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Effects of low-level laser therapy on epidermal oxidative response induced by wound healing

Efeitos da laserterapia de baixa potência na resposta oxidativa epidérmica induzida pela cicatrização de feridas

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Abstract

Background: Therapeutic use of low-level laser in physical therapy has increased significantly. **Objective:** To assess the effects of low-level laser therapy on the oxidative parameters of wound healing in rats. **Methods:** Eighteen Wistar rats were randomly divided into three groups (control, 5 days, n=6; 2 J/cm², 5 days, n=6; 4 J/cm², 5 days, n=6). A single circular wound measuring 8 x 8 mm was surgically created on the rats' backs. Thirty minutes after the last irradiation, the rats were euthanized and the irradiated tissue was surgically removed and stored at -70°C. We determined the activity of the respiratory chain enzymes DCIP oxidoreductase (complex II) and soluble succinate dehydrogenase (SDH); the activity of cytochrome c oxidase (complex IV); the production of superoxide anion; and the activity of superoxide dismutase (SOD) and catalase (CAT). Lipid peroxidation was assessed by means of the TBARS assay. **Results:** There was a decrease in the complex II activity in the groups irradiated for 5 days with 2 and 4 J/cm², while superoxide anion production decreased significantly in the group irradiated for 5 days with 4 J/cm² when compared with the control group. There was also a significant increase in CAT activity in the group irradiated for 5 days with 2 J/cm² as well as a decrease in lipid peroxidation activity in the two irradiated groups. **Conclusions:** The results of the present study indicate that laser stimulates antioxidant activity and protects cells against oxidative damage during the wound healing process in rats.

Keywords: wound healing; low-level laser therapy; oxidative stress; respiratory chain; free radical.

Resumo

Introdução: O uso terapêutico do laser de baixa potência na fisioterapia tem aumentado significativamente. **Objetivo:** Avaliar os efeitos da laserterapia de baixa potência nos parâmetros oxidativos na cicatrização de feridas em ratos. **Métodos:** Dezoito ratos Wistar foram divididos randomicamente em 3 grupos (controle 5 dias, n=6; 5 dias/2 J/cm², n=6; 5 dias/4 J/cm², n=6). Uma única ferida circular medindo 8 X 8 mm foi cirurgicamente realizada no dorso do rato. Trinta minutos após a última irradiação, os ratos foram submetidos à eutanásia, e o tecido irradiado foi removido cirurgicamente e armazenado a -70ºC. Foi determinada a atividade das enzimas da cadeia respiratória: DCIP oxirredutase (complexo II) e succinato desidrogenase solúvel (SDH), atividade do citocromo c oxidase (complexo IV), produção de ânion superóxido, atividade da superóxido dismutase (SOD) e catalase (CAT). A lipoperoxidação foi avaliada pela técnica de TBARS. **Resultados:** Os resultados mostram uma diminuição na atividade do complexo II nos grupos irradiados por 5 dias com 2 e 4 J/cm², enquanto a produção de ânion superóxido mostrou uma diminuição significativa no grupo irradiado por 5 dias com 4 J/cm² em relação ao grupo controle. Além disso, um aumento significativo na atividade da catalase foi observado no grupo irradiado por 5 dias com 2 J/cm², como também uma diminuição da peroxidação lipídica nos dois grupos irradiados. **Conclusões:** Os resultados do presente estudo indicam que o laser estimula a atividade antioxidante e protege a célula contra danos oxidativos durante o processo de cicatrização de feridas cutâneas em ratos.

Palavras-chave: cicatrização; terapia a laser de baixa intensidade; estresse oxidativo; cadeia respiratória; radicais livres.

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Introduction

The application of low-level laser therapy as therapeutic technology in the area of physical therapy has grown significantly. The healing properties of laser radiation, associated with the treatment's safety, seem to be the most responsible for that growth and for the increased interest by biomedical researchers in investigating the action mechanisms and the therapeutic effects of the low-level lasers[1]. Laser therapy has a significant effect on the ulcerative process and reduces healing time. This response allows the individual to resume normal activities more quickly[2]. However, some mechanisms involved in this response are still obscure, especially with regard to the effects of laser on the mitochondrial respiratory chain and on oxidative stress biomarkers[3].

