Elsevier Editorial System(tm) for Journal of Dermatological Science

Manuscript Draft

Manuscript Number: JDS-16-375R2

Title: Triolein reduces MMP-1 upregulation in dermal fibroblasts generated by ROS production in UVB-irradiated keratinocytes

Article Type: Research Paper

Keywords: Keywords: UVB; MMP1; Triolein; IL-6; ROS

Corresponding Author: Dr. Karin Hagelin,

Corresponding Author's Institution: Fundación Pablo Cassará, Centro de Ciencia y Tecnología Dr. César Milstein

First Author: Gustavo J Leirós, doctor

Order of Authors: Gustavo J Leirós, doctor; Ana G Kusinsky, licenciada; María E Balañá, doctor; Karin Hagelin

Abstract: ABSTRACT
Background: Cytokine production and oxidative stress generated by ultraviolet radiation B (UVB) skin exposure are main factors of skin photoaging. Interleukin-6 (IL-6) produced by irradiated keratinocytes is proposed to have a role in metalloproteinases (MMPs) expression activation in dermal fibroblasts.
Objectives: We examined the effect of triolein treatment of UVB-irradiated keratinocytes on MMP1 (interstitial collagenase) expression response of dermal fibroblasts. We assayed UVB-irradiated keratinocytes soluble signals, mainly IL-6 and reactive oxygen species (ROS).
Methods: IL-6 expression and ROS generation were assayed in UVB-irradiated keratinocytes. MMP1 mRNA expression response was assayed in fibroblasts grown in keratinocytes conditioned medium. We evaluated the effect of treating keratinocytes with triolein on IL-6 expression and ROS generation in keratinocytes, and MMP1 expression in fibroblasts.
Results: The irradiation of epidermal cells with sublethal UVB doses increased IL-6 expression and ROS generation. Conditioned culture medium collected from keratinocytes was used to culture dermal fibroblasts. MMP1 mRNA expression increase was observed in fibroblasts cultured in medium collected from UVB-irradiated keratinocytes. Triolein treatment reduced the IL-6 expression and ROS generation in keratinocytes and this effect was reflected in downregulation of MMP1 expression in fibroblasts.
Conclusions: Triolein reduces both the expression of IL-6 and ROS generation in irradiated keratinocytes. It seems to exert an anti-inflammatory and anti-oxidative stress effect on irradiated keratinocytes that in turn reduces MMP1 expression in dermal fibroblasts.
Collectively, these results indicate that triolein could act as a photoprotective agent.
Keywords: UVB MMP1 Triolein IL-6 ROS

Response to Reviewers:

Reviewer #2: The point of an argument of this revise manuscript is clear. However, only one point is not satisfactory. I want you to describe this point.

Comment
The relation between triolein and mast cell is not specified. If there is not result, please describe an idea with reference to other papers.

ANSWER TO REVIEWER #2 COMMENT.
As stated before, in our previous response, our experiments with triolein were performed only on keratinocytes and dermal fibroblasts in culture. We have not performed any experiment with other cell types as, for instance, mast cells.
In line, and according to Reviewer #2 request, we included the following new paragraph in the Discussion section:
…"Although we did not study the effect of UVB irradiated keratinocytes supernatants on other cell types, in vivo UVB effect on keratinocytes could be even more complex and also drive other mechanisms rather than the direct effect on dermal fibroblasts observed in this report. For instance, Van Nguyen et al (Scand J Rheumatol 2011;40:197–204) demonstrated that soluble mediators released by irradiated keratinocytes (IL-15, CCL5, etc.) induced mast cells migration and activation in human skin. This inflammatory mechanism in which these mast cells would be involved, may contribute to increase ROS environment of keratinocytes, release IL-6 themselves, as it has already been described by Sarchio et al. (Experimental Dermatology, 2012, 21, 241–248.), contributing to MMP1 increase expression by dermal fibroblasts and/or produce MMP1 themselves".
Hoping this text will be satisfactory to Reviewer#2. We are grateful to Reviewers attention.

Conflict of Interest

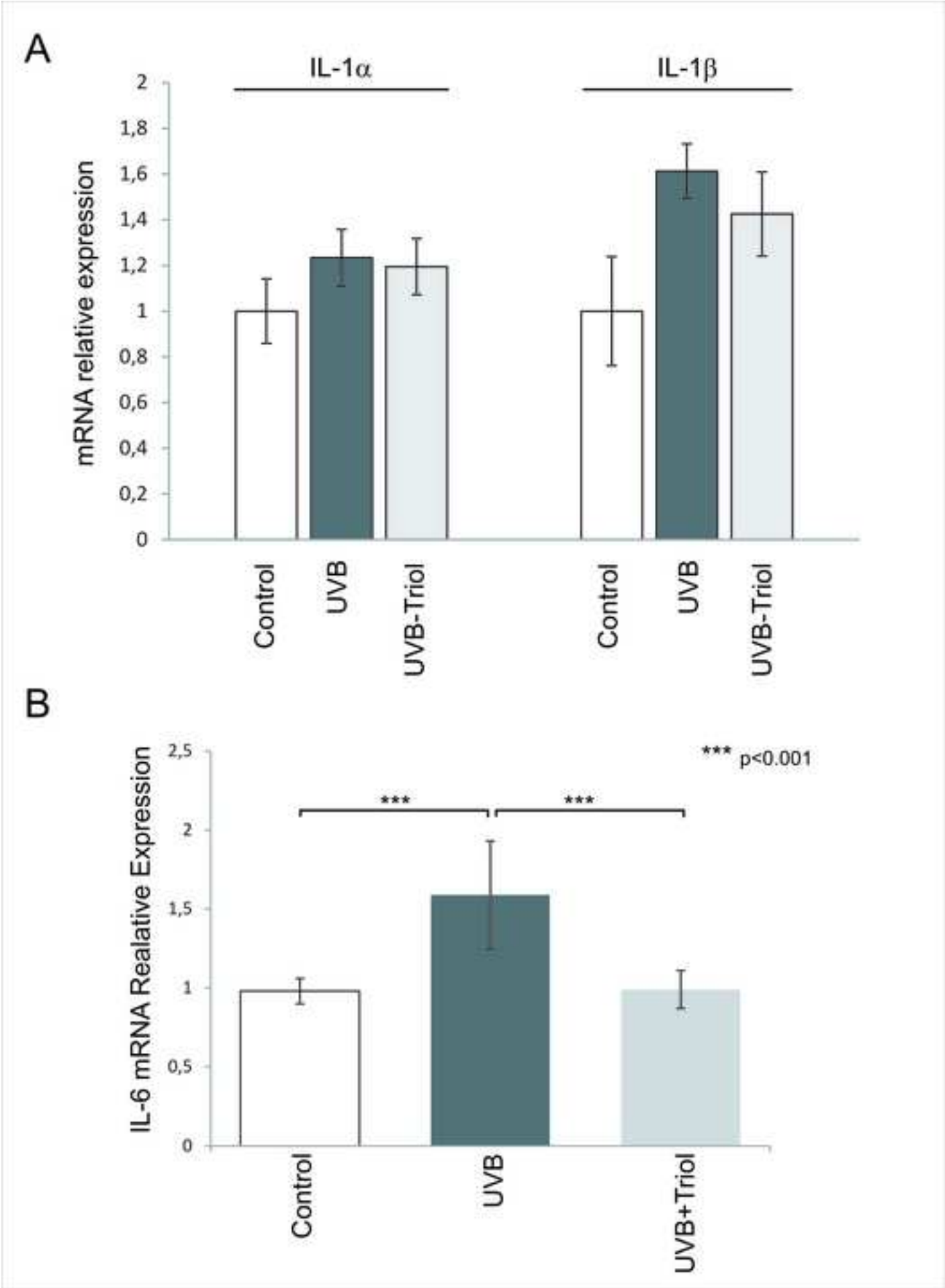
Click here to download Conflict of Interest: JDS Author Conflict of Interest Disclosure September.pdf

