



Laser Photobiomodulation in the acute inflammatory response of the calcaneal tendon injury in rats exposed to cigarette smoke

Fotobiomodulação Laser na resposta inflamatória aguda em lesão do tendão calcâneo em ratos expostos a fumaça de cigarro

Fotobiomodulación láser en la respuesta inflamatoria aguda en lesión del tendón calcáneo en ratones expuestos a humo de cigarrillo

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ABSTRACT | Nicotine delays the healing process and increases the levels of myeloperoxidase (MPO), an enzyme that plays a key role in the production of reactive oxygen species during the inflammatory process. Laser Photobiomodulation (PBM) is one of the most used electrophysical agents in the treatment of the calcaneal tendon, however, its effects on MPO activity need to be further elucidated. This study aimed to evaluate the effects of laser PBM on MPO activity after inflicting an injury to the calcaneal tendon of rats exposed to cigarette smoke. Thirty-four male Wistar rats with 90 days of age were used. After 14 days of exposure to cigarette smoke, the animals were divided into three experimental groups: control group (CG, n=12), not submitted to injury or treatment; sham group (ShG, n=10), submitted to partial calcaneal tendon injury and laser PBM simulation; and laser PBM group (PBMG, n=12), submitted to partial calcaneal tendon lesion and treated with laser PBM within the first minute after injury. PBM decreased MPO activity levels in PBMG compared to ShG (CG: 1.38 ± 0.69 pg/ml; ShG: 3.78 ± 1.09 pg/ml; PBMG: 2.58 ± 0.93 pg/ml; $p < 0.005$). In conclusion, applying laser PBM immediately after inflicting damage to the calcaneal tendon attenuates acute inflammatory activity in rats exposed to cigarette smoke.

Keywords | Low Level Laser Therapy; Achilles Tendon; Tendinopathy; Inflammation.

RESUMO | A nicotina retarda o processo de cicatrização e eleva os níveis da enzima mieloperoxidase (MPO), a qual possui um papel fundamental na produção de espécies reativas de oxigênio durante o processo inflamatório. A fotobiomodulação laser (FBM) é um dos agentes eletrofísicos mais utilizados no tratamento do tendão calcâneo, no entanto, os seus efeitos sobre a atividade da MPO carecem de maior elucidação. Este estudo objetivou avaliar os efeitos da FBM sobre a atividade da MPO, após lesão do tendão calcâneo em ratos expostos à fumaça de cigarro. Foram utilizados 34 ratos Wistar, machos, com 90 dias de vida. Após 14 dias de exposição à fumaça de cigarro, os animais foram divididos em três grupos experimentais: grupo controle (GC, n=12), não submetido à lesão ou tratamento; grupo sham (GSh, n=10), submetido à lesão parcial do tendão calcâneo e a simulação da FBM laser; grupo FBM laser (GFBM, n=12), submetido à lesão parcial do tendão calcâneo e tratados com FBM laser, no primeiro minuto após a lesão. A FBM diminuiu os níveis de atividade da MPO no GFBM em comparação ao

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GSh (GC: $1,38 \pm 0,69$ pg/ml; GSh: $3,78 \pm 1,09$ pg/ml; GFBM: $2,58 \pm 0,93$ pg/ml; $p < 0,005$). Conclui-se que a FBM laser aplicada imediatamente após lesão do tendão calcâneo, atenua a atividade inflamatória aguda em ratos expostos à fumaça de cigarro.

Descritores | Terapia a Laser de Baixa Intensidade; Tendão do Calcâneo; Tendinopatia; Inflamação.

