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Effects of Exclusively Developed Small Molecular Collagen Peptides (SMCPs) and Their Proprietary Formula on Skin Health in Cellular and Clinical Experiments

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Abstract Maintaining skin elasticity and moisture is crucial for preserving skin health and delaying skin aging. We have recently developed piscine-derived small molecular collagen peptides (SMCPs. MW < 1000 Daltons) and investigated their functionalities on skin health. Firstly, in the Caco-2 cell line that simulated gastrointestinal transport barriers, SMCPs exhibited superior absorption rates compared to other commercial collagen peptides. Secondly, a novel formula blended SMCPs with hyaluronic acid (HA) and an extract of *Phyllanthus Emblica* (EPE). Ultraviolet irritation-induced inhibition of elastin and hydroxyproline synthesis was attenuated by the formula in the HFF-1 cell line that was derived from human foreskin fibroblast. Thirdly, desiccation-induced inhibition of HA synthesis and water channels (AQP3) expression was attenuated by the same formula in HaCaT cell line that was the spontaneously immortalized human keratinocytes. Furthermore, the efficacy of the SMCPs formula as a beverage was evaluated in an eight-week intervention study involving human recipients. The SMCPs formula improved skin moisture, viscoelasticity, and wrinkle parameters, which sustained for the entire eight-week duration. Mechanistically the formula may enhance collagen stability and biosynthesis by increasing the bioavailability of key dipeptides or amino acids and help prevent cellular senescence induced by oxidative stress and chronic inflammation. In conclusion, these findings demonstrate the potential of SMCPs in combination with HA and EPE as a nutritional solution for skin care and skin aging prevention.

Keywords: *skin aging, skin elasticity, skin hydration, wrinkle, small molecular collagen peptides, Phyllanthus Emblica, hyaluronic acid*

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1. Introduction

The skin, our body's largest organ, plays a pivotal role in various essential physiological and biomechanical functions, including somatic sensing, regulating body temperature and water balance, and synthesizing vitamin D [1]. Moreover, it serves as an important barrier, shielding internal tissues and organs from environmental harm and pathogen invasion. Collagen, comprising 75% of the skin's dry weight and standing as its predominant protein, is indispensable for its strength, elasticity, and durability [2,3], thereby upholding the structural and functional integrity of the skin.

As individuals age, collagen gradually breaks down and its synthesis decreases, resulting in a loss of skin elasticity, thinning, dehydration, and the appearance of wrinkles [4]. Various approaches including dietary or nutritional interventions have been explored to prevent or slow down the aging process, and to keep the integrity and functionality of the skin, which, simultaneously, is also of great cosmetic or aesthetic importance to many individuals [5,6]. In recent years, scientific research has focused on the supplementation of collagen and hydrolyzed collagen peptides, also known as collagen hydrolysate or CH, as potential dietary interventions for promoting firm, smooth, and radiant skin [7]. It has been found that the small molecule peptides are especially promising because they are better absorbed by the body [8,9,10]. A handful of grouped analyses of human studies showed favorable results [11,12]. A very recent one, with 19 trials and 1125 participants included, found that hydrolyzed collagen supplementation improved skin

hydration, elasticity, and wrinkles compared with placebo controls. In the subgroup meta-analysis, the hydration and elasticity improvement remained present [12]. However, scientific data in this area are still considered limited in this field and further validation is needed [13,14]. In addition to collagen, other factors such as chronic low-grade inflammation and oxidative stress or reactive oxide species [13,14,15,16] are known to contribute to the cellular senescence process of the skin [15,16,17,18], which should also be targeted in the scheme of promoting skin health.

