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ORIGINAL ARTICLE

Antioxidants with proven efficacy and elastin-conserving vitamin C—A new approach to free radical defense

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Abstract

Background: This paper describes the background research and validation related to the formulation of a novel antioxidant product. Two defined outcomes were sought. Firstly, a combined efficacy of antioxidant ingredients in quenching free oxygen radicals. Secondly, the investigation into whether a vitamin C derivative sodium salt was elastin conserving in contrast to current vitamin C/L-ascorbic acid variations that have been reported to negatively affect elastin constitution and regeneration.

Materials and Methods: A leading L-ascorbic acid antioxidant available on the market was compared with the experimental new product in two studies. In the first experiment, the products were compared to assess their antioxidant properties. The evaluated products TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 were applied to human skin cultures (25–30 mg/cm^2) for a total of 72 h of treatment and exposed to oxidative stress. The generation of free radicals was semi-quantitatively assessed by measuring the fluorescence intensity of the deacetylation and oxidation of the probe dichlorofluorescein diacetate (DCFH-DA). In the second experiment, an ex vivo skin model (derived from patients undergoing facelift procedures) was used to assess elastin preservation. Three skin explants were topically subjected to the two formulations daily for 7 days. The skin was then prepared and fixed for immunofluorescent assessment after staining with CD44 and tropoelastin antibodies. Images were then analyzed using ImageJ.

Results: A full description of the different components selected for the new formulation is presented. In the first study, the experimental formulation performed with absolute equivalence to the comparator in its radical quenching capacity; both showed extremely effective antioxidant function. In the second study, the comparator negatively affected the existing elastin with areas of breakdown and diminished staining. In contrast, the new formulation showed good conservation of healthy elastin in all sections demonstrating elastin preservation.

Conclusion: A new antioxidant formulation was carefully designed with multiple actives that show an equivalent antioxidant capacity to a leading product on the market. More importantly, the vitamin C component shows direct elastin conservation

and improvement as opposed to the comparator, which had negative effects on elastin preservation. This is in keeping with little-known literature reports on vitamin C and its negative effects on elastin and validates the use of a sodium salt derivative, which appears to have protective effects on elastin. These findings support the overall regenerative extracellular matrix changes seen with TriHex® technology in other products.

KEYWORDS
antioxidant, elastin-conserving, reactive oxygen species, Vit C salt

1 | BACKGROUND

A potent stimulation of reactive oxygen species (ROS) levels in the body results in "oxidative stress", a major contributor to the pathogenic processes of many diseases. The skin is constantly exposed to ultraviolet (UV) radiation producing ROS and necessitating the production of antioxidants by the outer stratum corneum layer in an attempt to neutralize these ROS. This protective mechanism consists of lipophilic and hydrophilic low molecular weight antioxidants like carotenoids, vitamins (A, C, D, and E), enzymes (superoxide dismutase, catalase, and glutathione peroxidase), and other compounds (melanin, flavonoids, lipoic acid, selenium, and coenzyme Q10).[1] These agents work synergistically to protect one another from direct degradation while collectively neutralizing the ROS. However, with time, aging, and external stresses, the antioxidants are depleted, and the skin loses this important protective system.[1] Thus, there is a need for topical antioxidant replacements. These agents are derived from multiple sources and are essentially defined by three principal properties.[2]

1. Hydrophilic antioxidants:

- Vitamin C-like compounds and vitamin B derivatives (and the phenolic nature of polyphenols makes them relatively hydrophilic) mix with and protect the water-containing portions of cells, both within and outside of the cells.

2. Enzymatic antioxidants:

- Superoxide dismutase, catalase, glutathione peroxidase, and ubiquinone are proteins involved in the catalytic transformation of ROS and their by-products into stable nontoxic molecules against oxidative stress-induced cell damage supporting the body's internal defense system. The enzymatic antioxidants may also include organic or inorganic compounds that accelerate or facilitate chemical reactions by acting as catalysts or support the function of catalysts.

3. Hydrophobic (lipophilic) antioxidants:

- Vitamin E-like compounds are soluble in lipids, protecting the lipid-rich cell components, such as the cell membrane.

Using a combination of products with these varying properties, comprehensive skin protection can be achieved but certain chemical and structural nuances gleaned from advances in science are necessary to ensure added benefits when considering an advanced antioxidant formulation. Thus, a formulation was designed with actives that fall into these three basic categories. The actives are listed in Table 1 with their modes of action, but special consideration is given to some components with particular reference to new scientific knowledge.