RESUMEN | La nicotina retarda el proceso de cicatrización y eleva los niveles de la enzima mieloperoxidasa (MPO), que tiene un papel fundamental en la producción de especies reactivas de oxígeno durante el proceso inflamatorio. La fotobiomodulación con láser (FBM) es uno de los agentes electrofísicos más utilizados en el tratamiento del tendón calcáneo, sin embargo sus efectos sobre la actividad de la MPO carecen de mayor elucidación. Este estudio objetivó evaluar los efectos de la FBM sobre la actividad de la MPO después de lesión del tendón calcáneo en ratones

expuestos al humo de cigarrillo. Se utilizaron 34 ratones Wistar, machos, con 90 días de vida. Después de 14 días de exposición al humo de cigarrillo, los animales fueron divididos en tres grupos experimentales: grupo de control (GC, $n=12$), no sometido a la lesión o tratamiento; grupo *sham* (GSh, $n=10$), sometido a la lesión parcial del tendón calcáneo y a la simulación de la FBM láser; y el grupo FBM láser (GFBM, $n=12$), sometido a la lesión parcial del tendón calcáneo y tratado con FBM láser, en el primer minuto después de la lesión. La FBM disminuyó los niveles de actividad de MPO en el GFBM en comparación con el GSh (GC: $1,38 \pm 0,69$ pg/ml; GSh: $3,78 \pm 1,09$ pg/ml; GFBM: $2,58 \pm 0,93$ pg/ml, $p < 0,005$). Se concluye que la FBM láser aplicada inmediatamente después de la lesión del tendón calcáneo atenúa la actividad inflamatoria aguda en ratones expuestos al humo de cigarrillo.

Palabras clave | Terapia Láser de Baja Intensidad; Tendón del Calcáneo; Tendinopatía; Inflamación.

INTRODUCTION

Despite the decline in the number of smokers, smoking is still considered one of the biggest public health problems in the world, exposing individuals to various toxic substances that lead to dependence and development of chronic diseases, also being one of the main causes of delay in the tissue's healing process^{1,2}.

Nicotine, the main tobacco product, has been shown to cause vasoconstriction in an experimental model and, consequently, delay the healing process^{2,3}. Nicotine binds itself to nicotinic acetylcholine receptors and promotes effects on the central and peripheral nervous systems, altering the cardiovascular function via sympathetic stimulation and, when inhaled or injected, releases catecholamines that induce vasoconstriction and decrease tissue perfusion⁴. Moreover, it has been shown that cigarettes change cellular homeostasis by inducing a pro-inflammatory state, increasing reactive oxygen species (ROS) and unbalancing the antioxidant system, thus favoring the progression of cellular damage⁵. In rats submitted to rotational stress and to the application of nicotine patches, there was an increase in the number of inflammatory cytokines and reduction in the migration of fibroblasts, delaying the healing process of cutaneous lesions¹. In tendinous lesions, it has also been shown that nicotine leads to lower functional, vascular, and biomechanical properties² by reducing fibroblast proliferation and collagen synthesis³.

The calcaneal tendon is one of the strongest and most resistant tendons in the human body, but its low blood supply aggravates the occurrence of injuries and delays the healing process^{6,7}. Some therapeutic interventions, like Laser Photobiomodulation (PBM), have been widely used to repair these lesions for demonstrating several advantages, such as reduction in healing time, due to some important biochemical and cellular effects⁸, including increased proliferation of fibroblasts and collagen synthesis, antioxidant effects, increased mitochondrial metabolism, as well as stimulation of DNA synthesis and cell proliferation⁹⁻¹¹. Among the physiological mechanisms induced by PBM in wound healing, its influence is associated with the increase in the activation of redox-sensitive transcription factors and can induce the activation of intracellular signaling, as well as change the production of ROS^{12,13}. Thus, laser PBM has been used on the calcaneal tendon to decrease oxidative stress during the inflammatory phase and speed up the healing process¹⁴.

Initially, in the inflammatory phase, neutrophils and, subsequently, monocytes and lymphocytes are recruited to the site of inflammation, where they produce ROS and proteolytic enzymes involved in tissue repair¹⁵. The myeloperoxidase enzyme (MPO), present in leukocytes of the granulocytic and monocytic lineages, plays a fundamental role in the production of ROS and signaling of inflammation events¹⁶. Some studies indicate that MPO damages tissue by producing hypochlorous acid, which reacts with double lipid bonds leading to lipid

peroxidation¹⁷. In addition, in comparison to non-smokers, this enzyme is more present in the smoking population and is also capable of predicting systemic inflammation^{18,19}.

