

The onset and duration of mobilization affect the regeneration in the rat muscle

F.E.T. Faria¹, R.J. Ferrari¹, G. Distefano¹, A.C. Ducatti¹, K.F. Soares¹, M.I.L. Montebelo² and V.B. Minamoto¹

¹Department of Physical Therapy, Piracicaba Methodist University, Brazil and

²Department of Statistic, Piracicaba Methodist University, Brazil

Summary. The effects of different mobilization protocols for muscle regeneration after myotoxin injury was compared in the rat tibialis anterior (TA) muscle. Adult Wistar rats were divided into control (C); mobilized (M); injury (I); injury + late mobilization (LM) and injury + early mobilization (EM) groups. Muscle injury was induced by intramuscular lidocaine injection. The exercised animals were mobilized for 5 and 8 days during 15 and 45 minutes/session. The swimming started 1 hour or 3 days after injury. All animals were killed 8 days after the injury, together with the control group, when the TA muscles were weighted and excised. Cross sections were obtained by cryostat and submitted to Toluidine Blue stain. Qualitative morphological characterization of muscle regeneration and quantitative analysis of muscle fiber and non-muscle fiber area density were performed. The I and late mobilization groups showed decreased muscle mass when compared to all other groups. All injured animals showed signs of muscle fiber damage, although signs of early regenerated muscle fibers were more evident in injury + mobilization groups. Only the EM groups submitted to 45 minutes of exercise had increased muscle fiber and decreased non-muscle fiber area density values when compared to I group ($p < 0.05$). Conclusion: the regeneration process is related to the onset of exercise, since animals submitted to early mobilization showed improved regeneration when compared to LM groups. Besides, the length of session is also important for accelerating the regeneration process, as it was observed that 45 minutes was better than 15 minutes duration.

Key words: Swimming, Muscle damage, Muscle regeneration, Tibialis anterior

Introduction

Skeletal muscle regeneration basically consists of two events: myofiber regeneration and connective scar tissue production. These two processes are simultaneous and competitive events, as the latter is capable of completely inhibiting muscular regeneration when its production is excessive (Lehto et al., 1986; Jarvinen et al., 2002).

Thus, the physical therapy resources used for muscular lesion treatment must aim to promote fiber regeneration and prevent accentuated connective tissue production that acts as a mechanical barrier, which hinders or prevents muscular fiber regeneration and re-innervation (Kaariainen et al., 2000).

The following are among the resources most used after muscular injury: ice (Merrick et al., 1999), ultrasound (Rantanen et al., 1999), anti-inflammatory agents (Thorsson et al., 1998), mobilization (Jarvinen and Lehto, 1993) and immobilization (Lehto et al., 1985a).

For a long time, immobilization was the treatment of choice in soft tissue lesions. However, the use of this procedure causes muscular atrophy, accentuated connective tissue proliferation, neofiber disorientation and loss of muscular force (Kannus et al., 1992; Kaariainen et al., 2001), which discourages the use of this treatment.

At present, studies relate that mobilization is beneficial to muscular regeneration, not only because it accelerates this process, but also because it minimizes or prevents the appearance of deleterious signs related to immobilization, which promotes a more rapid return of the functional properties of the injured muscle (Kannus, 2000; Jarvinen et al., 2005).

In spite of the beneficial effects of mobilization, there is evidence that restriction of the affected member is important during the first few days after the lesions, since it protects the muscle from ruptures in the still weakened area (Lehto et al., 1985b; Lehto and Jarvinen,

1991; Jarvinen and Lehto, 1993).

However, a previous study reported that exercise started 1 hour after injury was more efficient for the muscular regeneration process when compared with exercise started 3 days after the injury (Gregory et al., 1995).

Thus, there is divergence among authors as regards the best period for starting mobilization, which justifies more studies in this area. Furthermore, the study of different duration time in days of mobilization, or duration of the daily exercise session deserves focus, since no reports were found related to these variables with muscular regeneration.

