



Research Article

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Antiaging Synergistic Effect of Vitamins C, A, and E Topical Organogel Formulation: Clinical and *ex vivo* Assessments Results

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Abstract

The aging process is modulated by both endogenous and exogenous factors, which can lead to the degradation of collagen and elastin, resulting in wrinkles, skin laxity, and alterations in skin texture. Age-related declines in melanin production contribute to irregular pigmentation and an elevated susceptibility to ultraviolet damage. Topical applications of vitamins, notably C, A, and E, are crucial in mitigating skin aging and enhancing dermal health. These vitamins act as antioxidants, protecting against oxidative damage, inhibiting melanogenesis, lowering melanin concentrations, and facilitating skin regeneration. Nevertheless, the stability of these vitamins within cosmetic formulations poses a considerable challenge, particularly vitamin C, whose instability may compromise the efficacy of topical preparations. This study explores the effectiveness of a formulation incorporating vitamins C, A, and E within an organogel system (VCAEO) to attenuate signs of skin aging. Clinical evaluations revealed that VCAEO markedly slowed the progression of aging indicators, resulting in improved skin texture and a diminution of visible aging signs over a 30-day treatment interval. *Ex vivo* assessments utilizing human skin models demonstrated a significant increase in the synthesis of collagen type I and elastin, alongside a reduction in melanin levels, thereby corroborating the observations from the *in vivo* studies and affirming the potential of VCAEO as a viable intervention for skin aging, underscoring its significance in enhancing skin health and appearance.

Keywords: Vitamin C; Vitamin A; Vitamin E; Aging; Ascorbic Acid; Retinoids; Tocopherol; Cosmetic; Wrinkles; Depigmentation

Abbreviation: VCAEO: Vitamins C, A and E in organogel system; D0: Initial time of treatment; D30: Day 30, end of treatment; COL-1: Collagen type I; COL-3: Collagen type III; MITF: Microphthalmic Aberrant Transcription Factor; TYR: Tyrosinase

Introduction

As life expectancy continues to rise, the quest for strategies to mitigate the effects of aging among the populace has similarly intensified. Aging is natural and unavoidable, and the skin, the most visible organ, can be considered the primary marker of aging [1,2]. In the skin, aging promotes various aesthetic changes, primarily the appearance of wrinkles and loss of elasticity, which are particularly evident in the facial region [3]. Aging is a multifactorial process influenced by intrinsic factors, such as genetics, and extrinsic factors related to the exposome [1,2]. The exposome, coined by Christopher Wild in 2005, encompasses environmental exposures throughout life, impacting the development of pathologies. In particular, skin aging is affected by internal and external factors, including solar radiation, pollution,

tobacco smoke, nutrition, and cosmetic products [4]. These factors trigger molecular events that degrade collagen and elastin, leading to wrinkles, sagging, and texture changes [2,4]. Solar radiation contributes to photoaging, resulting in deep wrinkles, loss of elasticity, dryness, and spots [3,5].

Studies conducted over the years have provided strong evidence that certain vitamins when applied topically, can play an essential and beneficial role in aged skin. Some protect the body's cells and tissues from damage caused by natural bodily processes, lifestyle choices, and environmental stress, while others provide the appropriate environment for correcting skin damage. Of particular interest are vitamins A, E, and C and their derivatives, which are valuable agents in the prophylaxis and

treatment of numerous skin disorders, partly due to their abilities as antioxidants and skin's regeneration and hydration enhancers [6,7]. Vitamin C, or L-ascorbic acid, is the most abundant natural antioxidant in human skin [8] and a powerful antioxidant that combats premature skin aging by promoting collagen synthesis and enhancing hydration [9]. Topical applications significantly improve photodamaged skin [10]. As humans cannot synthesize vitamin C, it must be obtained through diet [9]. Still, oral supplementation does not increase its active concentration in the skin due its limited absorption by intestinal transport mechanisms, making topical application more effective [11,12]. Vitamin A refers to a group of unsaturated organic compounds known as retinoids, including retinol, retinal, retinoic acid, and its derivatives. Retinoids bind to specific receptors, triggering actions related to vision, reproduction, inflammation, and cell proliferation, growth, and differentiation [13]. Introduced in dermatology in the 1960s, they are widely prescribed for conditions like acne, psoriasis, rosacea, hyperpigmentation, ichthyosis, and aging [13,14]. Topical vitamin A effectively prevent skin aging by promoting dermal regeneration and epidermal renewal [15]. These compounds help improve skin texture and elasticity, reducing the appearance of fine lines and wrinkles. Retinol is extensively used in treating various dermatological conditions, including acne and photoaging [9,14]. Vitamin E, primarily in the forms of alpha-tocopherol or its precursor tocopherol acetate, is a fat-soluble antioxidant that protects cell membranes from lipid peroxidation caused by free radicals, which contribute to skin aging [16]. Despite the skin's defense systems, excessive exposure to free radicals depletes antioxidants. Vitamin E mitigates this by scavenging free radicals, slowing aging, moisturizing the skin, and enhancing water-binding capacity [17,18]. Vitamin E contributes to skin health by providing antioxidant protection against UV damage and reducing photoaging. Its moisturizing properties further support skin barrier function, making it a valuable addition to topical formulations [19].

The stability of vitamins C, A, and E in cosmetic formulations is a significant concern that limits their effectiveness. The ascorbic acid stability in cosmetic formulations is challenged by its susceptibility to degradation, mainly through oxidation, which affects its effectiveness, appearance, and scent [20]. Retinyl palmitate also degrades rapidly due to light, heat, and oxygen, with studies showing degradation ranging from 0% to 80% over six months at 25°C and 40% to 100% at 40°C [21]. Vitamin E faces similar stability issues, with light, oxygen, and metal ions accelerating degradation [22,23].