Despite the increasing evidence regarding the effects of laser PBM, no reports on its role in inflammatory activity associated with tendon injury in rats exposed to cigarette smoke have been found. Thus, after the application of a model of exposure to cigarette smoke, this study aimed to evaluate the effects of laser PBM on MPO activity in the calcaneal tendon injury of rats.

METHODOLOGY

This study was approved by the Animal Ethics Committee (CEUA) of Centro Universitário Franciscano (Unifra) under process No. 003/2013. Thirty-six male adult Wistar rats weighing between 280 and 320 grams (three months of age) were used. The experimental procedures took place at the Unifra Biochemistry and Nanoscience Laboratory. The animals were acclimated to

the environment for seven days prior to the start of the experiment, having been kept in cages, with a 12-hour light-dark cycle and temperature controlled at 22°C, receiving food and water *ad libitum* throughout the acclimation and experimentation period, in accordance with the international ethical principles of the Research Ethics Committee of the International Association for the Study of Pain, and according to the determinations of Law 6638/79 and Decree 24665/34.

Experimental design

The animals were randomly sorted by the BioStat 5.0 software into three experimental groups. CG (n=12), exposed to cigarette smoke, was not submitted to partial damage of the calcaneal tendon and did not receive treatment. ShG (n=10) was exposed to cigarette smoke, submitted to partial damage of the calcaneal tendon, and to the simulation of treatment (with the laser turned off). PBMG (n=12) was exposed to cigarette smoke, submitted to partial calcaneal tendon injury, and treated with laser PBM (Figure 1).

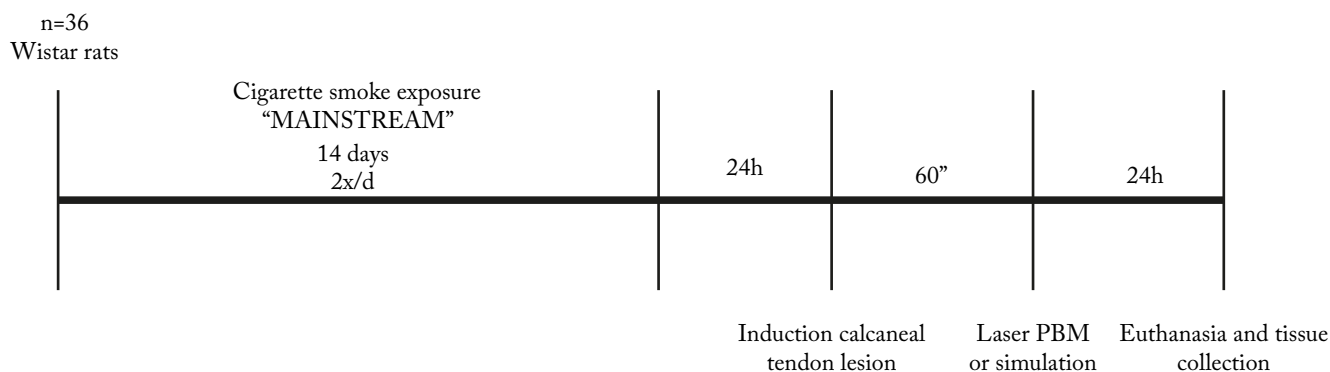


Figure 1. Experimental design of the different study groups

Cigarette smoke exposure protocol

All animals were exposed to commercial cigarette smoke at a concentration of 0.8 mg nicotine, 10 mg tar and 10mg carbon monoxide, for 14 days, in a closed polypropylene cage (41×34×16cm) containing only an orifice connected to a device for pumping the cigarette smoke into the box. This type of model exposes the rats to "mainstream" smoke, which corresponds to active smoking. During the first week, the rats were exposed to the smoke of two cigarettes for 20 min once a day, with one cigarette burned at a time for adaptation purposes. In the second week, exposure to smoke increased to

10 cigarettes for 20 min, twice a day, with a half-hour interval between each exposure²⁰.

Laser Photobiomodulation Protocol

One minute after the partial calcaneal tendon lesion's induction, the animals of PBMG were treated with laser PBM (Gallium Aluminum Arsenide; Imbramed, São Paulo, Brazil), $\lambda=830\text{nm}$, 30mW power, 50J/cm² energy density and 3J total energy, by punctually applying a continuous beam with a cross-sectional 0.06cm² area over the calcaneal tendon lesion for 100 seconds²¹, in a single application. In the animals of ShG, the treatment simulation was performed with the equipment switched off.

