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REGENERATION OF THE TIBIALIS ANTERIOR MUSCLE AT DIFFERENT TIMES FOLLOWING INJURY INDUCED BY NEUROMUSCULAR ELECTRICAL STIMULATION

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ABSTRACT

Background: Skeletal muscle injuries may be caused by contraction of the muscle concerned. Objective: To analyze the tibialis anterior muscle at different times following injury induced by electrical stimulation. Method: Male Wistar rats (298.2 ± 16.0g) were divided into two electrically stimulated groups evaluated after three and five days (n= 20) and two control groups, also evaluated after three and five days (n= 14). While stretched, the tibialis anterior muscle was injured by neuromuscular electrical stimulation (90 minutes, 30 Hz, 1 m/s, Ton/Toff 4 s and 4 mA). Three and five days afterwards, the animals were sacrificed and the muscles were removed. Histological sections were cut (10 μm) using a cryostat and were stained with toluidine blue. The body and muscle weights were statistically analyzed using Student's t test ($p \leq 0.05$). Results: The final body weight was higher than the initial weight for the 3-day control group (288.5 ± 18.3g vs. 308.5 ± 24.3g) and 5-day control group (288.4 ± 15.0g vs. 305.5 ± 20.7g) and lower for the 3-day stimulated group (305.0 ± 13.0g vs. 285.6 ± 13.2g) and 5-day stimulated group (306.1 ± 12.4g vs. 278.4 ± 20.9g). The relative muscle weight in the 5-day stimulated group was lower than in the 5-day control group (0.20 ± 0.001% vs. 0.22 ± 0.01%, respectively). The histological analysis showed variance between the animals regarding the extent and signs of fiber damage and/or regeneration, and the distal region was the most injured. The 3-day stimulated group presented predominance of cell infiltrate and myofilament hypercontraction, while the 5-day stimulated group presented predominance of cell infiltrate, basophils and fibrosis. Conclusion: A period of two days following electrical stimulation was sufficient for showing a difference in the regeneration process. The distal region of the tibialis anterior muscle was more susceptible to injury.

Key words: muscle regeneration, tibialis anterior, electrical stimulation, muscle injury.

INTRODUCTION

Muscle injury occurring in athletic practice is very common in active individuals, and may cause, very often, pain and incapacity, compromising the performance of occupational activities as well as leisure ones[1]. Thus, the treatment of this injury is very frequent in physical therapy clinical settings.

Physical exercises such as muscle strengthening or endurance, especially those performed in eccentric contraction, may cause musle injury[2], since this type of contraction is considered as a more common mechanism of injury[3], in physiological conditions, when compared with other forms of induction[4].

In addition of the high injury incidence, the skeletal muscle presents capacity of regeneration. It is well documented in the literature that the phases of the regenerating process are similar, despite of the different injury inducing mechanisms[5], but with duration and characteristics specific to the causes of the lesion[6].

Due to the fact that the muscle eccentric contraction is one of the causes of the injuries observed during sports activity, the objective of this study was to analyze the histology of the anterior tibial muscle in different periods after the muscle injury induced by neuromuscular electrical stimulation. The resulting data will allow an understanding of the muscle responses in different periods of time after the injury by contraction.

MATERIAL AND METHODS

The experiment was developed according to the 'Guide for the Care and Use of Laboratory Animals'[7]. The animals were kept in the Central Biotherium of the University in

individual cages made of polyethylene, submitted to a cycle light/dark of 12 hours and temperature of 23 ± 2ºC, and had free access to water and pellet ration. Thirty-four Wistar male rats, with average weight of 298,2 ± 16 g were divided into groups: electrically stimulated and analyzed after three (n= 10; EE3) and 5 (n= 10; EE5) days, and control: 3 (n= 7; C3); and 5 (n= 7; C5) days. In the groups electrically stimulated, the groups were submitted to a single session of neuromuscular electrical stimulation and analyzed 3 or 5 days after the same.

The electrical stimulation was done with the aim of causing an injury in the right anterior tibial muscle (RAT). This muscle was chosen because it presents fibers disposed longitudinally, which facilitates histological analyses[8].

