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Enhanced Skin Solar Protection and Repair via a Topical Antioxidant Complex

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Abstract

Background: To avoid the detrimental effects of solar skin exposure, including hyperpigmentation, skin laxity and loss of radiance, dermatologists routinely recommend sun avoidance and the daily use of sunscreens. Repair of sun damaged skin is usually addressed in terms of ablative or surgical procedures. Compared to the well documented effects of ultraviolet radiation, oxidative stress related to environmental conditions, including solar exposure, diet and lifestyle is often overlooked as a cause of skin damage and premature aging. **Purpose:** This paper aims to investigate the efficacy of a topical formulation comprised of a comprehensive antioxidant complex, an environmental protection complex and an optimized delivery system to enhance overall skin physiological and cellular antioxidant and anti-inflammatory protection from solar and environmental damage. **Methods:** The Formulation (#2LCRE.20) was tested for: *in-vitro* LDH cytotoxicity activity, qPCR gene expression on a 3-dimensional skin model, *in-vivo* efficacy on 21 patients introducing the Formulation into their pre-established skincare regimen with no other treatments and *in-vivo* experience testing on 7 subjects incorporating the Formulation into their pre-established skincare ritual including a weekly hyperpigmentation facial treatment in a professional skin clinic. **Results:** *In-vivo* data showed a significant improvement in skin hydration, hyperpigmentation, radiance, inflammation and redness after exposure to the Formulation. These results were supported by *in-vitro* testing, where six specific genes were upregulated. **Conclusion:** This study highlights the role that oxidation and antioxidant skin Formulations and Treatments can play in helping to prevent the damaging effects of solar and environmentally induced skin damage.

Keywords

Photoprotection, Photorepair, Hyperpigmentation, Gene Expression, Antioxidant, Rejuvenation

1. Introduction

As the largest external human organ, the skin is most highly exposed to external aggressors including sunlight, dryness, temperature extremes and airborne toxins. The damaging effects of sunlight are now known to extend beyond Ultraviolet Radiation (UVR) to include Visible Light (VL) and Near Infrared (NIR) Radiation [1]-[7]. Temperature and humidity affect skin hydration and enzymatic function, while airborne Carbon Particulate Matter (CPM) can penetrate the stratum corneum causing oxidative stress and inflammation [8]-[10]. All these factors affect overall skin health and beauty.

As an adaptive evolutionary response, the skin is endowed by a comprehensive endogenous antioxidant system designed to prevent and ameliorate the formation of harmful Reactive Oxygen Species (ROS) and Free Radicals (FR) generated by solar and environmental interactions with skin proteins, lipids and biomolecules [11].

Depletion of the skin's endogenous antioxidants is both intrinsic and extrinsic, with environmental aggressors largely responsible for defensive hyperpigmentation, loss of skin tone and firmness and rough texture [12].

This study reports on the efficacy of a topical Formulation comprised of a Primary Active Complex (PAC) of endogenous and exogenous antioxidants, peptides, lipids and botanicals as well as a complex blend of functional Delivery System (DS) ingredients designed to enhance in situ solar and environmental skin protection functions.

2. Materials and Methods

2.1. Topical Formulation

The Formulation #2LCRE.20 under evaluation as the Test Material (TM) in this study is comprised of the PAC, that includes ascorbates, tocopherols, tocotrienols, ubiquinone, enzymes and amino acids in an optimized DS, described in **Table 1**.

Table 1. List of PAC and DS ingredients in formulation #2LCRE.20.

Topical Formulation Ingredients Functions
Vitamin C, E
Complex and Essential Fatty Acids
16 Amino Acids, 2 Proteins and 1 Enzyme
Humectants & Penetration Enhancers
Australian Botanicals Extracts
Emollients
Stabilizers and Preservatives
Thickener & Emulsifiers
Extracts, Minerals & Sugars
Pigments

Four TMs were used to complete the study described below. The Untreated Control consists of tissue samples that have not been treated with any substances. This TM was used as negative control to assess cytotoxicity. Triton X-100 is a non-ionic surfactant which disrupts cell membranes leading to cellular death is used as a positive control for cytotoxicity assessment. Cells exposed to Triton X-100 are not expected to survive, yielding a 100% death rate. A 0.9% Saline solution was used as negative control for the assessment of the gene expression changes in the 3-dimensional reconstructed skin tissue model. Its cytotoxicity potential was measured to ensure full cell viability during the gene expression study and isolate the TM's cytotoxicity. This solution, considered neutral and inert to the skin cells, should induce very low to zero cytotoxicity or changes in gene expression. The results obtained with this solution serve as a comparison with the tested Formulation results. #2LCRE.20, also referred to as the Formulation, is described in Table 1. This is the main material to be assessed to determine the degree of change that will be induced after using the Formulation in the cytotoxicity, gene expression changes and *in-vivo* studies. Table 2 describes the approximate concentrations of actives in the PAC which are expected to provide most of the antioxidant activity of the TM. Table 3 expands further on the Test Materials design.

Table 2. List and concentrations of PAC ingredients in formulation #2LCRE.20.

Primary Active Complex	[% range]
Beta-alanyl-L-histidine dipeptide	2% - 4%
Ubiquinol	1% - 2%
Ascorbates	1% - 5%
Tocotrienols	0.1% - 1%
Tocopherols	1% - 5%
Superoxide Dismutase	0.001% - 0.01%
Glutathione	0.001% - 0.01%

Table 3. Test groups and result expectations.

