Dear Dr. Baldomero Antonio Kato da Silva:

The review of your manuscript, "Acute effects of low-laser therapy (660 nm) on oxidative stress levels in diabetics rats with skin wounds", submitted to the Journal of Experimental Therapeutics and Oncology for publication is completed. I am pleased to inform you that two associate editors have reviewed and accepted your article without further modification.

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With kind regards,

Dominic Fan

Dominic Fan, Ph.D.
Professor
Editor-in-Chief
The Journal of Experimental Therapeutics and Oncology
Department of Cancer Biology-173
The University of Texas M. D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, Texas 77030
U.S.A.

Regional Editors: June Biedler, Jean-Pierre Jaffrézou, Victor Ling, Robert Pirker, Eiichi Tahara
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For Tumor Progression and Cancer Metastasis Are Not Random - Treatments and Cure Are Logical and Eventual
Acute effects of low-level laser therapy (660 nm) on oxidative stress levels in diabetic rats with skin wounds

Amanda Silveira Denadai¹, ⁴, Ricardo Dutra Aydos¹, Iandara Schetttert Silva¹, Larissa Olmedo², Bruno Mendonça de Senna Cardoso³, Baldomero Antonio Kato da Silva⁴,⁵ and Paulo de Tarso Camillo de Carvalho⁶

¹Federal University of Mato Grosso do Sul (UFMS), Post Graduate Program in Health and Development of Midwest Region, Campo Grande/MS, Brazil
²Federal University of Mato Grosso do Sul (UFMS), Graduate Course of Physiotherapy, Campo Grande/MS, Brazil
³University Anhanguera-UNIDERP, Graduate Course of Veterinary Medicine, Campo Grande/MS, Brazil
⁴Federal University of Piauí (UFPI), Department of Health Sciences, Parnaíba/PI, Brazil
⁵Federal University of Piauí (UFPI), Postgraduate Program in Biomedical Sciences, Parnaíba/PI, Brazil
⁶University Nove de Julho (UNINOVE), Postgraduate Program in Rehabilitation Sciences and Postgraduate Program in Biophotonics, São Paulo/SP, Brazil

Correspondence to: Amanda Silveira Denadai, Federal University of Piauí, Av. São Sebastião, 2819 – Reis Velloso, Parnaíba/PI, Brazil; E-mail: a.denadai@yahoo.com.br

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Background: Laser therapy influences oxidative stress parameters such as the activity of antioxidant enzymes and the production of reactive oxygen species.

Objective: To analyze the effects of low-level laser therapy on oxidative stress in diabetics rats with skin wounds.

Methods: Thirty-six animals were divided into 4 groups: NDNII: non-diabetic rats with cutaneous wounds that did not receive laser therapy; NDI: non-diabetic rats with cutaneous wounds that received laser therapy; DNI: diabetic rats with skin wounds who did not undergo laser therapy; DI: rats with diabetes insipidus and cutaneous wounds and received laser therapy. The animals were treated with LLLT (660 nm, 100 mW, 6 J/cm², spot size 0.028 cm²). On the day of killing the animals, tissue-wrapped cutaneous wounds were collected and immediately frozen, centrifuged, and stored to analyze malondialdehyde (MDA) levels.

Results: Significant difference was observed within the groups of MDA levels (ANOVA, p = 0.0001). Tukey’s post-hoc test showed significantly lower values of MDA in irradiated tissues, both in diabetic and non-diabetic rats. ANOVA of the diabetic group revealed a significant difference (p < 0.01) when all groups, except NDI and DI, were compared.

Conclusions: LLLT was effective in decreasing MDA levels in acute surgical wounds in diabetic rats.

Key words: Laser, Malondialdehyde, Oxidative stress

INTRODUCTION

Reactive oxygen species (ROS) can have both beneficial and deleterious effects. Under normal physiological conditions, ROS production is tightly regulated, with ROS participating in both pathogen defense and cellular signaling. However, insufficient ROS detoxification or ROS overproduction generates oxidative stress, which results in cellular damage. It has also been linked to various inflammatory diseases. Inflammation is an essential response in protecting against injurious insults and is thus important at the onset of wound healing [1].

Excessive release of ROS by mitochondria, activated leukocytes, and endothelial cells in chronic inflammatory conditions can ultimately result in severe cell and tissue damage and may further promote and aggravate inflammatory injury. In many inflammatory diseases, currently available intervention strategies fail or show limited success, necessitating the development of novel strategies for the treatment of chronic inflammatory...
Numerous aspects of wound healing are subject to redox control. Thus, a thorough understanding of how endogenous ROS generated in wound-related cells may influence the healing process is important. Such an understanding could result in the development of novel redox-based strategies for treating wounds. Current therapies using growth factor therapy for treating wounds are not sufficiently effective. Many of these growth factors such as platelet-derived growth factor (PDGF) may serve as effective adjuncts to jump-start the healing of a chronic wound. Hypoxia is a characteristic feature of most problem wounds, and it is reasonable to assume that correction of wound pO2 may facilitate generation of endogenous ROS by NADPH oxidases in wound-related phagocytic and non-phagocytic cells [2].