Induction of the calcaneal tendon lesion

The animals were anesthetized proportionally to their body mass, with an intraperitoneal injection containing 100mg/kg ketamine and 50mg/kg xilazine. Subsequently, the animals were placed in the ventral decubitus position on the surgical plank for digital epilation via manual traction of the hair (on the region adjacent to the calcaneal tendon) of the right paw, and then placed on the previously sterilized lesion equipment. Traction was performed on the right hind limb, with the ankle kept dorsiflexed at a 90° angle in relation to the calcaneal tendon until the animal's hind paw was in contact with the equipment.

After positioning the animal, a 200g weight was perpendicularly released over the calcaneal tendon, at a 20 cm height^{22,23}. The tendon's potential sagging energy was 364.9mJ. Five seconds after the fall, the weight was removed and the lesion site was circled with a dermatographic pen to enable the localized application of laser PBM.

Tendon excision and tissue preparation

After 24h, the animals were anesthetized and their right tendons were removed via dissection, from the calcaneal insertion to the musculo-tendinous junction²³, standardized with a 0.8cm length, and stored at -80° until the moment of analysis. The animals were euthanized via anesthetic overdose and section of the cervical vessels.

Determination of myeloperoxidase (MPO) activity

MPO activity was measured by performing a spectrophotometric-coupled peroxidase assay involving the oxidation of phenol. After the animals were euthanized, the skin of the tendon region was removed and the calcaneal tendon was carefully transferred through a vat, and an equal volume of hexadecyltrimethylammonium bromide (HTAB, Sigma Chem. Co., USA) was added, followed by homogenization with a vortex mixer (Heidolph DiAx 900, Germany) and ultrasonification for 20 sec. The tubes were heated for 2 h at 60°C in a greenhouse for inactivation of the endogenous activity of Catalase²⁴, and then centrifuged at 12.000 rpm for 2 min. Ten microliters of the supernatant were pipetted (in duplicate) into a 96-well microplate and added to 200 of a solution of potassium phosphate buffer (pH 6) containing 0.164 mg/mL o-dianisidine dihydrochloride (Sigma Chemical Co., USA) and 0.0005% hydrogen

peroxide (Merck, Germany). The change in absorbance at 460 nm was measured on a microplate reader (Spectra Max plus 384, USA) for 10 min, and MPO activity was estimated from the maximum reaction rate per second. The result was expressed in MPO/tendon unit.

Statistical analysis

The mean values and standard deviation (\pm SD) were estimated for all the analyzed data. The Shapiro-Wilk normality test was performed. One-way Anova followed by the Kruskal-Wallis test were used to compare the effects between groups. In all tests, $p < 0.05$ was considered statistically significant. Data analysis was conducted with the SPSS software version 22 (IBM SPSS Statistics 22).

RESULTS

During the experimental period, two animals of ShG died, totaling thirty-four animals. Figure 2 shows the levels of MPO activity in the different experimental groups. In all groups, MPO activity was observed. There was an increase in MPO levels in ShG and PBMG compared to CG (CG: 1.38 ± 0.69 pg/ml; ShG: 3.78 ± 1.09 pg/ml; PBMG: 2.58 ± 0.93 pg/ml; $p = 0.000$ CG vs. ShG; $p = 0.040$ CG vs. PBMG). PBMG had lower MPO activity in the calcaneal tendon compared to ShG ($p = 0.045$).

