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Low-level laser irradiation protects the chick embryo chorioallantoic membrane from UV cytotoxicity

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Abstract: Low-level laser therapy or photobiomodulation is the medical use of a very low intensity light in the red to near infrared (wavelengths in the range of 630-940 nm). The present work was conducted to explore the effects of both UV and low-level laser irradiation (LLLI) on microcirculation using the *in vivo* model of the chick embryo chorioallantoic membrane (CAM). The effects were assessed by measuring lipid peroxidation and antioxidant enzyme activity. Cell cytotoxicity, survival and intracellular reactive oxygen species (ROS) of the CAM were also evaluated. We found that UV irradiation induced alterations of the vessels, leading to bleeding and extravasation. This effect was intensified after 60 min of exposure to UV irradiation, leading to marked edema. UVA irradiation increased cell cytotoxicity as assessed by lactate dehydrogenase (LDH) release (56.23% of control) and reduced cell viability as assessed by decreased fluorescein diacetate (FDA) fluorescence (56.23% of control). Pretreatment with LLLI prior to UV exposure protected the CAM tissue from UV-mediated cell death. This protective effect was supported by the observation of significantly inhibited lipid peroxidation (from 0.3±0.004 for UV, to 0.177±0.012 after LLLI pretreatment), ROS and O_2^-production, as indicated by respective dihydrorhodamine (DHR) and dihydroethidium (DHE) intensities (from 132.78% of control for UVA, to 95.90% of control for L-UV (DHR), and from 127.34% of control for

UVA, to 82.03% of control for L-UV (DHE)), and by preventing the increase in oxidative activities. LLLI efficiently protected CAM cells from UV-induced oxidative stress and appeared as a safe protective pretreatment against UV irradiation.

Key words: low level laser irradiation; UV irradiation; chorioallantoic membrane; cytotoxicity; vascular protection

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INTRODUCTION

The solar radiation reaching the earth's surface includes wavelengths ranging from 290 nm to 4000 nm and is comprised of UV radiation (UVA, 320-400 nm; UVB, 290-320 nm), visible light (400-700 nm) and infrared radiation (700-940 nm). It is commonly accepted that solar UV induces deleterious effects on cells such as photocarcinogenesis and photoaging. UV irradiation from sunlight causes photodamage of the skin, leading to the appearance of wrinkles, laxity, coarseness and mottled pigmentation [1]. Moreover, it causes histological alterations, including expanded epidermal thickness and changes in connective tissue [2]. Typically, the generation of ROS by oxidative pathways leads to these afflictions. For this reason, several anti-oxidative and anti-photoaging compounds, such as vitamin E, vitamin C and their derivatives, have been previously identified [3].

On the Earth's surface, all living organisms are continuously and positively affected by infrared radiation before being exposed to solar UV. The infrared radiation protective process against solar UV is conserved throughout evolution and constitutes an important factor contributing to life preservation. Understanding this mechanism would provide crucial insight into the protective mechanisms against UV damage to human skin.

Low-power, non-invasive lasers with an output up to 500 mW are collimated, monochromatic and coherent radiation sources. These devices have been described for their beneficial effects, including analgesic, anti-inflammatory and stimulatory ones [4]. They have

been used for the treatment of many disorders in soft and bone tissues at different power doses, densities and wavelengths [5]. At low-power densities and doses, low-level laser therapy (LLLT) is used in the so-called therapeutic window (600-1100 nm).

In the present study, we chose the chicken chorioallantoic membrane (CAM) as an experimental model. The CAM model constitutes a vascular organ derived from a fusion of the allantois and chorion of the chick embryo. It develops in direct contact with the egg shell, constituting a respiratory organ. It is a dense vascular bed that grows rapidly over the course of several days, allowing for rapid responses to environmental changes. The blood vessels and their architecture are easily accessible because they grow in a single layer, are surrounded by clear connective tissue and are situated directly under the shell.

This work was designed to determine the effects of pretreatment with low-level laser irradiation (LLLI) against UV irradiation on CAM microcirculation. We also determined the possible implication of oxidative stress and calcium as an intracellular mediator.

MATERIALS AND METHODS

CAM sources and transport

Fertilized chicken eggs were purchased from local farmers “Polina Falous” and transported to the laboratory in a covered box to maintain temperature and to prevent jostling during transport. Upon arrival at the laboratory, the eggs were surface sterilized and incubated at 37°C at 70-75% relative humidity. Sterile conditions were maintained throughout the experiments.

