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Fractal dimension in the evaluation of different treatments of muscular injury in rats

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ABSTRACT

Objectives: To evaluate alterations from different therapies in muscular injury using the Fractal Dimension (FD) method.
Methods: 35 animals were allocated in Control Group (C), Injury Control Group (IC), Injury Low Level Laser Therapy Group (ILT), Injury Platelet Rich Plasma Group (IP), and Injury LLLT and PRP Group (ILP). The animals suffered a stretch injury in gastrocnemius muscle and after that IP and ILP groups received PRP application. The ILT and ILP groups received daily LLLT applications for seven days. After seven days the animals were euthanized and the gastrocnemius muscle removed and frozen. The muscles were stained with Hematoxylin and Eosin (HE) and Picrosirius Red, for observation of the morphology of the injury and semi-quantitative and quantitative analysis through the Fractal Dimension (FD) method.
Results: In the qualitative and semi-quantitative analysis, in relation to IC group, the ILT presented a reduction in rounded fibers and the IP in angular fibers. The ILP group demonstrated a reduction in both polymorphic fibers and inflammatory infiltrate. The FD of the muscles stained with HE was higher in the groups that suffered the injury when compared to the C group ($p < 0.05$); the FD of the collagen demonstrated no statistical difference between the groups.
Conclusion: Both treatments were able to accelerate injury repair, and the association of both presented better results than the isolated applications. However, the FD method showed no sensitivity to differentiate the treatments, either in the histological aspects or the injury in collagen.

1. Introduction

Muscle tissue injuries are a common problem in every population. These can be caused by work, sports, or accidents and due to their frequency more efficient treatments are required (Edouard et al., 2016; Astur et al., 2014). Among the principal treatments for these lesions are low intensity laser therapy (LLLT) and platelet rich plasma.

LLLT decreases inflammation and oxidative stress in muscle injury, in addition to increasing the production of growth factors responsible for angiogenesis and myogenesis (Silveira et al., 2016; Adabbo et al., 2016). On the other hand, PRP contains in its composition a concentration of anucleated cells that release cytokines (TGF-1β), myogenic factors (MyoD1, MYF5), and growth factors (IGF-IEb) responsible for activating muscle repair and regeneration pathways during the process of inflammation (Dimauro et al., 2014; Li et al., 2016).

Evaluating the degree of injury and efficiency of treatments with precision has presented a challenge, since the majority of histological analysis methods are qualitative, and depend on the degree of instruction of the evaluator. In this context, fractal dimension (FD) presents itself as a viable tool to evaluate the cellular morphology with the minimum of interference from the evaluator.

FD is a mathematical measure that quantifies the complexity of a figure of irregular (non-Euclidean) geometry. In this way it fits as an ideal and reproducible tool to deal with the complexity and irregularity of muscle cells.

Analysis of fractal objects is based on the relationship between the resolution and scale at which the object is measured. The results can be expressed quantitatively by the equation:

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FD = (Log Nr / log r-1)

In this formula "Nr" is the number of elements necessary to overlap or fill the original object, "r" represents the scale applied to the object, and "FD" is the dimension of the structure or object.

The FD can be calculated in several ways, the “box-counting method” being the most common in histology. In this method the image is covered with progressively smaller squares of "r" sides and thus "Nr" is the number of squares of side "r" needed to cover the image, for each chosen size. The FD will then be obtained by the slope of the regression line of the two log-values, that is, the size of the "r" side and the number of squares “Nr”(7).

FD has been used in many ways, be it to analyze tissues such as neurons, retina, and bronchi, or detect carcinomas and level of rejection of cardiac cells after heart transplantation (Moreira et al., 2011; Ristanović et al., 2014; de Melo de Mendonça et al., 2007; Gupta et al., 2014; Lee et al., 2014). However information on its use for measurement of different types of treatment for muscle injury is still scarce. This technique could present a more sensitive and effective method to evaluate the degree of injury and consequently the degree of muscle, independent of the evaluator.

Therefore the objective of the present study is to verify the differences in different treatment protocols in the muscle injury of rats using the fractal dimension method.

2. Methods

Thirty-five male Wistar (Rattus novergicus) rats (aged 150 days) were used, acquired from the central Bioterium of Paulista State University (UNESP), Botucatu-SP Campus (Brazil). They were maintained at the bioterium of the Histology and Histochemistry Laboratory at the Faculty of Science and Technology, Presidente Prudente (FCT/UNESP), in collective cages (polyethylene), at a controlled temperature (22 ± 2 °C), humidity (50 ± 10%), and 12-hour light/dark cycle with access to food (standard laboratory chow) and water ad libitum.

All procedures were approved by the ethics committee for animal use from FCT/UNESP, Presidente Prudente (SP, Brazil) campus, protocol no. 01/2013.

