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Blue light-irradiated human keloid fibroblasts: an in vitro study

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ABSTRACT

Blue LED light irradiation is currently under investigation because of its effect in wound healing improvement. In this context, several mechanisms of action are likely to occur at the same time, consistently with the presence of different light absorbers within the skin. In our previous studies we observed the wound healing in superficial abrasions in an in vivo murine model. The results evidenced that both inflammatory infiltrate and myofibroblasts activity increase after irradiation. In this study we focused on evaluating the consequences of light absorption in fibroblasts from human cells culture: they play a key role in wound healing, both in physiological conditions and in pathological ones, such as keloid scarring. In particular we used keloids fibroblasts as a new target in order to investigate a possible metabolic or cellular mechanism correlation. Human keloid tissues were excised during standard surgery and immediately underwent primary cell culture extraction. Fibroblasts were allowed to grow in the appropriate conditions and then exposed to blue light. A metabolic colorimetric test (WST-8) was then performed. The tests evidenced an effect in mitochondrial activity, which could be modulated by the duration of the treatment. Electrophysiology pointed out a different behavior of irradiated fibroblasts. In conclusion, the Blue LED light affects the metabolic activity of fibroblasts and thus the cellular proliferation rate. No specific effect was found on keloid fibroblasts, thus indicating a very basic intracellular component, such as cytochromes, being the target of the treatment.

Keywords: Wound Healing, Blue LED light, human keloid fibroblast.

1. INTRODUCTION

Keloid scars are described as a fibrous tissue overgrowth in the site of skin lesion. They are formed only in predisposed individuals and, unlike normal scars, do not regress and often reappear after surgery. Keloids are benign dermal fibro proliferative tumors with no malignant potential, but can be quite annoying for the afflicted subjects in terms of pruritus, pain and restriction of movements in the most serious cases [1], without taking into account the psychological traumas. Keloids occur almost always on the chest, shoulders, upper back, back of the neck and earlobes, rarely on mucous membranes [2]. They are unique in humans, principally in dark-skinned people [3], with a high show up in specific ethnic populations and individuals with familiar heritability [4]. The keloids tissue is characterized by a high level of collagen in the site of the lesion that produce a scar extending beyond the boundaries of the original wound. [6]. The collagen fibers are larger, thicker and wavier than those in normal skin and they show a random orientation. The exaggerated synthesis of collagen is probably due to the increase of fibroblasts density and their proliferation rate which induces a rising in production of collagen and components of extracellular matrix (ECM) respect to the normal skin [7,8].

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Currently, no specific growth factors or proteins have been identified as the cause for keloid origin and development. In scientific bibliography it has been reported some regulators of the fibrotic cascade, such as transforming growth factor β (TGF- β) and fibronectin extra domain A (Fn-EDA) that are demonstrated to play a role in collagen deposition during keloid growth, however, the underlying mechanisms and how they are involved are still unclear [9].

Recently, a keratinocytes role has been suggested [10] in relation to the hypoxia, which is a typical condition of keloid scars and other solid tumors. Hypoxia inducible factor1 α (HIF1 α) is the main factor induced by hypoxia and it is also involved in proliferative activity. HIF1 α has been correlated with tumor invasion and metastasis process through vimentin and fibronectin expression upregulation and E-cadherin and ZO-1 junction expression downregulation in keloid keratinocytes under hypoxic conditions. Hypoxia promotes the endothelial-to-mesenchymal transition (EMT) process and enhance the invasion ability of keloid keratinocytes, allowing the keloids to extend beyond the wound boundaries [11-13].

The use of incoherent light like the one emitted by LEDs (light-emitting diodes) in order to care hyperproliferative skin diseases such as psoriasis, acne, keratosis and skin cancer is widely studied [14] and today it is clear that the irradiation with specific wavelengths has a biological effect. Even if the cellular mechanisms involved in these processes are still unclear or not fully investigated yet, the photobiomodulation is an undisputed biological phenomenon.

