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Synergistic antiaging and Dermal Restorative Effects of an Oral Bioactive Procollagen and Astaxanthin Supplement with A Topical Retinyl Palmitate, Vitamin C, Hyaluronic Acid and Alpha Hidroxy Acid Based Regimen

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Abstract

Aging is a natural process undergone by every organism, with progressive changes that lead to an impairment of many biological processes. The human skin ages through several mechanisms which may cause changes to the morphology of skin leading to its reduced thickness, lower dermis density and to the presence of a subepidermal low echogenic band. These morphological changes are responsible for the visible changes of the skin such as reduced elasticity, firmness, luminosity and hydration. This study was conducted to investigate the anti-ageing effect and to assess the qualities and efficacy of a topical cosmetic products and a nutricosmetic product, after 84 days. Sixty women were divided into three groups and assigned different treatments (Group A: topical treatment, Group B: oral treatment and Group C: combination of topical and oral treatments). The topical treatment consisted in the application of a facial gel with alpha hydroxy acids and a facial cream with retinyl palmitate, hyaluronic acid and Vitamin C, whereas the oral treatment consisted in the daily ingestion of a drinkable solution composed of collagen peptides, astaxanthin, hyaluronic acid, vitamins and active other ingredients. Finally, Group C received a combination of the treatments in Group A and Group

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B. Different parameters of the skin were assessed such as wrinkles, roughness, firmness, elasticity, hydration, luminosity and spots (brown and visible). Skin morphology was also studied by ultrasonography to analyze the thickness of a Subepidermal Low Echogenic Band (SLEB), skin thickness and dermis density. All parameters were measured at the baseline visit (D0), at day 28 (D28), at day 56 (D56) and at the end point visit (D84). Volunteers had to complete a self-assessment questionnaire at each follow up visit. Results showed that all groups had some anti-aging benefits but the women receiving the combination treatment (Group C) had a faster and synergic effect in comparison to the other two groups with regards to improvements in the markers assessed for skin ageing.

Keywords

Antiaging; Photoaging; Collagen Peptides; Cosmetic Treatment; Nutritional Supplement; Ultrasonography

Introduction

Ageing is well observed phenomenon with intrinsic and extrinsic causes, having a direct impact on the firmness and elasticity of the dermis [1]. Fibroblasts start to reduce collagen and elastin synthesis in the dermis, cellular metabolism slows and elastosis sets in. These changes induce a more flaccid and less elastic skin and the first wrinkles start to be visible. Other visible changes are less skin illumination, less hydration and more visible spots [2].

To minimize these ageing manifestations, the action of different active ingredients on the skin have been studied in recent years. Hydroxy acids, also called fruit acids, are among the organic acids used in the treatment of skin disorders [3]. Other well-known active ingredients are antioxidants, which are commonly used to improve skin conditions by preventing or treating photodamage [4]. Vitamin C is a non-enzymatic antioxidant known to have important benefits on skin aging through its antioxidative effect but also its role as a cofactor for enzymes involved in collagen synthesis [5-8]. Animal studies have shown Vitamin C's protective effects against UVA phototoxicity [9,10]. Moreover, topical Vitamin C's benefits with regards to aging have been demonstrated in a randomized placebo-controlled double-blinded studies [11,12]. Vitamin A, or retinol, is another potent antioxidant widely used to treat various skin conditions. Its derivatives retinaldehyde, retinyl palmitate, ester of retinol and palmitic acid cause less skin irritation than Vitamin A and a similar efficacy since these derivatives are converted into retinoic acid in the skin [13]. Retinol and its derivatives are thought to enhance collagen production through their inhibition of AP-1 (Activator Protein 1) and MMP-1 (Matrix Metalloproteinase 1 or interstitial collagenase), which normally induce collagen degradation.

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Multiple human studies have shown significant rejuvenating effects of topical retinoids on various parameters of aged skin [14-17].