Organogels, a semi-solid system in which an organic liquid (such as oils or non-polar solvents) is entrapped within a 3D network of a gelator, forming a stable gel structure, are capable of stabilizing fat-soluble vitamins like vitamin A and vitamin E by encapsulating them within their gel matrix. This system can protect these vitamins from environmental factors such

as oxygen, light, and heat, preventing their degradation and improving their shelf life. For vitamin C in its powdered form, which is water-soluble and highly prone to oxidation, organogels can work as an encapsulation system, along with other protective agents, like antioxidants or stabilizers, to create a barrier against moisture and a low-oxygen environment within their 3D matrix, which would help protect the highly oxidation-sensitive vitamin C. The organogel structure also allows for controlled and sustained release, which can enhance the bioavailability of these vitamins in pharmaceutical and cosmetic formulations [24-26].

In this work, a Vitamins C, A and E stabilized in an organogel system (VCAEO) has been tested for its effectiveness in reducing signs of age, specifically its ability to improve collagen and elastin and reduce wrinkles, fine lines and blemishes.

Materials and Methods

Vitamins C, A and E in organogel system (VCAEO)

A commercially available bulk cosmetic formulation (Active C MultiSkin, Chemyunion Ltda., Sorocaba, SP, Brazil) was evaluated for its efficacy in improving firmness and elasticity in the facial skin. This VCAEO formulation contains the combination of vitamins ascorbic acid (vitamin C), retinyl palmitate (vitamin A-palmitate), and tocopheryl acetate (vitamin E-acetate) at average concentrations of 20%w/w, 0.3%w/w, and 5% w/w, respectively. The vitamins are stabilized by a proprietary silicon-free organogel system composed of liquid emollient esters, solid fatty acid esters, antioxidants and sensorial modifiers.

In Vivo Assessment of Efficacy in Reducing Signs of Age

Twenty-two volunteers of both sexes were selected, phototype (Fitzpatrick) from II to V, aged between 40 and 60 years, showing signs of wrinkles and/or expression lines and facial blemishes. Twenty-two volunteers completed the study. Participants applied a sufficient amount of the test products to the face, spreading it gently once a day (evening). Before each measurement, participants remained in the laboratory for at least 20 minutes with controlled temperature and humidity (20°C and 50%) and performed skin cleaning and asepsis with water and neutral soap.

All participants were evaluated at the beginning (D0) and end of the study (D30) using Cutometer® (MPA 580; Courage & Khazaka Electronic GmbH). The measurements were performed in the periorbital region.

The raw data were treated using statistical analysis. To evaluate the possible differences among ages, the initial age group (40 -60 years) was divided into smaller ranges (40- 45 years, 40-50 years, and 50-60 years) and analyzed. The results were normalized for each volunteer and for each analysis individually. The calculation was based on the percentage of the difference between result (X) from D30 to D0 in relation to D0 (Equation 1).

$$Normalized\ result = \frac{(X_{D30} - X_{D0})}{X_{D0}} \cdot 100 \text{ (Equation 1)}$$

After processing the raw data, the outliers were identified using the interquartile difference method, which considered observations with a mean distance greater than 1.5 times the interquartile difference (Q3-Q1) as outliers. Responses identified as outliers were excluded. To assess the statistical significance of each analysis, the data were evaluated for normality using the Shapiro-Wilk test, for data that met the normal distribution, the Student t-test was applied for one sample ($\mu \neq 0$), and for data that did not meet the normal distribution, the Wilcoxon test was applied for one sample ($\mu \neq 0$). In all groups studied, it was considered statistically significant for those whose $p < 0.05$ (95% probability). Results with $p < 0.1$ (90% probability) were signed. The evaluation of cosmetic appreciably was determined using a simple and easy-to-understand standardized questionnaire with monadic interval scales to measure the performance of the product's sensory attributes at D30. The results were processed in terms of the percentage of approval, defined as the sum of the positive responses.

Firmness and Elasticity of the Skin by *ex vivo* Evaluation of Collagen Type 1 and Elastin Synthesis by Immunofluorescence Assay

An immunofluorescence assay was carried out to determine the expression of the collagen type 1 (COL-1) and elastin proteins in an *ex vivo* human skin model. Human female skin explants from blepharoplasty of healthy patients aged 35 to 70 were cut into 0.5 cm² fragments and treated for 72 hours with 12 mg/cm² of product VCAEO. In parallel, fragments of human skin incubated in a culture medium were assessed as the control group. Sections of 10 µm of the fragments were collected and incubated with primary anti-collagen 1 antibody (Sigma Aldrich, C2456) or anti-elastin (Santa Cruz Biotechnology, SC25736), followed by incubation with Goat Anti-Rabbit (Invitrogen; A11008) and DAPI (4'-6-Diamidino-2-Phenylindole; DNA marker). Fluorescence intensity was analyzed using the Optical Fluorescence Microscope (Leica, DM6000B), which is accompanied by a 2.8 MP camera (Leica, DFC7000T). Images were captured using LAS v.4.12 software (Leica Application Suite). The images obtained were processed with ImageJ® software to quantify the pixels generated by the target protein. The raw data were treated using statistical analysis of variance (ANOVA). The Unpaired T-test was used when the analysis of variance detected significant differences among groups. In all groups studied, it was considered statistically significant for those whose $p < 0.05$.

Total Depigmenting Capacity by *ex vivo* Fontana-Masson Technique

Melanin levels in the skin was evaluated by *ex vivo* human skin model assay applying Fontana- Masson technique. Human female skin explants from blepharoplasty of healthy patients from 35 to 70 years of age were cut into 0.5 cm² fragments and treated for 72 hours with 12 mg/cm² of product VCAEO. The control group, fragments of human skin incubated in a culture medium, was assessed in parallel. Sections of 10 µm of the fragments were collected for silanized slides and then stained using the Fontana-Masson technique with a specific kit (Fontana-Masson Kit 4X, FM07223SO, Scientific Exodus). Melanin levels were analyzed using the Optical Microscope (Leica, DM6000B), accompanied by a 2.8 MP camera (Leica, DFC7000T). Images were captured using LAS v.4.12 software (Leica Application Suite). The images obtained were processed with ImageJ® software to semi-quantify the pixels generated by melanin pigment. The raw data were treated using statistical analysis of variance (ANOVA). The Dunnet test was used when the analysis of variance detected significant differences among groups. In all groups studied, it was considered statistically significant for those whose $p < 0.05$.

