Evaluation by fractal dimension of muscle regeneration after photobiomodulation

Avaliação por meio da dimensão fractal da regeneração muscular após fotobiomodulação

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Abstract

Introduction: Many treatment modalities are used for muscle tissue recovery. Photobiomodulation is a modality that can be employed to improve the quality of tissue repair. The use of fractal dimension (FD) is an innovative methodology in the quantitative evaluation of treatment efficacy. Objective: Use FD as a quantitative analysis method to evaluate the effect of photobiomodulation of 904 nanometers (nm) in the initial phase of the muscle regeneration process. Method: Thirty male Wistar rats were divided into three groups: Control Group (CG), Injured and Untreated Group (IUT), and Injured and Treated Group (IT). Muscle injury was induced by cryoinjury in the central region of the anterior tibial (AT) belly of the left posterior limb. This was performed by an iron rod that was previously immersed in liquid nitrogen. Applications started 24 hours after the injury and occurred daily for five days. They were performed at two points in the lesion area. The rats were euthanized on the seventh day. The AT muscles were removed and frozen in liquid nitrogen. Then, the histological sections were stained using the Hematoxylin-Eosin (HE) technique and submitted
to FD analysis performed by the box-counting method using ImageJ software. The Kolmogorov-Smirnov test was used for data normality, and the Kruskall-Wallis test and Dunn's post-test were used for group comparison (p<0.05%). Results: Differences between IT and IUT groups were statistically significant, and it was possible to observe the reduction of fractability with p=0.0034. Conclusion: FD is a useful tool for the analysis of skeletal muscle disorganization in the initial phase of regeneration and confirms the potentially beneficial effects of photobiomodulation to this process.

Keywords: Analyze. Fractals. Laser Therapy. Regeneration. Striated Muscle.

Introduction

Muscle lesions frequently occur in sports. They are commonly caused by injuries or strains [1, 2]. They are one of the most common health problems in Brazil. Injuries to muscles account for approximately 90% of medical procedures in the country. This is worrying not only from an epidemiological point of view, but also in relation to the health system. Such procedures are often expensive and performed by the public health system (Sistema Único de Saúde, SUS) [3].

Striated skeletal muscle has a high capacity for morphological, metabolic, and functional adaptation in response to different stimuli. It demonstrates excellent capacity for regeneration after injuries from physical activity or various muscle diseases [4 - 6]. Muscle regeneration is a complex and synchronized process that involves a wide variety of cells. It occurs in several different stages that include degeneration, inflammation, regeneration, and remodeling [7 - 9].

Effective muscle recovery after injury and the maintenance of mechanical properties are important factors to the complete functionality of a patient. This is particularly the case for athletes, amateur or professional [4]. Several therapeutic resources have been used in the treatment of different conditions that affect skeletal muscle tissue. Among these resources, photobiomodulation stands out [9 - 14].

Photobiomodulation is a form of low power electromagnetic radiation with a wavelength between 600-1200...
Fractal dimension has been used to evaluate several types of tissues. However, studies that evaluate muscle tissue with photobiomodulation and FD are still scarce. Thus, there is a necessity for new studies [22, 23]. Photobiomodulation application optimizes the tissue regeneration process, and FD provides an ideal quantitative method for a reliable assessment of this therapeutic resource. Therefore, the objective of this study was to use FD as a quantitative analysis method concerning the effect of photobiomodulation of 904 nm during the initial stage of the muscle regeneration process.

Method

Ethical aspects

This study was developed after approval by the Committee on Ethics of the Use of Animals (CEUA). The procedures related to sample selection, experimentation protocol, euthanasia, and histological slides were performed at the Botucatu Campus of Sao Paulo State University (UNESP) and approved under protocol 713.

In this study, the slides were analyzed by FD at Campus 1 of the University of Western Sao Paulo (UNOESTE) in Presidente Prudente and approved by the UNOESTE Ethics and Research Committee under protocol 5075. The study followed the recommendations of Animal Research: Reporting of In Vivo Experiments - ARRIVE [24].

Rat Models and Experimental Group

The experiment used 30 male Wistar rats (Rattusnorvegicus) with approximately three months of age. The rats weighed between 210 and 340 grams. They came from the UNOESTE vivarium. Sample numbers were based on previous studies with similar experimental designs [8]. During the study, the rats were kept in individual cages in a temperature controlled room (22 to 24 °C) with 12 hour light/dark cycles. They had access to food and water ad libitum. The animals were randomly divided into three groups of 10, as shown below:

1) Control Group (CG): the rats were not injured or submitted to treatment.
2) Injured and Untreated Group (IUT): the rats were injured but not subjected to photobiomodulation therapy treatment.
3) Injured and Treated Group (IT): the rats were injured and subjected to photobiomodulation therapy treatment.

