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Up-Regulated Expression of SOD2 and HPRT1 Following Topical Photoprotection and Photorepair Skincare Formulations in A 3-Dimensional Reconstructed Human Skin Model

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Abstract

Photodamage continues to threaten human skin health despite worldwide sun awareness campaigns and the widespread use of sunscreens. To date, extensive research is lacking into the effects of sun avoidance and solar specific skincare regimens on gene expression changes and DNA repair activity. We have previously reported that photoprotection and photorepair formulations which minimize the harmful effects of ultraviolet, visible light and near-infrared radiation can provide photoprotection, anti-photoaging benefits and rejuvenating effects optically, clinically and genetically. To investigate gene expression changes, specifically antioxidant and DNA repair effects following the use of topical photoprotection and photorepair formulations (The Essential Six, RATIONALE, Victoria, Australia), we used epidermal keratinocytes and dermal fibroblasts derived from a 3-dimensional reconstructed human skin model, and assessed upregulation of SOD2 and HPRT1. Gene expression was assessed via the Genemarkers Standard Skin Panel and quantitative real-time PCR exploration. Tissues were inoculated with solar specific topical formulations, then collected after 24 hours following application of photoprotection formulations and 16 hours following photorepair formulations. The quantitative real-time PCR revealed that, in comparison to the control, the genes encoding SOD2 and HPRT1 have been significantly up-regulated following usage of the photoprotection formulations, 1.86, and 1.41, respectively. SOD2 and HPRT1 were up-regulated following use of the photorepair formulations, 2.15, and 1.28, respectively. We were able to substantiate that the photo protection and photorepair formulations upregulated genes involved in

antioxidant and DNA repair mechanisms in a 3-dimensional reconstructed human skin model, suggesting a promising anti-photoaging skin regimen.

Keywords

Antioxidant, Anti-Photoageing, DNA Repair, Gene Expression, Photoprotection, Photorepair

1. Introduction

Solar exposure negatively impacts the health and appearance of human skin. The composition of incident solar energy is over 50% Near-Infrared (NIR), 40% Visible Light (VL) and less than 10% Ultraviolet (UV) radiation [1] [2]. Solar damage and skin diseases including skin cancers had thought to be prevented by using sunscreen, and world-wide educational campaigns have been conducted with the aim of preventing solar skin damage, leading to the widespread adoption of sunscreen use. However, cutaneous photodamage continues to pose a threat to human health worldwide [2]-[8].

In prior studies, the authors optically reported the inability of global sunscreens (SPF50+, PA+++ or ++++) to universally protect skin against the entire solar spectrum (UV, VL and NIR) [6] [8]. Also, the authors optically, clinically and genetically reported that specific Australian skin health formulations including immune-enhancing, antioxidant, solar protective, barrier restoration, pH recalibration and DNA repair strategies provide comprehensive photoprotection, anti-photoaging and facial rejuvenating effects [6]-[11]. Interestingly, the photoprotection and photorepair formulations investigated in our prior studies provided significant improvements in terms of skin texture, appearance, clarity and firmness as demonstrated in multidimension assessments (2D and 3D) [6] [7]. Digital facial topography analyses were conducted as objective computer 2D assessments using VISIA Complexion Analysis (Canfield Scientific Inc., Fairfield, NJ) [6] [7]. Eight objective parameter analytics were executed immediately following image capture with photography modes including UV, color and cross-polarized light [6] [7]. Improvements in redness and pigmentation were evaluated by percentile which was calculated to include data variables of sex, age and ethnicity. 3-D imaging was used as 3D assessments, acquired with a VECTRA Handy Camera and associated software (Canfield Scientific Inc., Fairfield, NJ) [7]. Differences in facial tissue volume pre and post-treatment were demonstrated via 3-D schematics [7]. Furthermore, most patients participating in self assessment studies reported high levels of satisfaction with both the formulations and visible results [6] [7].