Ethical aspects

The studies were conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Surgical Center of the Eye Bank of the Sorocaba Ophthalmological Hospital in Sorocaba, São Paulo State, Brazil. The volunteers were informed and advised on the study's goals and methods; having agreed to participate, they signed a Term of Free and Informed Consent, as advocated by Resolution 466, 12/12/2012, Brazilian National Health Council.

Use of Assistants Based on Artificial Intelligence

Grammatical and style correction of the manuscript were performed using artificial intelligence-based language assistants, ChatGPT (OpenAI, 2024) [27], SciSpace [28] and Grammarly [29]. SciSpace [28] was used to read articles and check journal compatibility. ChatPDF [30] was used as a support tool for reading and reviewing articles in Korean [20] to ensure the accuracy of translation and comprehension.

Results and Discussion

In Vivo Assessment of Efficacy in Reducing Signs of Age

A clinical investigation was conducted to assess the effectiveness of VCAEO in decelerating the manifestations of aging by reducing wrinkles, reducing skin blemishes, and improving the overall appearance of the skin. The 30 day-treatment with

VCAEO resulted in visible changes in the skin's overall appearance, including reduced wrinkles and blemishes (Figure 1). In order to assess the enhancement of firmness and elasticity in the skin of participants, a comparative analysis of D0 and D30 (Table 1) was conducted utilizing the Cutometer®, a suction-based apparatus.

The procedure involves the skin being drawn into the probe's aperture; subsequently, the negative pressure is released, allowing the skin to revert to its baseline condition, thereby facilitating the evaluation of its viscoelastic characteristics [31].

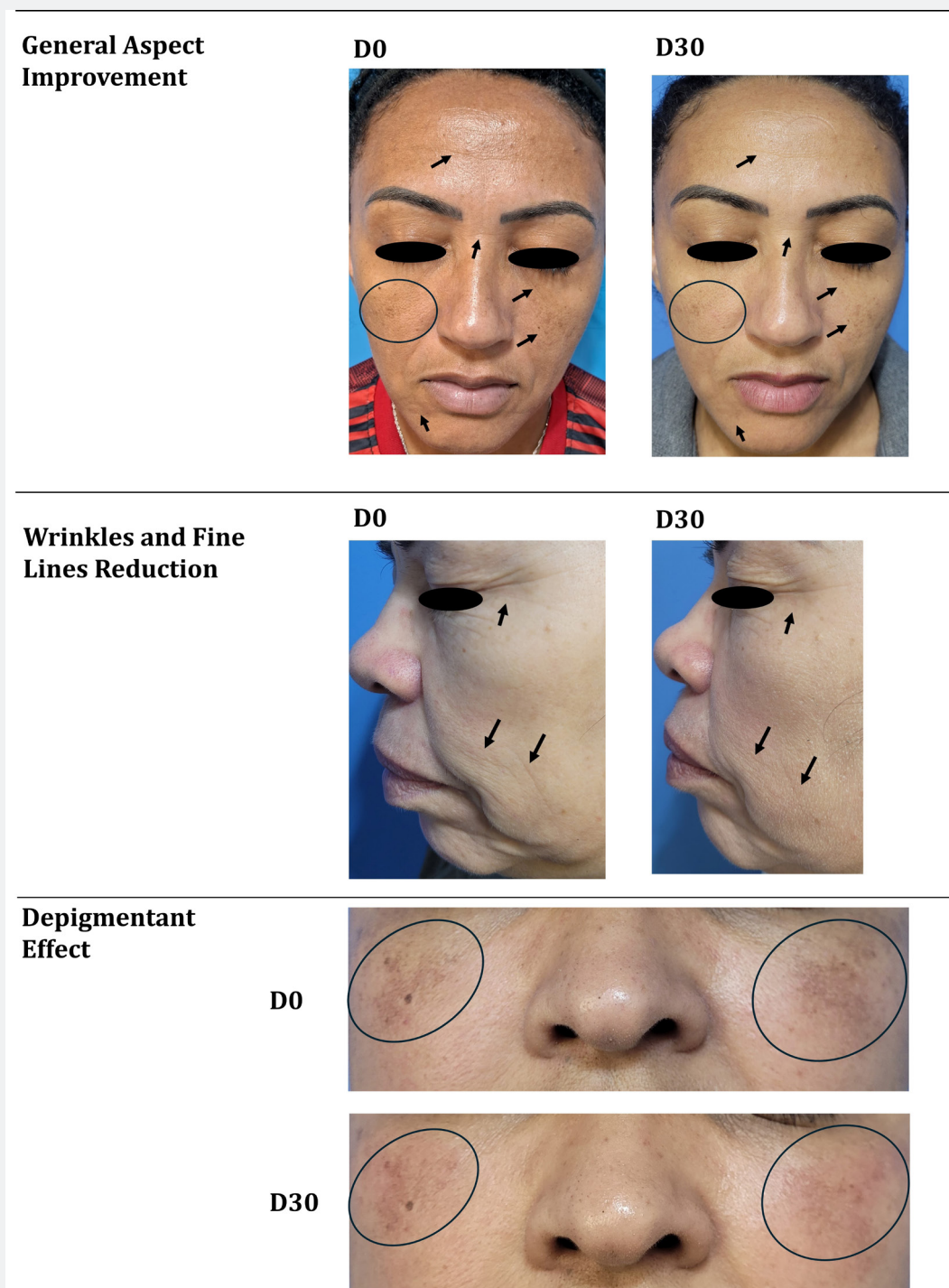


Figure 1: Photographic documentation of volunteers before (Day 0) and after 30 days of treatment with VCAEO.

Table 1: Cutometer® biomedical parameters. Values are expressed as mean $\pm$ SE. (Normality was evaluated with the Shapiro-Wilk test. Normally distributed data were analyzed using a one-sample t-test ($\mu \neq 0$), and non-normally distributed data were analyzed using the Wilcoxon test ($\mu \neq 0$). A p-value < 0.05 was considered statistically significant).