According to Karu[4], laser exposure causes an increase in mitochondrial electrochemical activity and a concomitant increase in ATP synthesis. Eells et al.[5] suggest that cytochrome c oxidase is the main photoreceptor of laser light. Additionally, the low-level laser has a cascade effect on the cell signaling, which promotes cellular proliferation and cytoprotection[6]. Some authors also postulate that laser therapy influences oxidative stress parameters such as changes in antioxidant enzyme activity and the production of reactive oxygen species (ROS)[7-10].

The absorption of laser light accelerates the transfer of electrons (respiratory chain) and induces an initial ROS production, specifically increasing the production of superoxide anion[7]. The excessive production of these species can damage cell components such as lipids, proteins and nucleic acids[11]. The cell membrane seems to be the first target. The effects of laser irradiation on the cellular mechanism and its influence on oxidative parameters are still unclear, with conflicting results in the literature[12]. However, it is possible that, depending on the dosage, exposure time and intensity, laser therapy can change the defense mechanisms that counter excessive ROS production[7]. Therefore, the aim of the present study was to evaluate the effects of low-level laser therapy on mitochondrial respiratory chain activity and oxidative stress parameters in response to wound healing in rats.

Methods

Animals

Eighteen adult male Wistar rats (250-300g) of the vivarium of Universidade do Extremo Sul Catarinense (UNESC) were used in this study. The rats were kept at a constant temperature of 22°C with a 12-hour light/dark cycle and free access to water and standard diet. The procedures have been approved by the Research Ethics Committee of UNESC, protocol number 167/2005.

Ulceration and low-level laser therapy

After anesthesia with ketamine (80 mg/Kg, i.p.), the animals' dorsal region was shaved and disinfected with alcohol 70%. In the mid-dorsal region, between the infrascapular line and the tail, a circular area of the skin of approximately 8 mm in diameter was removed with a punch[13]. The animals were randomly divided into 3 groups (n:6): injury without treatment (control); injury with treatment (2 J/cm^2); injury with treatment (4 J/cm^2). After the injury, the wounds were immediately treated for 5 consecutive days with low-level laser. All of the animals were anesthetized before each application, including the control group (ketamine - 80 mg/Kg, i.p.).

The low-level laser used in this study was gallium arsenide (GaAs), pulse waveform, invisible beam, wavelength of 904 nm, peak power of 15 mW, frequency of 2000 Hz, pulse time of 180 ns and beam cross-section of 0.07 cm^2 (Laserpulse - Ibramed). The application time was 40 seconds (2 J/cm^2) and 80 seconds (4 J/cm^2). Non-contact application (approximate distance of 1 mm) was used with the applicator perpendicular to the injury on five spots around the wound 1cm apart[14]. Thirty minutes after the last irradiation, all of the animals were euthanized (guillotine). The tissue around the wound was removed, processed, fractioned and stored at -70°C for subsequent biochemical analyses.

Biochemical analyses

Respiratory chain enzyme activity

Tissue preparation: the tissue around the wound was homogenized (1:10 w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/mL heparine). The homogenate was centrifuged at 800 x g for 10 minutes and the supernatant stored at -70°C for determination of enzymatic activity. The maximum period between the homogenization and the enzymatic analysis was five days.

Complex II + succinate dehydrogenase (SDH) activity: the enzymatic activities were measured according to the method described by Fischer et al.[15], in which the decrease in the absorbance of the 2.6-DCIP in 600 nm was used to calculate complex II activity. For the SDH calculation, the same system was used in the presence of phenazine methosulfate.

Complex IV activity: the complex IV activity was determined according to Rustin et al.[16] and calculated by the

decrease in absorbance caused by the oxidation of reduced cytochrome c, measured in 550 nm.