The animals submitted to electrical stimulation were anesthetized with chloral hydrate (460 mg/Kg – 1.4 ml/ 300 g, ip). Two electrodes were fixed in the fibular nerve for the stimulation of the AT muscle, using the tool Dualpex 961® (Quark), properly calibrated, with the following parameters: pulse width 1 m/s, intensity 4m/A, Ton/off 4 s and frequency 30 Hz, for a 90 minutes duration. During all the electrical stimulation, the right posterior member was kept at maximum permanent stretch in plantar flexion, using a small wood splint, as proposed and discussed in a previous article[9].

The animals were weighed at the first day of experiment, and 3 and 5 days after the electrical stimulation, when they were sacrificed. The left and right anterior tibial muscles of the electrically stimulated and controls groups were carefully dissected and weighed individually in analytical scale (Bel Enginnering, Umark®-210A) for posterior analyses of the relative muscular weight, obtained in relation to the body mass.

After dissection, the muscles were divided in three similar parts (proximal, medial and distal), glued with a mixture of *tissue tek* and *gum-tragacanth* to a piece of wood, cooled in isopentane and frozen in liquid nitrogen. After this procedure, the muscles were stocked in liquid nitrogen until processing and analysis.

The three parts of the muscles were sectioned using transversal histological cuts of 10 µm, obtained by means of cryostatic microtome (Ancap®, mod 300), and the cuts were stained with Toluidina Blue (10%). The signs of injured and regenerating fibers were identified using optical microscopes (Olympus®, BX-41), The presence of injury signs and of regenerating fibers were characterized by: a) fibers with cell necrosis, which present a well delimited border and the interior with an opaque glass aspect; b) border or generalized basophily, consequent to the ribossomical proliferation; c) muscle fibers with centralized nucleus and a preeminent nucleolus; d) presence of hyper contraction of the muscle filaments, and e) cell infiltrate[10,11].

The injuries encountered in the three parts of the electrically stimulated muscles were classified as light, moderate or severe, according to the extension of the signs of the analyzed cut, as reported previously[9]. In all regions, the percentage of indicative signs of injury was determined analyzing the presence or absence of these signs in each muscle of the electrically stimulated groups.

The *Origin* software version 6.0 was used for statistical analyses. In order to make the comparative analyses of body and muscle weights, the obtained results were analyzed by independent t - test (between groups) and paired t – test (within groups), and considered as significant when $p< 0.05$.

RESULTS

In the body mass analyses, it was observed that the final body weight, when compared to the initial, was larger in the groups C3 (288.5 ± 18.3 g x 308.5 ± 24.3 g; $p< 0.01$) and C5 (288.4 ± 15.0g x 305.5 ± 20.7g; $p< 0.01$). The groups electrically stimulated presented significant loss in the final body mass when compared to the initial state, in the EE3 groups (305.0 ± 13.0 g x 285.6 ± 13.2g; $p< 0.01$), as well as in the EE5 groups (306.1 ± 12.4 g x 278.4 ± 20.9g; $p< 0.01$) (Table 1).

There were no significant differences in the relative muscular mass analyses among the electric stimulated tibial muscles, the muscles of the non-stimulated limb and the control animals, analyzed after 3 days. The relative weight of the electric stimulated muscle of animals analyzed after 5 days was significantly smaller than the muscle of the non-stimulated limb and than the 5 days control (0.20 ± 0.001%

Table 1. Average and standard deviation of the initial and final body mass and relative muscle mass of the right tibialis anterior (RTA) and left tibialis anterior (LTA) of the control, 3 and 5 days (C3 and C5), and electrical stimulation 3 and 5 days (ES3 and ES5) groups.

Groups	Body Mass (g)		Muscle Mass (%)	
	Initial	Final	RTA	LTA
Control 3 days	288.5 ± 18.3	308.7 ± 24.3*	0.20 ± 0.009	-
Control 5 dyas	288.4 ± 15.0	305.4 ± 20.7*	0.22 ± 0.010	-
	305.0 ± 13.0	285.6 ± 13.2*	0.20 ± 0.013	0.21 ± 0.014
EE5	306.1 ± 12.4	278.4 ± 20.9*	0.20 ± 0.001**	0.25 ± 0.010

*significant difference between the final and initial body mass ($p< 0.01$).
**significant difference when compared to the LTA and control muscles ($p< 0.01$).

x 0.25 ± 0.01% x 0.22 ± 0.01%; respectively), as presented in Table 1.