Conflict of Interest

Click here to download Conflict of Interest: JDS Author Conflict of Interest Disclosure.pdf

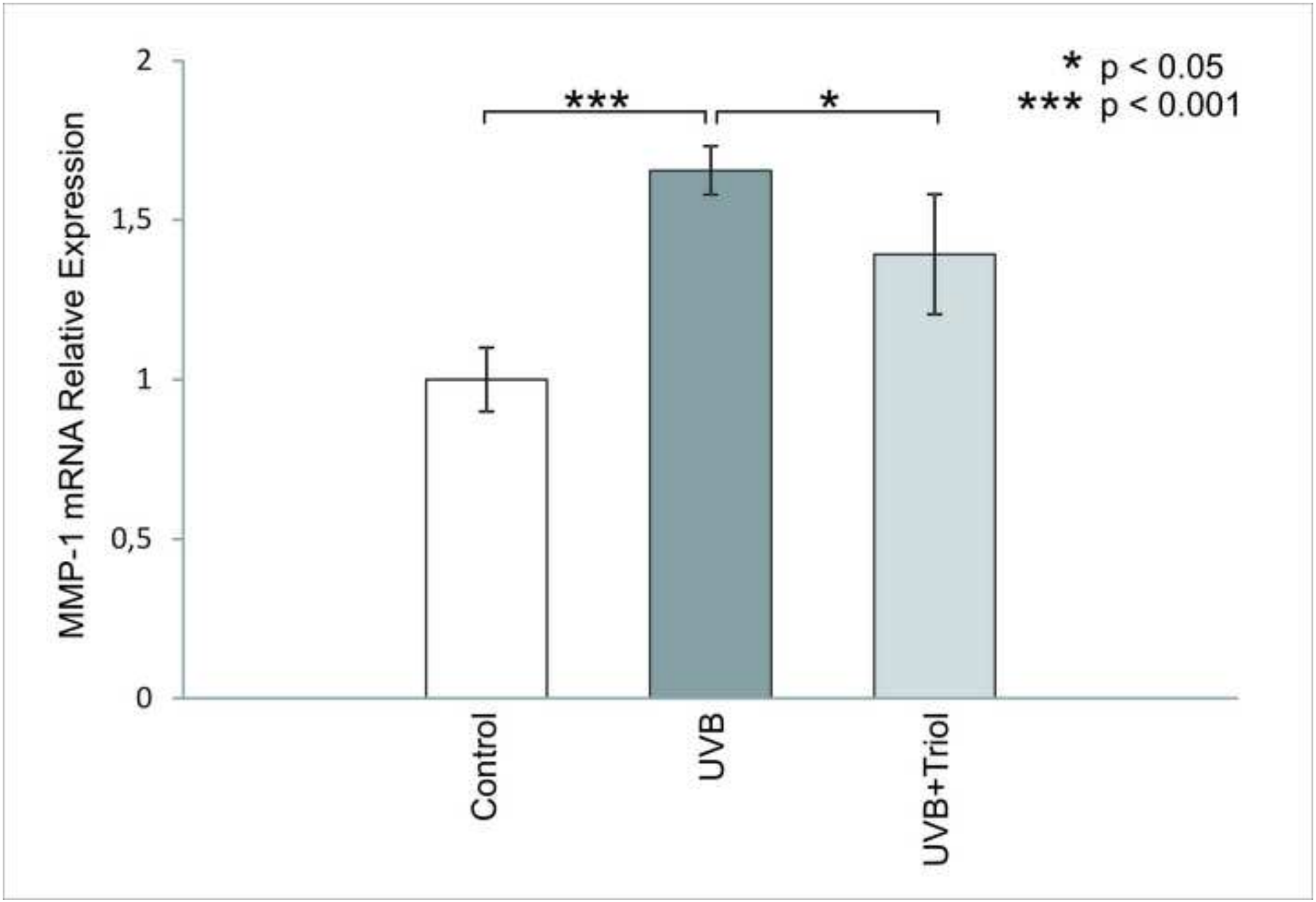
Figure(s)

Click here to download high resolution image



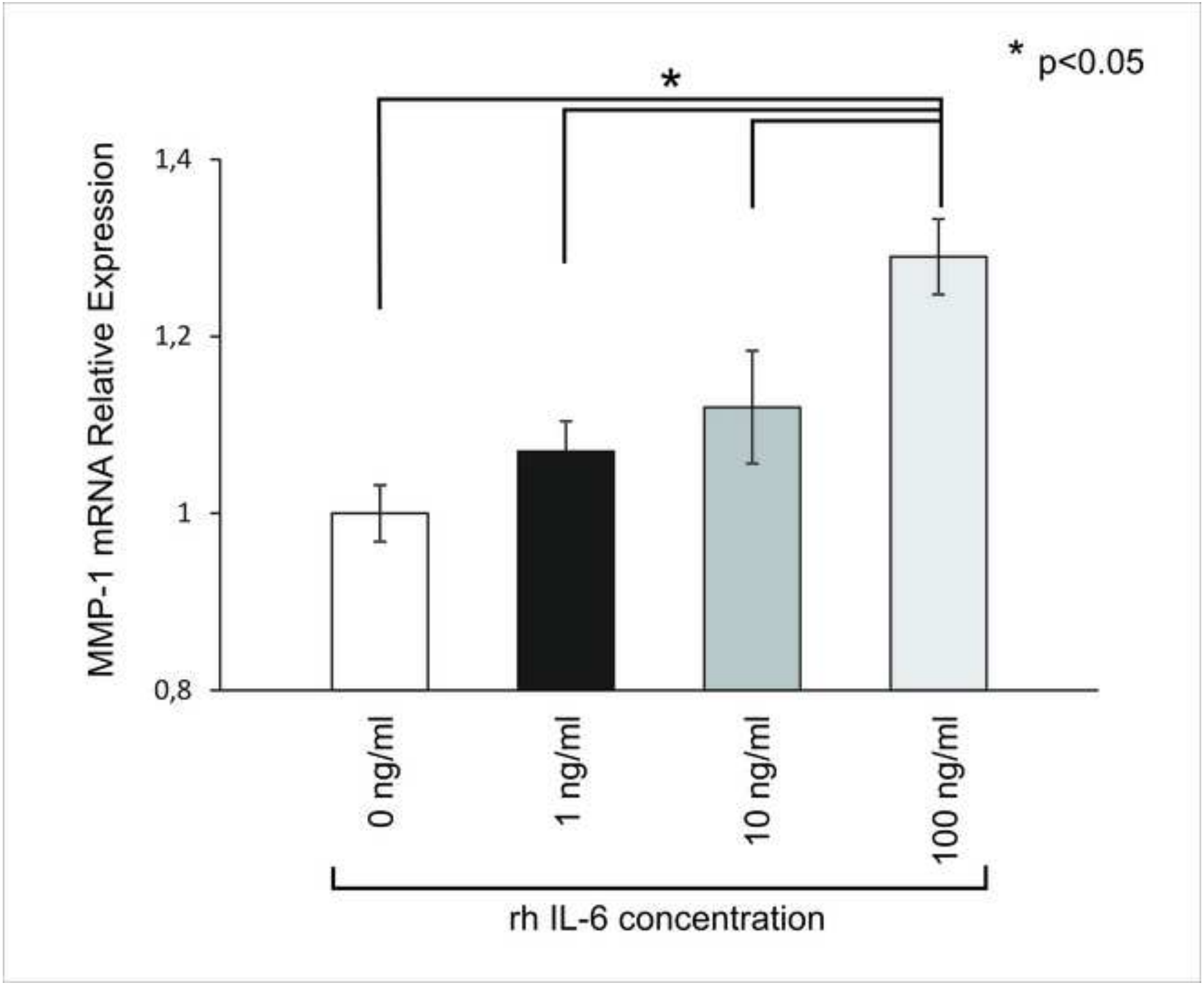
Figure(s)