Astaxanthin, a xanthophyll carotenoid extracted from *Haematococcus pluvialis* alga, is known to have multiple health benefits on skin through its photoprotective, anti-inflammatory and antioxidant activity [18]. In fact, owing to its chemical structure, astaxanthin has been found to have greater antioxidant properties than other carotenoids that get converted to retinol [19]. Astaxanthin has been studied in humans and significant antiaging effects have been observed when astaxanthin has been used topically or when ingested [20,21].

The nutricosmetic industry aims to engineer ingestible products that have a cosmetic benefit. This includes nutritional supplements and can be comprised of any bioactive ingredient from a lipid, to a peptide, a polysaccharide, minerals, vitamins and plant extracts [22]. These bioactive substances will most often be very safe and have minimal side effects. Considering the population's rising interest in leveraging their diet for health and wellness benefits along with the longstanding quest for a youthful appearance, it comes as no surprise that nutricosmetics have seen an increase in demand, particularly with regards to anti-aging products. In fact, randomized placebo-controlled trials have shown improvement of various skin aging parameters with an oral collagen peptide supplementation regimen [23].

The present study was designed to test the efficacy in improving cutaneous aging manifestations of a nutraceutical product that has collagen biopeptides and astaxanthin as its main active ingredients as well as two topical products, the first composed of Alpha Hydroxy Acids (AHA's) and the second composed of a retinol derivative, Vitamin C and hyaluronic acid. To determine if a synergic effect would be seen in skin treated with a combination of the nutritional supplement and the topical products, the combination of treatments was also studied.

Material and Methods

Study Design

The clinical trial was a single-center, randomized study conducted in healthy women. Sixty-six women were enrolled and only three did not finish the study for unrelated reasons. All patients were divided into three homogeneous groups.

In group A, women applied two topical cosmetic products on the face. One of the products was a gel in which the active ingredients were AHA's and the other topical product consisted of a cream with retinyl palmitate cyclodextrin, vitamin C and hyaluronic acid. The cream product was applied twenty minutes after the AHA gel to ensure that the gel had time to act and that the cream could penetrate more easily through the skin, thus potentiating the effects. Topical

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products were applied at night. In group B, women ingested one sachet of a nutritional supplement every morning in the form of a drinkable solution with collagen peptides and antioxidants such as astaxanthin as the main active ingredients. In group C, women applied the same topical products described in group A following the same protocol and also ingested the nutritional supplement every morning, as was done by group B (Table 1).

Control visits took place on days 0 (baseline visit), 28, 56 and 84 (end point visit). Treatment effects were assessed by comparing the results of efficacy parameters described below at the baseline visit with those obtained at days 28, 56 and 84, respectively. Comparisons were also drawn between the groups at each control visit (D0, D28, D56 and D84).

Different parameters related to ageing were studied by skin ultrasonography, which allowed for the assessment of the skin's morphology. The parameters measured were Sub-Epidermal Low-Echogenic Band (SLEB) thickness, dermis density and skin thickness [24-31]. These measurements were collected using a Derascan C ultrasound system (Cortex technology, Denmark) with a modified 20 MHz ultrasound probe. Skin luminosity was assessed by chromameter, specifically a Minolta Chromameter CR-400 (Minolta, Japan). Firmness and elasticity were measured through a skin biomechanical evaluation with a Dual-Cutometer MPA 580® (Courage and Khazaka, Germany). The measurement principle of these two physical parameters is based on a suction method. Hydration of the skin was studied by a Corneometer CM825 connected to a Dual-Cutometer MPA 580® (Courage and Khazaka, Germany). Hydration data was obtained by an electrometric system collecting capacitance measurements. Skin topography was assessed through 3D images (AEVA-HE, Eotech, France) allowing for the analyses of skin wrinkles (count, volume and depth) as well as skin roughness. The analysis of the facial region and calculation of the number of brown and visible spots was performed with the VISIA-CA (Canfield, USA), a cross-polarized and UV photography imaging system.

All evaluations were performed in a fully controlled and acclimated room (controlled temperature: $T=21 \pm 2^{\circ}\text{C}$; controlled relative humidity: $\text{RH}= 55 \pm 10\%$). Two evaluation periods were defined (in the morning, 9:00hr - 13:00hr and in the afternoon 13:00hr - 19:00hr). Each participant's evaluation was performed during the same period of the day to minimize intraindividual variations.

