Acute effects of low-level laser therapy (660 nm) on oxidative stress levels in diabetic rats with skin wounds

**Amanda Silveira Denadai[1,4], Ricardo Dutra Aydos[1], Iandara Schettert Silva[1], Larissa Olmedo[2],
Bruno Mendonça de Senna Cardoso[3], Baldomero Antonio Kato da Silva[4,5] and
Paulo de Tarso Camillo de Carvalho[6]**

[1]Federal University of Mato Grosso do Sul (UFMS), Post Graduate Program in Health and Development of Midwest
 Region, Campo Grande/MS, Brazil
[2]Federal University of Mato Grosso do Sul (UFMS), Graduate Course of Physiotherapy, Campo Grande/MS, Brazil
[3]University Anhanguera-UNIDERP, Graduate Course of Veterinary Medicine, Campo Grande/MS, Brazil
[4]Federal University of Piauí (UFPI), Department of Health Sciences, Parnaíba/PI, Brazil
[5]Federal University of Piauí (UFPI), Postgraduate Program in Biomedical Sciences, Parnaíba/PI, Brazil
[6]University 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*

(Received: February 23, 2015; accepted: April 14, 2015)

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: NDNI: non-diabetic rats with cutaneous wounds that not received 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/cm^2, spot size 0.028 cm^2). 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

conditions. The prolonged presence of oxidative stress is thought to promote and fuel these deleterious inflammatory processes and may form a novel target for treating chronic inflammatory conditions [1].

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 depend on ROS to function. Thus, redox-based strategies 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 pO_2 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%

glucose solution for 24 h after STZ injection. Successful induction of diabetes was confirmed by measuring the fasting blood glucose concentration in rats 6 h after injection of STZ. Rats with a fasting blood glucose level ≤250 mg/dL were considered diabetic and included in the present study [11].

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.

Malondialdehyde levels

MDA levels in the wound tissue homogenate were also measured using TBAR methods to determine the level of lipid peroxidation. Briefly, tissues were homogenized in 1.15% KCl and diluted 1:5 with distilled water before analysis. A 2.5-mL volume of trichloroacetic acid solution was then added to the hemolysate, which was incubated at room temperature for 15 min. Thiobarbituric acid (1.5 mL) was then added to the mixture, which was mixed and incubated in a water bath for 30 min. The tubes were then cooled and shaken vigorously for 30 min before adding 4 mL of *n*-butanol solution. The tubes were centrifuged at room temperature, and the absorbance of the resulting upper layer was then read using a spectrofluorometer [12].

Statistical analysis

Data were tabulated in Excel 2007 software and statistically analyzed in Software BioEstat 5.0. Initially, the distribution of measured data was observed. The D'Agostino test was used. The null hypothesis was rejected when the calculated value was less than or equal to the minimum or critical value equal to or greater than the critical value most. To determine the difference in magnitude of variation between groups in the measurement of "sample analysis" and "MDA" was used for the analysis of variance analysis of variance (ANOVA) with Tukey's post-hoc test. The level of significance was set at $p \leq 0.05$.

RESULTS

A significant difference was observed in the intragroup comparison of MDA levels (ANOVA, $p = 0.0001$). Tukey's post-hoc 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

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

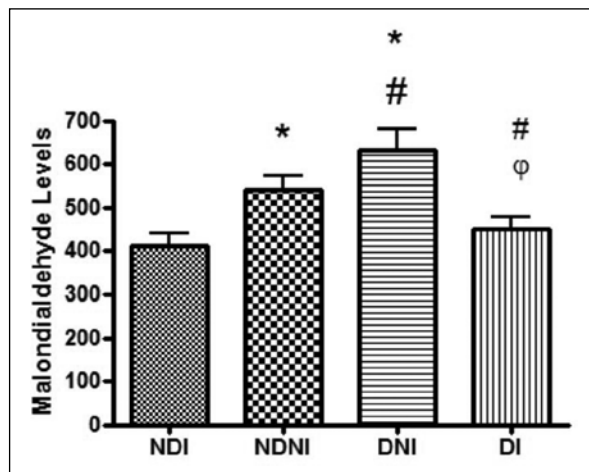| Groups	MDA	ANOVA	Tukey (<0,01)
NDI	410.3090 ± 28.8816		NDI *vs* NDNI
NDNI	541.1123 ± 31.8307		NDI *vs* DNI
DNI	631.9861 ± 49.1964	<0,0001	NDNI *vs* DNI
			NDNI *vs* DI
DI	450.2385 ± 29.0634		DNI *vs* DI

Figure 1. Comparison of mean and standard deviation of the concentration of the MDA. Induced cutaneous wounds in diabetic rats at 24 h after hours. *($p<0,01$) Tukey's test Compared with NDI. #($p<0,01$) Tukey 's test Compared with NDNI. φ($p<0,01$) Tukey's test Compared with DNI.

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

1. Wagener FA, Carels CE, Lundvig DM. Targeting the redox balance in inflammatory skin conditions. Int J Mol Sci. 2013; 14(5): 9126–9167.

2. Sen CK, Roy S. Redox signals in wound healing. Biochim Biophys Acta. 2008; 1780: 1348–1361.

3. Tang Y, Zhang MJ, Hellmann J, Kosuri M, Bhatnagar A, Spite M. Proresolution therapy for the treatment of delayed healing of diabetic wounds. Diabetes. 2013; 62(2): 618–627.

