High energy Gallium Arsenide laser does not facilitate collagen alteration in muscle skeletal extracellular matrix: experimental study

Laser de Arseneto de Gálio com alta energia não promove alteração colágena da matriz extracelular no músculo esquelético: estudo experimental

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Abstract

Introduction: Low intensity laser therapy has proven effective in treating different tissues, reducing inflammation, preventing the formation of fibrous tissue, and promoting muscle regeneration. **Objective:** To evaluate the effect of low intensity laser therapy, seven days after the injury, and verify whether the radiated energy chosen influences the formation of fibrous tissue. **Methods:** Thirty Wistar rats, adult male, average body weight 210-340 g were used. The animals were randomized into three groups: control group, untreated injured group (L), and injured and treated group (LT). After anesthetizing the animals, muscle injury was induced by freezing (cryoinjury) in the central region of the tibialis anterior muscle belly (TA) on the left hind limb, through an iron rod previously immersed in liquid nitrogen. A Gallium Arsenide laser, wavelength 904 nm was used. The applications were initiated 24 hours after injury, daily,

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for five days, at two points in the lesion area. After 7 days, the animals were euthanized; the TA muscle of the left hind limb was removed and frozen in liquid nitrogen and the obtained histological sections were subjected to Sirius Red staining. **Results:** Histological analysis showed no significant difference in relation to the area of fibrosis in the LT and L groups. **Conclusion:** The results suggest that the energy density of 69 J/cm² and final energy (4.8 joules) did not promote alterations in the area of collagen in the skeletal muscle extracellular matrix.

Keywords: Laser Therapy. Striated Muscle. Fibrosis.

Resumo

Introdução: *A Laserterapia de Baixa Intensidade tem se mostrado eficaz no tratamento de diferentes tecidos, diminuindo o processo inflamatório, prevenindo a formação de tecido fibroso e promovendo a regeneração muscular.* ***Objetivo:*** *Avaliar o efeito da Laserterapia de Baixa Intensidade, 7 dias após a lesão e verificar se a energia irradiada escolhida tem influência na formação de tecido fibroso.* ***Métodos:*** *Foram utilizados trinta ratos Wistar, machos, adultos, peso corporal médio de 210-340 g. Os animais foram randomizados em três grupos: Grupo Controle, Grupo lesado não tratado (L) e o Grupo lesado e tratado (LT). Após anestesia dos animais, a lesão muscular foi induzida por congelamento (criolesão) na região central do ventre do músculo tibial anterior (TA) do membro posterior esquerdo, por meio de uma haste de ferro previamente imersa em nitrogênio líquido. Foi utilizado o laser de Arseneto de Gálio, comprimento de onda de 904 nanômetros. As aplicações foram iniciadas 24 horas após a lesão, diariamente, durante cinco dias, em dois pontos na área da lesão. Aos 7 dias, os animais foram eutanizados; o músculo TA do membro posterior esquerdo foi retirado e congelado em nitrogênio líquido e os cortes histológicos obtidos foram submetido a coloração Picrosirius Red.* ***Resultados:*** *A análise histológica mostrou que não houve diferença significativa em relação á área da fibrose do LT e Grupo L.* ***Conclusão:*** *Os resultados sugerem que a densidade de energia de 69 J/cm² e energia final (4,8 joules) não promoveu alteração na área do colágeno da matriz extracelular do músculo esquelético.*

Palavras-chaves: *Terapia a Laser. Músculo Estriado. Fibrose.*

Introduction

Low intensity laser therapy (LILT) is the application of low power electromagnetic radiation, from 1 mW to 500 mW, used in the clinical practice of Physiotherapy for the treatment and rehabilitation of several tissues, where the wavelength lies in the red spectrum of 630 to 700 nanometers (nm) and the infrared spectrum of 700 to 1200 nm [1-4]. Red and infrared radiation can be used in the treatment of different types of tissue as the wavelength allows efficient penetration of the beam into the tissue. In this way, infrared radiation is widely used in the treatment of soft tissues such as ligaments, tendons, muscles, and capsules [2].