Study Groups		Type of Administration	Product Name	Product Directions of Use
Group C	Group A	Topical	Fluidbase Rederm 15% AHA	Product to be applied first, at night.
			Fluidbase Rederm Retinol + Vitamin C	Apply 20 minutes after previous product application.
	Group B	Oral	Fluidbase Rederm Drinkable Collagen	Ingest a content of one sachet a day.

Table 1: Treatment assignment per group and administration of treatment administration details.

Inclusion and Exclusion Criteria

Sixty-six women were enrolled in our trial, of which sixty-three completed the study. Women between the ages of 40 and 60 who presented signs of skin ageing were included. All skin types were included and most of the women were of phototype I to III per Fitzpatrick classification.

The main exclusion criteria were having an allergy or history of local or systemic reaction to any ingredient present in the products tested as well as having any cutaneous marks on the experimental areas that could interfere with skin assessments. Women with a history of malignant melanoma or carcinoma were also excluded, along with women known for a systemic disorder. Patients undergoing hormone therapy with ongoing adjustments to their treatment regimen were or women anticipating the initiation of hormone therapy during the study were excluded as well. Pregnant or breast-feeding women were excluded. Women having received a treatment containing Vitamin A or any of its derivatives were excluded if this treatment was discontinued less than 3 months prior to the initiation of the study, as were women having received anti-ageing or aesthetic treatments within 6 months of study initiation.

During the study period, the volunteers were instructed not use any other facial creams or facial sunscreens and to avoid direct or intense sun or UVA exposure (UV lamps).

At the start of the study, a data collection logbook was given to participants. Any reaction or sensation of discomfort detected was recorded. A skin examination of the experimental areas was performed by the dermatologist or the responsible technician, which was done visually under standard “daylight” source at each control visit (0, 28, 56 and 84 days).

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The study was performed according to the Declaration of Helsinki principles and subsequent amendments. It was conducted in the spirit of Good Clinical Practice Guidelines and general principles of Law 46/2004 of August 19th.

Statistical Analysis

Statistical analyses were performed with SPSS 23 (IBM). Non-parametric tests were used for all data comparisons between D0, D28, D56 and D84 and within groups themselves.

The efficacy results of skin changes within each group were analyzed with Wilcoxon tests. Comparisons between groups were studied with Mann-Whitney U-tests. For qualitative data, for instance subjective efficacy data, a binomial test was performed. For all statistical analyses, significance was considered when $p < 0.05$.

Results

The objectives of this study were to investigate the anti-ageing effects of a cosmetic and a nutricosmetic product applied under normal conditions of use. We also aimed to verify the compatibility and acceptability as well as to assess the qualities and efficacy of these treatments. The evaluation of patients was compared to their baseline evaluation and between the groups for the same time points.

Throughout the study, all products showed very good skin acceptability and compatibility as no volunteers reported reactions or a sensation of discomfort. At baseline (D0), all parameters evaluated showed no statistically significant differences between groups.

Results are expressed in absolute values of the unit specific to each parameter at a given experimental time as well as in percentage of change (%), representing the variation of the parameter in comparison to D0 for each experimental time. Area variation was also recorded for D84, D56 and D28 in comparison to D0. A comparison between the three groups was also drawn.

Skin Ultrasonography

Ultrasound skin imaging is a non-invasive diagnostic technique in which the physical properties of ultrasound are used to examine the skin and its appendages [32,33]. This technique allows for the detection of skin alterations through the analysis of the cross-sectional images obtained. Sub-epidermal Low-Echogenic Band (SLEB) thickness, density of dermis and skin thickness are the three parameters measured in this study with ultrasound. These

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sonographic parameters have a direct relationship with age and have been used in prior studies as markers of aging skin (Fig. 1) [24-31,34].

The malar area was studied by ultrasound and measurements were collected at the baseline visit (D0), at days 28, 56 and at the end point visit (D84).

Figure 1: Ultrasonography images the skin overlying the malar area at baseline (D0), day 28 (D28), day 56 (D56) and at the end point visit (D84).