Superoxide anion: determined by the adrenaline oxidation rate showed through the spectrophotometer at 480 nm, as described by McCord and Fridovich[17].

Superoxide dismutase (SOD) and catalase (CAT) activity: the enzymatic SOD activity was determined by the inhibition of adrenaline auto-oxidation measured through the spectrophotometer (480 nm)[18]. CAT activity was determined by the fall in absorbance (240 nm) corresponding to the consumption of hydrogen peroxide[19].

Lipid peroxidation: as an index of lipid peroxidation, we verified the formation thiobarbituric reactive substances (TBARS) measured with the spectrophotometer (532 nm)[20].

Protein determination: the amount of protein in biochemical trials was measured using the Lowry et al.[21] method.

Statistical analysis

The data were expressed as means and standard deviation and statistically analyzed using one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test. The α level considered for the analysis was set at 0.05. The software SPSS (Statistical Package for the Social Sciences) version 12.0 was used.

Results

Mitochondrial enzyme activity

According to Figure 1B, there was a significant decrease in complex II activity in the groups irradiated with 2 J/cm^2 (0.39±0.25) and 4 J/cm^2 (0.59±0.27) compared to the group without treatment (1.51±0.92), however no significant difference was observed in the SDH and complex IV activity.

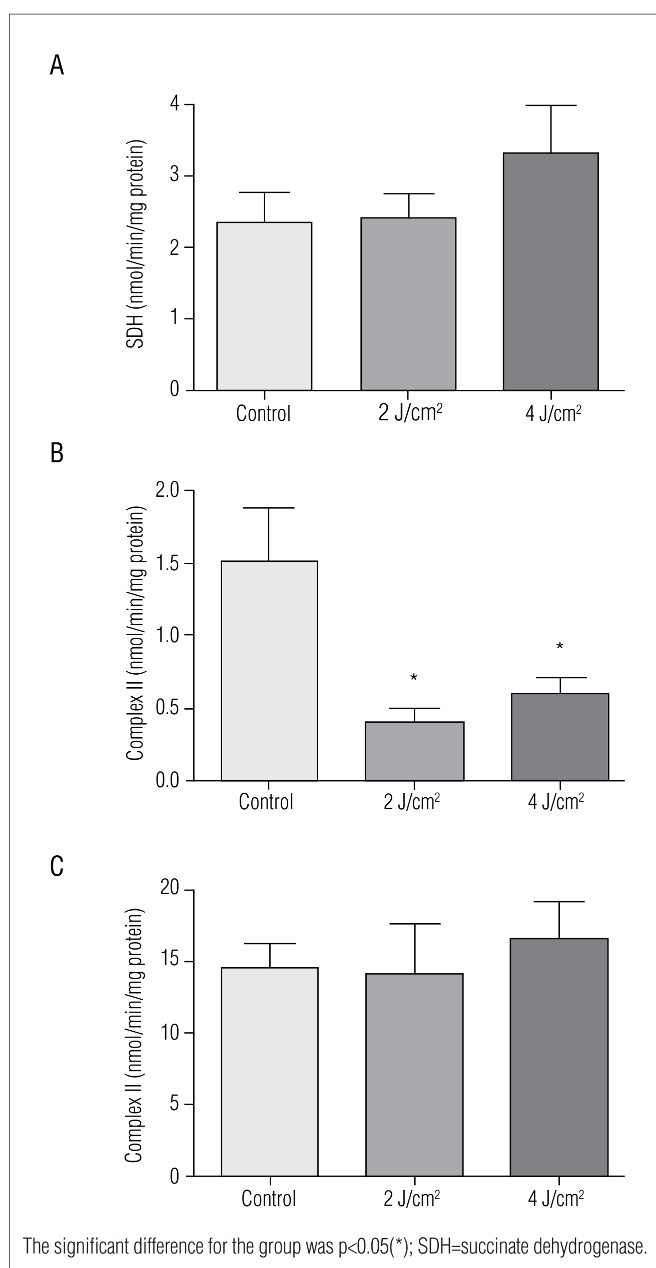

Figure 1. Effect of low-level laser therapy on (A) succinate dehydrogenase activity; (B) mitochondrial respiratory chain complex II; (C) mitochondrial respiratory chain complex IV. The values are presented as mean±SEM, and the results are expressed as nmol/min/mg protein.