The first area of consideration is the constituent that most physicians think about first when considering antioxidants, which is vitamin C (Vit C). Vitamin C is a potent antioxidant that neutralizes and removes oxidants after exposure to ultraviolet radiation. This activity appears to be of particular importance in the epidermis, where vitamin C is concentrated in the skin.[3]

Vitamin C uptake from the plasma and transport across the skin layers is mediated by specific sodium-dependent vitamin C transporters (SVCTs) that are present throughout the body. Sodium-dependent vitamin C transporter 1 is primarily responsible for the transport of epidermal vitamin C, while SVCT2 is responsible for intradermal transport.[3] Sodium-dependent vitamin C transporter 2 in dermal cells (such as fibroblasts) diffuse ascorbic acid transported from the plasma into the epidermis, and SVCT1 in the epidermis supplies ascorbic acid to keratinocytes.[3] The content of ascorbic acid in the epidermis is 425% higher than the content in the dermis, and there is a concentration gradient of ascorbic acid in the epidermal keratinocytes.[4] For topical application, the stabilization of Vit C from exposure to light and oxidation, conversion to ascorbic acid, and the challenge of skin penetration are paramount. In addition, UV light depletes Vit C content in the epidermis.[3] Thus, very few formulations are actually effective in topical application, and stabilization is therefore critical.[5]

A little appreciated fact is that Vit C, a known enhancer of collagen deposition, has also been identified as an inhibitor of elastogenesis, and it has been suggested that ascorbic acid destabilizes tropoelastin mRNA and causes excess hydroxylation on prolyl/lysyl residues of tropoelastin molecules, thereby promoting their intracellular accumulation and inhibiting their secretion.[6] However, sodium ascorbate (SA), a Vit C sodium salt, has been demonstrated to stimulate the production of both collagen and elastic fibers in all tested cultures with investigators endorsing the use of this potent stimulator of collagen and elastin production in the treatment

TABLE 1 Constituent actives of antioxidant formulation.

Antioxidant active	Mechanism of action
Hydrophilic	
Sodium ascorbate	Potent anti-oxidant, stimulating both collagen production and elastin preservation.[6] (See text)
Lactoferrin	Plasmin inhibitor and iron chelator – iron accumulates in post-menopausal women, as menstruation no longer occurs. Iron is stored in ferritin in the skin and when released, it catalyzes the creation of free radicals. By targeting excess iron, it is possible to prevent the formation of free radicals.[7] The oxidative damage created by excess iron and the promotion of ROS can result in skin aging and the increased stress may make skin more susceptible to UV damage.[7]
Ergothioneine	Naturally occurring amino acid, derivative of histidine found mainly in mushrooms. Protects skin cells from UV irradiation, partly through its UV-absorbing properties, but also by raising endogenous antioxidant levels in keratinocytes[8] and protecting against UV-induced mitochondrial DNA damage.[9]
Green tea	Antioxidant with highest concentration of polyphenols compared to other teas[10]—acts by scavenging ROS or indirectly by upregulating antioxidant enzymes.[10]
Oleuropein	The most abundant phenolic found in olive leaves and oil and has been shown to exhibit antioxidant and free radical scavenging activities.[11]
Pomegranate	Photoprotective, antioxidant, anti-inflammatory, and antiproliferative compound. The fruit extract has been shown to reduce UVB-induced oxidative stress, improving skin color, and restoring skin glow.[12]
Ectoine	A 'natural extremolyte' produced from several species of microorganisms under stressful conditions that significantly downregulates UVA-radiation-induced ROS production in keratinocytes.[13]
Centella asiatica	High antioxidant activity with high free radical scavenging activity[14] and increased levels of antioxidants (enzymatic and nonenzymatic) have been well defined.[15]
Carnosine	Hydrophilic dipeptide is a component of several tissues with pH buffering and excellent antioxidant properties due to metal ion chelation and scavenging reactive oxygen species (ROS) and peroxyl radicals.[16]
Enzymatic	
Ubiquinone (coenzyme Q10)	Endogenously synthesized lipid-soluble antioxidant involved in cellular energy production. It decreases with age and with external stress factors in the skin.[17] A combination of CoQ10 and carotenoids has been shown to work synergistically producing much greater inhibition of PGE-2 production than with either compound alone.[18]
Betaine	Known as trimethylglycine, widely distributed in animals, plants, and microorganisms. Sulfur amino acids, such as homocysteine and methionine, are involved in GSH synthesis.[19] Betaine stimulates homocysteine to form methionine, which plays an important role in antioxidation.[19]
Tremella fuciformis	Popular nutritious mushroom in China that has demonstrated enhanced activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), thus strengthening enzymatic defenses and antioxidant effects.[20]
Lipophilic	
Bisabolol	Derived from German chamomile and has potent antioxidant effects, protecting the skin from free radical damage.[21] It improves the visible signs of aging, has been used to lighten skin, and may also have antimicrobial properties.[21]
Phytoene and phytofluene	Colorless carotenoids found naturally in tomatoes and other vegetables and can be sourced in rich supply from saltwater microalgae. They absorb UV radiation and act as antioxidants, anti-inflammatory, and anticarcinogenic agents.[22]
Physalis angulata	An exotic fruit native to the Amazon region that exhibits an antioxidant activity that is substantially higher than other fruits traditionally regarded as antioxidants, such as papaya, pineapple, and plum, providing major benefits to the skin.[23]