Test Groups	Comments	Expectations
Untreated Control	Cytotoxicity Negative Control	No cytotoxicity should be observed
Triton X-100	Cytotoxicity Positive Control	Maximum cytotoxicity should be observed
0.9% Saline	Gene Expression Control	Very low cytotoxicity should be observed
#2LCRE.20	Formulation	Very low cytotoxicity should be observed

2.2. Skin Model and Gene Expression

The gene expression and cytotoxicity studies were performed on a commercially

available 3-dimensional in vitro skin model (Mattek EFT-400) composed of epidermal keratinocytes and dermal fibroblasts as described in Figure 1. Tissues were equalized prior to individual inoculation with 15 uL of one of the four TM samples. For each treatment group, four tissue samples were included. Post distribution of TMs, the tissue samples were placed in an incubator at 37˚C with 5% CO2 and ~95% relative humidity for 24 hours. Following 24 hour incubation, each tissue sample surface was washed and retreated with a fresh TM sample. This process was performed a total of three times. Tissue samples were collected and gene expression was assessed after 72 hours exposure to the TMs utilising Genemarkers' qPCR-based Standard Skin Panel containing 107 target genes (Appendix 1). After full incubation, tissue sample surfaces were washed, and each culture was placed in contact with a RNAlater solution ready for RNA isolation.

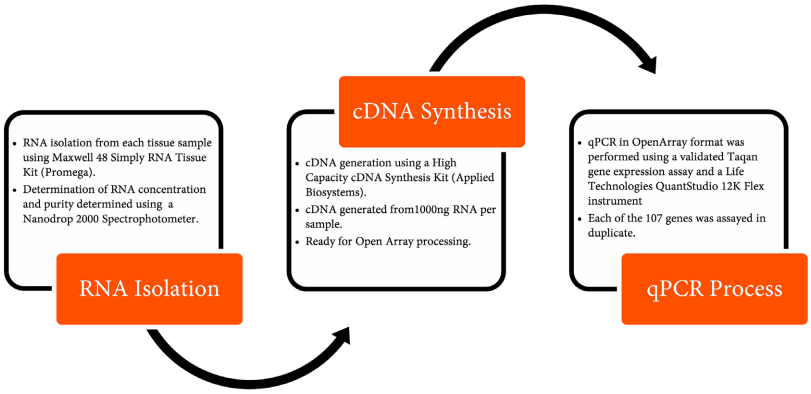


Figure 1. Gene Expression Process from inoculated tissue samples to raw data generation.

Following a qPCR process, statistical data analysis was performed using relative quantitation (RQ) method and converting any linear RQ values into linear fold-change values.

2.3. LDH Cytotoxicity Assessment

Controls:

- High Control: 1% Triton X-100 was inoculated on the surface of two tissue samples as per process above.
- Low Control: Four untreated (UNT) tissue samples.

To assess the LDH activity (cytotoxicity) of the TM, the culture medium of each treated tissue sample was used and diluted 1:10 with Phosphate Buffered Saline (PBS). Every dilution was then combined with the LDH reaction mix at a ratio 1:1 and followed by a 20minute incubation at room temperature in the dark. A 1.0N HCl solution was used to stop the reaction of each dilution and absorbance was measured at 492 nm with a reference filter at 620nm. The LDH activity or cytotoxicity was calculated relative to the absorbance of the low control (0% cytotoxicity) and high control (100% cytotoxicity) following the below formula:

%Cytotoxicity = [(Test Media Value – Low Control)/(High Control – Low Control)] * 100.

2.4. *In-Vivo* Clinical Evaluation

Subjects

Patients were selected based on inclusion and exclusion criteria defined in Tables 4-6. Patients were directed not to use any other skincare products or undergo any form of aesthetic procedure before and during the study. Additionally, they were instructed to continue with their normal diet throughout the study. This study involved a retrospective review of previously treated patients. All patients signed an informed consent form approving their inclusion following an explanation of the study design and execution and agreed to the publication of results and images.

Table 4. Subjects' inclusion criteria for selection.

Inclusion Criteria	Non-Inclusion Criteria
Specific • Sex: female and male; • Age: between 45 and 65 years old; • All skin type; • Type: Caucasian; • Subjects with dry, normal and combination skin. **General** • Healthy subject; • Subject having given his/her free informed, written consent; • Subject willing to adhere to the protocol and study procedures.	• Female Specific—pregnant or nursing women or women planning to get pregnant during the study; • Cutaneous pathology on the study zone (eczema, etc.); • Start, stop or change of hormonal treatment (including the contraceptive pill) within the last 1.5months; • Subject with make-up on the day of the visit at the laboratory; • Use of topical or systemic treatment during the previous weeks liable to interfere with the assessment of the cutaneous acceptability/efficacy of the study product; • Subject having undergone a surgery under general anaesthesia within the previous month; • Excessive exposure to sunlight or UV-rays within the previous month.

Application and Directions for Use. Each subject is his/her own control.

Table 5. Formulation application instructions.

Application Zone	Application Frequency	Directions for use
Face	At home. Once daily (in the morning).	Use as replacement of the usual morning facial care product. Each evening, only cleanse face and neck with usual cleanser and apply the usual moisturizer on a dry face and neck. Each morning, apply the Testing Product on a dry face and neck (no cleansing of face and neck with the usual cleanser—only water is allowed for face rinsing if needed).

Kinetics

Parameters tested and the time points are described below:

Table 6. Kinetics known as *in-vivo* tests parameters and check points.

Kinetics	Measurement zone	D0	D28	D56
Information of the subject about study conditions and collection of his/her informed consent.		●		
Verification of inclusion and non-inclusion criteria.		●		
Acclimatization for 15 minutes.		●	●	●
Clinical examination by the dermatologist in charge of the study in order to evaluate the cutaneous state of the face.	Face	●	●	●
Application of the product by the subject at home.		●	●	●
Distribution/collection of the daily log.		●	●	●
Distribution/collection of the studied product.		●	●	●
Subjective evaluation questionnaires.			●	●
Measurements using Corneometer®.		●	●	●

***In-Vivo* Case Study**

This assessment was performed on 7 patients using products within a personalized pre-established skincare ritual. The TM was introduced into patients existing skincare routine for a period of 4 weeks. Visia images (Visia, Canfield Scientific, NJ, USA) were taken on clean skin prior to commencement of the study then again after 4 weeks of introducing #2LCRE.20 into each subject's skincare routine. To measure and observe physiological changes, the following Visia-defined parameters were captured: Overview, Spots, Brown Spots, UV Spots and Red Spots. Detailed data are reported for one single representative case. Further details on the rest of the cohort are available in Appendix 2.