Injury Model

After group selection, all the animals were anesthetized intraperitoneally with 1 ml/kg of 1% ketamine HCl (Dopalen, Vetbrands, Sao Paulo, Brazil) and 2% xylazine (Anasedan, Vetbrands). Following anesthesia, trichotomy of the left posterior limb was performed. A longitudinal cut of 1 cm was made on the skin adjacent to the AT for its exposure. Cryoinjury was performed using a rectangular iron bar (0.8 X 0.8 mm²) that was previously frozen in liquid nitrogen for 30 seconds. Cross contact was then maintained for 10 seconds in the belly of the AT. This process was repeated twice consecutively with a 30 second interval between repetitions [25]. The skin was then sutured, and the rats were placed individually in plastic cages in a temperature controlled room.

Photobiomodulation treatments

Treatment began 24 hours after the cryoinjury, and five consecutive applications were performed for five
days. There was an interval of 24 hours between applications. The rats were manually restrained and kept with the injured limb in extension. Photobiomodulation (KLD Biossistemas®, Amparo, Brazil) of 904 nm was then used. The photobiomodulation was previously calibrated by the manufacturer. It was kept on pulsed mode with a 904 nm wavelength, an output power (average) of 50 mW (milliwatts), a density power of 1.42 W/cm², and a beam emission area of 0.035 cm². The laser was applied by direct contact on the skin in the damaged area at an angle of 90° in the irradiated surface directly in the muscle belly of the AT (Figure 1). The energy density was at 69 J/cm². It was applied at two points over the lesion and at a distance of 1 cm per point to reach the entire lesion area [26]. The irradiation lasted for 48 seconds at each point, and the final energy was irradiated by a point of 2.4 joules, totaling 4.8 joules of final energy in the lesion area [27]. The rats in the control group received the same application. However, the equipment was turned off.

Figure 1 - Photobiomodulation application procedure.

After seven days, the animals were weighed and then euthanized by an anesthetic overdose of an intraperitoneal injection (Ketamine). AT muscle was then removed. A fragment of the middle third of the muscle was removed and frozen with isopentane at -156 °C that was previously cooled in liquid nitrogen. The frozen material was stored in a freezer at -80 °C. The AT muscle fragments were then cut to widths of 8 µm using a cryostat microtome (JUNG CM1800, Leica, Germany) at -20 °C. They were then subjected to the HE staining technique.

Fractal Dimension

For FD, three fields of each slide were photographed per rat. The HE images were subjected to the binarization process. This requires the photos with normal colors to be converted to black and white. This makes it possible to perform fractal analysis. The colors were then inverted, which left only the black cell nuclei. This is a necessary procedure, as fractal analysis measures only the black area of the image [22, 23].

Fractal dimension analysis was later performed blindly by the box-counting method. This permits pixel quantitation in a given space without consideration to image texture. Thus, two images (binarized and gray scale) can have the same result in the fractal analysis. The calculation is performed using the formula $DF = \log N_r / \log \frac{1}{r}$, where $N_r$ is the amount of pixels needed to fill the image, and $r$ is the scale applied to the image under the microscope. After the calculation, the results may vary from 0 to 2. Values closer to 2 indicate that the evaluated tissue presents a greater state of cellular disorganization [28-30]. All procedures were performed using Image J software with the addition of the Fractal Dimension and Lacunarity plugins, all of which are freely available on the internet [22, 23].

Analysis of results

An electronic database was created to analyze the results. The evaluator was blinded during the analysis. The Kolmogorov-Smirnov test was used to determine sample normality. When data normality was violated, the Kruskall-Wallis test and Dunn’s post-test were used to assess the differences between groups. All analyzes were performed with a significance level of 5%. GraphPad® software (Graph-Pad Software, La Jolla, California) was used for analysis purposes.

Results

A significant difference in fractability was observed between the IUT and IT groups (Figure 2). This was closer to zero in the treated group, suggesting greater
tissue organization. Figures 3, 4, and 5 show images of the fractability process of each studied group.

**Figure 2** - Values expressed as the median and interquartile range (25% - 75%) of the Control Group (CG), Injured and Untreated Group (IUT), and Injured and Treated (IT) Group, p = 0.0034.

**Figure 3** - The scheme describes the fractalization process shown in the images. Note: Image A shows HE stains of the CG with 40x magnification. Image B shows the same slide with increased brightness. Image C shows the binary image in black and white. Image D shows the result after the box-counting method (D = 1.0887).

**Figure 4** - The scheme describes the fractalization process shown in the images. Note: Image A shows HE stains of the IUT group with 40x magnification. Image B shows the same slide with increased brightness. Image C shows the binary image in black and white. Image D shows the result after the box-counting method (D = 1.3700).

**Figure 5** - The scheme describes the fractalization process shown in the images. Note: Image A shows HE stains of the IT group with 40x magnification. Image B shows the same slide with increased brightness. Image C shows the binary image in black and white. Image D shows the result after the box-counting method (D = 0.8539).