Click here to download high resolution image



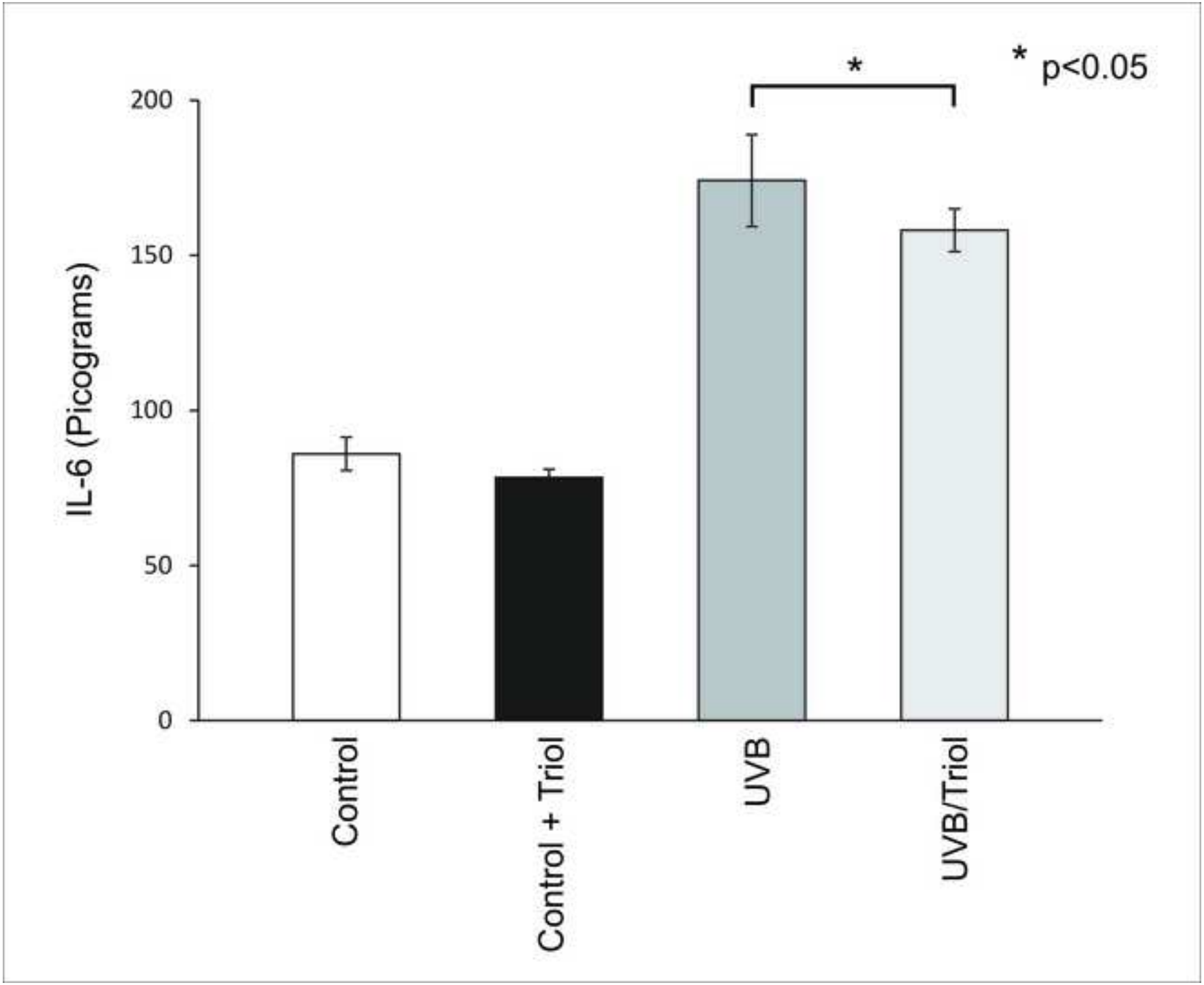
Figure(s)

Click here to download high resolution image



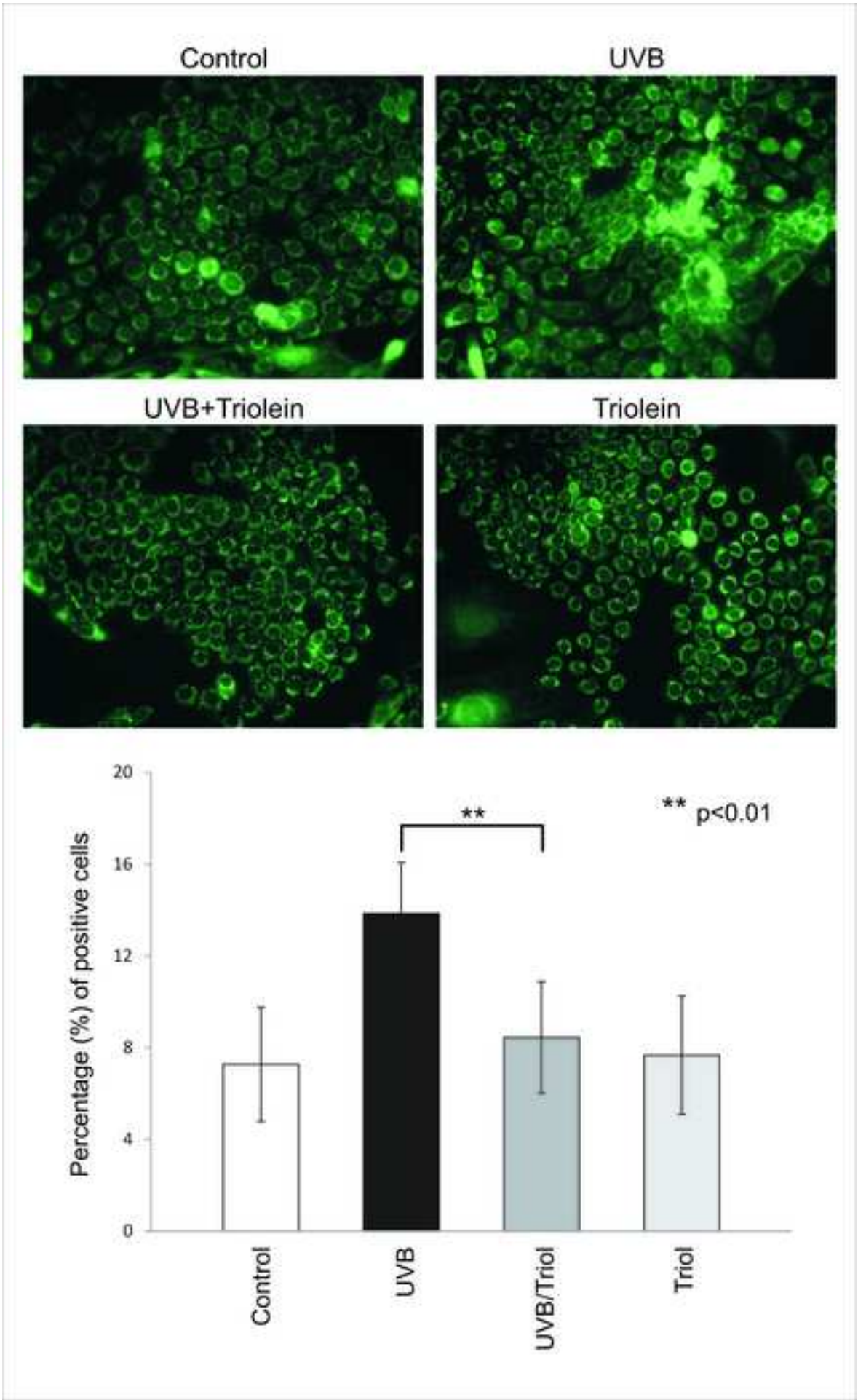
Figure(s)