3. Results

3.1. LDH Cytotoxicity Assessment

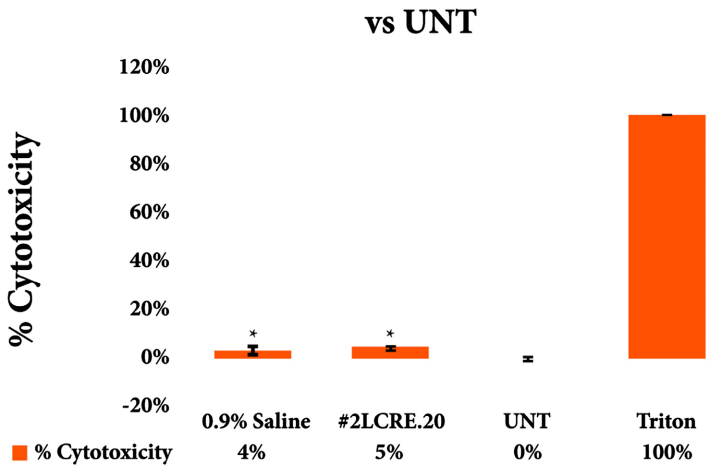
Cytotoxicity and gene expression studies were performed on the Test Groups described in Table 3. Increased LDH activity is interpreted as an indication of cell damage or cell death. The positive control, Triton X-100 is the reference for full cytotoxic LDH activity. Levels of cytotoxicity were assessed after 72 hours of treatment and were compared to the untreated, negative control.

The cytotoxicity level of the #2LCRE.20 was minimal and comparable to the 0.9% Saline solution results as shown in Figure 2. The LDH activity threshold allowing to conclude that the TM is affecting the gene expression results is set at 50% cytotoxicity.

3.2. Measured Changes in Gene Expression Following Treatment with #2LCRE.20

Of the 107 genes tested (Appendix 1), approximately 50 presented with significant

changes in genetic expression. Each gene change is reported to influence structural and functional activity within skin cells (anti-inflammation, DNA repair, survival, apoptosis, antioxidation etc). A selection of 6 genetic markers was curated and reported above as their functions and mechanism of action are most relevant to this study as displayed in Table 7.



Note. The * ($p \leq 0.05$) symbol designates statistical significance after performing unpaired t-test.

Figure 2. Relative % LDH activity (cytotoxicity) after 72 hours of treatment.

Table 7. Gene expression linear fold change 72 hours after TM application.

Gene ID	#2LCRE.20			
	Gene Name	FC	%C	Function in the skin
DCN	Decorin	**2.02**	**102%**	**Extracellular Matrix Breakdown**
NQO1	NAD (P) H:quinone oxidoreductase-1	**2.52**	**152%**	**Oxidative Stress Response**
OCLN	Occludin	**2.41**	**141%**	**Epidermal Barrier**
LCE3D	Late Cornified Envelope 3D	**2.17**	**117%**	**Epidermal Barrier**
CLDN7	Claudin 7	**9.12**	**812%**	**Epidermal Barrier**
AQP3	Aquaporin 3	**2.65**	**165%**	**Hydration**

3.3. *In-Vivo* Clinical Evaluation

Each patient's skin was assessed at Day 0, 28 and 56 under dermatologist supervision, thus representing its own control.

Each patient was provided with the TM to apply every morning, a self-assessment questionnaire and a daily log to report any changes and unusual or adverse occurrences.

The cohort recruited is detailed below in Table 8:

Table 8. *In-vivo* test demographics (total number of subjects n = 21).

Sex	N	%
Female	18	86%
Male	3	14%
Face skin type	**N**	**%**
Dry	5	24%
Normal	5	24%
Oily	0	0%
Combination	11	52%

Cutaneous Compatibility

This assessment was performed by a dermatologist. One patient experienced some clinical signs that were not attributed to use of the product and was therefore classified as not relevant. A second patient reported a functional sign of skin tightness during the trial period which was classified as relevant to use of the product (Table 9).

Table 9. Clinical and reported signs of non-acceptability.

Non-Acceptability Signs		
	N	%
No clinical signs	20	95%
Not relevant clinical signs	1	5%
Relevant clinical signs	0	0%
No reported signs	20	95%
Not relevant reported signs	0	0%
Relevant reported signs	1	5%

Cutaneous Hydration

Cutaneous hydration was measured with a corneometer [13], and results were compared before and after using the TM (Figure 3). 95% of the patients experienced an increase in hydration of 40% after applying #2LCRE.20 daily for 28days and continuing using it for 56 days, 78% of the patients experienced increased hydration. An average of 22% elevation in epidermal moisture levels was recorded after 56 days of continual use.

Subjective Questionnaire

Each patient was requested to complete a questionnaire related to perceived efficacy of the Formulation based on various criteria after 28 and 56 days. Affirmations were proposed and subjects were asked to rate their degree of agreement with the statement on a four-point scale from agree to disagree. Where patients agreed or strongly agreed, the results were considered positive. Twenty-one patients were considered for this assessment therefore 1 patient represents 4.8% of

the total result. To evaluate the significance of the answers, a 95% confidence interval (CI) was determined using the Wilson Methodology and compared to the lower limit of significance (Figure 4).

Overall, most patients observed and reported a global improvement in the appearance and feel of their skin including hydration, firmness, radiance, evenness of skin tone, suppleness and softness.

