Novel Hydrolyzed Porcine and Fish Collagen Beverage Improved Collagen, Elasticity, Moisture, Spots: A Randomized, Double-blind, Placebo-controlled Trial

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Abstract  Background: As the population ages, oral hydrolyzed collagen supplements are gaining traction. However, few clinical studies on the skin health of hydrolyzed porcine collagen combined with fish collagen.

Objective: This study investigates whether TripleUp collagen beverage has skincare and anti-inflammatory effects.

Methods: Using collagen beverage to treat epidermal keratinocytes and examine ROS, HA, inflammatory factor, and moisturizing-related gene expressions (Tgm1, Krt14, FLG-F, GBA, HAS3). Fifty subjects were recruited into a placebo and collagen beverage group for four weeks and then examined skin condition at 0, 2, 4 weeks.

Results: TripleUp collagen beverage decreased IL-8 and ROS expression and increased moisturizing-related gene expressions. In addition, collagen beverages increase skin collagen, elasticity, moisture, and decreased spots.

Conclusions: Thus, TripleUp collagen beverage included hydrolyzed porcine, and fish collagen and vitamin C can improve skin condition.

Keywords: collagen, fish, skin, porcine, vitamin C


1. Introduction

Healthy skin provides an active interface between the internal and external environments of the body. Many different factors can accelerate the skin aging process, including intrinsic aging, radiation, dietary intake, and nutrient deficiencies, resulting in the loss of skin collagen [1]. Excessive exposure to ultraviolet light on the skin can cause acute or chronic damage. Short-term exposure to ultraviolet light can cause sunburn and erythema [2]. Long-term exposure can increase cancer and accelerate aging. Irradiation of ultraviolet light will increase the reactive oxygen species (ROS) production, activate complex signaling pathways in skin cells, increase inflammation, and reduce the activity of some antioxidants such as superoxide dismutase and catalase [3]. Skin aging is also associated with loss of skin moisture. The key molecule involved in skin moisture is hyaluronic acid (HA). The skin’s hydration critically depends on the HA-bound water in the dermis, suggesting that HA homeostasis exhibits a distinct profile in intrinsic skin aging [4]. Therefore, as the population continues to age, the capabilities of nutritional supplements are receiving increasing attention.

Collagen, the most abundant component of the extracellular matrix, is the decisive protein that determines skin physiology by maintaining the skin structure and enabling its numerous functions [5]. The collagen structure is that three chains are intertwined to form the collagen triple helix. Collagen fibers with tremendous strength and tension that retain moisture and support smooth, firm, and toned skin [6]. Hydrolyzed collagen is commonly derived from cow, pig, and chicken sources and is more suitable for digestion because it dissolves in water or brine and is well absorbed [7]. Hydrolyzed collagen derived from fish has emerged as an alternative source due to lower environmental impact and risk of disease transmission. Further, fish collagen and collagen peptides have a high degree of homology to human structure and bioavailability through the gastrointestinal barrier [8]. Fish collagen is more easily absorbed than porcine collagen, has a low molecular weight, and is preferable to the industry due to low inflammatory
reactions. Also, type I collagen is abundant in marine organisms [9]. Studies showed that oral administration of specific bioactive porcine collage peptides has anti-aging effects, and oral fish collagen supplements increase skin hydration, elasticity, and dermal collagen density [10]. In addition to its role in collagen synthesis, vitamin C acts as a powerful antioxidant by neutralizing ROS responsible for cell apoptosis [11]. Vitamin C also plays an essential role in collagen fiber formation and cell differentiation [12]. Although several preclinical studies provide evidence for the beneficial effect of hydrolyzed collagen on skin health, less information is known about the clinical benefits.

In this study, we used TripleUp collagen beverage, formulated primarily with porcine and fish collagen and vitamin C, to explore whether collagen beverage can improve skin condition. First, we used collagen beverages to treat epidermal keratinocytes and examined ROS, HA, and inflammatory factors. Second, 50 subjects were divided into a placebo group (n = 25) and a collagen beverage group (n = 25) for four weeks and then examined skin condition at 0, 2, 4 weeks.

