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"Novel combination of antioxidants for key skin exposome regulation pathways: in vitro, ex vivo and in vivo studies"

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1. Introduction

Skin aging is a multifactorial biological process influenced by both intrinsic and extrinsic factors. While intrinsic aging is largely governed by genetic and chronological influences, extrinsic aging—commonly referred to as photoaging—is predominantly driven by environmental and lifestyle elements such as ultraviolet (UV) radiation, visible light (notably high-energy visible or blue light), air pollution, poor nutrition, and psychological stress [1]. These stressors accelerate skin aging through various cellular and molecular mechanisms, primarily involving oxidative stress and the formation of advanced glycation end products (AGEs). Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the skin's antioxidant defense, leading to DNA damage, lipid peroxidation, and degradation of dermal proteins such as collagen and elastin [2]. Similarly, AGEs—formed through non-enzymatic glycation of proteins and lipids—alter the extracellular matrix and promote chronic inflammation, further contributing to skin aging and loss of elasticity [3].

Blue light, a component of the visible light spectrum, has gained attention for its ability to penetrate the skin and induce ROS formation, promoting hyperpigmentation and mitochondrial damage [4]. Environmental pollutants such as particulate matter (PM) and ozone can also exacerbate ROS production and inflammatory responses in the skin [5]. Additionally, modern diets high in sugars and fats can enhance glycation processes, while chronic psychological stress disrupts skin barrier function and increases inflammatory cytokines [6].

In response to these aging mechanisms, numerous strategies have been developed to prevent and treat photoaged skin, with antioxidant therapy being a central approach. Topical application of vitamin C (ascorbic acid) is one of the most widely studied and used methods due to its potent antioxidant properties, its role in collagen synthesis, and its ability to inhibit melanogenesis [7]. However, vitamin C is highly unstable in aqueous formulations, easily degraded by

light, heat, and oxygen, which significantly limits its efficacy, shelf life and use at high concentrations [8].

To overcome these limitations, current research focuses on developing novel formulations that either stabilize vitamin C or combine it with other antioxidants to exert synergistic effects that maintain or enhance vitamin C anti-aging effects [9]. The advancement of such multifunctional delivery systems is critical to fully harness the therapeutic potential of vitamin C in skin aging interventions.

Here, we studied the *in vitro* skin exposome protection of vitamin C plus ferulic acid, glutathione and astaxanthin against blue light, AGEs and pollution damage. Based on the promising results of the combination, we carried out *ex vivo* and *in vivo* studies to study the effect on epidermal and dermal skin aging markers.

2. Materials and Methods

Human dermal fibroblasts o human epidermal keratinocytes were treated with different stimuli (blue light, AGEs or urban dust) plus the different combinations of compounds for 24h. Concentrations used for the compounds were 1 mg/mL, 100 µg/mL, 1 µg/mL and 0.5 mg/mL for vitamin C, ferulic acid, astaxanthin and glutathione, respectively. Gene expression of skin aging markers was quantified by qPCR. A serum containing the actives was applied to a 47-year old female skin explant and skin proteins were quantified by immunofluorescence after 3 days. The same serum was applied once daily for 30 days to 25 volunteers. Skin brightening and firmness were measured using Glossymeter® and Cutometer®, respectively.

3. Results

First, the effect of the combination of compounds on different skin exposome damages in dermal fibroblasts and epidermal keratinocytes was studied through gene expression analysis. Table 1 shows that the compounds upregulated *COL1A1* and *GDF11* while downregulated the expression of several matrix metalloproteinases (MMPs) such as *MMP1, MMP3* and *MMP10* in blue light irradiated fibroblasts.

