



High final energy of gallium arsenide laser increases MyoD gene expression during the intermediate phase of muscle regeneration after cryoinjury in rats

Caroline Pereira Santos¹ · Andreo Fernando Aguiar² · Ines Cristina Giometti¹ · Thaoan Bruno Mariano¹ · Carlos Eduardo Assumpção de Freitas¹ · Gisele Alborghetti Nai¹ · Selma Zambelli de Freitas¹ · Maeli Dal Pai-Silva³ · Francis Lopes Pacagnelli¹

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Abstract

The aim of this study was to determine the effects of gallium arsenide (GaAs) laser on IGF-I, MyoD, MAFbx, and TNF- α gene expression during the intermediate phase of muscle regeneration after cryoinjury. 21 *Wistar* rats were divided into three groups ($n = 7$ per group): untreated with no injury (control group), cryoinjury without GaAs (injured group), and cryoinjury with GaAs (GaAs-injured group). The cryoinjury was induced in the central region of the tibialis anterior muscle (TA). The region injured was irradiated once a day during 14 days using GaAs laser (904 nm; spot size 0.035 cm², output power 50 mW; energy density 69 J cm⁻²; exposure time 4 s per point; final energy 4.8 J). Twenty-four hours after the last application, the right and left TA muscles were collected for histological (collagen content) and molecular (gene expression of IGF-I, MyoD, MAFbx, and TNF- α) analyses, respectively. Data were analyzed using one-way ANOVA at $P < 0.05$. There were no significant ($P > 0.05$) differences in collagen density and IGF-I gene expression in all experimental groups. There were similar ($P < 0.05$) decreases in MAFbx and TNF- α gene expression in the injured and GaAs-injured groups, compared to control group. The MyoD gene expression increased ($P = 0.008$) in the GaAs-injured group, but not in the injured group ($P = 0.338$), compared to control group. GaAs laser therapy had a positive effect on MyoD gene expression, but not IGF-I, MAFbx, and TNF- α , during intermediary phases (14 days post-injury) of muscle repair.

Keywords Gallium arsenide laser · IGF-I · MyoD · MAFbx · TNF- α gene expression · Muscle regeneration

Introduction

Muscle injuries are common events in sports and may cause functional disability and compromise occupational and leisure activities [1, 2]. Depending on the degree of the injury, the

regeneration process can take several days, or even weeks, for a complete muscle recovery [3]. Therefore, non-pharmacological strategies used to accelerate muscle recovery after injury are crucial to a functional rehabilitation of individuals engaged in recreational sports and performance-related activities [4].

Regarding this issue, several therapeutic resources have been proposed to improve muscle recovery after injury. In particular, low-level laser therapy (LLLT) has received special attention in the last decade due to its probable positive effects on skeletal muscle gene expression [5], inflammatory response [6, 7], satellite cells proliferation [8], and extracellular matrix remodeling [9]. Nevertheless, there is still no consensus on the best technique and irradiation parameters (e.g., dose, period, wavelength, energy density, and final energy) to maximize regenerative responses.

The most LLLT used in *in vivo* and *in vitro* studies are helium-neon (HeNe), gallium arsenide (GaAs), gallium arsenide

✉ Andreo Fernando Aguiar
afaguiarunesp@gmail.com

¹ Department of Physical Therapy, University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo, Brazil

² Center of Research in Health Science, North University of Paraná (UNOPAR), Avenue Paris, 675, Jardim Piza, Londrina, PR 86041-120, Brazil

³ Department of Morphology, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil

aluminum (AsGal), and Indio-gallium-aluminum-phosphide (InGaAlP), and the wavelength ranges from 660 to 830 nm [10–13]. However, to date, only few studies investigated the effects of GaAs laser on skeletal muscle regeneration, with some studies showing positive results [7, 14–16], but not all [17]. Moreover, these studies used different irradiation parameters (e.g., final energy) and periods of treatment (e.g., very long 21 days, or very short 3 h up to 5 days) after injury, making it difficult to establish a consensus regarding the best application duration of GaAs laser.

