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"A new performance territory for skincare using targeted long UVA protection in association with powerful anti-aging actives"

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1. Introduction

Skin photoaging is a cumulative process where damages induced by chronic exposure to ultraviolet (UV) rays add to intrinsic aging. UVA and especially long UVA wavelengths (UVA1, 340-400 nm) are major contributors of this process, since they constitute almost 80% of total energy received on earth and exhibit high penetrating properties into the dermis [1,2]. We hypothesized that such chronic stress could weaken deep skin cells responsiveness to biologically active compounds.

Among them, ascorbic acid (vitamin C) has shown anti-aging efficacy both in vitro and in vivo thanks to its collagen biosynthesis enhancing action [3]. A new UVA1 absorbing filter, Methoxypropylamino Cyclohexenylidene Ethoxyethylcyanoacetate (MCE), exhibiting a peak of absorption at 385 nm, was approved by the Scientific Committee on Consumer Safety for use in sunscreen products or as light stabilizer and was shown to improve UVA1 photoprotection in vitro and in vivo [4]. It was previously shown that UVA could antagonize the effect of Vitamin C on collagen secretion, both in 2D and 3D in vitro models, and that filter MCE was able to significantly improve the anti-aging efficacy of Vitamin in a context of UV exposure [5].

Here, we evaluate for the first time the skin benefits of adding 1% MCE filter to a 12% vitamin C skincare product in real-life usage. In order to deepen the knowledge, we studied the effect of the association of vitamin C and MCE filter on gene expression *in vivo*. Finally, to go further, we investigated *in vitro* the added value of associating antioxidants such as resveratrol to MCE filter to improve its protective effect on vitamin C biological efficacy under repeated UVA stress.

2. Materials and Methods

2.1. Real-life study

2.1.1. Subjects

A panel of 130 women aged between 45-70 years old, with all skin types, phototypes II to IV in Fitzpatrick classification scale and users of anti-aging product was recruited in accordance with the following inclusion criteria:

- Subjects presenting wrinkles, dull complexion, and lack of firmness by self-declaration.
- Subjects presenting moderate signs of ptosis, frontal wrinkles, crow's feet wrinkles, underneath eye wrinkles, sagginess, and texture by atlas scoring.[6]
- Subjects presenting moderate skin firmness, moderate skin radiance, even skin tone, moderate visibility of fine lines and non-smooth skin according a 10-points scale.
- Subjects that have at least 2 hours of outside exposure in their daily routine (walking, waiting for the bus, having lunch at a terrasse, gardening, etc) but no sunbathing. In case of stronger exposure, a hat and the usual sun protection should be used.

All subjects were fully informed about the objective of the study, its design, its constraints with regard to duration and signed an inform consent.

2.1.2. Investigated products

The participants were distributed in two groups thanks to a randomization approach to evaluate the skin benefits of 12% pure vitamin C + 1% MCE filter (group 1) compared to 12% pure vitamin C (group 2), both formulated in an oleosome emulsion base (patented). This kind of robust lamellar emulsion structure allows to overcome the challenge of associating and stabilizing a high amount of ascorbate with a dispersion of polar oily phase containing the filter MCE, while keeping good sensorial attributes. Vitamin C and MCE concentration and stability in formula during storage were checked prior to the study. The products' usage convenience was validated in a preliminary qualitative consumer study to ensure a good observance of the treatment throughout the duration of the study. A neutral moisturizer was used by the participants in the wash-out of 14 days before the study. The investigational products were applied morning and evening by subjects on face and neck followed by the neutral moisturizer for 3 months, with a 15-days remanence phase (only application of neutral moisturizer).

2.1.3. Study design

The study was conducted in Mauritius between April and December of 2023 under dermatological control. In this season, Maximum Daily UV Index remained between 4.4 and 11.6 and Maximum Solar Energy (w/m2) remained between 874 and 1248.

Different skin benefits were investigated in a dual clinical and instrumental approach at baseline and 14, 28 and 84 days of treatment and at 99 days (15-days remanence).

2.1.4. Clinical scoring

The skin aging signs of ptosis, frontal wrinkles, crow's feet wrinkles, underneath eye wrinkles, sagginess and neck texture were graded by expert according to the Skin Aging Atlas [6].

