Effect of photobiomodulation (670 nm) associated with vitamin A on the inflammatory phase of wound healing

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Abstract

Wound healing is a complex biological process with specific phases. Photobiomodulation (PBM) decreases the inflammatory infiltrate, stimulating fibroblast proliferation and angiogenesis, and therefore, is indicated for wound healing. Vitamin A is used to reverse the inhibitory effects on wound healing and accelerate the healthy granulation tissue. The study aimed to evaluate the effect of topical vitamin A and PBM (GaAIs) in inflammatory phase of cutaneous wounds. Forty Wistar male rats were separated into four groups: (1) control (CG); (2) laser group (LG) GaAIs, 670 nm, 30 mW, energy per point of 0.9 J, radiating by 1 point in 30 s; (3) vitamin A group (VitAG); and (4) laser group plus vitamin A (LG + VitAG). Wounds were surgically made by a punch biopsy with 10 mm of diameter on the back of the animals and all treatments were started according to the experiment. The treatments were administered for four consecutive days and biopsy was performed on day 4. We performed both H&E and immunohistochemistry analysis. The results were compared between groups by one-way analysis of variance ANOVA test with post hoc Tukey ($p < 0.05$). Inflammatory infiltrate increased significantly in LG compared to CG and VitAG ($p < 0.05$). Regarding angiogenesis, VEGF expression was increased significantly in LG and LG + VitAG groups, $p < 0.01$. The results indicate that proposed treatments were effective on the healing process improved by LG and LG + VitAG. We show that laser plus vitamin A enhances healing by reducing the wound area and may have potential application for clinical management of cutaneous wounds.

Keywords GaAIs laser · Photobiomodulation · Retinol · Vitamin A · Wound healing

Introduction

Wound healing is a complex process divided in specific biological events such as inflammation, proliferation, and remodeling.

The literature shows evidence of mechanisms of action and response for each specific phase [1–3]. Thus, it is known that the inflammatory phase follows the trauma and involves vascular responses such as blood coagulation and hemostasis, causing local vasodilation, extravascular blood and fluid leakage, and blockage of lymphatic drainage, evidencing signs of inflammation. Such cellular events include fibrin network formation, which will aid the migration of cells that are essential for phagocytosis and chemotaxis. Neutrophils and macrophages are essential cells that participate in this early phase of the healing process, and are both responsible for tissue phagocytosis in the wounded area; they are also able to release specific growth factors that play a role in the subsequent phases [2–4].

Literature shows that wounds not properly treated increase the risk of contamination and may have physiologic consequences, impair the quality of life, and lead to high financial burden. These can be due to extrinsic factors such as pressure, impaired lymphatic function, mechanical stress, or to intrinsic factors such as immobility, infection, malnutrition, and peripheral vascular disease [3].

Due to the complexity of the tissue regeneration process, several resources as acupuncture [5], pharmacological products [6], and physiotherapeutic resources have been proposed

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as aids and/or stimulants of the healing process, with the aim to optimize the length of treatment and improve tissue quality. Among the physiotherapeutic resources, photobiomodulation (PBM) offers safe and efficient mechanisms to stimulate the wounded area and promotes positive response to the tissue based on the absorbed light by the mitochondrial chromophores [7, 8]. The primary effects of PBM in the initial repair process stimulate the macrophage phagocytic activity, increased proliferation of several cellular types, and maturation and locomotion of fibroblasts and lymphocytes, in addition to stimulating the subsequent phases of proliferation and remodeling [8–10]. Under the vascular point of view, PBM activates the proliferation of endothelial cells, stimulated by the vascular endothelial growth factors (VEGF). VEGF is characterized by the presence of multiple components that act in angiogenesis, through increased the vascular permeability, vasodilation, and the transport of specific substrates [10, 11].

Similarly, topic treatments such as vitamin A have been investigated for the same purpose. Vitamin A has an active ingredient that stimulates the tissue regeneration dynamics, including extracellular matrix formation, fibroplasia, and synthesis of collagen, glycoprotein, and proteoglycan [12, 13]. Its application in the wounded area contributes to wound reepithelialization, acting as a direct factor for collagen synthesis and influencing the immunological response [13–17], as well as preventing susceptibility to infections.

Although the literature shows both PBM and vitamin A as isolated resources to promote tissue regeneration, their association is little explored in scientific studies. Thus, this work aims to evaluate the PBM action both isolated and in association with vitamin A, in order to develop an efficient protocol for cutaneous wound healing in rats.

Material and methods

This study met the Guidelines for Care and Use of Animal Models and was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de São Carlos (UFSCar) (Protocol: 2-007/2014). Animals were kept in the vivarium of the Department of Physiotherapy of the UFSCar during the experimental period, totaling 4 days, and were allocated to appropriated standard polyethylene cages, in controlled environment (19–23 °C and day/night cycle of 12/12 h), with free access to water and adequate food.

