tolerability of CH (CH-a or CH-b) as an add-on nutritional supplement during the management of subjects with stage II or III pressure ulcers.

The subjects were enrolled according to the inclusion and exclusion criteria. After enrollment, a centralized allocation method was used to assign the study subjects to the CH-a, CH-b, or placebo group. The subjects were allocated to each group using computer-generated randomization code and the SAS® software. The trial sites were also subjected to centralized allocation. The randomization was balanced, and access to the code was strictly controlled. The study protocol, informed consent documents, and case reports, along with all of the secondary documents used for the study were reviewed and approved by an independent ethics committee. The study was conducted in accordance with the ethical principles laid out in the current version of the Declaration of Helsinki and the ICH-GCP guidelines and was registered (No. CTRI/ 2009/091/001097) with the Clinical Trial Registry, India. Written informed consent was obtained from all subjects who participated in this study. The subjects were advised to orally consume 5 g of CH or the placebo, dissolved in 250 ml water or milk in the morning and night after eating food. Regarding the standard therapy, the subjects were treated with antimicrobials, antiseptics, wound debridement, and wound dressing, as required.

A total of 137 subjects were screened using the inclusion and exclusion criteria. One hundred and twenty subjects were considered to be eligible for the study based on these criteria and so were enrolled. After enrollment, the subjects were randomized in a 1:1:1 ratio to receive CH-a, CH-b, or the placebo, as illustrated in Fig. 1. After the first visit (week 0), two subjects (one each from the CH-a and CH-b groups) dropped out citing travel difficulties. Hence, two more subjects were enrolled in the study. They were allotted to the placebo arm using the same randomization procedure. Eight more subjects subsequently dropped out of the study, but they were not replaced as at least one set of post-baseline data had been obtained for them. Therefore, a total of 112 subjects completed the study and were analyzed.

The variables used to assess the efficacy of the treatments included the PUSH score, the Pressure Score Status Tool (PSST) score, and wound area according to photographic measurements (length and breadth were measured in cm using a metric ruler, and the resultant area values were recorded in cm²).

The primary treatment efficacy endpoint was to compare CH group and placebo group for PUSH score, PSST score, and wound area at week 16. The secondary treatment efficacy endpoint was the following 2 steps: (1) the primary.

---

**Table 1. Characteristics of the collagen hydrolysates (CH-a and CH-b) and placebo.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unit</th>
<th>Placebo</th>
<th>CH-a</th>
<th>CH-b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>kcal/kg</td>
<td>3840</td>
<td>3860</td>
<td>3740</td>
</tr>
<tr>
<td>Protein</td>
<td>g/kg</td>
<td>965</td>
<td>894</td>
<td>961</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>g/kg</td>
<td>360</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Moisture</td>
<td>g/kg</td>
<td>40</td>
<td>33</td>
<td>41</td>
</tr>
<tr>
<td>Fat</td>
<td>g/kg</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ash</td>
<td>g/kg</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

**Materials & Methods**

The placebo, maltodextrin TK-16, was purchased from Matsutani Chemical Industry Co., Ltd. (Itami, Japan). Two pork skin gelatin-derived CH, which were composed of different proportions of free-form Pro-Hyp and Hyp-Gly, were used in this study.
improvement was a reduction in the PUSH score of ≥5 points and a reduction in the PSST score of ≥7 points between the baseline and week 16.

Clinical laboratory and biochemical evaluations, including blood and urine analyses, were used to assess the safety of CH-a and CH-b. Vital signs, such as temperature, pulse rate, respiratory rate, and systolic and diastolic blood pressure, and other parameters related to safety were analyzed.

