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Gene Expression Change Analysis Following Specific Comprehensive Solar Protection SPF50+ after Ultraviolet Light and Blue Light Exposure in a 3-Dimensional Reconstructed Human Skin Model

Tanaka Y[1], Parker R[2] and Aganahi A[2]*

[1]*Clinica Tanaka Plastic, Reconstructive Surgery and Anti-aging Center, Japan*

[2]*RATIONALE, Australia*

Abstract

Background: Clinical, optical and histological studies report that solar damage continues to pose a threat to skin health despite sunscreen usage and sun awareness campaigns.

Purpose: To investigate gene expression changes following topical solar protection after ultraviolet and blue light exposure.

Methods: Using epidermal keratinocytes and dermal fibroblasts derived from a 3-dimensional reconstructed human skin model, gene expression was assessed via the Genemarkers Standard Skin Panel deploying DNA microarray and quantitative real-time PCR exploration. All of the tissue samples were then inoculated with the solar protection formulation capable of blocking solar energy from ultraviolet, visible light (including blue light) and near-infrared energy (#3 The tinted Serum SPF50+, RATIONALE, Victoria, Australia).

Results: A DNA microarray analysis revealed 13 genes that were dramatically up-regulated by at least two-fold out of 107 genes after ultraviolet exposure, and 12 genes that were dramatically up-regulated by at least two-fold out of 52 genes after blue light exposure. The quantitative real-time PCR revealed that, in comparison to the control, the genes encoding Toll Like Receptor 5 (TLR5), Metallothionein 1G (MT1G), Tumor Protein 73 (TP73), Filaggrin (FLG), and Caspase 14 (CASP14) were up-regulated after ultraviolet light exposure, 4.57, 2533.59, 4.94, 13.46 and 2.13, respectively. Lecithin Retinol Acyltransferase (LRAT), Sequestosome 1 (SQSTM1), Loricrin (LOR), RAR Related Orphan Receptor A (RORA) were up-regulated after blue light exposure, 6.07, 2.3, 51.82, and 3.56, respectively.

Conclusion: This study demonstrates that the specific comprehensive solar protection upregulated genes involved in photoprotection and anti-photoaging mechanisms in a 3-dimensional reconstructed human skin model.

Keywords: Gene expression; Anti-photoaging; Antioxidant; Photoprotection; Barrier repair; DNA repair; Hydration; Near-infrared

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Correspondence:
Yohei Tanaka, Clinica Tanaka Plastic, Reconstructive Surgery and Anti-aging Center, M-1 Bld 1F, 3-4-3, Ote, Matsumoto, Nagano, 390-0874, Japan, Tel: +81-263-36-0016; Fax: +81-263-36-0016

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Introduction

The composition of the sun's incident solar energy is approximately less than 7% Ultraviolet (UV), 40% Visible Light (VL), and over 50% Near-Infrared (NIR) radiation [1,2].

Excessive UV exposure is heavily implicated in inducing both photoaging and carcinogenesis through direct and indirect DNA damage, with the UV dose absorbed by DNA chain base pairs in the DNA chain accounting for the differing mechanisms and degree of UVA and UVB damage. Characteristic mutations in nucleic acid chains, including the formation of pyrimidine base transversions and cyclobutane pyrimidine dimers result from the direct biological activity of UVB radiation on cellular DNA [3]. Likewise, UVA radiation bioactivity leads to damage and transition of DNA Bases in addition to the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts [4,5].

Through direct interaction with skin chromophores including opsins, nitrosated proteins,

porphyrins and flavins, Blue Light (BL) instigates changes in skin biomolecules by triggering changes from ground to activated state. This results in a cascade of undesirable *in situ* skin reactions, including the release of nitrogen monoxide and overproduction of Reactive Oxygen Species (ROS). A visible example of this process is solar induced hyperpigmentary conditions [6].

In recent decades, world-wide educational campaigns have been conducted with the aim of preventing photoaging and skin cancers, leading to the widespread adoption of sunscreen use. However, rates of photoaging, photoimmunosuppression and skin cancer continue to rise worldwide, posing a threat to human health despite widespread recognition of the dangers of solar skin damage [2,7-11].

