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RESEARCH ARTICLE

Evaluation of human skin response to solar-simulated radiation in an ex vivo model: Effects and photoprotection of L-Carnosine

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Abstract

Sunlight, and more specifically the UV component, induces several skin damages, including sunburns, erythema and photoaging. The purpose of this work is to set up an ex vivo human skin model to assess the capacity of active principles in protecting skin from the deleterious effects of solar radiation. Ex vivo human skin biopsies were cultured in an air–liquid interface and exposed to solar-simulated radiation (SSR, 300–750 nm). L-Carnosine (0.2% and 2%) was applied topically to be tested as photoprotective compound. The effect on oxidative stress induction, photoaging and skin transcriptional profile was assessed by evaluating reactive oxygen species, advanced glycosylation end products formation and gene expression changes. In our model, SSR increases ROS production and AGE accumulation and affects the expression of genes related to oxidative stress, pigmentation, immunity, inflammation and photoaging. Among these pathways, 11 genes were selected as biomarkers to evaluate the skin solar radiation response. Results showed that L-Carnosine provides effective prevention against solar radiation damages reducing ROS, AGEs and mitigating the modulation of the selected biomarker genes. In conclusion, we report that our ex vivo skin model is a valuable system to assess the consequences of solar light exposure and the capacity of topically applied L-Carnosine to counteract them.

KEYWORDS

AGEs, gene array, ex vivo human skin, L-Carnosine, photoprotection, ROS, skin, solar-simulated radiation

Abbreviations: AGEs, advanced glycosylation end products; DCFH-DA, 2'-7'dichlorofluorescin diacetate; ECM, extracellular matrix; IHC, immunohistochemistry; PBS, Dulbecco's phosphate-buffered saline; ROS, reactive oxygen species; SEM, standard error of mean; SSR, solar-simulated radiation.

INTRODUCTION

Human skin is the largest body organ acting as an outer barrier. It plays a key role in protecting the body against environmental aggression, including chemical products, microorganisms and radiations. In particular, radiations—predominantly made of solar light—affect the physiology of human skin in both beneficial and harmful ways.[1–3] Terrestrial solar radiation is composed of a continuous electromagnetic spectrum that can be divided into three main wavelengths portions: ultraviolet (UVA and UVB, 5%), visible (VIS, 50%) and infrared (IR, 45%). Skin physiology is primarily influenced by solar ultraviolet radiation (UV), with UVA (315–400 nm) constituting more than 95% of its spectrum, while UVB (~295–315 nm) accounts for less than 5%.[4,5] Exposure of human skin to solar UV rays can lead to short- and long-term consequences, including erythema, photoaging, photoimmunosuppression and skin cancers.[6,7] 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 photocarcinogenesis process.[8] On the contrary, UVA penetrates deeper into the skin, reaching the deep dermis, and is mostly responsible for the generation of reactive oxygen species (ROS) and, to a lesser extent, DNA damages.[1,9] UVA radiation is, in the long term, mostly involved in skin photoaging.[10,11] 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., photoaging, immunosuppression, collagen degradation).[1,6,7,12] The accumulation of glycation reaction products is considered one of the main factors that contribute to skin aging. Glycation is a nonenzymatic reaction between a reducing sugar and the amine-free function of an amino acid (lysine and arginine) to form "advanced glycosylation end products" (AGEs) via oxidative or nonoxidative pathways.[13] At cutaneous level, AGEs modify the mechanical and biological properties of human skin, modulating elasticity/stiffening, and the extracellular matrix synthesis/degradation.[14] Several studies demonstrate that oxidative stress generated in UV-exposed skin may accelerate the glycation products formation and amplify the local degenerative phenomena associated with the aging process.[15–17] Another important point consists in the molecular consequences of solar exposure, which may affect the transcriptional profile of the skin. Many groups investigated cutaneous gene expression changes induced by UV, visible light, sunlight or solar-simulated light exposure, reporting that the mainly modulated genes were related to epidermal differentiation and proliferation processes, which are in turn connected to skin surface alterations, such as hydration and thickening,[18,19] immunity and inflammation, showing the induction of cytokines and interleukins and the reduction of innate immunity genes,[1,4,20–26] stress and oxidative stress response with induced expression of genes encoding heat shock proteins,[27,28] and target genes of the cytoprotective to oxidative and electrophilic stress NF-E2-related factor 2 (Nrf2)-pathway.[1,29,30] Finally, the extracellular matrix (ECM) maturation and remodeling process, that is another important class of genes mainly involved in the aging/photoaging. In this case, the expression of ECM components was found down-regulated, whereas remodeling genes were up-regulated.[1,23,31,32] Based on these considerations on the impact of radiation on skin biology, it becomes crucial to study the impact of solar exposure on the skin and develop new photoprotection compounds that are able to soothe the deleterious effects of sunlight. To study the function of the skin, ex vivo skin models allow for a more physiologically relevant system compared to in vivo animal models and in vitro cell culture models and offer a more accurate prediction of drug and cosmetic efficacy and safety.[33–35]

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 actual capacity of different active principles, such as L-Carnosine, in protecting irradiated skin. This has been done using ex vivo human skin exposed to solar-simulated radiation (SSR) and evaluating the induction of reactive oxygen species (ROS) and its biochemical endpoints (AGEs), as well as gene expression changes. The modulation of these readouts following treatment with L-Carnosine was studied in our skin model in order to assess its photoprotective activity. Carnosine is a naturally occurring dipeptide (b-alanyl-L-histidine) present at high levels (up to 20 mM) in muscle and nervous tissues in many animals, especially long-lived species. Many functions of Carnosine have been previously described, including antioxidant, oxygen free-radical scavenging,[36,37] antisenescence[38,39] and antiglycating activities.[40,41] Consistent with literature, our findings confirm that our ex vivo skin model is a valuable system to assess the consequences of solar light exposure and that topically applied L-Carnosine provides effective protection against solar radiation negative effects.