2. Materials and Methods

2.1. Cell Culture

The normal adult human primary epidermal keratinocyte cell line was purchased from the ATCC, and cultured in dermal cell basal media supplemented with 0.4% bovine pituitary extract (BPE), 0.1% recombinant human (rh) TGF-α (0.5 ng/mL), 3% l-glutamine (6 mM), 0.1% hydrocortisone hemisuccinate (100 ng/mL), 0.1% rh insulin (5 mg/mL), 0.1% epinephrine (1.0 mM), 0.1% apotransferrin (5 mg/mL; ATCC PCS-200-400), and 0.1% penicillin (100 U/mL)-streptomycin (100 µg/mL; Thermo Fisher Scientific), in a humidified incubator of 5% CO2 at 37°C.

2.2. Cell Viability Assay

Primary epidermal keratinocytes (2 × 10^5 cells/ml) were seeded in 100 μl of 96-well plates for at least 24 h before use. The cells were treated with collagen beverage for 24 h. The cell viability was measured by the MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay according to the manufacturer's instructions (Promega, Madison, WI). After the cells had been incubated for the indicated times, they were incubated with MTT solution (0.5 mg/mL) for four h. The formazan precipitate was dissolved in 200 µl DMSO, and the absorbance at 570 nm was measured using an automated microplate reader (BioTek, SynergyTM2, USA).

2.3. Detection of IL-8 and Hyaluronic Acid

IL-8 and HA levels in cell supernatants were measured with ELISA. Samples were assayed in triplicate for each condition. The ELISA kits were obtained from Cloud-Clone Corp. (US). All experimental procedures followed the recommended protocols provided by the company.

2.4. Detection of ROS Production

The epidermal keratinocytes were seeded in 96-well plates (7× 10^3) and grown 24h. Using H2O2 (200 uM) induced ROS expression. Cells were then washed with PBS solution and incubated for 30 min with 2',7'-dichlorofluorescein diacetate probe (H2-DCFDA) (Merck, Milan, Italy) (10 µM). After one h of incubation, an excess of H2-DCF-DA was removed and replaced with PBS, and then ROS levels were measured using a microplate reader (Infinte 200, Tecan, Salzburg, Austria).

2.5. Quantification of Gene Expressions by Real-time PCR

The treated human primary epidermal keratinocytes were harvested, and total RNA was isolated from cells using a RNA purification kit (Geneaid, Taiwan). DNA-free total RNA was reversely transcribed to cDNA using a SuperScript™ Reverse Transcriptase kit (Invitrogen, Life Technologies Co., CA, USA). Quantitative real-time PCR was conducted using an ABI StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc., CA, USA) and the SYBR Green Master Mix (KAPA Biosystems, MA, USA) for transcript measurements. The gene-specific primers used in this study are listed in Table 1. The GAPDH gene was used as a normalization control.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Name</th>
<th>Sequence(5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgm1</td>
<td>TGM1-F</td>
<td>GATCGCATCACCCCTTGAGTTAC</td>
</tr>
<tr>
<td></td>
<td>TGM1-R</td>
<td>GCAGGTTCAATGATTGCC</td>
</tr>
<tr>
<td>Keratin 14</td>
<td>KRT14-F</td>
<td>TCTGAAAGAGATGGGGAC</td>
</tr>
<tr>
<td></td>
<td>KRT14-R</td>
<td>GCAGCTCAATCTCCAGTTTC</td>
</tr>
<tr>
<td>Filaggrin</td>
<td>FLG-F</td>
<td>GCAAATCTCTGAAGATACCA</td>
</tr>
<tr>
<td></td>
<td>FLG-G</td>
<td>TCTTCTTGTGCTTTTGC</td>
</tr>
<tr>
<td>Glucocerebrosidase</td>
<td>GBA-F</td>
<td>TCCGGTTGACAATTCTAGC</td>
</tr>
<tr>
<td></td>
<td>GBA-R</td>
<td>TGTGTCTGACATACGACTC</td>
</tr>
<tr>
<td>Hyaluronan synthase 3</td>
<td>HAS3-F</td>
<td>CGACGCAACTCTCATGAGG</td>
</tr>
<tr>
<td></td>
<td>HAS3-R</td>
<td>AGTCGCCACACCTGTAGTGT</td>
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<tr>
<td>GAPDH</td>
<td>GAPDH-F</td>
<td>CTGCGTACATGTGGGCAATG</td>
</tr>
<tr>
<td></td>
<td>GAPDH-R</td>
<td>AAGTGGTCTGTGGGCAATG</td>
</tr>
</tbody>
</table>