In their preceding work, the authors found that most globally available sunscreen formulations, whether they are SPF 50+, PA+++ or ++++ are incapable of providing skin protection from the complete solar spectrum, which includes not only UV but also VL and NIR radiation [2,7-15]. Most sunscreens only offer protection in the UV range, when in reality skin damage also results from VL and NIR exposure [2,7-15]. Increasingly, dermatologists are recommending comprehensive skin regimes that protects from UV, VL and NIR. This involves not only advanced sunscreens, but also topical immune boosters, antioxidants, barrier lipids; pH recalibration and DNA repair strategies [2,7,15].

The authors also reported that comprehensive solar protection and repair regimes provide significant improvements in terms of skin texture, appearance, clarity, and firmness as demonstrated in multidimension assessments (2D and 3D) [2,7]. As the effects of solar energy (UV and BL) on the skin are now understood to be the cause of skin ageing, it is reasonable to hypothesize that this skincare approach is able to provide optimal protection and repair of solar damage [2,7-15].

Despite this, there remain few studies that examine gene expression changes and DNA-mitigated skin photoprotection and anti-photoaging activity following comprehensive solar protection after UV and BL exposure.

To investigate gene expression changes following comprehensive solar protection which provides effective blocking from UV through to NIR, we assessed changes in gene expression activation or inhibition using various DNA assay and analysis methods on a 3D human skin model containing epidermal keratinocytes and dermal fibroblasts.

Methods

Skin model

A 3-D multilayered, full thickness reconstructed *in-vitro* human skin replica model containing normal. Human-Derived Fibroblasts (NHFB) and normal, Human-Derived Epidermal Keratinocytes (NKEK) (EpiDermFT, MatTek, MA, USA) were used in this study. At a mitotic and metabolic level, the EpiDermFT is biologically active. This replica contains a skin identical, highly differentiated dermis and epidermis. A collagen matrix composed of viable Normal Human Fibroblasts (NHDF) is contained in the dermal compartment, while organized cornified, granular, and spinous and basal cells reflect a normal epidermal layer structure. In addition, various markers native to mature *in vivo* epidermal processes of cellular differentiation, (including Type 1 Epidermal Transglutaminase, the K1/K10 cytokeratin pairing, Involucrin and Pro-Filaggrin) as well as specific intercellular lamellar lipid layers and structural markers (desmosomes, keratohyalin granules and tonofilament bundles) are located in positions analogous to the patterns seen in human epidermal tissue *in situ*. In this model, a well-defined basement membrane supports a skin Identical Dermal/Epidermal Junction (DEJ).

Structural and signaling proteins (integrin α6, anchoring fibrils, collagen V11 and collagen IV), as well as lamina densa, lamina lucida and hemidesmosomes are all present. Due to its accuracy in reflecting experimental changes in skin anatomy and physiology, the EpiDermFt is considered a reliable, non-invasive and humane replica for assessments involving skin photoprotection and photorepair, wound healing, anti-aging and collagen synthesis. Changes in skin histology, gene expression, protein expression, cytokine release and other skin-specific markers can all be studied and observed following modern-day techniques applied to the straightforward and clear protocols involving the EpiDermFt model.

Good Manufacturing Practice (GMP) has been deployed by EpiDermFT to ensure high accuracy and reproducibility of outcomes.

Consistent, reliable and replicable levels of intercellular activity and cellular differentiation can be observed structurally and morphologically using the NHEK and NHFB replicas, which are considered analogous to human skin tissue anatomically and physiologically.

Topical comprehensive solar protection

A comprehensive solar protective formulation (#3 the tinted Serum SPF50+, from RATIONALE, Victoria, Australia) was used in this study (Table 1). This technology involves comprehensively shielding the skin against the broader solar spectrum (approximately 200 nm to 3200 nm).

It contains vitamins B, D precursor and E, complex and essential fatty acids, 15 amino acids, humectants, penetration enhancers, Australian botanicals extracts, plant extracts, emollients, waxes, UV filter, protein, acids, minerals, sugars, extracts, pigments, texturizers, thickeners, emulsifiers, stabilizers and preservatives.