Sub-epidermal Low Echogenic Band (SLEB) Thickness

A variety of histological changes occurring with skin aging are thought to be responsible for the development of the SLEB. The accumulation of glycosaminoglycans at the sub-epidermal

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level as well as rearrangement of collagen and inflammatory reactions resulting in superficial edema and elastosis have been hypothesized to contribute to this SLEB.26,35 Venous hypertension may also play a role [36]. The thickness of this SLEB has been found to increase with age [24-29,34].

The SLEB thickness evolution is expressed in μm and is shown in Fig. 2. There was a statistically significant decrease in SLEB thickness in groups B and C after 28, 56 and 84 days. These groups had the nutritional supplement as part of their treatment.

The percentage of change in SLEB thickness was calculated for groups A, B and C and is shown in Fig. 3. Group C, which received a combination of topical and oral treatments, had a statistically greater change than group A at 28 and 56 days. Sub-epidermal low echogenic band thickness for Group C was found to decrease by $16,4\% \pm 17,6$ and $16,7\% \pm 20,4$ at days 28 and 56 days respectively. However, at the end point visit (D84), the percentage of change was not statistically different between groups, despite the reduction in SLEB thickness being greater in groups B and C in comparison to group A (topical cosmetic treatment).

In conclusion, after 84 days, results showed a reduction in SLEB thickness in all three treatment groups in comparison to their baseline visits. Mid-study trends suggested a possible advantage of treatments assigned to groups B and C over the treatments received by group A in reducing SLEB thickness, thus potentially having a greater impact on skin morphology.

Figure 2: SLEB thickness evolution. SLEB thickness at the baseline visit (D0), at 28 days (D28), at 56 days (D56) and at the end point visit (D84). An asterisk (*) above a treatment bar indicates a statistically significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon test).

Figure 3: SLEB thickness variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84). A bracket between bars indicates a significant difference between groups ($p < 0,05$, Mann-Whitney test).

Dermis Density

Dermis density is the second parameter that was measured by ultrasonography, which reflects the physical property of hardness. With age, a marked decrease in collagen and elastin can be noted in the skin, with an increase in collagen crosslink stability but a decrease in the organization of collagen bundles resulting in less firm skin [2,37,38]. Dermis density is related to firmness. The more youthful the skin, the more organized the collagen bundles, the greater the dermis density and firmness of the skin [24,29].

The results presented in Fig. 4 show that all treatments significantly increased dermis density at every control visit (D28, D56 and D84). Thus, both the topical treatments and oral treatment, alone or in combination, improved dermal density as of the first control visit.

The percentage of change in dermis density observed is illustrated in Fig. 5. Group C showed a higher percentage of change than groups A and B at 28 and 56 days with group C having a dermal density increase of a $45,3\% \pm 58,6$ and $49,8\% \pm 77,5$, at the two first follow ups respectively. At the end visit (D84), an improvement in dermis density of over 60% was noted for all treatment groups, although there was no statistically significant difference between groups at any of the time points.

Figure 4: Evolution of dermis density. Dermis density at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 5: Dermis density variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Skin Thickness

Skin thickness is the third parameter that was measured by ultrasonography. As skin ages, many studies have noted a decrease in its thickness at the epidermal and dermal levels as well as in total skin thickness [2,37,39].

The evolution in skin thickness is illustrated in Fig. 6. At the first control visit (D28), all groups presented a statistically significant improvement of this parameter. However, at day 56 (D56) only group A (topical treatments) and group C (topical and oral treatments) showed a statistically significant improvement in skin thickness. Finally, only the volunteers included in the combination group (group C) preserved this improvement in skin thickness until the end point visit at 84 days (D84).

A comparison of the percentage of change in skin thickness between groups is shown in Fig. 7. There was no statistically significant difference between groups for each time interval studied. It is worth noting that, despite the lack of significance, group C showed a greater reduction in skin thickness in comparison to groups A and B after 56 days of treatment.