Table 1. Gene expression changes in blue light exposed (1h irradiation) fibroblasts treated or not with the combination of active ingredients. T-student analysis was performed to evaluate significant differences between the conditions, comparing control vs irradiated cells and irradiated cells vs irradiated + treated cells. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

	Control	Irradiated	Irradiated + Treatment
COL1A1	1.00	0.72 (±0.06)**	1.31 (±0.02)**
GDF11	1.00	1.29 (±0.11)*	1.73 (±0.1)**
MMP-1	1.00	2.44 (±0.09)***	2.06 (±0.21)*
MMP-3	1.00	2.29 (±0.27)**	1.33 (±0.11)**
MMP-10	1.00	2.03 (±0.11)**	1.03 (±0.04)**

Table 2 shows that the compounds also exerted positive effects on blue light irradiated keratinocytes. The combination of compounds upregulated *CD44, GDF11* and *HAL*, while it downregulated *GDF15*. The expression of MMP1 remain unaffected after the treatment.

Table 2. Gene expression changes in blue light exposed (1h irradiation) keratinocytes treated or not with the combination of active ingredients. T-student analysis was performed to evaluate significant differences between the conditions, comparing control vs irradiated cells and irradiated cells vs irradiated + treated cells. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

	Control	Irradiated	Irradiated + Treatment
CD44	1.00	1.56 (±0.18)*	3.78 (±0.51)**
GDF11	1.00	1.17 (±0.19)	2.00 (±0.49)
HAL	1.00	0.90 (±0.10)	2.06 (±0.30)**
MMP1	1.00	0.88 (±0.17)	1.11 (±0.15)
GDF15	1.00	0.94 (±0.09)	0.63 (±0.09)*

Table 3 shows the effect of the compounds con urban dust-damaged keratinocytes. Here, the compounds downregulated the levels of proinflammatory markers such as *COX1, COX2*, *MCP1* and *TNFa*.

Table 3. Gene expression changes in urban dust (NIST1649b, 200 µg/mL) incubated keratinocytes treated or not with the combination of active ingredients. T-student analysis was performed to evaluate significant differences between the conditions, comparing control vs urban dust incubated cells and urban dust incubated cells vs urban dust incubated + treated cells. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

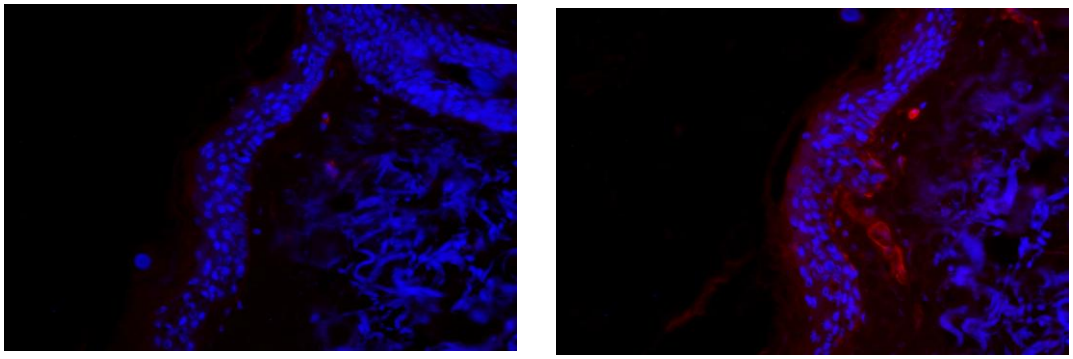
	Control	Urban dust	Urban dust + treatment
COX1	1.00	1.06 (±0.14)	0.20 (±0.06)**
COX2	1.00	11.68 (±0.46)***	9.46 (±0.96)*
MCP1	1.00	8.63 (±0.17)***	3.16 (±0.19)***
TNFa	1.00	1.17 (±0.06)	0.73 (±0.06)**

Table 4 shows the effect of the compounds on AGE-damaged fibroblasts. The compounds upregulated extracellular matrix proteins and growth factors such as *COL1A1, ELN* and *CTGF*, while it downregulated proinflammatory and tissue damaging factors such as *MCP1, COX1, MMP1, MMP3* and *TNFa*.