Superoxide anion production

According to Figure 2, only the group irradiated with 4 J/cm^2 (25.04±2.23) showed a significant decrease in superoxide anion production compared to the untreated group after laser treatment (51.33±3.95).

Superoxide dismutase and catalase activity

The results show that there was no significant change in SOD activity in the groups irradiated for five days with 2 and 4 J/cm^2 compared to the control group (Figure 3A). However, the CAT activity had a significant increase in the group irradiated with 2 J/cm^2 (6.85±0.45) compared to the group without treatment (4.31±0.71; Figure3B).

Lipid peroxidation

The TBARS results seen in Figure 4 show a significant decrease in lipid peroxidation in the groups irradiated for five days with 2 J/cm^2 (0.62±0.10) and 4 J/cm^2 (0.42±0.09) compared to the group without treatment (1.19±0.23).

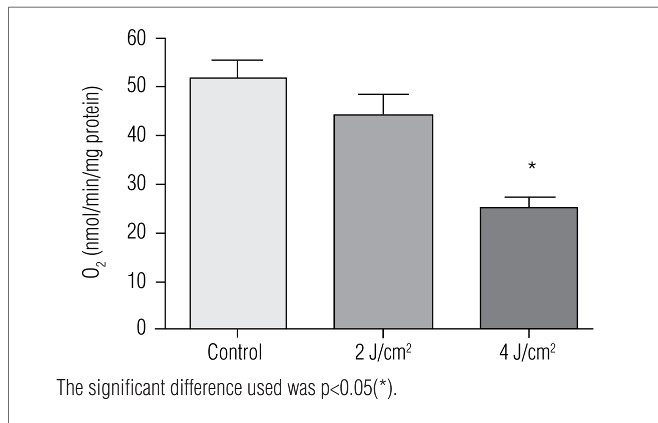

Figure 2. Effect of low-level laser therapy on superoxide anion production. The values are presented as mean±SEM, and the results are expressed as nmol/min/mg protein.

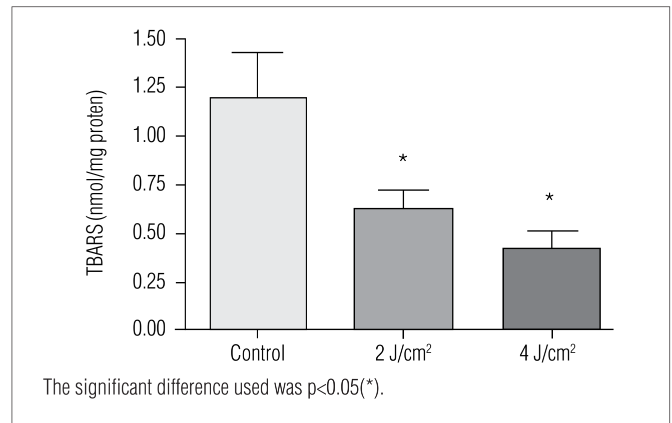

Figure 4. Effect of low-level laser therapy on levels of lipid peroxidation. The values are presented as mean±SEM, and the results are expressed as nmol MDA/mg protein.

