Evaluation of the human skin responses to solar-simulated radiation in an *ex vivo* model: effects and photoprotection.

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ABSTRACT

Introduction:
To date, it has been proven that sunlight, and more specifically the UV component, induces several skin damages, including sunburns, erythema, reactive oxygen species and photo-aging. The purpose of this work is to set up a methodology that could be used to verify the effects of solar radiation on human skin and to assess the capacity of different compounds in protecting the irradiated skin.

Methods:
Ex vivo human skin biopsies were cultured in an air-liquid interface and exposed daily to solar-simulated radiation (SSR, 300-800 nm) and to treatments (L-Carnosine). Reactive oxygen species (ROS), gene expression and protein modulation were assessed**.**

Results:
In our *ex vivo* skin model, SSR increases ROS production and affects the expression of several genes related to oxidative-stress, pigmentation, immunity/inflammation and photo-aging. Among these, twelve genes were selected and validated to evaluate the skin solar radiation response and to test L-Carnosine as possible protective compound. We found that L-Carnosine mitigates the adverse effects of solar exposure reducing ROS and helps to restore the expression level of most of the selected genes.

Discussion and Conclusion:
Our *ex vivo* skin model is a valuable system to assess the consequences of solar light. SSR induces oxidative stress and alterations of the expression of genes involved in skin functions, stress response and aging. Photoprotection studies showed that, L-Carnosine provides effective prevention against solar radiation damages reducing ROS and helps to restore the expression level of most of the selected biomarker genes.

Keywords: solar-simulated radiation; L-Carnosine; *ex vivo* human skin; ROS; gene array

INTRODUCTION

The human skin is the largest body organ that, acting as outer barrier, is essential to protect the body against environmental aggression, such as chemical products, microorganisms and radiations. The latter consists mainly of solar radiation, or just sunlight, that is one of the external factors to which human skin is acutely as well as chronically exposed. It influences the physiology of the human skin in either a beneficial or a harmful way [1].

Terrestrial solar radiation is composed of a continuous electromagnetic spectrum that could be divided into three main wavelengths portions: ultraviolet (UVA and UVB, 5 %), visible (VIS, 50 %), and infrared (IR, 45 %) [2]. Solar ultraviolet radiation (UV) is the most important environmental factor affecting skin physiology, which spectrum comprises $\leq$5% of UVB (~295–315 nm) and $\geq$95% of UVA (315–400 nm) [3, 4]. Exposure of human skin to solar UV rays can lead to short- and long-term consequences including erythema, photo-aging, photo-immunosuppression and skin cancers. Although UVB rays display beneficial effects such as production of vitamin D, they are more energetic than UVA, and can directly damage the DNA of epidermal cells, contributing to the photo-carcinogenesis process. On the contrary, UVA rays penetrate deeper in the skin, reaching the deep dermis, and are mostly responsible for the generation of reactive oxygen species (ROS) and, to a lesser extent in comparison to UVB, can also generate DNA damage [5]. UVA rays are, in the long term, mostly involved in skin photo-aging [6, 7]. To date, it has been proven that sunlight, and more specifically the UV component, induces skin damages, both acute (e.g., sunburns, erythema, reactive oxygen species (ROS) formation) and chronic (e.g., photo-aging, immunosuppression, collagen degradation) [1, 8-10]. For this reason, it is important to study the impact of solar exposure on the skin and develop new photoprotection compounds to avoid or reduce its damaging consequences.

The aim of this work was to set up a methodology that could be used to verify the effects of solar radiation on human skin and to assess the capacity of different compounds in protecting irradiated skin. In the first part of the study, *ex vivo* human skin exposed to solar-simulated radiation (SSR) was used as a model to establish the experimental conditions and readouts. In particular, based on literature data, a custom gene array was designed to analyse the expression of several genes related to skin biology and stress response in our *ex vivo* skin model exposed to SSR. Subsequently, human skin biopsies were topically treated with L-Carnosine at two different concentrations and exposed to SSR. The effect on oxidative stress induction, inflammatory response, and photo-aging was assessed by evaluating ROS formation, transcriptional response with the designed custom gene array, and protein level with immunohistochemistry staining.