	40-60 years (n: 22)		40-50 years (n: 15)		40-45 years (n: 13)		50-60 years (n: 7)	
	n	Mean $\pm$ SE	n	Mean $\pm$ SE	n	Mean $\pm$ SE	n	Mean $\pm$ SE
R0	16	25.78 $\pm$ 9.61***	14	45.67 $\pm$ 20.50* ^a	12	50.85 $\pm$ 23.65* ^a	6	15.02 $\pm$ 16.40
R1	17	-27.10 $\pm$ 5.10***	13	-37.63 $\pm$ 7.37***	12	-37.01 $\pm$ 7.99***	6	-20.98 $\pm$ 8.22#
1 - R2	21	-54.90 $\pm$ 6.09***	14	-49.75 $\pm$ 7.48***	13	-50.22 $\pm$ 8.07***	7	-65.19 $\pm$ 10.08***
R3	21	14.52 $\pm$ 11.77	14	4.70 $\pm$ 8.41	12	6.19 $\pm$ 9.92	7	34.15 $\pm$ 30.88
R4	20	4.33 $\pm$ 14.82	11	42.48 $\pm$ 18.51	12	19.27 $\pm$ 19.55	7	-36.27 $\pm$ 13.62
1 - R5	15 ^b	-47.77 $\pm$ 8.42***	12	-23.95 $\pm$ 20.30	3 ^b	-12.83 $\pm$ 23.84	4 ^b	-63.10 $\pm$ 16.29* ^a
R6	5 ^b	51.24 $\pm$ 20.54#	3 ^b	37.16 $\pm$ 5.02*	2 ^b	32.55 $\pm$ 3.44 ^a	1 ^b	130.65 NaN ^a
1 - R7	20	-70.20 $\pm$ 4.13***	14	-66.06 $\pm$ 7.37*** ^a	12	-66.25 $\pm$ 8.54*** ^a	7	-67.90 $\pm$ 6.54***
0 - R8	21	7.30 $\pm$ 12.51	14	-1.27 $\pm$ 14.33	12	0.53 $\pm$ 16.55	7	24.45 $\pm$ 24.52
R9	19	-7.30 $\pm$ 16.03	12	-12.44 $\pm$ 21.00	10	-3.85 $\pm$ 22.19	7	1.51 $\pm$ 26.18
0 - F0	20	-2.36 $\pm$ 10.63	13	-1.32 $\pm$ 8.90	12	-1.89 $\pm$ 9.66	6	-27.08 $\pm$ 16.99
0 - F1	16	-0.68 $\pm$ 5.69	12	11.00 $\pm$ 12.41	11	0.45 $\pm$ 15.45	6	-14.58 $\pm$ 11.53

Mean = Percentage of the difference from D30 to D0 in relation to D0.

SE = Standard error

Statistical significance: #p<0.1(90% probability); *p<0.05; **p<0.01; ***p<0.001

^aThe group did not present a normal distribution, so the p-value refers to the Wilcoxon test.

^bThe outliers exceed 25% of the group. Responses identified as outliers were excluded.

NaN = The group did not have enough variables and was excluded.

By analyzing the skin deformation profiles, one can derive various parameters: Ua (the differential between the peak deformation during the initial vacuum phase and the deformation observed after one second of normal pressure), Ue (the immediate expansion of the skin within the first 0.1 seconds of the initial vacuum phase), Uv (the differential between the deformation at 0.1 seconds and the peak deformation from the first vacuum phase), Uf (the terminal expansion at the conclusion of the initial vacuum phase), and Ur (the prompt relaxation observed within the first 0.1 seconds following the cessation of the initial vacuum phase), alongside R0, the ultimate expansion of the first curve (Uf); R1, the capacity to revert to the original condition (Uf-Ua); R2, the comprehensive elasticity of the skin, encompassing creep and creep recovery (Ua/ Uf); R3, the apex of the final curve; R4, the last minimal lowest point of the final curve; R5, the net elasticity (Ur/Ue); R6, the ratio of viscoelastic to elastic extension, commonly referred to as the viscoelastic ratio (Uv/Ue); R7, the ratio of elastic recovery relative to the total deformation (Ur/Uf); R8, the Ua from the initial curve; and R9, the residual deformation at the conclusion of the measurement cycle (R3-R0); F0 and F1 area parameters related to skin elasticity. F0 is the area calculated by Uf x suction time and F1 is the area calculated by Uf x relaxation time [31,32].

In (Table 1), the parameters R2, R5, and R7 are ideally as close as possible to 1, while the parameters R8, F0, and F1 are ideally as close as possible to 0. For these parameters, the absolute difference between them and their ideal point was evaluated, which presents a positive correlation with the improvement of the skin [31].

The data delineated in Table 1 demonstrated statistically significant findings for R0, R1, R2 (1-R2), and R7 (1-R7) when a comprehensive comparison was conducted among all participants (n=22). To ascertain potential differences across age demographics, the cohort was subsequently stratified into smaller age brackets for comparative analysis.

R0 pertains to the firmness of the dermis and signifies, for instance, the initial depth of the skin when subjected to an external force [31]. The findings suggest that a younger demographic exhibits a more pronounced enhancement in skin firmness. R1 is associated with the elasticity of the skin, reflecting the final distensibility of the epidermis post-force application; in other words, it measures the utmost extent the skin achieves under pressure [31]. The data in Table 1 indicate that while the enhancement in the final extensibility characteristic of the skin is statistically significant across the entire age spectrum examined, it is notably more accentuated in participants aged 50 and below. R2 relates to skin elasticity and assesses the dermal capacity to revert to its original shape immediately following the application of force, essentially measuring the rapidity with which the skin normalizes once pressure is alleviated [31]. Observations from Table 1 reveal that in contrast to R0 and R1, the immediate retraction capability in subjects over 50 years of age is comparably effective or marginally superior to that of individuals aged 40 to 50 years. R7 also pertains to elasticity and evaluates the skin's ability to recuperate within a brief time frame following the removal of the exerted force, specifically the duration required for the skin to entirely revert to its baseline state after enduring

prolonged pressure [31]. Table 1 indicates that akin to the findings for R2, the skin's delayed recovery capacity in individuals over 50 years is as proficient or slightly enhanced relative to those aged between 40 and 50 years. This finding is particularly interesting considering that a study by Ryu et al. (2008) reveals a significant age-related decline in skin elasticity, primarily in facial regions, as assessed through Cutometer® metrics. Notably, the R7 parameter demonstrates a pronounced negative correlation with age, underscoring the deterioration of skin elasticity over time [32].