Statistical Analyses

The Kolmogorov-Smirnov Z test was performed to assess the normality of the PUSH score, PSST score, and wound area data. For normally distributed data, two-way ANOVA was used during comparisons between groups (the CH-a, CH-b, and placebo groups), and repeated-measures ANOVA was employed for intra-group comparisons. Comparisons between the baseline and week-16 data were conducted using the paired t-test. ANCOVA was used to adjust for baseline differences among the treatment groups. For non-normal data, the Kruskal-Wallis and Friedman tests were used for intergroup and intra-group comparisons, and comparisons between the baseline and week-16 data were carried out with the Wilcoxon signed rank test. Evaluations of the proportions of subjects who exhibited improvements in their PUSH scores, PSST scores, or wound area were conducted using the chi square test or Fisher’s exact test.

Results

The demographic characteristics of the subjects are shown in Table 2. All of the subjects were advised to follow their normal diet; i.e., the diet they were consuming at the time of their inclusion in the clinical study. None of the patients involved in the clinical study had any pre-existing conditions.

Efficacy

The changes in the patients’ PUSH scores, PSST scores, and wound area measurements are shown in Fig. 2. The PUSH score, PSST score, and wound area values of the CH-b group were significantly lower than those of the placebo group at week 16 (PUSH score: 6.46 ± 0.98 vs. 9.26 ± 2.09, P < 0.01; PSST score: 19.71 ± 3.08 vs. 23.38 ± 3.85, P = 0.01; wound area: 3.19 ± 2.88 vs. 5.00 ± 3.88, P = 0.027). The PUSH score of the CH-a group was significantly lower than that of the placebo group at week 16 (8.21 ± 2.04 vs. 9.26 ± 2.09, P < 0.01; PSST score: 29.72 ± 3.51 vs. 35.21 ± 2.13, P = 0.028).

Table 2: Demographic data of the subjects selected for the clinical study. Data are expressed as mean (standard deviation) values.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Total (N= 30)</th>
<th>CH-a (N= 10)</th>
<th>CH-b (N= 10)</th>
<th>Placebo (N= 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td>44.4 (10.7)</td>
<td>44.0 (10)</td>
<td>45.1 (12.1)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>17</td>
<td>22</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>13</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
<td>158.0 (10.8)</td>
<td>158.0 (10.8)</td>
<td>158.0 (10.8)</td>
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<tr>
<td>Weight (kg)</td>
<td></td>
<td>55.2 (10.8)</td>
<td>55.2 (10.8)</td>
<td>55.2 (10.8)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td>24.5 (3.3)</td>
<td>24.5 (3.3)</td>
<td>24.5 (3.3)</td>
<td></td>
</tr>
<tr>
<td>PUSH score (points)</td>
<td></td>
<td>11.62 (2.9)</td>
<td>9.42 (1.7)</td>
<td>13.82 (1.7)</td>
<td></td>
</tr>
<tr>
<td>PSST score (points)</td>
<td></td>
<td>29.72 (3.51)</td>
<td>25.31 (2.13)</td>
<td>35.21 (2.13)</td>
<td></td>
</tr>
<tr>
<td>Wound area (cm²)</td>
<td></td>
<td>12.45 (2.28)</td>
<td>12.14 (2.20)</td>
<td>12.75 (2.20)</td>
<td></td>
</tr>
</tbody>
</table>

PUSH Score Calculation. The PUSH Score was calculated as per the PUSH Table.

(1) The following three individual variables were measured to calculate the PUSH Score: (1) Length X Width: The greatest length and the greatest width of the wound were measured in square centimetres. The two values were multiplied and corresponding score from the PUSH table (An instrument to measure healing, Version 3.0:98’ National Pressure Ulcer Advisory Panel) was noted as the first variable; (2) Exudate Amount: The amount of exudate, post removal of dressing and before the application of any topical agent was estimated. The corresponding score from the PUSH table was noted as the second variable; (3) Tissue Type: The tissue of the wound was examined. The corresponding score from the PUSH table was noted as the third variable.

(2) These three variables were added to get the PUSH Score.

(3) The PUSH score was calculated as baseline and periodically upon treatment to show improvement metrics.

PSST Score Calculation. The PSST Score was calculated based on the assessment of 13 different variables as follows: Size, Depth, Edges, undermining, necrotic tissue type, necrotic tissue amount, exudate type, exudate, skin color surrounding wound, peripheral tissue edema, peripheral tissue indurations, granulation tissue, epithelialization.