Click here to download high resolution image



Figure(s)

Click here to download high resolution image



Supplementary Material

Click here to download Supplementary Material: Figure S0.tif

***Article Checklist**

Click here to download Article Checklist: jds_oacl September.pdf

***Author Declaration**

Click here to download Author Declaration: JDS Author Declaration September.pdf

Highlights

- Triolein treatment of irradiated keratinocytes inhibits signals like IL-6 and ROS.
- Treating keratinocytes with Triolein inhibits MMP1 expression in fibroblasts.
- Triolein can be proposed as an active agent against skin photodamage.

Triolein reduces MMP-1 upregulation in dermal fibroblasts generated by ROS production in UVB-irradiated keratinocytes.

Gustavo J. Leirós[1], Ana Gabriela Kusinsky[1], María Eugenia Balañá[1] and Karin Hagelin[1*].

[1]Fundación Pablo Cassará -Instituto de Ciencia y Tecnología Dr. César Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina.

gleiros@fundacioncassara.org.ar

gkusinsky@fundacioncassara.org.ar

mbalana@fundacioncassara.org.ar

*Corresponding author at: Fundación Pablo Cassará -Instituto de Ciencia y Tecnología Dr. César Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 C1440FFX, Ciudad de Buenos Aires, Argentina. khagelin@fundacioncassara.org.ar.

This work was supported by Fundación Pablo Cassará.

The authors have no conflict of interest to declare.

Word count: 2950

Number of references: 40

Number of figures: 5

Number of supplemental files: 1 (figure S0)

ABSTRACT

Background: Cytokine production and oxidative stress generated by ultraviolet radiation B (UVB) skin exposure are main factors of skin photoaging. Interleukin-6 (IL-6) produced by irradiated keratinocytes is proposed to have a role in metalloproteinases (MMPs) expression activation in dermal fibroblasts.

Objectives: We examined the effect of triolein treatment of UVB-irradiated keratinocytes on MMP1 (interstitial collagenase) expression response of dermal fibroblasts. We assayed UVB-irradiated keratinocytes soluble signals, mainly IL-6 and reactive oxygen species (ROS).

Methods: IL-6 expression and ROS generation were assayed in UVB-irradiated keratinocytes. MMP1 mRNA expression response was assayed in fibroblasts grown in keratinocytes conditioned medium. We evaluated the effect of treating keratinocytes with triolein on IL-6 expression and ROS generation in keratinocytes, and MMP1 expression in fibroblasts.

Results: The irradiation of epidermal cells with sublethal UVB doses increased IL-6 expression and ROS generation. Conditioned culture medium collected from keratinocytes was used to culture dermal fibroblasts. MMP1 mRNA expression increase was observed in fibroblasts cultured in medium collected from UVB-irradiated keratinocytes. Triolein treatment reduced the IL-6 expression and ROS generation in keratinocytes and this effect was reflected in downregulation of MMP1 expression in fibroblasts.

Conclusions: Triolein reduces both the expression of IL-6 and ROS generation in irradiated keratinocytes. It seems to exert an anti-inflammatory and anti-oxidative stress effect on irradiated keratinocytes that in turn reduces MMP1 expression in dermal fibroblasts. Collectively, these results indicate that triolein could act as a photoprotective agent.

Keywords: UVB MMP1 Triolein IL-6 ROS

Abbreviations

UVR: UV radiation

UVB: UV-B radiation

IL-6: Interleukin 6

MMPs: metalloproteinases

MMP1: metalloproteinase 1; interstitial collagenase

ROS: reactive oxygen species

IL-1α: Inteleukin-1 alpha

IL-1β: Interleukin 1-beta

Triolein reduces MMP-1 upregulation in dermal fibroblasts generated by ROS production in UVB-irradiate keratinocytes

1. Introduction

The normal skin aging process comprises intrinsic and extrinsic components. The intrinsic component is the natural biochemical and molecular change of skin responding to passage of time. The extrinsic component is primarily linked to environmental factors that trigger other or similar biological processes of skin aging. Photoaging is an extrinsic related aging process associated to skin UV radiation (UVR) repetitive exposure [1]. Photoaged skin shows alterations mainly localized in dermal connective tissue. Major alterations include disorganized and damaged collagen fibrils and reduction of procollagen synthesis which result in net collagen loss, alterations of extracellular matrix and loss of skin structure. The metalloproteinases (MMPs) are a family of collagenolytic enzymes able to degrade the components of extracellular matrix. UVR exposure stimulates the expression of several MMPs in human skin [2]. Epidermal keratinocytes and dermal fibroblasts have shown to be the main source of MMPs induced by UVR in several models [3, 4]. The upregulation of these enzymes is responsible for the photoaging effect triggered by chronic and cumulative skin exposure to UVR.

MMPs expression is triggered by UVR in a cytokine regulated way. One of the first responses of keratinocytes to UVR is the release/production of pro-inflammatory and immunosuppressive cytokines [5, 6].

IL-6 cytokine release by human epidermal keratinocytes is known to be promoted by UVR, particularly the UVB portion of UVR reaching the earth surface [7, 8]. In addition, UVR including UVB does affect MMP expression in skin fibroblasts through the release of IL-6 mediated by reactive oxygen species (ROS) production [9]. Furthermore ROS production is involved in the

kinase cascade activation leading to MMPs activation/transcription [4, 10-12]. Altogether, cytokine production and oxidative stress generated by UVR skin exposure are main factors of skin photoaging by activation of Mitogen-Activated Protein Kinase (MAPK) signaling pathways leading to up-regulate collagenases gene expression [13, 14].

