A Collagen Formula for Anti-aging

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***Abstract*—This article demonstrates the synergistic effect of a collagen formula drink on improvement in Extracellular Matrix (ECM) protein synthesis and down-regulation of the collagen degradation gene and Reactive Oxygen Species (ROS) formation in human fibroblasts. The collagen formula drink mainly consists of porcine and fish collagens, which is an uncommon approach to skin repair and anti-aging. The collagen formula drink could enhance the collagen and elastin formation in UVA-treated CCD-966SK cells by 30% and 19%, respectively. In addition, it up-regulated collagen- and hyaluronate (HA)-associated genes (*COL1A1*, *COL1A2*, *LOX*, and *HAS2*) in the range of 75%-187% as well as down-regulated collagen-degradation gene (MMP-1) by 41%. Also, the collagen formula drink lowered the ROS formation in CCD-966SK cells by 26%. Accordingly, the collagen formula drink can substantially improve the skin repair efficiency after photodamage and provide a comprehensive protection effect on anti-aging.**

***Index Terms*—porcine collagen, fish collagen, anti-aging, collagen, elastin, ROS**

I. Introduction

Chronological aging is an inevitable phenomenon but unpleasant experience in human beings. Although the aging process of the whole body is hard to be elaborated by specific measurement indexes, skin appearance delineates a part of entities. Skin aging caused by either endogenous (e.g., cellular metabolism) or exogenous (e.g., UV radiation) factors and is generally manifested as dullness, dryness, laxity, wrinkles, and benign neoplasms [1], [2]. In particular, 80% of skin aging is associated with photodamage since UV radiation may result in premature skin aging [3]. Specifically, UVA (wavelength range: 315-400 nm) and UVB (wavelength range: 290-320 nm) are considered the primary causes to dermal and epidermal damages [4].

When UV radiation penetrates into the connective tissue of skin and interacts with cellular chromatophores and photosensitisers, the interactions may lead to an increase of oxidative stress in response to cumulative ROS and elicit DNA damage, cell death, and the degeneration of ECM proteins (i.e., collagen and elastin) [5]. Collagen and elastin impart elementally mechanical forces to the structural integrity of the skin [6]. Chronic oxidative stress up-regulates the expression of Matrix Metalloproteinases (MMPs) released from epidermal keratinocytes and dermal fibroblasts, which cleave collagens into collagen fragmentation and deformed collagen fibers [7], [8]. MMP-1 initiates the cleavage of collagen I and collagen III at a single site in the triple helix followed by the degradation of MMP-3 and MMP-9 [9]. Studies have reported that the increased levels of MMPs are subject to a function of age and the exposure time of UV irradiation [7], [10]. Moreover, MMPs and collagen fragment may impede the efficiencies of collagen synthesis and skin repair [11].

Collagen hydrolysates have been proved to be beneficial for antioxidant, anti-aging, anti-inflammation, and wound healing in animal/clinical studies [12]-[17]. Some research groups unveiled that consuming 10 g/day of collagen supplement over 6 weeks could significantly improve skin elasticity, hydration, and dermal collagen density [13], [17]. Thus, collagen supplement/drink is possibly available for anti-aging. The accessible collagen sources are from bovine, porcine, human, and marine collagens [18]. In this study, we attempt to explore the synergistic effect of a collagen formula, called TripleUp Collagen Drink, mainly containing porcine (pig) and fish collagens and vitamin C, for anti-aging and the improvement of photoaging through the *in-vitro* methodology. Despite porcine applied in a variety of medical applications such as vaccine, capsule, or wound depressing, to the best of our knowledge, few researches clearly point out the relationship between porcine collagen and anti-aging [19]-[21]. Additionally, fish collagen has recently drawn tremendous attention because of the reuse of fish wastes (e.g., bone) [22]. This research successfully demonstrated the positive outcomes

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of TripleUp Collagen Drink for remarkably slowing collagen degeneration, improving collage production, retarding cell ROS production, and enhancing the expression levels of collagens, elastin, and HA modulation genes.

