skin life
ANALYTICS
Making the Invisible Visible

The Skin Diary – Gene Expression in 3D-Full Thickness Living Skin Models

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Abstract

The aim of this study was to determine the effects of a physiological dose of solar UV irradiation on gene expression in 3D full-thickness human skin models in the presence and absence of The Skin Diary Age Defence day cream. Five genes were selected for analysis, alongside one housekeeping gene (GAPDH). The genes examined were MMP1, PTGES, and the senescence markers p21, p16, and GLB1. Expression levels were normalised to GAPDH to calculate fold change values.

The results show that The Skin Diary Age Defence day cream was able to provide statistically significant complete protection against increased expression of MMP1, p21 and PTGES when compared to irradiated in the absence of the day cream and also non-irradiated conditions. This 'protection' profile afforded by the formulation was also evident for GLB1 expression although the results did not reach formal significance. The magnitude of the fold protection of UV-induced gene expression provided by the formulation was different depending upon the gene being investigated with the highest fold protection observed for MMP1 followed by PTGES then p21 and GLB1. In contrast, the level of p16 expression was statistically unchanged either by the UV stressor or the intervention of the formulation, likely due to the fact that p16 is a late cellular senescent marker as opposed to a gene like p21 which is an earlier senescence marker.

Experimental Work Summary

The overall aim was to assess and compare the effects of a physiological dose of UV irradiation on gene expression in 3D full-thickness living human skin models in the presence and absence of The Skin Diary Age Defence day cream. Five genes plus a housekeeping gene (GAPDH) were examined in the study. The genes were selected based on their biological roles in photoageing and senescence-related processes.

All test formulations were applied to the stratum corneum of viable skin equivalents prior to UV irradiation. The same protocol was carried out in the absence of product. Twenty-four hours after irradiation, RNA was isolated from the skin equivalents followed by cDNA synthesis and then analysis of gene expression by RT-qPCR. Gene expression analysis was performed in triplicate.

Following discussion, it was agreed that 24hrs after irradiation the skin samples are analysed for expression levels of the following genes:

(1) GAPDH - housekeeping gene
(2) MMP1 - matrix metalloproteinase 1
(3) p21 - early cellular senescence marker
(4) p16 - later senescence marker than p21
(5) PTGES - prostaglandin E synthase, codes for the terminal enzyme which generates PGE2
(6) GLB1 - encodes for beta galactosidase and is a senescence marker

A greater description of the genes is included in the conclusion section.

Methods

Skin model methodology

Phenion® 3D in vitro, full thickness human skin models with an area of 1.5cm2 (Henkel, Germany), were cultured in sterile conditions following manufacturer's guidelines. Each skin equivalent was cultured separately in a 35mm petri dish containing 5ml Air-Liquid Interface (ALI) Medium (Phenion, Henkel, Germany), with sterile filter paper on top of a spacer. Each skin equivalent was transferred onto the filter paper with sterile forceps and cultures were then incubated at 37°C, 5% CO2 for 24 hours before proceeding with experimental conditions.

Following media change, for relevant experimental conditions 2mg/cm2 of product was added to the relevant skin equivalent using a positive displacement pipette (Gilson, USA) and spread using a formulation spreader. Following 30 minutes after cream application, skin equivalents were exposed to 5.0 SED (Standard Erythemal Dose) of solar UV irradiation using a Newport Oriel Solar Simulator (1000W output, MKS Instruments, Inc., USA). For the duration of the irradiation, the petri dish lids were removed to expose the skin equivalent, with non-irradiated controls wrapped in aluminium foil. Following irradiation, for each skin equivalent, the ALI medium was replaced with fresh ALI medium and all equivalents were returned to incubation at 37°C, 5% CO2 for 24 hours.