Based on the above, the aim of the present study is to compare the early (1 hour after the injury) with later (3 days after injury) beginning of exercise, as well as different mobilization duration periods (5 and 8 days of exercise) and exercise session duration (15 and 45 minutes per session) effects on the muscular regeneration process.

Materials and methods

The experiment was developed in accordance with the Guide for the Care and Use of Laboratory Animals, and was approved by the Federal University of São Carlos. The animals, housed in plastic cages in a room kept at $23\pm 2^{\circ}\text{C}$ and a 12h:12h dark-light cycle, had free access to water and standard food. Sixty Wistar rats (*Rattus norvegicus*, albinus), aged 2 months (weight ranging from 250 to 300 g) were randomly divided into: control (C; n=6); injury (I; n=7); non-injured mobilized group (M; n=5); injury + late mobilization (LM) and injury + early mobilization (EM) groups. There were 2 sub-groups in the LM group: animals that were injured and mobilized for 5 consecutive days during 15 (LM-15; n=7) and 45 (LM-45; n=7) minutes per session. For the EM group, there were 4 sub-groups: animals that were injured and mobilized for 5 (EM5) and also for 8 (EM8) consecutive days during 15 (EM5-15, n=7; EM8-15, n=7) and 45 (EM5-45, n=7; EM8-45, n=7) minutes per session. The mobilized group was performed to evaluate the possibility of exercise-induced muscle damage. These animals were mobilized for 8 days, during 45 minutes. Control rats were age-matched with experimental groups and performed no exercise, except for their normal movements in their cages.

Muscle injury was produced by intramuscular injection of lidocaine at 2%, in a dose of 0.2 mL. The injection was made on the media region of the right tibialis anterior (TA) muscle, with the animals held in a contention cage during the procedure. The TA muscle was chosen because it is accessible for intramuscular injection and possesses longitudinal fiber architecture (Lieber et al., 1991).

The exercise chosen for mobilization was swimming. Animals swam in a container with water at a temperature of around 30°C , in a group not exceeding 5 animals, as previously described (Ueno et al., 1997). To

make sure that the animals did not float during the mobilization procedure, they were supervised during the whole swimming session. When necessary, they were stimulated to swim using a wooden stick.

Mobilization started 1 hour (for the EM groups) or 3 days (for the LM groups) after the muscle injury. After exercise, the animals were dried using a hair dryer and kept in their cage until the next exercise session.

At the same time-point, that is, eight days after the beginning of the experiment, the anesthetized rats of all groups had their muscles removed and were then euthanized by cervical dislocation. The media region of the TA muscle was mounted in tragacanth gum and quickly frozen in isopentane cooled to the temperature of liquid nitrogen. Cross-sections ($10\ \mu\text{m}$) obtained using a cryostat-microtome were stained with Toluidine Blue. A qualitative analysis of the morphological muscle pattern was performed and the signs of damaged muscle fiber were characterized as cellular infiltration, basophilic fibers, hypercontracted fiber and fiber with central nuclei and prominent nucleolus. Regenerated muscle fibers were classified as split fibers and fibers with central nuclei (Schmalbruch, 1976; Minamoto et al., 2001, Hwang et al., 2006).

For the quantitative analysis of muscle regeneration, we measured the muscle fiber area density and the non-muscle fiber area density. Muscle fiber area included normal muscle fiber, hypercontracted fibers, basophilic fibers and early regenerated muscle fiber (split fibers and centronucleated regenerating myofibers). The term non-muscle fiber area was used to refer to cellular infiltration, the very acute sign of muscle damage, plus the connective tissue area density. It is worth noting that in control and mobilized groups no signs of cellular infiltration were found, thus, in these groups, the non-muscle fiber area included only the connective tissue area density. The quantitative analysis was made using one section from each muscle, which was photographed in its whole length and analyzed using the planimetry by point-counting method (Mathieu et al., 1981).

For the statistical analyses, the homogeneity of variance was initially analyzed, using the Levene Test. In addition, Anova Fisher's test was used to test for significant differences among the results. Because a significant value was observed ($p < 0.01$), multiple comparison testing was performed using the Tukey HSD or Tamhane; the level of statistical significance was set at 5%. All data are presented as mean and 95% confidence interval (CIs).