In conclusion, participants within the 40-50 age range seem to have experienced a more substantial improvement in the R0 and R1 metrics, indicating a more favorable reaction concerning initial skin depth and final extensibility. Conversely, individuals in the 50-60 age bracket exhibited a more significant enhancement in R2 and R7 metrics, suggesting a quicker response in immediate retraction and a more efficient recovery following extended periods of distension. These variations imply that the treatment response to VCAEO may exhibit age-dependent differences. Accordingly, the VCAEO methodology may be tailored towards preventive measures for individuals up to the age of 50 and restorative strategies for those exceeding 50 years. Once VCAEO was an organogel system, there was concern about the acceptability and appreciability of the formulation by the volunteers, and the possible negative effects on the skin of the high concentration of oily materials.

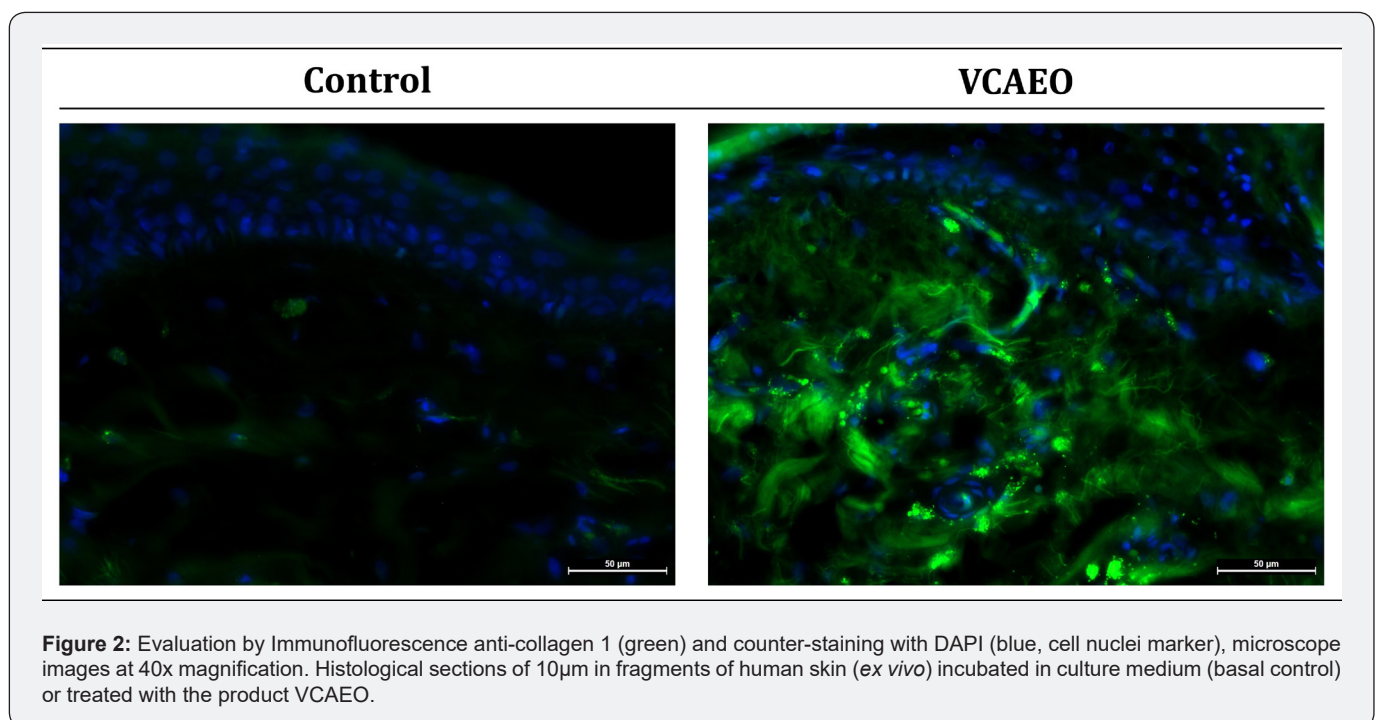
The result of the questionnaire applied at D30 to assess the volunteers' perception of the formulation indicated an acceptance of 82% of participants regarding the product being pleasant to the touch, 82% of the participants regarding the product having a pleasant texture, 82% of the participants regarding the ease of

application of the product, 82% of participants perceived that the product absorbs quickly through the skin, 64% of participants regarding the product not leaving a sticky appearance on the skin, 73% of the participants regarding the product not increasing the oiliness of the skin, 68% of participants regarding the product not making the skin drier, 86% of the participants regarding the product does not increase the appearance of pimples and blackheads, 82% of participants regarding using the product again.

Firmness and Elasticity of the Skin by *ex vivo* Evaluation of Collagen Type 1 and Elastin Synthesis by Immunofluorescence Assay

To understand the action of VCAEO in improving skin firmness and elasticity, an immunofluorescence assay was carried out on the expression of the collagen type 1 (COL-1) and elastin proteins in an *ex vivo* human skin model. The results of the evaluation of COL-1 protein expression for the test formula VCAEO are shown in Figures 2 and 3. The images demonstrate that VCAEO promoted a statistically significant increase ($p < 0.01$) in COL-1 synthesis, reaching up to 178% compared to the control group. These results were obtained through immunofluorescence analysis on human skin explants after 72 hours of incubation with the VCAEO.

Analysis of immunofluorescence images (Figure 2) reveals more intense coloring in samples treated with the VCAEO, indicating increased COL-1 production. This increase is relevant since collagen is the most abundant macromolecule in the skin, ensuring the mechanical properties and structural integrity of the tissue. Therefore, collagen is one of the main proteins responsible for maintaining the skin's architecture [33,34].