Standard Erythemal Dose (SED) is not linked to skin type, unlike minimal erythemal dose (MED), and is a skin type independent, weighted measurement of sun exposure equivalent to 100 Jm2, as opposed to MED which is the lowest dose required to produce erythema in an individual. The skin cells used in this current study do not exhibit erythema and so MED is not relevant, therefore SED represents the unit dose (Diffey 2021, Development and Validation of an Algorithm to Predict the Erythemal Ultra Violet Dose). This publication shows that 2 SEDs approximates to just less than 1 MED in the Mediterranean sun at noon in midsummer depending upon the light source. This is a dose equivalent to a physiological solar UV environment for the skin, therefore 5 SEDs represents 50-75 minutes in the sun.

RNA extraction and cDNA production

After 24 hours, each skin equivalent was homogenised for RNA extraction. RNA extraction of the equivalent was performed following the Phenion protocol. Reverse transcription was performed

using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, U.S) following manufacturer protocol.

qPCR to determine differences in gene expression levels

Gene expression qPCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, U.S) and the qPCR was performed using QuantStudio 3 PCR machine (applied Biosystems, U.S) following manufacturer instructions.

Statistical analysis was conducted using GraphPad Prism statistical software (GraphPad Software, Inc., San Diego, CA, USA). Results from gene expression qPCR were calculated using $2^{-\Delta\Delta Ct}$ method to give fold change. Analysis in the figures was by ANOVA.*, **, *** = statistical significance at less than or equal to 0.05, 001 and 0001 respectively.

Results

Full thickness human skin models were irradiated with 5 SED of solar UV in the presence or absence of the Skin Diary, Age Defence formulation. Gene expression levels of MMP-1, p21, p16, PTGES, and GLB1 were normalised to the housekeeping gene GAPDH using the 2-ΔΔCt method, to give fold change.

MMP1:

As can be seen in **Figure 1a**, the level of UV-induced MMP-1 expression showed a 13.69 fold increase (UVR only column) compared to the non-irradiated sample. This increase was highly statistically significant at *** as determined by ANOVA. This result is expected and shows that the experiment was technically successful.

The introduction of the Skin Diary Age Defence formulation caused a decrease in UV-induced gene expression to a fold change value of 2.78 when compared to the non-irradiated condition (normalised at 1, Figure 1a). **This represents a greater than 10 fold abrogation of the UV-induced MMP1 increase by the formulation.** This 'potency of protection' was statistically significant at ** by ANOVA.

In addition, the large degree of protection against induced MMP1 expression exhibited by the formulation meant that the expression values were not statistically significant from the non-irradiated conditions (i.e. non-irradiated vs Age Defence). This is very encouraging and together these results in Figure 1a) suggest that the Skin Diary formulation was able to provide statistically significant **complete** protection against UV-induced MMP1 expression.

P21:

A 'protection' profile similar to the MMP1 expression was observed for the effect of the Skin Diary formulation on p21 expression (although the overall magnitude of UV-induced expression was lower - Figure 1b). The level of UV-induced p21 expression showed a 2.29 fold increase compared to the non-irradiated sample (this increase was highly statistically significant, at *** for ANOVA, Figure 1b). This result is expected and shows that the experiment was technically successful.

The introduction of the Skin Diary Age Defence formulation caused a significant decrease in UV-induced gene expression to a fold change value of 1.07 when compared to the non-irradiated condition (normalised at 1). **The ≥1 fold abrogation of the UV-induced MMP1 increase by the formulation was highly statistically significant** at *** for ANOVA.

The degree of protection against UV-induced MMP1 expression exhibited meant that the expression values were not statistically significant from the non-irradiated conditions according to ANOVA (i.e. non-irradiated vs Age Defence). Again, this is very encouraging and suggests that the formulation provides statistically significant **complete** protection against increased p21 expression as induced by solar UV irradiation.

P16:

In contrast to the MMP1 and p21 expression data, the results in **Figure 1c** show that the level of p16 expression appears to be unchanged. This is either by the UV stressor or the intervention of the formulation. This is likely to be due to the fact that p16 is a late cellular senescent marker as opposed to p21 which is an earlier senescence marker which of course is detected within the 24 hour time window following UV-irradiation and RNA extraction.

