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Optimized oral supplementation: *in vitro* evidence for enhanced dermal collagen synthesis via physiologically digested collagen and micronutrients

Abstract

Introduction: Skin aging leads to structural degradation, particularly of collagens. In an integrative beauty approach, oral supplements complement conventional topical treatments and stimulate the skin from within. We thus developed a novel oral supplement combining eggshell collagen fragments with a micronutrients mix (Blend of vitamins C & E, zinc & copper), designed to enhance skin's natural collagen synthesis. To evaluate its efficacy, an *in vitro* study was conducted on human dermal fibroblasts. The collagen component underwent an *in vitro* enzymatic pre-digestion to mimic physiological assimilation, ensuring a more relevant assessment of its bioactivity.

Methodology: Collagen was digested using a standardized method. Human dermal fibroblasts were subsequently treated with the actives alone or in combination. Untreated cells served as controls, and a mixture of Vitamin C and TGF-β was included as a positive control. Collagen types I, III, IV, V, and VI expression were quantified by *in situ* immunolabeling and image analysis, followed by statistical analysis.

Results: Digested collagen significantly stimulated the expression of collagens I, III, IV, and VI. Micronutrients mix also demonstrated significant increases in collagens I, III, and V. Most importantly, the combined formulation potently increased the expression of all collagens I, III, IV, V, and VI. The effect on collagen I suggested an additive interaction, while the increases in collagens IV, V, and VI indicated a complementary action between the components.

Conclusion: Our *in vitro* findings robustly demonstrate that the combination of pre-digested collagen, vitamins, and minerals significantly enhances the production of multiple collagen types crucial for skin structure and health. This comprehensive approach, validated through a physiologically relevant *in vitro* digestion model, underscores the potential of our novel oral supplement to effectively stimulate the skin's natural collagen synthesis from within, thereby promoting integrative beauty and combating age-related signs due to dermal degradation.

Keywords: oral supplement, collagens, skin aging, eggshell, vitamins and minerals

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Introduction

Skin is constantly exposed to both intrinsic and extrinsic factors that contribute to its aging, leading to detrimental changes in its structure and overall health.[1] The primary modifications observed in the skin include an alteration of the barrier function, a decrease in epidermal thickness, a flattening and degradation of the dermo-epidermal junction, and an increased dermal atrophy characterized by a loss of the main components of the dermal extracellular matrix, including structural fibers (collagens and elastic fibers) and glycosaminoglycans. These modifications result in a loss of skin volume across all its compartments, which manifests on the surface as visible signs of aging such as wrinkles, fine lines, and loss of firmness and elasticity.

Modifications to the dermal extracellular matrix play a central role in skin aging, and in this context, collagens are crucial. Collagen, being the most abundant protein in the skin, accounting for approximately 80% of the skin's dry weight, is therefore particularly susceptible to age-induced damage.[2–3] It is extensively documented in the literature that the collagen fiber network undergoes considerable alterations during the aging process, which directly contributes to the manifestation of visible aging signs.[4–6]

While topical treatments offer strategies to modulate skin aging, an integrative beauty approach emphasizes the importance of nourishing the skin from within, through oral supplementation. In this field, nutritional strategies targeting the extracellular matrix are growing, especially with oral collagen supplementation described to counteract age-related changes in skin structure and function.[7–11] Although collagen extracts are increasingly used to improve skin quality systemically, they are not the only key ingredients. Vitamins such as Vitamin C and Vitamin E, and minerals like copper and zinc, also prove to be extremely important.[12] Vitamin C is crucial for skin health, playing a vital role in stimulating collagen and elastin synthesis and inhibiting melanin production. It also offers protection against UV-A and UV-B radiation by inhibiting proinflammatory cytokines and apoptosis. Vitamin C also enhances hydration in the epidermis.[13] Therefore, oral supplementation ensures its systemic availability for these critical functions. Vitamin E provides significant protection against oxidative stress in lipids, making it a valuable supplement for patients with conditions such as psoriasis and atopic dermatitis.[14] It actively protects against collagen breakdown in the skin and reduces skin inflammation. A deficiency in vitamin E has been linked to irregular collagen structure and the presence of skin ulcers.[13]

