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Proceeding paper

In vivo evidence of blue-LED-light photobiomodulation †

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Abstract: A study investigated the effects of blue LED light (410-430 nm) on wound healing in mice with superficial and full-thickness skin wounds. The wounds were treated with blue LED light (20.6 J/cm², 30 s), and biopsies were collected over 6 days. Results showed modulation of cytokine release, increased number of granulocytes and mast cells, increased density of vessels with mast cells expressing platelet-derived growth factor, and improved collagen deposition compared to untreated wounds. The study concluded that blue light leads to faster and more effective wound healing and improved skin morphology.

Keywords: photobiomodulation; wound healing; blue LED light; in vivo model

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1. Introduction

Photobiomodulation, also known as low-level light therapy, is a growing field of research that explores the use of light energy to induce therapeutic effects in living tissue. This non-invasive therapy involves applying low-intensity light in the visible to the near-infrared range to target specific cells or tissues [1]. Photobiomodulation treats various conditions, including pain, wound healing, and neurodegenerative diseases. It is also being studied for its potential to improve skin health, increase joint mobility, and enhance athletic performance. Photobiomodulation can be delivered using various devices, including light-emitting diodes (LEDs), laser therapy devices, and infrared lamps [2]. The exact mechanisms of photobiomodulation are still not fully understood, but the involvement of the synthesis of adenosine triphosphate (ATP), mitochondria and action on specific cellular targets appears evident [3]. Current research suggests that the absorption of light energy by specific cellular components triggers various biological responses [4]. Photobiomodulation has been shown to activate several key cellular processes, including i) modulation of energy production, where light energy is absorbed by cellular mitochondria, leading to an increase in ATP synthesis; ii) reduced oxidative stress by increasing the antioxidants and reducing the production of reactive oxygen species (ROS); iii) stimulated cell proliferation and promote tissue repair, making it practical for treating conditions such as wounds and neurodegenerative diseases [5].

In our previous research, we studied the effects of blue light in acute wounds, 24 hours after the treatment. Our results showed that treated wounds (TW) had an increase in inflammatory cells, starting 1 hour after injury till 6 hours later. Granulocytes, mast cells (MC), and endothelial cells showed an increase in the timeframe between 6 to 9 hours in TW, while in untreated wounds (UTW) the increase occurred later (12 to 24 hours). Additionally, the number of M2 macrophages increased earlier in TW. Dendritic cells (DC) played a crucial role in the organisation of the inflammatory infiltrate and increased after 6 hours, while decreased after 24 hours in NTW. The number of M2 macrophages was almost constant in TW. Plasmacytoid cells (pDC) modulate Treg lymphocytes, which are involved in tolerance and suppressing excessive immune responses [6,7]. We found that pDC cells increased at 9 hours in TW, but remained unchanged in NTW. However, TW and NTW showed a significant decrease in pDC cells 12 hours after injury [8]. In another study, 6 days after the treatment with blue light MC led to a well-coordinated cellular response, including an early inflammatory reaction, angiogenesis due to Tumor Necrosis Factor–alpha (TNF-α) secretion [9], and fibroblasts activation [10].

Here, we started studying the effect of blue LED light on full-thickness wound, which represents a model of chronic wound. Numerous clinical data support the hypothesis that chronic wounds manifest an excessive inflammatory phase, with high levels of inflammatory infiltrate cells, such as neutrophils and macrophages. The inflammatory environment stimulates cytokines, ROS and proteases and inhibits factors such as Vascular Endothelial Growth Factor (VEGF) and basic Fibroblasts Growth Factor (bFGF). The consequences lead to a degradation of the extracellular matrix (ECM) and therefore the impossibility of progression in the subsequent stages of healing, proliferation and remodelling [11]. Based on our previous findings regarding the modulation of the inflammatory phase in the acute wound [10,12], here we analysed cytokine release and growth factors on chronic wounds.

2. Materials and Methods

2.1. Animal model

Sixty-three CD1 male mice (15 and 20 g, Envigo, Udine, Italy) were used. The animals were fed with a standard pellet diet and housed in static filter top cages under a 12 hours light/dark cycle and controlled temperature (24°C). The experiments were carried out at the Centre for Laboratory Animal Housing and Experimentation, University of Florence, Italy. In each mouse, one or two full-thickness wounds were performed using a biopsy punch (4 mm in diameter). Irradiation with blue LED light was performed immediately after wound induction: one treatment was performed per animal (410 to 430 nm, 20.6 J/cm^2, 30 s treatment time). At 1 - 3 - 6 - 9 - 24 hours and finally 7 days after the treatment, the animals were sacrificed by CO_2 inhalation. All experimental procedures were performed in accordance with the European Community guidelines for animal care (86/609/EEC), and written consent was duly obtained from the Italian Ministry of Health (791/2016-PR).