CAM preparation and treatment

Fertilized 7-day-old eggs were pre-incubated at 37°C up to 48h. They were kept in a horizontal position for 30 min in the incubator to ensure the proper positioning of the embryo. The top surface of the shell was then carefully removed to reveal the embryo. The CAMs opened surface was covered with a plastic wrap. Experiments were carried out on four groups. The first

underwent 30- or 60-min periods of UV irradiation (0.003 W cm^{-2}), the times needed to deliver a total light dose of 5.4 J cm^{-2} or 10.8 J cm^{-2}, respectively. These total light doses were selected based on results from previous studies of our laboratory [6]. The fluence rate of 3 mW cm^{-2} was measured with a UVA radiometer. The UVA source, a UVA Hazard Detector SEL 033/UVA/W with a spectrum of emission in the UVA range (315-390 nm), was placed at a distance of 15 cm above the CAM surface.

The second group was irradiated at 55.54 J cm^{-2} for 60 min (15.428 mW cm^{-2}) only by LLLI. Laser irradiation was performed with an ABYONIK^{R} 500 system diode laser (on a probe designed and built by Beauty Lumis, Munich Germany), power 5 mW. Continuous wave radiation at simultaneous wavelengths of 655 nm and 780 nm, corresponding to red light and near infrared, was performed. A third group was treated with LLLI prior to UV irradiation. The control groups were subjected to the same treatment but were not irradiated either by LLLI or UV. Before and after each irradiation, pictures were taken by a numeric photograph from the microscope.

Cytotoxicity assay

Treated embryo membranes were carefully retrieved, weighed and immediately stored at -20°C. Before each experiment, the samples were thawed on ice and an appropriate amount of lysis buffer was added according to the sample weight. Then the samples were ground using a manual instrument. After centrifugation of the homogenates at 1000 x g for 10 min, the supernatants containing intracellular material were used for the measurements. Cell toxicity induced by UV and LLLI was determined by measuring the content of lactate dehydrogenase (LDH) in the CAM homogenate of each experimental group. CAM homogenates were obtained after cell lysis with 1% Triton X-100 in phosphate-buffered saline (PBS 0.1 M; pH 7.4). LDH activity was measured using a commercial kit (Biomaghreb; Tunisia) and

spectrophotometrically determined according to Bergmeyer [7]; the results were presented as a percentage of total LDH release.

Assessment of cell survival

Every set of differently irradiated CAM was treated with fluorescein diacetate (FDA) (15 µg/mL), washed twice with 0.1 M PBS, pH 7.4and lysed with a Tris/HCl solution containing 1% sodium dodecylsulfate. Fluorescence intensity was determined using a FL800TBI fluorescence microplate reader, with excitation at 485 nm and emission at 538 nm (BioTek Instruments, Winooski, VT, USA).

Assessment of intracellular ROS formation

The amount of ROS was determined by measuring the fluorescence of 2',7'-dichlorofluorescein (DCFH). DCFH is formed after hydrolysis and oxidation of the $DCFH_2$-DA, a non-fluorescent compound. CAM cells were seeded onto 24-well plates, incubated with 10 µM of cell-permeant $DCFH_2$-DA in serum-free loading medium at 37°C for 30 min and rinsed twice with PBS. Fluorescence was determined using a FL800TBI fluorescence microplate reader (BioTek Instruments), with excitation at 485 nm and emission at 538 nm.

Assessment of intracellular superoxide anion production

The amount of O_2^- was determined by measuring the fluorescence of ethidium derived from the oxidation of dihydroethidium (DHE), a non fluorescent compound. CAM cells were seeded onto 24-well plates, incubated with 2 µM DHE in serum-free loading medium at 37°C for 15 min and rinsed twice with PBS. Ethidium fluorescence was determined using a FL800TBI fluorescence microplate reader (BioTek Instruments), with excitation at 488 nm and emission at 575 nm.

Lipid peroxidation measurement

Lipid peroxidation was determined by measuring malondialdehyde (MDA) using an extinction coefficient for the MDA-TBA complex of $1.56\ 10^5 M^{-1}cm^{-1}$ and according to the double-heating

method [8]. Briefly, an aliquot from the CAM tissue was mixed with a BHT-TCA solution containing 1% butylated hydroxytoluene (BHT; m/v) dissolved in 20% trochloroacetic acid (TCA; m/v) and centrifuged at 100 x g for 5 min at 4°C. The supernatant was mixed with 0.5 N HCl and 120 mM TBA in 26 mM Tris and then heated at 80°C for 10 min.The absorbance was measured at 532 nm using a Bio-Rad spectrophotometer.