PTGES:

The results for PTGES expression in **Figure 1d** mirror more closely the results observed for MMP1 and p21 in Figures 1a and b respectively. The level of PTGES expression showed a 3.86 fold increase in UV-induced expression compared to the non-irradiated sample. These expected increases were statistically significant at ** for ANOVA and confirms the experiment was technically successful.

The introduction of the Skin Diary Age Defence formulation caused a statistically significant decrease in this UV induced gene expression to a fold change value of 1.64 when compared to the non-irradiated condition (normalised at 1). The greater than 3 fold abrogation of the UV-induced MMP1 increase by the formulation was **statistically significant at** ** as determined by ANOVA .

In terms of ANOVA the formulation data show restoration of the PTGES expression at a value that is not statistically different to those observed in the non-irradiated condition. Therefore, in a similar fashion to the MMP1 and p21 data, this strongly suggests that the UV induced PTGES expression can be completely abrogated by the inclusion of the Skin Diary formulation. This is very encouraging and suggests that the formulation provides statistically significant **complete** protection against increased UV-induced PTGES expression compared to non-irradiated conditions.

GLB1:

As can be seen in **Figure 1e**, the level of GLB1 gene expression showed an expected, but small in this case, increase in expression compared to the non-irradiated sample, when irradiated with solar UV. This confirms the experiment was technically successful.

The introduction of the Skin Diary formulation did indeed cause a small reversal of this induced increase but despite the protection looking clear in Figure 1e, it did not achieve formal statistical significance. The results indicate that the formulation provides protection against increased UV-induced GLB1 expression compared to non-irradiated conditions although this degree of protection was not formally statistically significant.

Figure 1a

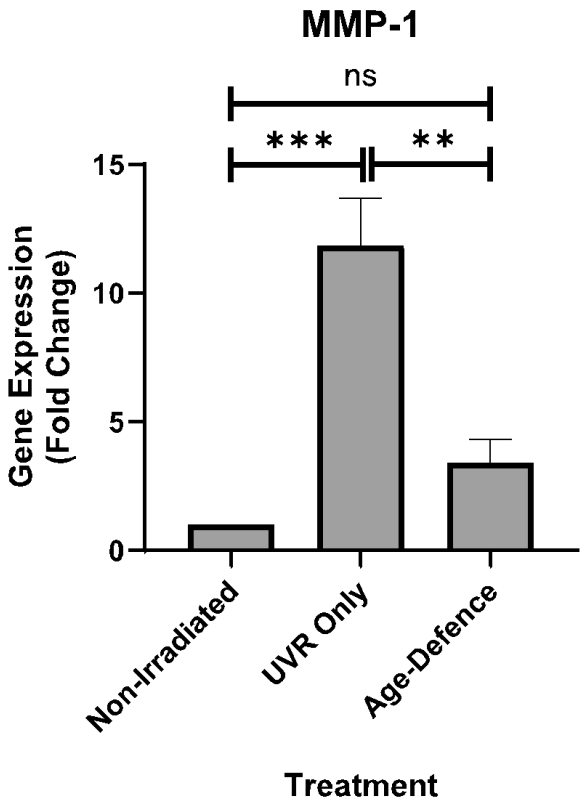
MMP-1
ns
***
**
15
10
5
0
Gene Expression
(Fold Change)
Non-Irradiated
UVR Only
Age-Defence
Treatment