Four tissue samples were inoculated in the center of each EFT-400 culture, one after the other using a calibrated positive displacement pipette with 5 uL of each of the Solar Protection Formulations. The Formulations were distributed across the sample surface using a calibrated positive displacement pipette and sterile glass spreader.

Tissues were exposed to UVB for 24 h (UVB group), and exposed to BL for 6 h per day for 5 consecutive days (BL group). Tissues were then inoculated with the Solar Protection Formulation.

Cultures were returned to an incubator maintained at 37°C with 5% CO_2 and approximately 95% relative humidity post application until the next day.

Four separate tissue samples were inoculated with 5 uL of each of the Solar Protection Formulations using the same application technique and equipment. These samples were then returned to the incubator and maintained at the same temperature and relative humidity as the first samples and collected along with the Solar Protection samples. The tissues were then washed and prepared with sterile DPBS to remove any surface culture.

Extraction of RNA

Following removal of samples from the incubator at 37°C and 5% CO_2 and approximately 95% humidity, each culture was incubated at room temperature for 1 h to 2 h in an RNA preservative solution tube. Each tube was then transferred to a 4°C refrigerator awaiting RNA isolation post-incubation.

Using the Maxwell RSC Simply RNA kit (Promega), highly differentiated cultures of reconstructed 3-D human epidermal keratinocytes and human dermal fibroblasts (Mattek EFT-400) were processed to extract and isolate the total RNA content of 12 (4 per test material plus the negative control) samples.

Qualitative and quantitative measurements of the RNA samples were determined using UV absorbance.

Synthesis of cDNA

A high-capacity cDNA Synthesis Kit (Applied Biosystems) was used to produce cDNA samples. To generate first-strand cDNA, a total of approximately 2000 ng of RNA was required.

Processing and analysis with OpenArray

Validated gene expression assays in a 394-well format and OpenArray were required to perform the relevant qPCR reactions. A Life Technologies QuantStudio 12K Flex instrument was used to conduct assays. Every isolated gene was assayed in duplicate to ensure accuracy of results. ThermoFisher Connect Software (Life Technologies) was used to obtain quality raw data and conduct the statistical analysis from the qPCR method. The difference (delta) of the quantification Cycle (dCq) values were ascertained by normalizing the quantification Cyle (Cq) value of target genes to the Cq value of an endogenous control gene using the relative (RQ) method to determine the statistical analysis. Variability occurrences during the experimental process between several samples are highlighted using this method.

Gene selection-endogenous control

In order to isolate a consistently expressed control gene, endogenous control gene selection is critical. For the OpenArray, Hypoxanthine Phosphoribosyltransferase (HPRT1), β-Glucuronidase (GUSB), Peptidylprolyl Isomerase A (PP1A), Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) and Polyubiquitin-C (UBC) were considered as the five candidates for the control genes. For the 384-well plate format, the two nominated control genes were GAPDH and PPIA. Normalized dCq values using statistical analysis (unpaired t-tests) were performed for each comparison. Analysis with the OpenArray method resulted in the selection of two genes (GAPDH and PPIA) for the 394-well plate method as the control genes. Calculations using the ThermoFisher Data Connect RQ software identified the stability score and range scores to determine the most consistent endogenous control gene, with gene expression being more consistent between different samples the lower the stability scores. Based on this data, PPIA emerged as the ideal endogenous control gene for both the 384-well plate and OpenArray formats.

Statistical data analysis and qPCR quality

A variety of verification techniques including visual assessment of the Cq value and qPCR curve were used to assess the quality of qPCR data. Typically occurring before cycle 30 in a 384-well plate format, qPCR amplification typically occurs over a sum of 40 cycles, while the quality of qPCR data can be influenced by the total transcript present, as indicated by Cq values. The Cq values are then linked to the relative quantity and quality of transcript:

- High quality, robust PCR data correlating to high transcript levels can be expected when Cq values are below 30
- Less robust data, which should be reviewed cautiously is generated when Cq values are above 30, corresponding to lower transcript levels

Results

A DNA OpenArray identified 74 genes that were statistically significantly up-regulated or down-regulated and 13 genes that were dramatically up-regulated by at least twofold out of 107 genes after UV exposure.