On the other hand, skin lipids, a mix of lipids synthesized by epidermal keratinocytes and sebaceous glands [15], are involved in many important aspects of skin biology including skin permeability barrier function [16] and photoprotection [17]. The reduction of free fatty acids and triglycerides content/synthesis in UV-irradiated and photoaged human skin *in vivo* has been reported by Kim et al [18] suggesting that triglycerides and free fatty acids may play a role in photoaging somehow relating to the expression of MMPs. For instance triolein, a symmetrical triacylglycerol derived from three units of the mono-unsaturated fatty acid oleic acid and one of the major components of sebum triglycerides [19] was pointed out as an attenuator of the UVR induction of MMP1 expression by keratinocytes [18]. Moreover, triolein seems to have an anti-cytotoxicity effect reducing apoptosis in a human endothelial cells model, showing a strong antioxidant response to oxidative stress and anti-inflammatory properties, which are mediated via the antioxidant defense system [20]. In this view we choose to analyze the effect of triolein on the interaction between the UVB irradiated immortalized adult epithelial stem cells line Tel-E6E7 and dermal fibroblasts. Tel-E6E7 is an immortalized human epithelial stem cells line derived from bulge stem cells of the hair follicle where most stem cells for epidermal regeneration and repair precursors reside. Tel-E6E7 cells represent the precursor cells of all three epidermal cell linage, including keratinocytes (25).

Our results indicate that triolein may exert a protective effect against the UVR-mediated MMP1 upregulation in dermal fibroblasts triggered by irradiated keratinocytes. We studied the UVR

mediated inflammatory response of keratinocytes on MMP-1 upregulation in dermal fibroblasts and discuss a model of potential signaling between these cells types where the ROS generation and IL-6 release could have a key signaling function.

2. Materials and Methods.

2.1. Cell cultures

The immortalized human bulge stem cell line Tel-E6E7 was kindly provided by Doctor Stephen Lyle (University of Massachusetts Medical School, MA, U.S.A.). Tel-E6E7 cells [21, 22] were cultured on mytomicin C-treated (10 µg/ml) 3T3-swiss cells (*feeder layer*) in cFAD medium (Dulbecco's Modified Eagle's Medium (DMEM), Ham´s F12 Nutrient Mixture (Gibco-Thermofhisher Scientific, Waltham, MA, USA) (3:1 v/v) supplemented with adenine (24.3ug/ml), insulin (5ug/ml), triiodotironine (0.2mM), hydrocortisone (0.4ug/ml), cholera toxin (10-7M) and 10% fetal bovine serum (Gibco-Thermofhisher Scientific, Waltham, MA, USA)) at 37°C in a humidified atmosphere at 10% CO_2. Human dermal fibroblasts were obtained from skin biopsies [22] and maintained in DMEM supplemented with 2 mM glutamine and 10% fetal bovine serum at 37°C in a humidified atmosphere at 5% CO_2.

2.2. UVB irradiation and triolein treatment

Tel-E6E7 cells were seeded on day one at a density of 2000 cells per cm^2 on 3T3-swiss cells feeder layer in cFAD medium. On day five the medium was change to DMEM and F12 (1:1) mixture containing 2 mM glutamine, hydrocortisone (0.4 µg/ml), 2 mM penicillin/streptomycin, Epithelial Growth factor (5ng/ml) and 10% fetal bovine serum. On day six the cells were irradiated on PBS using a UVM-57 UVP handheld UV lamp (302 nm Midrange) at sub-lethal doses of 3.4 or 6.8 mJ/cm^2. Immediately, PBS was replaced with medium containing triolein 20µM (Sigma, St. Louis, MO, USA) when indicated. 24 hours later mRNA was extracted from cells using Trizol Reagent (Invitrogen-Thermofhisher Scientific, Waltham, Ma, USA) according to manufacturer. Conditioned culture medium was collected and used to replace the medium of dermal fibroblasts cells plated

the day before (75000 cells/cm^2) in DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine. After 48 hours dermal fibroblasts mRNA was extracted as described

2.3. Real-time PCR analysis

cDNA was obtained from 0.5-1 µg of total mRNA using MMLV Reverse Transcriptase (Promega, Madison, WI USA) according to manufacturer. The mRNA relative expression was determined by Real Time PCR using FastStart Universal SYBR Green Master Mix (Roche, Basel, Switzerland) in a StepOne Real Time PCR-System machine (Applied Biosystems, Waltham, MA, USA) by standard procedures. Primers were as follow: MMP1 5´- AAGCGTGTGACAGTAAGCTA-3´ (Fw) and 5´-AACCGGACTTCATCTCTG-3´ (Rv)[1]; IL6 5´- AGGGCTCTTCGGCAAATGTA-3´(Fw) and 5'-GAAGGAATGCCCATTAACAACAA-3' (Rv); IL-1α 5´- TCCTGCCGCAACAGTTTTTT-3´(Fw) and 5'-TCGGCTTCAAGAAGTATTTTACCA-3' (Rv); IL-1 β 5´- GCACGATGCACCTGTACGAT-3´(Fw) and 5´-CACCAAGCTTTTTTGCTGTGAGT-3' (Rv); HPRT 5´-CTCAACTTTAACTGGAAAGAATGTC-3´(Fw) and 5´-GTCTGCATTGTTTTGCCA-3 (Rv). The HPRT gene expression was used as an internal control to relative mRNA quantitation.

2.4. IL-6-enzyme-linked-immunoassay

Conditioned media of Tel-E6E7 cell cultures was collected 24 hours after UVB irradiation and cytokine ELISA for IL-6 was performed using OptEIA™ Human IL-6 ELISA Kit II (Becton Dickinson, Franklin Lakes, NJ, USA) according to manufacturer´s protocol. Absorbance at 450 nm was measure in a Microplate Reader (Biorad, Hercules, CA, USA).

2.5. Detection of reactive oxygen species (ROS) production

Tel-E6E7 cells were treated with triolein 20 µM in cFAD medium for 24 hours. Control cells were incubated in medium alone. Then cells were washed with PBS and incubated with 2´, 7´-dichlorofluorescin diacetate (DCF-DA) 1 µM in cFAD without serum for 30 minutes at 37°C in a CO_2

incubator. Cells were washed with PBS and irradiated with UVB as indicated. ROS generation was visualized by green fluorescence of oxidized DCF and imaged immediately using fluorescence microscope (Olympus IX51, Shinjuku, Tokio, Japan) (excitation wavelength 490 nm, emission wavelength 520 nm). Pictures were analyzed with the Image J software (NIH, Bethesta, MD, USA).

2.6. Statistical analysis

All experiments were performed at least three times using triplicates. The data shown are representative results of the means ± standard deviation of triplicate experiments. Differences were judged to be statistically significant using the Turkey-Kramer Multiple Comparison test, when *p*-value was ≤0.05.

3. Results

3.1. Pro-inflammatory cytokines and MMP1 mRNA expression increases in human epidermal cells after UVB exposure but triolein treatment attenuates the effect.