**Discussion**

To our knowledge, this is the first study that used FD as a quantitative method to analyze the effects of photobiomodulation of 904 nm in the initial phase of muscular regeneration. The significant reduction of fractability in IT group cells is the most notable finding.

The statistical difference observed between the IT group and IUT group confirms the effectiveness of this resource for muscle injury treatment during the acute phase and solidifies its relevance in clinical practice [31].

Studies using gallium-aluminum-arsenide lasers for the muscle regeneration process have demonstrated reductions in inflammatory infiltrates and improvements to extracellular matrix reorganization after seven days of application [19, 21]. Other studies with
photobiomodulation have shown its efficacy in angiogenesis, and in the proliferation and differentiation of satellite cells [16]. Garcia et al. used a diode laser for the application of platelet-rich plasma, which resulted in the improvement of histological aspects in muscle regeneration [14].

Fractal dimension is a low cost and innovative tool that quantifies cellular and tissue disorganization. It is involved in important clinical applications of histopathological studies in the areas of neurology, ophthalmology, and hepatology [28, 32, 33]. Fractal dimension has also been evaluated in the analysis of cardiac and skeletal muscle [22, 29, 30, 34]. In cardiac muscles of rats with monocrotaline-induced pulmonary hypertension, FD showed an average value of 1.37 in the control group and an average value of 1.43 in the group with the disease. The small difference in the data of this study and our study data may be related to the muscle type, as the fractal evaluation performed by the histological HE technique in the heart identified the nuclei in the cross section and longitudinal section [29]. Fractal dimension has also been used in skeletal muscle for the characterization of fiber types, muscle fatigue evaluation, and muscle strain injuries [35-37]. A study evaluating soleus muscle of dystrophic mice showed that FD is an effective tool to analyze tissue disorganization resulting from this condition. The same study revealed fibroblast-myoblast changes that contribute to myoblast dysregulation observed in vitro. FD values had an average of 1 in the control group rats on slides stained with HE. The dystrophic rates had an average of 3. These values were very similar to those of our study, which found tissue disorganization [28].

Another study that involved skeletal muscle verified inflammatory infiltrate sizes at the injury site by measuring the nucleus and alterations of the inflammatory process [35]. In addition to the identification of tissue complexity, FD allows for the quantification of structural changes in acute inflammation [38]. These findings confirm the result of the present study and highlight the ability of FD to quantify the effect of photobiomodulation in the initial phase of the muscle regeneration process.

Garcia et al. found qualitative differences in histological slide comparisons while using a diode laser with a wavelength of 637 nm, 25 mW of power, a 1 mm beam diameter, a continuous emission for 10 seconds, and a dose of 0.25 J / 0.00785 cm applied in a single point for seven days. However, the study was unable to confirm a quantitative difference in the inflammatory process during slide analysis when using FD [22]. It is possible to observe the influence of the examiner’s bias on the slides when examining such findings. Such a factor, which does not occur with FD, may have influenced the incompatibility of the results. Also, the wavelength and parameters used in the intervention were different from those of our study. This same study by Garcia et al. used the application of isolated platelet-rich plasma in association with the diode laser. The fact that this method is not sensitive to the differentiation of applied groups was mentioned in the slide analysis section of the study [22].

After a muscle strain injury, Ozaki et al. used FD to analyze collagen with the picrosirius staining technique in gastrocnemius muscle. Fractal dimension identified and quantified collagen alterations. Control group values had an average of 1.2. Average values of 1.3 were observed two days after the injury. There was a significant drop on the third day (averages of around 1) when compared to the second day. Fractal dimension was unable to identify the different collagen fibers present on the slides due to the fact that all the fibers with different colors after binarization could be seen only in black. This can be considered a limitation to the technique [35].

In a study by Martins et al., cryotherapy with pulsed ultrasound therapy was used as a tissue regeneration treatment technique after musculoskeletal contusions in rats. This combination reduced the area of the injury and oxidative stress in muscles, and increased catalase production and antioxidant capacity against peroxyl radicals. This contributed to a reduction to adjacent injuries and significantly promoted tissue regeneration [39].

We did not use the gait assessment to evaluate the functionality of the affected limb in this study, which denotes the importance of other studies that use this approach and its correlation with FD.

Fractal dimension quantifies the degree of complexity of the image through the distribution of the nucleus in the tissue. It analyzes the slides as a whole without differentiating the nuclei of myocytes from inflammatory infiltrate cells. Despite demonstrating statistical significance, the present study is limited by this lack of differentiation.

Conclusion

Our results demonstrate that FD is a useful tool for the analysis of skeletal muscle disorganization in the
initial regeneration phase and reveal the potentially beneficial effect of photobiomodulation in this process.

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