Antioxidant enzyme activity assays

Spectrophotometric measurements of antioxidant enzyme activities were determined with an UV-visible spectrophotometer (SmartSpec 3000 BIORAD; Germany).

Superoxide dismutase (SOD) activity

SOD activity was measured by the modified epinephrine assays [9]. The O_2^- ion induces the autoxidation of epinephrine to adrenochrome. One unit of SOD was defined by the amount of CAM homogenate inhibiting by 50% the rate of adrenochrome formation. Samples were added to a reaction mixture containing epinephrine (5 mg/mL), bovine catalase (0.4 U/µL) and 62.5 mM sodium carbonate/sodium bicarbonate buffer (pH 10.2). Absorbance was measured at 480 nm.

Catalase (CAT) activity

CAT activity was determined by measuring the rate of H_2O_2 degradation at 240 nm for 3 min and using the extinction coefficient of 40 $Mm^{-1}cm^{-1}$ for H_2O_2 [10]. The reaction mixture consisted of 33 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0).One unit of CAT activity was determined by the amount of enzyme catalyzing the disappearance at 37°C of 1mmol of H_2O_2/min. Specific activity was expressed as mmol (H_2O_2)/min/mg protein.

Peroxidase (POD) activity

POD activity was determined at 25°C by measuring the guaiacol (hydrogen donor) oxidation [11]. One mL of the reaction mixture was comprised of 19 mM H_2O_2 in 50 Mm phosphate buffer pH 7.0, 9 mM guaiacol and 50 µL of CAM supernatant. The reaction was induced by the

addition of H_2O_2 and its progress was measured by the absorbance increase every 30 s for 3 min, at 470 nm. POD activity was calculated using a molecular extinction coefficient of 26.2 mM^{-1}. Specific POD was expressed as nmol (guaiacol oxidized)/min/mg protein.

Total protein measurement

Total proteins were determined according to the biuret method [12] and using an available commercial kit (Biomaghreb; Tunisia).

Ionizable calcium determination

Ionizable calcium was measured according to Stern and Lewis [13]. Briefly, at basic pH, calcium reacts with cresolphthalein, which is conducive to the formation of a purple complex, monitored by spectrophotometer at 570 nm. CAM homogenates were added to a mixture of cresolphthalein (0.62 mmol/L), 2-amino-2-methyl 1-propanol buffer (500 mmol/L) and hydroxy-8 quinoline (69 mmol/L).Incubation was carried out at room temperature for 5 min, with the assumption that the complex is stable for 1 h.

Statistical analysis

All statistical tests were 2-tailed. Data analyzed by unpaired Student's t-tests was expressed as means±standard error of the mean (SEM). Values were compared by one-way analysis of variance (ANOVA), followed by Bonferroni's test. Differences between results were considered as statistically significant when $p<0.05$.

RESULTS

Microscopic observation of the effects of LLLI pretreatment and UV irradiation on CAM microcirculation

UV irradiation induced alterations of the vessels, leading to rapid (after 30 min of irradiation) bleeding and extravasation (Fig. 1B). This effect was intensified after 60 min of exposure to UV irradiation, leading to marked edema (Fig. 1C). In Fig.1D we show the effect of the

pretreatment with LLLI on CAM microcirculation after 60 min of exposure. Results clearly showed normal structures of vessels, without any difference when compared to the control group (Fig.1A). Extravasation induced by 60 min of UV irradiation was abrogated by the LLLI pretreatment (Fig. 1E).

Protective effects of LLLI against deleterious actions of UV irradiation in terms of cell viability, cell cytotoxicity and ROS and O_2^- generation.

As can be seen in Fig. 2A, UV irradiation increased LDH release (181.42% of control) while pretreatment with LLLI significantly protected CAM tissue and abrogated all UV-induced alterations to the control level (83.03% of the control for UV, and 69.77% of the control for L-UV). We further evaluated the effects of UV irradiation and LLLI pre-irradiation on the percentage of CAM cell viability. Fig. 2B shows a significant decrease in the percentage of cell survival after UV exposure when compared to the control (56.23% of control). However, irradiation with LLLI alone did not change the rate of cell viability when compared to the control (93.08% of control). Pre-irradiation with LLLI protected CAM cells from UV toxicity (96.43% of control).