To induce a biological activity, the light must be absorbed by specific molecules, also called photoacceptors, leading them to an excited state. These activated-molecules then affect secondary targets inside the cell, transducing the light signal into a molecular response [15, 16]. In the visible-to-near-infrared spectral range, one of such acceptor is cytochrome c oxidase [17, 18]. In the blue range of wavelength, porphyrin-containing enzymes and flavoproteins are thought to be photoacceptors linking the mitochondrial respiratory chain to photostimulation [19-21].

From our previous studies in rodent models we demonstrated that 30 seconds of blue light at 420 nm treatment induce an early increase of inflammatory infiltrate improving wound healing in superficial abrasions [22-31]. In this work we use an in vitro model in order to investigate if human derma keloids fibroblasts are influenced by blue light LED treatment at 420 nm, for the purpose to consider an innovative application in cosmetic surgery.

2. MATERIALS AND METHODS

2.1 Primary cells cultures

Human keloid fibroblasts cells (HKFCs) cultures has been set up from human keloids tissues coming from aesthetic surgeries performed at the AOU Città della Salute e della Scienza di Torino. The keloid tissue has been used within 5 hours from the excision and during this time it has been maintained in Dulbecco Modified Eagle Medium (DMEM) at a temperature of 4°C. After several washes in Phosphate Buffer Saline (PBS) (Pan-React Applicchem, Milan, Italy), the cultures of HKFCs has been prepared with a surgical punch in order to obtain sections of approximately 2 mm in diameter. The sections have been collected in a scratch-Petri dish and maintained in DMEM low glucose medium (Pan-React Applicchem, Milan, Italy) supplemented with 10% Foetal Bovine Serum, 1% of Glutamine and 1% Penicillin-Streptomycin (EuroClone, Milan, Italy), keeping it at 37°C and 5% CO₂. In the two weeks following the preparation, the fibroblasts migrated from the tissue. The experiments have been performed between the second and fifth cell division; during this time, the cultures were maintained under standard culture conditions and the medium has been refreshed every 48 hours. Every keloids tissue has been divided in superficial derma and deep derma, in order to obtain a specific fibroblasts cell culture. When it was possible, also fibroblasts from the wound periphery have been harvested.

2.2 The Blue light

The blue light-based device uses a commercial LED, emitting 1 W power at 420 nm (see figure 1). The LED is coupled with a 1.2 m long flexible polymeric fiber and mounted on a benchtop device equipped with a touchscreen where it is possible to control all the irradiation parameters. The illuminated area corresponds to a 5 mm radius circle with a resulting power density of about 1.2 W/cm², homogeneously distributed on the spot area like as from a top-hat source.

2.3 Viability Assay

The viability assay has been performed using WST-8, a metabolic and colorimetric test from Sigma-Aldrich (St. Louis, MO, USA). 5x10³ HKFCs have been seeded in a multiwell plate (Corning, Sigma Aldrich, Milan, Italy) and triple of wells have been irradiated with the blue light respectively for 5, 10, 20, 30, 45 and 60 seconds. The power density of radiation has been set at 235 mW, while the irradiation was performed keeping the fiber tip 1 cm far from the bottom of

the well. Every treatment has been performed in Serum Free Medium without red phenol in order to avoid light absorption from cell medium. All the tests were recorded with a IR thermal camera (Nec Avio R300SR, Nippon-Avionics, Tokyo, Japan) in order to measure an eventual variation of temperature. Tests analysis has been performed 24 hours afterwards irradiation and absorbance has been read with an automatic microplate absorbance reader (LT-4000 Labtech, Heathfield, East Sussex, England) processing the values with a specific commercial software.



Figure 1. The blue-light LED device.