Our laboratory recently showed that application of GaAs laser during 5 days can improve muscle regeneration after cryoinjury in rats [7]. This beneficial effect of LLLT during the initial phase (i.e., 3–7 days) of regeneration appears to be associated with a decrease in proinflammatory cytokine expression (e.g., tumor necrosis factor α [TNF- α] and interleukin 6 [IL-6]) [18, 19] and increase in satellite cells markers (e.g., MyoD) [18]. However, the complete process of muscle regeneration generally requires 3–4 weeks [20], when multiple regeneration markers are involved, including myogenic regulatory factors (MRFs) (e.g., MyoD and myogenin), insulin-like growth factor-1 (IGF-I), TNF- α , and muscle atrophy F-box (MAFbx) [20, 21]. Therefore, it is important to identify whether GaAs laser therapy could also have beneficial effects during the more advanced stages of muscle regeneration (up to 3 weeks) when the major molecular markers of muscle regeneration are potentially involved.

To expand our previous findings with short-term GaAs therapy (5 days post-injury) [7], we analyzed the effects of GaAs laser therapy on collagen content and gene expression of IGF-I, MyoD, MAFbx, and TNF- α during the intermediate phase (14 days post-injury) of muscle regeneration after cryoinjury in rats.

Materials and methods

Animals and experimental groups

Male Wistar rats (120 days old, 244.8 ± 33.0 g) were obtained from the Central Animal Facility of the University of Western São Paulo (UNOESTE). They were kept in a temperature-controlled room (22 °C) with a 12-h light/dark cycle and provided with unlimited access to food and water. The animals were randomly divided into three groups ($n = 7$ per group): untreated with no injury (control group), cryoinjury without GaAs (injured group), and cryoinjury with GaAs (GaAs-injured group). All procedures were approved by the Ethics Research Committee of the University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo, Brazil (Protocol No. 713), and were conducted following the principles of laboratory animal care formulated by the Brazilian College of Animal Experimentation (COBEA).

Cryoinjury

The rats were anesthetized with ketamin (40 mg/kg, IP) and xilazin (20 mg/kg, IP). The right and left tibialis anterior (TA) muscles were surgically exposed and submitted to the cryoinjury procedure. A rectangular iron bar (0.8×0.8 mm²), previously frozen in liquid nitrogen for 30 s, was suavely applied to the surface of the central region (exposed) of the TA muscle and maintained in this position for 10 s. The procedure was repeated twice in both limbs, with a 30-s interval between applications, and the skin was sutured with nylon and cleaned with iodine alcohol [22]. The injured area was macroscopically identified as homogeneous, firm, white, disk-shaped region, as previously reported [19, 22].

Laser irradiation

A gallium arsenide (GaAs) diode laser (Endofoton-KLD Biosistemas®, Amparo, Brazil) with pulsed emission, wavelength of 904 nm, mean output power of 50 mW, and spot size of 0.035 cm² was used in the experiment (Table 1). Treatment was initiated 24 h after cryoinjury and performed during 14 consecutive days to evaluate the effects of GaAs during the intermediate stage of muscle regeneration. The irradiation time and final energy were automatically controlled by the previously calibrated laser equipment. The animals were manually restrained with the left hind limb positioned in extension. The laser pen was positioned at a 90° angle and placed directly on the skin (direct contact), on the surface of the middle region of TA muscle in both hind limbs. The energy density used was