Skin firmness, Skin tone, Fine lines visibility, Skin smoothness and Skin radiance were evaluated by a 10-points scale, where a decrease in scores means an improvement in skin attributes.

2.1.5. Instruments

Different instruments were used to complement clinical assessments. To evaluate the effect of the investigational products in improving skin firmness, Dermotrace was used. Dermascan - Ultrasound image of the skin was used to evaluate performance on skin density (non-echogenic proportion) and dermis thickness. Colorface was chosen to obtain illustration before and after treatment. Photographs with eyes closed were taken in cross polarization, parallel polarization, standard and UV lighting. The repositioning of the face was done in live using the referent image.

Finally, the self-assessment questionnaire was applied to collect participant's opinions on the acceptability, efficacy and cosmeticity of products.

2.1.6. Statistical analysis

All statistical tests were performed by considering bilateral tests and an alpha = 5% error rate (except for the Shapiro-Wilk test, where the alpha was set to 1%). For continuous parameters that pass normality, all comparisons between treatments at each time point (treatment effects) and all comparisons versus baseline (time effect) for each time point were obtained by using contrasts of least square mean estimations and least square means estimations respectively calculated by the linear mixed models. Benjamini-Hochberg's adjustment was used for multiple comparison of primary criteria.

2.2. *In vivo* RNAseq study

2.2.1. Subjects

A panel of 22 Caucasians subjects was included and randomized, aged between 30 to 60 years old, with phototypes III in Fitzpatrick classification scale and individual typology angles (ITA°) ranging from 28° to 41°. ITA° is derived from the L*a*b* color space. Subjects were requested not to expose themselves under solar or artificial sources during the entire study duration. 4 subjects withdrawn their consent during the study and 18 completed it.

At Day 1 (D1, Baseline), all subjects fulfilling the inclusion criteria were assigned a randomization number (provided by the sponsor via eCRF) in chronological order of inclusion. The study was conducted in Bucharest, Romania, under dermatological control.

2.2.2. Study design and investigated products

To investigate the efficacy of the formulae, 4 areas were delineated on the subjects' backs. The treatment applied to each zone was randomly allocated. Three zones were exposed under UVA1 (20J/cm^2), at D29, D30 and D31. One zone remained untreated and unexposed. Details are as follow:

- zone 1: no application ; no exposure (untreated control)
- zone 2: Vitamin C at 12,6% ; exposure under UVA1
- zone 3: Vitamin C at 12,6% + MCE filter at 2% ; exposure under UVA1
- zone 4: no application; exposure under UVA1

Products were applied at 4mg/cm2, twice daily at site, 5 days a week without any UVA1 exposure from D1 to D28, then at D29, D30 and D31, products were applied before exposure, immediately after UVA1 exposure, and at least 4h after UVA1 exposure.

Exposures to UVA1 (20J/cm^2) were done on the investigational area. They were delivered on the back of the volunteer by a solar simulator (ORIEL 1600 Watt lamp; Oriel Instruments) equipped with a dichroic filter and a WG360 filter (Schott). The spectrum delivered contains UVA1 starting at 340 nm up to 400 nm and residual photons of Visible light up to ~450 nm.

2.2.3. Skin biopsies and Next Generation Sequencing

Skin biopsies (3 mm) were performed by a Dermatologist on the 4 selected investigational zones on the back area at visit D32. Analysis of gene expression profiles were performed using RNA Sequencing after RNA extraction from the biopsies. RNA sequencing library was prepared using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). The sequencing libraries were multiplexed and loaded on the flowcell on the Illumina NovaSeq 6000 instrument according to manufacturer's instructions. Quality of raw reads was assessed; adapter sequences and low-quality reads were trimmed, and sample contamination was assessed. Quantification and summarization

were based on version 41 of the *Homo Sapiens* GENCODE. After filtering, the normalized signals were generated using the package DESeq2.
Gene expression profiles were analysed in the different treatment zones after normalization and statistical analysis of raw data.
Unsupervised hierarchical clustering was first performed to compare global gene expression profiles in the different treatment zones. Differential analyses were then performed between the control zone (1) and the other zones to validate the UVA1 effects under the different treatments. To identify differential gene expression between two conditions, we applied the following criteria: an FDR-adjusted p-value of less than 0.05 and an absolute fold-change greater than 1.5. These different signatures were then analysed comparatively.