MATERIAL AND METHODS

Study design

In order to evaluate the effects of solar light on our *ex vivo* skin model, and the efficacy of L-Carnosine on protecting skin from SSR, oxidative stress, gene expression and proteins were analysed.

Ex vivo human skin biopsies were topically treated with L-Carnosine at different concentrations (0.2% and 2.2%), and daily exposed to 250 J/cm^2 of SSR. Reactive oxygen species (ROS), gene expression and protein modulation were assessed respectively at day1, day 2 and day3. The radiation doses were selected following the literature [11-13], and represent the approximative amount of solar light acquirable under natural conditions by sunbathing.

Tissue culture

Human skin samples were obtained from plastic surgery of healthy patients (aged between 25 and 60), without any kind of cutaneous diseases or intensive sun exposure. The informed consent was obtained from patients and retained by clinic. For the studies, twelve 8 mm diameter punch biopsies were used for each treatment. The skin samples were cultured in modified Williams' E medium at air-liquid interface and, when not irradiated, maintained in incubator at the following conditions: 37°C, 5% CO_2 and 95% humidity.

Treatments

For these studies L-Carnosine was dissolved in vehicle (DMSO/PBS 1:25) at the following concentration 0.2% and 2.2%. Undiluted a-Tocopherol acetate (VIT-E) (Merck KGaA, Germany) was used as positive control. Treatments were applied topically and renewed daily as follows: skin biopsies were cleaned with cotton buds and 4 µl of treatment were applied on the biopsy's epidermis. All treated and the control samples were covered with 6 mm diameter delivery membranes.

Solar Simulated Radiation

Skin irradiation was performed daily, with Suntest XLS+ (Ametek Inc., USA) equipped with the provided IR filter and a DAY LIGHT filter, to obtain an emission spectrum between 300 and 800 nm. Every day the skin samples were irradiated with a SSR dose of 250 J/cm^2. Control samples were sham-irradiated under the same conditions.

ROS evaluation

To perform ROS evaluation, 2'-7'dichlorofluorescin diacetate (DCFH-DA) was used. Before irradiation, skin samples were treated overnight (about 16 hours) with vehicle or test compounds; then skin biopsies were incubated with DCFH-DA probe for 30 minutes, finally the samples were exposed to 250 J/cm^2 of SSR. Immediately after irradiation skin samples were harvested and cryo-fixed. The images of the skin sections were acquired, and the upper dermis analysed by evaluating the fluorescence through Image-J application (NIH, USA). The obtained value has been normalized upon the dimension of the selected area.

RNA Isolation and Quantitative Real-Time

Human skin biopsies were collected in cryo-vial containing RNAlater stabilization solution (Merck KGaA, Germany) and stored at -80°C. Total RNA was extracted using an Rneasy mini-kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA). Reverse transcription reactions were performed using 1 µg of total RNA with Quantitect Reverse transcriptase kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. mRNA levels of selected genes were detected using custom TaqMan® Array 96-Well Fast Plates (Thermo Fisher Scientific Inc., USA). Real-time RT-PCR was performed on QuantStudio 3 (Thermo Fisher Scientific Inc., USA) using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc., USA) according to the manufacturer's recommendations. The $2^{-\Delta\Delta Ct}$ method was used to determine the gene expression changes of the target genes in experimental samples versus controls (fold-change; up-reg. $\geq$2 and down-reg. $\leq$0.5). The Ct values were normalized versus the endogenous control genes YWHAZ, PPIA, B2M, TBP (geometrical mean), previously selected using a TaqMan™ Array Human Endogenous Control (Thermo Fisher Scientific Inc., USA).

IHC staining

Ex vivo human skin biopsies were topically treated with L-Carnosine at different concentrations (0.2% and 2.2%) and exposed daily to SSR. At day 3 skin samples were collected and fixed in formalin and paraffin embedded. Skin sections were immuno-stained with CYR61 antibody 1:200 and PTGS2 antibody 1:100 (Thermo Fisher Scientific Inc., USA), 1h at RT. The images of the skin sections were acquired, and the analysis were performed through Image-J application (NIH, USA) considering the skin epidermis (CYR61) and the basal lamina region (PTGS2). The obtained value has been normalized upon the dimension of the selected area.