We have recently developed SIRIO-exclusive small molecular collagen peptides (SMCPs) that are piscine-derived with a molecular weight of smaller than 1000 Daltons. Using SMCPs, we designed and conducted a series of experiments *in vitro* and *in vivo* to investigate the functionality and effectiveness of the new product on skin health. First, we tested the transportation or absorption rate of the SMCPs across the cell membrane in the Caco-2 cell line; second, we tested the improvement of hyaluronic acid synthesis and the expression of the water channel aquaporin-3 (AQP3) with a proprietary formula that combined the SMCPs, sodium hyaluronate and the extract of *Phyllanthus Emblica* in the HaCaT cell line that was subject to the stress of dryness; third, we tested the improvement of elastin and collagen synthesis with the formula in HHF-1 cell line that was subject to UV irradiation; and finally, we conducted a clinical trial of eight weeks to evaluate the effect of the oral intake of the formula as a beverage in human subjects on skin moisture, viscoelasticity, and wrinkle parameters.

2. Materials and Methods

2.1. Materials of Cell Assay

The cell lines of HFF-1, HaCaT and Caco-2 were purchased from Procell Life Science&Technology Co.,Ltd., China. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma, USA. Epidermal growth factor (EGF) was procured from Gibco, ThermoFisher, USA. ELISA kits were procured from CUSABIO and CusAb Co. Pvt. Ltd., USA. Antibiotics solution (penicillin-streptomycin) was procured from Himedia, India.

2.2. Investigational Product

The small molecule collagen peptides (SMCPs) were derived from the skin or scales of *Oreochromis mossambicus* and registered as PEPTIVATOR®. The average molecule weight of the collagen peptides is less than 1000 Da. SMCPs was developed by SIRIO Pharma Co., Ltd (Guangdong, China). There were five characteristic peptides in PEPTIVATOR®, including dipeptides prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly), tripeptides glycyl-prolyl-alanine (Gly-Pro-Ala) and glycyl-prolyl-hydroxyproline (Gly-Pro-Hyp) and tetrapeptide glycyl-prolyl-hydroxyprolyl-glycine (Gly-Pro-Hyp-Gly).

The formula used in the present study contained three ingredients: SMCPs, sodium hyaluronate (Bloomage Biotechnology Co., Ltd., Shandong, China) and the powder of *Phyllanthus Emblica* extract (SIRIO Pharma Co., Ltd, Guangdong, China). There was 20% of total polyphenols in *Phyllanthus Emblica* extract. The ratio of the three ingredients in the beverage was: SMCPs: Sodium hyaluronate: *Phyllanthus Emblica* extract=41.7:1:1.7. Three ingredients were mixed and dissolved in deionized water. The pH of the formula was adjusted to 3.8 with citric acid monohydrate, then put into a water bath at 90°C for 1 hour.

2.3. Method of Cell Assay

2.3.1. Cell Culture

Under the microscope, the cultured cells should grow to 80%-90% for cell passage. The old medium was discarded and washed with PBS 1-2 times. One mL of trypsin was added. The medium was put into a 37°C incubator for digestion and taken out every 1 min to observe the degree of cell digestion under the microscope. It was shaken gently by hand if cells were falling off or the cells became round under the microscope to stop the digestion. The digestion was terminated with 2 mL of complete medium (medium doubled with trypsin). The cells were collected and centrifuged at 1000 rpm for 5 min, the supernatant was discarded, the cells were resuspended with medium, shaken well, and inoculated into new culture dishes at a ratio of 1:3~4. The new culture dishes were placed in a 37°C, 5% CO_2 incubator nourish. The medium was replaced every 2-3 days thereafter.

2.3.2. Collagen Transportation Model in Caco-2 Cells

During the incubation, the culture medium was changed every 2 days. The integrity of Caco-2 cells monolayers was monitored by transepithelial electrical resistance (TEER). The TEER of each well of the Caco-2 monolayer was measured by EVOM2, Epithelial Voltohmmeter. Generally, the TEER value is in the range of 200-1000 $\Omega \cdot cm^2$, and the larger the value, the cell monolayer is considered dense and complete. In this transportation study, the transportation of collagen will be conducted on cell monolayers with TEER value greater than 350 $\Omega \cdot cm^2$