2.2.4. Safety

Safety was assessed throughout as adverse event reporting, including local tolerance.

2.3. In vitro 2D

2.3.1. Cell culture and treatments

Normal human dermal fibroblasts (NHDFs) derived from breast surgery of a 28-year-old Caucasian female (Thermo Fisher Scientific, Waltham, MA, USA) were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine solution, 1% sodium pyruvate solution, 1% non-essential amino acid solution, and 0.2% penicillin/streptomycin solution (all from Thermo Fisher Scientific) at 37°C in a humidified atmosphere containing 5% CO_2. 24 hours after seeding, cells were pre-treated, or not, for 24 hours with the following antioxidants: resveratrol (1 µM in DMSO) and vitamin E (1 µM in DMSO), individually or in combination. Antioxidants were added directly to the culture medium (0.1% DMSO final). On days 2, 3, and 4, cells were washed with phosphate-buffered saline (PBS) containing calcium and magnesium and maintained in PBS during UVA exposure. Vitamin C (ascorbic acid, 10 µM in DMSO) was added to the culture medium (0.1% DMSO final) after each UVA exposure and maintained for 60 hours after the final irradiation (Fig. 1A).

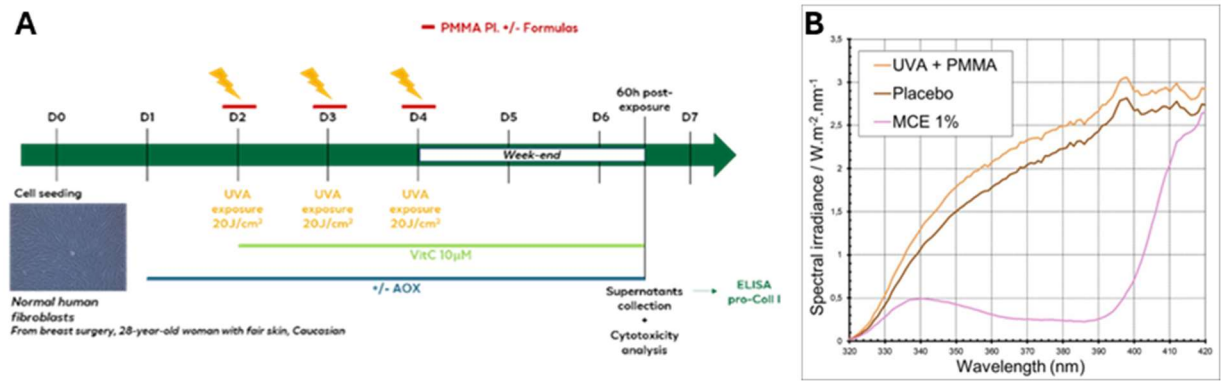

Figure 1. Experimental setup for assessing the impact of vitamin C, antioxidants, and MCE filter on procollagen I production in UVA-exposed NHDFs. (A) Schematic representation of the experimental protocol. (B) Spectral irradiance measured under PMMA plates coated with placebo or 1% MCE formulation during UVA exposure using a solar simulator equipped with a WG335 filter.

2.3.2. UVA exposure and MCE formulas

UVA irradiation (20 J/cm²) was delivered using a solar simulator (ORIEL 1600 Watt lamp, Oriel Instruments) equipped with a WG335 filter. Poly(methyl methacrylate) (PMMA) plates (Helioscreen, Creil, France), coated with 1.3 mg/cm² of either an oleosome placebo or an oleosome formulation containing 1% MCE UVA1 filter, were placed above the cells during UVA exposure.

The spectral irradiance under the PMMA plates was measured using a spectroradiometer (Irradian, Tranent, UK) (Fig. 1B). Control, unexposed cells were maintained in PBS and protected from light during the exposure period.