MATERIALS AND METHODS

A schematic overview of the experimental design is shown in Figure S1.

Study approval

The Padua Ethical Committee for Clinical Research approved the study confirming that all methods were performed in accordance with the relevant guidelines and

regulations (2658 P approved 10/04/2014); each subject gave informed written consent.

Skin samples

Discard tissue from abdominal plastic surgery was obtained from anonymized healthy patients. In total, 12 individuals aged between 25 and 60, without cutaneous diseases or evidence of intensive sun exposure, were considered. The skin phototype was assayed with a spectrocolorimeter and classified following Del Bino et al.[42] Table S1 summarizes donors' details (see Data S1).

Tissue culture

Fat and hypodermis were removed from the skin explant, and 8 mm diameter biopsies were excised using a sample punch. Skin samples were cultured in modified Williams' E medium (Merck KGaA) at air-liquid interface under classical cell culture conditions (37°C, in a 5% CO_2/95% air-humidified incubator).

Treatments

L-Carnosine was purchased from Merck KGaA and dissolved in vehicle (DMSO/PBS 1:25) at the following final concentrations 0.2% and 2.0%. The lower treatment (0.2%) corresponds to the concentration used in several cosmetic products, see also Narda et al.[43] whereas the 2% was selected to evaluate any enhanced effects in our model. Undiluted a-Tocopherol acetate (VIT-E) (Merck KGaA) was used as positive control. Treatments were applied topically and renewed daily until sampling as follows: skin biopsies were cleaned with cotton buds, and a 4 µL treatment was applied on the epidermis. All samples, both treated and control, were covered with 6 mm diameter delivery membranes. To perform ROS evaluation or immunohistochemistry, each treatment condition and control was tested in 6 replicates (6 biopsies) per donor. Gene expression modulation was assessed in a pool of 3 replicates (3 biopsies) per donor.

Solar simulated radiation

Skin irradiation was performed with Sun test XLS+ (AMETEK Inc.) equipped with a xenon lamp type NXE1700. A daylight filter (#56079174, AMETEK Inc.) was used to filter UV. The spectral irradiance of the SSR source used is shown in Figure S2 (see Data S1), and the spectral distribution was as follows: 0.6% UVB (300–320 nm), 10.2% UVA (320–400 nm), 76.9% visible light (400–700 nm), and 12.4% IR (700–800 nm) radiation. For these studies, a custom IR filter (SCHOTT, Germany) was also applied to filter IR from the light source and to obtain a final irradiance in the range of 300–750 nm. During the testing, the irradiance is automatically measured by the instrument and controlled electronically to achieve a constant value. Skin samples were irradiated at the selected SSR dose (range of 50–250 J/cm^2). During SSR exposure, the skin medium was replaced by Dulbecco's phosphate-buffered saline (Merck KGaA, Germany). Control samples were sham-irradiated under the same conditions. After SSR exposure, PBS was removed and fresh medium was added. Skin biopsies were incubated at 37°C, 5% CO_2. The irradiation step was repeated every day until sampling, and skin samples were collected at different time points based on the performed analysis. ROS were evaluated immediately after irradiation (day 1), and gene expression analysis was done on samples collected at day 2, whereas IHC staining at day 3.

ROS evaluation

To perform ROS evaluation, 2′-7′dichlorofluorescin diacetate (DCFH-DA) method was used. Before irradiation, skin samples were treated overnight (about 16 h) with vehicle or test compounds; then, skin biopsies were incubated with DCFH-DA probe (Merck KGaA) for 30 min; lastly, the samples were exposed to the selected SSR dose (range 50–250 J/cm^2). Immediately after irradiation, skin samples were harvested, cryo-fixed, and cut into 7 µm-thick sections using a Leica CM 1850 cryostat (Leica Microsystems). Two skin sections for each skin biopsy were mounted on Superfrost® plus glass slides (Menzel Gläser) and acquired at 200× magnification with a Leica DMi8 light microscope equipped with Leica DFC7000T digital camera. For each condition tested, the upper dermis of 12 digital images, deriving from 6 skin biopsies, was analyzed by evaluating the fluorescence through ImageJ application (NIH). The obtained value has been normalized upon the selected area.