The analysis of the expression of pro-inflammatory cytokines IL-1 alpha, IL-1 beta (figure 1a) and IL-6 (figure 1 b) mRNA in human multipotential hair follicle stem cells line Tel-E6E7 show that these cytokines were upregulated after UVB irradiation related to control and Interestingly, the presence of triolein in the culture medium partially inhibited the induction of IL-6 mRNA expression ($p<0.001$) (figure 1b). The triolein effect was not significant in the case of IL-1 alpha and IL-1 beta (Figure 1a).

MMP1 mRNA expression was also induced in Tel-E6E7 cells 24 hours after both 3.4 or 6.8 mJ/cm^2 doses of UVB irradiation as shown in a representative experiment depicted in supplemental figure (figure S0). MMP1 induction was partially inhibited by culturing cells after UVB exposure with triolein 20 µM. Triolein did not show any effect on cell viability at several concentrations tested (data not shown).

3.2. Triolein presence in the culture medium after keratinocytes UVB irradiation reduces its inductive action on MMP1 mRNA upregulation in dermal fibroblasts.

To analyze the effect of UVB irradiation driven by epidermal cells on dermal cells, we irradiated Tel-E6E7 cells and cultured them in the presence or absence of triolein 20µM. After 24 hours, the conditioned culture medium was collected and used to replace the medium of human dermal fibroblast primary cultures for 48 hours. Conditioned medium from UVB irradiated keratinocytes stimulated MMP1 expression on dermal fibroblasts as shown in figure 2 ($p<0.001$). Moreover, when irradiated keratinocytes were treated with triolein, this conditioned medium attenuated its MMP1 expression inductive activity on dermal fibroblasts ($p<0.05$).

This result indicates that UVB irradiated keratinocytes could drive upregulation of MMP-1 in dermal fibroblasts in a paracrine fashion and triolein could protect against this deleterious effect.

3.3. IL-6 as the key paracrine factor driving UVB damage into the dermis.

To evaluate if IL-6 could act as a soluble signal released by Tel-E6E7 cells affecting MMP1 expression in fibroblasts, we incubated dermal fibroblast cells in the presence of human recombinant IL-6 protein (rh IL-6) (Akron Biotech, Boca Ratón, FL, USA) at the indicated concentrations for 48 hours. As shown in figure 3, IL-6 at 100ng/ml significantly induced the expression of MMP1 mRNA in fibroblasts (p <0.05). In view of this result IL-6 expressed by keratinocytes in response to UVB could be thought as part of a paracrine signaling (as well as autocrine) pathway activating MMP1 expression in dermal fibroblasts contributing to photoaging.

In order to further analyze the role of IL-6, we analyze the IL-6 release of the epidermal cells into culture medium. We analyzed the IL-6 protein expression of Tel-E6E7 cells under UVB challenge and its correlation with the MMP1 expression by fibroblasts cultured under Tel-E6E7 conditioned medium. As depicted, ELISA analysis showed that IL-6 release increases after UVB irradiation in the Tel-E6E7 conditioned culture medium used to induce MMP1 expression in fibroblasts (p <0.001). Moreover, triolein was able to inhibit IL-6 protein secretion in irradiated epidermal cells (see figure 4) (p <0.05). This inhibition of IL-6 secretion is in accordance with mRNA MMP1 expression inhibition in dermal fibroblasts (see figure 2).

3.4. Triolein reduces intracellular ROS generation by UVB.

In view of the inhibitory effect of triolein, first on IL-6 expression by epidermal cells under UVB irradiation and second on MMP1 mRNA expression by dermal fibroblasts exposed to soluble signals derived from epidermal cells, we evaluated the effect of triolein on UVB intracellular ROS generation in epidermal cells. The aim was to test a possible inhibitory mechanism of triolein in

ROS generation, as ROS could be a trigger event on IL-6 production [14]. With this goal, Tel-E6E7 cells were incubated with or without triolein for 24 hours, and then treated with the fluorescent probe DCF-DA as explained in Materials and Methods. Cells were immediately subjected to UVB irradiation and fluorescence was observed right away under the microscope to asses ROS generation. The percentage of fluorescent positive cells representing ROS generation status was higher than control under UVB treatment (p <0.01) but when triolein was applied before UVB treatment the percentage of fluorescent cells was similar to that of non-irradiated cells (control) or to that of cells in the presence of triolein only (p <0.01) (figure 5).

4. Discussion

The earth incidence of the UVB component of solar light is the most affected by ozone layer disruption [10]. Also UVB induces the most cytotoxic effects. Sunburn, characterized by erythema, edema, and skin sensibility, is caused by excessive UVB radiation [23]. UVB (280–320 nm) is mostly absorbed by the epidermis, but given its low penetration into the dermis and the fact that photoaged skin shows alterations mainly localized in dermal connective tissue, UVB-exposed epidermal keratinocytes should be the initiator of a molecular crosstalk with dermal fibroblasts (and other skin cells such as Langerhans cells and melanocytes) through diffusible signaling molecules. These signaling molecules elicit a pro-inflammatory status that triggers induction of the proteinases responsible for dermal extracellular alterations resulting in the histological and molecular traits of photoaged skin [24]. MMP-1 (interstitial collagenase) seems to be the predominant UVR induced enzyme that enhances collagen breakdown in UVB irradiated human skin, thus MMP-1 is suggested to be the major collagenolytic enzyme responsible for collagen destruction in photodamaged skin [3, 25, 26].

The multipotent stem cells from the hair follicle bulge can contribute to the repair of the damaged epithelium, as well as contributing to growth of hair follicles and sebaceous glands [27]. Thus, the immortalized adult epithelial stem cells line Tel-E6E7 derived from bulge stem cells is a good model of epidermal keratinocytes to study skin photodamage *in vitro* due to its repairing potential and the significance that UV damage could have on precursor cell linages.

In our experiments, the pro-inflammatory cytokine IL-6 was release by Tel-E6E7 cells *in vitro* when exposed to 6.8 mJ/cm^2 of UVB. This sublethal UVB dose resulted in cell survival of 70% at 5 days (data not shown) and corresponds to around half a minute of sunbathing at sea level [28]. *In vivo* keratinocytes are usually exposed to UVR, nevertheless photodamage seems to affect mostly the

dermal portion of skin [29]. MMP-1, a metalloproteinase involved in UV dermal damage induction is release by epidermal as well as dermal cells. It is thought to be mostly expressed by fibroblasts [30], although this is controversial [3]. Thus, signals from irradiated keratinocytes are supposed to affect fibroblasts leading to metalloproteinases expression increase [30]. Cytokines are good candidates for such role [5, 30-32]. Also MMPs produced by keratinocytes can translocate to the dermis to exert their collagenolytic effect by diffusion through direct binding to the collagenous extracellular matrix [33, 34]. Although we did not study the effect of UVB irradiated keratinocytes supernatants on other cell types, *in vivo* UVB effect on keratinocytes could be even more complex and also drive other mechanisms rather than the direct effect on dermal fibroblasts observed in this report. For instance, Van Nguyen et al [35] demonstrated that soluble mediators released by irradiated keratinocytes (IL-15, CCL5, etc.) induced mast cells migration and activation in human skin. This inflammatory mechanism in which these mast cells would be involved, may contribute to increase ROS environment of keratinocytes, release IL-6 themselves [36] contributing to MMP-1 increase expression by dermal fibroblasts and/or produce MMP-1 themselves.

