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Low level laser therapy increases angiogenesis in a model of ischemic skin flap in rats mediated by VEGF, HIF-1α and MMP-2

Vivian Cury [a], Ana Iochabel Soares Moretti [b,c,*], Lívia Assis [a], Paulo Bossini [a], Jaqueline de Souza Crusca [a], Carlos Benatti Neto [d], Renan Fangel [a], Heraldo Possolo de Souza [b], Michael R Hamblin [e,f,g], Nivaldo Antonio Parizotto [a]

[a] Department of Physiotherapy, Federal University of São Carlos, São Carlos, São Paulo, Brazil
[b] Emergency Medicine Division, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil
[c] Post-Graduate Health Sciences Program, Instituto de Assistência Médica ao Servidor Público Estadual—IAMSPE, São Paulo, SP, Brazil
[d] Department of Physiology and Pathology, Universidade Estadual de São Paulo, Araraquara, São Paulo, Brazil
[e] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston, MA 02114, United States
[f] Department of Dermatology, Harvard Medical School, Boston, MA 02115, United States
[g] Harvard–MIT Division of Health Sciences and Technology, Cambridge, MA 02139, United States

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ABSTRACT

It is known that low level laser therapy is able to improve skin flap viability by increasing angiogenesis. However, the mechanism for new blood vessel formation is not completely understood. Here, we investigated the effects of 660 nm and 780 nm lasers at fluences of 30 and 40 J/cm^2 on three important mediators activated during angiogenesis. Sixty male Wistar rats were used and randomly divided into five groups with twelve animals each. Groups were distributed as follows: skin flap surgery non-irradiated group as a control; skin flap surgery irradiated with 660 nm laser at a fluence of 30 or 40 J/cm^2 and skin flap surgery irradiated with 780 nm laser at a fluence of 30 or 40 J/cm^2. The random skin flap was performed measuring 10 × 4 cm, with a plastic sheet interposed between the flap and the donor site. Laser irradiation was performed on 24 points covering the flap and surrounding skin immediately after the surgery and for 7 consecutive days thereafter. Tissues were collected, and the number of vessels, angiogenesis markers (vascular endothelial growth factor, VEGF and hypoxia inducible factor, HIF-1α) and a tissue remodeling marker (matrix metalloproteinase, MMP-2) were analyzed. LLLT increased an angiogenesis, HIF-1α and VEGF expression and decrease MMP-2 activity. These phenomena were dependent on the fluences, and wavelengths used. In this study we showed that LLLT may improve the healing of skin flaps by enhancing the amount of new vessels formed in the tissue. Both 660 nm and 780 nm lasers were able to modulate VEGF secretion, MMP-2 activity and HIF-1α expression in a dose dependent manner.

1. Introduction

Skin flaps are frequently used to reconstruct large areas of skin, damaged after accidental trauma or surgical procedures, like tumor resection. Although this maneuver is generally regarded as safe, when correctly performed, complications may still occur. Considering that these grafts might represent the last resource available for treatment of injured patients, the loss of the skin flaps are devastating events.

The mostly feared of these events is skin flap necrosis, caused by inadequate blood flow [1–4]. Under ideal conditions, hypoxia resulting from improper tissue perfusion should lead to an adaptive response, inducing angiogenesis, that is, the formation of new vessels from pre-existing ones, a necessary step to ensure adequate blood supply during the healing process [5–7]. The mechanism of angiogenesis is very complex, involving several cell types and dozens of mediators and signaling pathways. This cascade of events is initiated by migration and invasion of endothelial cells, followed by lumen formation, connection of new vascular segments with pre-existing circulation, and remodeling of extracellular matrix (ECM), a process dependent on adequate matrix metalloproteinase (MMP) activity [7]. Moreover, among the many signaling pathways that are involved in the whole process, some of them seem to be particularly critical such as vascular endothelial growth factor (VEGF) and hypoxia inducible factor 1α (HIF-1α) [5].

* Corresponding author. Address: Laboratory of Medical Research, Division of Emergency Medicine, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Arnaldo, 455 sala 3189, CEP 01246-903 São Paulo, SP, Brazil. Tel.: +55 11 30648756; fax: +55 11 30617170.
E-mail address: aismoretti@yahoo.com.br (A.I.S. Moretti).

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Low level laser therapy (LLLT) has been demonstrated to be able to modulate biological mechanisms in wound healing [8,9], angiogenesis [10,11], and inflammation [12,13]. Besides that, it has also been reported to induce angiogenesis in several experimental models where blood vessel formation is critical to success [10,11]. Although LLLT has a history of widespread use and good results, the mechanism(s) for its actions are not yet completely understood. Our group has previously reported that LLLT can increase the viability of skin flaps, increasing local vascularization and reducing the area of necrosis [3]. These results have been further confirmed by other authors [2,14]. However, the mechanisms responsible for LLLT-induced revascularization have not yet been explored in detail.

Therefore, the main objective of this study was to analyze molecular mechanisms involved in LLLT-induced angiogenesis in a model of ischemic skin flap in rats. We focused our attention in some of the most important mediators involved in angiogenesis, such as VEGF and HIF-1α expression and MMP activity.