II. Materials and Methods

A. Experimental Materials

Triple Up Collagen Drink [Triple Up® Collagen, Melaleuca (China), ingredients: porcine collagen, fish collagen, brown rice fermented powder, apple polyphenols, ascorbic acid, soy power, west Indian cherry, spinach powder, chondroitin, sea buckthorn juice, yeast extract, sucralose, β-carotene, citric acid monohydrate, roxburghii extract, corn powder, walnut extract, stevioside, apple juice, water, blackcurrant/blackberry /strawberry flavors, and fructose], human skin fibroblast CCD-966Sk (ATCC, CRL-1881), cell growth media (Gibco®; minimum essential media, 10% fetal bovine serum, 1 mM sodium pyruvate, and 1% penicillin/streptomycin), ultravioler radiation chamber (Vilber), Sircol™ Soluble Collagen Assay kit (Biocolor), Fastin™ Elastin Assay kit (Biocolor), RNA extraction kit (Genaid Biotech), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), nCounter® platform (NanoString Technologies).

B. Collagen Assay

2×10^4 CCD-966Sk cells in 0.5 mL of the media were seeded in each well of 24-well plates for 24 hours incubation. Then, we replaced the media with serum free media with 0.25% or 0.125% of collagen formula and shined the cells with 10 J/cm^2 UVA (note that the normal culture group without this step). After the UVA exposure, we cultured the cells for another 24 hours and collected the culture media for the analysis of collagen content. In the end, the collagen level was analyzed by the collagen assay.

C. Elastin Assay

1×10^5 CCD-966Sk cells in 2 mL of the media were seeded in each well of 6-well plates for 24 hours incubation. Afterward, we replaced the media with a fresh batch of media with 0.25% or 0.125% of collagen formula and shined the cells with 10 J/cm^2 UVA. After the UVA exposure, we cultured the cells for another 48 hours and used RIPA buffer to lyse the cells. Finally, the elastin content of the cells was analyzed by the elastin assay.

D. Analysis of mRNA Expression

1.5×10^5 CCD-966Sk cells in 2 mL of the media with 0.25% of collagen formula were seeded in each well of 6-well plates and incubated for 24 hours. Then, we collected the cells and used the RNA extraction kit for RNA collection. Finally, we adjusted the RNA concentration to 75 ng/μL for mRNA expression analysis. The mRNA expression level was analyzed by a nCounter® platform, and all the operation steps were referenced by the recommend protocol.

E. ROS Assay

2×10^5 CCD-966Sk cells in 2 mL of culture medium were added to each well of 6-well plates and incubated for 24 hours. Afterwards, the medium were replaced with the 0% or 0.25% the collagen drink. After another 24 hour incubation, the cells were washed with PBS twice and interacted with DCFH-DA (5 μg/mL) dye solution for 15 minutes. Next, the cells were treated to 0.5 mM hydrogen peroxide (H_2O_2) for an hour. The fluorescence signals were obtained by a flow cytometer (excitation wavelength: 450-490 nm; emission wavelengths: 510-550 nm).

F. Statistical Analysis

All the measurement results were analyzed by t-test, with $p < 0.05$ considered significant.

III. Resuls and Discussion

A. Evaluation of Collagen Production

We firstly investigated the collagen production capacity in fibroblasts in the normal culture condition (Fig. 1A). 0.125% and 0.25% of collagen formula drinks could facilitate the increase of collagen production in CCD-966Sk cells by 32% and 40%, respectively, as compared to the control group. The collagen production showed a dose-dependent trend. We proved that the collagen formula drinks would not impede the cell vitality and could significantly facilitate the collagen synthesis in the fibroblasts. Further, we evaluated the anti-UV capacity of collagen formula drink in the condition of 10 J/cm^2 UVA treatment (Fig. 1B). 0.125% and 0.25% of collagen formula drinks increased the collagen production in the CCD-966Sk cells after treatment with UVA by 17% and 30%, respectively. Although the collagen production efficiency (0.25% of collagen drink) was 10% lower than the result without UVA treatment, the collagen formula drinks still imparted a significant improvement in UV-affected fibroblasts. In addition, the result also unveils a dose-dependent effect for collagen production. Accordingly, these encouraging outcomes suggest that the collagen formula drink may provide the characteristics of delaying chronological skin aging and improving skin repair.