Table 4. Gene expression changes in AGEs (bovine serum albumin + glyceraldehyde 0.1 mg/mL) incubated fibroblasts treated or not with the combination of active ingredients. T-student analysis was performed to evaluate significant differences between the conditions, comparing control vs AGEs incubated cells and AGEs incubated cells vs AGEs incubated + treated cells. * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

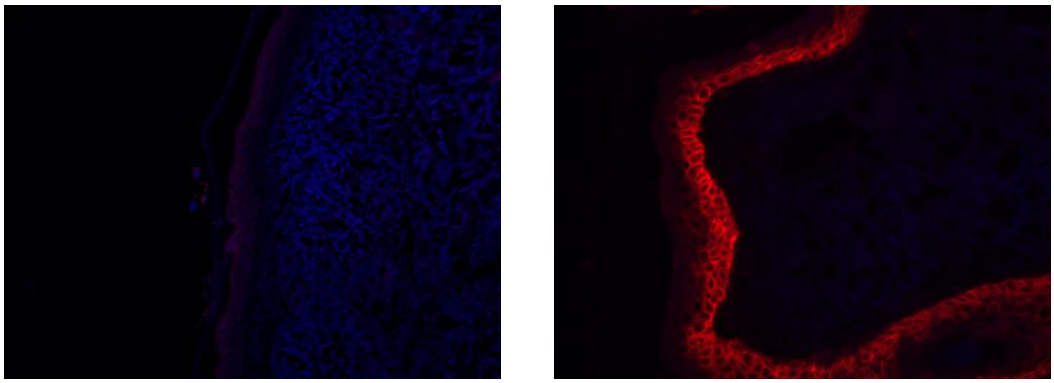
	Control	AGEs	AGEs + treatment
COL1A1	1.00	0.77 (±0.06)*	1.33 (±0.06)**
ELN	1.00	0.40 (±0.02)***	1.29 (±0.09)**
CTGF	1.00	0.71 (±0.03)**	1.65 (±0.07)***
COX1	1.00	3.21 (±0.15)**	1.17 (0.10)***
MCP1	1.00	1.56 (±0.09)**	0.28 (±0.02)**
MMP1	1.00	5.32 (±0.13)***	0.87 (±0.08)***
MMP3	1.00	6.60 (±0.39)**	0.37 (±0.03)**
TNFa	1.00	1.21 (±0.06)**	0.82 (0.11)*

Based on these in vitro positive results, a serum including the 4 compounds was formulated (15% ascorbic acid, 0.5% ferulic acid, 0.5% glutathione and 0.1% astaxanthin) and applied to a skin explant and several skin markers were studied by immunofluorescence. As observed in Figure 1 and Figure 2, the serum increased the levels of collagen I and cytokeratin 14 in skin after 3 days of treatment.



(a) **(b)**

Figure 1. Collagen I immunostaining in skin explants after 3 days of treatment with (b) or without (a) the application of a serum containg vitamin C, ferulic acid, astaxanthin and glutathione.



(a) (b)

Figure 2. Cytokeratin 14 immunostaining in skin explants after 3 days of treatment with (b) or without (a) the application of a serum containg vitamin C, ferulic acid, astaxanthin and glutathione.

Finally, the serum was applied daily for 30 days to 25 volunteers and skin quality was studied using Glossymeter and Cutometer. After the treatment, the serum increased 15% and 26% skin brightness righ after application and 30 days after daily use, respectively, and 13% skin firmness in volunteers (Table 5).

Table 5. Skin brightness and firmness improvement after the topical application of the serum. D0 representes day 0 (before application), D0 after product use representes day 0 right after product application, and D30 representes day 30 after daily use of the product.

	D0	D0 after product use	D30
Skin brightening after application	-	15%	-
Skin brightening after 30 days	-	-	26%
Skin firmness after 30 days	-	-	13%

4. Discussion

Topical formulations that incorporate elevated concentrations of vitamin C are often regarded as the optimal strategy for addressing the manifestations of skin aging, attributable to the vitamin's antioxidant properties and its role in collagen synthesis [10]. Nevertheless, the inherent instability of vitamin C within cosmetic formulations poses significant challenges to its application and effectiveness in the treatment of skin photoaging. Various methodologies have been proposed to mitigate these challenges, including encapsulation techniques, the utilization of vitamin C derivatives, or the synergistic combination with alternative antioxidants. In this study, we opted for the latter approach and postulated that an innovative combination of vitamin C, ferulic acid, astaxanthin, and glutathione represents a promising strategy for addressing the deleterious effects of the skin exposome.