The transepithelial transport of collagen peptides was simulated by the following method. The cell monolayers were rinsed three times with 37°C of HBSS buffer to remove the remaining culture medium and incubated with HBSS at 37°C in 5% CO_2 for 30 min to stabilize the cells before transport studies. Different types of collagen peptides (0.5 mL) dissolved in HBSS and diluted to the same concentration (2 mg/mL) were then added to the apical side, while 0.6 mL of fresh HBSS was added at the basolateral side of the monolayer. After 2 h incubation at 37°C in a 5% CO_2 atmosphere, the samples both from the apical side and basolateral side were collected. The collected collagen samples were hydrolyzed under vacuum by 3 M H_2SO_4 at 105°C for 16 h. The hydrolyzed samples were filtered and diluted. The hydroxyproline concentrations of the hydrolyzed samples were calculated by comparing the UV absorption of the samples at 558 nm with the standard curve established using different concentrations of hydroxyproline standard solutions. The collagen contents of the samples were estimated using the amount of hydroxyproline calculated from the standard curve and the conversion factor of hydroxyproline to collagen. The transportation rate was expressed as:

$$Transport\ \ rate\% = \frac{Collagen\ \ contents\ \ transported\ \ to\ \ the\ \ basolateral\ \ side}{Total\ \ collagen\ \ contents\ \ applied\ \ at\ \ the\ \ apical\ \ side}$$

2.3.3. Cell Viability Test

One hundred μL of cell suspension (10,000 pcs/well) was seeded in a 96-well plate and placed in a 5% CO_2 37°C incubator for 24 h. The medium was discarded, and 100 μL of different concentrations of the material was added to each well, and the plate was incubated for 24 h. The tested materials were discarded and 100 μL of 10% CCK-8 reagent was added and configured by complete medium per well. Then the cells were incubated for 1~4 h. The absorbance was measured at a wavelength of 450 nm. The cell viability was calculated according to the following equation:

Cell viability (%) = $100*[A_{Material}-A_{Blank}]/[A_0-A_{Blank}]$.

2.3.4. Ultraviolet A-induced Stress in HFF-1 Cells

The six-well plate plates with HFF-1 (human fibroblast) cells were incubated in a 5% CO_2 37°C incubator for 24 h. Then the cells were treated with different combinations of the three ingredients for 2 h before irradiation. Ten J/cm^2 UVA was used to irradiate the cells. The medium was removed, and the cells were washed with sterile PBS for 3 times. The cells were covered with a thin PBS layer and irradiated with a dose of 10 J/m^2 UVA again. After that, the cells were placed in a 37°C, 5% CO_2 incubator and continued to culture for 24 h. The concentration of elastin and hydroxyproline were measured using the ELISA kits from Sigma.

2.3.5. Air Exposure Model in HaCaT Cells

The production of hyaluronic acid and the expression of aquoporin-3 was measured in HaCaT cells (human keratinocyte cells). The cell culture method was similar to that used for the HFF-1 cells. The model group was created by exposing HaCaT cells on the ultra-clean bench without wind for 30 min at 25℃. After that, the cell viability was reduced to 50-60%. Then the tested mixture was added to the cells for 24 h. The cells were then digested with preheated trypsin and fixed with 4% paraformaldehyde for 10 minutes. The cells were washed with PBS after permeabilized with 0.5% Triton-100 at room temperature for 5 minutes. Then HaCaT cells were blocked with 1% BSA at room temperature for 60 minutes. One μL AQP3 primary antibody and 0.5 μL AQP3 secondary antibody were added to the cells and incubated at room temperature for 30 min. Finally, the mean fluorescence intensity of the cells was measured by the flow cytometer. The production of hyaluronic acid by HaCaT cells was measured by the ELISA kit from its optical density value.