2.4 Electrophysiology

Electrophysiological recordings were performed on freshly isolated HKFCs. Membrane potentials and ion currents were measured with standard whole cell current - clamp and voltage - clamp techniques. Each coverslip was transferred to a recording chamber (1 mL volume), mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of 2 mL·min⁻¹ with a standard extracellular solution containing (in mM): HEPES 10, D-glucose 10, NaCl 147, KCl 4, MgCl₂ 1 and CaCl₂ 2 (pH adjusted to 7.4 with NaOH). Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 4–6 MΩ. Pipette solution used contained the following (in mM): K-gluconate 134, KCl 10, EGTA 11 and HEPES 10 (pH adjusted to 7.4 with KOH). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), stored and analyzed with a pClamp 9.2 software (Axon Instruments, CA, USA). Cell membrane capacitance was calculated in each cell throughout the experiment by integrating the capacitive currents elicited by a -10mV voltage pulse. Peak currents activated by blue light were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results.

2.5 Protocols

Unless otherwise stated, cells were voltage-clamped at -70 mV. Capacitive transients generated by the electrode and by cell membrane were digitally subtracted by the amplified circuit. Series resistance (Rs), membrane resistance (Rm) and membrane capacitance (Cm) were routinely measured by fast hyperpolarizing voltage pulses (from 70 to 75 mV, 40 ms duration). Only cells showing a stable Cm and Rs before, during, and after light treatment were included in the analysis. Immediately after breakthrough into whole-cell configuration, cell resting membrane potential (V_{rest}) was determined by switching to the current-clamp mode. A voltage ramp protocol (800 ms depolarization from -120 to +80 mV) has been used to evoke a wide range of overall voltage-dependent membrane currents before, during and after treatments.

3. RESULTS

3.1 Viability Assay

Two samples of human keloids have been examined (figure 2). From WST-8 tests, we discovered that fibroblasts derived from different sections of the keloid tissue show a different behavior with irradiation times. In particular cells

metabolism increases at short irradiation time (5 seconds), while for long irradiation times it decreases with increasing treatment time. The reduction of metabolic activity is related to fibroblasts death.

The analysis of superficial and deep fibroblasts populations also suggests that different sections of keloid tissue respond in different ways to blue light treatment. In particular fibroblasts from superficial derma seem to be more sensitive than ones from deep derma, but these results must be clarified with further assays and experiments.

Moreover, fibroblasts derived from perilesional tissue react in a different way respect to fibroblasts of deep and superficial derma. In fact, the viability of these kind of cells does not increase after 5 seconds of irradiation but, rather, it immediately decreases. On the other hand, for long irradiation time, the behavior of fibroblasts from wound boundary is the same of other fibroblasts subpopulations.

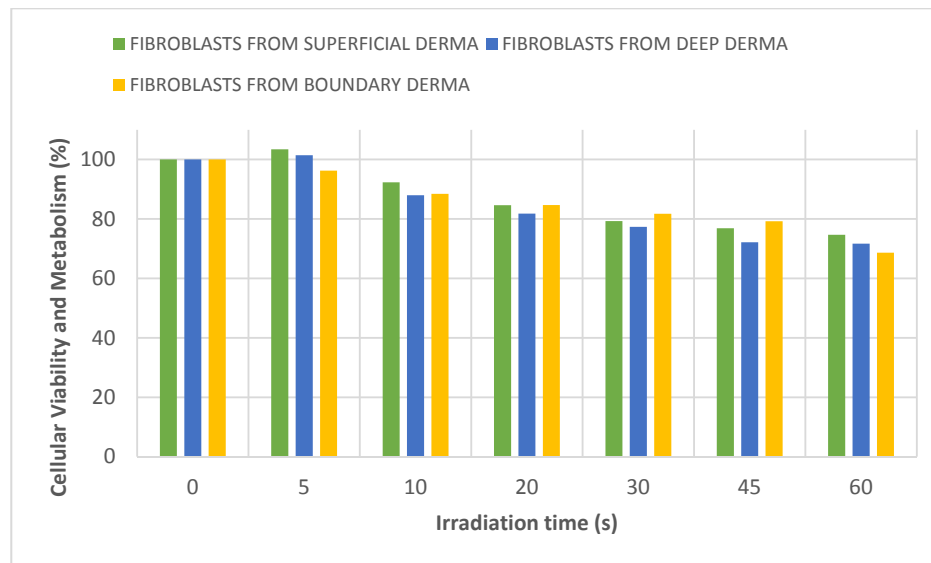


Figure 2. The results obtained from WST-8 tests. Metabolism of fibroblasts from wound boundary decreases immediately after blue light irradiation at 420 nm, while superficial and deep fibroblast metabolism increases. For longer irradiation times, all fibroblasts shows a homogeneous behavior.