Studies have shown that LILT is able to accelerate the tissue repair process, using Helium-Neon (HeNe), and semi-conductor diode lasers of Gallium-Aluminum-Arsenide (AsGaAl), Gallium Arsenide (AsGa), and Gallium-Indium-Phosphorus Arsenide (InGaAlP) [3].

LILT has been used in the treatment of different types of tissues, including skeletal muscle, and the results point to control of the inflammatory process, increase in mitosis, and promotion of angiogenesis, as well as favoring the deposition of collagen, which can promote tissue repair and/or regeneration [4-7].

Adult skeletal muscle, under normal conditions, is a stable tissue with remarkable regeneration capacity in response to injury, physical overload, or genetic muscle disease. During muscle repair and/or regeneration, an inflammatory process, cell proliferation and differentiation, remodeling of connective tissue, angiogenesis, and functional recovery of injured muscles occur [8].

Among several beneficial effects, LILT can prevent the formation of fibrous tissue in muscle tissue after injury. Fibrosis is characterized by abnormal and exaggerated deposition of extracellular matrix and may compromise muscle function [9]. In a study in rats using the Gallium-Aluminum-Arsenide semiconductor diode laser (AsGaAl), wavelength (808 nm) and energy density 180 J/cm^2, reductions in type 1 collagen and fibrous tissue were observed in the lesion area after injury to the anterior tibial muscle induced by cryosurgery [10]. In another study, performed in the anterior tibial muscle of rats after cryoinjury, using the same type of laser, with wavelength (780 nm) and energy density of 10 J/cm^2, it was observed that LILT had a significant influence on the organization of collagen in the area of the injury, minimizing the formation of fibrosis [11].

Other studies using a Gallium Arsenide (AsGa) laser, wavelength (904 nm), energy density of 5 J/cm^2, and final energy of 1.6 joules, with an experimental model of cryoinjury in the anterior tibial muscle of Wistar rats found that LILT is capable of stimulating collagen synthesis and avoiding excessive production of collagen [12-14].

LILT has also been used in the treatment of other alterations, such as: treatment of pressure ulcers [15-17], treatment of glaucoma [16], in the area of dermatology in the treatment of keloids, and in odontology for the treatment of orofacial pain [18, 19]. The possible mechanisms suggest that LILT promotes an increase in mitochondrial adenosine triphosphate (ATP) and tissue oxygenation, inhibits pain as a consequence of the anti-inflammatory effect [20, 21], and reduces the release of inflammatory cytokines (prostaglandin, histamine, serotonin, and bradykinin) [22]. LILT also induces the release of beta-endorphin (endogenous analgesic), which favors the sensation of well-being.

Although LILT is an excellent therapeutic option in the treatment of different tissues, its effects seem to be related to the appropriate choice of irradiation parameters [3]. A study with laser equipment from different manufacturers showed that energy density is not the most appropriate parameter to use when choosing the ideal dose, as depending on the area of the beam and power of the equipment, the final radiated energy presents great variations and this appears to be the most relevant parameter [23]. The energy radiated is one of the most important

parameters and is dependent on the power of the equipment and the radiation time [24].

According to the World Association of Laser Therapy, the final energy recommended in the treatment of different tissues ranges from 2 to 4 joules [25]. The majority of studies that evaluated the effects of LILT used mean energy of 1.6 joules and observed that the laser was able to modulate collagen synthesis [14].

In several investigations using experimental models of rats, with a Gallium Arsenide (AsGa) laser, wavelength 904 nm, the final energy presented variations between 0.04 and 3.0 joules. In this context we chose to evaluate the effects of a Gallium Arsenide (AsGa) laser, 904 nm, 50 mW, and beam area of 0.035 cm^2 in the area of fibrosis, after cryoinjury, using a high final energy (4.8 joules).

In this way, the present study is highlighted as it presents the use of higher final energy than that observed in other studies found in the literature, which could enrich the knowledge on this subject and contribute to clarification of the therapeutic benefits of LILT.