Parameter	Kinetics		D Dx-D0 (mean ± SEM)	Statistic			% of efficacy	% of subjects with an improvement in the cutaneous hydration (NB: if variation > 1)
				p=	Statistical test	Statisticall y Significant		
Cutaneous hydration rate	Delta	D28	18.8 ± 2.5	<0.0001	Wilcoxon	Yes	40%	95%
	Delta	D56	10.8 ± 3.1	0.0027	Student	Yes	22%	78%

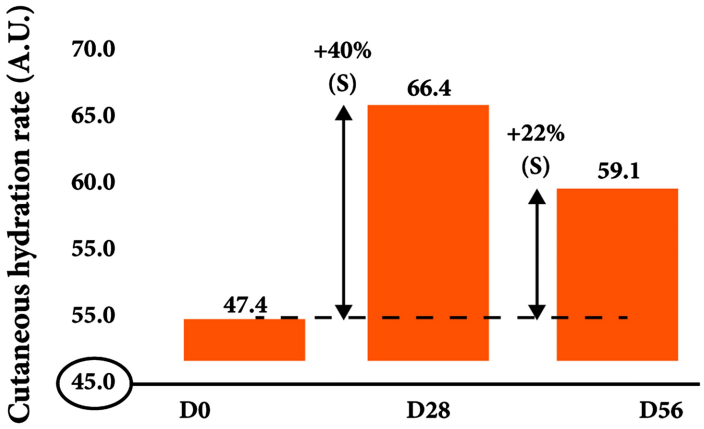
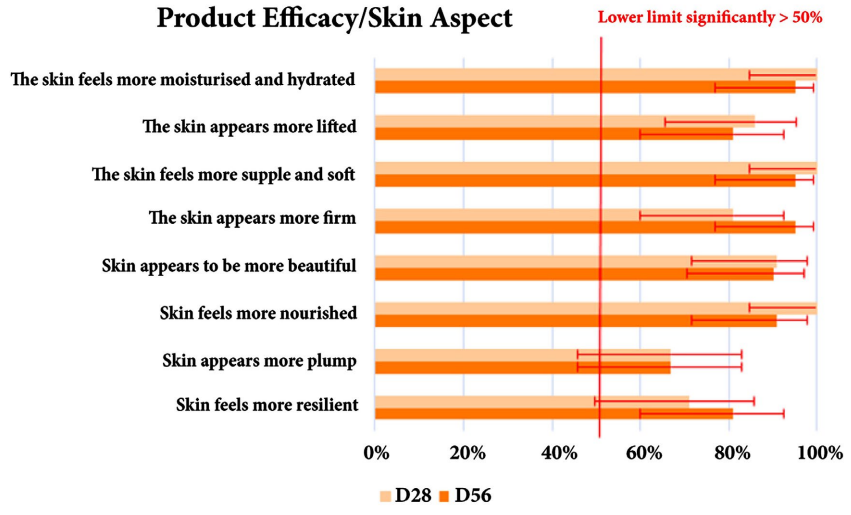


Figure 3. Variations of cutaneous hydration rate measured with a corneometer (in arbitrary units) after using the Formulation for up to 56 days compared to baseline. A significant increase in hydration is interpreted as enhanced moisturization attributable to use of the TM, while no change of hydration status indicates a non-drying effect.



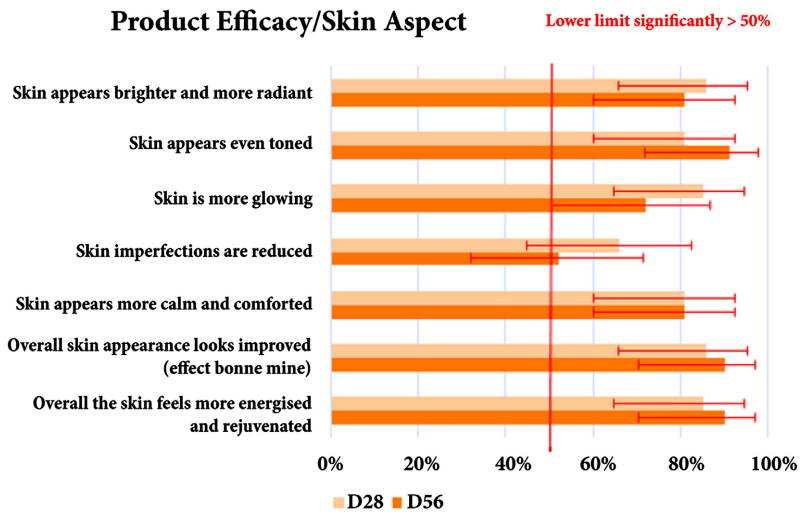


Figure 4. Patient self-assessment after using the Formulation for 28 and 56days. * "effect bonne mine" meaning healthy glow.

3.4. *In-Vivo* Experience Test

Out of the 7 patients participating in this study (**Table 10**), one subject was selected to be presented in this report based on their results. The patient is of Type III on the Fitzpatrick scale and has a combination skin.

Prior to starting the study, a skin therapist assessed all subjects skin conditions and concerns as well as goals and current skincare ritual.