Immunohistochemistry (IHC)

Skin samples were treated daily with L-Carnosine 0.2% and 2.0% and irradiated 250 J/cm^2 of SSR for 3 days; at the selected time point (day 3), skin biopsies were fixed in 4% formaldehyde (KALTEK srl), paraffin-embedded and cut into 5 µm-thick sections using a Leica RM 2255 microtome. Two skin sections for each skin biopsy were mounted on

Superfrost® plus glass slides (Menzel Gläser). IHC staining was performed using an automated IHC stainer (Autostainerlink48, Agilent Technologies Inc.), following the manufacturer protocol. Briefly, sections were pretreated using heat-mediated antigen retrieval with EnV FLEX TRS, High pH (cat. # K800421-2, Agilent Technologies Inc.) for 10 min at 80°C with PTlink (Agilent Technologies Inc.) and then incubated 1 h at RT with rabbit polyclonal anti-AGE (1:2000 dilution, cat. # ab23722, Abcam), rabbit polyclonal anti-CYR61 antibody (1:200 dilution, cat. PA5-78022, Thermo Fisher Scientific Inc.) and rabbit monoclonal anti-PTGS2 antibody (1:100 dilution, cat. MA5-14568 Thermo Fisher Scientific Inc., USA). Detection was performed using the Dako-Alkaline phosphatase/RED kit (Dako REAL Detection System, cat. K5005). Hematoxylin was used for counterstaining. Sections were mounted with Fluoromount aqueous mounting medium (cat. F4680, Merck KGaA) and observed with a Leica DMi8 light microscope at 200× magnification. Twelve skin sections deriving from 6 skin samples for each tested condition have been immunostained. Digital images of 12 fields per condition were captured with Leica DFC7000T digital camera, and the antigen level was manually evaluated in the epidermis region using ImageJ application (NIH, USA). The obtained values have been normalized upon the dimension of the selected area.

Statistical analysis

Results obtained from the image analysis were expressed as mean value 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.

Target genes selection

To perform gene expression analysis in our model, we decided to utilize the large amount of published data regarding gene expression modulation in skin exposed to different sources of light (UV, VIS, Blue light, Sunlight, SSR). In the end, 42 genes were selected and grouped based on their main function. Belonging to the genes involved in the apoptotic process, AEN and BAX were selected.[1,4] Among the main components of the immunity and inflammation pathway, 11 genes, previously found modulated by irradiation, were selected: IL1A, IL20, IL8, IL6, PTGS2, TNF, S100A9, S100A8, CSF2, CCL2 and CXCL6.[1,4,20–24] Eight of the genes involved in the pigmentation process, and reported to be modulated by irradiation, were considered: DCT, TYR, TYRP1, OCA2, PMEL, ASIP, EDN3 and MITF.[4,22,44] To analyze the oxidative stress response, 6 genes demonstrated to be positively modulated by irradiation were chosen: HO1, SOD1, SOD2, NOS2, CAT and NRF2.[29,30,45] Regarding the extracellular matrix remodeling genes modulated by irradiation, 4 genes were selected: MMP9, COL1A1, CYR61 and TIMP-1.[46–49] Finally, 11 genes belonging to other functions were also included based on their previously demonstrated correlation with the skin irradiation response[1,4,20,21,50,51]: BYSL, EPHB1, GRIP1, NOLC1, PRKCB, FOSL1, OPN3, FGF7, ICAM1, HBEGF and GDF15. The list of the selected genes is provided in Table S2.

RNA isolation and quantitative real-time

Skin samples were topically treated with L-Carnosine at low and high concentrations (0.2% and 2.0%) and exposed daily to 250 J/cm^2 of SSR up to day 2. At the selected time point (day 2), skin biopsies were collected in cryo-vials containing RNAlater stabilization solution (Merck KGaA) and stored at −80°C. Total RNA was extracted from a pool of 3 biopsies/treatment using an RNeasy mini kit (Qiagen GmbH) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.). Reverse transcription reactions were performed using 1 μg of total RNA with QuantiTect Reverse Transcriptase Kit (Qiagen GmbH) 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.). Real-time RT-PCR was performed on QuantStudio3 (Thermo Fisher Scientific Inc.) using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific Inc.) 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 not-irradiated control or vehicle (fold change; up-regulated ≥2 and down-regulated ≤0.5). The Ct values were normalized to the endogenous control genes YWHAZ, PPIA, B2M and TBP (geometrical mean), previously selected using a TaqMan™ Array Human Endogenous Control (Thermo Fisher Scientific Inc.).

RESULTS

SSR induces oxidative stress in our ex vivo skin model

Since the purpose of this work was to set up a useful methodology to assess the consequences of solar light exposure and the capacity of applied compounds to counteract

them, different irradiation doses were tested for their potential to induce oxidative stress. Based on literature data,[23,31,32] a dosage range of 50–250 J/cm^2 was selected as representative of the approximative amount of solar light acquirable by few hours of sunbathing under natural conditions. Ex vivo human skin samples from two donors were exposed to 50 J/cm^2, 100 J/cm^2 or 250 J/cm^2 of SSR, and ROS production was evaluated by DCFH-DA assay. The fluorescence signal obtained, corresponding to the ROS level, was evaluated in the upper dermis, and the mean value of the two independent studies was calculated. As reported in Figure 1, only the high dosages of SSR significantly induced oxidative stress; thus, 250 J/cm^2 of SSR was selected as stimulus to investigate oxidative stress induction, inflammatory response and photoaging in our ex vivo skin model.

L-Carnosine promotes a reduction of ROS production induced by SSR stimulus

In order to evaluate the ability of L-Carnosine to modulate the oxidative stress induced by SSR, ROS generation was assessed in the samples treated with L-Carnosine overnight and then exposed to 250 J/cm^2 of SSR. Figure 2 shows the mean values of two independent studies, and the ROS level is expressed as ratio with respect to the not-irradiated vehicle. After exposing the samples to SSR stimulus, a noticeable increase in oxidative stress was detected in the group treated with the "vehicle." On the other hand, the group treated with L-Carnosine showed a significant reduction in the production of ROS already with 0.2% treatment (−60%). The positive antioxidant control (VIT-E) confirmed the antioxidant effect of L-Carnosine in our model (−73%).