of wrinkled and stretch-marked skin.[6] In vitro studies involving cultured fibroblasts demonstrate that SA induces insulin-like growth factor 1 receptor (IGF-1R) activation, which is always followed by an upregulation of the consecutive stages of elastogenesis.[24] This important differentiator of the sodium salt of Vit C provides a new approach to antioxidant protection that takes advantage of the antioxidant effect of Vit C with an added protective and stimulatory effect on elastin formation, a crucial element in skin health maintenance and rejuvenation. There are currently multiple potent antioxidants on the market that compete and in some cases surpass the activity of Vit C, but there are scant Vit C formulations that claim conservation of elastin. Thus, combining a multitude of well-investigated antioxidants together with a Vit C elastin-conserving component is a new strategy.

Antioxidant enzymes can be used to protect the skin, but the instability of enzymes makes them hard to formulate.[25] The skin's intrinsic enzymes can be rapidly overwhelmed by excessive sun exposure. To counteract this impact, we can either stimulate or protect the existing enzymes or supply the lacking enzymes by a topical application. An alternative solution to supplying the enzymes themselves is to use antioxidant enzymes originating from organisms that live and thrive under extreme conditions of heat, namely "extremophiles".

These bacteria live close to the hydrothermal vents at the bottom of the ocean at extremely high temperatures (Table 1).[25]

Delivery:

- Different types of antioxidant compounds have been topically administered to the skin, such as carotenoids, coenzyme Q10, essential oils, polyphenols, and vitamins. Since only a few active ingredients are effective after topical application due to the barrier function of the skin, lipid nanoparticles have been explored as carriers to enhance the bioavailability, skin penetration, and retention of the active ingredients.[1]
- Liposomes have been used as carriers for different applications in dermatology and cosmetics, particularly related to Vit C.[26] Due to its instability and low skin penetration, ascorbic acid needs an appropriate carrier to obtain desirable efficacy. A unique sodium ascorbate phosphatidylcholine (PC) liposome was developed to overcome the barrier of the stratum corneum and deliver the active agent into the dermis to prevent photodamage. The delivery system allows up to 12 times the amount of the sodium ascorbate to reach the interior of the skin without undergoing molecular structure changes ensuring functionality at the point of delivery.[27]

2 | VALIDATION TESTING

2.1 | Antioxidant activity

The antioxidant efficiency studies were conducted at Kosmoscience Technologies (Brazil). Two products were compared: antioxidant 1 (New formulation—AX-1) and antioxidant 2 (leading antioxidant on the market—AX-2). AX-2 ingredients include water, ethoxydiglycol, L-ascorbic acid, propylene glycol, glycerin, laureth-23, alpha tocopherol, phenoxyethanol, triethanolamine, ferulic acid, panthenol, sodium hyaluronate. In effect, the difference between the two products are as follows: AX-1, new formulation has fourteen active antioxidant constituents plus the sodium ascorbate variation of Vit C (as listed in Table 1); AX-2 has the L-ascorbate variation of Vit C, alpha tocopherol (Vit E) and ferulic acid as its actives. Thus the two products are much differentiated. The aim was to evaluate their protective effect against free radicals by semi-quantitatively measuring reactive oxygen (ROS) intermediates using the dichlorofluorescein diacetate (DCFH-DA) probe, which readily crosses the cell membrane through passive diffusion and undergoes deacetylation, producing an oxidant sensitive 2′,7′-dichlorofluorescein (DCHF). In the presence of ROS, DCHF is oxidized to form the highly fluorescent dichlorofluorescein (DCF). The skin fragments used in this study came from healthy female individuals who underwent elective plastic surgery in the abdominal region. (The use of human skin fragments from elective surgeries for this study was submitted to the Research Ethics Committee of Universidade São Francisco – SP, CAAE 56005722.8.0000.5514, under the opinion 5.503.565.) The skin fragments were fractionated into pieces (1.5 cm^2), packed in culture plates with specific culture medium, and kept in an incubator at 37°C in the presence of 5% CO_2. They were treated once with 25–30 mg/cm^2 of the evaluated products.