Various factors contribute to delayed diabetic wound healing, such as growth factors, nitric oxide (NO), ROS, matrix metalloproteinases (MMPs), microRNA, and endothelial progenitor cells (EPCs) [3].

In acute wounds, a temporary increase in the level of oxidants occurs. Antioxidant defense mechanisms are based on gradual detoxification of oxidants and a gradual return of cells to the state of redox homeostasis. However, in chronic wounds, the detoxification process is hindered because of persistent and uncontrolled production of ROS and reactive nitrogen species during the inflammatory phase. Oxidative stress is thought to be an important pathogenic factor in diabetic wound complications [4].

Evidence indicates that hyperglycemia and advanced glycoxidation products contribute to impaired wound healing in diabetes; hyperglycemia is a hallmark of diabetes and leads to increased ROS and cellular damage. The hyperglycemia augments superoxide anion generation in skin tissue by activating NADPH oxidase and protein kinase C, resulting in delayed wound healing in diabetic mice. Accumulation of ROS leads to widespread cellular damage and poor wound neovascularization [5].

Currently, the management of diabetic wounds is focused primarily on debridement, off-loading, antibiotic therapy, and, in some cases, surgical revascularization [3]. However, studies [6–10] have indicated that low-level laser therapy (LLLT) can be used to improve and accelerate wound healing even in diabetic patients. These studies indicate an improvement in fibroblast proliferation [6,7], increased production of both collagen type I and type III [8], increased neovascularization [9], and modulation of pro-inflammatory cytokines and anti-inflammatory effects [10].

According to Siveira [11], LLLT influences oxidative stress parameters by changing the activity of antioxidant enzymes and the production of ROS. Absorption of laser light accelerates electron transfer (respiratory chain) and induces an initial production of ROS, specifically by increasing the production of superoxide anion.

Because oxidative stress is changed on a large scale in diabetic patients and the use of LLLT can act very effectively in pathways important in the production of ROS, this study evaluated the potential for using LLLT as an antioxidant.

**MATERIALS AND METHODS**

**Animals**

The sample population consisted of 90-day-old male Wistar rats (n = 36) (*Rattus norvegicus albinus*) weighing 310–350 g. The animals were obtained from the animal housing facility of the Federal University of Mato Grosso do Sul – UFMS (Brazil) and kept under controlled light and temperature conditions with free access to water and chow. All experimental procedures were approved by the Institutional Research Ethics Committee (439/2012) and were conducted according to the guidelines of the Brazilian College for Animal Experimentation as well as the standards of the International Council for Laboratory Animal Science.

**Experimental groups**

Rats were randomly divided into 4 groups of 9 animals each. The NDNI group included non-diabetic rats with skin wounds that did not receive laser therapy, the NDI group included non-diabetic rats with cutaneous wounds that received laser therapy, the DNI group included diabetic rats with skin wounds who did not receive laser therapy, and the DI group included diabetic rats with cutaneous wounds that received laser therapy.

**Induction of diabetes**

Animals were fasted overnight, and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ; 65 mg/kg; Sigma Aldrich, St. Louis, MO, USA) in 0.1 mol/L citrate buffer (pH 4.5). The dosing volume was 1 mL/kg. To prevent fatal hypoglycemia, rats were kept on 5%
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Production of surgical wounds

Surgical wound production was performed after anesthesia with intraperitoneal injection of ketamine (10%) associated with xylazine (2%), combined in a single syringe at a ratio of 1 mL of ketamine (100 mg) to 1 mL xylazine (20 mg) and administered 0.1 mL of the mixture per 100 g body weight of the animal under aseptic conditions. An incision was made on the animal’s back in a round shape by using a jig with plastic 2 cm² to include the skin as well as the muscle fascia.

Laser irradiation indium gallium aluminum phosphide (InGaAlP)

Laser irradiation was conducted using indium gallium aluminum phosphide (InGaAlP) DMC Photon Laser III model at a power of 100 mW (power density of 3.57 W/cm²), beam area of 0.028 cm², and wavelength of 660 nm. The application was in the form of two-point using the transcutaneous method (one retractor used for the irradiated area was equivalent to 1 cm²) in the wounds, with a fluency (energy density) of 6 J/cm² of energy, at 60 s per point. The application was initiated immediately after surgery.

Euthanasia and collection and storage of samples

Animals from each group were sacrificed after 24 h by intraperitoneal injection with a lethal dose of thiopental sodium (150 mg/kg). Samples were collected and = tissues were washed in a solution of 1.15% potassium chloride (KCl), and then frozen in liquid nitrogen.