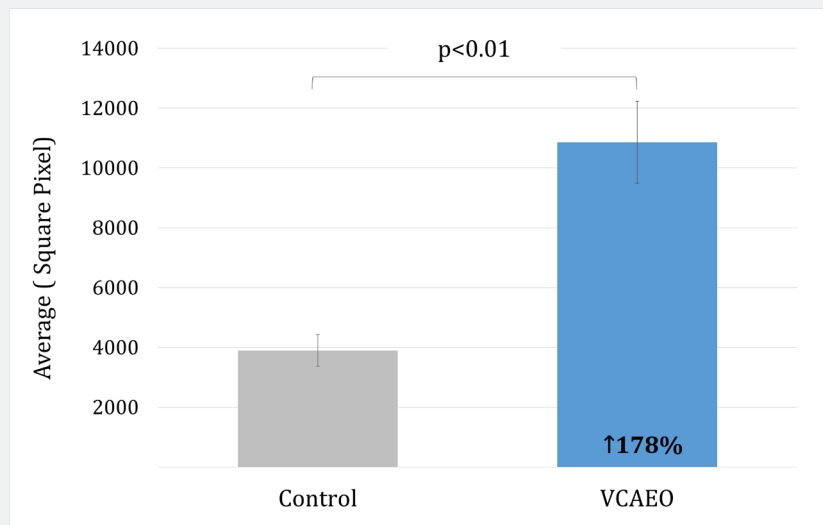


Figure 3: Quantification of the representative images of COL-1 synthesis in human skin fragments incubated with the product VCAEO for 72 hours, in comparison to control (basal control) group. The mean pixel intensity of the green channel was measured for each image using the Image J® software, with results expressed in units of square pixels. Data represent mean $\pm$ standard deviation (ANOVA, Dunnet post-hoc test). ** $P < 0.01$ compared to basal control group.

The different types of collagen exhibit variations that allow them to perform distinct functions within the skin structure. However, all are formed by a basic molecule, tropocollagen, a fiber composed of three high molecular weight amino acid chains [35]. Collagen types I, III, and IV are present in the dermis. Type I (COL-1) and type III (COL-3) are fibrillar structures found in large amounts in the dermis [33,35]. Specifically, COL-1 is found in the

thickest collagen fibers [34,35], and its reduction is associated with intrinsic aging [36]. Vitamin C is also essential for collagen biosynthesis and is a cofactor for the enzymes responsible for its stabilization and cross-linking [12,37]. Additionally, it directly stimulates collagen synthesis [12] and prevents the overproduction of matrix metalloproteinase-1 (MMP-1), which degrades collagen [38].

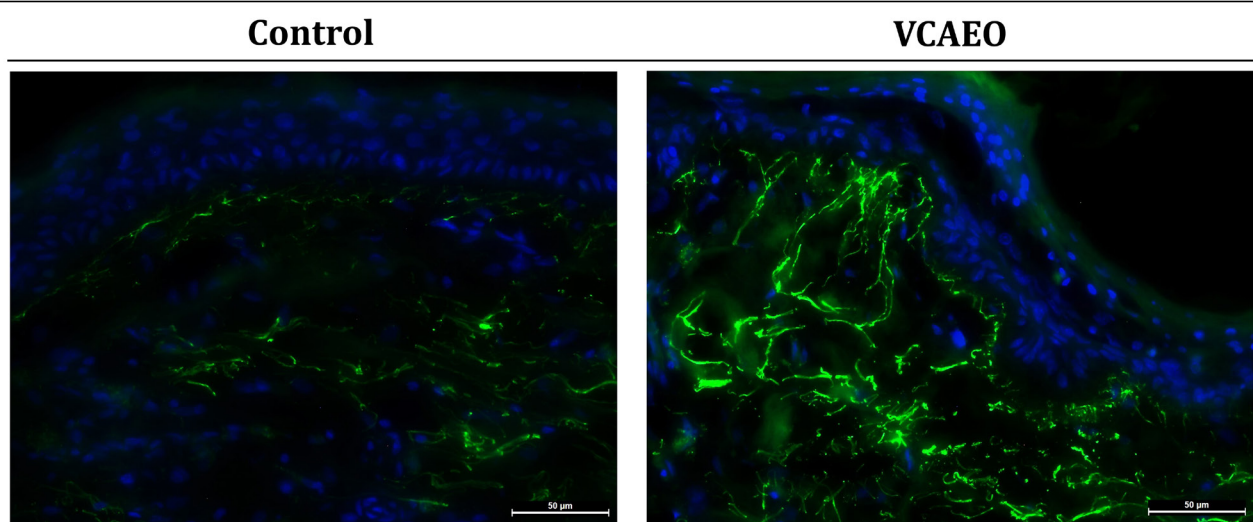


Figure 4: Evaluation by Immunofluorescence anti-elastin (green) and counter-staining with DAPI (blue, cell nuclei marker), microscope images at 40x magnification. Histological sections of 10 μ m in fragments of human skin (*ex vivo*) incubated in culture medium (basal control) or treated with the product VCAEO.

Retinoids significantly stimulate the synthesis of collagen type I (COL-1), the most abundant collagen in the dermis, responsible for skin structure and firmness. Retinoids enhance collagen production while regulating the activity of metalloproteinases, enzymes that degrade collagen, ensuring its preservation in aging skin [39]. By boosting COL-1 synthesis and protecting against collagen breakdown, retinoids are highly effective in treating

photoaging and reducing signs of aging, such as wrinkles and loss of skin firmness [14]. VCAEO was not only able to stimulate the synthesis of COL-1, but also acted positively on elastin synthesis (Figures 4 and 5). The result obtained through immunofluorescence analysis on human skin explants after 72 hours of incubation with the VCAEO showed a statistically significant increase ($p < 0.01$) of 126% in elastin synthesis compared to the control group.