Results

Muscle mass

As observed in Fig. 1, the I and injury + late mobilization groups showed decreased muscle mass in the injected right TA muscle when compared to all other groups ($p < 0.05$). In addition, no difference was observed among the other groups.

Morphological pattern of the tibialis anterior muscle

A normal morphological muscle pattern (Fig. 2A) can be observed in control and mobilized groups. Signs of damaged muscle fibers are present in the I group (Fig. 2B), with a predominance of cellular infiltration, basophilic fibers and hypercontracted fiber. These signs can also be observed in the groups submitted to mobilization (Fig. 2C-F), although with lower intensity and mixed with signs of early regenerated muscle fibers, mainly centronucleated regenerating myofibers. These results suggest a more advanced stage of regeneration in the mobilization groups, which was more evident in the group submitted to early mobilization for 8 days and during 45 minutes of daily mobilization (Fig. 2F). In this exercised group it was possible to note better muscle fiber organization, as seen by the marked bundles, although the muscle fibers showed smaller cross-sectional area when compared with normal muscle fibers (Fig. 2A).

Percentage of muscle fiber and non-muscle fiber area density

The quantitative analysis of muscle regeneration was measured through the muscle fiber and non-muscle fiber area density values. As observed in Figure 3, the non-injured mobilized group showed similar values when compared to the control group. All other groups showed larger non-muscle fiber and lower muscle fiber area density in comparison to control and non-injured mobilized group ($p \leq 0.01$).

In a comparison among I group and injury + mobilization groups it was observed that only the early mobilization groups submitted to 45 minutes of mobilization, in both the 5- and 8-day groups, showed different values when compared to I group. These groups showed a lower value in the non-muscle fiber area ($p \leq 0.008$) and increased fiber occupancy per field ($p \leq 0.01$; Fig. 3) when compared to I group. No

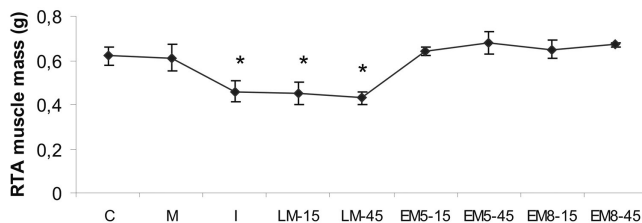


Fig. 1. Muscle mass from all experimental groups. Note that the late mobilization groups were the only treatment groups which were not different from I group. *: when compared to all other groups ($p < 0.05$). Control (C), mobilization (M), injury (I), late mobilization during 15 minutes (LM-15), late mobilization during 45 minutes (LM-45), early mobilization for 5 days during 15 minutes (EM5-15), early mobilization for 5 days during 45 minutes (EM5-45), early mobilization for 8 days during 15 minutes (EM8-15) and early mobilization for 8 days during 45 minutes (EM8-45) groups.

statistically significant difference was observed for LM-15, LM-45, EM5-15 and EM8-15 groups when compared with the I group ($p > 0.05$; Fig. 3).

Moreover, the EM8-45 group had a lower value in the non-muscle fiber area and larger muscle fiber area density when compared to EM5-45 group ($p = 0.000$; Fig. 3).

