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Effects of short-wavelength blue light on fibroblasts. Experimental evidence in wound healing and cutaneous fibrosis †

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Abstract: Fibroblasts play a crucial role in wound healing and skin fibrosis. It is also probably the cell model used to study in vitro photobiomodulation. Our previous in vivo results evidenced a faster recovery in blue light-treated wounds (410-430 nm). In vitro experiments demonstrated that the lower dose increases cell metabolism, while higher doses (30.9 and 41.2 J/cm^2) reduce it. Furthermore, 20.6 J/cm^2 affects outward currents and Cytochrome C. Here, we described our preliminary results on the effects of blue LED light on mitochondria and reactive oxygen species production. Globally, our results demonstrated that short-wavelength blue LED light has PBM properties.

Keywords: photobiomodulation; blue light; wound healing; fibroblasts; skin fibrosis

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1. Introduction

More than 50 years have passed since Endre Mester identified the first medical evidence of Low Level Laser Therapy (LLLT). Experimental observations such as the faster hair regrowth in rats and the promotion of wound healing were categorized as "laser biostimulation" [1]. Soon it became clear that it was not necessary to use the laser and its peculiarities (monochromatic, coherent, etc.) to obtain an effect at a biological level. Non-coherent light-emitting diodes (LEDs) with comparable application parameters could also be exploited with lower production costs and better handling. For these reasons, the term LLLT has been replaced with Photobiomodulation (Therapy), PBM(T) [2]. Following the debates about the appropriateness of the terms, a nomenclature consensus meeting was organized by the North American Association for Light Therapy and the World Association for Laser Therapy. The term LLLT (cited in the Medical Subject Headings (MeSH)) led to thinking of laser as the only usable source, and gathered together terms and techniques significantly different from each other without any distinction. In November 2015, the term "Photobiomodulation Therapy" officially entered in the MeSH. This event represents an advance not only from a technological and methodological point of view, but also considering a rigorous organization of the scientific literature and the experimental protocols [1,2].

Today, the mechanism underlying PBM effects is still partially unclear. The most widely accepted theory is based on "hormesis", a dose-response relationship caused by a

biphasic effect. From a biological point of view, it is considered an adaptive response: a very low dose, or a treatment applied for a very short time does not induce any effect, while a high dose or a prolonged treatment time induces an inhibitory response.

Our research is focused on the effects of short-wavelength blue LED light (emission range 410-430 nm) in the field of wound healing and skin fibrosis. For these reasons, we dedicated our studies to analyze the behaviour of fibroblasts, both through in vivo and in vitro experiments.

In vitro experiments were performed on several cultures of fibroblasts isolated from keloids and their perilesional tissue, compared to fibroblasts obtained from normal human skin and purchased-cell cultures of adult human dermal fibroblasts (HDFa). Using colourimetric tests, we showed that blue LED light (3.43 – 6.87 – 13.75 – 20.6 – 30.93 – 41.2 J/cm^2) affects in different ways on fibroblasts with different origins, but the response is always dose-dependent. Electrophysiological recordings in patch-clamp showed that only keloid-derived fibroblasts increased the outward current after applying 20.6 J/cm^2. Furthermore, micro-Raman spectroscopy performed on single cells demonstrated that the same dose of blue LED light directly affects Cytochrome C, inducing a transition from oxidized to reduced form [3]. Scratch-test executed in co-culture of healthy fibroblasts and human keratinocytes (HaCaT cells) showed that the application of 20.6 J/cm^2 of blue light induces an acceleration of the closure of the scratch, compared with unirradiated samples [4].

From in vivo studies performed on superficial wounds induced in CD1 mice, we demonstrated that the application of 20.6 J/cm^2 of blue LED light stimulates the activation of fibroblasts into myofibroblasts, without inducing any changes in the cell density. Myofibroblasts were detected using alpha-smooth muscle actin antibody (alpha-SMA, Sigma-Aldrich, Milano, Italy) and revealed by fluorescence microscopy. Furthermore, the distribution and morphology of type I collagen in the wounded and treated tissue appears to be more similar to a never-injured one, when compared to the wounded but untreated tissue [5,6].

Wound healing is an intricate process consisting in three different phases: inflammation, tissue formation and remodeling [7]. Reactive oxygen species (ROS) play a key role in the tissue regeneration. Indeed, regulated production of ROS is crucial to maintain physiological processes and adaptive responses such as chemotaxis, cytoskeletal remodeling and calcium homeostasis [8,9].