Regarding the effects of UV and laser irradiations on oxidative stress, we studied ROS generation as indicated by DHR fluorescence intensity. The results in Fig. 2C show the effects of different irradiation on the ROS content in CAM tissue. Our results show that UV irradiation significantly increased the ROS level in CAM tissue (132.78% of control). The LLLI pretreatment significantly protected against UV-induced oxidative stress (95.90% of control). Our results showed that UVA irradiation induced a significant increase (127.34% of control; $p<0.05$) in O_2^- production, as indicated by DHE fluorescence intensity, and that LLLI pretreatment restored the levels to near the control values (82.03% of control; Fig. 2D).

Protective effects of LLLI against oxidative stress generated after UV exposure

The data presented in Fig.3Ashow that UV irradiation induced lipid peroxidation, as indicated by the increase in the MDA level in the CAM tissue (0.3±0.004 for UV vs 0.166±0.001 for the control). The laser pretreatment reduced significantly the MDA levels and reversed UV-induced lipid peroxidation (0.177±0.012 for L-UV).

The effects of UV irradiation and LLLI pretreatment on CAM homogenate antioxidant enzyme activities were also determined and the results are presented in Fig. 3B. UV irradiation significantly increased CAM tissue antioxidant enzyme activities, including SOD (Fig. $3B_1$), CAT (Fig. $3B_2$) and POD (Fig. $3B_3$). More importantly, LLLI pretreatment significantly reversed all UV-induced antioxidant enzyme increases.

Finally, we investigated the effects of these treatments on calcium (which regulates several signaling pathways) levels (Fig. 3C) in CAM tissue. Neither UV irradiation nor the LLLI pretreatment changed the CAM tissue Ca^{2+} concentration.

DISCUSSION

Living cells are continuously exposed to solar polychromatic radiations, which are absorbed by intracellular chromophores. Mitochondrial cytochromes, flavins, porphyrins and the plasma membrane NADPH oxidase system, including flavoproteins and cytochrome b, have been suggested to be potential candidates for endogenous chromophores [14,15]. Interactions damage these molecules and challenge the maintenance of hereditary information and survival through the generation of photochemical reactions. Cells have developed adaptive strategies aimed at preserving these vital functions by reaching a balance between damage and repair. However, cellular reactions to solar radiation are complicated because of the interactions at certain wavelengths. Although sunlight is polychromatic, its final effect on human skin is the result of not only the action of each wavelength individually, but also the interactions between these wavelengths [16].

Due to its clinical efficacy for the enhancement of wound healing and pain treatment, improved circulation induced by LLLI is demonstrated to be one of the possible mechanisms by which wounds could be repaired [17,18]. In respect to the effects of LLLI on large vessels, a small number of studies have been conducted on the aorta [19]. Thus far, no scientific studies have been conducted on microvessel modulation during exposure to LLLI. In the present study we chose the CAM model, which can be used to carry out different analyses, including toxicological ones [20]. During avian development, the mesodermal layers of the allantois and chorion fuse to form the chorioallantoic membrane. This structure develops rapidly and generates a rich vascular network that provides an interface for gas and waste exchange [21]. In this study, we assessed the impact of UVA exposure on CAM microcirculation. UV irradiation altered CAM vessels and caused vascular damage that intensified with time, with edema appearing after 60 min of exposure. This vascular damage was observed on large and small vessels. Previous studies have shown that UVA irradiation is responsible for the liberation of NO from chemical stores in the skin. It is well known that NO can be produced enzymatically in the skin from a family of NO synthases, some of which are constitutively expressed and one of which is inducible. There are also chemical stores of NO in the skin, including nitrite, which can decompose to produce NO [22]. The endothelium of blood vessels uses NO to signal the surrounding smooth muscle to relax, resulting in vasodilatation and increased blood flow [23].

LLLI causes potent dilation in the laser-irradiated arteriole, leading to a pronounced increase in arteriolar blood flow. Our investigation showed that at the intensity used for therapeutic applications, LLLI defends CAM cells against the destructive action of UV irradiation. This vasodilator protective effect of LLLI may be mediated by NO production [24], which is a two-edged sword in that it can serve either as a prooxidant or as an antioxidant. Furthermore, NO has been described for its myriad biological functions. In fact, it is used in the treatment of different disease states, including pain and inflammation [25]. NO was also found

to play an important role in wound-healing mechanisms by the modulation of defined cytokine cascades [26].