Statistical analysis

Results obtained from the image analysis were expressed in terms of mean value or mean score, and the measures of variation as standard error of mean (SEM). Differences between groups were evaluated by One-way ANOVA with permutation test followed by Tukey's test with permutations. For all statistical tests, $p<0.05$ was considered significant.

RESULTS

L-Carnosine promotes a reduction of ROS production

In order to evaluate the ability of L-Carnosine to modulate the oxidative stress induced by SSR, ROS generation was assessed in sample treated with L-Carnosine overnight (about 16h) and then exposed to 250 J/cm^2 of SSR. Fig. 1 shows the mean values of two independent studies, and the ROS level is expressed as a ratio to the not-irradiated vehicle. The obtained results confirmed a significant induction of oxidative stress after SSR stimulus, whereas a significant decrease of ROS production was observed in samples treated with L-Carnosine at both concentrations tested. Specifically, mean values of -60% and -63% were respectively found with 0.2% and 2.2% treatments. The positive antioxidant control (VIT-E) confirmed the antioxidant effect of L-Carnosine in our model, with a reduction of 73%.

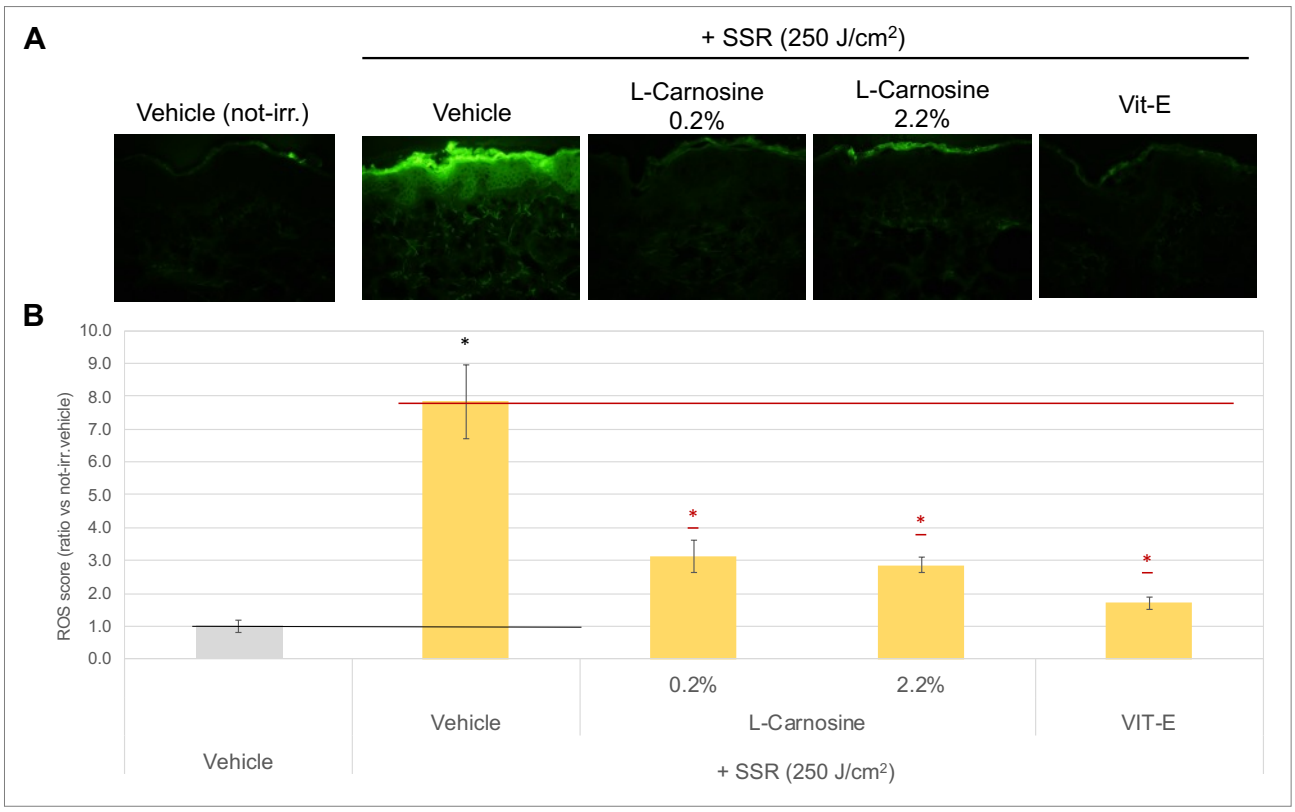

Fig. 1. Decrease of ROS production in L-Carnosine treated samples. Skin samples were treated 16h with vehicle or test compounds and stimulated with 250 J/cm^2 of SSR. ROS production was assessed by DCFH-DA assay and fluorescence obtained was evaluated in the upper dermis. A) Representative images. B) Graph showing ROS mean level (expressed as ratio vs the not-irradiated vehicle) obtained in two independent studies (n=2). Error bars indicate the standard error of mean (SEM). * Significantly different from not-irradiated vehicle, * Significantly different from irradiated vehicle (Tukey's test, $p<0.05$).

L-Carnosine helps to counteract the effect of SSR on gene expression

In order to proceed with the gene expression analysis in our model, we have decided to exploit the large amount of published data regarding gene expression modulation in skin exposed to different sources of light. Between the numerous genes reported to be modulated after irradiation (UV, VIS, Blue light, Sunlight, SSR) some genes were selected as most interesting to study stress response, inflammation, and skin biology