Here, we irradiated human dermal fibroblast cells with three different doses of blue light (5 – 21 – 42 J/cm^2) to study ROS production and changes in the shape and morphology of mitochondria.

2. Materials and methods

2.1. Cell cultures

Adult Human Dermal Fibroblast cells (HDFa, Lot# 2207322) were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and used following the recommendation of the manufacturer. The primary cultures of human dermal fibroblasts were obtained from 7 patients subjected to mole removal, while keloid-derived fibroblasts were obtained from 11 patients subjected to keloid tissue removal. In each case, the samples were fragmented into small pieces (2-4 mm in diameter), collected in a scratched-Petri dish (Greiner Bio-One, GmbH) and kept under laminar flow until the adhesion to the plate occurred. Within three weeks from the preparation, fibroblasts migrated out of the tissue. All the cells were cultivated in Dulbecco Modified Eagle Medium (DMEM, 1.5 g/L in glucose) added with 10 % of Fetal Bovine Serum (FBS), 1% of Glutamine and Streptomycin (PAN-Biotech GmbH, Aidenbach, Germany) and detached using Trypsin-EDTA solution (Sigma-Aldrich, Milan, Italy) when necessary. DMEM was refreshed every 48 hours.

2.2. Irradiation protocols in cultured cells

Cells were counted using Neubauer chamber (Karl Hecht Assistent GmbH, Sondheim vor der Rhön, Germany), and an appropriate number of cells were seeded in a specific support for the analysis to be performed. The following fluences of blue LED light were applied: 5-21 and 42 J/cm^2.

2.3. ROS analysis

Reactive oxygen species were detected using a cell-based, fluorescent kit purchased from AbCam (ab113851, Cambridge, UK) and was used according to the manufacturer's recommendations. The signal was revealed by confocal microscopy (Leica SP8, Martinsried, Germany).

2.4. Electron microscopy

Twenty-four hours after irradiation, fibroblasts were detached using trypsin EDTA 0.25% (Pan-React Applichem, Milan, Italy). After 3 centrifuges in phosphate buffer saline, the pellet was resuspended in an appropriate volume of Karnovsky's fixative. Electron microscopy was conducted as follows. Briefly, the specimen were washed with cold 0.01 M phosphate-buffered saline, pH 7.4, and were directly fixed in cold 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C and post-fixed in cold 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 hour at room temperature. The samples were dehydrated in graded acetone, passed through propylene oxide and embedded in Epon 812. Ultrathin sections were stained with uranyless and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV.

3. Results

3.1. Blue LED light modulates Reactive Oxygen Species

Confocal microscopy (Figure 1A-E) showed that the application of 5 and 21 J/cm^2 (Figure B,C) did not induce a significant ROS increase, compared to the control (unirradiated cells, Figure A). The application of 41 J/cm^2 (Figure D) induces an increase in ROS signal. Figure 1E represents the signal after the addition of 100 μM of H_2O_2.

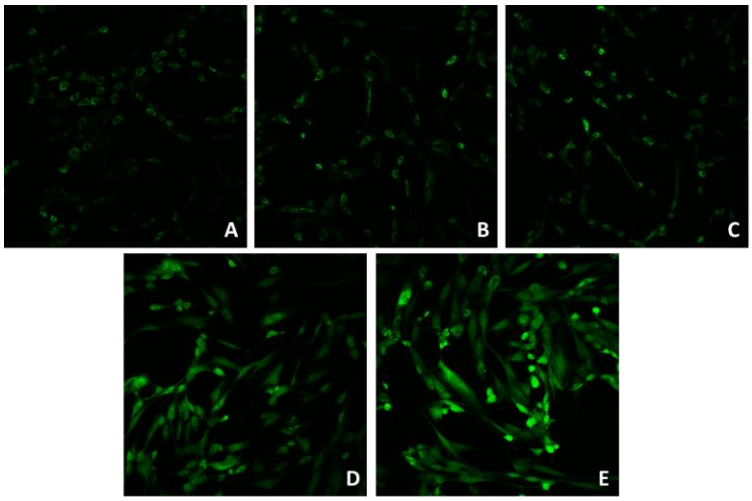

Figure 1. Confocal microscope imaging of unirradiated cells (**A**), cells irradiated with 5 (**B**), 21 (**C**) and 42 (**D**) J/cm^2. Positive control (**E**) was obtained by incubating cells in 100 μM of H_2O_2. Magnification: 20x.