Table 1 Parameters of gallium arsenide diode laser

Equipment model laser	Endofoton LLT 0107
Manufacturer	KLD Biosistemas; Amparo, São Paulo, Brazil
Active medium	Gallium arsenide (GaAs)
Wavelength (nm)	904
Energy density (J/cm ²)	69
Power density (W/cm ²)	1.42
Power (W)	0.05
Time of irradiation (s)	96
Spot size (cm ²)	0.035
Spot diameter (cm)	0.022
Emission mode continuous or pulsed	Pulsed
Number of points	Two
Area of application (cm ²)	1
Contact or not contact	Direct contact
Frequency of treatment	1 time per day- 5 consecutive days- every 24 h
Number of sessions	5
Accumulative dose (J/cm ²)	138

69 J cm⁻², applied in two spots of the injured area, during 48 s per point (2.4 J per dot), at a distance of 1 cm per point [23] to reach the entire area, promoting a final energy of 4.8 J. This dose and final energy were chosen based on a previous study using a GaAs laser in the early phase of muscle regeneration process [15].

Euthanasia

Animals were euthanized with an overdose of anesthetic (ketamine, IP), 24 h after last laser application. The left and right TA muscles were collected, weighed, and immediately frozen using n-hexane cooled in liquid nitrogen (-156 °C) for histological and molecular analyses. Muscles were stored at -80 °C until use.

Histological analysis

TA muscle histological sections (8- μ m thick) were obtained in a cryostat (JUNG CM1800; Leica, Wetzlar, Germany) at -20 °C and stained with picosirius red [24] for collagen density analysis. The stained sections were used for photographic documentation of three histological fields (\times 20 lens) from each animal, using a light microscope connected to a computer. The collagen density (%) was measured by the total area (μ m²) of collagen corresponding to three histological fields, using an image analysis system (software QWin Plus; Leica, Germany).

Real-time polymerase chain reaction (RT-qPCR) analysis

The total RNA was extracted from the TA (100 mg) using Trizol (Invitrogen), then treated with DNase to remove any DNA present in the sample, and quantified by measuring optical density (OD) at 260 nm. Degradation of RNA samples was monitored by the observation of appropriate 28 to 18 S ribosomal RNA ratios, as determined by the GelRed staining

of the agarose gels. A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA) was used for the synthesis of complementary DNA (cDNA) from 1000 ng of total RNA. The cDNA samples were stored at -20 °C and submitted to real-time PCR amplification. The reactions were run in duplicate using 0.4 μ M of each primer and 29 Power SYBR Green PCR master mix (applied biosystems). RT-PCR was used to quantitatively measure the relative levels of mRNA to IGF-I, MyoD, MAFbx, and TNF- α (Table 2). Cycling conditions were defined as follows: 50 °C during 2 min, 95 °C during 10 min, followed by 40 cycles of 95 °C during 15 s and 60 °C during 1 min in QuantStudio (applied biosystems). Control reactions were run lacking cDNA template to check for reagent contamination. Relative gene expression was calculated using the comparative CT method [25] and the control group was used as a reference. The choice of reference genes was experimentally determined according to Vandesompele et al. [26]. The qPCR data were imported into real-time StatMiner Software (Integromics, Spain), which calculates normalizing genes based on geNorm calculations. The normalization factor was obtained as the geometric mean of expression data of the three most stable reference genes (PpIa, PpIb and B2M). The normalization factor, generated by geNorm, using the most stable genes (PpIa and PpIb) was used to normalize the RT-qPCR data.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). One-way ANOVA tests were used to identify differences in body weight, muscle weight, fibers CSA, collagen density, and gene expression among experimental groups (control, injured, and GaAs-injured). Tukey *post hoc* test was used to identify the differences confirmed by ANOVA. The level of significance was set at $P < 0.05$. Statistical analyses were performed using SPSS statistical analysis software (SPSS version 20.0; Chicago, IL, USA).