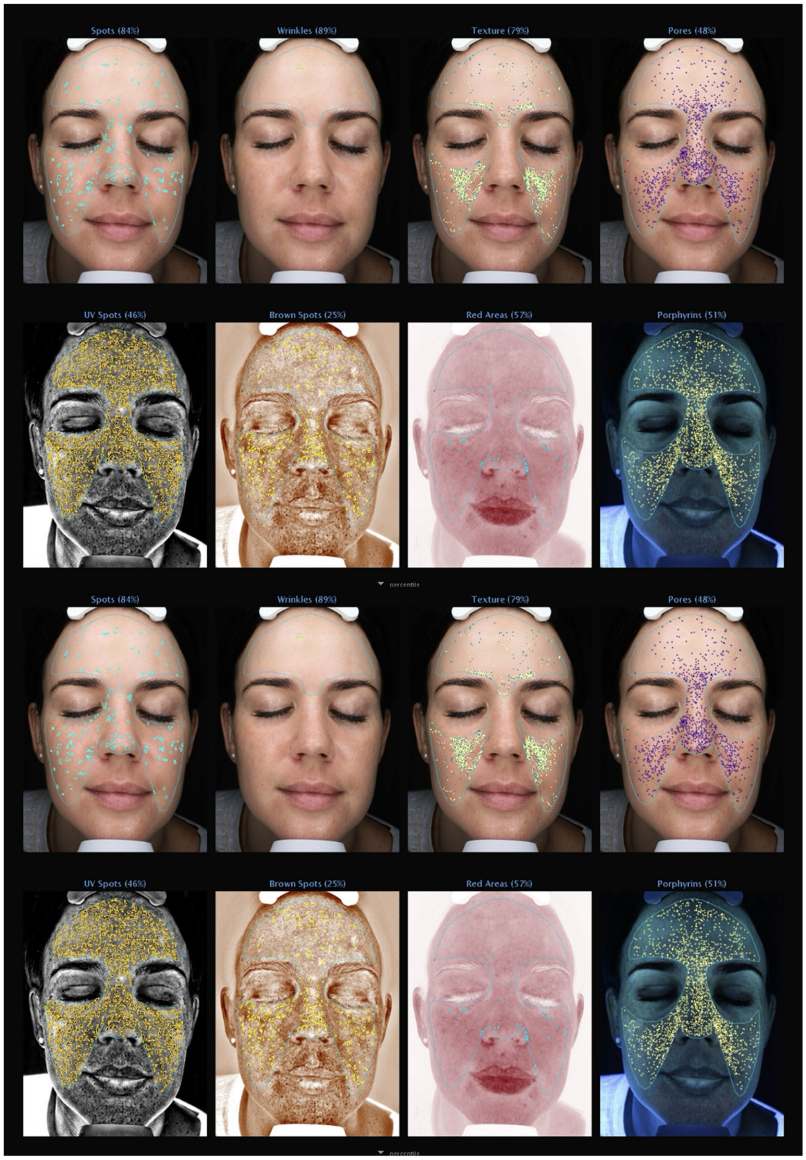
Pre-assessment results for all patients are presented in **Table 10**; baseline and final Visia images after 4 weeks of Formulation usage are presented in **Figure 5** and 6 for the selected participant (#1).

Visia (Canfield Scientific, NJ) Skin Analysis imaging deploys a non-invasive measure of skin changes using cross polarized and UV light to reveal superficial, epidermal and dermal changes in skin tone and texture. The Visia images and measurements reported in **Figure 5**, **Figure 6** and in **Figure 7** for the selected participant #1 provide results for 8 different skin markers including an improvement percentage score and visible assessment. An increase in score results indicate an improvement of the specific skin marker.

The first parameter, Spots, typically identifies brown or red skin lesions including freckles, acne scars, hyper-pigmentation and vascular lesions. The before and after scores are very close, from 82% to 84%. The second parameter examined is the appearance of Pores. They are identified by their circular shape and their size being much smaller than a spot. The reduction in pore size score in this subject's skin increased by 34%. Wrinkles are defined as folds or creases in the skin which are known to be associated with a decline in skin elasticity and are often considered to be a result of excessive sun exposure over time. The baseline results for this patient were 89% and increased to 99% over a 4-week period of using the Formulation. The Skin Texture parameter, assessed through Visia imaging is essentially an analysis of skin smoothness. It measures skin colour and smoothness by identifying gradients in colour from the surrounding skin

Table 10. Pre-selected patients pre-assessment performed by a Skin Therapist including skin type, conditions and goals.

ID	Gender	Skin Type	Fitzpatrick Type	Skin Conditions	Skin Goals	Skincare Ritual
1	Female	Combination	III	Dehydration; Hyperpigmentation; Congestion; Sensitivity.	- More youthful looking skin; - Even out skin tone; - Brighten a dull looking complexion; - Smooth texture; - Improve hydration.	Extensive: - 6 leave on products for day and evening use, including serums and moisturisers; - 6 rinse off including cleansers, masks and make up remover; - 3 tinted SPF serums.
2	Female	Combination	II	Sensitivity; Dematitis; Eczma; Congestion.	- Maintain skin health; - Brighten a dull looking complexion; - More youthful looking skin.	Extensive: - 7 leave on products for day and evening use, including serums and moisturisers; - 4 rinse off including cleansers, masks and make up remover; - 4 tinted SPF serums and crème.
3	Female	Combination	IV	Hyperpigmentation; Dehydration; Congestion.	- Reduce breakouts; - Improve hydration; - Brighten a dull looking complexion.	Extensive: - 8 leave on products for day and evening use, including serums and moisturisers; - 2 rinse off including cleansers, masks and make up remover; - 4 tinted SPF serums and cremes.
4	Female	Combination	IV	Hyperpigmentation; Dehydration; Congestion.	- More youthful looking skin; - Smooth texture; - Brighten a dull looking complexion.	Extensive: - 9 leave on products for day and evening use, including serums and moisturisers; - 5 rinse off including cleansers and masks; - 3 tinted SPF serums and cremes.
5	Female	Combination	IV	Congestion; Dehydration.	- More youthful looking skin; - Maintain skin health; - Brighten a dull looking complexion; - Improve clarity; - Smooth texture; - Improve hydration.	Extensive: - 6 leave on products for day and evening use, including serums and moisturisers; - 3 rinse off including cleansers, masks and make up removers; - 3 tinted SPF serums.
6	Female	Combination	V	Dehydration; Hyperpigmentation; Congestion; Dematitis.	- Smooth texture; - Improve hydration; - More youthful looking skin.	Extensive: - 11 leave on products for day and evening use, including serums and moisturisers; - 3 rinse off including cleansers and masks; - 3 tinted SPF serums and cremes.
7	Female	Combination	IV	Congestion; Acne; Dehydration; Hyperpigmentation.	- Even out skin tone; - Brighten a dull looking complexion; - Improve clarity; - Reduce breakouts; - Smooth texture.	Extensive: - 10 leave on products for day and evening use, including serums and moisturisers; - 4 rinse off including cleansers, masks and make up remover; - 2 tinted SPF serums and cremes.