3.2 Electrophysiology

Whole-cell patch clamp recordings were performed on 10 cells showing a $C_m = 13.3 \pm 2.8$ pF; $R_m = 772 \pm 260$ M Ω ; $V_m = -45 \pm 7.4$ mV (mean \pm SEM). Preliminary voltage-clamp results on HKFCs show that 30 seconds of blue light application increases the amplitude of outward currents evoked by a voltage ramp protocol. Not all cells react in the same way after the blue light treatment, probably depending on the different metabolic state of fibroblasts. In order to facilitate result interpretation, we plan to integrate patch clamp recordings with investigations into fibroblasts cell-cycle, i.e. by performing electrophysiological experiments in 24 h starved cells in order to synchronize cell cycle in G0.

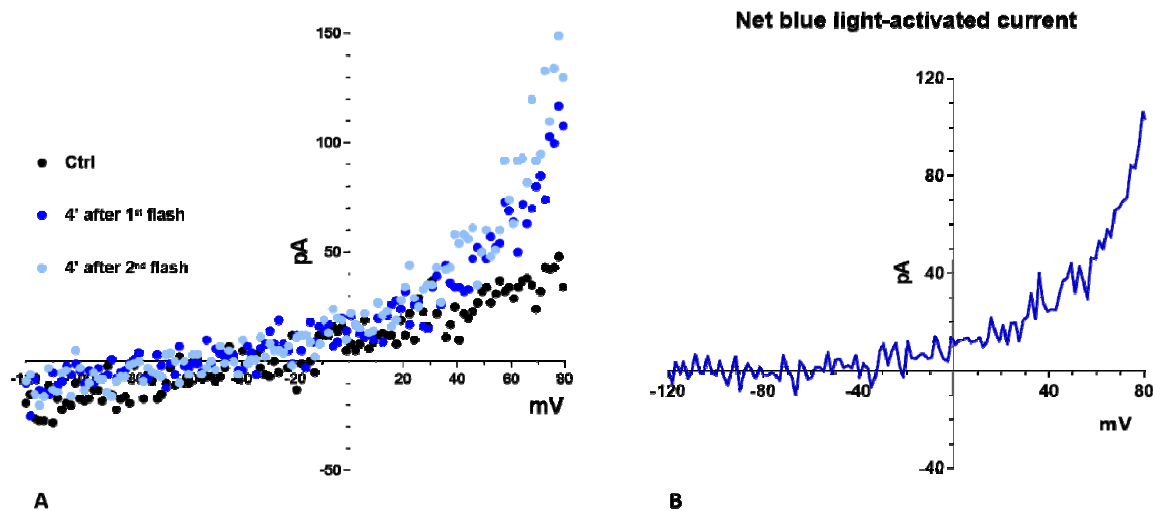


Figure 3. Preliminary electrophysiology experiments results. Figure 2A shows the original ramp-evoked currents in a representative cell before (Ctrl), and after blue light irradiation. Figure 2B shows the net blue light-activated-sensitive current obtained in the same cell by subtraction of the ramp recorded after the treatment from that recorded in control. Note the different scale of y axis.

4. CONCLUSIONS

In this work we have studied the effects of blue-light LED irradiation on human fibroblasts keloids cells, showing that fibroblasts from wound boundaries shows a different metabolic response respect to superficial and deep ones. Our experiment also suggest that the superficial fibroblasts have a different reaction to the light respect to the deep ones. The electrophysiology experiments evidenced a result in line with the current scientific literature: light-sensitive channels are not expressed by fibroblasts cells, in fact a few seconds after the treatment there is a modification of the ramp-evoked currents, suggesting that the light exposure has an effect on the activation of other signal transduction ways. Further studies will be performed in the next future, in order to investigate the effects of the blue light on human keloids, corroborating these preliminary evidences.

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