The objective of the present study was to evaluate the effects of a Gallium Arsenide (AsGa) laser on the formation of fibrous tissue, using final energy of 4.8 joules in the injury area of rats submitted to injury in the tibialis anterior muscle.

Methods

Aspects of an ethical nature

This study was developed after approval from the ethics committee on animal use (CEUA). The procedures related to the selection of the sample, protocol of experimentation, euthanasia, and preparation of histological slides were already carried out at the Universidade Estadual Paulista (UNESP) — Campus Botucatu, and approved under protocol 713. In the present study, techniques of histomorphometric analysis of the results were performed.

Sample selection

The experiment was performed with 30 rats (*Rattus norvegicus*), lineage (Wistar), males, weighing

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between 210 and 340 grams, aged approximately 3 months, from the bioterium of the Universidade do Oeste Paulista. The sample number was based on a previous study with a similar experimental design [26]. During the study period the rats were kept in individual cages, given access to feed and water *ad labitum*, with controlled ambient temperature between 22 to 24°C and light reversed light/dark 12 hours. The rats were randomly subdivided into three groups containing 10 animals each:

Control Group (C): the animals were not subjected to injury or treatment.
Injured/Untreated Group (L): the animals were injured, but not treated with LILT.
Treated Group (LT): the animals were injured and treated with LILT.

Induction of muscle injury (Cryolesion)

After separation of the groups, the animals were submitted to intraperitoneal (IP) anesthesia with 1 ml/kg of 1% ketamine HCI (dopalen; Vetbrands; São Paulo, Brazil) and 2% xylazine (Anasedan; Vetbrands; São Paulo, Brazil). After anesthesia, the tricotomy of the left hind limb was carried out; a 1 cm longitudinal cut along the anterior tibial muscle was performed, thus exposing the anterior tibialis muscle (TA). Muscle injury was induced using a rectangular iron bar (0.64 mm^2) previously frozen in liquid nitrogen for 30 seconds, placed in direct contact, transversely, in the central region of the TA muscle and maintained for 10 seconds, this process was repeated two times consecutively with an interval of 30 seconds [10]. The skin was then sutured and the animals individually housed in plastic cages under controlled temperature conditions.

Experimental design

A Gallium Arsenide (AsGa) semiconductor diode laser was used (KLD Biossistemas®, Amparo, Brazil), pulsed emission, wavelength 904 nanometers, output power (mean) 50 mW (miliwatts), beam emission area 0.035cm^2.
Treatment started 24 hours after the cryosurgery, and five consecutive applications were performed

for five days, with a 24-hour interval between applications. The animals were manually restrained with the injured member in extension. The laser was applied through direct contact with the skin in the area of the lesion directly in the belly of the TA muscle, at a 90° angle on the radiated surface. The energy density used was 69 J/cm^2, applied at two points over the area of the injury, at a distance of 1 cm per point, to reach the entire area of the injury [27]. The radiation time was 48 seconds at each point, and the final energy radiated per point was 2.4 joules, totalizing 4.8 joules of final energy in the area of the injury [28].
After seven days the animals were weighed and then euthanized intraperitoneally by anesthetic overdose (Ketamine). The TA muscle was removed, and fragments of the medial third of the muscle removed and frozen using isopentane at -156°C, previously cooled in liquid nitrogen. The frozen material was stored in a freezer at -80°C.
After freezing, the TA muscle fragments were cut into 8 μm thick strips using a cryostat microtome (JUNG CM1800, Leica Germany) at -20°C and then stained with Picro-sirius Red [29] to perform the measurements of the area of fibrosis.

Analysis of the area of fibrosis

The picro-sirius stained slides were submitted to microscopy, and the images captured using an optical microscope coupled to a camera connected to a desktop computer containing the Leica application suite LAS 4.2.0 (Leica Microsystems, Switzerland). One image of the lesion area was captured per animal (one slide per animal), and then the entire cut was captured and the ratio of the diameter of the fibrosis area to the cut area was analyzed (the areas of fibrosis found in the TA muscle were corrected for the total area of the histological cut). After this phase, the images obtained were analyzed by Image pro plus software, version 6.0. The values are expressed in μm^2 [29].