Discussion

The skeletal muscle is shown to be capable of spontaneous regeneration by means of activating satellite cells (Hurme and Kalimo, 1992). However, regeneration may result in inadequate muscular function, due to the development of scar tissue (Huard et al., 2002). Thus, professionals make a great effort with regard to rehabilitation, to determine treatment that promotes rapid and complete skeletal muscle regeneration, which allows athletes an early return to their sporting activities. The use of exercise and stretching after muscular lesion has been studied a great deal (Lehto and Jarvinen, 1991; Jarvinen and Lehto, 1993; Kannus, 2000; Kaariainen et al., 2001; Hwang et al., 2006) and the importance of mobilization in accelerating the regeneration process is supported by the physiological effects of the exercise. It is known that mechanical stress is a powerful regulator of the cell phenotype, which influences many cell functions, such as orientation, replication, growth factor production and collagen synthesis (Bishop et al., 1993). Recently, authors also state that physical forces, notably mechanical and electric, have a direct effect on the structure and composition of extracellular matrix, through regulation of gene expression and the synthesis of structural proteins, as well as signaling proteins (Aaron et al., 2006). Besides, the importance of contractile activity for avoiding connective tissue accumulation, which can impair muscle regeneration, has been previously reported (Williams et al., 1988). It was also reported that exercise promotes tensile strength, which is important for myotube alignment and guidance and which result in a better morphological pattern of the muscle (Lehto and Jarvinen, 1991).

Another important effect of exercise is related to vascularization in the injured area (Jozsa et al., 1980; Lefaucheur and Sébille, 1995). Previous studies showed that exercise therapy induces more rapid and intense capillary growth (Jarvinen, 1976; Jarvinen and Lehto, 1993) and the authors concluded that the new capillary sprouts have an important role in offering oxygen for the adequate metabolism of the regenerating tissue.

Despite all the encouraging reports in using exercise after muscle injury, the effects of some mobilization variables on muscular regeneration, such as its beginning, duration and length of session, have not yet been well defined.

Thus, the aim of this study was to assess different mobilization protocols on the rat tibialis anterior muscle regeneration process. The hypothesis that different beginning times (early or late), duration in days (5 and