2.2. Samples preparation for Multiparametric ELISA tests

After tissue excision, samples were dissolved with a manual homogenizer. The protein content was assessed by comparison with the albumin calibration curve obtained by Bicinchoninic acidic (BCA protein assay, Sigma-Aldrich, Milan, Italy). The samples were then diluted with radioimmunoprecipitation assay buffer (RIPA buffer) so prepared: 10 mM Tris-HCl (tris(hydroxymethyl)aminomethane), 1 Mm EDTA (Ethylenediaminetetraacetic acid), 0.5 mM EGTA (egtazic acid), 1% Triton X-100, 0.1% SDS, 140 mM NaCl (Sodium Chloride). The RIPA buffer was used to obtain a different total protein content. The absorbance at 570 nm was read using a reference wavelength at 630 nm and was evaluated using an automatic microplate absorbance reader (LT-4000 Labtech, Heathfield, East Sussex, England). Samples were then placed in glass slides and sent to Raybiotech

(GA, USA) service for multiparametric ELISA essay. Results were analyzed for means and SD; the Kolmogorov-Smirnov test was then performed.

3. Results

3.1. Modulation of cytokines

ELISA analysis revealed a differential modulation of signal molecules involved in the inflammatory pathways underlying the healing process. Figure 1 A,B shows an overall increase in TNF-α and Epidermal Growth Factor (EGF) in TW, except for the first hour after wound induction. At the same time, Matrix MetalloProteinase-2 (MMP-2) and pro-Matrix MetalloProteinase-9 (pro-MMP-9) (figure 1 C,D) remained constant or slightly augmented.

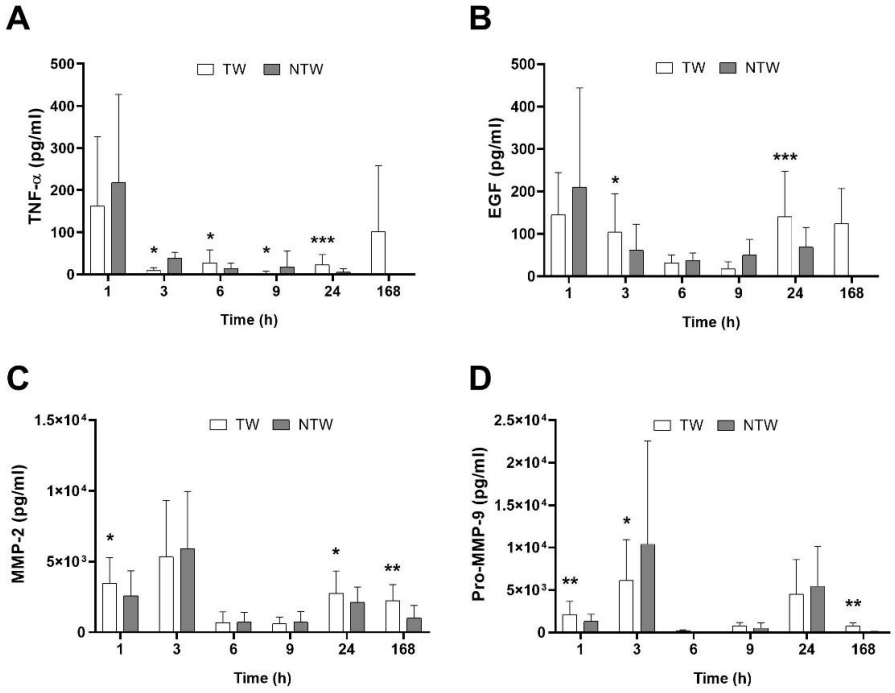

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Figure 1. ELISA test results in samples from TW and NTW in a mice model: TNF-α (**a**), EGF (**b**), MMP-2 (**c**), and pro-MMP-9 (**d**) trends. Data are expressed as mean ± SD. Significant p-values: *p < 0.1; **p < 0.05; ***p < 0.01 vs. NTW at the same time point.