Biological Evaluations

As part of the safety assessment, laboratory analyses of various serological and urinary biochemical parameters were performed. The baseline and week 16 data are shown in Table 4. Although many biochemical parameters differed significantly between the baseline and week 16, there were no clinically significant abnormalities. These findings demonstrated the safety of CH-a and CH-b in humans. Moreover, the US Food and Drug Administration (US FDA) has classified gelatin and CH as Generally Recognized as Safe (GRAS) products.

As an indicator of the subjects’ nutritional status, we focused on the serum albumin level. Compared with those seen at the baseline, the serum albumin levels of the CH-a and CH-b groups were significantly increased at week 16, indicating that the nutritional status of these two groups had significantly improved. Furthermore, the serum albumin levels of the CH-a group and placebo group differed significantly at week 16, indicating that the nutritional status of the CH-a group was significantly better than that of the placebo group at week 16. On the other hand, in the placebo group there was no significant increase in the serum albumin level, and hence, the patients’ nutritional status did not significantly improve during the study period.

Adverse Events

Among the 39 subjects treated with CH-a, two experienced moderate constipation, and one suffered mild diarrhea. Of the 39 subjects who received CH-b, two developed moderate diarrhea, and one of the 42 subjects treated with the placebo experienced a mild headache. It is possible that all of these adverse events were caused by the administered treatment/placebo. However, they only persisted for one day and were resolved with concomitant medication. Upon re-challenge, none of the adverse events reappeared.

Discussion

The present study demonstrated that the oral ingestion of CH-b, which contains higher concentrations of the free-form bioactive peptides Pro-Hyp and Hyp-Gly, resulted in significantly greater improvements in the PUSH score, PSST score, and wound area compared with the ingestion of a placebo. Moreover, the ingestion of CH-a, which contains lower concentrations of these bioactive peptides, produced significantly greater improvements in the PUSH score than the placebo. This effect of CH-a was supported by the previous finding that the ingestion of CH together with an amino acid mixture led to a significant improvement in the rate of change in the PUSH score within 8 weeks. These results suggest that although all CH are derived from similar raw materials, it might be possible to control the healing effects of CH on pressure ulcers by altering their dipeptide content, e.g., by modifying the processes used to produce CH.

Although the examined CH and placebo contained similar amounts of energy (Table 1), it is considered that the improvements in the nutritional status of the patients seen in the CH-a and CH-b groups at week 16 contributed to the improved healing of their pressure ulcers.

(Study continues on to the next page)
The Ingestion of Bioactive Collagen Hydrolysates Enhanced Pressure Ulcer Healing (continued)

16-week clinical study that aimed to determine the effectiveness of collagen hydrolysate during the management of stage II or III pressure ulcers

The significant improvements in the PUSH and PSST scores and wound area induced by CH-b can be attributed to the physiological functions of the oligopeptides absorbed into the subjects’ blood. We previously reported that large amounts of Pro-Hyp and Hyp-Gly were absorbed into the blood after the ingestion of CH-b by healthy subjects. Furthermore, in an experimental study in which [14C]-Pro-Hyp was orally administered to rats it was reported that the Pro-Hyp reached the skin within 30 minutes. Pro-Hyp and Hyp-Gly increased the proliferation of fibroblasts that had migrated from skin pieces on a collagen gel culture system, and Pro-Hyp promoted hyaluronan synthase 2 mRNA expression and hyaluronic acid synthesis by hyaluronan synthase 2 in cultured skin fibroblasts. Furthermore, an experimental study involving a pressure ulcer rat model reported that Pro-Hyp and Hyp-Gly were absorbed into the blood in the CH oral administration group, and the wound area ratio was significantly lower in this group than in the control group. These findings suggest that the Pro-Hyp and Hyp-Gly absorbed into the blood after the ingestion of CH act on fibroblasts in the dermal layers of pressure ulcers and also might affect stem cells, resulting in re-epithelialization and improved healing. In order to confirm that the ingestion of CH promotes the healing of pressure ulcers, it will be necessary to (1) elucidate the mechanism responsible for the accelerated pressure ulcer-healing induced by oligopeptides, such as CH-derived Pro-Hyp and Hyp-Gly, and (2) carry out a large-scale double-blind study.