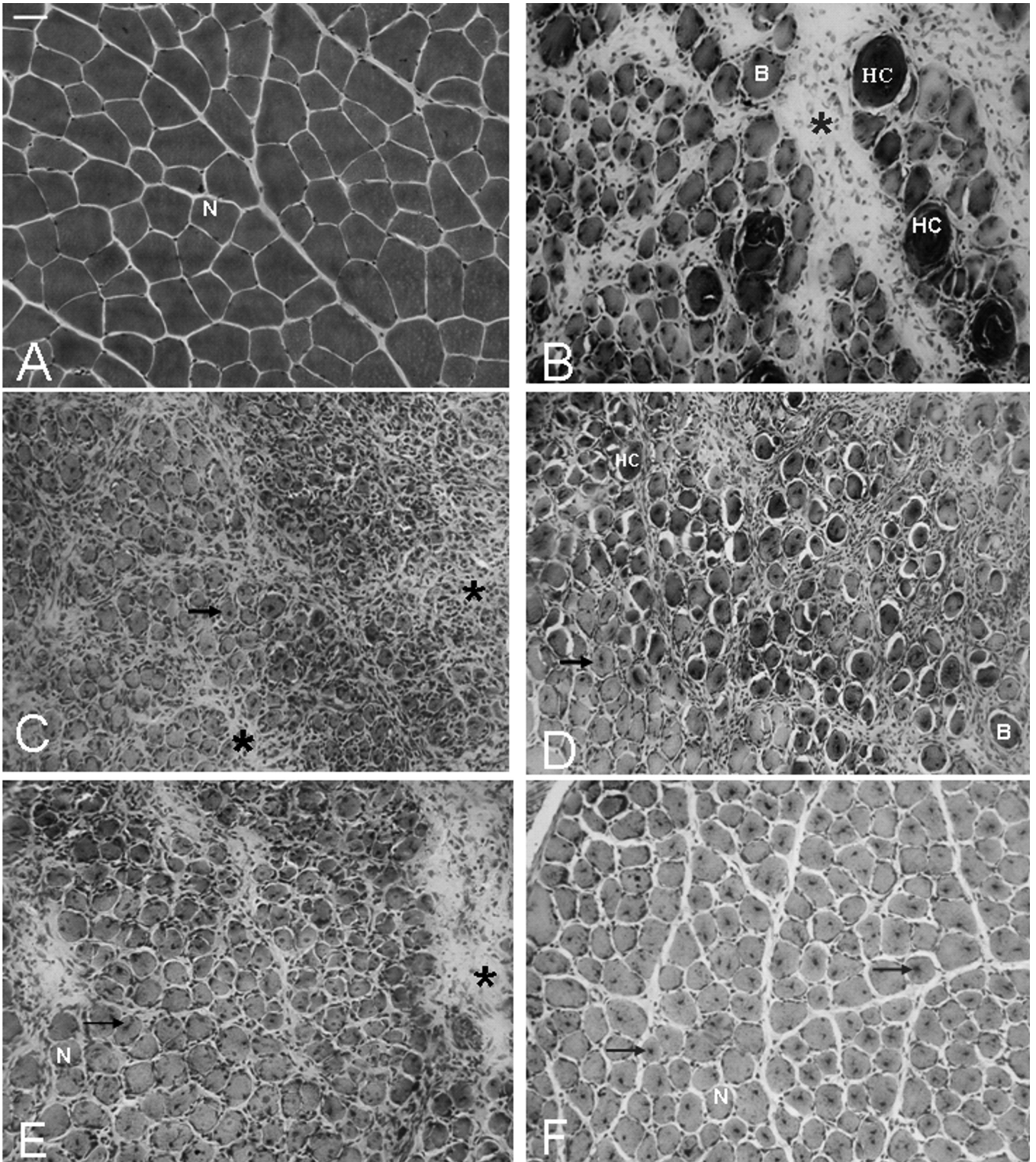


Fig. 2. Cross sectional area of the M (A), I (B), LM-15 (C), LM-45 (D), EM5-15 (E) and EM8-45 (F) groups. Note that in the mobilized group the muscle fibers show normal aspect with peripheral nuclei in the polygonal-shaped fibers (N). Signs of muscle damage, such as cell infiltration (asterisk), basophilic fibers (B), and hypercontracted fibers (HC) are observed in all muscle submitted to the injury, although signs of regenerated muscle fibers, such as muscle fiber with centralized nucleus (arrow) are found only in the groups submitted to the mobilization. Toluidine Blue, x 20. Bar: 50 μ m.

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8) and daily session duration (15 or 45 minutes) of mobilization have an influence on muscle regenerative response was tested.

It is worth noting that the muscle injury induced in this study is not similar enough to what one would encounter in clinical practice. However, the use of models such as muscle contusion or strain, which is easily found among athletes, promote an inconsistent muscle damage between experiments, making comparisons difficult. So, muscle damage induced by local anesthetic was the injury muscle model chosen, due to the similarity in the muscle damage among the animals. Swimming was the exercise of choice, since it is widely used to rehabilitate patients with orthopedic disorders. The benefits from weight reduction in water make the exercise safe and less stressful to the joints and soft tissues, thus preventing secondary lesions (Burns and Lauder, 2001). Furthermore, it was chosen because it is a natural activity of rodents (Dawson and Horvath, 1970).

The results of our study showed that different mobilization protocols resulted in different morphological patterns of the muscle. The best exercise treatment for morphological muscular pattern was observed in early mobilization groups, during 45-minute daily duration, since these were the only groups that showed a difference in the quantitative analysis of muscle regeneration in comparison to I group.

Several studies have been conducted with the aim of determining the best period for starting mobilization (Jarvinen, 1975; Lehto et al., 1985a,b; Jarvinen and Lehto, 1993; Gregory et al., 1995; Hwang et al., 2006). The majority of them reported that mobilization started late, that is, some days after the injury, results in a better regenerative pattern when compared to early mobilization, starting some hours after the injury. The hypothesis for the best results found after late mobilization is that restriction of movement for around 2 to 5 days after injury would be necessary to prevent muscular ruptures in the still weakened tissue. However, Menetrey et al. (1999) showed that restriction of movement was not favorable to muscle healing after

muscle laceration, since the animals immobilized for 5 days after muscle injury, followed by free mobilization, presented a decrease of muscular force, development of a large fibrotic tissue area and slower regeneration, when compared to control animals.