Antiaging strategies using skincare topicals require scientific proof of efficacy, and an important investigation in this realm involves qualification and quantification of gene expression changes [11] [12] [13] [14]. However, there remain few studies that examine gene expression changes and DNA-mitigated photo-

protection and photorepair activity following the use of topical photoprotection and photorepair formulations ranging from UV through to NIR exposure [11].

Superoxide dismutase 2 (SOD2) is a manganese containing enzyme located in mitochondria that protects cells against oxidative stress [15]. Mutations in this gene have been associated with idiopathic cardiomyopathy, premature aging, sporadic motor neuron disease, and cancer [16]. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) is thought to function in cell cycle and proliferation mechanisms, DNA replication and repair, and RNA metabolism [17] [18].

To investigate gene expression changes regarding antioxidant and DNA repair effects following topical photoprotection and photorepair formulations, we used epidermal keratinocytes and dermal fibroblasts derived from a 3-dimensional reconstructed human skin model, and assessed SOD2 and HPRT1 upregulation.

2. Materials and Methods

2.1. Skin Model

For this study, a reconstructed multilayered 3D human skin model containing normal, human-derived epidermal keratinocytes (NHEK) and normal, human-derived dermal fibroblasts (NHFB) was used. (EpiDermFT, MatTek, MA, USA). The cultures of NHEK and NHFB are configured to replicate a human multilayer and highly differentiated epidermis and dermis. The skin model is composed of epidermally organized basal, spinous, granular, and cornified epidermal layers similar to those in human (in-vivo) skin anatomy. The dermal compartment contains a collagen matrix incorporating viable normal human dermal fibroblasts (NHDF). The EpiDermFT system is active on a mitotic and metabolic level. Structurally and morphologically, the NHEK and NHFB array is accurate in replicating consistent and reliable levels of differentiation and intercellular activity analogous to human skin.

2.2. Topical Skincare Formulations

The skincare formulations used in this study (The Essential Six from RATIONALE, Victoria, Australia) represent a comprehensive daily photoprotection and nightly photorepair regimen. The photoprotection formulations used in this study provide extensive protection against the broader solar spectrum beyond UV (approximately 200 nm to 3000 nm). The Essential Six daily regime is composed of three photoprotective formulations for day (#1 The Serum, #2 The Serum, and #3 The Tinted Serum SPF50+) and three nightly photorepair formulations (#4 The Crème, #5 The Serum, and #6 The Night Crème). Each formulation is a task-specific specific combination of up to 100 compounds grouped and listed by function or nature below (**Table 1**).

2.3. Photo Protection Formulations

#1 The Serum

Vitamins B complex and Australian botanical extracts enhance skin immune

Table 1. Each RATIONALE Essential Six formulation contains a specific combination of upto 100 photoactive compounds grouped and listed by function or nature below.

Photoprotection Formulations			Photorepair Formulations		
#1 The Serum	**#2 The Serum**	**#3 The Tinted Serum**	**#4 The Crème**	**#5 The Serum**	**#6 The Night Crème**
Vitamin B, E	Vitamin A, C, E	Vitamin B, E, D presursor	Vitamin C, E	AHA, BHA	Vitamin A, E
Complex and Essential Fatty Acids			Complex and Essential Fatty Acids		
15 Amino Acids			15 Amino Acids		
Humectants & Penetration Enhancers			Humectants & Penetration Enhancers		
Australian Botanical Extracts			Australian Botanical Extracts		
Plant Extracts			Plant Extracts		
	Emollients and Waxes	Emollients and Waxes	Emollients and Waxes	Anti-Inflammatories	Emollients and Waxes
Stabilizers and Preservatives			Stabilizers and Preservatives		
Thickener & Emulsifiers			Thickener & Emulsifiers		
Minerals, Peptides, Ferments,	Enzymes, Minerals, Protein,	UV Filter, Protein, Acids, Minerals, Sugars, Extracts	Minerals, Peptides, Sugars	Minerals	Extracts, Ferments, Minerals
		Pigments	Pigments		
		Texturizers			Texturizers

responses to protect from photoimmunosuppression.