2.1. Experimental groups

The animals were randomly divided into five groups: Control (C); Control Injury (IC); Injury and Low Level Laser Therapy (ILT); Injury and Platelet Rich Plasma (IP); Injury with both LLLT and PRP (ILP). The animals in group C remained in the bioterium and were euthanized paired with the other groups. The animals in groups IC, ILT, IP, and ILP suffered the muscle injury and were euthanized seven days after injury. The ILT group animals received laser application daily for seven days. The IP animals received PRP application immediately after the injury. The ILP group received application of both protocols mentioned above.

2.2. PRP preparation

The animals of the IP and ILP groups underwent cardiac puncture for preparation of PRP and were then submitted to the muscle injury protocol. Blood collection was performed through cardiac puncture in the animals of the IP and ILP groups. The animals were submitted to anesthesia by intraperitoneal administration of ketamine (70 mg/kg) and xylazine (15 mg/kg), and after confirmation of anesthesia a cardiac puncture was performed using a 0.2 ml disposable syringe containing sodium citrate at 10%, 4 ml of blood was obtained from each animal. Immediately after the puncture, saline solution was injected to restore blood volume (Li et al., 2012).

The collected blood was centrifuged at 200 *g* for 15 min, splitting the sample into three parts: red bottom fraction, composed primarily of red blood cells; intermediate yellow-straw fraction (buffy coat), with the serum component; and the top fraction, composed of the blood plasma. The top fractions were pipetted, including the buffy coat, and the pipetted contents were centrifuged again at 500 *g* for 10 min. Next, 0.2 ml of the bottom content PRP was pipetted.

Blood and PRP samples were analyzed in the laboratory of the Veterinary Hospital of the Universidade do Oeste Paulista (UNOESTE) by means of an automatic blood cell analyzer (pocH 100iy Diff, Sysmex®). The analysis was performed on two blood samples and three PRP samples, for confirmation of platelets.

2.3. Muscle injury protocol

In the IP and ILP groups the muscle injury was performed immediately after cardiac puncture, avoiding the application of new doses of anesthesia. In the IC and ILT groups, the animals received an intraperitoneal injection of xylazine and ketamine, as described above. After confirmation of anesthesia, each animal was placed on the damage inductor equipment, in a supine position, with the hip in slight flexion, knee extension, and ankle in plantar flexion, the right leg attached to the machine with adhesive tape (duct tape). After positioning the animal in the equipment, two electrodes were placed on the paw, on the calcaneal tendon and popliteal fossa of the animal, respectively. Electrical stimulation was applied suddenly to the positioned animal until full contraction of the lower limb in plantarflexion. Immediately afterwards, the equipment was fired, which promoted abrupt dorsiflexion of the lower limb while it was stimulated, the electrical current was stopped immediately after dorsiflexion. The dorsiflexion stimulation caused by the equipment and interruption of the current, in total, took an average of 2 s to complete. This procedure was repeated until totaling 10 series, with a 30 s interval between applications. In each series 2.25 J was released, totaling 22.5 J of energy applied to the muscle injury. This protocol was adapted from Pachioni et al. (2009).

2.4. PRP application

In animals of the IP and ILP groups, 100 µl of PRP was injected using a sterile syringe. The syringe with the needle was placed on the injured limb in the distal third of the tibia in order to be applied in the belly of the gastrocnemius muscle. The application of PRP was performed within six hours of the muscle injury protocol and withdrawal of blood (Hammond et al., 2009).

2.5. Low level laser therapy application

LLLT was applied by means of diode laser equipment (Coherent, Laser Cube) and previously calibrated with a wavelength of 637 nm, power of 25 mW, beam diameter of 1 mm, and continuing emission for 10 s. Therefore, the dosage was 0.25 J/0.00785 cm (Astur et al., 2014) or 31.85 J/cm (Astur et al., 2014). The laser was applied to a single point, daily, starting on the day of injury until the completion of seven applications (Iyomasa et al., 2009; Luo et al., 2013). The laser was applied at the same point as the PRP injection, both to the muscle belly region.

2.6. Histological process and analysis

After the experimental period, the animals were euthanized with an overdose of anesthetic xylazine and ketamine. Next, the right gastrocnemius muscle was removed from each animal and immersed in n-hexane; cooled in liquid nitrogen, using the freezing method for non-fixed tissues (JCSC et al., 2011); and stored in an ultra-low temperature freezer, Coldlab CL580-80 V, at −75 °C. The slides for histological analysis were stained with the hematoxylin and eosin (HE) and picrosirius methods (Castoldi et al., 2013).

Histological analysis was performed in a qualitative and semi-quantitative way on the slides stained with HE, using a Nikon Eclipse