2.3.3. ELISA and cell viability analysis

60h after the third UVA irradiation, cell culture supernatants were collected for quantification of pro-collagen I secretion by ELISA (Abcam, Cambridge, UK). Concurrently, cell viability was assessed using the XTT colorimetric assay (Roche, Basel, Switzerland). Experimental conditions resulting in XTT levels below 70% of the untreated control (set at 100%) were excluded from analysis to avoid misinterpretation of results due to cell death (data not shown). Following cell lysis, total cell number was quantified using the CyQUANT™ cell proliferation assay (Thermo Fisher Scientific) (Fig. 1A). Pro-collagen I levels in the supernatants were normalized to the total cell number.

2.3.4. Statistical analysis

Normalized mean pro-collagen I content was compared between different experimental conditions using a non-parametric Mann-Whitney test (GraphPad Prism, GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$. All experiments were performed in triplicate for both technical and biological replicates.

3. Results

3.1. Real-life study

We have studied the added value of 1% MCE filter on 12% vitamin C skin benefits in real-life usage.

After only 14 days of use of MCE filter + Vitamin C (group 1), we observed a performance in the skin firmness by clinical scoring while no difference versus baseline for group 2 at this timepoint. Both products showed performance in this parameter after 28 and 84 days of use and after 15-days remanence, with higher deltas in group 1. Moreover, we observed superior efficacy on skin plump (capacity of skin to bounce back “pillow marks”, decreasing with age) measured by Dermotrace after 84 days treatment for group 1 (11%) versus group 2 (7%), and this superiority remains even 15 days after treatment stop. In these two parameters, the formula containing MCE filter and Vitamin C (group 1) demonstrated superior skin benefits and also more responders to treatment than the formula containing vitamin C alone (Fig. 4).

Besides, the participants strongly agree that the skin looks firmer and plumper from the third day of application (88% for group 1 and 81% for group 2) until the end of the treatment (94% for group 1 and 91% for group 2) and even 15 days after the end of the treatment (91% for group 1 and 89% for group 2). Data obtained with the aid of Dermascan ultrasound complement these results (no significant difference between the two groups): an increase in skin density was measured after 28 (6-12%) and 84 (27-33%) days of treatment and even 15 days after the end of treatment.

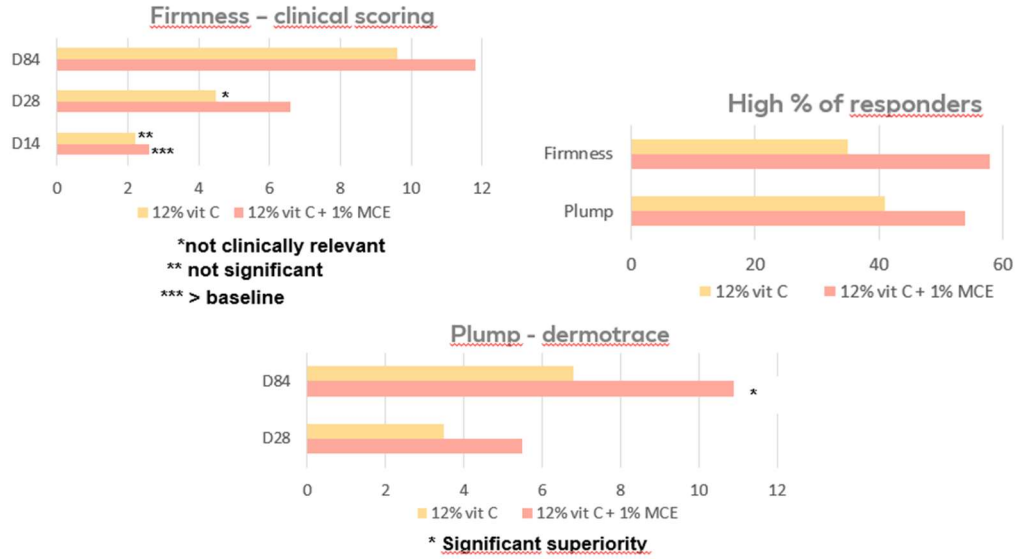

Figure 4: Performance of the treatments containing 12% vitamin C vs 12% vitamin C + 1% MCE filter vs baseline represented by the deltas and % responders for skin firmness and plump attributes.