#2 The Serum

An extensive complex of skin identical vitamins, minerals and enzymatic antioxidants help prevent the formation of Reactive Oxygen Species (ROS) and free radicals induced by solar energy and environmental pollution.

#3 The Tinted Serum SPF50+

A daily photoprotection formulation (UV + VL and NIR radiation) containing zinc oxide, iron oxides, provitamin D, melanin and heat shock proteins.

2.4. Photo Damage Repair Formulations

#4 The Crème

A skin identical composition of stratum corneum lipids including specialised ceramides, triglycerides and cholesterol that assists in restoring and augmenting skin barrier function.

#5 The Serum

This complex of alpha and beta hydroxy acids at low pH maintains stratum corneum pH at optimal acidic levels. Acidic pH of the skin's surface and superficial layers creates the ideal physiological conditions for skin barrier repair and maintenance [19] [20]. All major skin enzymes, including DNA repair enzymes

involved in photo protection and repair are active at acidic pH and are deactivated as skin pH becomes increasingly alkaline.

#6 The Night Crème

A synergistic combination of vitamin A and DNA repair enzymes assists in promoting skin cellular DNA repair processes in response to solar and environmental damage. In human skin, the activity of DNA repair enzymes is linked to retinoid metabolism and bioavailability [21]. This formulation contains a complex of vitamin A (retinol) and DNA repair enzymes obtained from bacterial ferments.

Four cultures were used with each treatment group (photoprotection and photorepair formulations). Four tissue samples were inoculated with 5 uL of each of the photorepair formulations at the centre of each EFT-400 culture, one after the other using a calibrated positive displacement pipette and a sterile glass spreader to distribute the topical materials across the surface in between each application.

Post application, the cultures were returned to the incubator at 37˚C with 5% CO_2 and ~95% relative humidity. Eight hours later the tissues were rinsed off to clear tissues and returned to the incubator until the next day. Four other tissues were inoculated with 5u L of each of the photoprotection formulations at the centre of each EFT-400 culture, one after the other using a calibrated positive displacement pipette and a sterile glass spreader to distribute the topical materials across the surface in between each application. The cultures were then returned to the incubator and collected 24 hours later the next morning. Photoprotection samples were also collected on that morning.

2.5. RNA Extraction

After collection, each sample culture was placed in a RNAlater preservative solution and inclubated at room temperature for 1 - 2 hours then transferred to refrigeration at 4˚C awaiting RNA isolation.

Using the Maxwell RSC Simply RNA Kit (Promega), extraction and isolation were conducted on 12 highly differentiated cultures (encompassing the negative control and 4 samples per test material) of 3D reconstructed epidermal keratinocyte and dermal fibroblast models (Mattek EFT300). Quantitative and qualitative measurements of RNA sample composition was determined using UV absorbance.

2.6. cDNA Synthesis

A High Capacity cDNA Snthesis Kit (Applied Biosystems) was used to produce cDNA samples. Approximately 2000 ng of RNA per sample was required to generate first-strand cDNA.

2.7. OpenArray Processing and Analysis

The use of validated gene expression assays in an OpenArray and 384-well for-

mat was required to perform qPCR reactions. QuantStudio 12 K Flex instrumentation (Life Technologies) was used to conduct all assays. Every gene assay was duplicated to ensure accuracy. ThermoFisher Connect Software (Life Technologies) was deployed to generate statistical analysis from raw data obtained from the qPCR method.

The relative quantification (RQ) methodology was used to ascertain the statistical analysis, while the difference of quantification Cycle (dCq) values were calculated by standardizing the quantification Cycle (Cq) of the target genes to the Cq value of an endogenous control gene. Using this method, potential variability potentially occurring between various samples during the experiment was highlighted.