2.4. In Vivo Study Protocol

The human study was conducted in Micro-spectial Chemical Analysis and Test Technology Co., Ltd (Shanghai, China). A total of 16 Chinese women were recruited in the study (Figure 1). The recruited subjects were screened by the following criteria: 1) those who were considered to be suitable for the study on visual examination (presence of wrinkles and absence of wounds, pimples, warts or burns); 2) those who were health and ages from 25-55 years old. 3) those with a moisture content in the stratum corneum of the inner forearm (Corneometer value) lower than 60 A.U.; 4) those facial skin meets wrinkle grade 2~5 (basic value); 5) those who were able to cooperate well with the subjects and maintain the regularity of life during the study. The excluded criteria were: 1) those who with facial skin diseases that may affect the judgment of test results; 2) those who with high allergy; 3) those who are pregnant, breastfeeding or intend to become pregnant during the test period; 4) patients with severe heart, liver, and renal function damage and severe immune dysfunction; 5) those who with mental illness, serious endocrine diseases and oral contraceptives; 6) Participants in drug clinical trials or other trials within 30 days, or those who have systematically used drugs that have an impact on the test results within one week; 7) Oral and topical beauty products within 2 weeks that may have an impact on the test results. A total of 10 subjects were included in the study. The studies involving human participants was conducted in accordance with the Declaration of Helsinki, reviewed and approved by the Chinese Ethics Committee of Registering Clinical Trials (ChiECRCT20210582). Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the subjects to publish this paper. At the start of the trial, the skin parameters of the subjects were measured to establish the experimental baseline. After that, the subjects orally ingested the tested beverage once daily for 8 weeks. The subjects were advised to avoid excessive eating, drinking, exercise, strong sunburn, change in lifestyle, and change cosmetics.

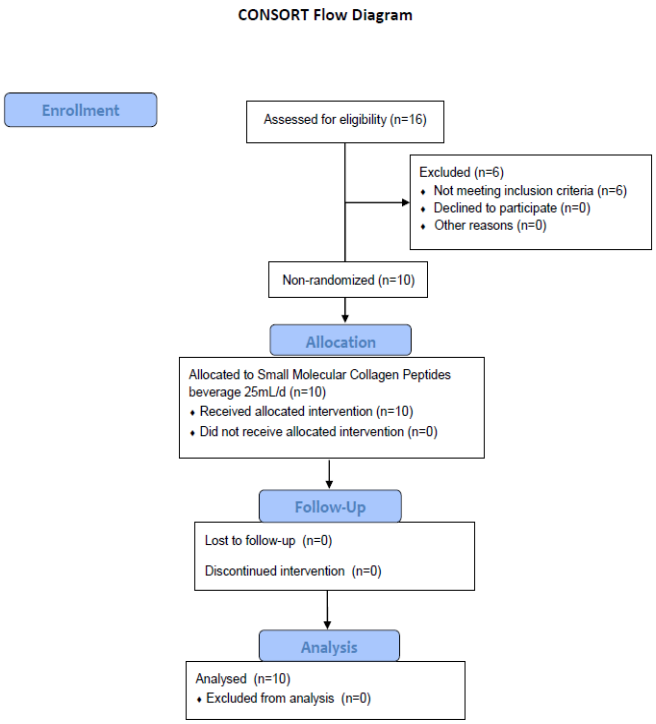

Figure 1. CONSORT Flow Diagram. Flow-chart showing inclusion, participation throughout the study

2.5. Physiological Measurements of the Skin

Instrumental measures of skin condition were assessed at five points: at baseline before the intervention, and at 2, 4, 6 and 8 weeks after the intervention. The facial make-up was removed by conventional methods and the subjects were acclimatized for 20 minutes in a waiting lounge with a constant temperature of 24 ± 2℃and humidity of 55 ± 5% before facial skin assessment.

2.5.1. Skin Hydration

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 CK Multi probe adaptor (MPA580) with a corneometer probe CM825 (Courage and Khazaka, Cologne, Germany). Three measurements were taken and averaged and the standard deviations were calculated.

2.5.2. Skin Elasticity

The change of skin elasticity on the cheek of the subjects was assessed using a skin deformation curve measured by Cutometer MPA580 (Courage & Khazaka, Germany) at the baseline, 2, 4 and 8 weeks after taking the product. The test method draws skin with suction into the aperture of the probe and after 2 seconds releases it again. The method is noninvasive using a 2 mm of probe pressed into the skin. The results were calculated from three measurements with Mode 1, 450 mbar of constant suction for 2 s of suction time and 2 s of relaxation time. Ua represents the difference between the maximum deformation of the first vacuum period and the deformation after 1 second of normal pressure and Uf represents the final distension at the end of the first vacuum period. The skin elastic parameter R_2 is equivalent to Ua/Uf, which represents the overall elasticity of the skin. Another skin elastic parameter R_5 represents the net elasticity of the skin, which is equivalent to the immediate relaxation within the first 0.1 second after the end of the first vacuum period Ur divided by Ue, the immediate distension of the skin within the first 0.1 second of the first vacuum period. R_2 and R_5 are parameters highly related to skin elasticity.