processes [1,14-23]. Based on the results obtained in previous study, 18 genes were found to be modulated as expected by SSR stimulus in our model (data not shown). To assess the skin solar irradiation response and to test possible protective compounds, 12 of 18 genes were evaluated as markers of SSR exposure. These genes were selected because mainly involved in the processes of inflammation (IL20, IL6, PTGS2, TNF), aging/photo-aging

(MMP1, CYR61), pigmentation (OCA2) and other important cellular functions that regulate skin biology (GRIP1, PRKCB, FOSL1, FGF7, GDF15) [1, 3, 10, 11, 18-22].

The analysis of gene expression was performed on *ex vivo* human skin obtained from three healthy donors, without cutaneous diseases or intensive sun exposure. Skin samples were topically treated with L-Carnosine at different concentrations (0.2% and 2.2% in vehicle) and exposed to 250 J/cm^2 of SSR daily. At the selected end point (day 2), samples were collected and processed for the analysis with the TaqMan® custom gene array. The gene expression change of target genes was expressed as fold-change value compared to the not-irradiated vehicle (fold-change = 1.0). Table A in fig. 2 shows the results obtained for each donor. In particular, the transcriptional response to SSR of the selected marker genes was confirmed (fold-change values in *grey*), with 10 of 12 genes modulated at least in two out of three donors. Interestingly, L-Carnosine treatment protects from the effect of SSR restoring the expression level of the most of the selected biomarker genes (fold-change values in *bold italics*). This photo-protective effect was observed at least with one concentration tested, resulting in a reduced modulation of 8 genes in at least 2/3 donors (fig. 2B). These genes, all up-regulated by SSR, are mainly involved in the processes of immunity and inflammation (IL20, IL6, PTGS2, TNF), extracellular matrix remodeling (MMP1, CYR61), and other biological functions, including cell growth, morphogenesis, tissue repair and pigmentation (FGF7) or stress response and cellular senescence (GDF15) [18, 20-22]. Graphs in fig. 2C show the gene expression results obtained in donor 1 for the eight SSR up-regulated genes. The protective effect of L-Carnosine against SSR is demonstrated by the reduction of the gene expression level ranging from -21% of PTGS2 to -91% for FGF7.