3.1. Blue LED light affects mitochondria in human dermal fibroblasts

No substantial changes in cell morphology were evidenced by electron microscopy, with any of the blue light doses in the range 0-21 J/cm². The size of the mitochondria increases significantly when 42 J/cm² is applied. Mitochondrial morphology also slightly changes from rod-shaped in the controls to oval-shaped in the irradiated cells. Mitochondrial cristae show no significant changes in their overall extent.

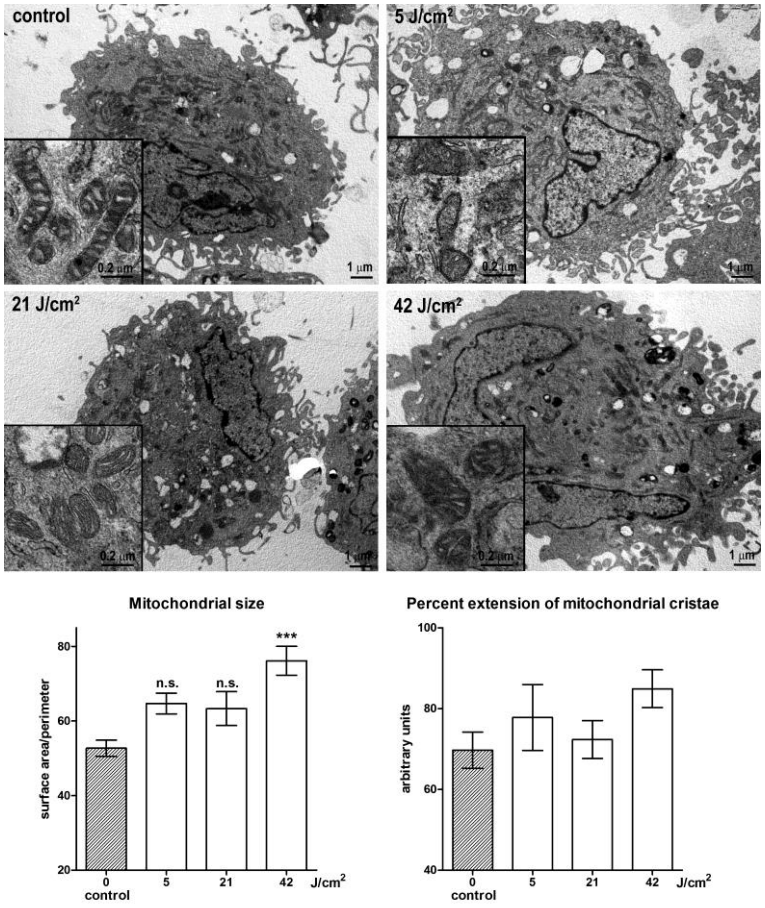

Figure 2. Electron microscopy of unirradiated cells (**A**), cells irradiated with 5 (**B**), 21 (**C**) and 42 (**D**) J/cm². Magnification: 5000x.

4. Discussion

Despite the limitation, in vitro experiments are still helpful in studying some biological processes. Here, we applied three fluences of short wavelengths of blue LED light: a low, a medium and a high dose, in human dermal fibroblasts to investigate eventual effects on the mitochondria shapes and ROS synthesis. Our preliminary results show that at low and medium fluences (4 and 21 J/cm², respectively), blue LED light does not stimulate ROS and does not damage the mitochondria, while the higher fluence (42 J/cm²) induces a significant increase in ROS and mitochondrial size. However, mitochondrial cristae show no significant changes in their overall extent, suggesting substantially similar patterns of energy metabolism. Together with our previous observations [3,4,6,10] and clinical evidence [11–13], these findings suggest that blue LED light-photobiomodulation (410 - 430 nm) represents a safe and effective treatment in the management of skin wounds and fibrosis.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of A.O.U Città della Salute e della Scienza di Torino – A.O. Ordine Mauriziano di Torino – A.S.L. Città di Torino (protocol code 0073787, 26/07/2017) and by the Hospital Ethical Board of A.O.U di Perugia (protocol code 16806/19/AV, 07/17/2019). The animal study protocol was approved by the Institutional Review Board of the Italian Ministry of Health (protocol code 791/2016-PR).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are available upon reasonable request to the corresponding author.

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