2.5.3. Skin Wrinkles

Primoslite imaging was performed on the crow's feet area. The Primoslite 45 × 30 mm system (GFMesstechnik GmbH, Teltow, Germany) is a hand-held 3D imaging device for assessing the microtopography of skin. The field of view is 45 × 30 × 20 mm with a resolution of 61 × 61 × 6 µm. Primoslite images were analyzed using the Primoslite wrinkle analysis software built-in function. The changes in each subject's area, volume and length of crow's feet wrinkle were measured at the baseline, 2, 4 and 8 weeks after taking the product.

2.6. Statistical Analysis

Comparisons of cellular assay results of different samples; skin moisture, and elasticity data at different time points of the subjects were carried out with One-way ANOVA analysis. Significance was defined as $p < 0.05$ using the data analysis software SPSS Ver. 13.0 (IBM Inc., Armonk, NY, USA). Each value was expressed as the mean ± standard deviation (SD) of triplicate of the experiments.

3. Results

3.1. Cell Assay Experiment

The transportation rate of the SMCPs was compared with 6 other commercial collagen peptides in the Caco-2 cell line. The results of the transport rates are shown in Figure 2. The results indicate that the SMCPs had the highest transport rate among the selected collagen peptides from the market. The transport rate of SMCPs was about 1.54 to 2.87 times higher than the other collagen peptides and the differences were statistically significant. The high transport rate of the SMCPs demonstrated that it might be absorbed better than the other collagen peptides by the intestine.

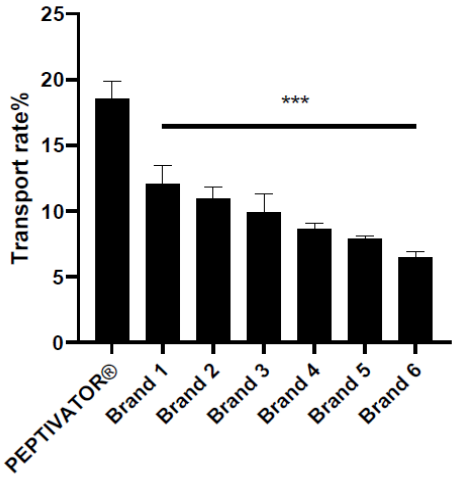

Figure 2. Transportation rate of different commercial collagen peptides to Caco-2 cells. ***p<0.001(compared with SMCPs)

The effects of functional beverage containing SMCPs, the extract of *Phyllanthus Emblica* and sodium hyaluronate on the cytotoxic viability of human foreskin fibroblasts (HFF-1) and human immortalized keratinocyte (HaCaT) were detected by CCK-8 method. As shown in Figure 3(a), after the treatment of different concentrations of the functional beverage, the cell viability was above 100%, which indicated that at these concentrations the functional beverage had no significant cytotoxicity to HFF-1 cells. Moreover, at concentrations of 200µL and 800µL, the functional beverage can promote the proliferation of HFF-1 cells significantly. Figure 3(b) shows that the formula did not have significant cytotoxicity on HaCaT cells also.

The elastin produced by HFF-1 cells is shown in Figure 4(a). Compared with the control group, the elastin content of the model group was significantly decreased by UVA exposure, indicating that the modeling of the UVA-induced stress in HFF-1 was successful. While all peptide drinks increased elastin levels, the collagen drink containing SMCPs exhibited a pronounced effect on

elastin synthesis, i.e., a 49.3% increase from the level of the model group. Figure 4(b) shows the hydroxyproline content generated by the HFF-1 cells. Among different groups of collagen drinks, collagen drinks 2 and 3, and the collagen drink containing SMCPs exhibited an improving effect on hydroxyproline content compared with the model group. Again, the collagen drink containing SMCPs exhibited a greater effect on hydroxyproline concentration.