After 48 h of incubation with evaluated product TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2, the skin specimens were subjected to a dose of 13 J/cm^2 of UV radiation, using the devices UVA Cube 400, SOL 500 H1 filter, and UV Meter (Honle UV America Inc.). The skin fragments were then again exposed to products TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 and maintained in culture condition for another 24 h.

After the treatment period, the skin fragments were embedded in Tissue-Tek® O.C.T.™ and then serial histological sections of 12 µm were collected directly on slides silanized with a cryostat (GER—CRYOCUT 1800; Leica). Subsequently, the sections were washed with phosphate buffer (PB) and incubated for 1 min with a solution (1:10000 in PB) of DCFH-DA (Sigma).

Immediately after this incubation, the slides were mounted using specific mounting media and analyzed under the microscope (JAP—BX53; OLYMPUS) using the CellSens software (©2010 OLYMPUS CORPORATION). The fluorescence intensity emitted by the oxidation of the DCFH-DA probe was evaluated. After obtaining the images, the fluorescence intensity was quantified using the ImageJ software (version 1.48, Arbitrary Units—A.U.).

A semi-quantification of free radical (FR) synthesis using DCFH-DA was done using an ANOVA, which also allowed for measuring the variation of the results and comparing the data between the groups. Then, Bonferroni's posthoc test was applied. A significance level of 5% was used in both evaluations (GraphPad Prism v6).

2.2 | Assessment of Elastin Conservation

The following studies were conducted by 3D Genomics (Carlsbad, CA) as an independent laboratory investigation. The new antioxidant (AX-1) was compared to a leading antioxidant comparator (containing L-ascorbic acid) (AX-2) in an ex vivo skin model. Initial skin culture and histology preparation involved photodamaged skin derived from patients undergoing facelift procedures (study approved under Veritas Institutional Review Board—study ID # 3192). Discarded skin was received within 2 h of removal from patients. All skin processing was conducted under BSL2 laboratory conditions. The skin was washed in PBS and defatted if necessary. Any visible hairs were shaved using a scalpel. The skin was then cut into ~5 mm × 5 mm to ~8 mm × 8 mm square pieces and placed into transwells suspended in 12 well plates.

A total of 1.0 mL of Skin Media (composed of DMEM/Ham's F-12 50/50 mix, adenine hydrochloride hydrate, calcium chloride dihydrate, T3 tri-iodothyronine, insulin-transferrin-selenium-ethanolamine (ITS-X), penicillin/streptomycin fetal bovine serum (FBS), glutaGRO,

and gentamicin sulfate) was added to each well, and about 200–300 μL was added to each transwell to surround the skin sample while maintaining an air-exposed epidermal surface. Media was changed daily. Three replicates were treated with AX-1 or AX-2 (L-ascorbic) or left untreated. Formulations were applied every 24 h for 7 days, with media changes. The skin was then washed, fixed, and prepared for formalin fixation and paraffin embedding following standard procedures. Sections were cut and prepared for staining with tropoelastin and CD44 primary antibodies followed by fluorescent-conjugated secondary antibodies. DAPI was used to identify the nuclei. Multiple images were captured by immunofluorescence microscopy (Zeiss) to obtain a stitched image of the entire section. The intensity of tropoelastin and CD44 was measured using ImageJ. The color channels were split, and the intensity of the red and yellow in the dermal-epidermal junction (DEJ) was measured across the entire section. The average values were assessed by a student's *t*-test and $p < 0.05$ was considered significant.

3 | RESULTS

Figure 1 represents the antioxidant effects of the evaluated products TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 in human skin cultures subjected to UV radiation. As expected, UV exposure increased the green fluorescent signal by 141.85% ($p < 0.001$; Figures 1 and 2) when compared to the unexposed control, thus revealing the induction of oxidative stress and FRs. Treatment with the evaluated product TOPICAL ANTIOXIDANT 1 decreased the green fluorescent signal (FR detection) by 74.06% ($p < 0.001$) compared with the UV group. Likewise, the evaluated product TOPICAL ANTIOXIDANT 2 decrease the green fluorescent signal (FR detection) by 74.27% ($p < 0.001$) compared with the UV group. There was not a statistically significant difference between the evaluated products (TOPICAL ANTIOXIDANT 1 vs. TOPICAL ANTIOXIDANT 2).