We demonstrated that UV irradiation alone decreased the viability of CAM cells, suggesting that the UV light triggered toxic events in CAM cells. The toxic effects of UV light on cells are mediated by generated free radicals [27,28]. Moreover, it has been established that the UV light with shorter wavelengths is one of the most potent inducers of DNA damage, which is responsible for cell death [29]. However, the pretreatment with LLLI induced an increase in cell survival after exposure to UV light irradiation (Fig. 2B). These results suggest that the LLLI light source is not only non-toxic to CAM cells but can also prevent cell death.

Assessment of ROS formation using the Cellular Reactive Oxygen Species Detection Assay (DCFDA) showed that the exposure to UVA radiation for 60 min significantly increased ROS generation when compared to the control. All these deleterious effects were significantly suppressed by the LLLI pretreatment. These results were obtained with different experimental conditions such as exposure time, incubation period between the LLL exposure, subsequent UV irradiation doses and wavelength and types of cells [27,28,30].

At higher levels, ROS react indiscriminately with proteins, membrane lipids, carbohydrates, nucleic acids and other cellular components, resulting in various cytotoxic effects [31-33]. Moreover, lipid peroxidation is considered one of the most relevant mechanisms of cellular oxidative damage, and MDA, an end product of lipid peroxidation, is commonly used as an indicator of lipid oxidative damage [34,35].

In the present study, we found that UV irradiation was responsible of the generation of oxidative stress on CAM cells, as shown by the high MDA level and SOD, CAT and POD activities. The LLLI pretreatment induced a significant decrease in lipid-peroxidation level and restored SOD, CAT and POD activities to near control levels, which argues in favor of CAM cell protection from oxidative stress. The significant increase in the activity of these antioxidant

enzymes is an indicator of increased oxidative stress since these defensive enzymes function cooperatively to handle the relatively high amounts of ROS inside the cell [36]. In the present study, the activity of SOD increased after 60 min of exposure to UVA radiation, revealing increased production of superoxide radicals. These results are in agreement with previous studies that also reported variations in SOD activity in response to increased ROS production [37-39]. CAT is a light-sensitive antioxidant enzyme that is directly controlled by the amount of H_2O_2 in the cell [40]. Our results showed that the activity of CAT increased in CAM cells after 60 min of exposure to UVA light, revealing the increased activity of SOD. However, the LLLI pretreatment induced a significant decrease in lipoperoxidation level and restored SOD, CAT and POD activities to control level, which argues in favor of protection of CAM cells from oxidative stress. The protective process of the LLLI pretreatment is still unclear. It may be a biological adaptive response of CAM cells to LLLI, making them more resistant to UV irradiation through an increase in the cellular electron transfer process [41].

We next sought to highlight the putative implication of calcium in the mechanism of action of these effects. Only a few studies have dealt with calcium/ROS changes following LLLI. In this study, we have determined that neither LLLI nor UV changed total calcium concentrations in CAM tissue. Although we did not find any effects, a previous study demonstrated that illumination of cardiomyocytes with low-energy visible light results in a transient increase in intracellular calcium concentration [42]. Because intracellular calcium concentration is a well-known cellular mediator responsible for the stimulation of many processes [43,44], it was assumed that it can participate in pathways responsible for the preventive effect of LLLI. The increase in intracellular calcium concentration after laser irradiation characterizes the adaptive process of cells to oxidative stress and is without any morphological damage. In these cells, a pathway is initiated to restore the normal oxidation levels within the cell [45,46]. Although these reactions appear to be coordinated, further work

is required to determine in detail the effects of the LLL and UVA irradiations on the intracellular calcium in CAM cells.

In conclusion, the present study demonstrates that laser pretreatment protected CAM cells from UV damage by reducing oxidative stress. The protective effect of LLLI is partly attributable to activation of antioxidant enzymes.

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Authors' contribution: Amira Hammami and Mohamed Amri conceived and designed the experiments. Amira Hammami performed the experiments. Meherzia Mokni and Amira Hammami wrote the manuscript. Meherzia Mokni revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors report no declarations of interest.

