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Mitochondrial dynamics (fission and fusion) and collagen production in a rat model of diabetic wound healing treated by photobiomodulation: comparison of 904 nm laser and 850 nm light-emitting diode (LED)

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ABSTRACT

Objective: Mitochondrial dysfunction has been associated with the development of diabetes mellitus which is characterized by disorders of collagen production and impaired wound healing. This study analyzed the effects of photobiomodulation (PBM) mediated by laser and light-emitting diode (LED) on the production and organization of collagen fibers in an excisional wound in an animal model of diabetes, and the correlation with inflammation and mitochondrial dynamics.

Methods: Twenty Wistar rats were randomized into 4 groups of 5 animals. Groups: (SHAM) a control non-diabetic wounded group with no treatment; (DC) a diabetic wounded group with no treatment; (DLASER) a diabetic wounded group irradiated by 904 nm pulsed laser (40 mW, 9500 Hz, 1 min, 2.4 J); (DLED) a diabetic wounded group irradiated by continuous wave LED 850 nm (48 mW, 22 s, 1.0 J). Diabetes was induced by injection with streptozotocin (70 mg/kg). PBM was carried out daily for 5 days followed by sacrifice and tissue removal.

Results: Collagen fibers in diabetic wounded skin were increased by DLASER but not by DLED. Both groups showed increased blood vessels by atomic force microscopy. Vascular endothelial growth factor (VEGF) was higher and cyclooxygenase (COX2) was lower in the DLED group. Mitochondrial fusion was higher and mitochondrial fusion was lower in DLED compared to DLASER.

Conclusion: Differences observed between DLASER and DLED may be due to the pulsed laser and CW LED, and to the higher dose of laser. Regulation of mitochondrial homeostasis may be an important mechanism for PBM effects in diabetes.

1. Introduction

Photobiomodulation (PBM) is used to improve wound healing [1], promote pain relief [2], and improve muscle performance [3]. One of the basic mechanisms is to stimulate mitochondrial function when the red or near-infrared light is absorbed by cytochrome c oxidase [4].

Diabetes mellitus (DM) is a chronic disease characterized by high glycemia due to insufficient production or utilization of insulin [5]. DM reduces collagen synthesis [6–8] caused by an inflammatory state associated with oxidative stress [9]. DM can lead to dysregulation of the expression of vascular endothelial growth factor (VEGF). Lower VEGF levels in diabetic wounds impair angiogenesis in cutaneous wound healing [10]. On the other hand, up-regulated COX-2 expression (11) is associated with increased expression of VEGF (particularly in the eyes

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leading to diabetic retinopathy) [12–14].

Mitochondrial homeostasis depends on the balance between fusion and fission processes. Mitochondrial fusion helps reduce mitochondrial stress by mixing the contents of partially damaged mitochondria [15]. Fission allows the creation of new mitochondria, but it also enables the removal of damaged mitochondria. Mitochondrial fission 1 protein (FIS1) is a component of a mitochondrial complex called the "ARCosome" that promotes mitochondrial fission [16]. Fusion between mitochondrial outer membranes is mediated by membrane-anchored dynamin family members called "mitofusins" MFN1 and MFN2. Fusion is diminished in the muscles of obese and diabetic individuals and is associated with insulin resistance after bariatric surgery [17]. Disorders of mitochondrial function may lead to overproduction of ROS [17] and have been implicated in the development of insulin resistance and type 2 diabetes [18, 19].

This goal of this study was to investigate collagen production in the wounded skin of diabetic rats treated with PBM mediated either by a pulsed laser (904 nm) or a continuous wave LED (805 nm). Atomic force microscopy, and COX-2, VEGF, FIS1 and MFN2 immuno-expression were measured to understand the mitochondrial mechanisms.

2. Methods

2.1. Experimental Design

The Federal University of Sao Carlos ethics committee approved this research under protocol no. 01/2013. In this study, 20 male Wistar rats were used (45 days of age and weight 200–250 g), housed in controlled temperature and humidity with 12 h light-dark cycle, with water ad libitum and a commercial diet. Rats were randomized into groups (n = 5 each): (SHAM) a control non-diabetic wounded group with no treatment; (DC) a diabetic wounded group with no treatment; (DLASER) a diabetic wounded group irradiated by 904 nm pulsed laser; (DLED) a diabetic group with an incision irradiated by continuous wave LED 850 nm.