<table>
<thead>
<tr>
<th>Efficacy improvement*</th>
<th>PUSH score</th>
<th>PSST score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo (N = 39)</td>
<td>CH-a (N = 38)</td>
</tr>
<tr>
<td>Primary (%)</td>
<td>9.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Secondary (%)</td>
<td>11.9</td>
<td>28.2</td>
</tr>
<tr>
<td>Primary + secondary (%)</td>
<td>21.4</td>
<td>46.2</td>
</tr>
</tbody>
</table>

Table 3. Frequencies of each treatment efficacy improvement. *The primary treatment efficacy improvement was a reduction in the PUSH score of ≥5 points and a reduction in the PSST score of ≥10 points between the baseline and week 16. The secondary treatment efficacy improvement was a reduction in the PUSH score of 3–4 points and a reduction in the PSST score of 5–9 points between the baseline and week 16.
REFERENCES


Table 4. Results of the biochemical evaluations. Data are expressed as mean ± SD values. #P < 0.05 vs. baseline; *P < 0.05 vs. placebo. SGOT, serum glutamic oxaloacetate; SGPT, serum glutamic pyruvate transaminase; ALP, alkaline phosphatase.
Collagen-Derived Dipeptides Has Been Shown to Improve the Condition of the Skin in Humans

1. Introduction
Collagen is one of the most abundant proteins in animals, and is a major constituent of connective tissues. Gelatin is a denatured form of collagen, and collagen hydrolysate is produced from gelatin by enzymatic hydrolysis. After the oral administration of radiolabeled CH, radioactivity was shown to accumulate in mouse skin for up to 96 h [1]. The administration of CH also reportedly improved the loss of epidermal barrier function and skin viscoelasticity in hairless mice following UV irradiation[2,3]. Proksch et al. recently demonstrated that the oral ingestion of CH had beneficial effects on skin hydration, transepidermal water loss, and elasticity in a double-blind, placebo-controlled study[4]. These findings from experimental animals and humans suggest that the administration of CH has beneficial effects on the health of skin.

Several peptides have been detected in human peripheral blood following the oral ingestion of CH. Of these collagen-derived peptides, prolyl-hydroxyproline (PO) was identified as the most prevalent dipeptide[5-7]. On the other hand, previous studies detected not only PO, but also hydroxyprolyl-glycine (OG) in human peripheral blood after the ingestion of CH [8,9]. PO and OG are resistant to intracellular dipeptide hydrolysis and were shown to be transported into intestinal cells via peptide transporter-1 (PEPT-1) [10]. Therefore, PO and OG may play an important role in the improving skin barrier dysfunction. However, the effects of the pure peptides, PO and OG, on skin function have not yet been elucidated.

The HR-AD diet-fed hairless mouse has dry skin and more prominent transepidermal water loss [12]. The effects of some food ingredients have already been evaluated on skin barrier function in this animal model [13-15].

The aim of the present study was to examine the effects of ingesting PO and OG on skin barrier dysfunction in HR-1 hairless mice fed a HR-AD diet. Furthermore, a gene expression analysis was performed on the skin using DNA microarrays.

2. Materials and methods

2.1. Peptides
The synthetic dipeptides, prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG), were purchased from Bachem AG (Bubendorf, Switzerland).

2.2. Animals
Male Hos:HR-1 mice (4 weeks old) were purchased from Hoshino. Experimental Animals Inc. (Ibaraki, Japan). Mice were housed in plastic cages, and were kept in an air controlled room maintained at a temperature of 23 ± 1°C, humidity of 50 ± 10%, and a 12-h light–dark cycle (light on 7:00–19:00). They had free access to de-ionized water and semi-purified purified stock diet (Labo MR stock diet, Nosan Corporation, Yokohama, Japan). Animal care and experiments were approved by the Animal Committee of Josai University. After a 1-week acclimatization period, mice were divided into three groups (n = 6) and assigned experimental diets.