We used Wistar 40 rats (*Rattus norvegicus albinus*), male, approximate weight of 250 to 300 g, randomly divided in four groups ($n = 10$):

- Control group (CG): Wounds and simulated PBM;
- Laser group (LG): Wounds and treatment with PBM;
- Vitamin A group (VitAG): Wounds and topic application of Vitamin A;

- Laser group + vitamin A group (LG + VitAG): Wounds and treatment with PBM (670 nm, 30 mW, 14.28 J/cm²) + topic application of vitamin A.

Surgical procedure

The animals were weighted and anesthetized with ketamine (40 mg/kg, Agener, SP, Brazil) and xylazine (15 mg/kg, Dopaser, SP, Brazil). The animals were placed in ventral decubitus for digital trichotomy of the dorsal area, followed by washing with saline solution. A punch of 10-mm diameter was performed in the back of each animal (Fig. 1).

Photobiomodulation treatment

The groups treated with PBM received the first application 1 h after the surgery. During the experiment, the animals received daily applications in the morning, in a single point over the wound, totaling a maximum of four applications. The laser was positioned perpendicularly and applied in continuous emission mode, with the beam covering the entire wounded area. PBM was performed with red laser (LASERPULSE, IBRAMED, Brazil), wavelength of 670 nm, power of 30 mW, and power density of 14.28 J/cm² [18]. The equipment was calibrated by a qualified technician of Instituto de Física da Escola de Engenharia de São Carlos da Universidade de São Paulo (EESC-USP), before the beginning of the experiment. Laser parameters are shown in Table 1. At the time of treatment, animals were immobilized with a cotton blanket to improve the therapy application and avoid stress.

Fig. 1 Illustration of the wound caused by surgical procedure using a 10-mm punch. (a) indicates the wound boundaries and (b) indicates the central area of the wound

Table 1 Detailed parameters used for treatment with PBM

Parameters	Values
Mark	LASERPULSE, IBRAMED, Brazil*
Power (mW)	30*
Irradiance (W/cm ²)	0.47*
Wavelength (nm)	670*
Mode of action	Continuous*
Beam transverse area (cm ²)	0.063*
Time (s)	30*
Energy (J)	0.9*

*Otterço, 2018

Topic lotion with vitamin A

Groups treated with topic lotion received the application 1 h after surgery. The experimental period totaled four applications and the animals received 5 mg of lotion on the wound, daily, in the morning, covering the whole area of the wound. Lotion was composed of 10.000 UI of vitamin A/volume-based with high hydrolipid content (Essenziali®).

The LG + VitAG group first received PBM, immediately followed by the lotion application, covering the whole wound area.

Euthanasia

Animals were euthanized by decapitation with guillotine, in day 4 after surgery, when the samples were collected for posterior analysis. The analysis was performed in a transversal section of the material removed by the 10-mm punch, encompassing the center and the margin of the initial wound and portion of the healthy tissue. The choice of euthanasia in day 4 was made based on investigation of the inflammatory period (Fig. 2).

Fig. 2 Illustration of the timeline with surgical procedures, treatment, and sample collection for analysis

*PS: post-surgery

Histopathology

Samples collected by punch of the central and border area were fixed in 10% formalin and stored in 70% ethanol. For the slides preparation, samples were embedded in paraffin and the tissue was cut to a thickness of 5 µm.

We obtained three sections of each sample, which were then stained with hematoxylin and eosin (H&E, Merck) and analyzed. Histological evaluation was performed using an optical microscope (Zeiss Axioskop, Carl Zeiss, 40× objective) to check the intensity of the inflammatory infiltrate.

We performed a semi-quantitative analysis through the scores [18, 19], considering the values of 0–4 described in Table 2. The analysis was blinded with regard to the experimental groups and was performed by three reviewers.

Immunohistochemical analysis

Samples were inserted in silanized slides for better adherence of the biological material studied and kept at 37 °C for 24 h. After dewaxing and hydration, the histological sections were marked with hydrophobic pen and washed in tween-enriched buffer solution twice, for 3 min. The sections were then submerged in hydrogen peroxide for 10 min and washed in phosphate buffer solution (PBS) twice in 3 min, and finally submerged in endogenous peroxidase for 30 min. The slides were then incubated with the primary antibody. We used vascular endothelial growth factor (VEGF) primary antibodies: polyclonal anti-VEGF primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at concentration 1:4000; and cyclooxygenase-2 (COX-2): anti-COX-2 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:200. Both were incubated for 2 h and washed twice in PBS. The slides were then incubated with the second

Table 2 Histopathological classification scale for semi-quantitative analysis of the inflammatory infiltrate in slides stained with hematoxylin and eosin

Histopathological classification scale for evaluation of inflammatory infiltrate

1	Acute inflammation (pyogenic membrane is formed)
2	Predominance of diffuse acute inflammation (predominance of granulation tissue)
3	Predominance of chronic inflammation (fibroblasts beginning to proliferate)
4	Resolution and healing (decrease or absence of chronic inflammation, with occasional round cells)

antibody (anti-rabbit IgG) (Vector Laboratories, Burlingame, CA) at a concentration of 1:200 in PBS for 30 min.