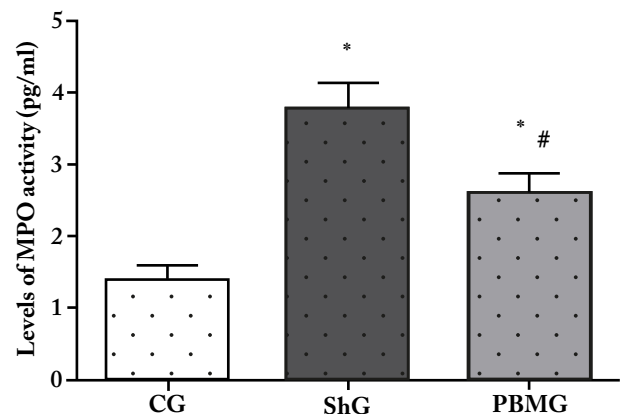


Figure 2. Levels of myeloperoxidase activity in the calcaneal tendon in CG, ShG and PBMG
CG: control group; ShG: sham group; PBMG: Laser Photobiomodulation group. * $p < 0.05$ vs. CG; # $p < 0.05$ vs. ShG.

DISCUSSION

The main finding of this investigation was the reduction in MPO activity levels caused by the application of laser

PBM in the acute inflammatory phase after the induction of a partial injury to the calcaneal tendon of rats exposed to cigarette smoke. MPO activity was present in all groups of this study, indicating that the time of exposure to cigarette smoke was able to promote tissue damage, even in animals not submitted to tendinous lesion.

Other studies also analyzed MPO activity after exposure to cigarette smoke and verified results similar to ours, but in lung tissue²⁵. To date, no assessment of MPO activity in the tendons of rats exposed to cigarette smoke and treated with laser PBM has been found in the literature. However, a study using corticosteroid therapy presents results similar to ours, showing improvement in the calcaneal tendon's healing process, evidenced by the decrease in MPO activity²⁶. MPO is related to the production of ROS, which increase oxidative stress. Recently, it has been demonstrated that laser PBM is able to decrease lipid peroxidation in the tibial muscles on the first day of application, indicating a positive modulation in the activity of antioxidant enzymes and reduction in oxidative stress markers²⁷. Similar results were also observed in analyses of the calcaneal tendon¹⁴, which corroborate our finding of lower MPO activity in PBMG compared to ShG, indicating an antioxidant effect of laser PBM.

In addition, an explanation for the decrease in MPO activity levels in PBMG compared to ShG would be the angiogenic effect of laser PBM²⁸, which influences the balance of MPO activity, decreases the inflammatory cellular influx^{29,30}, and stimulates neovascularization and antibacterial activity, leading to the improvement in the tendon's healing process^{21,28,31}. Although these hypotheses have already been demonstrated, they were not evaluated in the present study; laser PBM was applied only once, and the evaluation was immediate (24 hours later), which may have prevented the obtaining of greater anti-inflammatory effects and other effects such as angiogenesis. Our results suggest that the application of laser PBM with a 3J dose for 100 seconds may be related to the increase in the tendon's immediate blood flow, being able to decrease MPO activity after the injury's induction.

Other results also highlight the improvement in the quality of tendon repair immediately following the lesion's induction in response to laser PBM^{23,32}. However, most studies associate this therapy with the improvement in the deposition of collagen fibers at the lesion site^{11,23,33}, without evaluating the effect of laser PBM on MPO activity in total or partial ruptures of the calcaneal tendon.

Although our findings demonstrate a decrease in MPO activity in PBMG compared to ShG, further studies

should be performed with different duration and doses of laser PBM application, to verify similar results. A better understanding of the ideal dose of laser PBM in a smoking model is needed, taking into account its clinical applicability in situations of tendon lesions that occur without the patient avoiding smoking, as in elective surgeries, which may result in worse recovery and tissue repair. As demonstrated, laser PBM is a resource that improves this process, and dose is one of the important factors in these cases.

In addition, one of the limitations of our study was not having included the histological evaluation of new vessels and of the vascular growth factor. Further studies should also be conducted to measure other inflammatory markers and oxidative stress for a better understanding of the mechanisms responsible for the effects of laser PBM on the calcaneal tendon's acute inflammatory response. The analysis of neovascularization and tendinous realignment may also be part of future investigations.

CONCLUSION

The attenuation of the acute inflammatory response obtained with the application of laser PBM in rats exposed to cigarette smoke demonstrates its potential therapeutic effect in the immediate management of the calcaneal tendon injury. Modulation of MPO activity and analysis of tendon repair markers in response to laser PBM, in the acute and chronic inflammatory phases, should be the subject of further experimental and clinical investigations.

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