A

Target	Expected modulation after SSR	Fold-change (vs Not-irr. VEHICLE)								
		Don_1			Don_2			Don_3		
		+ SSR (250 J/cm2)			+ SSR (250 J/cm2)			+ SSR (250 J/cm2)		
		L-Carnosine			L-Carnosine			L-Carnosine		
		VEHICLE	0.2%	2.2%	VEHICLE	0.2%	2.2%	VEHICLE	0.2%	2.2%
GRIP1	-	0.3	0.1	0.2	0.2	0.1	0.2	0.5	0.4	***0.7***
PRKCB	-	0.3	0.1	0.2	0.0	0.1	***0.2***	0.4	0.4	0.3
IL20	+	8.2	***4.1***	***4.6***	14.7	***12.8***	***5.7***	1.5	***0.8***	***0.9***
IL6	+	4.2	***1.4***	***2.4***	13.3	***9.4***	***10.4***	2.6	2.2	***2.1***
TNF	+	12.9	***8.2***	***7.3***	6.6	7.7	***4.7***	1.4	1.4	1.2
OCA2	-	0.5	0.2	0.3	0.7	0.4	0.4	0.3	0.3	0.3
MMP1	+	3.3	***2.4***	***1.8***	4.0	5.4	***1.9***	0.2	0.7	0.1
PTGS2	+	5.3	***4.2***	***4.0***	13.9	***11.3***	***7.7***	2.0	***1.4***	***1.4***
CYR61	+	8.1	***2.7***	***4.0***	6.1	6.2	***4.5***	2.3	2.9	***1.5***
FOSL1	+	4.8	4.6	4.5	11.2	***9.6***	***5.7***	2.2	3.1	4.8
FGF7	+	14.9	***1.9***	***1.3***	1.8	2.0	***1.0***	0.7	4.2	1.0
GDF15	+	67.4	***22.0***	***22.4***	16.5	***11.9***	***14.1***	57.3	***25.3***	***36.8***

B

L-Carnosine photo-protective effect			
Target	Don_1	Don_2	Don_3
GRIP1			✓
PRKCB		✓	
IL20	✓	✓	✓
IL6	✓	✓	✓
TNF	✓	✓	
OCA2			
MMP1	✓	✓	
PTGS2	✓	✓	✓
CYR61	✓	✓	✓
FOSL1		✓	
FGF7	✓	✓	
GDF15	✓	✓	✓