The muscles of the control group and the counterlateral muscles of the electrically stimulated groups presented a normal morphological muscular pattern (Figure 1A). The electrically stimulated muscles analyzed after three and five days presented signs of injured and in-regeneration fiber, such as infiltration, hyper-contraction, fibers with basophilia, fibers with centralized nucleus and preeminent nucleolus, as well as fibers with areas of smaller caliber (Figures 1B and 1C).

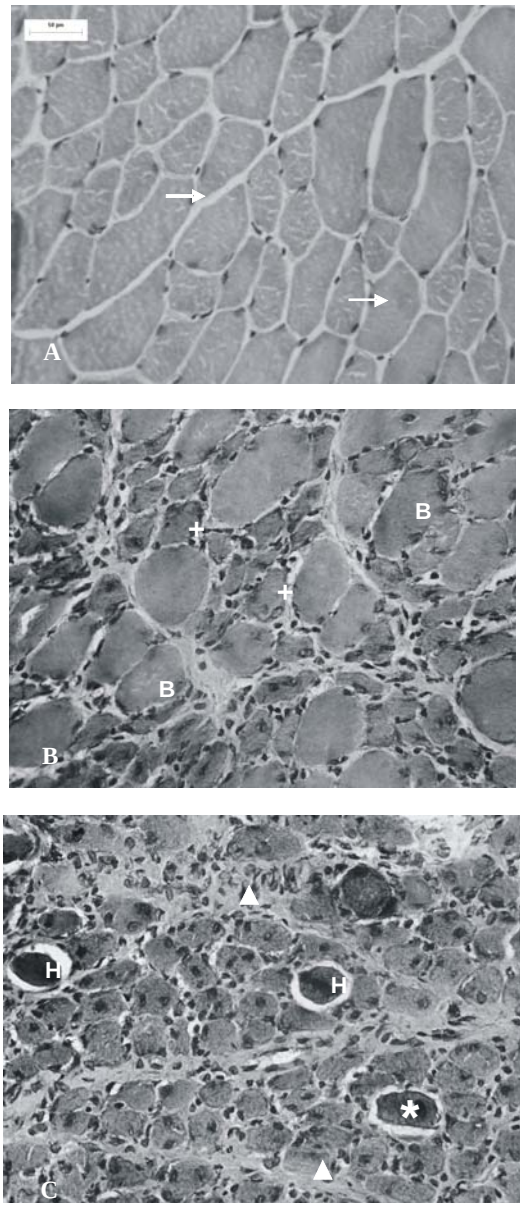
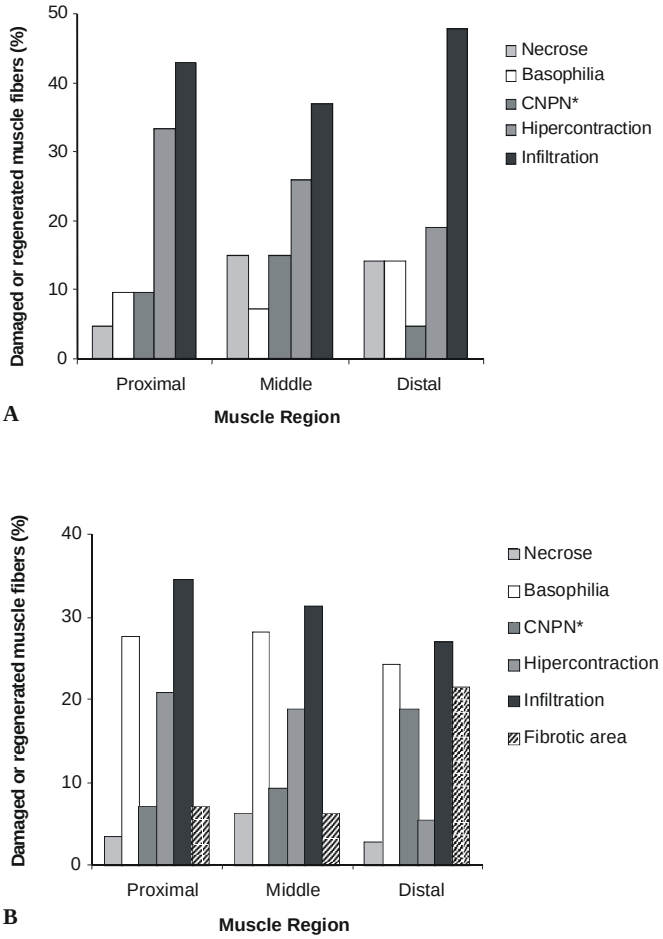


Figure 1. A: Tibialis anterior muscle of the control group. Observe fiber with peripheral nuclei (arrow) and hexagonal shape. Electrically stimulated muscle analyzed after 3 (B) and 5 (C) days. B: note fiber with signs of basophilia (B) and fiber with smaller cross sectional area (cross). C: infiltration (triangle), myofilaments hypercontraction (H) and fiber with centralized nucleus and prominent nucleolus (asterisk). Toluidine Blue staining, 400x.