Table 2 Primer sets

Gene	Forward primer	Reverse primer
IGF-I	TTTTTCATGCGACTCACAGC	GAAGGCAGGGCTTAAGTGTG
MyoD	TTTTTCATGCGACTCACAGC	GAAGGCAGGGCTTAAGTGTG
MAFbx	GACCTGCATGTGCTCAGTGAAG	GGATCTGCCGCTCTGAGAAGT
TNF- α	TGATCGGTCCCAACAAGGA	GGGCCATGGAAGTCTGATGAGA
PpIa	TCAACCCACCGTGTCTTCTC	ACTTTGTCTGCAAACAGCTCG
PpI	CAAGACCTCCTGGCTAGACG	CCGTACCACATCCATGCCTT
B2M	CGAGACCGATGTATATGCTTGC	GTCCAGATGATTCAGAGCTCCA

IGF-I insulin-like growth factor-1, *TNF- α* tumor necrosis factor-alpha, *PpIa* peptidylprolyl isomerase A (cyclophilin A), *PpIb* peptidylprolyl isomerase B (cyclophilin B), *B2M* beta-2-microglobulin

Results

Anatomical analysis

Body and muscle weight and relative muscle weight are shown in Table 3. There were no significant ($P > 0.05$) differences in initial body weight (IBW), final body weight (FBW), and muscle weight-to-body weight ratio (MW/FBW) among the control, injured, and GaAs-injured groups.

Collagen density

A representative picrosirius red staining analyzing the collagen density is shown in Fig. 1a–c, and the corresponding data are shown in Fig. 1d. There were no significant ($P > 0.05$) differences in collagen density among the control, injured, and GaAs-injured groups at 14 days after injury.

IGF-I, MyoD, MAFbx, and TNF- α gene expression

The gene expression data are presented in Figs. 2 and 3. There were no significant ($P > 0.05$) differences in IGF-I gene expression among the control, injured, and GaAs-injured groups after 14 days of the injury (Fig. 2). In addition, there was a significant ($P < 0.05$) increase in MyoD gene expression in the GaAs-injured group compared with the control group (Fig. 2). There were no significant ($P > 0.05$) decreases in MAFbx and TNF- α gene expression in the injured and GaAs-injured groups, compared to the control group (Fig. 3).

Discussion

The purpose of this study was to analyze the effects of GaAs laser therapy (904 nm, 50 mW, and high final energy of 4.8 J) on collagen content and muscle gene expression (i.e., IGF-I, MyoD, MAFbx, and TNF- α) during the intermediate phase (first 14 days) of muscle regeneration after cryoinjury in rats. The major findings of this study were that (1) there was a complete remodeling of the collagen content in both injured groups (with and without GaAs), (2) the IGF-I gene expression remained unchanged in both injured groups (with and without GaAs), (3) the MyoD gene expression was

significantly increased in GaAs-injured group, compared to control, and (4) the MAFbx and TNF- α gene expressions were reduced in both injured groups (with and without GaAs), compared to control.

Skeletal muscle repair is a complex process that includes four independent phases: (1) degeneration, (2) inflammation, (3) regeneration, and (4) fibrosis [19, 27]. Several molecular factors are involved in these four stages, and they are considered potential therapeutic targets for improving muscle repair, such as IGF-I, MyoD, MAFbx, and TNF- α [7, 19]. For example, it has been shown that local expression of a muscle-restricted IGF (mIGF-I) transgene accelerates the regenerative process of injured skeletal muscle by modulating the inflammatory response, remodeling connective tissue, and restoring functional properties [28]. In addition, mIGF-1 can improve muscle regeneration by increasing recruitment of bone marrow stem cells to sites of muscle injury [29]. IGF-I can also activate the myogenic stem cells (called satellite cells) during muscle regeneration/repair [30] by regulating the activity of MRFs family proteins, in particular MyoD and myogenin [30–32]. In light of these evidences, we expected an increase of IGF-I and MyoD gene expression after 14 days of the cryoinjury (injured group). However, there were no significant differences in IGF-I and MyoD gene expression between control and injured group. A possible explanation for these findings is that the repair process could be in its final stages, when IGF-I and MRF probably are not upregulated [20, 33, 34]. Despite this, the GaAs laser therapy increased MyoD gene expression, but not IGF-I, in GaAs-injured group compared to control group, indicating that GaAs therapy may be an efficient strategy to improve expression of regenerative myogenic makers. The increase in MyoD gene expression, but not IGF-I, suggests that the beneficial effects of GaAs laser may be more associated with modulation of MRFs than growth factors.