Compared with the control group, air exposure created dryness that was accompanied by the reduction of both hyaluronic acid synthesis and the expression of aquaporins three (AQP3) in HaCaT cells as seen in Figure 5(a) and Figure 5(b). All collagen drinks improved hyaluronic acid production and the expression of aquaporins three (AQP3). In terms of efficacy, the collagen drink containing PEPTIVATOR® was greater than collagen drinks 1, 2 and 3 for hyaluronic acid and AQP3 expression.

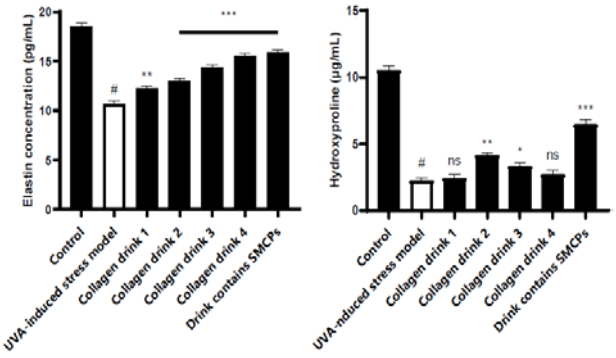

Figure 4. Ultraviolet A irritation-induced inhibition of elastin and hydroxyproline synthesis was attenuated by SMCPs formula in the HFF-1 cell line: (a). the production of elastin; (b) the production of hydroxyproline. *p<0.05, **p<0.01, ***p<0.001(compared with model), #p<0.01(compared with control)

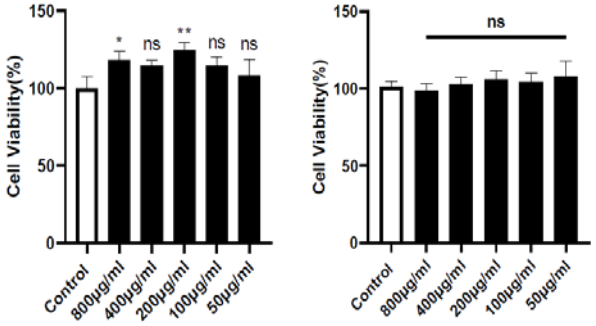

Figure 3. Evaluation of cell viability in the HFF-1 and HaCaT cell lines that were exposed to varying concentrations of formula containing SMCPs: (a) in HFF-1 cell; (b) in HaCaT cell. *p<0.05, **p<0.01(compared with control)

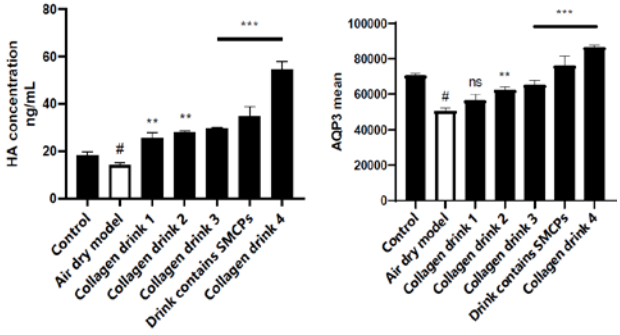

Figure 5. Desiccation-induced inhibition of HA synthesis and water channels (AQP3) expression was attenuated by the same formula in the HaCaT cell line: (a). the production of hyaluronic acid; (b) the expression of AQP3. **p<0.01, ***p<0.001(compared with dry model), #p<0.01(compared with control)