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Figure Legends

Fig. 1. Microscopic observation of the effects of UV and LLLI vs control on *in vivo* microcirculation. Photos of CAM microcirculation irradiated by UVA for 0 min (**A**; control), 30 min (**B**) and 60 min (**C**). The opened surface of CAM was pretreated by LLLI for 60 min (**D**) and pretreated with LLLI then irradiated by UV for 60 min (**E**). The arrows point to normal structure of vessels (A, D and E) and extravasation (B and C). Representative results of three independent experiences photographed at magnification ×18. Scale bar; 5 cm.

Fig. 2. Protective effects of LLLI against the deleterious actions of UV irradiation in terms of cell toxicity (**A**), cell viability (**B**), ROS generation (**C**), and O_2^- generation (**D**). **A** – LDH levels expressed as the percentage relative to the control (100%). **B** – Cell viability indicated by FDA fluorescence intensity expressed as the percentage relative to the control (100%). **C** –Cellular ROS formation indicated by DHR fluorescence intensity expressed as the percentage relative to the control (100%). **D** – Cellular O_2^- generation indicated by DHE fluorescence intensity expressed as the percentage relative to the control (100%). CAM tissue (**A**) and CAM cells (**B**, **C** and **D**) were pre-treated or not with laser for 60 min, followed by UV irradiation (for 60 min). Results are presented as the mean±SEM of at least four different wells from three independent experiments. One-way ANOVA followed by Bonferroni's comparison test. ** indicates $p<0.01$ vs CTR. CTR–control group; LASER– laser-treated group, UV – UV irradiated group; L-UV –laser pretreated then UV irradiated group.

Fig.3. Protective effects of LLLI against oxidative stress generated after UV exposure. LLLI improves the antioxidant status in CAM tissue, as shown by MDA levels (expressed in pmoL/mg of protein) (**A**), antioxidant enzyme activities: SOD (expressed in UI/min/mg of protein) (**B_1**), CAT (expressed in nmoL/min/mg of protein) (**B_2**) and POD (expressed in mM/min/mg of protein) (**B_3**) and CAM calcium (expressed in mmoL/mg of protein) (**C**). CAM

tissue was pre-treated or not with laser for 60 min followed by UV irradiation for 60 min. Results are presented as the mean±SEM (n=6);one-way ANOVA followed by Bonferroni's comparison test. * indicates $p<0.05$ *vs* CTR; ** indicates $p<0.01$ *vs* CTR. CTR – control group; LASER – laser-treated group, UV – UV irradiated group; L-UV – laser pre-treated then UV irradiated group.