Figure 6: Skin thickness evolution. Skin thickness at the baseline visit (D0), at 28 days (D28), at 56 days (D56) and at the end point visit (D84). An asterisk (*) above a treatment bar indicates a statistically significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon test).

Figure 7: Skin thickness variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Skin Luminance

Luminance, representing skin's ability to reflect light, diminishes with age. It is compromised by irregular pigmentation, an uneven surface texture, decrease in water content and changes in collagen cross-linking, which are hallmarks of aging skin [2,40,41]. Youthful appearing skin is more luminous.

There was a significant increase in skin luminosity for group C after 56 and 84 days, with no significant changes observed for groups A or B at any time point. Results are illustrated in Fig. 8.

To better evaluate skin luminosity changes after 28, 56 and 84 days of treatment, the percentage of change in luminosity between D0 and each control time point was calculated for each group. It is represented in Fig. 9. At the first control visit (D28), a statistically significant difference was noted between the combination treatment group (group C) and both groups A and B. However, after 56 and 84 days, a significant increase in skin luminosity was only found when comparing group C to group B (oral treatment).

Figure 8: Evolution of skin luminance. Skin luminance at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 9: Skin luminance variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84). A bracket between bars indicates a significant difference between groups ($p < 0.05$, Mann-Whitney test).

Skin Firmness and Elasticity

Firming Effect

Skin firmness is one of the parameters most affected by the aging process [1]. As mentioned previously, the organization and quantity of collagen and elastin in the dermis determine the firmness of the skin [2,37,38]. Skin density is proportional to firmness and inversely proportional to elasticity [24,29].

Firmness was measured using a suction-based technique: the greater the millimetres (mm) of suction, the lesser the firmness. Therefore, a decrease in mm measured represents an improvement in skin firmness and a negative variation in change indicates a rejuvenating effect on the skin.

Results showed that group A, receiving the topical treatments, presented a statistically significant improvement in skin firmness at every time point studied. Group C (combination of topical and oral treatments) showed a significant improvement at days 28 and 84 but not at day 56. Finally, group B, receiving the oral treatment, showed statistically significant benefits on skin firmness only at the final follow up visit.

In group A, firmness over the malar area showed variations of $11,0\% \pm 18,9$, $13,2\% \pm 16,7$ and $12,6\% \pm 14,9$ after 28, 56 and 84 days of treatment, respectively. Comparisons between groups did not show a significant difference at any of the time points but showed a trend in which group A, receiving the topical treatments only, had greater skin firmness improvements than groups B and C (Fig. 10 and 11).

Figure 10: Evolution of skin firmness. Skin firmness at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 11: Skin firmness variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Skin Elasticity

Skin elasticity, much like skin density, reflects the organization of dermal constituents. Alterations in collagen bundle configuration cause the skin to become more rigid. Elastic fibers are responsible for the skin's ability to recover after mechanical depression, which a youthful dermis can do within minutes, but aged skin may require over 24 hours [2,42].

After receiving treatment for 28 days (D28), 56 days (D56) and 84 days (D84), volunteers from all groups presented a statistically significant increase in skin elasticity in comparison to their baseline at D0. These results are illustrated in Fig. 11 and 12.

When assessing the percentage of change in skin elasticity over time, no group stood out as having a significantly greater change than others, despite results at 28 days suggesting larger changes in groups A (topical treatments) and C (topical and oral treatments)- both groups including the topical treatments. Changes in skin elasticity of $22,4\% \pm 17,4$ and $25,2\% \pm 24$ were found for these groups, respectively.

Figure 12: Evolution of elasticity. Elasticity at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 13: Elasticity variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Skin Hydration

The skin's natural water content, or hydration, is a key characteristic reflecting skin's health. Ageing skin is known to have less water content as well as a decreased ability to bind water and dehydrated skin causes visible changes compromising the smoothness of the skin's appearance [43-46].

All groups presented a statistically significant increase in skin water content after 28, 56 and 84 days of treatment as illustrated in Fig. 14.