Each of the treatments (group 1 and group 2) performed on the clinical parameters of radiance, softness, smoothness, dark circles, skin tone evenness, firmness, plump, skin density and fine lines and showed 15 days of remanence. A weaker effect was observed on skin aging signs of ptosis, forehead wrinkles, crow's feet wrinkles, under eyes wrinkles, neck sagging and neck texture. The products were very well appreciated and well tolerated, and the effects perceived by the participants.

3.2. *In vivo* RNAseq study

A clinical study was performed to assess the effect of repeated UVA1 exposure on gene expression, without treatment, after 1 month treatment with Vitamin C or after 1 month treatment with Vitamin C and protection with MCE filter during UV exposure.

Initial unsupervised hierarchical clustering analysis of gene expression profiles revealed a pronounced donor effect, outweighing treatment effects. Considerable inter-sample variability within individual donors, attributed to the presence of skin appendages (notably hair follicles), was also noted. Therefore, the analysis focused on comparative differential gene expression between each treatment condition and the untreated control. The number of significantly modulated genes for each treatment was then quantified.

Following UVA1 exposure, a significant number of genes were significantly modulated. Specifically, 257 genes displayed a fold-change (FC) > 1.5 (adjusted p-value < 0.05), with 67 exhibiting a FC > 2. Interestingly, pre-treatment with 12% Vitamin C reduced the number of significantly modulated genes following UVA1 exposure to 149 with FC > 1.5 (Fig. 2A). This suggests that the Vitamin C formulation confers partial protection against UV-induced skin damage. Moreover, the combined treatment with 2% MCE filter and 12% Vitamin C markedly reduced the number of modulated genes to 39 (85% reduction) with FC > 1.5 (Fig. 2A).

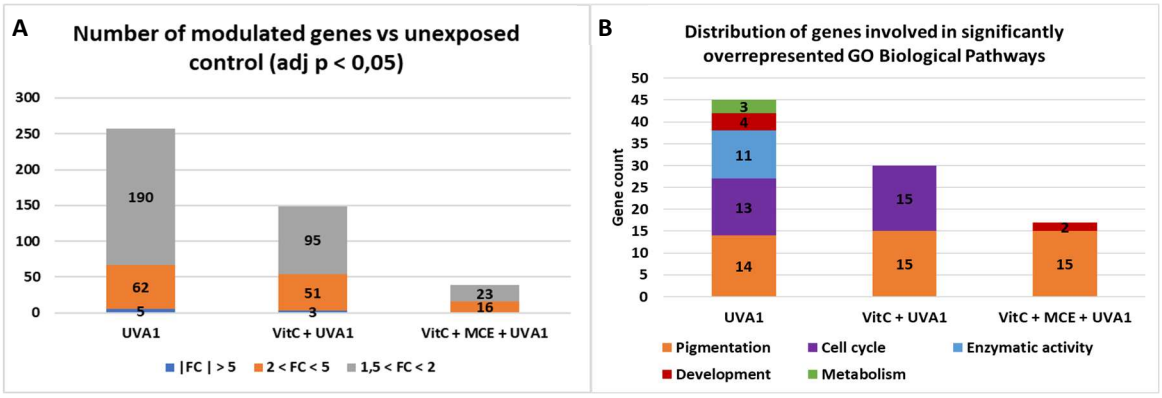

Figure 2. Gene expression modulation and pathway enrichment analysis after *in vivo* repeated UVA1 exposure and topical application of Vitamin C and/or MCE filter. (A) Number of significantly modulated genes (fold-change > 1.5 ; 2 < FC < 5 ; FC > 5 ; adjusted p-value < 0.05) in each treatment group (UVA1, Vitamin C 12% + UVA1, Vitamin C 12% + MCE 2% + UVA1) compared to the untreated control. (B) Distribution of genes involved in significantly enriched GO terms across the same treatment groups.

Subsequent Gene Ontology (GO term) enrichment analysis identified pigmentation, cell cycle, enzymatic activity, tissular and cellular embryo development, and metabolism as the primary biological pathways modulated by UVA1 (Fig. 2B). After vitamin C 12% treatment, metabolic-related GO terms were not enriched anymore, while over-representation of pigmentation and cell cycle pathways was still observed (Fig. 2B). In contrast, after treatment with Vitamin C + MCE filter, only the over-representation of the pigmentation category was still observed, indicating a robust protective effect of MCE against UVA-induced cell cycle alterations (Fig. 2B).