C

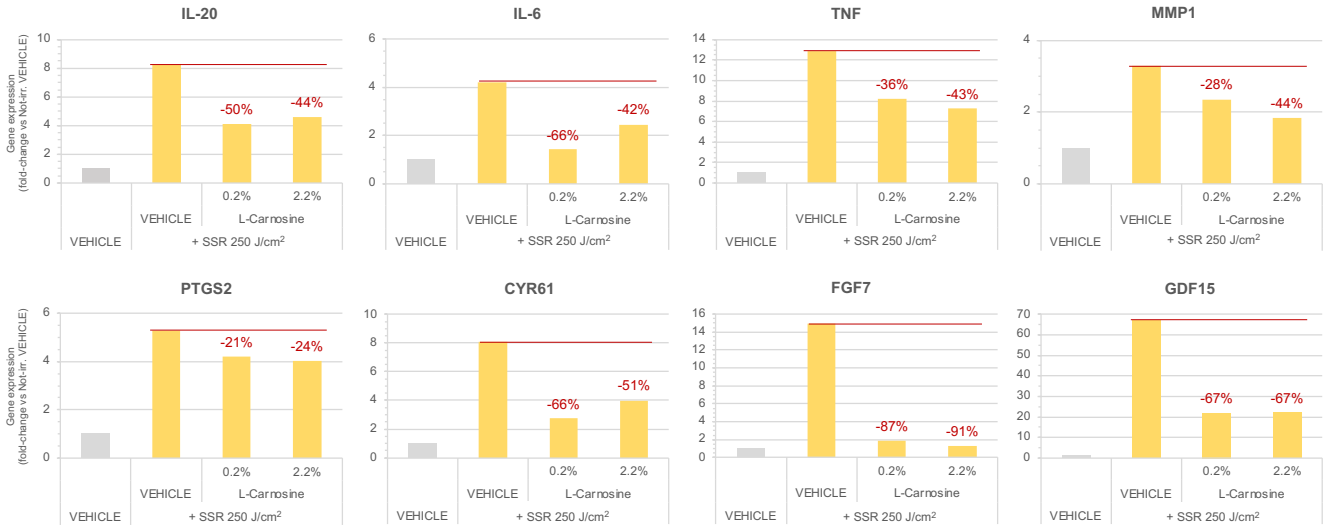

Fig. 2. L-Carnosine mitigates the gene expression modulation. Gene expression of the 12 selected genes was evaluated in skin biopsies from 3 donors treated with L-Carnosine 0.2% or 2.2% and exposed to SSR up to day 2. L-Carnosine treatment protects from the effect of SSR reducing the gene expression modulation of several genes. A) Gene fold-change values obtained in relation to the not-irr.vehicle (fold-change = 1.0). The expected gene modulation following SSR is highlighted in *grey* (fold change up-reg. ≥2 and down-reg. ≤0.5); in *bold italics* the fold-change values mitigated by L-Carnosine treatment (photo-protective effect, Δfold-change ≥0.5). B) Summary of the gene expression results; √ indicates L-Carnosine photo-protective effect at least with one concentration tested, and in *grey* if this effect is confirmed at least 2/3 donors. C) L-Carnosine reduces the effect of SSR on gene expression of 8/12 genes. Example of results obtained (Don_1).

After SSR, PTGS2 and CYR61 protein induction is mitigated by L-Carnosine treatments

Among the SSR marker genes restored by L-Carnosine treatments, CYR61 and PTGS2 were selected and validated at protein level.

Skin samples were treated daily with L-Carnosine 0.2% and 2.2% and irradiated 250 J/cm^2 of SSR for three days; at the selected time point, samples were collected, and protein level was evaluated by IHC. The obtained results, reported in fig. 3, showed an increased protein level of CYR61 and PTGS2 after SSR exposure compared to the control, +82% and +448% respectively. L-Carnosine treatment protects from the effect of SSR reducing significantly CYR61 protein level at both concentrations tested. Specifically, mean values of -45% and -49% were found with 0.2% and 2.2% treatments respectively. PTGS2 protein level seems to be mitigated by L-Carnosine 2.2% (-26%).