B. Evaluation of Elastin Content

Fig. 2 displays the relative elastin content in fibroblasts after treatment with the collagen formula drink and 10 J/cm^2 UVA. 0.125% and 0.25% of collagen formula drinks increased the elastin synthesis in the CCD-966Sk cells by 3% and 19%, respectively. The elastin synthesis was positively correlated with the concentration of the collagen formula drink. The significant improvement results indicate that the collagen formula drink could expedite the elastin synthesis after skin damage, which is a prominent effect on skin repair. Considering the promising experimental results of collage and elastin synthesis, we believe that our collagen formula drink can substantially help skin to repair the damaged connective tissue and minimize the influence of UV damage and confer a beneficial anti-aging effect.

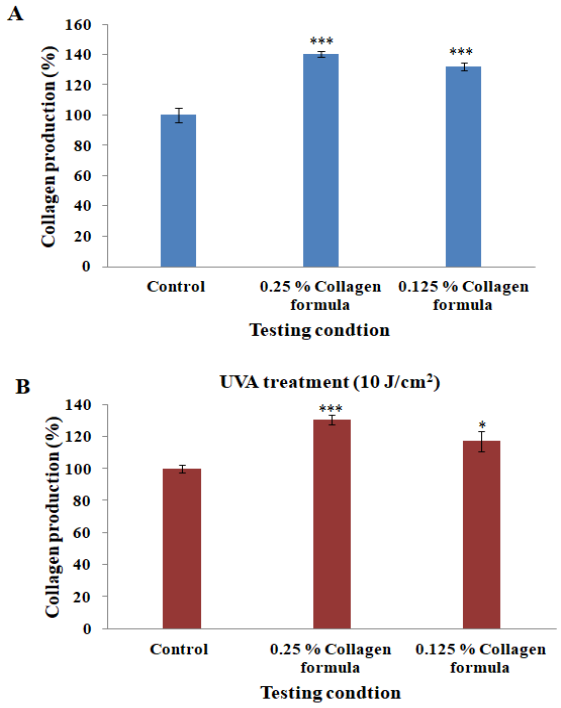

Figure 1. Evaluation of relative collagen production in CCD-966SK cells after treatments with 0.125% /0.25% of collagen formula. (A) Normal culture conditions. (B) UVA treatment. (n = 3, mean value ± S.D.) (*, p < 0.05; ***, p < 0.001).

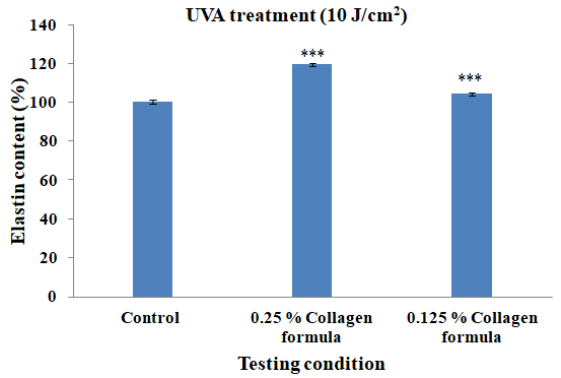

Figure 2. Evaluation of relative elastin content in CCD-966SK cells after treatments with 0.125%/0.25% of collagen formula and UVA treatment. (n = 3, mean value ± S.D.) (***, p < 0.001).