2.8. Endogenous Control Gene Selection

In order to isolate a consistently expressed control gene, endogenous control identification and selection is critical. For an OpenArray, five candidate control genes-Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH), Peptidylprolyl Isomerase A (PPIA), Hypoxanthine Phosphoribosyltransferase (HPRT1), Polyubiquitin-C (UBC) and β-Glucuronidase (GUSB) were analysed. For the 381-well format, two candidate control genes were selected-(GAPDH and PPIA). Normalized (dCq) values were used to perform each comparison for statistical analysis (unpaired t-tests). ThermoFisher Data Connect RQ software was used to calculate the most consistent endogenous control gene, based on stability score and range scores. Gene expression is more consistent between different samples where the stability score is lowest. For both the OpenArray and 384-well plate formats, PPIA was selected as the endogenous control based on these criteria.

2.9. qPCR Data Quality and Statistical Data Analysis

A variety of techniques, including visual assessment of the qPCR graph and the Cq value were used to assess the quality of qPCR data.

The quality of the qPCR data can be impacted by the transcript total of the sample, indicated bby the Cq values.

Typically occuring before cycle 30 in a 384-well plate, qPCR amplification takes places over a total of40 cycles. Cq is linked to the relative quantity and quality of transcript:

- Robust, high quality PCR data corresponds to high transcript where Cq values are below 30
- Less reliable quality qPCR data corresponds to lower transcript levels, where Cq values are above 30.

3. Results

The quantitative real-time PCR identified that, in comparison to the control, the genes encoding SOD2 and HPRT1 have been up-regulated following usage of the photoprotection formulations, 1.86, and 1.41, respectively. SOD2 and

HPRT1 were up-regulated following use of the photorepair formulations, 2.15, and 1.28, respectively (Figure 1).

4. Discussion

The restoration of youthful, healthy skin and facial profile are amongst the most desired benefits of patients seeking aesthetic medical procedures including facelifting, cosmetic injectables and phototherapy [6]. Aggressive procedures and ablative treatments induce tissue damage, and have been largely replaced by non-invasive procedures due to risk of complications and inconsistent outcomes. Laser or light therapies may provide the expected results as long as the treatment is continuous in-clinic. Nevertheless, these results often come with significant inflammation and downtime. Medical procedures such as, facelifting, thread lifting and fillers provide some level of improvement as skin and subcutaneous tissues are lifted but would not provide actual, visible skin rejuvenation [6]. Physical and mental health implications caused by solar damage and skin ageing highlight the imperative for home-based, non-invasive treatments [6].

Solar exposure generates ROS that damage DNA, lipids, membranes, mitochondria and proteins [22]. To protect against such damage, skin cells have evolved protective antioxidant enzyme systems including endogenous skin antioxidants glutathione peroxidase (GSH-Px), copper and zinc-dependent superoxide dismutase (SOD1), SOD2, and catalase [22]. Human skin cells prepare for subsequent solar exposure by upregulating this antioxidant defence network, in particular mitochondrial SOD2 [22]. UVA induces the generation of ROS that indirectly cause oxidative damage to DNA, leading to nucleic acid mutations and potentially skin cancer [23] [24]. UVB damages cell membranes and proteins of the skin, leading to sunburn erythema that can result in skin cancer in extreme cases [25] [26]. ROS can activate the expression of Matrix Metalloproteases that degrade the extracellular matrix including collagen and elastin fibers in the

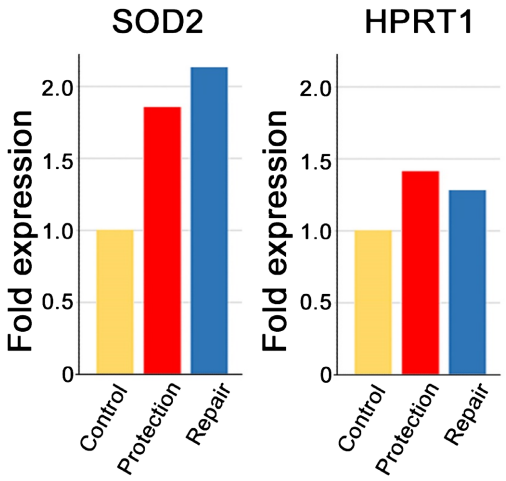

Figure 1. Quantitative real-time PCR validation of SOD2 and HPRT1 gene expressions. Fold-change in expression was calculated by setting the median value of expression seen in the control to 1.0.