2.2. DM Induction

The animals of SHAM group received an intraperitoneal injection with 0.022 mL NaCl 0.9% solution. Animals of diabetic groups (DC, DLASER and DLED) received an injection with streptozotocin (70 mg/kg in citrate buffer, pH = 4.5). For induction of diabetes we used a previously described methodology [20, 21]. Rats were prepared fasting for 6 h before anesthetized by thiopental (40 mg/kg/2.5% IP) and after this, received an intraperitoneal injection of streptozotocin in a prone position (solution 70 mg/kg - STZ) associated to citrate buffer solution (pH 4.5). Glucose levels were measured with a glucometer in blood samples taken from the tail vein (AccuChek, Roche Diagnostics, Indianapolis, IN). Although in 10 days to 14 days is sufficient to obtain a hyperglycemic condition, treatments starts 90 days after diabetes induction by STZ to guarantee a chronic diabetic state to simulate clinical conditions. Reason for this it is because diabetic foot and another skin diseases in diabetes are more common in elderly people, our main motivation for this research and the results of this research can provide evidence for future translational research.

2.3. Surgical Procedures

On the 90th-day post DM induction, blood was collected from tail vein and animals with glycemic levels of 300 mg/dL or above were included in the study [22]. Anesthesia was performed intraperitoneally (dose of thiopental 40 mL/kg IP Cristália®, Itapira, Brazil). The skin on the dorsal region was disinfected with povidone-iodine and was cut with a surgical scalpel until the depth reached the hypodermis. An incision was made 2 cm wide and 2 cm long, on the posterior iliac crest of the animal and the skin was removed [23]. The animals were housed in pairs.

Table 1
Photobiomodulation parameters.

Parameters	Laser	LED
Wavelength	904 nm	850 nm
Average radiant power	40 mW	48 mW
Pulse structure	Duty cycle 20%; Pulse duration 60 nsec; Frequency 9500 Hz	Continuous wave
Fluence	18.33 J/cm^2	14.69 J/cm^2
Time	1 min	22 s
Energy	2.4 J	1.0 J
Beam spot size at target	0.1309 cm^2	0.196 cm^2
Technique	Contact	Contact
Number of points irradiated	1	1
Frequency of treatment	1 ×/day for 5 days	1 ×/day for 5 days

2.4. Treatment

The animals were randomized by lottery using opaque envelopes. The group denominated DLASER received laser treatment and animals denominated DLED received LEDT treatment. The diode laser 904 nm GaAs Laserpulse model (IBRAMED, Amparo, Brazil), and a LED prototype was delivered on the wound edges.

The first application of PBM was delivered immediately after wounding, and PBM was repeated once a day for the next five days. Immediately after the last application of PBM the rats were anesthetized and the entire area of the wound was removed for histological analysis. The rats were then sacrificed by carbon dioxide euthanasia. Table 1 shows the parameters of the laser and LED devices.

2.5. Sample Collection and Processing

The removed skin of the rats was fixed in 10% buffered formalin solution (Merck, Darmstadt, Germany) for 24 h. and embedded in paraffin blocks. The paraffin blocks were sectioned in series of 3 cuts (5 μm) each transversely (Microtome Leica Microsystems SP 1600, Nussloch, Germany) and attached to glass slides.

2.6. Collagen Analysis

For collagen analysis, the glass slides were stained with picrosirius red and cross-sectioned for analysis by fluorescence microscopy using a 20× objective and a software capture system AxioVision 4.7.2.0 v (Carl Zeiss, Germany). The images were processed using ImageJ 1:49 software, 64-bit version (National Institutes of Health, Bethesda, USA), using plugin color deconvolution. The images were analyzed for the relative percentage of collagen in the total tissue [24, 25].

2.7. Atomic Force Microscopy

For atomic force microscopy, the slides were observed using a Flex-Axiom Nanosurf device (Nanosurf, Liestal, Switzerland). The tissue was rehydrated, and no histological staining was used. The samples were scanned in vibrational mode using NanoWorld tip model NCSTR with a typical resonance frequency of 160KHz.

2.8. Immunohistochemistry

Xylene and rehydration in graded ethanol were used to remove paraffin from the blocks. Samples were preincubated with 0.3% hydrogen peroxide in PBS for 10 min and blocked with goat serum in PBS (20 min). The following primary rabbit antibodies and dilutions were used for a 2 h incubation. Polyclonal anti-cyclooxygenase-2 (COX2, 1:500, Santa Cruz Biotechnology, Santa Cruz, USA), anti-vascular