* CNPN: centralized nucleus and prominent nucleolus.

Figure 2. Incidence of signs of damaged and regenerated muscle fibers observed in the proximal, middle and distal region of the electrically stimulated tibialis anterior muscle analyzed after 3 (A) and 5 (B) days.

Regarding the injury signs, the electrically stimulated group analyzed after three days presented predominance of cell infiltrate and myofilaments hyper-contraction, which are signs of early injury (Figure 2). The group stimulated electrically that was analyzed after five days presented, in addition to high cell infiltrate incidence, greater percentage of fibers in process of regeneration, characterized by basophilia and fibers with centralized nucleus and preeminent nucleolus. Fibrosis was observed in the injured muscles, but it was encountered only in the group analyzed after five days of lesion and with greater incidence at the distal portion (Figure 2B).

In relation to the analyzed muscular regions, it was observed quantitative difference of injury signs between the different regions and between the electrically stimulated groups. In this way, in both electrically stimulated groups, the proximal, medial and distal regions presented injuries classified as light or moderate. However, only the distal region presented injury classified as severe. Sever injuries were more pronounced in the group analyzed after five days.

In the group stimulated and analyzed after three days, the muscle injury classified as light was present at 70, 60 and 50%, and the moderate at 30, 40 and 40% of the animals, on the proximal, medial and distal regions, respectively. The severe muscle injury was observed at the distal region in only 10% of the animals of this group (Figure 3A). On the group electrically stimulated and analyzed after five days, the light injury was encountered in 80, 70 and 40%, and the moderate in 20, 30 and 10% of the animals, at the proximal, medial and distal regions respectively. Only the distal region presented signs of lesion classified as severe. This type of injury was encountered in 50% of the animals of this group (Figure 3B).

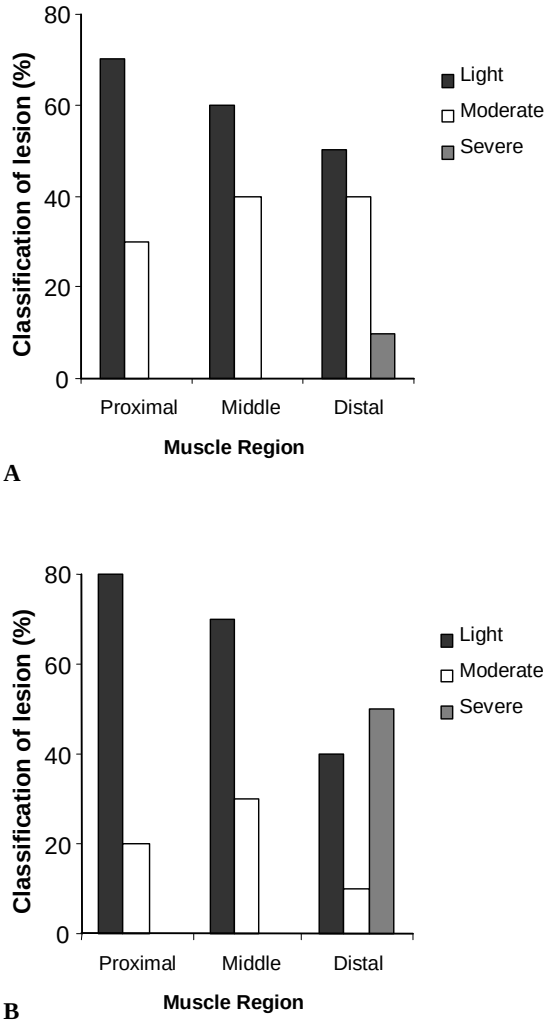


Figure 3. Signs of muscle damage observed in the proximal, middle and distal regions of the electrically stimulated tibialis anterior muscle analyzed after 3 (A) and 5 (C) days.