Table 1. Primer sequence
2.6. Clinical Trial Design

The clinical study had been approved by Antai-Tian-Sheng Memorial Hospital Institutional Review Board (TSMH-IRB 19-098-B), and the study had been registered on ClinicalTrials.gov Identifier: NCT04225091. Fifty adult subjects (20-65 years old) were recruited in this trial between November 2019 and November 2020. Informed consent was obtained from all subjects before the study at Chia Nan University of Pharmacy & Science. The subjects were divided into a placebo group (n=25) and a TripleUp collagen beverage group (n=25). Each subject was informed about intaking a bottle of TripleUp collagen beverage labeled 50ml or a placebo drink daily for four weeks and was not allowed to take any other supplement during the intervention period. The exclusion criteria included: i) skin disease, liver cirrhosis, or chronic renal failure; ii) allergy to cosmetics, drugs, or foods; iii) pregnant and breastfeeding; iv) taking chronic drugs; v) people who had any cosmetic procedures (intense pulse light, medical peelings, or laser therapy) before four weeks of the study.

2.7. Test Sample

TripleUp collagen beverage (Triple Up® Collagen, Melaleuca (China)) contains 15% porcine and 0.1% fish collagen, and 0.1% vitamin C, Rosa rugosa extract, citric acid, sucralose, water. Placebo beverage of the main ingredient: citric acid, sucralose, water. Each subject was required to examine skin condition checks at 0, 2, and 4 weeks.

2.8. Clinical Skin Efficacy Assessment

DermaLab® Series SkinLab Combo was utilized to scan and analyze skin collagen density. The color scale indicates collagen density; white reflects the highest collagen density, and black reflect the lowest. Cutometer® dual MPA580 was utilized to measure skin elasticity, and the higher the relative value, the more significant the improvement. Corneometer® CM825 was utilized to measure skin moisture content, and the higher the relative value, the more significant the improvement. VISIA Complexion Analysis System was utilized to measure visible skin spots, and the lower the relative value, the more significant the improvement.

2.9. Statistical Analysis

The comparison of measurement results for skin parameters among groups and between groups was analyzed by one-way repeated measurement ANOVA and one-way ANOVA, respectively, followed by Tukey’s post hoc test through GraphPad Prism, as P < .05 was considered statistical significance.

3. Results

3.1. TripleUp Collagen Beverage Decreased IL-8 and ROS Expression

First, to explore whether TripleUp collagen beverage had anti-inflammatory and ROS ability, we used collagen beverage to treat epidermal keratinocytes and examined IL-8 and ROS expression. We used LPS (200ng/ml) induced IL-8 expression, and then treated with 0.25%, 0.5%, 1% collagen beverage. TripleUp collagen beverage significantly decreased IL-8 expression, 91.4%, 91.7%, 100.7% respectively, compared to LPS group (Figure 1A). We also used H2O2 (200 μM) induced ROS expression and then treated with 0.25% 0.5% collagen beverage. TripleUp collagen beverage significantly decreased ROS expression, 49.2%, and 32.1%, respectively, compared to the H2O2 group (Figure 1B). In addition, 0.25%, 0.5%, 1% collagen beverage did not cause cytotoxicity (Figure 2).