C. mRNA Expression Analysis for Collagen-, HA-Related Genes

Furthermore, we demonstrated the collagen formula drink could significantly increase the mRNA expression levels of *COL1A1*, *COL1A2*, *LOX*, and *HAS2* genes (Fig. 3). 0.25% of collagen formula drink could up-regulated *COL1A1*, *COL1A2*, *LOX*, and *HAS2* genes by 187%, 75%, 78%, and 84%, respectively, as compared to the UVA group. *LOX* gene encodes a member of the lysyl oxidase family of proteins, which engage in the crosslinking of collagen and elastin in ECM. Increased expression level of *HAS2* represents the up-regulation of HA synthesis. The elevated levels of *COL1A1* and *COL1A2* as well as *LOX* genes positively corresponded with the results of collagen production and elastin content (Fig. 1, 2). Therefore, based on the molecular level evidences, we confirmed that our collagen formula drink noticeably up-regulated the expression of the important ECM proteins and glycosaminoglycan and provide a comprehensive effect on skin tissue repair.

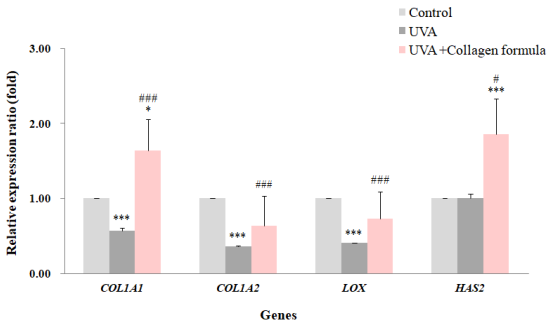

Figure 3. mRNA expression analysis of collagen-/HA-related genes in CCD-966SK cells after treatment with 0.25% of collage formula drink and UVA treatment. (n = 3, mean value ± S.D.) (Corresponding to the control group: *, p < 0.05; ***, p < 0.001) (Corresponding to the UVA group: #, p < 0.05; ###, p < 0.001).

D. Inhibition of MMP Gene Expression

Fig. 4 show the inhibition result of *MMP-1* expression. MMPs involve collagen degradation and ECM remodeling in connective tissue, so their expression levels directly influence skin aging, especially MMP-1. The collagen formula drink could down-regulate the relative expression of *MMP-1* gene by 41% as compared to the UVA group. This significant improvement highly supported the efficacy of our collagen formula drink for anti-aging. In light of the cell behavior and molecular results, we presume that our collagen formula drink may also down-regulate the upstream modulator activator protein-1 (AP-1). AP-1 dominates the expression of MMPs via the MAPK signaling pathway [23].

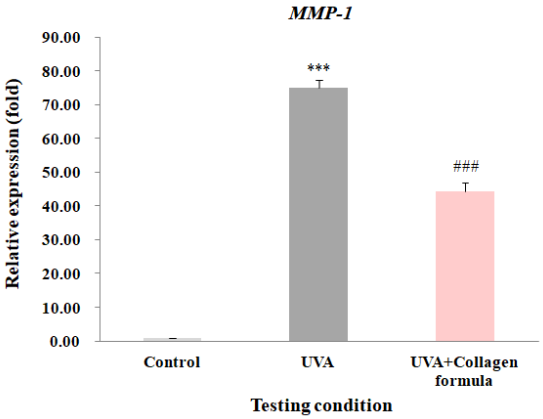

Figure 4. mRNA expression analysis of *MMP-1* gene in CCD-966SK cells after treatment with 0.25% of collage formula drink and UVA treatment. (n = 3, mean value ± S.D.) (Corresponding to the control group: ***, p < 0.001) (Corresponding to the UVA group: ###, p < 0.001)

E. ROS Inhibition

In addition to the improvement in collagen synthesis, we are also curious about the aspect of oxidative stress inhibition in the cells treated with the collagen formula drink because ROS are associated with collagen synthesis

and degradation through the regulation of AP-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and transforming growth factor beta (TGF-β) [24]. Fig. 5 shows the ROS production results in CCD-966Sk cells. As compared to the positive control (H_2O_2 group), CCD-966Sk cells treated with collagen formula drink lowered the ROS synthesis by 26%. The notable improvement of ROS formation indicates that our collagen formula drink is beneficial to minimize the influence of oxidative damage and improve ROS scavenging ability. Thus, the collagen formula drink may be possible to delay cell/tissue aging cause by endogenous and exogenous stressors.