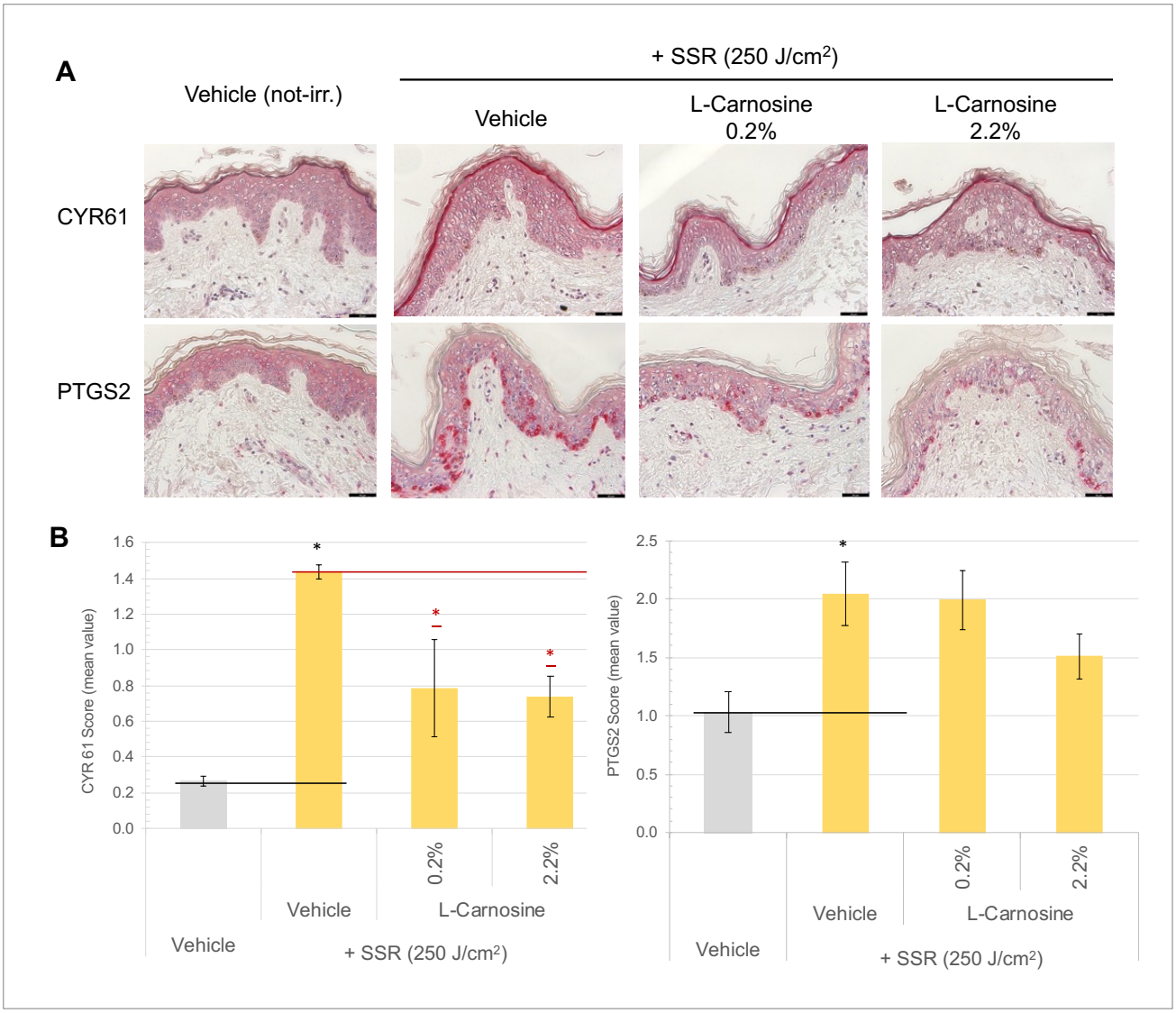

Fig. 3. L-Carnosine mitigates PTGS2 and CYR61 protein induction. PTGS2 and CYR61 protein level was evaluated in skin biopsies treated with L-Carnosine 0.2% or 2.2% and exposed to SSR up to day 3. Protein level was assessed by immunohistochemical staining. L-Carnosine treatment protects from the effect of SSR reducing the protein induction of both markers. A) Representative images. B) Graphs showing CYR61 and PTGS2 level (mean score). Error bars indicate the standard error of mean (SEM). * Significantly different from not-irradiated control vehicle; * Significantly different from irradiated vehicle (Tukey's test, $p<0.05$).

DISCUSSION

Skin is the largest organ of the human body, which protects it from the harmful external stimuli including chemical products, microorganisms and electromagnetic radiations.

In detail, terrestrial solar radiation, and more specifically the UV component, is the most important environmental factor which affects the skin physiology, inducing sunburns, erythema, photoaging, immunosuppression and skin cancer [1; 4; 5; 7-10]. For this reason, it is important to study the impact of solar exposure on the skin and develop new photo-protective compounds to avoid or reduce its damaging consequences.

The purpose of this work was to set up a methodology that could be used to verify the effects of solar radiation on human skin and to assess the capacity of different components in protecting the irradiated skin. Specifically, *ex vivo* human skin exposed to solar-simulated radiation (SSR) was used as a model to test L-Carnosine as photo-protective treatment.

Human skin biopsies were topically treated with L-Carnosine at two different concentrations and exposed to 250 J/cm^2 of SSR. According to literature [11-13], this dose represents the approximative amount of solar light acquirable under natural conditions by sunbathing.

The effect on oxidative stress induction, inflammatory response, and photo-aging was assessed by evaluating ROS formation, transcriptional response with custom gene array and marker protein levels by immunohistochemistry.

We demonstrated that our SSR model on ex vivo human skin is a valuable system to assess the consequences of solar light and the capacity of applied compounds to counteract them. The selected dose of SSR significantly induces oxidative stress, increasing ROS (fig. 1), as well as it modulates the expression of genes involved in the processes of inflammation (IL20, IL6, PTGS2, TNF), aging/photo-aging (MMP1, CYR61) and other important cellular functions that regulate skin biology (OCA2, GRIP1, PRKCB, FOSL1, FGF7, GDF15) (fig.2A).

More importantly, we showed that L-Carnosine treatments (0.2% and 2.2%) decrease SSR induced ROS production (fig.1) and helps to counteract the effect of SSR on gene expression of the most of the selected biomarker genes (fig. 2A and 2B). These genes, all up-regulated by SSR, are mainly involved in the processes of inflammation (IL20, IL6, PTGS2, TNF), extracellular matrix remodeling (MMP1, CYR61), and other biological functions, including cell growth, tissue repair, stress response and cellular senescence (FGF7, GDF15) [1, 3, 10, 11, 18-22].