Figure 2 is a graphical representation of the anti-oxidant effects of the tested products with the fluorescence for the DCFH-DA probe quantified by Image J following exposure to ultraviolet radiation. The data represent the mean ± standard deviation of six replicates. Statistical significance was determined by ANOVA followed by Bonferroni correction.

The evaluated products TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 reduced oxidative stress in the skin tissue by exerting a protective effect against the excessive increase in the synthesis of free radicals induced by exposure to UV radiation. Thus, these results reveal that TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 exerted a substantial antioxidant and antiaging effect, protecting the skin from the deleterious effects of exposure to solar radiation.

Elastin conservation assessment in an ex vivo model results (Figures 3A,B): Skin explants from discarded face lift tissue were cultured in an ex vivo system and treated with AX-1 formulation or a comparator's (L-ascorbic) AX-2 formulation or left untreated. The formulations were applied every day for 7 days, and then the skin samples were immunostained to detect tropoelastin (red) and CD44 (yellow). The nuclei were detected using DAPI staining (blue). Images were captured (A), and the intensities of the signals were quantified using ImageJ (B). The samples were tested in triple replicates, and assessment was made across all the specimens.

The comparator's L-ascorbic acid formulation had a direct effect on elastin with a significant reduction in tropoelastin accompanied by a weakened fiber appearance in the DEJ and papillary dermis compared with AX-1 ($p < 0.05$). CD44 was also diminished in the AX-2 (L-ascorbic) formulation compared with AX-1, but the difference was not significant ($p = 0.06$). AX-1 showed not only conservation of elastin but healthier-looking elastin matrix with increased healthy tropoelastin fibers and conservation and stimulation of CD44.

4 | DISCUSSION

Extrinsic stresses on the skin, mainly in the form of UV radiation, release a host of free oxygen radicals that can be potentially harmful to all segments and functions of the skin. This oxidative stress necessitates the production of antioxidants by the outer stratum corneum layer in an attempt to neutralize ROS, but with time, the antioxidants become depleted and the skin loses this important protective system.[1] Traditionally, the backbone of antioxidant therapy revolved around Vit C and its derivatives or variations. Thus, it was well recognized as the leader in antioxidant therapy.[3] However, over the past decade or two, multiple alternatives to Vit C have been uncovered with antioxidant activity and other ancillary advantages (telomere effect[9]) that provide good alternatives and adjuncts to Vit C.

In addition, the important work by Hinek et al.[6] and other investigators not only demonstrated that traditional ascorbic acid used in 99% of market formulations can be detrimental and destructive to elastin, but that the sodium salt of ascorbate in carefully designed concentrations can be protective and stimulatory to elastin production.[6] This represents a major advance in the quest for an ideal antioxidant formulation – use of multiple new antioxidants with added benefits, together with a Vit C variation that in fact conserves and stimulates elastin in the extracellular matrix. It is well known that Vit C in all its forms stimulates collagen formation, but the addition of elastin stimulation is a significant added bonus.

Aside from this important function of the sodium salt of ascorbate on elastin conservation, the overall antioxidant efficacy was ensured by using antioxidants active in all three traditional areas of activity – hydrophilic, hydrophobic, and enzymatic categories. Individual functions of these actives are described in Table 1, but a few highlights are relevant. In the hydrophilic category, aside from the sodium ascorbate described above, well-recognized active ingredients were selected such as ergothioneine, oleuropein, Centella