DISCUSSION

Despite the fact that eccentric contraction is the most harmful type of muscle contraction, the present work has used a model of injury by isometric contraction with the muscle immobilized in stretched position, as proposed in a previous article[9]. This kind of contraction simulates the morphological alterations induced by eccentric contraction[3,12]. In this type o contraction, in addition to the tension generated by the own muscle during contraction, occurs an increase in muscular tension produced by the stretched position[1,13,14]. In eccentric contractions, the muscle contracts to generate tension and is then passively elongated by an external force, generating additional tension[15].

Regarding body mass, it is supposed that the significant loss in weight on the electrically stimulated groups analyzed after 3 and 5 days was a consequence of the stress to which the animals were submitted, which may interfere on their diet and/or metabolism[16,17]. Furthermore, it is observable that the five-day period after muscular injury was not enough for the animals to reach the body weight observed in the control group.

There was significant weight loss on the electrically stimulated muscle analyzed after 5 days when compared to the counterlateral limb and to the control group. This result is suggestive of muscular atrophy. This weight loss was also observed in previous work[3], when the anterior tibial was analyzed after five days (11.6% weight loss when compared to the counterlateral limb), but without weight alteration when analyzed one day after injury. No decrease in muscular weight, observed in the group analyzed after 3 days of lesion, was due to the fact that muscular atrophy was masked by presence of an edema[18], since the inflammatory process has its peak between 24 and 47 hours after the muscle injury[19].

The Muscle injury was produced by contraction. In this case it was induced initially by a mechanical component (contraction), and, subsequently, exacerbated due to the inflammatory component, secondary to the initial lesion[2,20]. This injury characteristic may explain the fact that the group analyzed after 3 days presented greater incidence of injured fibers signs, since the analyses of the muscles were made during the peak of the inflammatory process[19], while the group analyzed after 5 days presented greater incidence of regenerating fibers. Previous studies also reported greater muscular damage during the development of the inflammatory process[3,20,21,22].

The injury classified as severe, because it presents greater extension of the injured area, was more evident on the electrically stimulated group analyzed after 5 days. However, the predominant signs in this group were the presence of fibers in process of regeneration. This result demonstrated that the regenerating process after lesion by electric stimulation is relatively fast, because in only two days there was a significant evolution of this process. Similar data were found in a previous study, in which the anterior tibial of the analyzed animals after seven days of electrical stimulation also presented significantly reduced injury when compared to animals analyzed after 5 days. In this study, after seven days, the medial region of the muscle presented a pattern very similar to that of the control group[3].

At the present study, the most severe injury was observed at the distal region. Previous works also report greater injury occurrence at the myotendinous region[1,3]. This is explained by the fact that each muscular fiber is connected to its terminal regions by conjunctive tissue, and the contraction of the miofibers is transformed in movement, via myotendinous junction[23]. Thus, the lesion, which is observed more intensely at this area, may occur due to the concentration of stress at the distal region of the muscular fiber. Since the ankle joint was immobilized, the movement restriction did not permit adequate transmission of force towards the tendon[8].

Muscular fibrosis was observed only in animals analyzed after five days of injury, which suggests that the 3 days period was not sufficient for a significant proliferation of fibroblasts that could lead to the formation of scar tissue. Despite the fact that previous works had shown collagen types I and II synthesis at the first days after lesion, greatest levels were observed at the period five to seven days[24,25], what may reinforce the results found in the present study.

One hypothesis for fibrosis occurrence is the presence of ischemia and low oxygenation, which may favor fibroblast proliferation[26]. It is important to highlight that muscular regeneration and fibrosis formation at the injured area are competitive events and fibrosis may lead to complete inhibition of muscle regeneration[27]. In this case, physical therapeutic resources, such as mobilization, should be applyied during the process of muscle regeneration in order to prevent fibrosis formation[23].

CONCLUSIONS

The results of this study, within the experimental conditions utilized, showed that injury induced by electrical stimulation promoted greater damage at the distal muscular region, suggesting that the myotendinous region is more susceptible to injury. Furthermore, histological analysis demonstrated that regeneration after electrical stimulation is relatively fast. After five days from the initial injury, it was observed greater incidence of regenerating fibers, and qualitatively better morphological pattern, when compared to the group analyzed three days after the electrical stimulation.

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