3.2. TripleUp Collagen Beverage Increased Moisturizing-related Gene Expressions

Next, we want to know whether TripleUp collagen beverage had a moisturizing effect; we used collagen beverage to treat epidermal keratinocytes and examined HA and moisturizing-related gene, Transglutaminase 1(Tgm1), Keratin 14 (Krt14), Filaggrin (FLG-F), Glucocerebrosidase (GBA), Hylauronan synthase 3 (HAS3). 1% collagen beverage slightly increased HA expression compared to mock group (Figure 3A), and 0.25% collagen beverage significantly increased Tgm1, Krt14, FLG-F, GBA, HAS3, 27.4%, 1.8%, 30.6%, 40.6%, 17.7% respectively, compared to mock group (Figure 3B).

3.3. TripleUp Collagen Increases Skin Collagen, Elasticity, Moisture, and Decreased Spots

Finally, we want to know whether TripleUp collagen beverages improve skin collagen, elasticity, moisture, and spots. Subjects were required to consume 50 mL of the collagen beverage or a placebo beverage daily for four weeks. After consuming the collagen beverage for 2, 4 weeks, the skin collagen density significantly increased 7.2%, 13.1% compared to baseline (week 0), and significantly increased 6.3%, 11.6% compared to the placebo group (Figure 4A). The skin elasticity significantly increased 3.9%, 5.9% compared to baseline (week 0), and significantly increased 3.4%, 5.8% compared to the placebo group (Figure 4B). In addition, the skin elasticity was increased 5.5%, 4% compared to baseline (week 0), and significantly increased 7.3%, 8.6% compared to the placebo group (Figure 5A). The skin spots decreased 5.3%, 5.1% compared to baseline (week 0), and significantly reduced 8.5%, 13.9% compared to the placebo group (Figure 5B). These results indicated that the TripleUp collagen beverage increased skin collagen, elasticity, moisture, and decreased spots.
Figure 1. TripleUp collagen beverage decreased IL-8 and ROS expression. (A) Using LPS (200ng/ml) induced IL-8 expression in primary epidermal keratinocytes, then treated with different concentrations of collagen beverage, the IL-8 was measured by ELISA. ##P,0.01, compared to mock. ***P,0.001, compared to LPS. Error bars represent ± standard deviation. (B) Using H2O2 (200 uM) induced ROS expression in primary epidermal keratinocytes, then treated with different concentrations of collagen beverage, the ROS was measured by 2'-7'dichlorofluorescin diacetate (DCFH-DA). *P,0.05, **P,0.01, compared to mock+ H2O2. #P,0.05, compared to mock. Error bars represent ± standard deviation.

Figure 2. TripleUp collagen beverage not affected cytotoxicity. Using primary epidermal keratinocytes incubated for 24 hours with different concentrations of collagen beverage, then cytotoxicity was assessed by MTT assay. Error bars represent ± standard deviation.
Figure 3. TripleUp collagen beverage increased HA and moisturizing-related gene expressions. (A) Treated with different concentrations of collagen beverage in primary epidermal keratinocytes, the HA was measured by ELISA. Error bars represent ± standard deviation. (B) The moisturizing-related gene (Tgm1, Krt14, FLG-F, GBA, HAS3) was measured by Q-PCR. *P < 0.05, **P < 0.01, ***P < 0.001 compared to mock. Error bars represent ± standard deviation.

Figure 4. The TripleUp collagen beverage improved skin collagen and elasticity. The (A) collagen, (B) elasticity (n = 25; mean value ± S.E.M.) (*) compared with before using (week 0), #, compared with placebo) (*, p < 0.05, **, p < 0.01, ***, p < 0.001) (#, p < 0.05, ###, p < 0.001)
4. Discussion

This study found that a TripleUp collagen beverage containing porcine and fish collagen and vitamin C decreased IL-8 and ROS expression and increased moisturizing-related gene expressions in vitro. In the clinical trial, TripleUp collagen beverage improved skin collagen, elasticity, moisture, spots, suggesting the potential of collagen beverage for improving skin moisture.