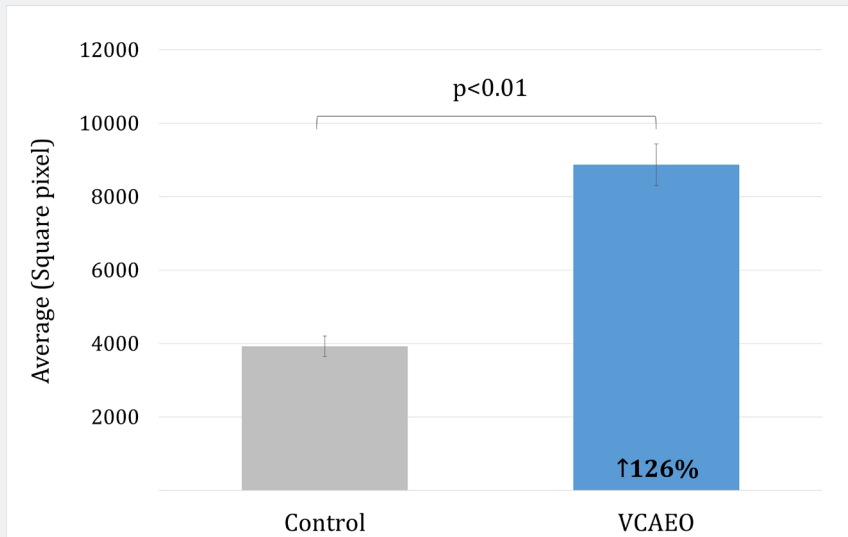


Figure 5: Quantification of the representative images of elastin synthesis in human skin fragments incubated with the product VCAEO for 72 hours, in comparison to control (basal control) group. The mean pixel intensity of the green channel was measured for each image using the Image J® software, with results expressed in units of square pixels. Data represent mean $\pm$ standard deviation (ANOVA, Dunnet post-hoc test). ** $P < 0.01$ compared to basal control group.

Skin elasticity is a crucial factor in maintaining overall skin health. The reduction in collagen and, especially, elastin fibers significantly impacts the skin's tensile strength and ability to resist deformation, leading to wrinkles and sagging [5]. Wrinkles can be classified into two types: superficial and deep. While fine lines result from epidermal distortion due to water loss, deep wrinkles are primarily caused by dermal deformation due to the loss of elasticity, strongly related to the decrease in collagen and elastin fibers [40].

Collagen, which constitutes 70-80% of the dermis [35], is key in providing skin structure and strength [33, 35]. At the same time, elastin gives skin resilience and the ability to return to its original shape after stretching [41]. Elastin, in particular, is essential for skin elasticity. As we age, collagen and elastin fibers undergo significant degradation [42]. Signs of aging arise from the decreased function of the connective tissue. Collagen becomes more rigid, and elastin fibers lose their ability to stretch, reducing skin elasticity. This loss of elastin is a major contributor to sagging and deeper wrinkles. Additionally, aging is marked by a decrease in glycosaminoglycans, reduced water content, and diminished cellular function, all exacerbating the visible signs of skin aging [43,44].

The combination of vitamins A (retinoids), C (ascorbic acid), and E (tocopheryl acetate) plays a significant role in enhancing elastin production and improving skin elasticity. Retinoids stimulate fibroblast proliferation and elastin synthesis, which is crucial for maintaining skin elasticity and reducing wrinkles [39]. They also strengthen the extracellular matrix, effectively preventing and treating stretch marks by improving skin resilience [14]. Ascorbic acid directly stimulates collagen and elastin production in fibroblasts, contributing to the skin's structural integrity. Additionally, it acts as an antioxidant, protecting the skin from oxidative stress, which can degrade elastin and collagen, leading to aging and reduced elasticity [45,46]. However, maintaining the appropriate concentration of ascorbic acid is crucial, as excessive amounts may inhibit elastin synthesis [47]. Vitamin E, particularly in the form of tocopheryl acetate, supports elastin production indirectly through its potent antioxidant properties. By protecting skin cells from oxidative damage and maintaining fibroblast health, vitamin E creates a favorable environment for elastin and collagen synthesis [48]. Although direct evidence linking tocopheryl acetate to elastin synthesis is limited, its role in delaying skin aging and preserving elasticity is well recognized [19,49].

Total Depigmenting Capacity by *ex vivo* Fontana-Masson Technique

To evaluate the product's effectiveness in reducing melanin levels in the skin, an *ex vivo* human skin model assay was carried out applying Fontana-Masson Technique. The results of the

evaluation of reduction melanin levels for the test formula VCAEO are shown in Figures 6 and 7. The images demonstrate that VCAEO promoted a statistically significant decrease ($p < 0.01$) in melanin levels, reaching up to 54% compared to the control group. These results were obtained through immunofluorescence analysis on human skin explants after 72 hours of incubation with the VCAEO.