Statistical analysis

For data analysis, the statistical software GraphPad Prism was used and to analyze the normality of the data, the Shapiro Wilk's test. In the comparison of

independent samples, the Mann Whitney test was used, due to the nonparametric distribution of the data. Data are expressed as median and interquartile range 25%-75%. The level of significance was 5% and test power 80%.

Results

There were no sample losses. In the injured (untreated and treated) groups, there was an increase in collagen synthesis, arranged in bundles in a disorganized and compact manner. In addition, the percentage of collagen in the injury area increased 57.3% and 63.6% in the injured and untreated group and the injured group treated with LILT, respectively (Data already published) [26]. The smallest lesion area (fibrosis) in the group treated with the laser corresponded to the highest percentage of collagen (63.6%).

Histological analysis of the injured muscle showed that there was no significant difference in relation to the fibrosis area of the LT group and L group (Table 1 and Figure 1).

Table 1 - Histological parameters (morphometric) of both groups. Data expressed as median and interquartile range 25-75%

	LT	L	p-value
Total area of the cut (μm^2)	178683 (132680-183937)	174746 (143596-192678)	0.9591
Area of the injury (μm^2)	33531 (19719-43789)	38945 (31493-43482)	0.6454

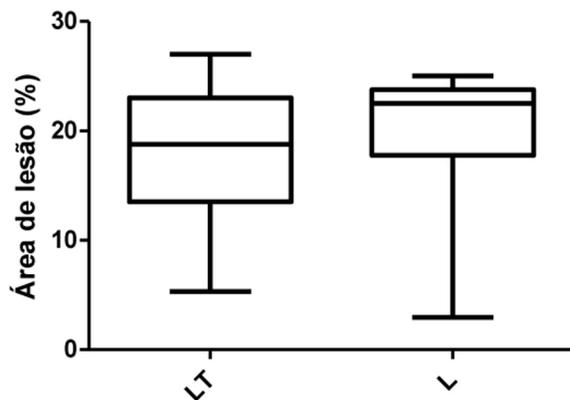Note: LT (injured treated group), L (injured untreated group). Mann Whitney Test.

Figure 1 - Box plot, median and interquartile range values (25%-75%) of the injured treated group (LT) and injured untreated group (L).

Discussion

The present study demonstrated that treatment with the Gallium Arsenide (AsGa) laser, 904 nanometers (nm), 50mW, and a final energy of 4.8 joules, applied for 5 consecutive days on the tibialis anterior muscle after cryoinjury, presented no influence on the decrease in the area of fibrous tissue.

The present study is highlighted for demonstrating the use of high energy with an AsGa laser, and although no statistical difference was detected in the results, clinical relevance can be observed in these findings.

The World Association of Laser Therapy recommends the use of final energy between 2 and 4 joules for the treatment of various tissues, including skeletal muscle tissue [25]. Laser is an excellent therapeutic option in the treatment of injuries in skeletal muscle tissue [11].

However, there is no consensus regarding the parameters of the laser, principally regarding the dose (energy density) and radiated energy. One study evaluated the parameters in equipment from different manufacturers and demonstrated variations in the final radiated energy, indicating that the energy density alone does not seem to be the best parameter for choosing the appropriate dose to be used in treatment, it is also important to observe the final energy radiated to obtain the best therapeutic result [23].

Experimental studies in animals have shown that low intensity laser therapy is able to reduce the inflammatory process, increase cellular mitotic activity, stimulate angiogenesis and collagen synthesis, and promote muscle regeneration [11, 7]. The possible mechanisms suggest that laser therapy can reduce oxidative stress and the production of reactive oxygen species [30, 13, 10], improve mitochondrial function [31], and activate microcirculation [32].