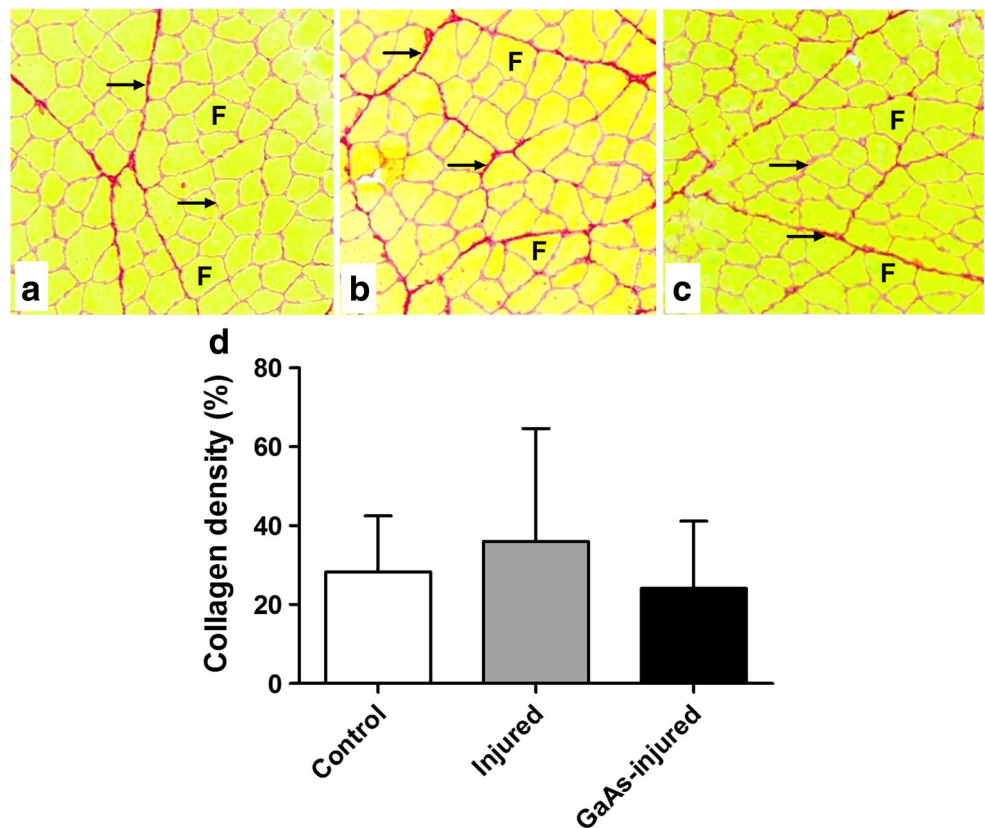
Consistent with our results, Rodrigues et al. [5] used an indium gallium aluminum phosphorus laser (660 nm, 40 mW) at of 50 J/cm² (2.0 J) and showed an increase in the MyoD gene expression after 7 days of the cryoinjury in the TA muscle. This beneficial effect of LLLT on MyoD expression appears not to be dose-dependent since the LLLT in the early phases of muscle repair (4–7 days after injury) had better effects at lower final energy (1.4–2.0 J) [5, 9] than higher final energy (3.0–4.8 J) [7, 15]. Although the World Association for