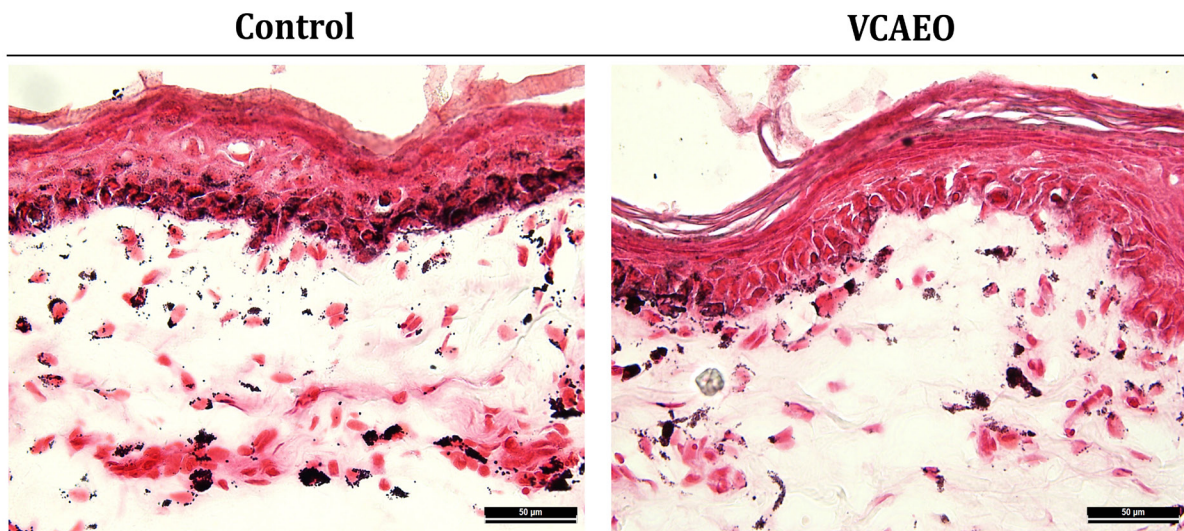


Figure 6: Evaluation of melanin synthesis by Fontana-Masson Technique, microscope images at 400x magnification. Data were obtained by recorded images of human skin histological cuts, previously incubated in a culture medium (basal control), and treated with the product VCAEO.

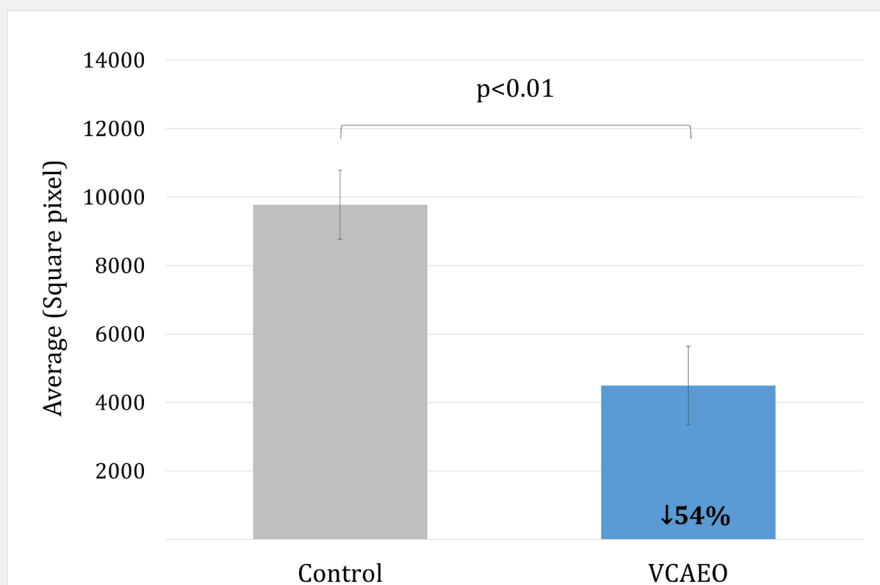


Figure 7: Semi-quantification of melanin pigment formation. Data obtained from microscopic images of human skin fragments (*ex vivo*) at 400X magnification, incubated in culture medium (control), treated with the test product VCAEO. Data represent mean $\pm$ standard deviation (Unpaired T-test). ** $P < 0.01$ compared to basal control group.

Melanin levels in the skin decrease with age, leading to irregular pigmentation and an increased risk of UV damage. Melanocyte density drops by 10-20% per decade after age 25-30, contributing to uneven skin coloration and hyperpigmentation in photoaged skin [50]. As melanin production becomes irregular, older individuals are more vulnerable to UV damage and skin cancer [51]. Aging also increases dyspigmentation, characterized by age spots and uneven skin tone, particularly in sun-exposed areas [52, 53]. Sun exposure further stimulates melanocytes, causing hyperpigmentation and uneven skin tone [54]. While overall pigmentation decreases with age, hyperpigmentation and hypopigmentation indices rise [53]. Melanin levels can also increase due to the hyperactivation of melanocytes, leading to age spots and accelerating the aging process [52].

Combining ascorbic acid, retinyl palmitate, and tocopheryl acetate can effectively reduce melanin levels in the skin, contributing to blemish whitening through their complementary mechanisms [54,55]. Ascorbic acid inhibits melanin production by interacting with copper ions at the active site of tyrosinase, the key enzyme responsible for converting tyrosine into melanin. By inhibiting tyrosinase activity, ascorbic acid reduces pigment formation and has been shown to significantly decrease hyperpigmentation in conditions like melasma [56]. Its anti-inflammatory properties further enhance its effectiveness in treating skin aging and pigmentation disorders [57].

Retinyl palmitate also plays a role in reducing melanin levels by promoting epidermopoiesis, which leads to melanin loss and inhibits melanogenesis. This makes retinyl palmitate useful for treating pigmentary disorders by reducing melanin accumulation in the skin [58]. In addition, it stimulates keratinocyte and fibroblast proliferation, enhancing skin repair and contributing to improved skin texture and pigmentation [59]. While retinyl palmitate can also stimulate melanin synthesis in certain cases by activating MITF and TYR expression, its role in reducing pigmentation is significant in blemish whitening [60]. With its potent antioxidant properties, tocopheryl acetate reduces melanin levels by mitigating oxidative stress, which is known to influence melanogenesis. It inhibits tyrosinase activity and other melanogenesis-associated proteins, reducing melanin production [61,62]. Additionally, tocopheryl acetate protects the skin from UV-induced damage, which can indirectly impact melanin production, thus helping maintain balanced melanin levels and preventing hyperpigmentation [63].

Conclusion

Aging is an inevitable process; however, the manifestations of aging can be alleviated through the application of topical vitamins. The synergistic formulation of vitamins C, A, and E, stabilized within an organogel system (VCAEO), demonstrated significant efficacy in diminishing the appearance of wrinkles, fine lines, and imperfections by modulating biomarkers such as collagen-1 and elastin while also reducing melanin levels. *In vivo* instrumental

assessments suggest that the response to VCAEO may differ in an age-dependent manner. Thus, the VCAEO approach could be optimized for preventive interventions for individuals aged 50 and younger, while restorative strategies may be more suitable for those over 50. VCAEO is not only an effective formulation for addressing the visible signs of skin aging, with significant benefits in improving skin texture and reducing pigmentation, but its user-friendliness is a critical factor for consumer endorsement.

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