To further elucidate the effects of UVA1 and each formulation, a targeted comparative analysis focused on gene lists involved in extracellular matrix regulation (163 genes), oxidative stress (87 genes), and epidermal proliferation/differentiation regulation (71 genes). The results demonstrated that (i) these gene families were minimally affected by UVA1 24h after the last exposure (3-15% of genes significantly modulated), (ii) Vitamin C 12% treatment afforded partial protection against UVA1-induced modulations, and (iii) the Vitamin C + MCE formulation provided near-complete protection against modulation of these genes (Fig. 3).

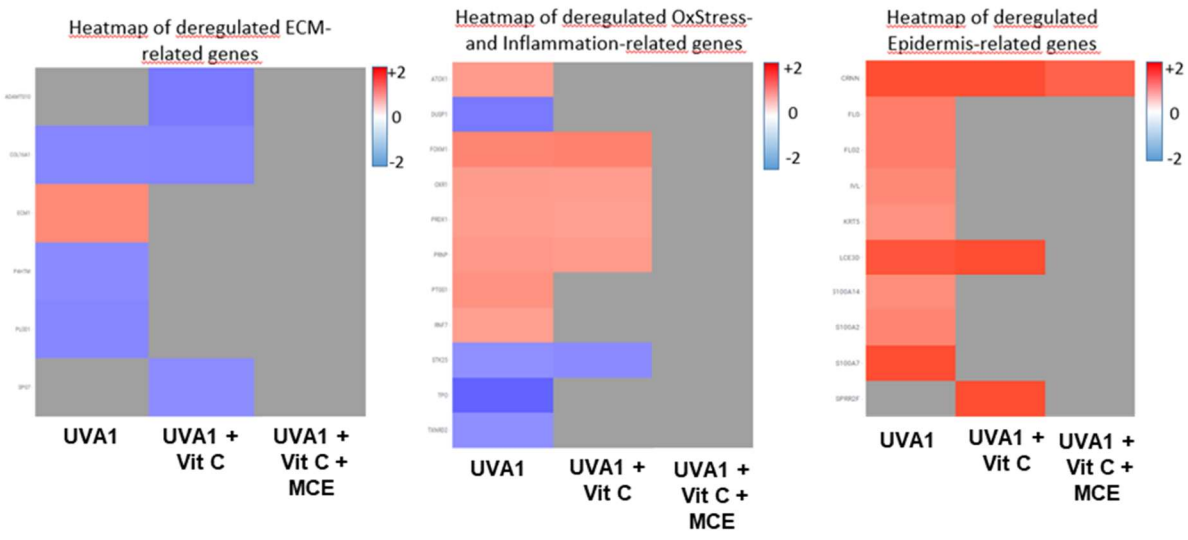

Figure 3. Heatmap visualization of differentially expressed genes after in vivo repeated UVA1 exposure and topical application of Vitamin C and/or MCE filter. Expression levels of selected genes from curated lists related to extracellular matrix regulation (left), oxidative stress (center), and epidermal proliferation/differentiation (right), across the different treatment groups (UVA1, Vitamin C 12% + UVA1, Vitamin C 12% + MCE 2% + UVA1) compared to the untreated control. Increased expression is represented by red, and decreased expression is represented by blue.

3.3. *In vitro* study

We then hypothesized that the protecting effect of MCE on Vitamin C's anti-aging efficacy could be enhanced if combined with antioxidants (such as resveratrol and vitamin E), which would contribute to mitigate UVA induced oxidative stress in deep skin layers. This was tested in vitro on a 2D model by measuring pro-collagen I production, as a marker of skin aging, under repeated UVA exposures. As expected, vitamin C induced a significant increase of 203 % and 191 % in the secretion of pro-collagen I compared to untreated and DMSO-treated controls, respectively (Fig. 5). In contrast, repeated UVA exposure decreased pro-collagen I secretion in DMSO-treated fibroblasts (under PMMA + oleosome placebo) by 38%, validating our protocol as a relevant in vitro model for studying photoaging-associated dermal alterations (Fig. 5). Furthermore, after 3 × 20 J/cm² of UVA exposure, the stimulating effect of vitamin C decreased by 49% compared to the unexposed control treated at the same concentration, demonstrating a photo-induced alteration of the anti-aging efficacy of vitamin C (Fig. 5).