2.3. Diets
Labo MR stock powder diet was used for the normal (N) group. A HR-AD diet (Nosan Corporation) was used for the control (C) group. The ingredients of this diet have been described in detail previously [16]. In the PO and OG group (PO + OG), PO and OG were added to the HR-AD diet at 0.15% each (total 0.30%). Mice received the diets and drinking water ad libitum for 35 days.

2.4. Measurement of transepidermal water loss, water content of stratum corneum, and skin viscoelasticity
Transepidermal water loss (TWEL) in the dorsal skin was assessed once every week using VAPOSCAN AS-VT100 RS (Asahi Techno Labo. Ltd., Kanagawa, Japan). Water content of stratum corneum and skin viscoelasticity were measured with a Siskon 200DEX (IBS Co. Ltd., Shizuoka, Japan) spectrophotometer -50% (Courage & Khazawa Electronics, Cologne, Germany), respectively. Skin viscoelasticity (R7) indicated the percent return at 0.1 s after the skin is subjected to reduced pressure at 300 mb for 4 s with a probe. To avoid applying stress to the skin, water content of stratum corneum and skin viscoelasticity were measured before the final day of the experimental period. Skin condition was assessed in triplicate at each dorsal skin spot. All skin condition measurements were performed on conscious animals, and the room was kept at a temperature of 23 ± 1°C and humidity of 50 ± 10%.

2.5. Histology of dorsal skin samples
After the 35-day experimental period, mice were sacrificed under diethyl ether anesthesia, and skin samples were obtained. A portion of the skin was fixed with 10% neutral buffered formalin, and skin sections (5 μm) were stained with hematoxylin and eosin.

2.6. Isolation of total RNA and DNA microarray analyses
Total RNA from the dorsal skin was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer’s instructions. In each group, an equal amount of total RNA was pooled into one sample to normalize individual differences. Pooled total RNA was labeled by Cy3 using the Agilent Low RNA Input Liner Amplification Kit, one-color (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer’s instructions. Cy3-labeled cRNA were purified using the RNasey mini kit (Qiagen, Venlo, Netherlands). The concentration and quality of the labeled cRNA were assessed using the Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bio Analyzer (Agilent Technologies, respectively). After the fragmentation step, labeled cRNA was hybridized to the Agilent Whole Mouse Genome Oligo DNA microarray (44 K, Product No. G4122F) according to the manufacturer’s instruction. Microarray slides were analyzed using an Agilent Microarray Scanner (Model G2565BA). The fluorescence intensities of microarray images were digitized using Agilent Feature Extraction software (version 10.7).

3. Results
3.1. Food intake, water intake, and final body weight
Table S1 shows the final body weights, food intakes, and water intakes for the 35 days of the experimental period. No significant differences were observed in food intake or final body weight between the C group and PO + OG group.

3.2. Changes in TWEL
To clarify the influence of the administered PO + OG on the barrier function of the skin, TWEL was measured weekly. Fig. 1(A) shows changes in TWEL following the administration of the PO + OG diet. Fig. 1(B) shows TWEL after 35 days. TWEL in the N group did not vary throughout the study, but had increased after 21 days in the C and PO + OG groups. TWEL was significantly lower in the PO + OG group than in the C group after 35 days.

3.3. Water content of stratum corneum and skin viscoelasticity
Water content of stratum corneum and skin viscoelasticity were measured to clarify the influence of the PO + OG diet on the water holding capacity and flexibility of the skin, respectively. Fig. 2(A) and (B) shows water content of stratum corneum and skin viscoelasticity after 34 days, respectively. Water content of stratum corneum was significantly higher in the PO + OG group than in the C group. No significant difference was observed in skin viscoelasticity between the C group and PO + OG group.