Percentage of change in skin hydration was positive in all groups, although the difference between groups was not statistically significant. Results suggest that groups A and C, the groups including the topical products in their treatment, presented a higher increase in hydration level at all time points. The topical treatment group had an increase in skin water content of 35% $\pm$ 25,7 and 35,2% $\pm$ 24,2 at days 56 and 84 respectively. The increase in skin hydration at these same time points for the combination treatment group was of 37,6% $\pm$ 23 and 39,4% $\pm$ 18,5. These results are shown in Fig. 15.

Figure 14: Evolution of skin hydration. Skin hydration at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 15: Skin hydration variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Skin Wrinkles and Roughness

The development of wrinkles in the skin is one of the hallmark changes during aging. They are thought to occur due to the loss of certain collagens at the dermo-epidermal junction, the flattening of this area as well as the alterations in skin thickness, density and elasticity [2,47]. Crow's feet wrinkles are very structured wrinkles lateral to the eye resulting from the orbicularis muscle contractions [48].

Roughness of the skin reflects changes in the epidermis and is also a characteristic sign of ageing skin. These uneven surface texture changes can also reveal an uneven tone and compromise luminance, as mentioned previously [2,40,41].

Crow's Feet Wrinkle Count and Area

Wrinkle count in the crow's feet area decreased in all groups after 28, 56 and 84 days, although this reduction was not statistically significant.

When assessing the percentage of change in wrinkle count throughout the study, a decrease of $8,3\% \pm 24,5$ was observed for group C (topical + oral treatment) after 28 days of application. The topical treatment group demonstrated a higher percentage of change at the 56- and 84-day visits with a reduction of $9,4\% \pm 30,7$ and $7,3\% \pm 25,4$ in crow's feet wrinkles, respectively. However, there was no statistically significant difference between groups. The results are shown in Fig. 16.

There was a reduction in the volume of crow's feet wrinkles in all groups at all control visits except for group B (oral treatment group) at day 28. These decreases in wrinkle volume were not statistically significant.

A comparison of the percentage of variation in crow's feet volume between groups was drawn and showed that groups A and C (both groups including the topical treatments) had the greatest reduction in wrinkle volume after 28 and 56 days of treatment, despite this improvement not having statistical significance. Group C (combination treatment group) showed a percentage of reduction in wrinkle volume of $6,6\% \pm 18,9$ and $-6,6\% \pm 31,1$ after 28 days and 56 days respectively, although this change was not significantly greater than in other groups (Fig. 17 and 18).

Finally, wrinkle depth was studied. This parameter was assessed in the area lateral to the eye and did not show statistically significant changes in either of the three groups at any of the time points studied. When looking into the percentage of change of wrinkle depth, it can be noted that group C started to show some improvement in this parameter after 84 days, with a reduction of $-2,4\% \pm 7,8$ in wrinkle volume.

Figure 16: A. Evolution of wrinkle count. Wrinkle count at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). B. Wrinkle count variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Figure 17: A. Evolution of wrinkle volume. Wrinkle volume at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). B. Wrinkle volume variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Figure 18: A. Evolution of wrinkle depth. Wrinkle depth at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). B. Wrinkle depth variation from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Roughness of the Skin

Skin roughness was expressed in Ra and Rz indices, parameters that were initially derived for the metal industry but have since been studied and validated for quantification of roughness in skin [49,50]. The Ra index is an arithmetic mean of the skin surface and Rz is a mean of the 5 biggest peaks and the 5 lowest valleys in the image area [49]. In this study, the malar area was evaluated. The combination of both parameters the roughness of the epidermis, where a decrease in Ra and Rz indices suggests smoother skin with an even tone [49].

Results revealed a slight decrease in the Ra and Rz indices in groups B and C at each control visits, as shown in Fig. 19. This reduction was not statistically significant.

Group A (topical treatments) had a greater improvement in roughness parameters in comparison to groups B and C, which all showed some improvement in skin roughness although none of these improvements were statistically significant at any time point (Fig. 20 and 21).

Figure 19: Evolution of skin roughness (Ra and Rz indices). A. Ra index at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). B. Rz index at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84).