VEGF and COX-2 immunolabeling were semi-quantitatively [18, 19] and qualitatively [10] analyzed by the average of three reviewers using optical microscope (40×, Leica Microsystems AG, Wetzlar, Germany). The qualitative analysis was made according to the amount of brown immunolabeling found in the tissue. The semi-quantitative analysis considered the score 1–4, where 1–absent, 2–mild, 3–moderate, and 4–intense [10]. The analyses were performed by a pathologist in blind mode.

Statistical analysis

The results were expressed as average $\pm$ standard deviation. The result analysis was performed with the Software GraphPad Prism 5.0. We performed the Shapiro-Wilk test to assess the data normality. Intergroup comparisons were performed with ANOVA one-way. For multiple comparisons, we used the Tukey post hoc test with significance level of $p < 0.05$.

Results

Histopathological analysis

Histopathological analysis revealed that groups LG and LG + VitAG present lower amount of inflammatory infiltrate when compared to the remaining groups, especially LG, which characterize a more advanced phase of the healing process. However, the groups CG and VitAG showed intense inflammatory infiltrate suggesting acute inflammatory phase (Fig. 3).

The quantitative analysis of the inflammatory infiltrate revealed significant differences that prove the descriptive analysis findings, in which the comparison between groups evidenced that the LG is in a more advanced phase, with predominant chronic inflammation, according to Table 2 and Fig. 4.

Immunohistochemical analysis

COX-2 Immunoexpression

The results of the descriptive analysis of COX-2 expression immunolabeling were analyzed according to the brownish hue found in the cytoplasm of inflammatory cells (Fig. 5). The best COX-2 immunolabeling was observed in the groups CG and VitAG, with decreasing COX-2 in the groups LG and LG + VitAG; however, the difference was not statistically significant among the studied groups (Fig. 6).

VEGF factor immunoexpression

The analysis of the VEGF group was evaluated by the expression of the brownish hue, with the groups VitAG, LG, and LG + VitAG presenting higher immunolabeling compared to CG (Fig. 5).

Only the group VitAG showed significant difference compared to CG; however, groups LG and LG + VitAG showed increased labeling expression (Fig. 6).

Discussion

This study evaluated the action of PBM associated or not to vitamin A in the initial phase of the tissue healing process of surgical wounds in rat skin. We show tissue, structural, and morphological differences among the experimental groups, suggesting that each treatment provided distinct stimuli in the wounded area.

The wound healing process is related to a series of mechanisms, interlinked and highly regulated by cellular, humoral and molecular signaling, growth factors, and cytokines in the extracellular matrix, initializing a cascade process that attracts and activates fibroblasts, endothelial cells, and macrophages [3, 4, 20, 21]. PBM leads to important pro-inflammatory effects that directly contribute to the adequate evolution of the process. In addition, its association with other treatments is predicted to intensify the release of important and specific factors of the inflammatory phase [10].

Guirro [22] evaluated the action of PBM (670 nm, 30 mW, and 4 and 7 J) in dorsal wounds of 1 cm² in rats and observed positive effects in the wound healing process in day 7 after the wound. These findings corroborate with our study, which used similar parameters and also found pro-inflammatory action with the use of PBM, such as decrease of the inflammatory infiltrate and increase of VEGF expression in the groups LG, VitAG, and LG + VitAG. A study evaluating the PBM action with a laser of 660 nm and power of 30 mW in wound healing [23] found a significant improvement regarding the initial phase of inflammation compared to the control group.

Fig. 3 Photomicrographs representing the inflammatory infiltrate of the experimental groups. **a** CG (control group). **b** VitAG (vitamin A group). **c** LG (laser group). **d** LG + VitAG (laser + vitamin A group) ($n = 10$). Black arrow indicates presence of inflammatory infiltrate (40 $\times$)

On the other hand, a study by Mendes [24], investigating the PBM effects with two distinct wavelengths (685 and 830 nm, 35 mW, 20 and 50 J/cm²), showed negative results with wavelength 685 nm. However, the power applied was lower when compared to the power used in our study, which suggest that the power was insufficient to stimulate the cellular mechanism in the wound.