3.4. Appearance and histopathological findings of the skin
HE-stained dorsal skin sections from HR-1 hairless mice were shown in Fig. 3(A)–(C). The thickness of the epidermis was shown in Fig. 3(D). Epidermal thickening was noted in the C group and PO + OG groups. Although the thickness of the epidermis was higher in the C group and PO + OG group, no significant differences were detected.
Clustering. Annotation cluster 1 included myofibril (GO: 00030016), contractile fiber (GO: 0043292), sarcromere (GO: 0030017), and contractile fiber part (GO: 0044449), and all GOs were related to muscle function.

On the other hand, in the analysis of the down-regulated genes, DNA binding (GO: 0003677) was found in annotation cluster 1; however, it was impossible to identify genes related to skin function (Table S2).

4. Discussions

Previous studies have examined the efficacy of oral administration of PO + OG on skin, especially in rodents. We hypothesized that adsorbed PO and OG reached the skin and improved skin barrier dysfunction. In the present study, we investigated the effects of the administration of PO + OG on diet-induced skin barrier dysfunction in HR-1 hairless mice. The results obtained for the first time that the administration of PO + OG significantly improved skin barrier dysfunction.

However, the mechanism by which PO + OG improved skin barrier function currently remains unclear. It has been suggested that PO may have a bioactively improved barrier function. Kawaguchi et al. reported that some orally administered PO reached the skin [17]. PO was previously found to stimulate cell proliferation and induce the synthesis of hyaluronic acid in mouse skin fibroblasts and human dermal fibroblasts [18-19]. The mRNA expression of hyaluronic acid synthase was suppressed and the content of hyaluronic acid was decreased in mouse skin chronically irradiated with ultraviolet B [3,20]. In the present study, we could not identify any skin function-related genes, such as hyaluronic acid synthase, in genes whose expression was changed by twofold or greater. Hyaluronic acid is a component of the extracellular matrix, and has been shown to play crucial roles in the skin barrier, elasticity, and hydration [21].

Improvements in skin barrier function in the present study may be related to the synthesis of hyaluronic acid by PO and the slightly altered expression of its gene.

On the other hand, OG has been detected at a relatively high concentration in human peripheral blood following the ingestion of CH [8,9]. Shigemura et al. reported that OG enhanced the proliferation of fibroblasts derived from mouse skin more than PO [8]. These findings suggest that not only PO, but also OG modulated cells and the extracellular matrix in the skin. However, information regarding the function of OG is limited. Further studies are needed to investigate the effects of PO and/or OG on skin barrier function. The findings of these studies will provide an insight into whether PO and/or OG play an important role in the function of CH, and also if these peptides are candidates as bioactive peptides.

Many numerical genes are related to skin function. We hypothesized that PO + OG upregulated gene expression related to the maintenance and repair of skin. Therefore, we adopted a DNA microarray analysis to investigate changes in gene expression in the skin. We extracted genes whose expression levels were higher or lower by twofold or greater in the PO + OG group than in the C group. To clarify the extracted genes, up- or down-regulated genes were obtained by searching against the Gene Ontology (GO) using the functional annotation clustering program, DAVID [22]. However, the results obtained showed that no gene was related to skin function. The present study demonstrated that PO + OG did not improve skin barrier function by altering gene expression. The top 3 ranked clusters of up-regulated genes were muscle-related genes. In this study, we used dorsal skin as a sample for the DNA microarray, and mice did not perform exercise. The results of this experiment suggested that PO and/or OG have the potential to enhance muscle function. Further studies are required to investigate the effects of PO and/or OG on muscle function and search for new food-derived bioactive peptides.

In conclusion, the results of the present study demonstrated that the daily administration of PO + OG improved skin barrier dysfunction in HR-1 hairless mice. Furthermore, PO + OG affected muscle-related gene expression. These results demonstrated that PO and/or OG may have important beneficial effects on skin and muscle. Foods containing PO and/or OG have the potential to be used as food supplements for skin and muscle.
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