Figure 20: Skin roughness variation A. Variation of Ra index and B. Variation of Rz index from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Figure 21: Illustrative 3D images of the crow's feet area of a volunteer from group B at the baseline (D0), after 28 days (D28), 56 days (D56), and 84 days (D84).

Skin Pigmentation Homogeneity

Aging skin characteristically tends to have uneven pigmentation and has been found to have a reduction in active melanocytes. Despite this, these pigment irregularities are thought to stem from melanocyte hyperactivation and a change in the distribution of pigment [51].

The assessment of skin pigmentation homogeneity was studied by counting spots and measuring their surface area in the malar region. The spots were classified as brown or visible. Brown spots were defined as dark macules and visible spots was defined as any macule of a different tone than that of the background skin. The absence of brown and visible spots is characteristic of homogeneous, youthful appearing skin.

Brown Spot Count and Area

A statistically significant reduction in the number of brown spots in the group receiving the combination treatment (Group C) after 28 days and 56 days was noted. Surface area of these brown spots decreased significantly in all groups after 28 days of treatment. However, on subsequent control visits (D56 and D84), changes were no longer statistically significant.

There was a 4.7% decrease in the number of brown spots at the 28 day follow up time point in group C, which was a greater than the variations in groups A and B. After 56 and 84 days of

treatment, the oral treatment (group B) and the combination treatment (group C) groups showed a similar reduction in number of brown spots. With respect to the changes in surface area of brown spots, all three groups showed a reduction between 4 and 6% at the first control visit (D28). However, this reduction was less important after 56 and 84 days of treatment.

Overall, results showed that there was some reduction in brown spot count and area in the different groups. Trends suggest that groups B and C had a greater reduction in the number of brown spots relatively to group A and that at the final follow up (D84), group A had a greater reduction in brown spot surface area relatively to groups B and C, although none of these differences were statistically significant (Fig. 22 and 23).

Figure 22: Evolution of brown spot count and surface area. Brown spot count (A) and surface area (B) at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 23: Variation in brown spot count and surface area. Brown spot count (A) and surface area (B) from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Visible Spots

The number of visible spots was significantly reduced in groups A (topical treatments) and C (topical and oral treatments) after 28 days. There was a similar trend for groups A and C regarding the reduction in surface area of these visible spots, but this decrease was significant after 28 days (D28) and after 56 days (D56).

The visible spot count was reduced by $3,9\% \pm 4,7$ in group A presented and by $6,4\% \pm 12$ in group C, both changes being statistically significant in comparison to group B (oral treatment). However, after 56 and 84 days, the difference between groups was no longer statistically significant, but the trend of groups A and C showing greater change than group B persisted.

Additionally, the surface area of visible spots was reduced to a greater extent in group C (topical and oral treatments) in comparison to groups A and B. The combination group presented a reduction of $8,1\% \pm 5,8$ of the visible spot surface area after 28 days. However, the difference between groups was not statistically significant at any of the follow up time points.

Self-Assessment

At each evaluation time point (D28, D56 and D84), all volunteers included in the study completed a patient self-appraisal questionnaire about perceived skin changes and satisfaction regarding the changes of various properties of their skin. A defined grading scale was used and results were expressed in percentage of satisfied patients.

After 84 days, 90 % of the volunteers in group A noted an enhancement in skin firmness, 85,7% reported softer and more uniform appearing skin and 81% found their skin had a more youthful appearance. As for patients in group B who were assigned the oral treatment only, 95,5% found their skin to be more hydrated and softer, 90,9% noted their skin was smoother and firmer and 86,4 % noted an anti-aging effect. Finally, in group C, 100% of the volunteers reported more hydrated, softer and more uniform appearing skin and a 95% noted a rejuvenated, smoother and firmer appearance to their skin (Fig. 24 and 25).

Figure 24: Evolution of visible spot count and surface area. Visible spot count (A) and surface area (B) at the baseline visit (D0), 28 days (D28), 56 days (D56) and the end point visit (D84). An asterisk (*) above a treatment bar indicates a significant difference in the improvement noted at that time ($p < 0.05$, Wilcoxon signed ranks test).