Reduction of AGE formation in sample exposed to SSR and treated with L-Carnosine

Several studies reported the inhibition of AGEs formation as a potential anti-aging strategy.[14,16] Therefore, after evaluating the ability of L-Carnosine to reduce the generation of ROS, its anti-aging potential was also investigated in our model. AGEs formation was assessed by IHC staining in samples treated daily with L-Carnosine 0.2% or 2.0% and exposed to SSR up to day 3. The obtained results are reported in Figure 3. Image analysis confirmed a significant strong formation of AGEs after SSR stimulus, whereas a significant decrease was observed in samples treated with L-Carnosine at both concentrations tested, with evidence of a dose–response relationship. Specifically, mean values of −46% and −73% were respectively found with 0.2% and 2.0% treatments.

Gene expression analysis on ex vivo skin model exposed to SSR

Of all the genes reported to be modulated after irradiation (UV, VIS, Blue light, Sunlight, SSR), 42 genes were

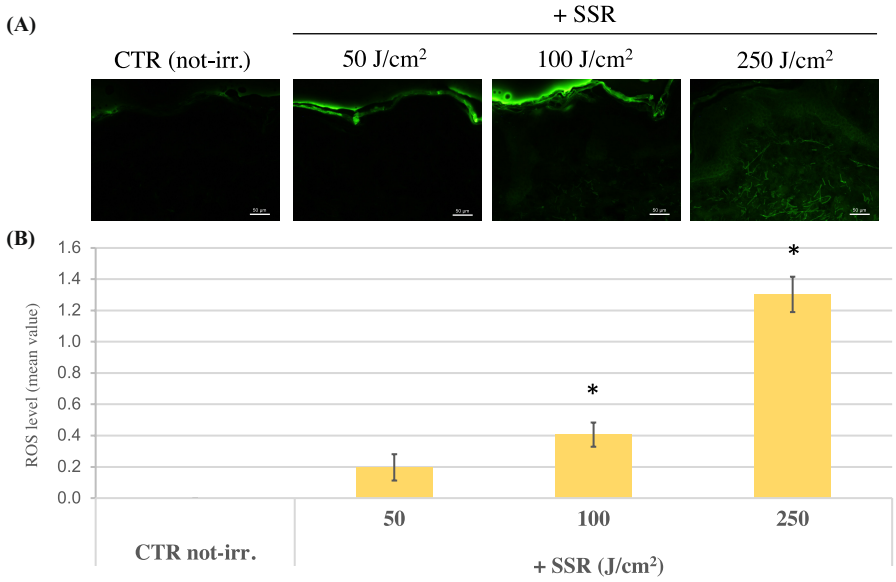

FIGURE 1 SSR induces ROS production. Skin samples from two different donors were stimulated with 50-100-250 J/cm^2 of SSR. ROS production was assessed by DCFH-DA assay, and fluorescence signal obtained was evaluated in the upper dermis. (A) Representative images. (B) Graph showing ROS mean level obtained in two independent studies ($n = 2$). Error bars indicate the standard error of mean (SEM). * Significantly different from not-irradiated control (Tukey's test, $p < 0.05$).

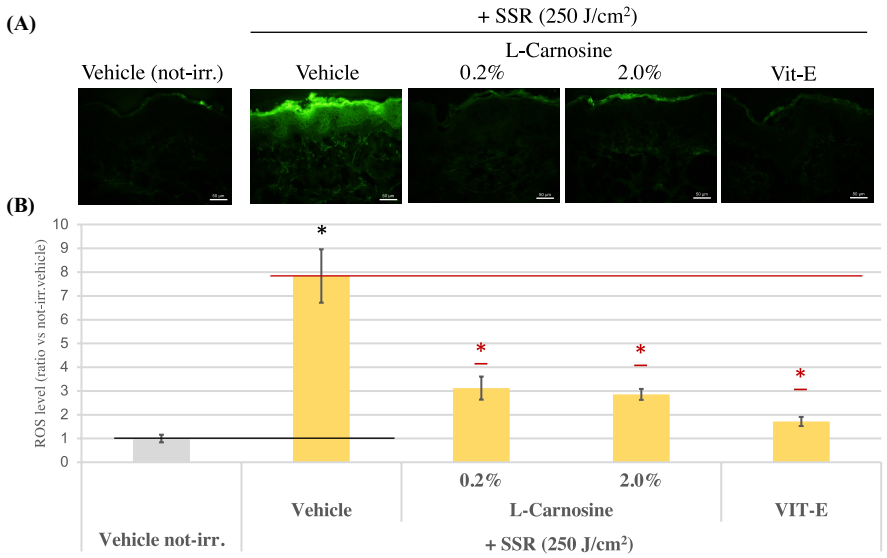

FIGURE 2 Decrease of ROS production in L-Carnosine-treated samples. ROS production was assessed by DCFH-DA assay in two independent studies (2 donors). Skin samples were treated ON with vehicle or test compounds and stimulated with 250 J/cm^2 of SSR. The fluorescence signal obtained was evaluated in 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, $p < 0.05$).