4. Tie L, Li XJ, Wang X, Channon KM, Chen AF. Endothelium-specific GTP cyclohydrolase I overexpression accelerates refractory wound healing by suppressing oxidative stress in diabetes. Am J Physiol Endocrinol Metab. 2009; 296(6): E1423–1429.

5. Marrote EJ, Chen D, Hakim JS, Chen AF. Manganese superoxide dismutase expression in endothelial progenitor cell accelerates wound healing in diabetic mice. J Clin Invest. 2010; 120(12): 4207–4219.

6. Houreld N, Abrahamse H. Low-intensity laser irradiation stimulates wound healing in diabetic wounded fibroblast cells (WS1). Diabetes Technol Ther. 2010; 12(12): 971–978.

7. Carvalho PT, Silva IS, Reis FA, Perreira DM, Aydos RD. Influence of ingaalp laser (660 nm) on the healing of skin wounds in diabetic rats. Acta Cir Bras. 2010; 25(1): 71–79.

8. Silva AA, Leal-Junior EC, Alves AC, Rambo CS, Santos SA, Vieira RP, Carvalho PD. Wound-healing effects of low-level laser therapy in diabetic rats involve the modulation of MMP-2 and MMP-9 and the redistribution of collagen types I and III. J Cosmet Laser Ther. 2013; 15(4): 210–216.

9. Santos NR, Santos JN, Reis Jr JA, Oliveira PC, Sousa AP, Carvalho CM, Soares LG, Marques AM, Pinheiro AL. Influence of the use of laser phototherapy (lambda 660 or 790 nm) on the survival of cutaneous flaps on diabetic rats. Photomed Laser Surg. 2010; 28(4): 483–488.

10. Sekhejane PR, Houreld NN, Abrahamse H. Irradiation at 636 nm positively affects diabetic wounded and hypoxic cells in vitro. Photomed Laser Surg. 2011; 29(8): 521–530.

11. Silveira PC, Silva LA, Pinho CA, Souza PS, Ronsani MM, Scheffer DL, Pinho RA. Effects of low-level laser therapy (GaAs) in an animal model of muscular damage induced by trauma. Lasers Med Sci. 2013; 28(2): 431–436.

12. Graham ML, Janecek JL, Kittredge JA, Hering BJ, Schuurman H. The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources. Comp Medic. 2011; 61: 356–360. PMID: 22330251.

13. Al-Bayaty F, Abdulla MA. A comparison of wound healing rate following treatment with aftamed and chlorine dioxide gels in streptozotocin-induced diabetic rats. Evid Based Complement Alternat Med. 2012; 2012: 468764.

14. Forgiarini Junior LA, Kretzmann NA, Tieppo J, Picada JN, Dias AS, Marroni NA. Lung alterations in a rat model of diabetes mellitus: effects of antioxidant therapy. J Bras Pneumol. 2010; 36(5): 579–587. PMID: 21085823.

15. Lopes JP, Oliveira SM, Soares FJ. Oxidative stress and its effects on insulin resistance and pancreatic beta-cells dysfunction: relationship with type 2 diabetes mellitus complications. Acta Med Port. 2008; 21(3): 293–302. PMID: 18674421.

16. Karu T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J Photoch Photob. 1999; 49: 1–17. PMID: 10365442.

17. Yagi K. A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. 1976; 15: 212–216. PMID: 962904.

18. Antunes-Neto JM, Toyama MH, Carneiro EM, Boschero AC, Silva LP, Macedo DV. Circulating leukocyte heat shock protein 70 (HSP70) and oxidative stress markers in rats after a bout of exhaustive exercise. Stress. 2006: 9(2): 107–115. PMID: 16895834.

19. Sibbald RG, Woo KY. The biology of chronic foot ulcers in persons with diabetes. Diab Metab Res Rev. 2008; 24: 25–30.

20. Vessby J, Basu S, Mohsen R, Berne C, Vessby B. Oxidative stress and antioxidant status in type 1 diabetes mellitus. J Int Med. 2002; 251: 69–76. PMID: 11851867.

21. Lindgård A, Hultén LM, Svensson L, Soussi B. Irradiation at 634 nm releases nitric oxide from human monocytes. Lasers Med Sci. 2007; 22(1): 30–36. PMID: 17120165.

22. Firat ET, Dağ A, Günay A, Kaya B, Karadede MI, Kanay BE, Ketani A, Evliyaoğlu O, Uysal E. The effects of low-level laser therapy on palatal mucoperiosteal wound healing and oxidative stress status in experimental diabetic rats. Photomed Laser Surg. 2013; 31(7): 315–321.

23. Ferraresi C, Hamblin MR, Parizotto NA. Low-level laser (light) therapy (LLLT) on muscle tissue: performance, fatigue and repair benefited by the power of light. Photonics Lasers Med. 2012; 1(4): 267–286. PMID: 23626925.

24. Assis L, Moretti AI, Abrahão TB, Cury V, Souza HP, Hamblin M R, Parizotto N A. Low-level laser therapy (808 nm) reduces inflammatory response and oxidative stress in rat tibialis anterior muscle after cryolesion. Lasers Surg Med. 2012; 44(9): 726–735.