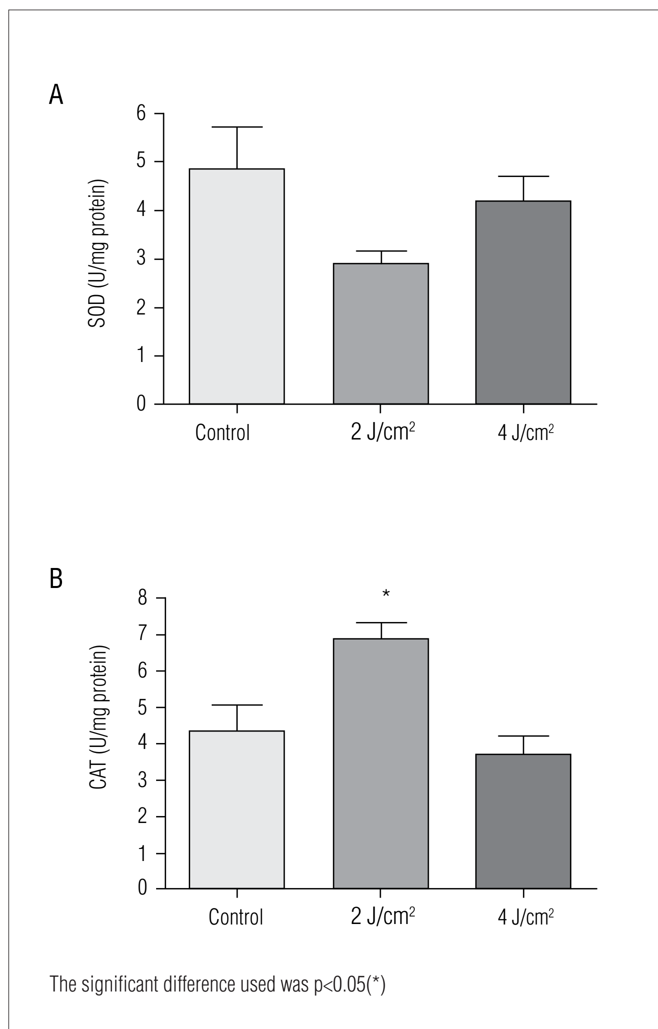

Figure 3. Effect of low-level laser therapy on (A) superoxide dismutase activity and (B) catalase activity. The values are presented as mean±SEM, and the results are expressed as U/mg protein.

Discussion

The healing process has an essential role in the protective response of the epidermal injury through tissue repair[22]. This process stimulates inflammatory mediators, such as cytokines and ROS, which have harmful effects on the tissue[23]. Recent studies have reported evidence of the important role of ROS in microvascular dysfunction, tissue damage and inflammatory processes which precede the tissue healing[24-27].

The present study evaluated the effects of low-level laser therapy (904 nm) with varied irradiation intensity on mitochondrial respiratory chain activity and some oxidative stress markers. With regard to the respiratory chain activity, Figure 1, the results show a significant decrease in complex II activity in the groups irradiated with 2 J/cm^2 and 4 J/cm^2, respectively (Figure 1B). It is possible that the inhibitory effect of complex II is directly linked to the excessive irradiation, which can produce complete oxidation and consequently a reduction in the complexes[28]. This inhibition is not directly associated with the electron transfer, but with a partial or total structural rearrangement, which leads to a bioinhibitory effect[29].

Previous studies indicate that irradiation with energy density above 4 J/cm^2 has a high energy fluence with inhibitory characteristics[30]. However, it was observed that this inhibitory characteristic also occurred with the energy density of 2 J/cm^2 and 4 J/cm^2 on the complex II activity. It is possible that this differentiated response results from the laser type and wavelength used in the studies, responsible for the high energy fluence.

According to Kreisler et al.[31], the stimulation of the photoreceptors by the laser in the mitochondrial respiratory

chain and the change in the ATP levels are not well established, and they are the subject of several discussions. It is commonly accepted that both the stimulatory and inhibitory effects of laser on cells are dependent on dosage and wavelength. Low-level irradiation performs biomodulative functions in cellular activity[32]. The initial molecular absorption of laser light is still unknown and, depending on the wavelength, the effects are changed due to the different chromophores which can be available as photoreceptors. It is likely that the process of photomodulation is only one among many photosignaling phenomena[33].