During the first 24 h after the treatment, we observed a modulation of the Vascular Endothelial Growth Factor-A isoform (VEGF-A) level, as shown in Figure 2. During the first hours after the wound, the VEGF-A level seemed not to be affected by the treatment, but 9 hours later, an increase showed up.

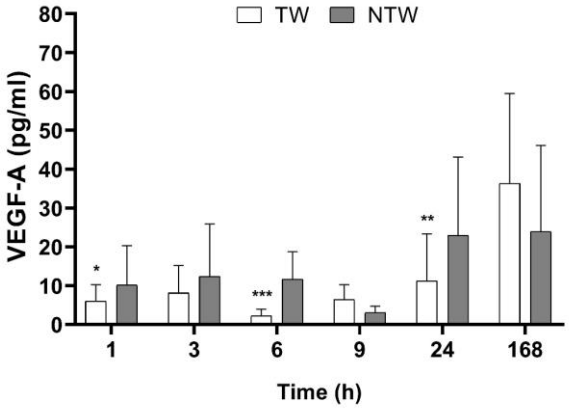

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Figure 2. ELISA test results in samples from TW and NTW in a mice model: VEGF-A levels over time. Significant *p*-values for VEGF-A data were: $p < 0.1$ at 6 h and 9 h, $p < 0.05$ at 24 h. Data are expressed as mean ± SD. Significant *p*-values: $^{*}p < 0.1$; $^{**}p < 0.05$; $^{***}p < 0.01$ vs. NTW at the same time point.

4. Discussion

Wound healing is a complex and dynamic process. A variety of cell types are involved, orchestrated by several growth factors and cytokines. Blue LED light is successfully used in wound healing, notwithstanding its mechanism of action is still unclear. In our previous work, we demonstrated the beneficial effects of blue LED light irradiation in the healing of superficial abrasions in animal models [10,12,13]. In particular, we studied cell inflammatory infiltrate and new tissue formation. Here, our attention was focused on specific cytokines and growth factors involved in the healing process in chronic wounds.

ELISA assay shows that the blue LED light also affects pro-MMP-9 and MMP-2. We pointed out that in the TW, both markers quickly increase significantly after the wound induction (1 hour) while reversing at 3 hours (significantly only for pro-MMP -9), showing a significant increase in NTW. After 24 h, only the MMP-2 appears significantly increased in the TW, while after 7 days, both markers are significantly expressed in the TW, when compared to the NTW. The involvement of MMP-2 and MMP-9 in wound healing has been demonstrated [14]. The MMP-2 is expressed at the edge of the acute wounds. Moreover, it is correlated with the appearance of laminin-332 and the increase in keratinocyte migration [15]. Furthermore, MMP-9 knockout mice display a delay in wound closure [16], demonstrating that MMP-9 is necessary for the final phase of the wound healing process. Accordingly with the literature, our findings indicate that both the pro-MMP-9 and MMP-2 levels are increased at the initial and in the final stage of the wound healing process. We suppose that at early times after the wound induction, the blue LED light induces an increase in matrix degradation to prepare the wound bed for new collagen deposition. In contrast, at a later time in the healing process, this increase may become an indicator of the angiogenetic process and the closure of the wound. Both MMP-2 and MMP-9 play a role in regulating angiogenesis during wound healing through the activation of proangiogenic cytokines, including TNF-α [17–19]. TNF-α is a cytokine involved in acute inflammation, known to be produced by several immune cells, primarily by macrophages, but also by neutrophils and mast cells. As demonstrated in our previous work, an increase in the TNF-α level in treated tissues indicates an activation of the inflammatory cells [12]. Surprisingly, we found that blue LED light reduces the levels of VEGF-A in chronic wounds. We suppose these effects may be linked to an accelerating inflammation in TW compared to the NTW and different time courses of these growth factors during our observations. VEGF affects blood vessel formation and might be enhanced by the

treatment, consistently observing a better skin morphology in the later phases [10,20]. Obviously, the considerations made are only preliminary. Experiments relating to the responses of individual cellular components of the cellular infiltrate and angiogenesis are currently being carried out in the laboratory. Globally, no evidence was pointed out in these experiments concerning the cross-talk between TW and NTW in the same animal.

The beneficial effects of blue light, especially in dermatology and wound healing, are indisputable. However, further experiments are necessary to better understand the mechanism behind the light action. Anywhere, the proposed method might provide a helpful approach in skin wound management.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board of the Italian Ministry of Health (protocol code 791/2016-PR).

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

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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