Table 3 Anatomical analysis

	Control ($N = 7$)	Injured ($N = 7$)	GaAs-injured ($N = 7$)	P value
IBW (g)	260.4 \pm 34.5	230.1 \pm 23.6	244.0 \pm 36.2	0.15
FBW (g)	314.8 \pm 42.4	277.2 \pm 27.2	276.5 \pm 42.1	0.07
MW/BW (mg/g)	2.10 \pm 0.13	2.10 \pm 0.12	1.97 \pm 0.20	0.16

Values are mean \pm SD. IBW initial body weight, FBW final body weight, Tibialis muscle weight MW, MW/FBW ratio. There were no significant differences among groups

Fig. 1 Collagen density (%) in the control (a), injured (b), and GaAs-injured (c) groups. Fiber (f); Collagen (→). Data are means \pm SD. There were no significant differences among groups



Laser Therapy (WALT) recommends final energy of 2 to 4 J for the treatment of muscle tissue [35], the optimal final energy of GaAs laser to maximize the activation of myogenic markers (e.g., MyoD) during muscle repair remains uncertain. Several GaAs laser-studies using different final energy (0.04 to 4.8 J) [14–16, 36–38] have shown improvement in muscle repair process, but these studies did not analyze the activation or expression of MRFs, particularly MyoD. Here, we showed for the first time that GaAs laser therapy using a high final energy of 4.8 J during the intermediate phase (first 14 days) of muscle repair might be efficient to improve MyoD gene expression. Nevertheless, it is important to note that the improvement in the muscle repair process with LLLT has been associated with no change [7, 15] or increase [5, 9, 18] in

MyoD expression, raising the question whether a further LLLT-induced increase in MyoD gene expression is, in fact, required for muscle repair. Although we did not analyze the morphological aspects of injured myofibers (e.g., number and area of regenerating fibers) after GaAs laser therapy, our results showed no positive effect of GaAs laser on collagen content. This indicates that the further GaAs-induced increase in MyoD gene expression may not contribute to the extracellular matrix remodeling during intermediary phases of muscle repair.

While it remains unclear whether GaAs-induced increase in MyoD expression may contribute to the muscle repair, it is of particular interest to understand what factors are associated to this increase in MyoD expression during muscle regeneration process. Previous studies have shown that Muscle

Fig. 2 IGF-I (a) and MyoD (b) gene expression in the control, injured, and GaAs-injured groups. Data are means \pm SD. * $P < 0.05$ compared to control

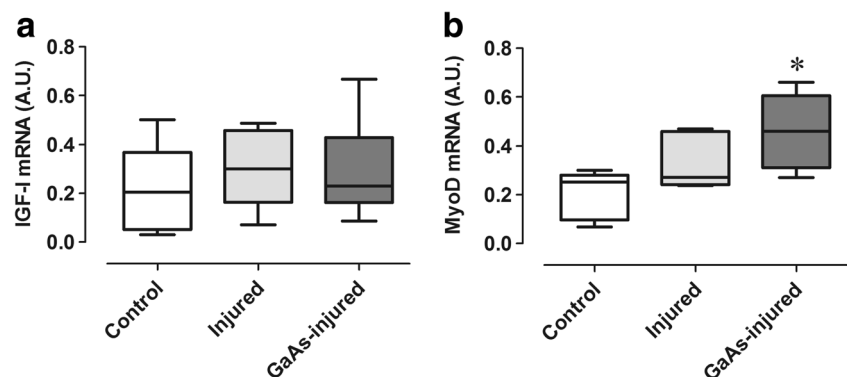
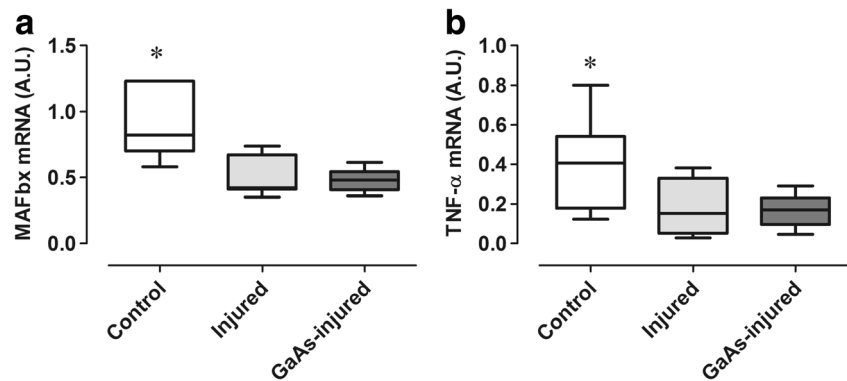