Figures 1A and 1C show that the complex IV and SDH activity did not change after laser treatment. The reasons for these results are still unknown and merit further investigation. However, previous studies show that irradiation with 2.4 and 3 J/cm^2 increases complex II and IV activity after 10 days of irradiation[27,28]. It is believed that this fact occurs due to the longer exposure time, which activates the chromophores of the respiratory chain, especially cytochrome c oxidase. The results of this study also show a significant reduction in superoxide anion production (Figure 2) five days after injury in the group irradiated with 4 J/cm^2.

The inflammatory response induced by the epidermal injury provokes the migration of neutrophils and macrophages, leading to rapid oxygen consumption. This mechanism activates the NADPH-oxidase, catalyzing the electron transfer from the NADPH to the oxygen to form superoxide[34]. Laser therapy reduces this migration of neutrophils and macrophages and stimulates leukocyte phagocytosis and shortening the inflammatory phase thus reducing superoxide anion production[35]. It is believed that the reduction observed in the present study can also be associated with a mitochondrial mechanism of reassertion, suggesting that the superoxide anion can be a source of electrons for the oxidative phosphorylation of ADP[7].

It must also be pointed out that there was no significant difference between the group irradiated with 2 J/cm^2 and the control group. Therefore, there may be a dosage and time-dependent ratio of laser therapy on superoxide anion production. The SOD enzyme represents the first line of enzymatic defense against the intracellular production of free radicals, catalyzing the dismutation of the superoxide anion. The resulting product of the reaction catalyzed by SOD is hydrogen peroxide (H_2O_2), which is catalyzed by the catalase and other peroxidases[11].

It has been postulated that the use of low-level laser induces an increase in SOD activity in different models, contributing to a decrease in tissue damages and to the maximization of the healing process[36,37]. As demonstrated in Figure 3A, there was no significant difference in the SOD activity, however there was an important increase in the CAT activity in the group irradiated with 2 J/cm^2 (Figure 3B). Regarding the CAT activity, the results are in agreement with Fulton and Shitabata[38] in that the activity increased in the rats' skin after irradiation with low-level laser.

Clinically, this change in the CAT activity in the epithelial tissue after laser exposure can be produced by the generation of free radicals caused by rotational changes in the macromolecules due to the photostimulation. This stimulation can be important because antioxidants enzymes are controlled by dosage or exposure duration[39]. Low-level laser irradiation has been efficient in the reduction of oxidative damages in different models and situations[40].

The results, according to Figure 4, show a significant decrease in lipid peroxidation in the groups treated with 2 J/cm^2 and 4 J/cm^2. These results suggest that low-level laser therapy stimulates the defense mechanisms against the oxidative damages to membrane lipids. Although SOD activity did not increase, and CAT activity only increased in the group irradiated with 2 J/cm^2, it is possible that other enzymatic and non-enzymatic antioxidants are involved in the protection against lipid oxidation. Additionally, photostimulation can increase tissue resistance against lipid peroxidation. These variables can justify the observed results.

Fillipin et al.[36] observed significantly reduced TBARS values in rats tendons irradiated and treated with low-level laser for 14 and 21 days, which shows that the irradiation had a protective effect and that the cells developed a positive antioxidant role in the deactivation of ROS excesses. Using irradiation in blood tissue, Stadler et al.[10] demonstrated that the lipid peroxidation levels were high after low-level irradiation, suggesting an increase in the ROS and hydroperoxide production. This study sustains the hypothesis that the hemoglobin in red blood cells can serve as a photoreactive substance and thus cause high levels of ROS when irradiated. It is possible that the difference in the lipid peroxidation results in different tissues after the low-level irradiation is directly associated with the time of exposure, irradiation intensity and method used for the determination of lipid peroxidation.

Conclusions

Low-level laser irradiation reduces the complex II activity of the mitochondrial respiratory chain, possibly related to the laser type and wavelength. We concluded that, due to

the decrease in the superoxide anion production, the low-level laser could protect the cell against oxidative damage to membrane lipids. There may be a dosage to time-dependent ratio of laser therapy on antioxidative enzyme activity. Therefore, additional studies are necessary to elucidate these mechanisms.

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