Figure 25: Variation in visible spot count and surface area. Visible spot count (A) and surface area (B) from the baseline visit to day 28 (D0-28), day 56 (D0-D56) and to the end point visit (D0-D84).

Discussion

There has been an increasing demand for nutricosmetics from aesthetic medicine patients and the public. However, these products are not devoid of adverse effects and most preparations lack efficacy trials to support the biological effects they claim.

We sought to assess the effect on skin ageing of an oral collagen supplements, alone and in combination with topical treatments with proven skin antiaging properties such as AHAs, topical retinoids and Vitamin C. Various validated technologies were used to gather quantitative data on the skin for subsequent analyses. It is worth mentioning that ultrasound has been widely used to characterise skin and allows for insights on a variety of parameters regarding the epidermis, dermis and subcutaneous tissue.

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Collagen is a bioactive substance that has been well studied as a dietary supplement and nutricosmetic product. The digestion and absorption mechanisms of orally ingested collagen peptides have been elucidated in animal studies [52,53]. Further investigations on plasma kinetics have shown collagen peptide metabolites to appear in blood after 1h of ingestion [54]. Studies on orally ingested radiolabelled collagen peptides confirmed that these metabolites reach the skin and can stay there for as long as 2 weeks [55,56]. Importantly, human studies have revealed that orally ingested collagen peptides increase hydration and collagen density in skin *in-vivo* and *ex-vivo* can increase collagen and glycosaminoglycan content of the skin [23].

Our results showed that the SLEB thickness was reduced in all groups, but more so in group C. Dermis density was increased in all 3 treatment groups. Skin luminosity improved in all groups, with a significantly greater improvement seen in the group treated with both oral and topical agents. At the final follow up visit, there was a significant improvement in firmness in all groups. Elasticity improved significantly in all groups as of the first follow up visit, as did skin hydration. There was a non-statistically significant reduction in the number of crow's feet wrinkles in all groups, with no significant changes seen in the volume or depth of these wrinkles or in roughness of the skin. There was a statistically significant reduction in the number of brown spots for group C at the 28 day and 56 day follow ups, although a statistically significant reduction in the surface area of brown spots was seen in all groups at 28 days. As for visible spots, they decreased significantly in number in groups A and C at the first follow up visit and in area at the first and second follow ups. Finally, self-assessments revealed that rejuvenating changes of the skin were observed by patients in 81% of those treated topically, in 86.4% treated with the oral supplement and 95% of those receiving the combination treatment. Respect to skin thickness parameter, which decreases in our study, although in most clinical studies regarding antiaging treatments it tends to increase, in our study, the use of AHAS in both treatment arms, may have an influence in this paradoxical skin thickness reduction, as it also includes corneal layer, directly affected by the keratolytic properties of these substances. Despite this reduction, physical properties such as elasticity or firmness increased in the treatment arms of the study.

While all treatment groups showed improvements in a variety of parameters, group C stood out from other treatment arms with respect to SLEB thickness improvement, luminosity changes and in brown and visible spot parameters. Subjective changes were also most important in group C.

Some parameters began showing changes between groups at the last follow up visit, which we can hypothesize may have been significant if the trial had been conducted over a longer period- such as the improvement in wrinkle depth seen in group C. Prolonging follow up times after discontinuation of treatments would be important to assess the duration of the objectified benefits. As the male population has shown an increasing interest in skin care products and

antiaging treatments in recent years and knowing certain parameters of skin and aging vary in men, it would be important to assess the effects of these treatments in this population [57].

Strengths of our study include the use of validated tools for objective and quantitative data collection, the large number of parameters assessed to understand effects on skin aging and the standardized approach in conducting follow up studies to minimize intraindividual variability.

Limitations of this study are the limited number of patients, the absence of a control placebo group and the limitation to female patients between 40 and 60 years which does not permit its generalization to men or other age groups.

Conclusion

In conclusion, our randomized controlled trial revealed that all groups had some anti-aging benefits but that women receiving the combination treatment (group C) had a faster and synergic effect in comparison to the other two groups with regards to improvements in the markers assessed for skin ageing.

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