Given the dual functionality of vitamin C as both a promoter of collagen I biosynthesis (essential for anti-aging) and a reactive oxygen species (ROS) quencher, we hypothesized that the increase in ROS following UV exposure could contribute to attenuate its positive effects on collagen synthesis. We therefore treated the vitamin C-stimulated and UVA-exposed cells with antioxidants (endogenous protection) or with the MCE 1% UVA1 filter spread on a PMMA plate (exogenous protection, versus placebo). Under UV exposure, the addition of resveratrol 1 µM, vitamin E 1 µM, or the association of both increased the action of vitamin C alone (up to + 23%) on pro-collagen I production (Fig. 5). MCE filter alone also significantly increased the stimulating effect of vitamin C under repeated UVA exposure. Indeed, the addition of MCE at 1% during UVA exposure increased pro-collagen I secretion by 14% compared to cells exposed to UVA in the presence of the placebo formula (Fig. 5).

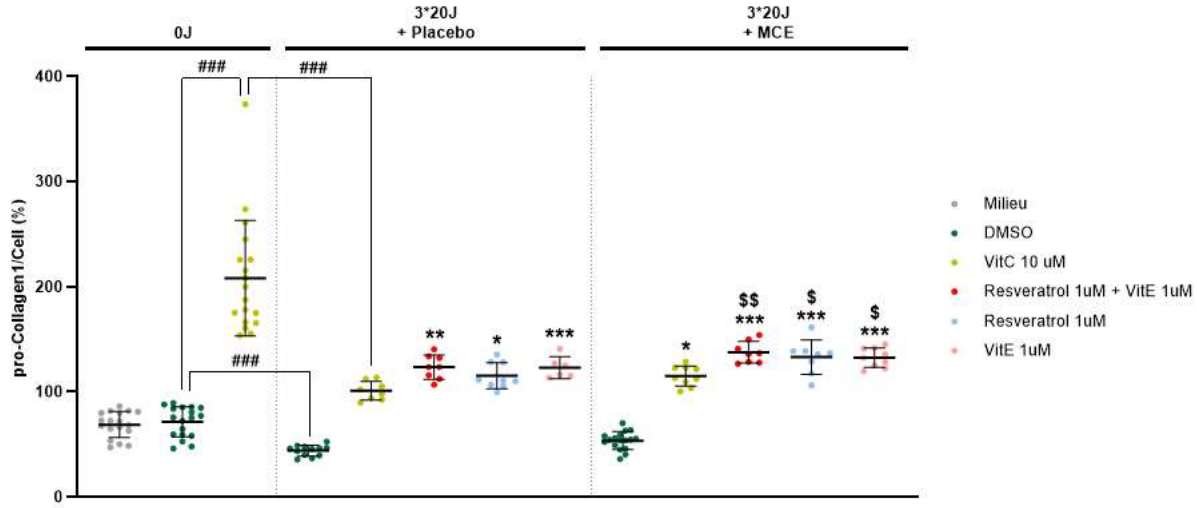

Figure 5. Protective effects of antioxidants and MCE filter on the efficacy of vitamin C in stimulating pro-collagen I production in UV-exposed NHDFs. Quantification of pro-collagen I secretion in NHDF cell supernatants following 4.5-day treatment with Vitamin C (10 µM, 0.1% DMSO) in non-irradiated and UVA-irradiated (3 x 20 J/cm²) cells. The impact of co-treatment with antioxidants (Resveratrol 1 µM +/- Vitamin E 1 µM) or UVA1 filtration with MCE (1% ; vs Placebo) on Vitamin C-mediated stimulation of pro-collagen I production under UV is also shown. Results are presented as percentage change relative to Vitamin C treatment under UV exposure.* significativity vs 3*20J + Placebo + Vitamin C ; $ significativity vs 3*20J + Vitamin C + MCE.