Figure 1b

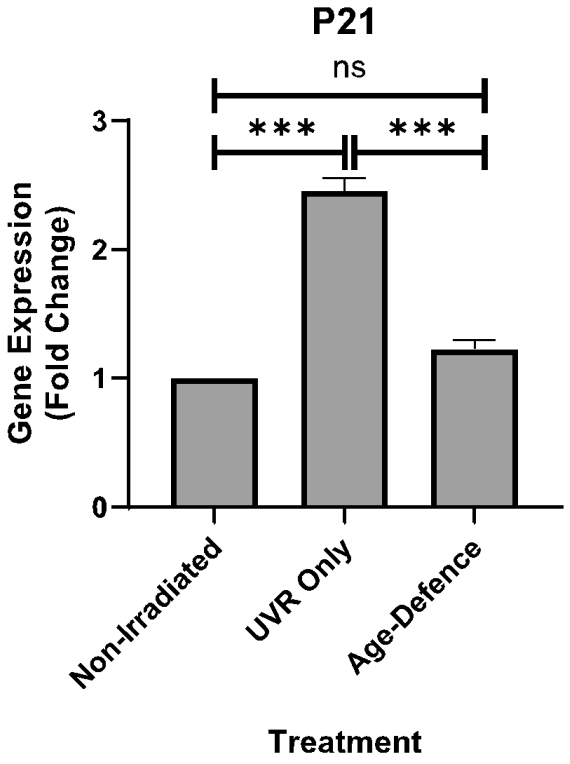
P21
ns
***
***
Gene Expression
(Fold Change)
3
2
1
0
Non-Irradiated
UVR Only
Age-Defence
Treatment

Figure 1c

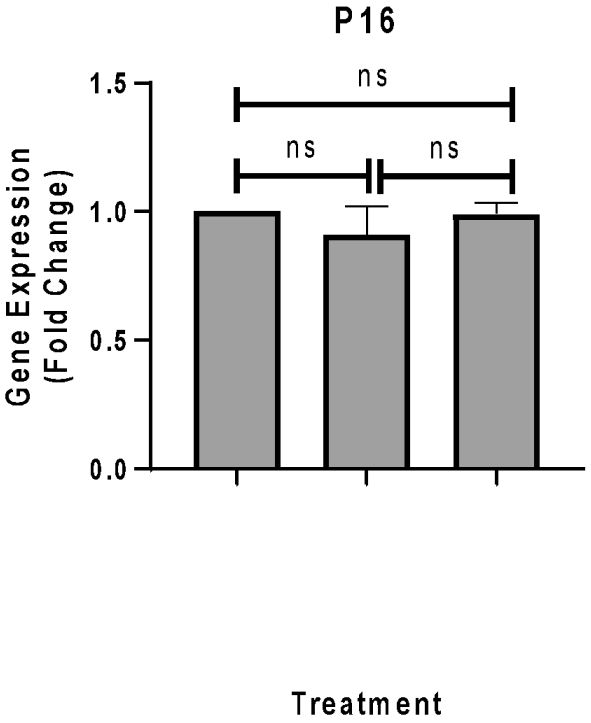
P16
ns
ns
ns
1.5
1.0
0.5
0.0
Gene Expression
(Fold Change)
Treatment

Figure 1d

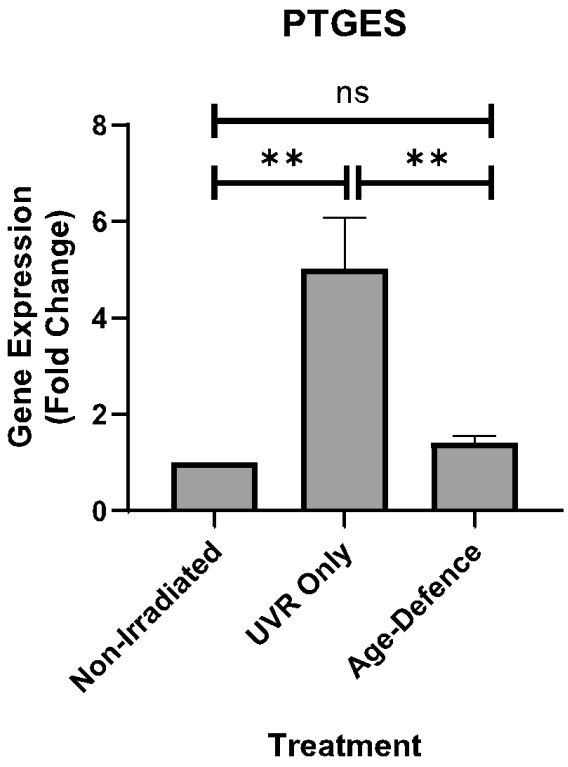
PTGES
ns
**
**
8
6
4
2
0
Gene Expression
(Fold Change)
Non-Irradiated
UVR Only
Age-Defence
Treatment