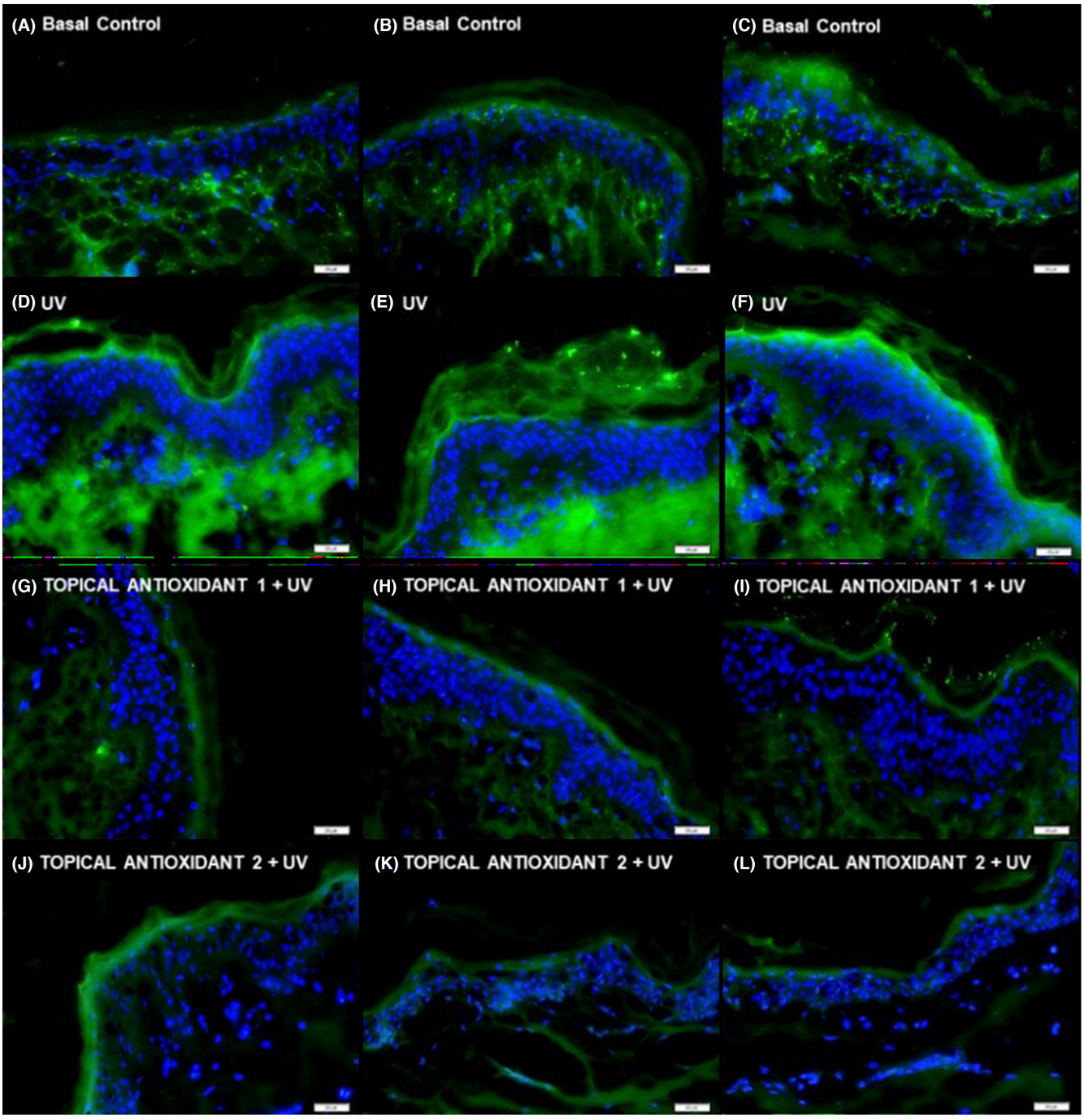


FIGURE 1 Antioxidant effect of TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 in human skin culture submitted to ultraviolet (UV) radiation. The DCFH-DA probe undergoes deacetylation, producing an oxidant DCHF. In the presence of ROS, DCHF is oxidized to form the highly fluorescent DCF, shown by the green fluorescent signal. The nuclei were detected by DAPI staining (blue). The scale bar represents 20 μm. The top row represents untreated samples, 2nd row represents UV stress and ROS induction (green), and 3rd and 4th row represents treated samples (AX-1, 3rd row, experimental; AX-2, 4th row, comparator).

asiatica, and lactoferrin. Ergothioneine not only demonstrates excellent antioxidant activity with mitochondrial DNA protection[9] but also protects against telomere shortening under oxidative stress.[28] Oleuropein, a known anti-oxidant, has also shown efficacy against UV irradiation,[29] and stimulates autophagic and proteasome activity for replacement of damaged proteins.[30] Centella asiatica is well known as an antioxidant,[31] but in addition, it has shown activity in wound healing, scar prevention, and fibroblast senescence.[32] Lactoferrin is also a novel addition to an antioxidant formulation. In addition to antioxidant activity, lactoferrin as a plasmin inhibitor and iron chelator, shows excellent activity relating to hyperpigmentation.[33–35] Thus, the dual action of these selected actives not only