In samples treated with L-Carnosine a reduction of the PTGS2 expression level was found in all donors tested, with mean values of -23% and -33% respectively after 0.2% and 2.2% treatments. Results obtained in donor 1 are reported as example (fig. 2C); after SSR stimulus, L-Carnosine helps to reduces the expression of this gene approximately of 20-25% at both the tested concentrations.

PTGS2 is the key enzyme in prostaglandin biosynthesis. It is strongly induced by many stimuli including growth factors, cytokines and solar radiation (UV), resulting in the production of prostaglandins during inflammatory processes [22, 23]. Also at protein level, our findings confirm previous published data that report a PTGS2 induction following UV exposure, predominantly in the basal keratinocyte layer [24] (fig. 3A). Inhibition of PTGS2 has been reported to prevent skin inflammation, aging and carcinogenesis, representing a potential strategy for preventing solar UV related skin disorders [25]. In our model, L- Carnosine 2.2% helps to reduce PTGS2 protein (fig. 3B).

Another important marker, modulated by L-Carnosine, is CYR61. L-Carnosine 2.2% mitigates the expression level of this gene in all donors tested (-37% average) (fig. 2A and 2B). In donor 1, we report a reduction of 66% and 51% respectively with 0.2% and 2.2% treatments (fig. 2C). CYR61 is an extracellular matrix protein reported to be a negative regulator of collagen homeostasis by inhibiting type-I collagen production and promoting its degradation. It is markedly induced by ROS in irradiated human skin and contributes to human skin connective tissue aging [26-28]. For this marker, the photo-protective effect of L-Carnosine was confirmed also at protein level, with a reduction of 45% and 49% respectively with 0.2% and 2.2% treatments (fig. 3).

As for PTGS2 and CYR61, also FGF7 gene is modulated by the topical application of L-Carnosine. We found a reduced expression level of FGF7 after SSR stimulus in the presence of L-Carnosine 2.2% in two of the three donors tested (fig. 2A and 2B); we reported in detail the results obtained in donor 1, where a reduction of -87% and -91% was obtained respectively with 0.2% and 2.2% treatments (fig. 2C). This gene is a growth factor protein mainly involved in the repair and remodeling of the dermis during the skin aging process and is reported to be a positive regulator of skin pigmentation [29-31].

Finally, in all donors tested, the expression level of GDF15 after irradiation is reduced in samples treated with L-Carnosine, with average values of -50% and -37%, respectively for 0.2% and 2.2% treatments (fig. 2A and 2B). In the more responsive donor (Don_1), a reduction of 67% was obtained with both treatments (fig. 2C). This gene was selected for its role in the stress-response and cellular senescence processes, and because it is known to be strong up-regulated in response to UV irradiation, as we confirmed [1, 21].

CONCLUSION

The UV component of terrestrial solar radiation is the most important environmental factor affecting skin physiology with either acute, or chronic skin damages. For this reason, it is important to study the impact of solar exposure on the skin and develop new photo-protective compounds to avoid or reduce its damaging consequences.

The reported findings on our *ex vivo* skin model confirm the literature data on solar-simulated radiation (SSR) effects. Samples exposed to SSR shows oxidative stress induction and alterations of the expression of genes involved in skin functions, stress response and aging.

Specifically, a biomarker genes panel was developed to evaluate the effects of solar radiation on skin, and to test the capacity of applied compounds to counteract them. Among the selected genes, PTGS2 and CYR61 are particularly interesting because respectively involved in the inflammation and skin aging processes.

Photoprotection studies showed that, L-Carnosine could be used in addition to UV-filter to provide better prevention against solar radiation. Our results shown that L-Carnosine helps to mitigate ROS production and to counteract the modulation of genes involved in inflammation, extracellular matrix remodeling, tissue repair, stress response and cellular senescence. The induction of PTGS2 and CYR61 following SSR stimulus is mitigated by L-Carnosine treatment at both gene and protein level. In conclusion, our *ex vivo* human skin model proved to be a valuable system to assess the consequences of solar light and the capacity of applied compounds (L-Carnosine) to boost the efficacy of sunscreen products.

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