Table 1. Analysis of variance (ANOVA) between studied groups (NDI, NDNI, DNI and DI).

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA</th>
<th>ANOVA</th>
<th>Tukey (&lt;0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDI</td>
<td>410.3090 ± 28.8816</td>
<td></td>
<td>NDI vs NDNI</td>
</tr>
<tr>
<td>NDNI</td>
<td>541.1123 ± 31.8307</td>
<td>&lt;0.0001</td>
<td>NDI vs DNI</td>
</tr>
<tr>
<td>DNI</td>
<td>631.9861 ± 49.1964</td>
<td></td>
<td>NDNI vs DNI</td>
</tr>
<tr>
<td>DI</td>
<td>450.2385 ± 29.0634</td>
<td></td>
<td>DNI vs DI</td>
</tr>
</tbody>
</table>

RESULTS

A significant difference was observed in the intra-group comparison of MDA levels (ANOVA, p < 0.0001). Tukey’s post-hoc test showed significantly lower values of MDA in irradiated tissues, both in diabetic as well as non-diabetic rats. ANOVA in the diabetic group...
revealed a significant difference (p<0.01) across all groups, except for comparison between the NDI and DI groups. Additionally, the most significant difference was compared between the NDI and DNI groups (Figure 1).

**DISCUSSION**

The present study examined the effect of LTTP on signal levels of oxidative stress (MDA) in diabetic and non-diabetic rats. Our results agree with those of previous studies [13], in which STZ-induced diabetic animals showed increased oxidative stress compared to controls, as well as reduced activity of antioxidant enzyme. This study also revealed increased expression of inducible nitric oxide synthase in the lung tissue of diabetic animals.

This study was conducted to examine the effects a low-power laser on oxidative stress induced by diabetes. The study was based on the following considerations: (1) After the onset of symptoms of diabetes mellitus (DM), oxidative stress is increased because of the numerous potential sources of ROS generation within the body injured [14], (2) LLLT acts on the parameters affecting oxidative damage [15], or (3) LLLT significantly increases the synthesis of ATP in injured tissue [16].

Quantification of MDA in biological systems is important for assessing cellular oxidative stress in which higher values of MDA indicate a higher level of oxidative stress, which is consistent with the higher values observed in the diabetic groups in our study [17,18].

In the present study, we analyzed MDA levels in diabetic animals to determine how oxidative stress changes in the presence of DM. Growing evidence suggests a causal link between hyperglycemia and oxidative stress, which leads to cell damage and various complications associated with diabetes [19, 20].

Within groups, laser irradiation in diabetic rats significantly decreased MDA levels compared with other groups that were not subjected to irradiation. However, this does not confirm LLLT to be an antioxidant. A study by Silveira et al. [11] on muscle injury in Wistar rats concluded that LLLT was effective for increasing the activity of the mitochondrial respiratory chain, likely by stimulating ATP synthesis and accelerating the muscle healing process. Other parameters must be evaluated to confirm these findings.

Sibbald and Woo [19] found that the superoxide anion produced in excess DM limits the biodiversity of vasodilators that can react with NO. Lindgard et al. [21] used a laser with a wavelength of 634 nm for irradiation and observed elevated levels of NO as well as an effect on inducible nitric oxide synthase or endothelial nitric oxide synthase. Irradiation also caused a decrease in the levels of intracellular ROS and affected cell viability. The authors concluded that their studies indicate that irradiation at 634 nm releases NO, possibly from a preformed store, without affecting cell viability. Irradiation at 634 nm may have a wide range of clinical applications, including the reduction of oxidative stress-mediated injury in the vasculature.

In this study, we observed decreased levels of MDA, which indicates a decrease in the levels of oxidative stress. These results are supported by several other studies that have investigated the effect of LLLT on oxidative stress [11,22–24].

Silveira et al. [11] found that increased superoxide anion production in the injured tissue and laser-induced reduction can be attributed to 2 main mechanisms, primarily due to cellular respiration because the tissue repair process shows a greater demand for energy, and this results in a concomitant increase in superoxide anion. LLLT can reduce this demand.

In our study, we analyzed the performance of LLLT alone in non-diabetic rats and diabetic rats not subjected to LLLT and found a greater reduction in MDA level in the non-diabetic rats, thereby confirming the high rates of oxidative stress marker production in DM. We also found that when diabetic animals were subjected to laser therapy, the results were not as significant, indicating that the laser treatment can modulate oxidative stress but should still be considered an antioxidant because it cannot eliminate the cascade of oxidation triggered by diabetes.
CONCLUSION

LLLT was effective for decreasing oxidative stress parameters, but other parameters must be evaluated to confirm this finding. A non-significant increase was observed in analyzing MDA among groups. In both groups, the diabetic animals had a higher level of MDA compared to non-diabetic animals. Both laser-irradiated groups showed significantly lower levels of MDA.

REFERENCES