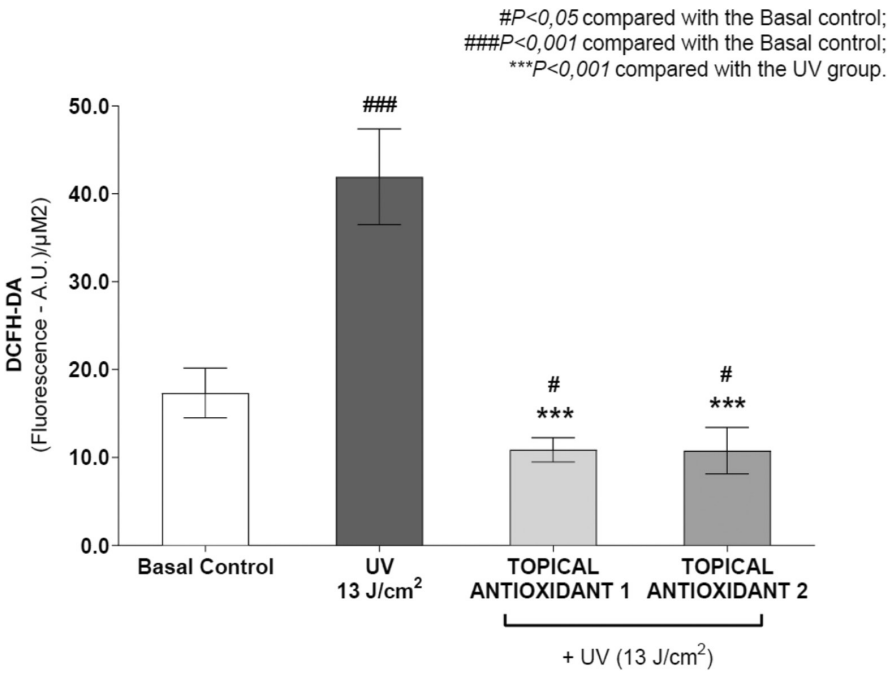


FIGURE 2 Graphical representation of Figure 1 – Evaluation of the synthesis of free radicals (FRs) in cultures of human skin fragments incubated with the evaluated products TOPICAL ANTIOXIDANT 1 and TOPICAL ANTIOXIDANT 2 and exposed to UV radiation. The fluorescence for the DCFH-DA probe was quantified by Image J.

ensures anti-oxidant activity but also contributes to general skin protection and overall skin health.

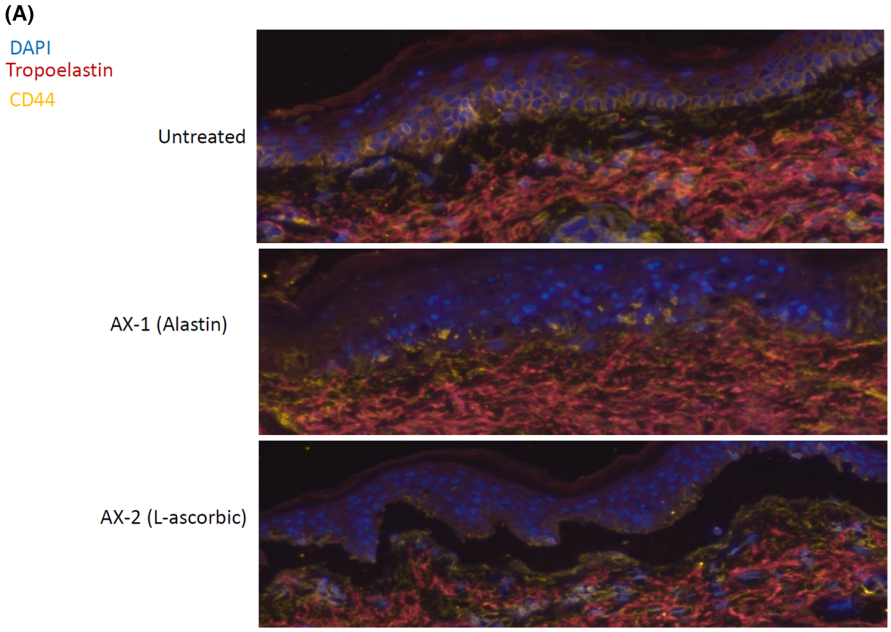
In the hydrophobic category, apart from well-recognized anti-oxidants bisabolol and *Physalis angulata* described above in Table 1, phytoene and phytofluene have additional activity against PGE 2, a potent inducer of post-inflammatory hyperpigmentation.[35] On the enzymatic front, all the actives categorized there provide indirect enzymatic synergy in anti-oxidant activity, but in addition, *Tremella fuciformis* has been show to improve hyaluronic acid levels and protect against UV damage.[36] Thus, in all categories described above, we have been careful to select many agents with dual activities, primarily as antioxidants but secondarily as effecting various components of skin health.