Recent studies with different types of lasers and radiation parameters have shown beneficial effects, specifically in the process of muscle regeneration modulating the inflammatory process [33, 34];

remodeling the extracellular matrix [11]; and stimulating the proliferation and differentiation of satellite cells [35].

Studies using LILT with different parameters (emphasizing that there is no consensus regarding the parameters used) of irradiation such as: wavelength, peak power, energy density, and radiated final energy in the injury area were shown to be beneficial in the synthesis and organization of collagen fibers; in the majority of these studies the final radiated energy varied between 1.4 and 3.2 joules [12-14].

According to Alves et al. [36], the synthesis of collagen in muscle tissue may be related to the type of laser and radiation parameters. Studies using the same type of laser (904 nm), energy density of 5 J/cm^2, observed that in the group injured and treated with the laser there was a significant reduction in collagen synthesis [37, 13]. Another study, also with the same type of laser and same energy density, in a muscle injury induced by anesthetics, demonstrated a reduction in the percentage of collagen and fibrosis [38]. In the present study, we chose to use a Gallium Arsenide laser (AsGa), 904 nm, 50 mW, 0.035 cm^2 (beam area), and final energy of 4.8 joules in the injury area and it was observed that LILT presented no influence on the area of fibrous tissue at the site of the injury, however with alterations that may be considered of clinical relevance as we observed that the injury area of the LILT group was smaller after the intervention.

The process of muscle regeneration is complex, organized, and coordinated. This can principally be divided into three interrelated phases which are: degeneration, inflammation, and regeneration, involving the repair or formation of new muscle fibers and collagen synthesis and remodeling, which lead to structural and functional recovery of the injured muscle [39]. The recovery of contractile capacity of skeletal muscle after injury depends on the balance between regeneration of muscle fibers and collagen synthesis [40].

Increased collagen synthesis induces the formation of fibrous tissue in skeletal muscle tissue after injury, impairing muscle contraction and predisposing to the appearance of pathological muscle contractures and, as a consequence, of chronic muscular pain, compromising normal muscle function [41].

LILT has been shown to be an effective resource in the modulation and formation of fibrous tissue during the tissue repair process after injury, by inhibiting the expression of TGF-β. This effect does not have a relation with the parameters of irradiation [10, 11, 14]. TGF-β expression reduction was observed after cryolesion in the anterior tibial muscle of rats using different radiation parameters; in the studies the radiated energy at the injury site was 1.4 and 1.6 joules, respectively. However, the synthesis of collagen seems to be related to the radiation parameters [11]. Other studies using a laser, with a 660 nanometer wavelength, red radiation, and radiated energy at the lesion site of 1.6 Joules, showed an increase in collagen deposition in the injury area [14, 42].

In contrast, Assis et al. and Alves et al. [10, 11], using a laser with wavelengths of 708 and 808, infrared radiation, radiated energy in the injury area of 1.4 and 3.2 joules, observed reduction and better organization of the collagen fibers at the injury site in rats. The results of Tidball et al. [39] show that using methodology similar to the present study (Gallium Arsenide laser, 904 nanometers, and final energy of 4.8 joules in the injury area), there was no significant difference in TGF-β expression between the injured and treated and injured and untreated groups or in the organization of the collagen fibers.

Although there was no statistically significant difference between the groups analyzed in the present study, which may be related to the sample size (study limitation), a smaller injured area could be observed in the treated group, concomitant with an increase in collagen synthesis, which may be considered clinically relevant. In this way, it is suggested that future studies are performed including an experimental design with a larger sample number and with placebo application in the injured control group (application with disconnected equipment), which could refine the experiment. In addition, other tools could be used such as determination of types of collagen with an immunohistochemical technique. In this way, it is believed that we can expand the knowledge and further expose the mechanisms indicated in the present study.

Conclusion

It is concluded that laser therapy using a Gallium Arsenide laser, final energy 4.8 joules, did not facilitate alterations in the collagen area of the skeletal muscle extracellular matrix.

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