A DNA OpenArray identified 51 genes that were statically significantly up-regulated or down-regulated and 12 genes that were dramatically up-regulated by at least two foldout of 52 genes after BL exposure.

When compared to the control cells, quantitative real-time PCR identified that, the genes encoding Toll Like Receptor 5 (TLR5), Metallothionein 1G (MT1G), Tumor Protein 73 (TP73), Filaggrin (FLG), and Caspase 14 (CASP14) were up-regulated after UV exposure, 4.57, 2533.59, 4.94, 13.46 and 2.13, respectively (Figure 1).

Lecithin Retinol Acyltransferase (LRAT), Sequestosome 1 (SQSTM1), Loricrin (LOR), RAR Related Orphan Receptor A (RORA) were up-regulated after BL exposure, 6.07, 2.3, 51.82, and 3.56, respectively (Figure 2).

Discussion

Patients exploring antiaging cosmetic medical procedures including phototherapy, facelifting and injectables list improvements in skin tone, clarity, texture and firmness as their most desired benefits [2]. The risks involved, combined with the inconsistent outcomes, undesirable complications and common side effects of such aggressive ablative and surgical operations are one reason why such procedures are not increasing in popularity after several decades of growth. An additional problem is that these modalities (laser, injectables) only have short to medium benefits and must be repeated in the doctor's office at regular intervals, which is expensive and carries a continual risk of complications, significant inflammation and undesirable downtime. Facelifting may offer some visible improvement due to

Table 1: Ingredients of comprehensive solar protective formulation (#3 The tinted Serum SPF50+, from RATIONALE, Victoria, Australia) used in this study.

Comprehensive solar protective formulation (#3 The tinted Serum SPF50+)
Zinc Oxide
Iron Oxide
Erioglaucine
Vitamins B, D (precursor) E
Essential Fatty Acid Complex
15 Amino Acids
Humectants & Penetration Enhancers
Australian Botanicals Extracts
Plant Extracts
Emollients and Waxes
Stabilizers and Preservatives
Film Formers, Thickeners, Emulsifiers, Texturizers

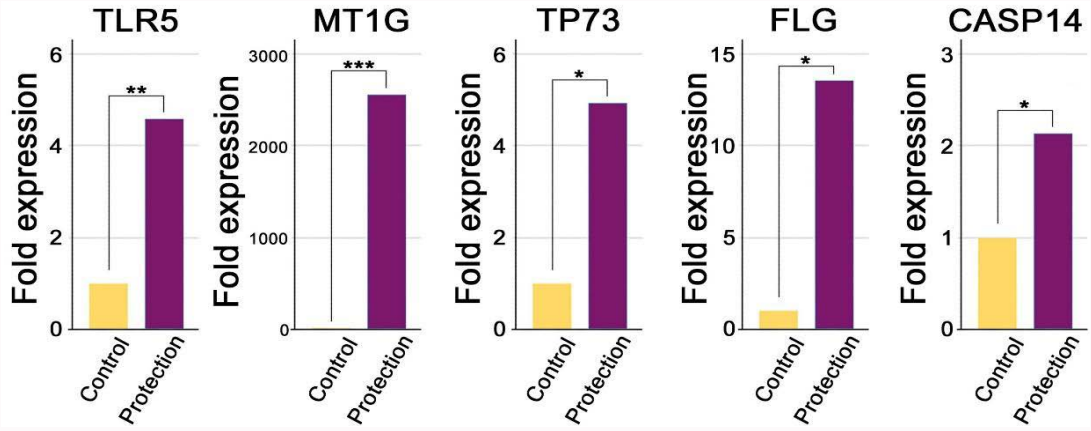

Figure 1: Quantitative real-time PCR validation of TLR5, MT1G, TP73, FLG, and CASP14 gene expressions. Fold-change in expression was calculated by setting the median value of expression seen in the control to 1.0. *p<0.05, **p<0.01, ***p<0.001.