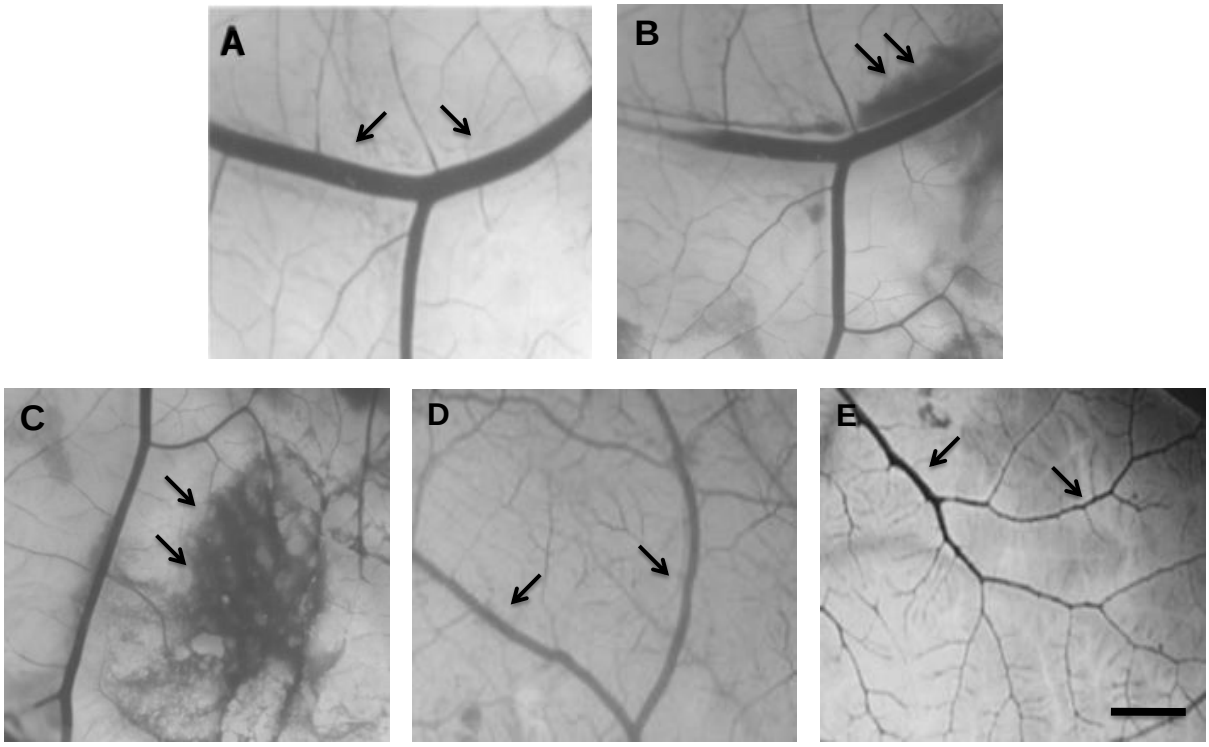

Fig. 1.

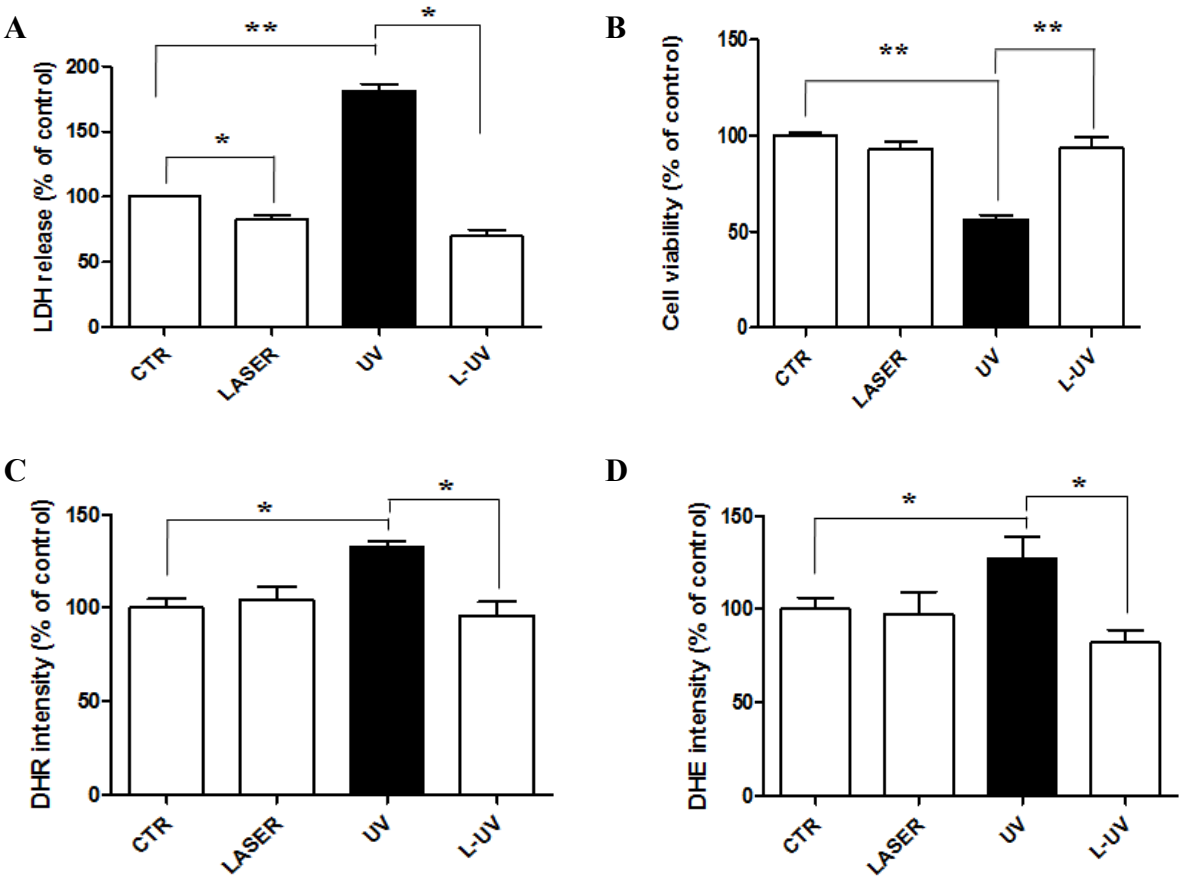
A
LDH release (% of control)
200
150
100
50
0
**
*
*
CTR
LASER
UV
L-UV
B
Cell viability (% of control)
150
100
50
0
**
**
CTR
LASER
UV
L-UV
C
DHR intensity (% of control)
150
100
50
0
*
*
CTR
LASER
UV
L-UV
D
DHE intensity (% of control)
150
100
50
0
*
*
CTR
LASER
UV
L-UV

Fig. 2.