Fig. 3 MAFbx (a) and TNF- α (b) gene expression in the control, injured, and GaAs-injured groups. Data are means \pm SD. * $P < 0.05$ compared to injured and GaAs-injured groups



Atrophy F-box (MAFbx) may mediate the degradation of MyoD during muscle regeneration [39, 40] and that a downregulation in MAFbx may be required to increase MyoD expression in this process [40]. Indeed, it has been shown an increase in MyoD mRNA expression [41] and decrease in MAFbx mRNA expression [42] during muscle recovery after resistance exercise (8 h after exercise) in humans. Consistent with these studies, we found a decrease in MAFbx mRNA expression and reciprocal increase in MyoD gene expression in the injured and GaAs-injured groups compared to control (although not statistically significant in the injured group) after 14 days of the cryoinjury. However, it is important to note that this further increase of MyoD gene expression was not accompanied by an additional reduction of MAFbx gene expression in the GAAs-injured group. Therefore, there is likely to be a downregulation limit of MAFbx gene expression during the intermediate phase (first 14 days) of muscle regeneration and that MyoD expression may continue increasing even when this threshold is reached. It remains unknown whether other mechanisms could be regulating the increase in MyoD gene expression when this downregulation limit of MAFbx expression is reached.

In addition to MAFbx, TNF- α has also been shown to decreased MyoD protein stability and abundance [43, 44]. This result is consistent with the muscle atrophy in skeletal myocytes exposed to TNF- α [45] due to activation of nuclear factor κ B (NF κ B)—a transcription factor promoter of proteolysis [20]. Thus, a decrease in TNF- α expression could enhance muscle repair by increasing MyoD expression and thus facilitating muscle cell differentiation. However, we have previously shown that the decrease in TNF- α gene expression with GaAs laser 5 days post-injury was associated with improved repair of myofibers (e.g., higher number of regenerating fibers) but not with increased MyoD gene expression [7]. These results suggest that GaAs-induced improves in myofiber repair may be more associated with decreased TNF- α gene expression than increased MyoD gene expression. However, our results showed no difference in TNF- α gene expression between GaAs-injured group and injured group 14 days

post-injury. Previous studies have shown a reduction in TNF- α gene expression 7 days post-injury [7, 19] but not 14 days post-injury [19], indicating a possible time-dependent effect of GaAs laser on decreasing TNF- α gene expression. A possible explanation for the lack of effect of GaAs laser on TNF- α gene expression may be the fact that muscle morphology (e.g., increase in fiber diameter and decrease in inflammatory infiltrate, and collagen remodeling) is almost entirely restored at this time [46].

In conclusion, our results indicate that GaAs laser therapy has a positive effect on the MyoD gene expression, but not IGF-I, MAFbx, and TNF- α , during intermediary phases (14 days post-injury) of muscle repair. However, this effect may not be associated with decreased TNF- α and MAFbx gene expression and improved collagen remodeling. This was a preliminary study, and further studies are needed to confirm whether the increased MyoD exhibits a causal relationship with the decreased TNF- α and MAFbx and improved morphological adaptations during muscle repair.

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Compliance with ethical standards

Conflicts of interest The authors declare that there is no conflict of interest.

Ethical approval All procedures were approved by the Ethics Research Committee of the University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo, Brazil (Protocol No. 713).

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