Figure 1e

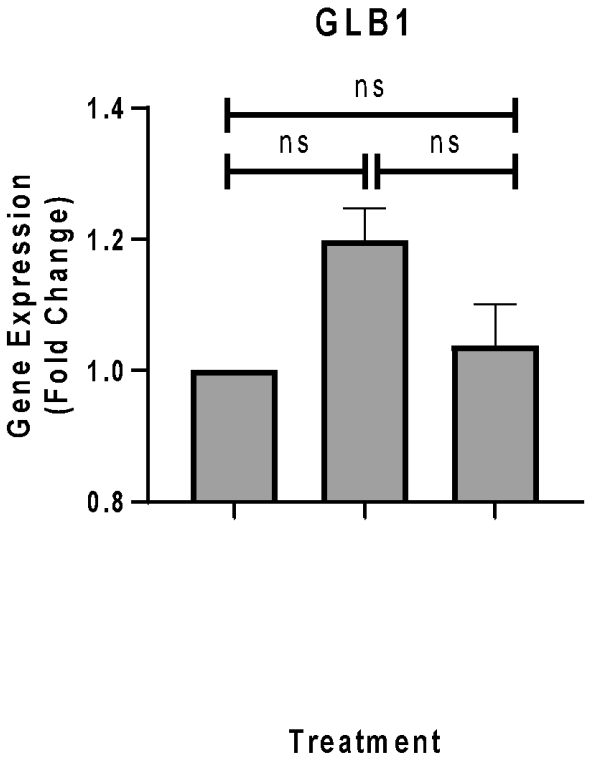

Figure 1. Gene expression and statistical analysis following 5 SEDs of solar simulated UV irradiation in the presence of The Skin Diary formulation. Human skin models were irradiated with 5 SED of solar simulated UV irradiation, following application of Test formulation. Gene expression levels of MMP-1, p21, p16, PTGES, and GLB1 were normalised to the housekeeping gene GAPDH using the 2-ΔΔCt method, to give fold change. Non-irradiated samples were wrapped in aluminium foil. Three biological repeats were performed for each condition (3x skin samples for each condition), with three technical qPCR repeats performed per biological repeat; therefore each bar on the graphs represent 9 data points. For the statistical analysis of data, GraphPad Prism statistical software was used with one-way ANOVA to compare each of the columns. *, **, *** = statistical significance at less than or equal to 0.05, 001 and 0001 respectively.

Conclusions

Studies on human skin in vitro and in vivo have consistently reported a decrease in collagen levels within the dermis due to an increase in collagenase production upon acute solar exposure; the most prominent of which includes MMP-1 (Lowe et al., 1995; Scharffetter et al., 1991; Nakyai et al., 2018). Matrix metalloproteinases (MMPs) are enzymes which break down the structural components of the extracellular matrix (ECM) and play a significant role in the maintenance of the dermis. They break down the existing collagen fibres which provide stability to skin and thus accelerate symptoms of photo-aging. The results in the current study show that The Skin Diary Age Defence day cream was able to provide statistically significant complete protection against increased MMP1 expression as induced by solar UV irradiation (Figure 1a).