2. Methods

2.1. Skin flap surgical procedure

This study was conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of the Federal University of São Carlos [4]. Sixty adult male rats (Wistar, 12 weeks old, 260–320 g) were anesthetized by intraperitoneal injection of ketamine (95 mg/kg) and xylazine (12 mg/kg) and shaved on the dorsal region. A skin flap measuring 10 × 4 cm was raised with a cranial base on the back of each rat (Fig. 1A and B). A plastic barrier with the same dimensions was inserted between the flap and its donor site (Fig. 1C). Flaps were closed with simple nylon 4/0 stitches(Fig. 1D). Laser irradiation was performed immediately after the surgery and for 4 consecutive days after surgery (Fig. 1E). A low-energy 660 nm and 780 nm laser (Twin Laser, MM Optics, São Carlos, Brazil), continuous wave (CW), 4 mm beam diameter, 40 mW total power was used. Laser irradiation was performed at fluences of 30 J/cm^2 (30 s, total energy 28.8 J) and 40 J/cm^2 (40 s, total energy 38.4 J). Twenty-four points on the skin flap surface and surrounding it were irradiated through the punctual contact technique. The irradiation was performed with a plastic template that overlaid the skin flap with demarcation points covering the entire flap and 1-cm of normal skin on all sides (Fig. 1E).

All animals were submitted to a skin flap (SF) surgery procedure and randomly divided into five groups, with 12 animals each. Groups were as follows:

(1) SF-NI group, skin flap surgery non-irradiated group.
(2) SF-R30 skin flap surgery irradiated with 660 nm laser, 30 J/cm^2.
(3) SF-R40 skin flap surgery irradiated with 660 nm laser, 40 J/cm^2.
(4) SF-IV30 skin flap surgery irradiated with 780 nm laser, 30 J/cm^2.
(5) SF-IV40 skin flap surgery irradiated with 780 nm laser, 40 J/cm^2.

2.2. Collection of skin sample

Skin samples were collected on the seventh day after surgery. In order to standardize sample collection, skin tissue was obtained at the borderline between viable tissue characterized by soft skin (reddish, warm, and haired) and necrotic tissue (stiff, dark, cool, and hairless) (Fig. 1F). This tissue was divided into three parts: right, central and left. The right piece was used for western blotting and zymography assay. The central piece was fixed in formalin and used in histological analysis.

2.3. Blood vessels count

We performed morphometric analysis in order to estimate the number of blood vessels in the skin after the procedures. The skin tissue was fixed in formalin (10%) and embedded in paraffin. Sections of 6 μm were cut and stained with the hematoxylin and eosin method (HE). Blood vessel count was performed with light

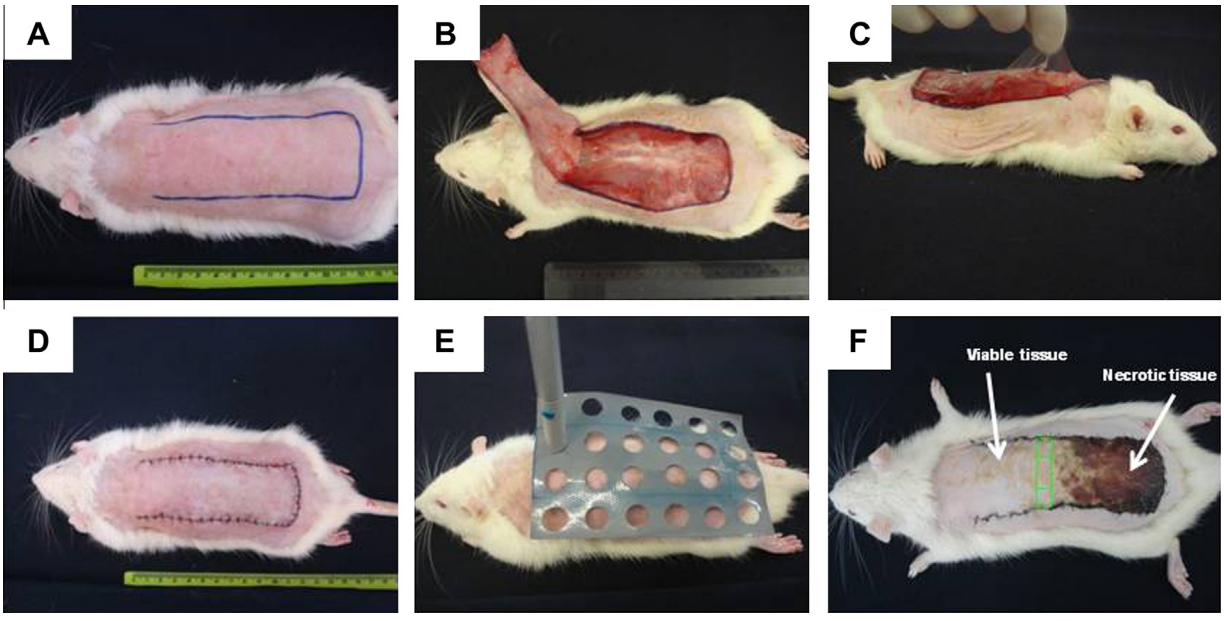

Fig. 1. Dorsal skin flap surgical procedure and laser irradiation. (A) Outline of flap intended (10 × 4 cm). (B and C) Dorsal skin flap with cranial base with interposition of a plastic barrier. (D) Sutured flap after surgical procedure. (E) Template for laser irradiation with 24 points. (F) Green box at border of viable/necrotic tissue area was utilized for analyses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)