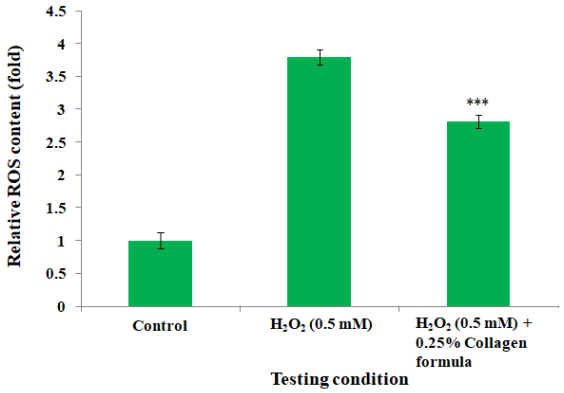

Figure 5. ROS inhibition result in CCD-966SK cells after treatment with 0.25% of collage formula drink. (n = 3, mean value ± S.D.) (Corresponding to the positive control group: ***, $p < 0.001$)

IV. Conclusions

This study successfully demonstrates the synergistic effect of porcine and fish collagens on improvements in collagen synthesis and the repair efficiency for photodamage in human fibroblasts. The collagen formula drink increased the collagen and elastin synthesis in UVA-treated CCD-966SK cells by 30% and 19%, respectively. Moreover, gene expression results proved that collagen formula drink up-regulated *COL1A1*, *COL1A2*, *LOX*, and *HAS2* genes in UVA-treated CCD-966SK cells in the range of 75%-187% as well as down-regulated *MMP-1* gene by 41%. Also, it inhibited ROS formation by 26%. In short, the collagen formula is able to substantially enhance the expression and production of ECM components and confer a comprehensive protecton effect on anti-aging—skin repair and skin firmness.

Conflict of Interest

The authors declare there is no ant conflict of interest in this research.

Author Contributions

Shu-Ting Chang, Yung-Hsiang Lin, and Yung-Kai Lin concocted the ideas, designed the experiments, and supervised the project. Kai-Wen Kan performed the experiments and analyzed the experimental data. Chen-Meng Kuan analyzed the experimental data and prepared the manuscript.