Although there are several studies showing the PBM action in cutaneous wound healing, the protocols are discrepant and there is a lack of details on the parameters used [6, 8, 10,

25–28], in addition to the use of distinct types of wounds, which impairs the comparison of results and understanding of the actual cellular mechanisms involved and engaged by the therapy. The present study investigates and suggests a new treatment protocol showing the association of the PBM properties and the beneficial topic use of vitamin A.

We chose to use vitamin A due to its stimulation to form a new extracellular matrix, and proteoglycans and glycoprotein synthesis [29–31]. In addition, vitamin A has biochemical mechanisms that act in angiogenesis and can potentialize the stimuli triggered by the PBM. Although the vitamin A mechanisms of action are not completely understood, studies show that supplementation with vitamin A results in increased interleukin-10 (IL-10) and decrease of tumor necrosis factor- α (TNF α), levels of serum IgA, and proliferation of mononuclear cells of peripheral blood, improving the inflammatory response [16, 17].

Reinke [4] analyzed the mechanisms of the cellular cascade in the inflammatory phase of human wounds and reported macrophages degranulation, which contributes to the release of several growth factors essential for the satisfactory evolution of the healing process, VEGF being one of them. Studies [11, 17, 32] have reported the increased VEGF expression in epidermal cell macrophages, inducing and keeping the early angiogenic phases in days 2 or 3 after the wound, in agreement with our findings, which also showed moderate labeling when using this treatment.

It has been shown that PBM is a resource capable of direct acting and improving the angiogenic process, stimulating the early release of VEGF, which is produced by keratinocytes at the beginning of the wound healing process [18, 29, 33, 34].

Fig. 4 Semi-quantitative analysis of the inflammatory infiltrate in the fourth day after wound, with significance level of $*p < 0.05$ ($n = 10$)

Fig. 5 Immunohistochemical analysis of the COX-2 and VEGF expression by immunolabeling in day 4 of treatment. **a** CG (control group). **b** LG (laser group). **c** VitAG (vitamin A group). **d** LG + VitAG (group laser + vitamin A). Asterisk indicates brownish immunolabeling where both factors are expressed (40×)

The same was observed in our results in the groups LG and LG + VitAG.

Our study evaluated the fourth day after the wounding and observed COX-2 expression in all groups, corresponding to its

physiological response to the inflammatory process [35–39]. The CG group showed the higher immunolabeling, suggesting that the proposed treatments, isolated or in association, had positive effects in this immunoexpression and improved the

Fig. 6 **a, b** Semi-quantitative analysis of COX-2 and VEGF (respectively) in the fourth day after wound, with level of significance of $*p < 0.05$ ($n = 10$)

resolution of the inflammatory phase. In addition, it is noteworthy that LG had the lowest immunoexpression, which can be associated to the pro-inflammatory effects due to the photobiomodulation. The same has been previously described in other studies [4, 34].

It is important to highlight that both treatments in this study showed specific and distinct characteristics that are able to help and stimulate the wounded cellular environment, especially in the studied process phase. The laser pro-inflammatory effects were observed through the analysis of COX-2 and its complementary effects to the healing process due to the higher expression of VEGF. Moreover, vitamin A also played a positive role in the wound environment with increased VEGF immunoexpression and better evolution of the inflammatory phase when compared to controls. Therefore, we suggest that both treatments have interesting isolated properties that are complementary when associated. Our study used VitA to intensify the neoangiogenesis stimuli, one of the pro-inflammatory effects of the laser. Interestingly, our results lead to the idea that the vitamin A can potentialize both VEGF release and the cellular mechanisms involved in angiogenesis provided by the PBM stimuli; however, such action needs further investigation. Thus, we emphasize that the treatments used, both isolated and in association, have positive effects that contribute to improved evolution of the inflammatory phase in this type of punch wound.

Conclusion

This study, using 670-nm low-level laser therapy, concludes that the treatment promoted the necessary stimuli for the satisfactory evolution of the wound healing process that were beneficial to the cellular mechanisms involved in the inflammatory phase. In addition, we observed the pro-inflammatory effects due to the use of PBM, as well as the action of VitA as inducer of neoangiogenesis. Thus, we conclude that these treatments, isolated and associated, promote relevant effects in the cellular environment that help and even accelerate the mechanisms involved in the resolution of the acute inflammatory phase in this type of wound.

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Compliance with ethical standards

This study met the Guidelines for Care and Use of Animal Models and was approved by the Ethics Committee in Animal Experimentation of the Universidade Federal de São Carlos (UFSCar) (UFSCar - Protocol: 2-007/2014)

Conflict of interest The authors declare that they have no conflict of interest.

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