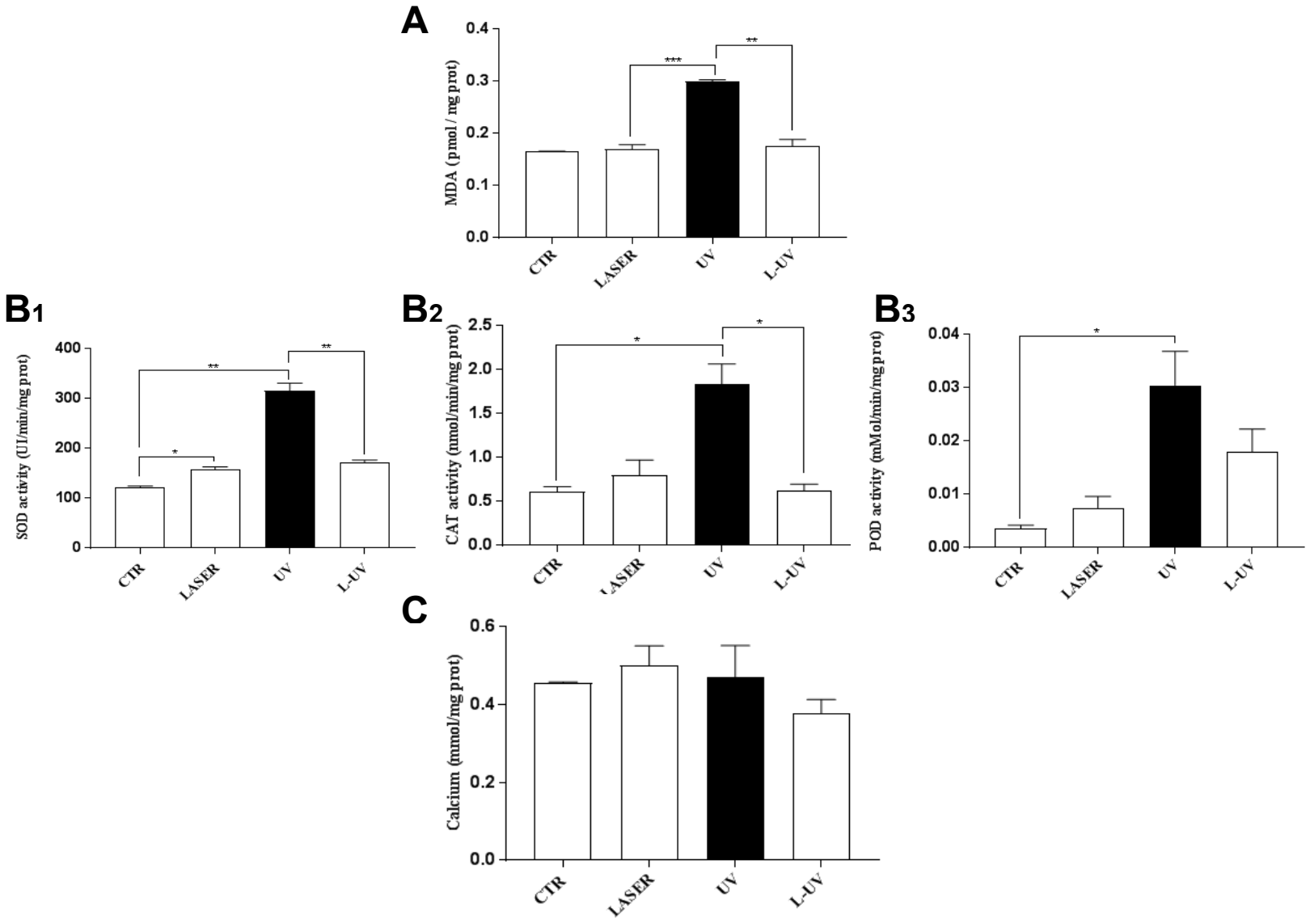

Fig. 3.

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