This vitamin has a long-standing role in dermatology, improving hyperpigmentation, and enhancing the integrity of epidermal and dermal structures, thereby delaying skin aging.[15] Zinc is an essential mineral and a crucial cofactor for various metalloenzymes, with its role in skin health being extensively reviewed.[15–17] Zinc deficiency can lead to skin disorders, including dermatitis, highlighting its importance in maintaining skin homeostasis.[18] Concentrated in the epidermis, zinc possesses anti-inflammatory properties and modulates apoptosis.[16] It also contributes to wound healing and exhibits microbial properties and has been used topically for these reasons.[17] Beyond topical applications, zinc, as a systemic nutrient, has been shown to be effective in treating inflammatory dermatoses and pigmentation disorders, as well as improving conditions like actinic keratoses and alopecia areata,[15] and enhancing wound healing.[18] Its fundamental role in skin health and systemic processes strongly supports its inclusion in an oral supplement. Finally, copper is a vital micronutrient that offers protection to the skin from UV damage. It is also essential for collagen maturation and melanin synthesis.[19] Systemically, it increases the expression and binding of hypoxia-inducible factor 1-alpha (HIF-1α) for angiogenesis and induces vascular endothelial growth factor (VEGF) for the regeneration of new skin during wound healing.[20] These properties underscore its importance in wound dressings and suggest its systemic value.

Furthermore, a lower Zn–Cu ratio observed in males with acne vulgaris indicates a potential therapeutic role for copper supplementation.[21] As a micronutrient involved in critical skin functions and repair processes, copper is highly beneficial for an oral cosmetic supplement.

Today, we are convinced that all these elements are important for improving skin health. We have thus developed a novel oral supplement combining collagen fragments extracted from eggshell, a blend of vitamins (Vitamins C & E) and minerals (Zinc and Copper), designed to enhance the skin's natural capacity for collagen synthesis.

To demonstrate the efficacy of this formulation in stimulating collagen production, an *in vitro* study was conducted on human dermal fibroblasts. This study assessed the individual and combined effects of hydrolyzed collagen, the vitamin/mineral mix, and their association on the production of various collagen types (I, III, IV, V, and VI). A key aspect of our experimental design involved a pre-digestion step for the collagen component, utilizing enzymes that mimic gastric and intestinal digestion processes in vitro. This innovative approach ensures that our study more accurately reflects the physiological reality of collagen assimilation and subsequent cellular interaction within the body, providing a more relevant assessment of its bioactivity.

Materials and methods

Enzymatic digestion

Eggshell collagen (Ovomet ®, Eggnovo, Spain) was digested according to the method describe by Minekus et al.[22] Briefly, to mimic the gastric phase, collagens were incubated with pepsin (2000 U/ml) for 2 hours at 37°C, pH3 and then neutralized with NaOH. Then, to mimic the intestinal phase, the previous mix was then incubated with Trypsin (100 U/ml) + Chymotrypsin (25 U/ml) + pancreatic lipase (2000 U/ml) + colipase (2:1 molar ratio with lipase) + pancreatic amylase (200 U/ml) + bile (10 mM) for 2 hours at 37°C, pH7. At the end of the incubation time, enzymes were inactivated by incubating the mix for 5 minutes at 90°C.

Collagens expression, *in situ* immunofluorescent labeling and images analysis

Fibroblasts were seeded in 96-well plates and incubated for 24 hours in culture medium. The medium was then replaced by assay medium containing or not (control) the digested collagen, the micronutrients, alone or combined to the digested collagen, or the reference (20 µg/ml Vitamin C + 10 ng/ml TGF-β) and the cells were incubated for 72 hours (Table 1). The digestion medium was also tested in parallel as a placebo control. All experimental conditions were performed in n=3.

The cells were rinsed with a PBS solution, fixed and permeabilized. The cells were then labeled using a specific primary antibody (Anti-collagen I, Abcam #138492; Anti-collagen III, Finetest #FNab01838; Anti-collagen IV, R&DSystems #MAB6308; Anti-collagen V, Rockland #600-401-107; Anti-collagen VI, Abcam #ab6588). The primary antibody was then revealed using an appropriate fluorescent secondary antibody (GAR-Alexa 488, Invitrogen #A11008; GAM-Alexa 488, Invitrogen #A11001) and the cell nuclei were colored using Hoechst solution 33342 (bis-benzimide, Sigma, # B2261) in parallel.

The image acquisition (5 photos/well) was performed with an ImageXpress Micro Confocal (Molecular Devices, x20 objective lens). The labeling was quantified by the measurement of the fluorescence intensity of protein normalized to the total number of nuclei identified by staining with Hoechst (Integration of numerical data with the MetaXpress software, Molecular Devices).

Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons test. Statistically significant differences are indicated by asterisks as follows: *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Results

The combination of 10 ng/ml TGF-β + 20 µg/ml Vitamin C, used as a test reference, strongly stimulated the expression of collagens I, III, IV, and V (Figure 1 – Figure 2), which was expected and validates the assays.