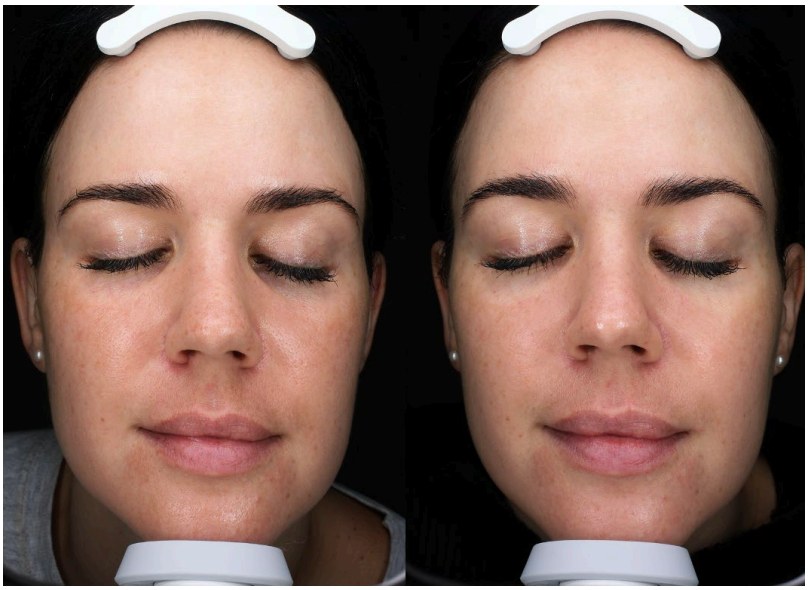


The patient signed the informed consent form.

Figure 5. Selected patient #1 visible improvements in the appearance of wrinkles, texture, brown spots and pores after 4 weeks (below) of using the Formulation. A score (%) increase from baseline (above) to end results, indicates an improvement in skin appearance.

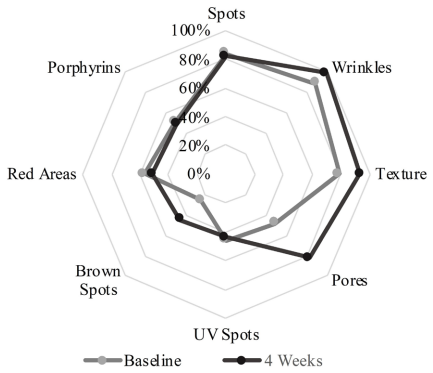
tone, as well as peaks (shown in yellow) and valleys (shown in blue) on the skin surface that indicate variations in the surface texture [Canfield Visia]. This patient has seen her skin texture improve from 79% to 94% after 4 weeks of using #2LCRE.20. The assessment of Porphyrins investigates the bacterial excretions that can settle in pores, potentially leading to acne. Canfield Visia imagery measures the fluorescence of UV light, manifesting as circular white spots and appear in yellow on the images. The porphyrin score remained somewhat similar post usage of the Formulation. UV Spots is a parameter directly linked to melanin coagulation below the skin surface and is considered to be a result of sun damage.

They are generally invisible under normal lighting conditions but the selective absorption of the UV light by epidermal melanin enhances its display and detection by VISIA. In this study, there was not a significant improvement in the appearance of UV Spots. Red Areas were examined in this study to identify improvement in a variety of potential skin conditions that could be exacerbated by sun damage (inflammation, rosacea etc.). This patient experienced a 6% decrease in Red Spots. The last parameter assessed is Brown Spots or skin lesions that could be considered as hyperpigmentation, freckles, lentigines or melasma. These conditions occur due to an excess of melanin which is produced by the melanocytes in the bottom layer of the epidermis in response to solar exposure over time. This patient experienced a 20% increase in the improvement score in Brown Spots.



The patient signed the informed consent form.

Figure 6. Selected patient #1 overall visible improvements in skin tone, radiance and pigmentation before incorporating the Formulation (left) and after 4 weeks (right). Visual assessment from baseline (left) to end results (right), indicates an improvement in skin appearance.



Skin Markers	(4weeks—Baseline) %
Spots	−2%
Wrinkles	10%
Texture	15%
Pores	34%
UV Spots	−2%
Brown Spots	20%
Red Areas	−6%
Porphyrins	−2%

Figure 7. Selected patient #1 skin markers measurements at baseline and after 4 weeks of using the Formulation.

The Visia images presented in **Figure 6** highlight the improved skin tone and radiance overall after 4weeks of incorporating the TM in the skincare regimen as well as a diffused pigmentation around the eye area.

4. Discussion

Dermatologist safety assessment protocols of the Formulation on different skin types revealed a high level of skin tolerance with no clinical signs of adverse reaction.

95% of patients reported not experiencing any perceived signs of adverse reaction after using the Formulation, with more than 80% of patients describing their skin as calmer and more comforted. A correlation could be made between the soothing activ ity of #2LCRE.20 *in-vivo* with the increased expression of DCN (+102%) specifically as it is a proteoglycan involved in wound healing and skin cells regeneration [14]. Through supporting the skin barrier function integrity and structure, other highlighted genes like LCE3D, CLDN7 and OCLN may impact positively the skin and keep it soothed and functional. This would demonstrate that achiving calmer and soothed skin could be achieved through formulations containing active ingredients targeting not only inflammatory pathways but also focusing on skin structural functions such as intercellular communication [15]-[18], epidermal thickness [19] [20] and tight junctions interactions [21].