The gene CDKN1A codes for p21, a cyclin-dependent kinase inhibitor. Like p53, the p21 protein is a tumour suppressor and is involved in multiple cell cycle related signalling pathways. UV causes direct DNA damage which triggers p53 stress response. p21 is consequently activated, which inhibits CDK2. The inhibition of CDK2 causes hypo-phosphorylation of Rb, which E2F-mediated transcription is dependent on. Due to the repression of E2F-target genes, cell cycle arrest occurs at the G1/S checkpoint. p21 also plays a crucial role in cellular senescence, acting as a key regulator of cell cycle arrest and contributing to the senescent phenotype (Englund et al., 2022). While it can promote senescence, p21 also maintains the viability of senescent cells under certain conditions. Furthermore, p21's expression levels and dynamics can influence whether a cell enters senescence or proliferation after a stress event. The results in the current study show that The Skin Diary Age Defence day cream was able to provide statistically significant complete protection against increased p21 expression as induced by solar UV irradiation (Figure 1b). The 'protection' profile afforded by the formulation was similar to the MMP1 expression results although the overall magnitude of UV-induced p21 expression was lower.

p16, specifically the protein encoded by the CDKN2A gene, is a key player in cellular senescence, a state of stable cell cycle arrest. It acts as a tumour suppressor by inhibiting cyclin-dependent kinases (CDKs) and preventing the progression from G1 to S phase in the cell cycle. Increased p16 expression is often observed in senescent cells and is considered a marker of this process. p16 and p21 are both cell cycle inhibitors and tumour suppressor proteins, but they function differently. p16 primarily inhibits the G1 to S phase transition by blocking CDK4/6 kinases. p21, on the other hand, can inhibit cell proliferation by blocking various cyclin-CDK complexes, including

those involved in both G1 and G2 phases. Additionally, p21 can inhibit DNA replication. p21 is often considered an early marker of senescence, while p16 is frequently associated with a more established or late-stage senescence (Wagner and Wagner, 2022). This latter fact is important when one considers the expression results for p16 in the current report. In contrast to the MMP1 and p21 expression data, the results (Figure 1c) show that the level of p16 expression is statistically unchanged either by the UV stressor or the intervention of the formulation. This is likely to be due to the fact that p16 is a late cellular senescent marker as opposed to p21 which is an earlier senescence marker which of course is detected within the 24 hour time window following UV-irradiation and RNA extraction.

Prostanoid biosynthesis is a pathway which is affected by UV irradiation. PTGES catalyses the conversion of prostaglandin endoperoxide H2 (PGH2) into Prostaglandin E2 (PGE2) during inflammation (Greaves, 1982) which impacts dermal vasculature in vivo and the ECM of the dermis in vitro (Funk, 2001). Therefore, the results in Figure 1d demonstrate the expected increase in PTGES gene expression following irradiation by UV. The Skin Diary Age Defence day cream data demonstrate a restoration of the increased gene expression to those observed in the non-irradiated condition. This is underlined by the fact that the formulation data are not statistically different to the non-irradiated condition and **confirms that the UV induced PTGES gene expression can be fully abrogated by the inclusion of the formulation. This latter statistically significant conclusion can also be applied to the effect of the formulation on both MMP1 and p21 expression**.

GLB1, which encodes the enzyme beta-galactosidase, is a key marker for cellular senescence. Increased GLB1 expression correlates with senescent cell morphology, as well as the expression of other senescence markers like p16 and p21. This makes GLB1 a useful tool for detecting senescent cells both in vitro and in vivo (Kurz et al., 2000). The introduction of The Skin Diary Age Defence day cream did indeed cause a small reversal of the UV-induced GLB1 expression but despite the protection looking clear in Figure 1e, it did not achieve formal statistical significance. Therefore, the GLB1 data reflects the similar protection profile pattern afforded by the formulation as observed with MMP1, p21 and PTGES. The fact that the GLB1 did not reach formal statistical significance is likely to be linked to the fact that the magnitude of UV-induction of GLB1 is much lower than the corresponding effects on MMP1, p21 and PTGES expression. As such, the

‘window of response’ for GLB1 expression is comparatively much smaller upon which to see an intervention effect by the formulation.

This study highlights the value of using full-thickness 3D skin models and gene expression profiling to objectively assess photoprotective efficacy and suppression of genetic markers of cellular ageing in the skin. The Skin Diary Age Defence day cream demonstrates a potent protective effect at the molecular level against solar UV-induced photodamage.

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