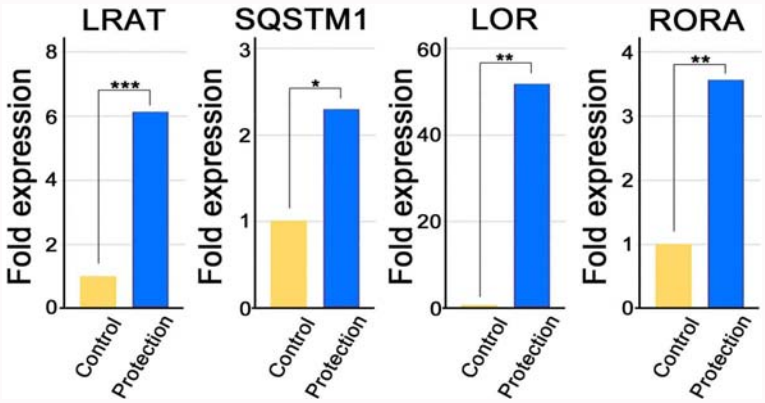

Figure 2: Quantitative real-time PCR validation of LRAT, SQSTM1, LOR, and RORA gene expressions. Fold-change in expression was calculated by setting the median value of expression seen in the control to 1.0. *p<0.05, **p<0.01, ***p<0.001.

the elevation of skin and sub-tissues, but skin rejuvenation is not achieved, creating an incongruous mismatch between lifted muscle and sun damaged skin. The visual rejuvenation will not be achieved by face lifting alone [2].

Increasingly, home-based, non-invasive treatments to prevent and repair negative solar effects are being recognized and recommended to reduce the physical and psychological burden of solar skin damage [2,7].

As reported in our previous studies, significant improvements in skin tone, texture and profile were achieved using this solar protection formulation and repair regime, with results validated and documented *via* 3-D volumetric assessment and objective analysis by digital facial surface imaging technology [2,7]. Results were impressive, visible and measurable, including patient and physician evaluation [2,7].

Most patients reported high satisfaction with their complexion post treatment, reporting that their skin looked and felt firmer, uplifted and generally rejuvenated [2,7]. Patients also expressed pleasure in using the formulations, with no discomfort, side effects or downtime reported during treatment [2,7].

In this study, the genes encoding *TLR5*, *MT1G*, *TP73*, *FLG*, *CASP14*, *LRAT*, *SQSTM1*, *LOR*, and *RORA* were up-regulated following specific comprehensive solar protection (Figure 1, 2).

***TLR5* was up-regulated following the solar protection; 4.57**. This gene encodes a member of TLR family, which plays a fundamental role in pathogen recognition and activation of innate immune responses. *TLR5* within keratinocytes functions to boost the immune system by defending against pathogens [16]. The protein encoded by this gene recognizes bacterial flagellin, the principal component of bacterial flagella and a virulence factor. The activation of this receptor mobilizes the nuclear factor NF-kappaB, which in turn activates a host of inflammatory-related target genes [17].

Furthermore, TLR5 induces thymic stromal lymphopoietin expression when exposed to bacteria with flagellin, which is an important factor in creating immune responses in allergies, and skin lesion of dermatitis, as well as promoting an effective skin barrier [18]. Up-regulation of TLR5 may enhance immune responses and protection from inflammation.

MT1G was up-regulated following the solar protection; 2533.59. Metallothioneins are small cysteine-rich proteins that play important roles in metal homeostasis and protection against heavy metal toxicity, DNA damage, and oxidative stress [19]. MT1G binds divalent metal ions, acts as an antioxidant, and is up-regulated after exposure to airborne particulates and other skin irritants [20]. Several metallothioneins, including MT1G have been shown to be down-regulated in proliferating keloid fibroblasts [21]. The dramatically increased activity of MT1G seen in this study may indicate potent antioxidant ability, and may be potentially beneficial

for skin protection from photoaging, as metallothionein is related to antiapoptotic properties and the ability to scavenge ageing free radicals [22].