Table 1. Measured skin parameters and changes for the subjects

Item		Unit	Measured value							
			Before ingestion	2 weeks		4 weeks		6 weeks		8 weeks	
			Mean±SD	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value	Mean±SD	P value
Moisture value		A.U.	43.95±4.16	52.69±4.30	0.0002	58.13±3.55	<0.0001	62.17±4.85	<0.0001	70.83±3.71	<0.0001
Viscoelasticity	R_2	%	59.64±4.89	61.26±5.08	0.8882	64.37±3.96	0.1374	67.35±4.24	0.0056	70.22±5.84	0.0001
	R_5		62.41±8.31	66.10±7.65	0.6811	70.61±7.11	0.0803	76.02±6.95	0.0014	78.78±7.37	0.0001
Wrinkles	area	mm^2	43.04±4.50	34.84±4.08	<0.0001	33.66±3.83	<0.0001	35.57±3.17	0.0002	33.33±2.35	<0.0001
	volume	mm^3	3.32±0.48	2.51±0.36	<0.0001	2.69±0.30	0.0020	2.49±0.45	<0.0001	2.43±0.24	<0.0001
	depth	mm	78.02±7.01	62.12±4.63	<0.0001	59.21±5.88	<0.0001	59.22±6.32	<0.0001	60.61±2.76	<0.0001

The p-value of the measured parameters on the ingestion week is calculated compared with the baseline.

3.2. Human Experiments

Compared with the baseline levels, the intervention with the SMCPs formula drink increased skin moisture by 19.89% at 2 weeks in the stratum corneum of the right cheek. The increases had kept at 32.26%, 41.46% and 61.16% at week 4, week 6 and week 8, respectively (Table 1). As shown in Table 1, the overall skin elasticity R_2 improved in response to the intervention with the SMCPs formula drink, the increases became statistically significant at week 6 and week 8, which were 12.93% and 17.74% higher than the baseline. The improvement of skin net elasticity R_5 followed a similar fashion, i.e., the increases of R_5 reached statistical significance at week 6 and week 8, which were 21.81% and 26.23% higher than the baseline. Skin wrinkles measured by the area, depth, or volume were all significantly improved as well throughout the experiment period by the intervention (Table 1).

4. Discussion

The major novel findings from our in vitro and in vivo experiments are several. First, in cultured Caco-2 cells, SIRIO-exclusive small molecular collagen peptides (PEPTIVATOR®) exhibited a better transport rate than other commercial products. Second, this formula was shown to improve the cellular synthesis of hyaluronic acid and the expression of water channels (AQP3) to desiccation exposure in the HaCaT cell line, which is a spontaneously transformed aneuploid immortal keratinocyte cell line derived from adult human skin. Third, the same formula was able to improve elastin and collagen synthesis in response to UV irradiation in the HFF-1 cell line, derived from human foreskin fibroblasts. Finally, intervention with this formula in the form of a beverage effectively improved skin moisture, viscoelasticity, and wrinkle parameters in healthy human recipients.

The gastrointestinal tract's absorption of collagen peptides is a crucial determinant of their (metabolites) deliverability to the target sites in the body. Previous studies have demonstrated that low or small molecular collagen peptides are better absorbed in both rats [10] and human recipients, [19] and raised the circulating level of prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-glycine (Hyp-Gly) - the two major dipeptides for collagenic stability, biosynthesis [20,21,22]. The Caco-2 cell line, originally derived from the epithelial cells of the human colon, serves as a model to study the transport of collagen peptides across the intestinal barrier [23]. In comparison to other commercial products available in the market, SMCPs significantly improved absorption rates in cultured Caco-2 cells. The advantage of this exclusive SMCPs derived from piscine source should be attributed to its small molecular weight, which is less than 1000 Da, whereas most collagen hydrolats in commercial products are larger and with molecular weight greater than 2000 Da [24]. In the meantime, this improved absorption did not compromise the survival rate of the cultured cells but was accompanied by an overall improvement in cell viability.