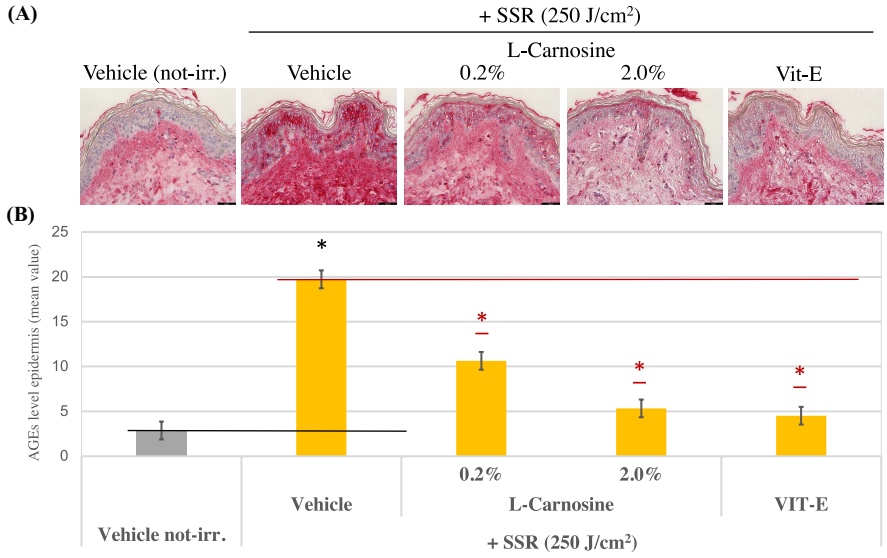

FIGURE 3 Decrease of AGE formation in L-Carnosine-treated samples. AGE level was assessed by IHC staining in two independent experiments (2 donors). Skin samples were treated daily with L-Carnosine 0.2% or 2.0% and exposed to SSR up to day 3; the signal obtained was evaluated in the epidermis. L-Carnosine treatment protects from the effect of SSR reducing AGE formation. (A) Representative images. (B) Graph showing AGE mean levels of two independent studies ($n = 2$). Error bars indicate the standard error of mean (SEM). * Significantly different from not-irradiated control vehicle; * Significantly different from irradiated vehicle (Tukey's test, $p < 0.05$).

selected as the most interesting to study stress response, inflammation and skin biology processes in our model (Table S2) and therefore included in a custom TaqMan® array. The analysis of gene expression was performed on ex vivo human skin obtained from four healthy donors (Table S1). Skin samples were exposed daily to 250 J/cm^2 of SSR and collected at day 2 for the analysis with the custom TaqMan® gene array. The modulation of gene expression of target genes is expressed as fold change value compared to the not-irradiated control. Table 1 shows the results obtained for each donor. Based on the published data and the fold change values obtained, 23 genes showed the expected trend (up-regulated ≥2, down-regulated ≤0.5, 0.5 ≥ not-modulated ≤2. Table 1 in

TABLE 1 SSR induces gene expression modulation.

Target	Fold-change (vs not irradiated)				Expected modulation	Function
	Don #1	Don #2	Don #3	Don #4		
AEN	9.7	3.1	3.1	1.3	+	Apoptosis
BAX	2.9	3.7	2.9	1.7	+	
IL1A	1.3	3.6	3.4	1.3	// or +	Immunity and inflammation
IL20	***15.3***	1.8	***19.4***	***2.2***	+	
CXCL8	6.3	1.7	19.2	4.9	++	
IL6	***6.9***	***3.9***	***9.7***	***2.4***	+	
PTGS2	***6.4***	1.8	***17.2***	***2.2***	+	
TNF	***6.5***	***4.4***	***7.8***	***1.0***	+ or //	
S100A9	1.1	3.2	1.9	0.3	+	
S100A8	0.9	3.8	4.3	0.7	+	
CSF2	24.2	ND	3.0	3.8	++	
CCL2	1.9	0.2	2.9	3.6	++	
CXCL6	5.2	29.3	4.0	0.5	+	
DCT	0.4	1.1	0.5	0.6	− or //	Pigmentation process
TYR	1.0	2.3	0.9	1.0	//	
TYRP1	0.5	1.5	0.3	0.5	//	
OCA2	***0.2***	***0.5***	0.6	***0.4***	−	
PMEL	1.3	2.1	1.0	1.0	//	
ASIP	3.9	1.3	0.5	0.9	− or //	
EDN3	ND	ND	0.3	ND	− or //	
MITF	0.9	1.3	0.9	0.7	//	
HO1	0.5	6.3	0.0	0.5	+	Oxidative stress response
SOD1	1.1	1.1	1.1	1.1	+	
SOD2	0.9	1.0	0.9	0.4	+	
NOS2	ND	ND	ND	ND	NA	
CAT	0.2	1.0	1.1	1.2	+	
NRF2	0.5	1.7	1.9	1.1	+	
MMP9	0.6	3.3	1.3	0.5	+	Extracellular matrix remodelling
COL1A1	5.3	0.7	0.6	1.4	−	
CYR61	***20.6***	***2.4***	***10.3***	***7.4***	+	
TIMP1	1.2	2.6	1.4	0.4	NA	
BYSL	2.7	2.3	1.5	0.7	+	Other functions
EPHB1	ND	ND	ND	ND	−	
GRIP1	***0.3***	***0.1***	***0.1***	***0.2***	−	
NOLC1	2.2	2.1	1.6	0.6	+	
PRKCB	***0.4***	***0.2***	***0.2***	***0.1***	− or //	
FOSL1	***28.3***	***6.2***	***325.6***	***6.6***	+	
OPN3	0.8	1.4	2.4	0.8	+	
FGF7	***9.4***	***3.2***	0.9	***2.7***	++	
ICAM1	1.9	1.9	1.3	0.6	++	
HBEGF	13.0	2.1	12.7	7.8	++	
GDF15	***15.7***	***259.6***	***5.1***	***4.8***	++	