In our hands, IL-6 expression was increased at mRNA and protein level when epidermal cells were UVB-irradiated (fig 1b; fig. 4). IL-1 alpha, IL-1 beta (figure 1a) and other analyzed cytokines and chemokines (Monocyte Chemoattractant Protein-1 (MCP1), Nucleosome Assembly Protein-2 (NAP2), Regulated on Activation Normal T cell Expressed and Secreted (Rantes), Tumor Necrosis Factor-alpha (TNF-alpha), data not shown) mRNA expression did not change significantly either upon UVB and/or triolein treatment of epidermal cells). Culture medium conditioned by irradiated epidermal cells led to an increase in MMP1 expression mRNA in primary dermal fibroblasts after 48 hours. The reduction of IL-6 secretion by irradiated keratinocytes treated with triolein is in accordance with the decrease of MMP1 expression by fibroblasts exposed to keratinocytes

conditioned medium. Accordingly we hypothesized that IL-6 could be responsible in an autocrine/paracrine way for the modulation of collagenolytic enzyme production in dermal fibroblasts under epidermal UV exposure as a part of the photoaging processes in agreement with others [14, 30, 32, 37]. Kim et al. [18] showed that triolein reduced basal and UV-induced MMP1 mRNA expression in cultured human epidermal keratinocytes and related this effect to skin lipid metabolism. We propose also an antioxidant role for triolein. UV irradiation elicits in skin the generation of ROS that participates in kinase cascade activation that triggers MMPs genes expression [13, 14, 38-40]. Our results showed that triolein inhibited the IL-6 mRNA/protein upregulation in UVB-treated epidermal keratinocytes. In addition IL-6 protein UVB-induced expression inhibition by triolein was reflected in MMP1 mRNA expression in dermal fibroblasts cultured in medium conditioned by UVB/triolein-treated epidermal cells. The decrease in cell fluorescence in UVB-irradiated epidermal keratinocytes under triolein treatment loaded with the DCF-DA probe suggests that triolein is reducing the UVB generation of ROS. This redox state modulation could in turn be regulating IL-6 expression [14]. Although we cannot conclude any exact mechanism, we suggest that upon UVB radiation exposure, epidermal keratinocytes first rapid response would be the generation of ROS, which in turn could be attenuated/modulated by triolein. ROS could trigger the pro-inflammatory cytokine release from keratinocytes, namely IL-6 (among others) which in turn would affect dermal fibroblasts response leading to MMP1 secretion. In an in vivo setting this MMP1 increase would allow an abnormal disorganized dermal matrix remodeling, a typical feature of photoageing. On the other hand the mechanism by which triolein affects ROS generation and IL-6 expression could be independent mechanisms converging on MMPs expression. Further studies beyond our cell culture experiments are needed to elucidate and confirm the exact mechanism and putative link. Nonetheless, in view of our and others results

[18, 31] triolein could be considered as a potential safe ingredient for daily moisturizers and photoprotective skin mixtures given the effect of triolein on UVB induced MMPs expression.

Acknowledgements

We thank Dr. Andrea V. Dugour (Fundación Pablo Cassará) for her kind technical assistance and Dr. Julieta Ceruti (Instituto de Ciencia y Tecnología, ICT-Milstein, Fundación Pablo Cassará, CONICET, CABA, Argentina) for her valuable discussion. This work was supported by Fundación Pablo Cassará.

Legends to figures and supplemental file

Figures

Fig.1. **Triolein effect on cytokine mRNA expression induced by UVB**. Tel-E6E7 cells were irradiated with UVB 6.8 mJ/cm^2 (UVB), irradiated with UVB 6.8 mJ/cm^2 and treated with triolein 20µM (UVB+Triol) or non-irradiated (control). Cells were cultured for 24 hours. mRNA was analyzed by Real Time PCR relative to control. IL-1 alpha and IL-1 beta mRNA representative analysis was shown in figure 1a. IL-6 mRNA analysis is shown in figure 1b.

Fig.2. **Keratinocytes conditioned culture medium affects MMP1 mRNA expression in primary dermal fibroblasts**. Tel-E6E7 cells were irradiated with UVB 6.8 mJ/cm^2 (UVB), irradiated with UVB 6.8 mJ/cm^2 and treated with triolein (20 µM) (UVB+Triol) and cultured for 24hs. Non-irradiated cells were used as control (control). MMP-1 mRNA was analyzed by Real Time PCR relative to control.

Fig.3. **Recombinant human IL-6 induces MMP1 mRNA expression in fibroblast.** Primary human dermal fibroblasts were cultured for 48 hours in the presence of increasing concentrations of human recombinant IL-6 (rh IL-6). MMP-1 mRNA was analyzed by Real Time PCR relative to fibroblasts cultured in absence of rh IL-6.

Fig.4. Triolein reduces IL-6 secretion in UVB-irradiated Tel-E6E7 cells. IL-6 secretion was analyzed in Tel-E6E7 conditioned culture medium by ELISA. Cells were previously irradiated with UVB 6.8 mJ/cm^2 and left untreated (UVB) or treated with triolein 20 µM for 24 hs (UVB/triol). Culture medium from non-irradiated cells (control) and cells treated with triolein only (control+triolein) were used as control.

Fig.5. **Triolein inhibits ROS generation in UVB-treated Tel-E6E7 cells.** Tel-E6E7cells were treated with triolein 20 µM (Triol) or kept untreated (Control) for 24 hours. Then cells were incubated with the fluorescent probe DCF-DA for 45 minutes. Afterwards, half of the triolein treated and control cell cultures were irradiated with UVB 6.8 mJ/cm^2 ((UVB/Triol), (Control/UVB)). The other half of the cultures was not irradiated. The cells were analyzed under the epifluorescence microscope. Upper panel shows pictures of fluorescence microphotoghaps at 400x magnification. Lower panel shows bar-graphic of percentage of positive cells for fluorescent probe DCF-DA.

Supplemental file:

Fig.S0. MMP-1 mRNA relative expression in UVB-irradiated Tel-E6E7 cells. Tel-E6E7 cells were irradiated with UVB 3.4 mJ/cm^2 (UVB 3.4) or 6.8 mJ/cm^2 (UVB 6.8) and maintained in culture for 24hs. The same irradiation protocol was performed with these cells but maintained them in culture with 20 micromolar triolein (UVB 3.4 + triol; UVB 6.8 + triol). Non irradiated cells were cultured for 24 hours (control) or cultured in presence of triolein (triol). MMP1 mRNA expression relative to control was analyzed by Real Time PCR.