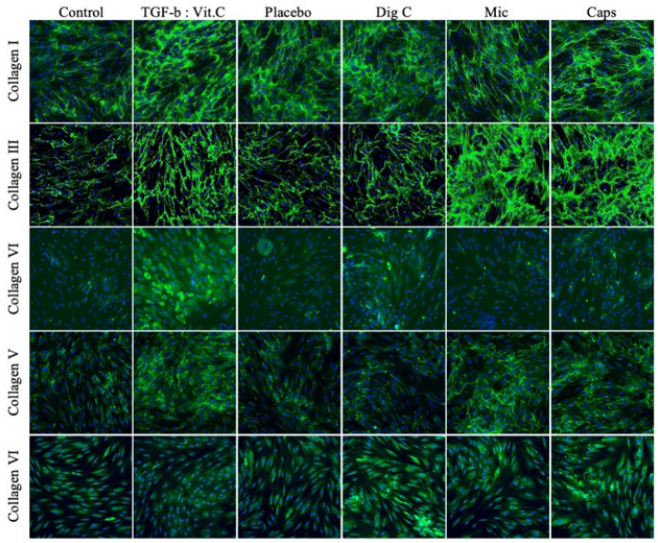

Figure 1 Effects of digested eggshell collagen (Dig C), micronutrients mix (Mic) and their combination (Caps) on the expression of collagens I, III, IV, V and VI by human dermal fibroblasts – Representative images of *in situ* immunolabelling.

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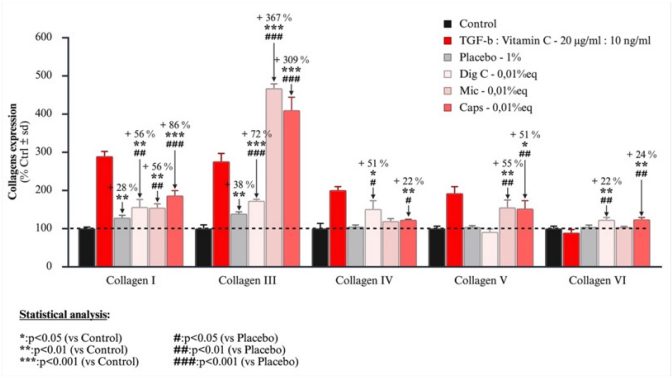

Figure 2 Effects of digested eggshell collagen (Dig C), micronutrients mix (Mic) and their combination (Caps) on the expression of collagens I, III, IV,V and VI by human dermal fibroblasts – Image analysis after *in situ* immunolabelling.

The placebo, corresponding to the reaction mixture used for eggshell collagen digestion, moderately stimulated the expression of Collagens I and III (+28% and +38% respectively, p<0.01, Figure 1 – Figure 2) without modulating the expression of collagens IV, V, and VI (Figure 1 – Figure 2).

Eggshell collagen (Dig C), after enzymatic digestion mimicking the natural digestion process, significantly stimulated the expression of collagens I, III, IV, and VI (+56%, +72%, +51%, and +22% respectively, Figure 1 – Figure 2) without modulating the expression of collagen V. In all cases, the effects were statistically superior to those of the placebo (Figure 2).

The micronutrient mix (Mic), composed of Vitamins C and E, copper, and zinc, very strongly stimulated the expression of collagen III (+367%, p<0.001) and, to a lesser extent, the expression of collagens I and V (+56% and +55% respectively, p<0.01, Figure 1 – Figure 2) without modulating the expression of collagens IV and VI.

The combination of digested eggshell collagen and the micronutrient mix (Caps) significantly stimulated the expression of all collagens I, III, IV, V, and VI (+86%, +309%, +22%, +51%, and +24% respectively, Figure 1 – Figure 2). The effects were statistically superior to those of the placebo (Figure 2). While the effect on collagen I expression is the result of an additive effect between the compounds, the effects observed on the other collagens are the result of complementarity between the active ingredients. Indeed, the observed effects on the expression of collagens III and V are mainly driven by the micronutrient mix, while the effects observed on collagens IV and VI are primarily driven by the digested eggshell collagen (Figure 2).

Discussion

The primary objective of this study was to evaluate the efficacy of combining an eggshell collagen fraction with essential vitamins (C and E) and minerals (copper and zinc) within a dietary supplement. The aim was to investigate its potential to systemically stimulate cutaneous collagen production, thereby positively influencing the visible signs of aging. To this end, an *in vitro* study was conducted using normal human dermal fibroblasts cultured in a monolayer.

In vitro biological assays are typically designed to measure the pharmacological activity of molecules. While tests commonly employed for topically applied substances can also be adapted for nutraceutical projects, particular attention must be paid to the nature of the ingredient, especially for proteins or protein fractions. *In vivo*, following ingestion, proteins undergo a digestive process in the stomach and intestines before being absorbed into the bloodstream via diffusion across the intestinal barrier. Therefore, directly testing our eggshell collagen extract *in vitro* in its native form would not accurately reflect its bioavailability and subsequent distribution to target cells in the body after ingestion. To address this, we adapted an *in tubo* enzymatic digestion method to mimic the natural digestive process.[22] In this study, only the protein fraction underwent this digestion process. Literature data indicate that vitamins C and E concentrations in plasma increase after ingestion,[23–24] suggesting these molecules are largely unaffected by digestion. Similarly, minerals are not chemically altered by the digestive process. Our digestion protocol involved a heat-inactivation step for enzymatic activities; however, this could have potentially degraded the vitamins and minerals, introducing analytical bias. Consequently, these micronutrients were added directly to the cell cultures without prior *in tubo* digestion.