Nutrition is essential for maintaining skin structure and function, as healthy skin depends on an adequate supply of various nutrients and on their interactions. Previous investigations have demonstrated the necessity of combining different functional ingredients for an additive or synergistic effect that a single ingredient cannot achieve [25,26,27,28]. In the formula we developed, one of the important constituents is *Phyllanthus Emblica* which is rich with phytochemical ingredients, for example, chlorogenic acid, ascorbic acid, ellagic acid, gallic acid, and quercetin, which are antioxidant and anti-inflammatory [29-32]. Additionally, chlorogenic acid has been shown to be vasodilatory in vivo, which may help blood circulation in the skin [33]; and ascorbic acid has been known for stimulating collagen synthesis and protection against UV-induced photodamage to the skin [34,35]. The extract of *Phyllanthus Emblica* is also found to inhibit the activity of matrix metalloproteinase (MMP), which is a family of enzymes that contribute to the degradation of collagen. Hyaluronic acid is another essential constituent in our formula, which is a major component of the skin's extracellular matrix known for its many benefits in promoting skin health [36]. When applied topically or administered through injection or implantation, it has demonstrated anti-wrinkle, anti-nasolabial fold, anti-aging, face rejuvenating, wound dressing, and space-filling properties, making it a popular ingredient in skin care products [37,38,39]. The anti-oxidative activity of *Phyllanthus Emblica* and hyaluronic acid may be another reason for their use in supplements to prevent skin aging.

The HaCat cell line was a spontaneously transformed aneuploid immortal keratinocyte cells derived from human skin [40]. In the present study we found that air exposure induced hyaluronic acid reduction and AQP3 down expression in HaCat cells were not only prevented but also improved by the formula intervention; we also found that UV-irradiation inhibited elastin and hydroxyproline in the HFF-1 cell line, which was derived from human foreskin fibroblasts, treatment with the formula attenuated the inhibition. The exposure to dryness and to UV-irradiation represents the environmental insults that accelerate the skin aging process [41,42], and the evidence from these experiments in vitro provided a premise for conducting a clinical trial in human subjects.

The ingredients and their ratio in the formula for human trials were the same as what was used in studies in cultured cells, but the formula was in a beverage form. To what have been anticipated, the beverage was functional and effective in improving skin moisture and viscoelasticity, wrinkle depth, and area size. The improvement appeared 2 weeks after the intervention and lasted 8 weeks at which the experiment finished. Our results not only confirmed previously stated functions and efficacies of *Phyllanthus Emblica* and hyaluronic acid in promoting skin health and aging prevention when used alone or in combination with other ingredients [26,27,28,29,30,31,32]. [36,37,38,39], but also made advancement by offering an effective recipe with even smaller molecular peptides for skin care in combination with *Phyllanthus Emblica* and hyaluronic acid.

The present study may have some limitations. In our clinical trial, for example, first, we observed a significant improvement in skin health parameters, the beneficial results were from the comparison between the values before and after the interventions. In the future experiment, we can try to design a placebo formula and have a placebo control group; second, the human trial was conducted in an age group of 35-50 and the effectiveness of the SMCPs formula may be different in other age groups.

5. Conclusion

In conclusion, SIRIO-exclusive small molecular collagen peptides (PEPTIVATOR®) exhibited a better absorption rate in a cellular experiment. The proprietary formula containing SMCPs, hyaluronic acid and *Phyllanthus Emblica* improved cellular hyaluronic acid level and water-channel expression in cells that were exposed to desiccation; in the meantime, the formula also improved elastin and hydroxyproline concentrations in cells that were exposed to UV-irradiation. Furthermore, the formula in the form of drinking beverage improved skin moisture, viscoelasticity, and wrinkle properties in human recipients. Given these positive results, we conclude

that the SMCPs and its combination with hyaluronic acid and the extract of *Phyllanthus Emblica* provide a promising solution in the framework of skin care and skin aging prevention through a nutritional approach.

Conflict of Interest

Authors Danyang Yu, Jiani Zheng, Jing Sun, Shukai Huang and Peng Chen were employed by the company SIRIO Pharma Co., Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Data curation, formal analysis, writing—original draft preparation, D.Y.; methodology, J.Z., resources, S.H., J.S.; investigation, J.Z., J.S., D.Y.; writing—review and editing, W.Z.; Conceptualization, supervision, P.C.; All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The studies involving human participants was conducted in accordance with the Declaration of Helsinki, reviewed and approved by the Chinese Ethics Committee of Registering Clinical Trials (ChiECRCT20210582). The authors confirm that all ongoing and related trials for this study were registered at Chinese Clinical Trial Registry, ChiCTR (ChiCTR2200055598). Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the subjects to publish this paper.

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