As far as validation aspects were concerned, a number of factors were considered. The work of Hinek and others relating to sodium ascorbate and its elastin-conserving effects was extremely thorough and well validated in in vitro studies,[6] patent applications,[37] and thesis work. Thus, the efficacy of the sodium salt of ascorbate as an individual component was well validated. What was needed was confirmation of antioxidant activity of the full formulation in comparison to a well-accepted competitor on the market, and then importantly, utilizing a full-thickness skin ex vivo model for both formulations to determine if the product was being absorbed (check for changes in ECM) and to confirm the elastin-conserving nature of this variation of Vit C. Using this model, we confirmed absorption by observing the effects in the papillary dermis, and we were able to show enhancement of the elastin fibers. A limitation of this approach may be the fact that the sodium ascorbate was not tested on its own to prove that this was the component responsible for these effects. However, consideration must be given to the fact that the reduced elastin seen with AX-2 is in line with the work referenced above as is the stimulation of elastin by the AX-1 formulation. No components within the antioxidant actives have been demonstrated to be involved in elastin destruction or this degree of elastin stimulation. Thus, it was deemed logical that the component responsible for these actions was very likely to be the sodium ascorbate.

Armed with this knowledge, we set about the design and production of a novel antioxidant formulation with two objectives in mind – that of establishing substantial equivalence of antioxidant activity to a leading brand and the demonstration of elastin conservation and stimulation, rather than destruction, with the sodium salt variation of Vit C. The studies elucidated above demonstrate success in both areas of validation – antioxidant efficacy and elastin preservation. In addition, the formulation and its derivatives have shown remarkable stability in conventional settings, a constant problem with some Vit C preparations. This relates to the unique liposomal technology used with the sodium ascorbate ensuring increased stability and efficacy.[27] This constitutes a significant advance in antioxidant formulation science.

5 | CONCLUSION

This paper details the approach to formulating a novel antioxidant product that combines the introduction of new antioxidant actives with excellent ROS quenching capabilities, together with a stable sodium salt variation of ascorbic acid that preserves elastin. The background thought processes, choices of active ingredients, and validation steps are presented, culminating in a product with added benefits over traditional and leading antioxidants on the market. Clinical studies are to follow.



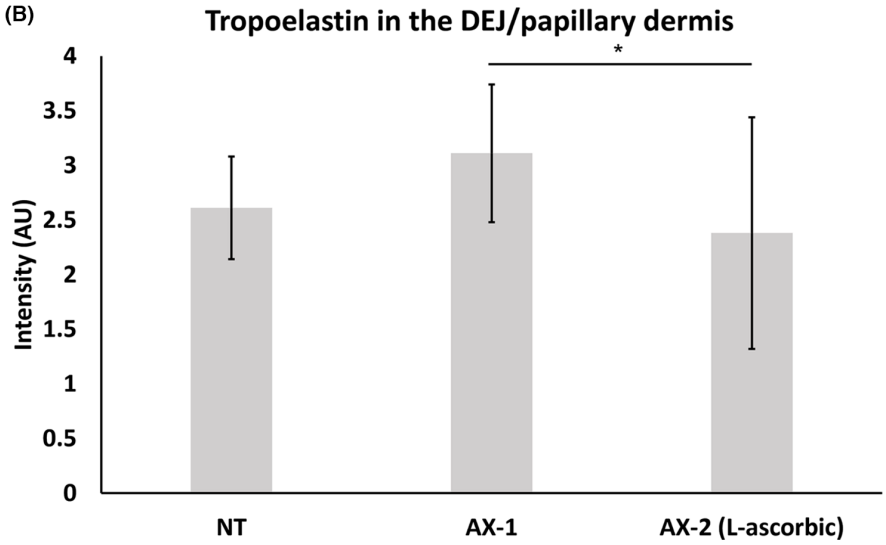


FIGURE 3 (A) Skin samples in an ex vivo model were immunostained to detect tropoelastin (red) and CD44 (yellow). The nuclei were detected using DAPI staining (blue). Top slide represents untreated sample with reasonable amount of tropoelastin present. Middle slide shows healthy and improved elastin fiber presence following treatment with AX-1 new anti-oxidant. While lower slide shows the effect of the competitor product on elastin (moth eaten appearance of tropoelastin and elastin fibers in papillary dermis). The intensities of the signals were quantified using ImageJ (B). AX-1 showed significantly more tropoelastin staining than competitor in the dermo epidermal region (DEJ) and papillary dermis. It was also increased over baseline in contrast to competitor, which reflected less elastin than at baseline when assessed across all replicates.

AUTHOR CONTRIBUTIONS

A.D.W. – developed the science, designed studies, analysis, paper writing. M.Z. – data analysis, paper writing, study design. J.G. – formulation design. F.S. – paper writing.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journals author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received (ex-vivo models).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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