Under these simulated physiological conditions, we demonstrated that digested eggshell collagen significantly stimulated the expression of collagens I, III, IV, and VI. Although the placebo, corresponding to the reaction mixture used for digestion, showed a weak, yet statistically significant, effect on collagen I and III expression, this effect was considerably lower and statistically inferior to that observed with the digested collagen. Collagen hydrolysates, commonly used in dietary supplements, are well-documented for their beneficial effects on the extracellular matrix in *in vitro* human fibroblast cultures.[25] However, regardless of the collagen's origin, previous studies typically utilized native forms, which may not be entirely relevant for assessing the efficacy of dietary supplements. To our knowledge, this is the first time a skin benefit has been demonstrated *in vitro* with a collagen extract that has undergone an artificial digestion process, thus more accurately reflecting *in vivo* conditions.

Furthermore, our study highlighted the significant role of our micronutrient mix, comprising vitamin E, vitamin C, copper, and zinc. This blend effectively stimulated the expression of collagens I, III, and V. While the precise contribution of each individual ingredient cannot be definitively determined in this study, these observed effects are consistent with the well-established benefits reported in the literature for each of these molecules.[13–21]

Finally, the combination of digested eggshell collagen with the micronutrient mix resulted in the stimulation of all studied collagen types: I, III, IV, V, and VI. These results unequivocally underscore the advantage of combining both an eggshell collagen fraction and micronutrients to stimulate all four key collagen families: junctional (collagen IV), pillar (collagens I and III), initiator (collagen V), and bonding (collagen VI).[26] While an additive effect was observed for collagen I expression, a clear complementary effect was evident for the other collagen types. Specifically, the effects on collagens IV and VI were primarily driven by the eggshell collagen, whereas the effects on types III and V were predominantly attributed to the micronutrient mix.

It is important to acknowledge certain limitations in our study. While the digestion process is a crucial parameter, it is not the sole factor determining efficacy. The diffusion of collagen degradation products across the intestinal barrier is equally vital. Although *in vitro* methods exist to evaluate this diffusion, such as using Caco-2 cells,[27] these methods were incompatible with our experimental design due to the absence of analytical methods to identify and quantify the specific degradation products and the incompatibility of conditioned media from different models. To mitigate this uncertainty, we tested low concentrations, representing only 0.01% of the estimated daily ingested dose (Table 1).

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Table 1 Summary of tested conditions and concentrations

Name (Abbreviation)	Tested concentration (0.001%eq*)
Digested eggshell collagen (Dig C)	3.3 µg/ml#
Micronutrients (Mic)	0.43 µg/ml Vitamin E
	1.14 µg/ml Vitamin C
	0.28 µg/ml Zinc
	11.91 µg/ml Copper
Digested eggshell collagen + Micronutrients (Caps)	3.3 µg/ml#
	0.43 µg/ml Vitamin E
	1.14 µg/ml Vitamin C
	0.28 µg/ml Zinc
	11.91 µg/ml Copper

After *in tubo* enzymatic digestion

*Concentration expressed as a percentage of the daily intake, taking into account the dosage (2 capsules/day).

Despite these *in vitro* results being preliminary, they robustly demonstrate the efficacy of our combination in stimulating all four collagen families. This suggests a significant potential benefit in combating visible signs of aging, such as wrinkles, fine lines, and the loss of skin firmness and bounce.

Conclusion

This *in vitro* study successfully demonstrated that a combined formulation of enzymatically digested eggshell collagen and a micronutrient blend (vitamins C and E, copper, zinc) effectively stimulates the expression of multiple collagen types (I, III, IV, V, and VI) in human dermal fibroblasts. This comprehensive collagen stimulation, particularly through a complementary mechanism between the collagen fraction and micronutrients, underscores the therapeutic potential of this innovative dietary supplement. By utilizing an *in tubo* digestion model, this research offers a more physiologically relevant *in vitro* assessment of nutraceutical efficacy. While acknowledging the preliminary nature of *in vitro* results, these findings strongly suggest that this combination represents a promising strategy to counteract the visible signs of skin aging by enhancing cutaneous collagen production. Further *in vivo* investigations are warranted to validate these observed benefits.

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None.

Conflicts of interest

The authors declare there is no conflict of interest.

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