Hydrolyzed collagen was a natural protein that was the primary substance in animals and was abundant in skin, tendons, cartilage, and bones [7]. There were several types of collagen, and type I and type III collagen were the most abundant collagen in the skin [13]. Hydrolyzed collagen had perfect biocompatibility and was widely used as medical and nutraceutical. Currently, commercial hydrolyzed collagen is mainly extracted from mammalian skin and organs. Studies showed that hydrolyzed porcine collagen could promote wound healing and stimulate human keratinocyte proliferation and differentiation [14]. In addition, hydrolyzed porcine collagen increased collagen synthesis and stem cell proliferation [15]. Compared with hydrolyzed collagen from land animals, hydrolyzed fish collagen had unique molecular and biological properties in amino acid composition, antioxidative activity, and anti-skin aging activity due to low temperature and high salt conditions [16]. Studies have shown that hydrolyzed fish collagen repaired skin collagen and elastin protein fibers and significantly thickened the outer skin surface [17]. Hydrolyzed collagen from shark cartilage was also rich in glycosaminoglycans and various proteins [18]. Studies had shown that cartilage contained active substances that inhibited angiogenesis and anti-inflammatory [19], and 0.25%, 0.5% hydrolyzed fish collagen inhibited ROS formation and increased mitochondrial activity in human skin fibroblasts [20]. Vitamin C is a water-soluble reducing agent and antioxidant [21]. Vitamin C had potential effects in alleviating inflammatory status by reducing high-sensitivity C-reactive protein (hs-CRP), IL-6, in obese patients [22]. Consistent with our results, TripleUp collagen beverage decreased IL-8 and ROS expression significantly. Tgm1 was an indispensable enzyme for the formation of the keratinocyte membrane; Tgm1 stabilized proteins by the formation of ε(γ-glutamyl)lysine cross-links [23]. The primary function was to prevent skin water loss. Krt14 was keratin, the main building block of the skin [24]. FLG was composed of polykeratin filaments (filaggrin), which enzymes can decompose to form a natural moisturizing factor on the skin [25]. GBA was an enzyme that synthesizes ceramide, a lipid between cells in the skin’s stratum corneum [26]. HAS3 promoted hyaluronic acid synthesis [27]. Some studies showed oral administration of hydrolyzed collagen promoted filaggrin expression in mice [28], and oral ingestion of hydrolyzed collagen induced an increase in water content, partially due to the rise in the natural moisturizing factor (NMF) level in the skin surface layer [29]. Consistent with our results, TripleUp collagen beverage significantly increased moisturizing-related gene expressions.

Administration of collagen peptides can positively impact various skin conditions and skin aging [5]. Orally
administered hydrolyzed collagen was absorbed in the small intestine and bloodstream as peptides and free amino acids and distributed into the dermis for up to 14 days [30]. In the dermis, hydrolyzed collagen provided amino acids for forming collagen and elastin and stimulating endogenous production of new collagen, elastin, and HA [7]. Oral supplementation with hydrolyzed collagen had shown more pronounced skin effects than topical products. Studies have shown that taking 10g of hydrolyzed collagen daily for 56 days increased skin moisture and collagen density compared to placebo [31]. Skin hydration, elasticity, wrinkles, and roughness were also significantly improved in subjects taking 10 mg [32]. Consistent with our results, TripleUp collagen beverage significantly increased skin collagen, elasticity, moisture, and decreased spots.

5. Conclusion

This study was demonstrated the TripleUp collagen beverage included porcine and fish collagen and vitamin C for the substantial improvements in collagen, elasticity, moisture, spots in the skin. In addition, collagen beverages can also increase the moisturizing-related gene expressions and anti-inflammatory effect. TripleUp collagen beverage can be used as one of the health care products for skin moisturizing in the future.

Conflict of Interest

The authors declare no conflict of interest.

References


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