Thus, antioxidants and the MCE filter are able to increase the tissue response to vitamin C after UVA exposure, but it is interesting to note that the MCE filter only partially restored the basal activity of vitamin C. To further boost the anti-aging action of UV-diminished vitamin C, we decided to combine both endogenous and exogenous protection. Interestingly, the combination of resveratrol 1 µM and vitamin E 1 µM, alone or in combination, with the MCE 1% formula showed a significant improvement in vitamin C activity compared with the MCE formula alone (+37% for resveratrol + vitamin E), demonstrating the benefits of this dual protection strategy for preserving the efficacy of anti-aging active ingredients under UV exposure (Fig. 5).

4. Discussion

In real-life usage, the results confirm the anti-aging efficacy of a highly concentrated pure vitamin C treatment, as already shown in previous clinical studies [7]. Interestingly, these benefits were lasting even after the end of the treatment. Moreover, the association of the new UVA1 filter MCE to vitamin C significantly promoted the treatment efficacy on skin plumpness, and in a slighter way firmness, than product containing Vitamin C only. This is coherent with MCE protecting deep skin cells target of vitamin C biological benefit on pro-collagen production previously shown *in vitro* [5] and shows than vitamin C skin benefits of anti-aging products may be enhanced.

Taken together, the *in vivo* RNAseq data confirm the advantage of combining Vitamin C and MCE filter for optimal protection against damage resulting from repeated UVA1 exposure. Although a direct evaluation of the additive effects of Vitamin C and MCE was not feasible due to the limited number of biopsies per volunteer, the results suggest partial protection conferred by Vitamin C, supplemented by important protection from the MCE filter. Although the combined treatment preserved gene families related to extracellular matrix regulation and epidermal proliferation/differentiation, these biological families were not the most represented ones, suggesting that the observed clinical improvements are likely multifaceted and not solely attributable to enhanced collagen production. Besides, the short 3-day UVA1 exposure may have been insufficient to induce detectable long-term dermal alterations. Pathways related to cell cycle and enzymatic activity may also contribute to the overall improvement in skin firmness and offer promising avenues for further mechanistic investigation.

Going further, our new *in vitro* results revealed that antioxidants alone could enhance vitamin C-induced pro-collagen I- secretion in a context of UV exposure, underscoring the critical role of ROS in the underlying mechanism. This is further supported by our *in vivo* data showing modulation of oxidative stress-related genes under UVA1, that were preserved by the

combined MCE + Vitamin C intervention. Interestingly, vitamin C alone partially protected against UVA1-induced gene modulations probably thanks to its antioxidant effect.

Importantly, combining MCE and antioxidants *in vitro* provided the most robust protection of vitamin C efficacy on pro-collagen I production, indicating the benefits of a combined exogenous and endogenous protection strategy. However, even this combined approach did not fully restore vitamin C's efficacy to pre-UVA exposure levels, suggesting other factors, such as the contribution of shorter UVA wavelengths and their associated biological effects (e.g., DNA damage, inflammation), may also play a role and warrant further investigation. This observation suggests nevertheless that combining MCE, vitamin C, and antioxidants *in vivo* may yield even greater benefits than those observed with MCE and vitamin C alone, representing a promising direction for future research and the development of more effective anti-aging strategies.

5. Conclusion

We showed for the first time that the association of the new UVA1 filter MCE with vitamin C was able to significantly improve the skin benefits of pure Vitamin C in real-life usage. This superiority, shown on skin firmness attributes, is coherent with the *in vitro* results that were obtained on biomarkers linked to collagen production enhancement with the association of MCE and vitamin C [5]. Besides, the association markedly reduced the number of skin genes expression modulated *in vivo* under chronic UVA1 exposure (-85%). These boosting effects could be pushed further adding antioxidants to MCE filter, as shown *in vitro* using vitamin C as anti-aging active. In the future, other biological active than vitamin C & other biological endpoints than pro-collagen could be considered to prove UVA1 protection added value on skin benefits.

These results highlight the interest of a new generation of anti-photoaging products, associating a strong UVA1 photoprotection to efficient anti-aging compounds, to provide enhanced skin benefits.

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