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Effect of ultrasound therapy on the repair of Gastrocnemius muscle injury in rats

Maria Cristina Balejo Piedade [a], Milene Sanches Galhardo [a], Cláudia Naves Battlehner [b], Marcelo Alves Ferreira [b], Elia Garcia Caldini [b], Olga Maria Szymanski de Toledo [a,*]

[a] *Discipline of Histology and Structural Biology, Department of Morphology, The Federal University of São Paulo, Rua Botucatu 790, 04023-062 São Paulo (SP), Brazil*
[b] *Laboratory of Cell Biology (LIM 59), Department of Pathology, The University of São Paulo School of Medicine, São Paulo, Brazil*

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Abstract

The aim of this study was to evaluate the effect of the pulsed ultrasound therapy (PUT) in stimulating myoregeneration and collagen deposition in an experimental model of lacerative gastrocnemius muscle lesion in 30 Wistar rats. Fifteen rats were treated (TG) daily with 1 MHz pulsed ultrasound (50%) at 0.57 W/cm^2 for 5 min, and 15 were control animals (CG). Muscle samples were analyzed on postoperative days 4, 7 and 14 through H&E, Picrosirius-polarization and immunohistochemistry for desmin. The lesions presented similar inflammatory responses in both treated and control groups. The areal fraction of fibrillar collagen was larger in the TG at 4 days post-operatively ($17.53 \pm 6.2\%$ *vs* $6.79 \pm 1.3\%$, $p = 0.0491$), 7 days ($31.07 \pm 7.45\%$ *vs* $12.57 \pm 3.6\%$, $p = 0.0021$) and 14 days ($30.39 \pm 7.3\%$ *vs* $19.13 \pm 3.51\%$, $p = 0.0118$); the areal fraction of myoblasts and myotubes was larger in the TG at 14 days after surgery ($41.66 \pm 2.97\%$ *vs* $34.83 \pm 3.08\%$, $p = 0.025$). Our data suggest that the PUT increases the differentiation of muscular lineage cells, what would favor tissue regeneration. On the other hand, it is also suggested that there is a larger deposition of collagenous fibers, what could mean worse functional performance. However, the percentage of fibers seems to have stabilized at day 7 in TG and kept increasing in CG. Furthermore, the collagen supramolecular organization achieved by the TG is also significant according to the Sirius red staining results.

Keywords: Myoregeneration; Fibrosis; Collagen; Myotubes; Desmin; Morphometry

1. Introduction

Despite having different injury mechanisms, the process of muscle repair follows a rather constant pattern. The histopathological pattern of the three phases of the healing process of an injured muscle (destruction, repair, and remodeling) has already been described in detail [1].

Even though the muscle retains its ability to regenerate following injury, muscle healing has been found to be very slow, sometimes, depending on the severity of the muscle injury, with an incomplete muscle recovery. Because fast and complete repair of the injured muscle is the obvious target, one challenging problem in traumatology and in sports medicine is to find clinically feasible treatment modalities that enhance the cell proliferation phase and prevent the occurrence of fibrosis during the reparative process [2].

Therapeutic ultrasound (US) is one of the most frequently used treatment modalities for a variety of skeletal muscle injuries. In spite of over 60 years of a wide range of clinical use, authors affirmed that it is difficult to provide sufficient evidence to establish the clinical efficacy of ultrasound therapy [3]. *In vitro* studies of fibroblast culture reveal that the benefit of using US could be related to its stimulation on collagen deposition [4] and there are

* Corresponding author. Tel.: +55 11 55764268; fax: +55 11 55764271.
E-mail address: olmatoledo.morf@epm.br (O.M.S. de Toledo).

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experimental evidences that US increases collagen fibers synthesis and organization in tendon repair [5].

Specifically concerning the effectiveness of US for treating muscular injuries, it has been shown that pulsed US enhances both myogenic precursor cell and fibroblast proliferation in an experimental contusion injury to the rat gastrocnemius muscle [6]. Moreover, it has been shown that US treatment improves muscle extensibility [7] and force production after contraction-induced muscle injury [8]. However, other authors using an experimental animal model were unable to demonstrated statistically significant increase of muscle mass, total protein concentration or cross section area of myofibers in the US treated lesions [9]. This controverse supports the idea that there is a lack of scientific evidence on the effectiveness of US in muscle healing enhancement [10].