Diving into hydration levels, the *in-vivo* studies showed that after 28 days, 95% of the patients experienced a 40% (mean) increase of skin hydration as measured by corneometry. In addition, all the patients reported their skin feeling more moisturised and hydrated. At 56 days, corneometry measurements revealed that 78% of patients experienced a 22% (mean) increase in hydration while more than 90% of the subjects perceived their skin to be more moisturised and well hydrated. While a direct link can be drawned between these results and the increased expepression of AQP3 (+165%) responsible for the transport of water within the epidermal cellular matrix [22]-[24], the improved skin barrier function as suggested by the gene expression results of LCE3D (+117%), OCLN (141%) and CLDN7 (+812%) could be a factor of improved hydration. Effectively, improving the permeability, structure and integrity of the skin barrier function would lead to a better retention of the water levels in the skin. Studies have shown a relationship between the expression of AQP3 and wound healing [25] [26], suggesting a interdependent link between soothing and hydration, and more widely with the ability of the skin to regenerate itself especially in stressing situations [27]-[29].

Further *in-vivo* results demonstrated that at 56 days, improvements in skin radiance, brightness and evenness were noticed by all patients. These results can be considered to corroborate the findings of the Experience Test reported on the single subject with hyperpigmentation and enlarged pores, prone to acne and redness, the study highlighted significant improvement of her brown spots by 20%, pores by 34%, and texture by 15%. These results could be linked directly to the

increased expression of NQO1 (+152%) considering its detoxifying activity combined with cell protection functions and responsibility in cellular proliferation/differentiation regulation leading to potential improvement in skin texture, pigmentation and radiance [30]-[33].

Overall, the expression of the 6 selected genes is showing to have complementary positive effects on the skin when upregulated. Protein expression testing would be required to further correlate the in-vitro and *in-vivo* results.

Although there is very limited data available attributing these positive results to the individual or synergistic complex of antioxidants (vitamins, peptides, protein and enzymes), the study shows a relation between the complex delivered via an optimised delivery system and improved skin barrier functions, hydration and overall skin appearance including radiance, textures and pores.

Based on the overall objective and subjective responses of all patients, it can be concluded that the Formulation has the potential to counteract negative environmental damages (including redness, dullness, sun damages…) by improving the skin barrier function, antioxidation and hydration which in turn assist in improving skin appearance (potential reduction of signs of hyperpigmentation, improvement of glow and radiance, evening out of skin tone and refinement of skin texture).

5. Summary

When initiating this study, it was hypothesised that the Formulation would impact the 3D skin model by increasing some of its antioxidant functions due to the composition of the PCA. Ubiquinol and ascorbates, for example, are known for their supporting functions against reactive oxygen species (ROS), promoting molecular damage caused by excessive environmental exposure [34]-[36]. Studies have also shown that Beta-alanyl-L-histidine dipeptide has the potential to significantly reduce UV and blue-light induced ROS [37], due to its composition resulting in the improvement of skin texture and appearance.

This study demonstrates that a topical antioxidant formulation may provide measurable benefits in mitigating environmentally induced oxidative stress in skin. The observed upregulation of genes involved in epidermal barrier function, hydration and oxidative defence (including DCN, CLDN7, LCE3D, OCLN, NQO1 and AQP3) suggests that the formulation may support endogenous protective mechanisms in keratinocytes and dermal fibroblasts resulting in visible results. *In-vivo* assessments further demonstrated improvements in hydration, radiance and overall skin appearance across the study population.

Reactive oxygen species generated through the exposure to ultraviolet radiation, visible light, infrared radiation and airborne particulate matter can damage the molecular structure of the skin, ultimately contributing to photoaging and pigmentary changes. Topical antioxidant systems have therefore been proposed as a complementary strategy to traditional photoprotection (via antioxidant-mediated support against oxidative pathways associated with environmental expo-

sure). Independent studies have demonstrated that combinations of antioxidants including vitamins C and E, ubiquinone and enzymatic antioxidants can reduce oxidative damage induced by UV radiation and environmental pollutants. The present study expands by demonstrating a synergistic effect of antioxidative components within a complex on gene expression changes associated with barrier integrity and hydration pathways both in a reconstructed human skin model and on human beings.

6. Conclusions

On an epigenetic level, the combination of the antioxidant Primary Active Complex within a specifically designed Delivery System has the potential to protect the skin from oxidation and environmental damage as well as improve the overall quality of skin health, vitality and appearance.

Of note is the fact that the standardised *in-vivo* method, confirmed by dermatologist assessment, aligned with the results obtained by the gene expression study on a physiological level. The skin appears brighter, calmer, more hydrated and rejuvenated, with all these attributes linked to a healthy, functioning and environmentally protected skin.

Limitations

The absence of a placebo or a comparator group, together with an open-label design, limits the ability to attribute observed effects solely to the intervention. Additionally, no UV, visible light, or infrared exposure model was used, and therefore, direct protective effects cannot be established. A further protein expression experiment would be beneficial to confirm the gene expression study.