Note: The analysis of gene expression was performed at day 2 on ex vivo human skin obtained from 4 healthy donors. Gene fold change values of SSR samples relative to the not-irradiated control are reported. Highlighted in *gray* are the 23 genes whose modulation is in line with expectations in at least 3/4 donors, with fold change ≥2 up-regulated (+ or ++), fold change ≤0.5 down-regulated (−) and 0.5≥fold change ≤2 not-modulated (//). The 11 genes selected for the photoprotection studies with L-Carnosine are reported in *bold* italics. NA, not available; ND, not determined.

gray). In particular, 17 of these 23 genes were found to be positively or negatively modulated after SSR stimulus, in at least three out of four donors. These genes are mainly involved in the processes of apoptosis (AEN, BAX), immunity and inflammation (IL20, CXCL8, IL6, PTGS2, TNF, CSF2, CXCL6), extracellular matrix remodeling (CYR61), and other important cellular functions that regulate skin biology (GRIP1, PRKCB, FOSL1, FGF7, HBEGF, GDF15). Regarding the pigmentation process, only OCA2 gene was modulated, whereas no gene belonging to the category stress response was identified. This study was performed on different skin phototypes to evaluate the importance of this parameter on SSR response in our model. In general, we observed that in light phototypes SSR stimulus modulates between 20 genes (donor 1 and 2) and 22 genes (donor 3), whereas in the pigmented donor (donor 4) only 14 genes were modulated. This can be attributed to the different melanin content as observed in other studies, which report that melanin scavenges ROS and decreases oxidative cellular damages.[52–56]

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

To assess the skin solar irradiation response and to test possible protective compounds, 11 of the 17 genes were evaluated as markers of SSR exposure (Table 1, *italics*) and included in a second custom TaqMan® array. These genes were selected considering both modulation level obtained (fold change) and their relevance for the biological skin response to light exposure. The gene expression analysis was performed on ex vivo human skin from three donors (Table S1). In this case, light skin phototypes were preferred as they showed a strong response to SSR in terms of modulation of gene expression in the previous experiments. Among the 11 selected biomarkers, 10 genes confirm the expected response to the SSR stimulus in two out of three donors (Figure 4A, fold change values in *gray*). Skin samples were topically treated with L-Carnosine at low and high concentrations (0.2% and 2.0%). As reported in Figure 4A, L-Carnosine treatment reduces the gene expression modulation induced by SSR confirming its photoprotective activity (values in *bold italics*). This effect was observed for 7 genes in at least 2/3 donors (Figure 4B, *bold*), and four of these show this effect already at 0.2% treatment. The markers genes mentioned above, all up-regulated by SSR, are mainly involved in the processes of immunity and inflammation (IL20, IL6, PTGS2, TNF),[4,22,57] ECM remodeling (CYR61)[48,49,58] and other biological functions, including cell growth, morphogenesis, tissue repair and pigmentation (FGF7)[1] or stress response and cellular senescence (GDF15).[59,60] The results obtained in donor 1 are reported in detail in Figure 4C; in this case, after SSR stimulus, L-Carnosine effectively reduces gene expression modulation of all the 7 marker genes already at the lower concentration tested (0.2%).

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

For their importance in the skin biology response to light exposure and photoaging, among the SSR marker genes mitigated by L-Carnosine treatment, CYR61 and PTGS2 were selected to be validated at protein level by IHC. Skin samples were treated daily with L-Carnosine 0.2% and 2.0% and irradiated at 250 J/cm^2 of SSR for 3 days. The results reported in Figure 5 showed an increased protein level of CYR61 (+448%) and PTGS2 (+82%) after SSR exposure compared to the control. L-Carnosine treatment protects from the effect of SSR reducing significantly CYR61 protein level already at the lower concentration tested (−45%). Regarding PTGS2 protein modulation following L-Carnosine treatments, a mild effect of L-Carnosine was observed. The level of this protein, induced by SSR stimulus, is reduced by L-Carnosine only with 2% treatment (−26%).

DISCUSSION

Skin is the largest organ of the human body, protecting it from the harmful external stimuli, including chemical products, microorganisms and electromagnetic radiations. In particular, terrestrial solar radiation, and more specifically the UV component, is the single most important environmental factor affecting the skin physiology, inducing sunburns, erythema, photoaging, immunosuppression and skin cancer.[1,10,11] For this reason, it is important to study the impact of solar exposure on the skin and develop new photoprotective compounds to avoid or reduce its damaging consequences. The purpose of this work was to set up an ex vivo methodology that could be used to verify the effects of solar radiation on human skin and to assess the capacity of different active principles in protecting the irradiated skin. Ex vivo skin models provide a valuable tool to study skin biology and pathology, offering several advantages over in vivo animal models and in vitro cell culture models. These models allow for a more physiologically relevant system to study skin functions and offer a more accurate prediction of drug and cosmetic efficacy and safety.[33–35] In this study, ex vivo human skin exposed to solar-simulated radiation (SSR) was used as

(a)