TP73 was up-regulated following the solar protection; 4.94. TP73 is a transcription factor involved in apoptotic activity, tumor suppression, and DNA damage repair. TP73 is found in basal keratinocytes that aid wound healing and skin development, and increases levels of the proliferation biomarker Ki67 in wounded epidermal keratinocytes, which indicates that proliferation correspondingly increases in response to damage [23]. Up-regulation of TP73 may indicate enhanced cell survival and DNA damage repair.

FLG was up-regulated following the solar protection; 13.46. FLG plays a key role in aggregating keratin proteins in the epidermis, and its metabolism is crucial for the development of Natural Moisturising Factors in the skin [24]. FLG is critically involved in skin barrier function through assembly and structural organization of keratins [25]. Up-regulation of FLG appears to be beneficial for skin barrier repair.

CASP14 was up-regulated following the solar protection; 2.13. CASP14 expression is restricted almost exclusively to the suprabasal layers of the epidermis, and is associated with epidermal differentiation and cornification [26]. CASP14-deficient epidermis is characterized by reduced epidermal barrier function, increased sensitivity to UVB radiation, [27] reduced skin hydration levels and increased water loss [26]. Up-regulation of CASP14 is involved in maintaining healthy epidermal barrier function and skin hydration.

LRAT was up-regulated following application of the solar protection formulation; 6.07. LRAT is an important enzyme in vitamin A metabolism that converts all-trans-retinol into all-trans-retinyl esters, the storage form of vitamin A [28]. Depletion of vitamin A has been shown to cause oxidative stress. LRAT is critical for retention of retinoids in the retina, circulation, and certain peripheral tissues [29]. Significant up-regulation of LRAT may indicate enhanced photoprotection and potent antioxidant ability.

SQSTM1 was also up-regulated in this study; 2.3. SQSTM1, which is also known as p62, acts as an autophagy adaptor and substrate for the selective inclusion of cargo [30].

p62/SQSTM1 has roles in the ubiquitin-proteasome system, cellular metabolism, signaling, and apoptosis [31]. p62 has context-dependent impacts on organismal aging and turnover of p62 usually reflects active proteostasis [31]. Up-regulation of SQSTM1 may contribute cellular metabolism and cell survival.

LOR was up-regulated following application; 51.82. The major function of LOR is to reinforce the cornified envelope and to enhance its defensive barrier function [32]. Interaction of LOR with the keratin intermediate filaments provides flexibility to the cornified envelope [33]. LOR also protects against mechanical stress by its association with nectin and calcium induction levels [34]. The dramatically increased activity of LOR may indicate potent barrier repair ability, and is thought to be beneficial for solar skin protection and repair processes.

RORA was also up-regulated; 3.56. RORA is a retinoid-related orphan nuclear receptor that regulates inflammation, lipid metabolism, and regulating proliferation and differentiation of keratinocytes [35]. The identification of RORA ligands may prove useful for treating skin disorders that are associated with abnormal keratinocyte differentiation, including cancer [35]. RORA is also involved in preventing inflammation may promote healthy skin [36]. Up-regulation of RORA is involved in maintaining an optimal skin circadian rhythm and preventing inflammation.

For this study, an *in vitro* skin model was deployed, specifically epidermal keratinocytes and dermal fibroblasts forming a multilayered 3-dimensional cultured human skin replica. This reconstructed skin model is highly comparable to that of living human skin. 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 this specific topical solar protection formulation.

Our findings that specific genes involved in enhanced photoprotection and repair of solar skin damage, namely, *TLR5*, *MT1G*, *TP73*, *FLG*, *CASP14*, *LRAT*, *SQSTM1*, *LOR*, and *RORA* warrants further investigation, particularly *in vivo* studies. Although significant upregulation of the gene expressions occurred following this skincare regime, further research is needed to determine whether other skincare ingredients, treatments or medical procedures could promote enhance further desirable changes in gene expression. Furthermore, this study was a preliminary assessment, suggesting that a larger pool of samples as well as a protein expression study could follow.

Conclusion

This study demonstrates that a specific comprehensive solar protection formulation is capable of up-regulating genes that are significantly active in solar skin protection and repair processes involving the UV and BL spectra.

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