dermis, causing wrinkles, seborrheic dermatitis, barrier dysfunction and dullness [27] [28]. Oxidative stress is caused by an excess of free radicals in cells and contributes to diseases such as photoaging and inflammation [29]. The redox homeostasis in mitochondria is tightly regulated by antioxidant enzymes such as SOD2 [30], and acetylation of SOD2 plays an important role in regulating its antioxidant activity [31]. Mutations in SOD2 gene have been associated with idiopathic cardiomyopathy, premature aging, sporadic motor neuron disease, and cancer [16].

The protein encoded by HPRT1 gene is an enzyme transferase, which plays a crucial role in the generation of purine nucleotides through the purine salvage pathway [17]. The HPRT1 enzyme recycles nucleotides to activate DNA and RNA synthesis in vital and actively dividing cells, explaining the ubiquitous presence of HPRT1 in most tissues [17]. HPRT1 plays an essential role in cell functions, such as cell cycle and proliferation mechanisms, DNA replication and repair, and RNA metabolism [18].

In this study, the genes encoding SOD2 and HPRT1 have been up-regulated following usage of the photoprotection formulations, 1.86, and 1.41, respectively. Additionally, SOD2 and HPRT1 were up-regulated following use of the photorepair formulations, 2.15, and 1.28, respectively (**Figure 1**).

SOD2 was up-regulated following the photoprotection, and photorepair formulations; 1.86, and 2.15, respectively. SOD2 protects cells against oxidative stress by scavenging ROS, and SOD2 activity is inhibited through acetylation under conditions of stress such as solar exposure [15]. Up-regulation of SOD2 seen in this study indicates enhanced photoprotection and potent antioxidant ability, and may be potentially beneficial for photoprotection and anti-photoaging.

HPRT1 was up-regulated following tissue treatment with the photoprotection, and photorepair formulations; 1.41, and 1.28, respectively. HPRT1 functions in cell cycle and proliferation mechanisms, DNA replication and repair, and RNA metabolism [17] [18]. Up-regulation of HPRT1 appears to be beneficial for skin repair and rejuvenation, and may also indicate enhanced photoprotection and anti-photoaging potential.

For this study, an *in vitro* skin model was used, incorporating epidermal keratinocytes and dermal fibroblasts from a multilayered 3-dimensional cultured human skin model. This reconstructed skin model is highly comparable to that of living human skin, and the *in vitro* results appear to indicate the living human skin reaction following the photoprotection and photorepair formulations. This was established through histological analysis which revealed a fully stratified epidermis containing all major epidermal layers and component cells as well as a dermal compartment and its collagen matrix. This highly analogous human skin model proved highly predictive and accurate in understanding the biological impacts of the photoprotection and photorepair formulations.

Our findings that specific genes involved in enhanced antioxidant and repair mechanisms namely, SOD2 and HPRT1 were upregulated using the selected so-

lar protection and repair formulations warrants further investigation, particularly *in vivo* studies. Although significant upregulation of the gene expressions occurred following tissue treatment with the photoprotection and photorepair formulations, further research is needed to determine whether other skincare ingredients, treatments or medical procedures could promote enhance further changes in gene expression. Furthermore, it shoud be noted that this study was a preliminary assessment, suggesting that a larger pool of samples as well as a protein expression study could follow.

5. Conclusion

This study demonstrates that the tested photoprotection and photorepair formulations are capable of upregulating genes that are significantly active in antioxidant and repair mechanisms in human skin, potentially representing a promising anti-photoaging skin regimen.

Disclosure

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Conflicts of Interest

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

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