The combination of the four compounds demonstrated protective effects against a variety of external stimuli (such as blue light, environmental pollution, and advanced glycation end products) administered to skin cells *in vitro*. Notably, our findings indicated that these compounds facilitated a positive modulation of the TGF-b signaling pathway in fibroblasts subjected to blue light irradiation, including the upregulation of *GDF11*, which has been previously documented to possess significant skin rejuvenation properties [11]. Besides, the upregulation of COL1A1 and downregulation of MMPs could be also attributed to an enhance activation of TGF-b pathway. Regarding blue light-damaged keratinocytes, the compounds upregulated the hyaluronic acid receptor (*CD44*), *GDF11* and the photoprotection factor *HAL*, which indicates tha these compounds are inducing the restoration of the epidermal function in damaged keratinocytes [12].

As expected, urban dust damage increased proinflammatory markers in keratinocytes (Table 3). Interestingly, the compounds downregulated the levels of these proinflammatory cytokines, including COX1, COX2, MCP1, TNFa. This effect could be explained by the already described anti-inflammatory properties of some of the selected compounds [2]. Finally, AGE-induced alteration in proinflammatory cytokines and extracellular matrix proteins was reversed by the compounds. This result confirms the antiinflamatory and restructuring effect on extracellular matrix of the compounds in a different context.

As far as we are concerned, none of the compounds, when considered independently, have been documented to exert an influence on GDF11, HAL, or CTGF levels within the dermal layer; consequently, our investigation sheds more light on the existing understanding of the mechanisms underlying the action of these compounds. Subsequent inquiries aimed at elucidating the specific contributions of each ingredient to these biological pathways are warranted.

The experiments on skin explants proved that the formula including the compounds can both stimulate epidermis (through the upregulation of cytokeratin 14) and dermis (through the upregulation of collagen I) compartments. Cytokeratin 14 is commonly expressed in proliferating keratinocytes [13], and thus the increase in this marker by the compounds supports our previous hypothesis of enhanced epidermal regeneration. Although topical vitamin C combinations have shown beneficial effects when applied to the skin, previous research has shown that vitamin c concentration can be critical. Interestingly, topical formulations including high doses fo vitamin C combined with ferulic acid reduce extracelular matrix components and exert a negative impact on skin ex vivo models [14]. As our combination included the same concentrations of vitamin C and ferulic acid plus the addition of glutathione and astaxantin, the observed increase in collagen I levels in our ex vivo model prove that the combination of the four compounds might be more beneficial than using vitamin C plus ferulic acid alone. The previous observations on the effect of these compounds on the TGF-b pathway (*GDF11, CTGF, COL1A1*) could explain this upregulation of collagen I levels in the treated skin explants.

Clinical studies have shown that high concentrations (more than 20%) of vitamin C do not exert better efficacy and can even have adverse effects such as mild irritation [10]. Besides, 20% vitamin C formulation has not demonstrated superior efficacy than formulations containing 15% vitamin C [14]. Thus, up to 15% vitamin C formulations combined with other compounds that can complement its skin protection and rejuventation are a preferred approach for the new topical products that are under development. Here, our proposed combination of vitamin C, ferulic acid, glutathione and astaxanthin shows a significant positive changes in skin brightness and firmness after 28 days of daily topical application, thus proving to be an suitable formula that can include vitamin C at as high as 15% for skin external damage protection.

5. Conclusion

Our investigation demonstrated that the positive interaction of vitamin C, ferulic acid, astaxanthin, and glutathione can proficiently address various forms of damage induced by the skin exposome *in vitro*. These observed effects were replicated in *ex vivo* and *in vivo* models, wherein the serum formulated with these compounds promoted epidermal cell proliferation, enhanced dermal collagen synthesis, and improved skin luminosity and elasticity..

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