Considering the above-mentioned evidences that US stimulates collagen deposition *in vivo* and *in vitro*, and also stimulates proliferation of myogenic precursor cells, it was thought to be of interest to proceed to a quantitative evaluation of the balance between myogenic cells and fibrillar collagen in a muscle laceration treated by US, considering that the muscle recovery after injury can turn to be incomplete in cases of excessive collagen deposition [2]. This task was undertaken and, in order to know the tissue area occupied by these tissue components, we applied morphometric techniques in association with the Picrosirius-polarization method according to a classical study in gracilis muscle injury [11] and with an immunohistochemical method for desmin detection [1]. All these techniques were carried out in tissue sections obtained from US treated and control lesions at three post-injury time spans.

2. Materials and methods

2.1. Subjects

The Ethics Committee of the São Paulo Federal University (UNIFESP) reviewed and approved the procedures of this study (# 0300/04). Adult male Wistar rats (approximately 90 days old and weighting 350–400 g) were used. The rats were randomly assigned into six groups (Table 1).

2.2. Surgical procedure

All surgical procedures were carried out under general anesthesia induced by intraperitoneal injection of 100 mg/kg ketamine (Vetbrands, São Paulo, Brazil) and 20 mg/kg xylazine (Agribrands, São Paulo, Brazil). All of the hair on the posterior side of the right calf was removed and the skin was incised and retracted. Subcutaneous dissection was performed to permit good exposure of the gastrocnemius muscle. The site of the lesion was standardized for all animals. The gastrocnemius muscle hemitransection was made at 2.5 cm from the calcaneus flexed at 90°. The laceration was approximately 1.0 cm wide × 0.3 mm deep, located laterally to the vessel-nervous bundle (popliteal artery and tibialis nerve). All measurements were made using a caliper. After controlling the bleeding by compression, the subcutaneous tissue was closed by suture (simple catgut 5.0) and then the skin wound was sutured with stitches of nylon.

Table 1
Number of pulsed US sessions for each animal group under study

Group ($n = 5$)	Number of pulsed US sessions
Control 4 days (4C)	Nil
Control 7 days (7C)	Nil
Control 14 days (14C)	Nil
Treated 4 days (4US)	2
Treated 7 days (7US)	5
Treated 14 days (14US)	12

After, animals were kept in individual cages with unlimited activities. The room was operated with a 12-h daylight cycle and temperature was maintained at about 25 °C. Food and water were available *ad libitum* during the study.

2.3. US parameters and treatment

Therapeutic US at 1 MHz with a near field extending for approximately 10 cm from the treatment head, a beam nonuniformity ratio (BNR) below 6.0 and an effective radiating area (ERA) = 3.5 $cm^2 \pm 20\%$ was applied; pulses of 5 ms duration were repeated at 100-Hz frequency at a setting of 1:1 (50% duty cycle), with a tissue speed of sound estimated in 1500 m/s. The treatment temporal average intensity was of 0.57 W/cm^2 for 5 min. This application time was determined because the lesion was smaller than the treatment head [12]. The machine had been calibrated by the manufacturer (Quark, US ProSeven 977 Standard, Brazil). A commercially available US gel was used as a coupling agent and all animals were depilated prior to the application of the ultrasound treatment. (There was thus no fur present between the animal skin and the appliance.) We estimated a tissue thicknesses between the transducer and the focal region of approximated 1.5 cm and a tissue attenuation of 24%/cm. During application, the rat was manually stabilized and care was taken to maintain the proper angle and coupling between the transducer face and tissues. Circular movement of the treatment head was employed to avoid the damages of standing waves.

We compared animals treated with pulsed US daily sessions (beginning 2 days post-trauma) with untreated but operated controls. The number of US sessions for the treated groups is shown in Table 1. The animals were sacrificed at 4, 7 or 14 days post-trauma; five treated and five control animals were sacrificed each time.

2.4. Histology and immunohistochemical study

The gastrocnemius muscles were removed and a fragment containing the injured site was fixed without stretching in 4% paraformaldehyde in PBS. Serial sections (5 μm) were studied using either the Hematoxylin and Eosin