References

1. Kim, E.J., et al., *Skin aging and photoaging alter fatty acids composition, including 11,14,17-eicosatrienoic acid, in the epidermis of human skin.* J Korean Med Sci, 2010 25(6): p. 980-3.
2. Fisher, G.J., et al., *Mechanisms of photoaging and chronological skin aging.* Arch Dermatol, 2002. 138(11): p. 1462-70.
3. Quan, T., et al., *Matrix-degrading metalloproteinases in photoaging.* J Investig Dermatol Symp Proc, 2009. 14(1): p. 20-4.
4. Pillai, S., C. Oresajo, and J. Hayward, *Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation - a review.* Int J Cosmet Sci, 2005. 27(1): p. 17-34.
5. Schwarz, T. and T.A. Luger, *Effect of UV irradiation on epidermal cell cytokine production.* J Photochem Photobiol B, 1989. 4(1): p. 1-13.
6. Takashima, A. and P.R. Bergstresser, *Impact of UVB radiation on the epidermal cytokine network.* Photochem Photobiol, 1996. 63(4): p. 397-400.
7. Petit-Frere, C., et al., *Induction of interleukin-6 production by ultraviolet radiation in normal human epidermal keratinocytes and in a human keratinocyte cell line is mediated by DNA damage.* J Invest Dermatol, 1998. 111(3): p. 354-9.
8. Kirnbauer, R., et al., *Regulation of epidermal cell interleukin-6 production by UV light and corticosteroids.* J Invest Dermatol, 1991. 96(4): p. 484-9.
9. Cherng, J.Y., L.Y. Chen, and M.F. Shih, *Preventive effects of beta-thujaplicin against UVB-induced MMP-1 and MMP-3 mRNA expressions in skin fibroblasts.* Am J Chin Med, 2012. 40(2): p. 387-98.
10. Brenneisen, P., H. Sies, and K. Scharffetter-Kochanek, *Ultraviolet-B irradiation and matrix metalloproteinases: from induction via signaling to initial events.* Ann N Y Acad Sci, 2002. 973: p. 31-43.
11. Hong, Y.F., et al., *Lipoteichoic acid isolated from Lactobacillus plantarum down-regulates UV-induced MMP-1 expression and up-regulates type I procollagen through the inhibition of reactive oxygen species generation.* Mol Immunol, 2015. 67(2 Pt B): p. 248-55.
12. Martin, R., et al., *Photoprotective effect of a water-soluble extract of Rosmarinus officinalis L. against UV-induced matrix metalloproteinase-1 in human dermal fibroblasts and reconstructed skin.* Eur J Dermatol, 2008. 18(2): p. 128-35.
13. Son, Y., et al., *Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways?* J Signal Transduct, 2011. 2011: p. 792639.
14. Chen, C.C., et al., *EGb-761 prevents ultraviolet B-induced photoaging via inactivation of mitogen-activated protein kinases and proinflammatory cytokine expression.* J Dermatol Sci, 2014. 75(1): p. 55-62.
15. Pappas, A., *Epidermal surface lipids.* Dermatoendocrinol, 2009. 1(2): p. 72-6.
16. Radner, F.P. and J. Fischer, *The important role of epidermal triacylglycerol metabolism for maintenance of the skin permeability barrier function.* Biochim Biophys Acta, 2013 1841(3): p. 409-15.
17. De Luca, C.a.V., G, *surface lipids as multifunctionl mediators of skin responses to environmental stimuli.* Mediators of Inflammation, 2010. 2010(321494).

18. Kim, E.J., et al., *UV decreases the synthesis of free fatty acids and triglycerides in the epidermis of human skin in vivo, contributing to development of skin photoaging.* J Dermatol Sci, 2010. 57(1): p. 19-26.
19. Downing, D.T., J.S. Strauss, and P.E. Pochi, *Variability in the chemical composition of human skin surface lipids.* J Invest Dermatol, 1969. 53(5): p. 322-7.
20. Luo, T., et al., *Triolein and trilinolein ameliorate oxidized low-density lipoprotein-induced oxidative stress in endothelial cells.* Lipids, 2014. 49(5): p. 495-504.
21. Roh, C., et al., *Multi-potentiality of a new immortalized epithelial stem cell line derived from human hair follicles.* In Vitro Cell Dev Biol Anim, 2008. 44(7): p. 236-44.
22. Leiros, G.J., A.I. Attorresi, and M.E. Balana, *Hair follicle stem cell differentiation is inhibited through cross-talk between Wnt/beta-catenin and androgen signalling in dermal papilla cells from patients with androgenetic alopecia.* Br J Dermatol, 2012. 166(5): p. 1035-42.
23. Rigo, L.A., et al., *Nanoencapsulation of rice bran oil increases its protective effects against UVB radiation-induced skin injury in mice.* Eur J Pharm Biopharm, 2015. 93: p. 11-7.
24. Imokawa, G., *Mechanism of UVB-induced wrinkling of the skin: paracrine cytokine linkage between keratinocytes and fibroblasts leading to the stimulation of elastase.* J Investig Dermatol Symp Proc, 2009. 14(1): p. 36-43.
25. Brennan, M., et al., *Matrix metalloproteinase-1 is the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin.* Photochem Photobiol, 2003. 78(1): p. 43-8.
26. Fisher, G.J., et al., *Collagen fragmentation promotes oxidative stress and elevates matrix metalloproteinase-1 in fibroblasts in aged human skin.* Am J Pathol, 2009. 174(1): p. 101-14.
27. Blanpain, C., et al., *Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche.* Cell, 2004. 118(5): p. 635-48.
28. Yoshizumi, M., et al., *Release of cytokines/chemokines and cell death in UVB-irradiated human keratinocytes, HaCaT.* Cell Biol Int, 2008. 32(11): p. 1405-11.
29. Fisher, G.J., et al., *Pathophysiology of premature skin aging induced by ultraviolet light.* N Engl J Med, 1997. 337(20): p. 1419-28.
30. Fagot, D., D. Asselineau, and F. Bernerd, *Matrix metalloproteinase-1 production observed after solar-simulated radiation exposure is assumed by dermal fibroblasts but involves a paracrine activation through epidermal keratinocytes.* Photochem Photobiol, 2004. 79(6): p. 499-505.
31. Kim, E.J., et al., *UV modulation of subcutaneous fat metabolism.* J Invest Dermatol, 2011. 131(8): p. 1720-6.
32. Kondo, S., *The roles of cytokines in photoaging.* J Dermatol Sci, 2000. 23 Suppl 1: p. S30-6.
33. Overall, C.M., *Matrix metalloproteinase substrate binding domains, modules and exosites. Overview and experimental strategies.* Methods Mol Biol, 2001. 151: p. 79-120.
34. Rohani, M.G., et al., *Cdc42 inhibits ERK-mediated collagenase-1 (MMP-1) expression in collagen-activated human keratinocytes.* J Invest Dermatol, 2014. 134(5): p. 1230-7.
35. Van Nguyen, H., et al., *Ultraviolet radiation-induced cytokines promote mast cell accumulation and matrix metalloproteinase production: potential role in cutaneous lupus erythematosus.* Scand J Rheumatol, 2011. 40(3): p. 197-204.
36. Sarchio, S.N., et al., *Dermal mast cells affect the development of sunlight-induced skin tumours.* Exp Dermatol, 2012. 21(4): p. 241-8.

37. Brenneisen, P., et al., *Ultraviolet-B induction of interstitial collagenase and stromelyin-1 occurs in human dermal fibroblasts via an autocrine interleukin-6-dependent loop.* FEBS Lett, 1999. 449(1): p. 36-40.
38. Nishisgori, C., *Current concept of photocarcinogenesis.* Photochem Photobiol Sci, 2015. 14(9): p. 1713-21.
39. Sander, C.S., et al., *Photoaging is associated with protein oxidation in human skin in vivo.* J Invest Dermatol, 2002. 118(4): p. 618-25.
40. Kang, S., et al., *Topical N-acetyl cysteine and genistein prevent ultraviolet-light-induced signaling that leads to photoaging in human skin in vivo.* J Invest Dermatol, 2003. 120(5): p. 835-41.