Disclosure

The authors disclose that this study was entirely funded by RATIONALE Skincare Pty Ltd, Victoria, Australia. Amaryllis Aganahi, Richard Parker, Katie Matten are paid employees of RATIONALE. Yohei Tanaka is a paid consultant plastic surgeon for RATIONALE.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix 1

STANDARD SKIN PANEL GENE LIST	
ANTI AGING	**EXTRACELLULAR MATRIX BREAKDOWN**
» FOX03: forkhead box 03	» F2RL1: F2R like trypsin receptor 1
» HNRNPD: heterogeneous nuclear ribonucleoprotein D	» KLKS: kallikrein related peptidase 5
» HSPG2: heparan sulfate proteoglycan 2	» KLK7: kallikrein related peptidase 7
» IGFlR: insulin like growth factor 1 receptor	» MMPl: matrix metallopeptidase 1
» MFNl: mitofusin 1	» MMP10: matrix metallopeptidase 10
» MFN2: mitofusin 2	» MMP2:matrix metallopeptidase 2
» NMRKl: nicotinamide riboside kinase 1	» MMP9: matrix metallopeptidase 9
» PANK4: pantothenate kinase 4	» SERPINB3: serpin family B member 3
» POLG1/MDP1: DNA polymerase gamma, catalytic subunit	» SPINKS: serine peptidase inhibitor, Kazal type 5
» SIRTl: sirtuin 1	**EXTRACELLULAR MATRIX INTEGRITY**
ANTIOXIDANT/ RESPONSE TO STRESS	» COL17A1: collagen type XVII alpha 1
» AHR: aryl hydrocarbon receptor	» COL1A1: collagen type I alpha 1
» ARNT: aryl hydrocarbon receptor nuclear translocator	» COL3A1:collagen type Ill alpha 1chain
» CAT:catalase	» COL4A2: collagen type IV alpha 2
» GPXl: glutathione peroxidase 1	» COL?A1: collagen type VII alpha 1
» HMOXl: heme oxygenase 1	» DCN: decorin
» MTlA: metallothionein 1A	» DPT: dermatopontin
» MT2A: metallothionein 2A	» DSCl: desmocollin 1
» NFE2L2: nuclear factor, erythroid 2 like 2	» DSGl: desmoglein 1
» NQOl: NAD(P)H quinone dehydrogenase 1	» DSG3: desmoglein 3
» S001: superoxide dismutase 1, soluble	» ELN: elastin
» S002: superoxide dismutase 2, mitochondrial	» FBNl: fibrillin 1
» TXN: thioredoxin	» FNl: fibronectin 1
» TXNRDl: thioredoxin reductase 1	» SERPINHl: serpin family H member 1
CELL RENEWAL/ REGENERATION	» TIMPl: TIMP metallopeptidase inhibitor 1
» CALMLS:calmodulin like 5	» TIMP2:TIMP metallopeptidase inhibitor 2
» CASPl 4: caspase 14	» TNC: tenascin C
» CASP3: caspase 3	» VCAN: versican
» GSK3B: glycogen synthase kinase 3 beta	**GROWTH FACTOR**
» KRTl 4: keratin 14	» BMP4: bone morphogenetic protein 4
» KRTS: keratin 5	» EDNl:endothelin 1
» PCNA: proliferating cell nuclear antigen	» KITLG:KIT ligand
» PPARD: peroxisome proliferator activated receptor delta	» CTGF: connective tissue growth factor
» PPARG: peroxisome proliferator activated receptor gamma	» EGFR: epidermal growth factor receptor
» TGFBl: transforming growth factor beta 1	» HBEGF: heparin binding EGF like growth factor
» TP63: tumor protein p63	» ICAMl: intercellular adhesion molecule 1
EPIDERMAL BARRIER	» VEGFA: vascular endothelial growth factor A
» CDSN: corneodesmosin	**HYDRATION**
» CLDNl: claudin 1	» AQP3: aquaporin 3 (Gill blood group)
» CLDN7: claudin 7	» CAPNl: calpain 1
» FLG: filaggrin	» CD44: CD44 molecule (Indian blood group)
» GRHL3: grainyhead like transcription factor 3	» GBA:glucosylceramidase beta pseudogene 1; glucosylceramidase beta
» ITGBl: integrin subunit beta 1	» HAS2:hyaluronan synthase 2
» ITGB4: integrin subunit beta 4	» SMPDl: sphingomyelin phosphodiesterase1
» IVL: involucrin	**INFLAMMATION / IMMUNE**
» KRTl: keratin 1	» ADAM17: ADAM metallopeptidase domain 17
» KRTl0: keratin 10	» CSF2: colony stimulating factor 2
» LCE3D: late cornified envelope 30	» CXCLB/ILB: C-X-C motif chemokine ligand 8
» LOR: loricrin	» DEFBl: defensin beta 1
» OCLN: occludin	» IFNA1: interferon alpha 1
» PKPl: plakophilin 1	» IL10: interleukin 1O
» ST14: suppression oftumorigenicity14	» IL1A: interleukin 1 alpha
» TGMl: transglutaminase 1	» Ill B: interleukin 1beta
	» IL1RN: interleukin 1receptor antagonist
	» IL23A:interleukin 23 subunit alpha
	» IL6: interleukin 6
	» PTGSl: prostaglandin-endoperoxide synthase 1
	» PTGS2: prostaglandin-endoperoxide synthase 2
	» TLR2: toll like receptor 2
	» TLR3: toll like receptor 3
	» TNF: tumor necrosis factor

Figure A1. Genemarkers standard gene panel list.

Appendix 2

Table A1. Visia skin markers measurements for the full cohort at baseline and after 4 weeks of using the Formulation within pre-established skincare regimen.

Skin Markers	(4weeks—Baseline) %							
	Participant 1	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Mean
Spots	−2%	21%	6%	−10%	−7%	11%	28%	6.71%
Wrinkles	10%	0%	−5%	−33%	67%	0%	−29%	1.43%
Texture	15%	−1%	−3%	−2%	31%	41%	−7%	10.57%
Pores	34%	−10%	28%	−7%	−18%	5%	0%	4.57%
UV Spots	−2%	−4%	5%	−12%	−32%	2%	9%	−4.86%
Brown Spots	20%	9%	10%	−12%	−3%	−2%	18%	5.71%
Red Areas	−6%	2%	−17%	7%	6%	−6%	8%	−0.86%
Porphyrins	−2%	−10%	4%	−21%	−28%	49%	−17%	3.57%