Function	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)		
			VEHICLE	L-Carnosine		VEHICLE	L-Carnosine		VEHICLE	L-Carnosine	
				0.2%	2.0%		0.2%	2.0%		0.2%	2.0%
Inflammation	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***
	PTGS2	+	5.3	***4.2***	***4.0***	13.9	***11.3***	***7.7***	2.0	***1.4***	***1.4***
	TNF	+	12.9	***8.2***	***7.3***	6.6	7.7	***4.7***	1.4	1.4	1.2
ECM remodelling	CYR61	+	8.1	***2.7***	***4.0***	6.1	6.2	***4.5***	2.3	2.9	***1.5***
Pigmentation	OCA2	-	0.5	0.2	0.3	0.7	0.4	0.4	0.3	0.3	0.3
Other functions	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***
	FOSL1	+	4.8	4.6	4.5	11.2	***9.6***	***5.7***	2.2	3.1	4.8
	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

(b)

L-Carnosine photoprotective effect			
Target	Don #1	Don #2	Don #3
IL20	✓	✓	✓
IL6	✓	✓	✓
PTGS2	✓	✓	✓
TNF	✓	✓	
CYR61	✓	✓	✓
OCA2			
FGF7	✓	✓	
GDF15	✓	✓	✓
FOSL1		✓	
GRIP1			
PRKCB			

(c)

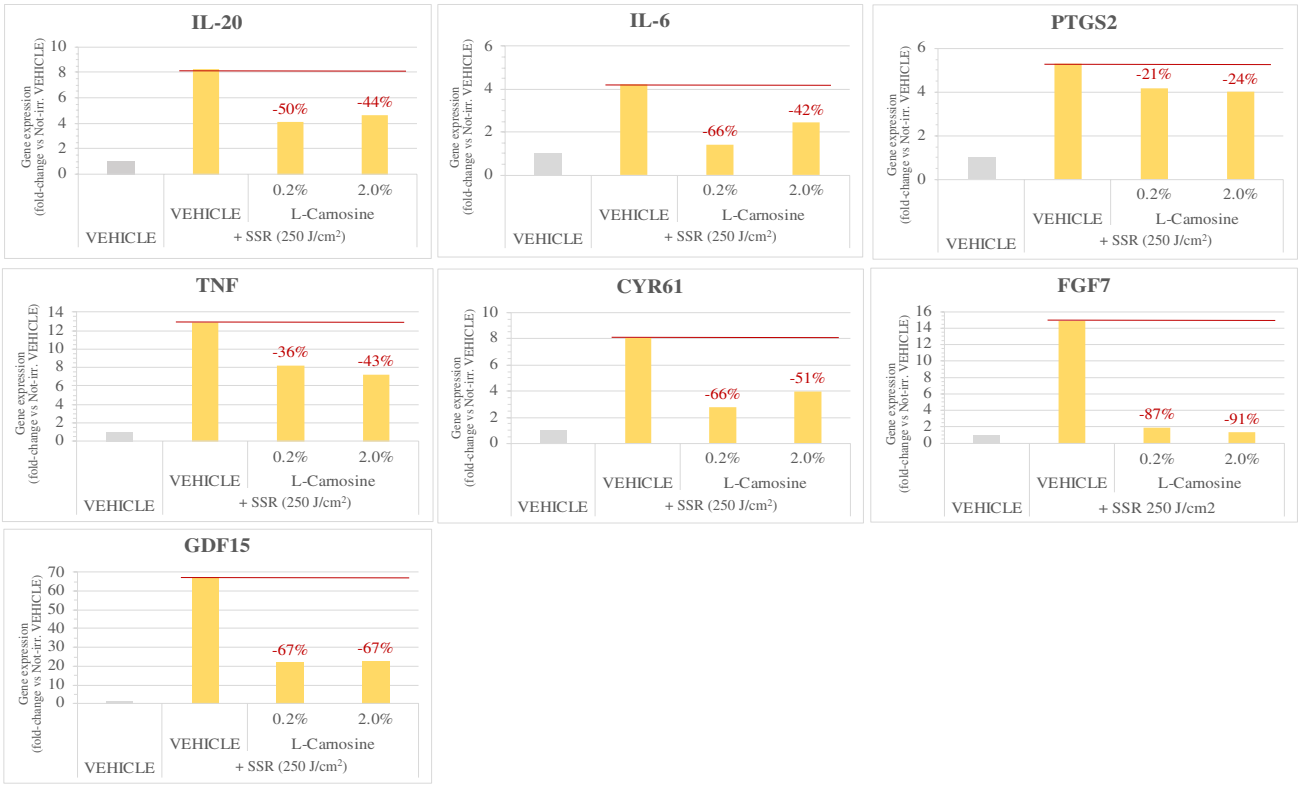

FIGURE 4 L-Carnosine reduces gene expression modulation. Gene expression of the 11 selected genes was evaluated in three independent experiments (3 donors). Skin biopsies were treated with L-Carnosine 0.2% or 2.0% and exposed to SSR up to day 2. L-Carnosine treatment protects from the effect of SSR reducing the gene expression modulation of several genes. (A) Gene fold change values obtained in relation to the not-irradiated vehicle. The gene expression modulation following SSR is highlighted in *gray* when it corresponds to the expected (fold change ≥2 up-regulated and ≤0.5 down-regulated); in *bold italics* the fold change values mitigated by L-Carnosine treatment (photoprotective effect, Δfold change ≥0.5). (B) Summary of the gene expression results; ✓ indicates L-Carnosine photoprotective effect in at least one concentration tested. (C) Graphical representation of the results obtained in donor 1 (Don #1). L-Carnosine reduces the effect of SSR on gene expression of 7/11 genes.

model, and L-Carnosine was tested as example of photoprotective treatment. We show 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.