References

[1] I. Binic, V. Lazarevic, M. Ljubenovic, J. Mojsa, and D. Sokolovic, "Skin ageing: Natural weapons and strategies," *Evid. Based Complement. Alternat. Med.*, vol. 2013, p. 827248, 2013.
[2] R. Ganceviciene, A. I. Liakou, A. Theodoridis, E. Makrantonaki, and C. C. Zouboulis, "Skin anti-aging strategies," *Dermatoendocrinol.*, vol. 4, pp. 308-319, 2012.
[3] J. Uitto, "Understanding premature skin aging," *N. Engl. J. Med.*, vol. 337, pp. 1463-1465, 1997.
[4] A. Amaro-Ortiz, B. Yan, and J. A. D'Orazio, "Ultraviolet radiation, aging and the skin: Prevention of damage by topical cAMP manipulation," *Molecules*, vol. 19, pp. 6202-6219, 2014.
[5] J. Fan, Y. Zhuang, and B. Li, "Effects of collagen and collagen hydrolysate from jellyfish umbrella on histological and immunity changes of mice photoaging," *Nutrients*, vol. 5, pp. 223-233, 2013.
[6] M. Tzaphlidou, "The role of collagen and elastin in aged skin: an image processing approach," *Micron.*, vol. 35, pp. 173-177, 2004.
[7] S. E. Fligiel, J. Varani, S. C. Datta, S. Kang, G. J. Fisher, and J. J. Voorhees, "Collagen degradation in aged/photodamaged skin in vivo and after exposure to matrix metalloproteinase-1 in vitro," *J. Invest. Dermatol.*, vol. 120, pp. 842-848, 2003.
[8] Y. R. Jung, *et al.*, "Anti-wrinkle effect of magnesium lithospermate B from Salvia miltiorrhiza BUNGE: Inhibition of MMPs via NF-kB signaling," *PLoS One*, vol. 9, p. e102689, 2014.
[9] G. J. Fisher, *et al.*, "Mechanisms of photoaging and chronological skin aging," *Arch. Dermatol.*, vol. 138, pp. 1462-1470, 2002.
[10] J . Varani, "Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin," *J. Invest. Dermatol.*, vol. 114, pp. 480- 4862, 2000.
[11] J. Varani, *et al.*, "Decreased collagen production in chronologically aged skin," *Am. J. Pathol.*, vol. 168, pp. 1861-1868, 2006.
[12] A. Alemán, B. Giménez, E. Pérez-Santin, M. C. Gómez-Guillén, and P. Montero, "Contribution of Leu and Hyp residues to antioxidant and ACEinhibitory activities of peptide sequences isolated from squid gelatin hydrolysate," *Food Chem.*, vol. 125, pp. 334-341, 2011.
[13] T. F. Juher and E. B. Pérez, "An overview of the beneficial effects of hydrolysed collagen as a nutraceutical on skin properties: scientific background and clinical studies," *Nutr. Hosp.*, suppl. 1, pp. 62-66, 2015.
[14] Z. Zhang, J. Wang, Y. Ding, X. Dai, and Y. Li, "Oral administration of marine collagen peptides from Chum Salmon skin enhances cutaneous wound healing and angiogenesis in rats," *J. Sci. Food Agric.*, vol. 91, pp. 2173-2179, 2011.
[15] A. Hartog, M. Cozijnsen, G. D. Vrij, and J. Garssen, "Collagen hydrolysate inhibits zymosan-induced inflammation," *Exp. Biol. Med.*, vol. 238, pp. 798-802, 2013.
[16] Z. Wang, *et al.*, "Improvement of skin condition by oral administration of collagen hydrolysates in chronologically aged mice," *J. Sci. Food Agric.*, vol. 97, pp. 2721-2726, 2017.
[17] F. D. Choi, C. T. Sung, M. L. Juhasz, and N. A. Mesinkovsk, "Oral collagen supplementation: A systematic review of dermatological applications," *J. Drugs Dermatol.*, vol. 18, pp. 9-16, 2019.
[18] M. I. A. Rodríguez, L. G. R. Barroso, and M. L. Sánchez, "Collagen: A review on its sources and potential cosmetic applications," *J. Cosmet. Dermatol.*, vol. 17, pp. 20-26, 2018.
[19] T. Nakayama and C. Aizawa, "Change in gelatin content of vaccines associated with reduction in reports of allergic reactions," *J. Allergy. Clin. Immunol.*, vol. 106, pp. 591-592, 2000.
[20] B. K. Yap and L. H. Gam, "Differentiation of bovine from porcine gelatin capsules using gel electrophoresis method," *Food Chem.*, vol. 274, pp. 16-19, 2019.
[21] C. A. Acevedo, "Re-epithelialization appraisal of skin wound in a porcine model using a salmon-gelatin based biomaterial as wound dressing," *Pharmaceutics*, vol. 11, p. 196, 2019.
[22] K. Jayathilakan, K. Sultana, K. Radhakrishna, and A. S. Bawa, "Utilization of byproducts and waste materials from meat, poultry and fish processing industries: A review," *J. Food Sci. Technol.*, vol. 49, pp. 278-293, 2012.
[23] J. Lu, J. H. Guo, X. L. Tu, C. Zhang, M. Zhao, Q. W. Zhang, *et al.*, "Tiron inhibits UVB-Induced AP-1 binding sites transcriptional activation on MMP-1 and MMP-3 promoters by MAPK signaling

pathway in human dermal fibroblasts" *PLoS One*, vol. 11, p. e0159998, 2016.
[24] L. Horev, "Environmental and cosmetic factors in hair loss and destruction," *Curr .Probl. Dermatol.*, vol. 35, pp. 103-117, 2007.

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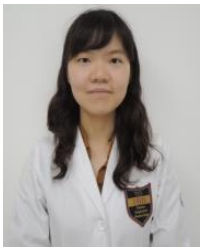

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