Using our ex vivo model, we were able to assess the harmful effects of solar radiation by measuring ROS production, AGEs formation and changes in the expression of genes related to apoptosis, inflammation, extracellular matrix remodeling and other critical cellular functions

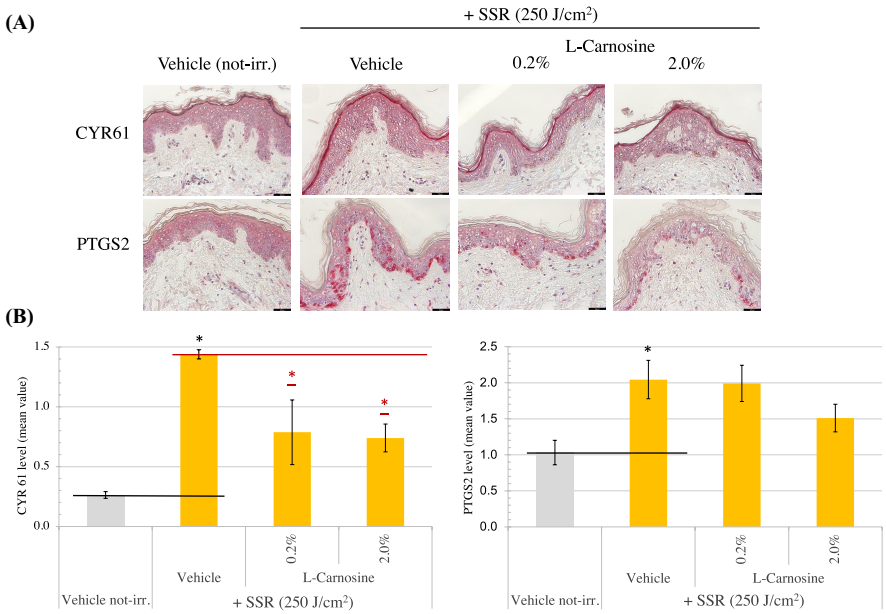

FIGURE 5 L-Carnosine reduces PTGS2 and CYR61 protein induction. PTGS2 and CYR61 protein level was evaluated in skin biopsies from one donor treated with L-Carnosine 0.2% or 2.0% and exposed to SSR up to day 3. Protein level was assessed by IHC staining, and the signal obtained was evaluated in the epidermis. 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 value). Error bars indicate the standard error of mean (SEM). * Significantly different from not-irradiated control vehicle; * Significantly different from irradiated vehicle (Tukey's test, $p < 0.05$).

that regulate skin biology. During the photoprotective experiments, the application of L-Carnosine treatments at concentrations of 0.2% and 2.0% resulted in a decrease of ROS production and AGEs formation induced by SSR (Figures 2 and 3). Since SSR induces alterations of the expression of genes involved in skin functions, stress response and aging, a panel of 11 biomarker genes was identified to test the photoprotective capacity of applied compounds. Results obtained showed that L-Carnosine protects from the effect of SSR reducing the expression modulation of 7/11 selected biomarker genes already at 0.2%. (Figure 4A,B). This is in accordance with the use of Carnosine at 0.2% for cosmetic purpose. PTGS2, belonging to the inflammation process, is strongly induced by many stimuli, including growth factors, cytokines and solar radiation (UV), resulting in the production of prostaglandins during inflammatory processes.[24] In all the three analyzed donors, L-Carnosine has proven to be effective in reducing the gene expression modulation of PTGS2. Also at protein level, our findings confirm previously published data that report a PTGS2 induction following UV exposure, predominantly in the basal keratinocyte layer[61,62] (Figure 5A). Inhibition of PTGS2 has been reported to prevent skin inflammation, aging and carcinogenesis, representing a potential strategy for preventing solar UV-related skin disorders.[48,63] In our model, L-Carnosine 2.0% seems to reduce PTGS2 protein (Figure 5). Another important marker, modulated by L-Carnosine, is CYR61. This gene encodes for 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.[48,49,64] In our experiments, the photoprotective effect of L-Carnosine was confirmed at transcriptional and protein level. As for PTGS2 and CYR61, also GDF15 expression level after irradiation is reduced. This gene was selected for its role in the stress response and cellular senescence processes, because it is known to be strongly up-regulated in response to UV irradiation, as we confirmed.[1,64] In all tested donors, the highest protective effect is observed in samples treated with L-Carnosine 0.2%. Finally, FGF7 gene is also modulated by the topical application of L-Carnosine. This gene is a growth factor protein mainly involved in the repair and remodeling of the dermis during the skin anti-aging process and is reported to be a positive regulator of skin pigmentation.[57–59] We found a reduced expression level of FGF7 after SSR stimulus in the presence of L-Carnosine.

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 topically applied compounds (L-Carnosine) to counteract them. Our findings confirm the literature data, demonstrating that skin samples exposed to SSR show oxidative stress induction 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 reducing ROS, AGEs and mitigating the modulation of genes involved in inflammation, extracellular matrix remodeling, tissue repair, stress response and cellular senescence.

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