In the present study, early mobilization was shown to be superior to late mobilization, and this superiority is supported by the larger muscle fiber area density, and consequent lower non-muscle fiber area, found in the early mobilization group, which suggests a regeneration process in a more advanced stage. The lower non-muscle fiber area, characterized by cellular infiltration plus connective tissue, indicates a better regeneration process, since it is known that cellular infiltration impairs satellite cell activation (Merly et al., 1999). The understanding of mechanisms involved in the effects of early versus late mobilization is beyond the scope of this study, but we can suggest that the early mechanical stimulus triggers, in a more appropriate way, all the physiological effects of the exercise.

Our findings are in agreement with the results of Gregory et al. (1995). These authors studied two kinds of exercise, swimming and running, started 1 hour or 3 days after muscle injury. They reported that any of the given exercises is better than no exercise and that immediate onset of exercise is preferable to delayed onset.

While some studies focused on determining the best period for starting mobilization, there are no reports relating different durations and lengths of session of mobilization to muscle regenerative process. This concern was raised in 1983, by Carlson and Faulkner, and until today no reports related to this subject were found. Thus, the present study also proposed to study this variable.

The results presented suggest that the daily length of exercise session is more relevant to regeneration than the duration of mobilization in days. This is because animals that were exercised for 15 minutes per session, both during 5 and 8 days, did not present differences in muscular fiber area and non-muscle fiber area density when compared with the I group. However, these

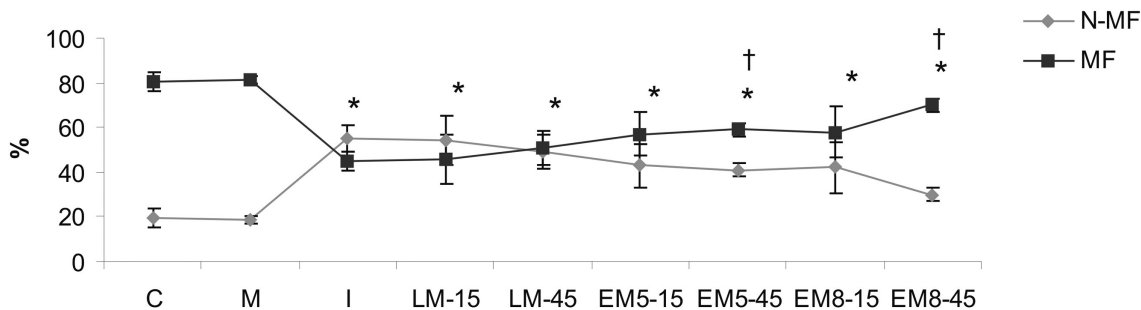


Fig. 3. Percentage of muscle fiber (MF) and non-muscle fiber (N-MF) area density of TA muscles. Note the early mobilization groups during 45 minutes were the only treatment group different from I group. *: when compared to C and M groups. †: when compared to

I group. Control (C), mobilization (M), injury (I), late mobilization during 15 minutes (LM-15), late mobilization during 45 minutes (LM-45), early mobilization for 5 days during 15 minutes (EM5-15), early mobilization for 5 days during 45 minutes (EM5-45), early mobilization for 8 days during 15 minutes (EM8-15) and early mobilization for 8 days during 45 minutes (EM8-45) groups.

differences were observed in the groups mobilized for 45 minutes, irrespective of mobilization having been done for 5 or 8 days, although the precise mechanisms of these effects are not fully understood.

With respect to muscle mass, it is suggested that the model of muscle injury caused an alteration in the protein metabolism, as observed by the decreased muscle mass in I group. In addition, it is noted the importance of early, but not late, mobilization for maintenance of muscle mass, since only the early mobilization groups showed a muscle mass value similar to the control group. Because muscle mass is not a good predictor of cross-sectional muscle fiber area, since non-contractile material (inflammatory cells, fluid, connective tissue) can also contribute to muscle mass, the best results found in the early mobilization groups should be interpreted carefully.

The results of the non-injured mobilized group showed no difference in all variables analyzed when compared to the control group. These findings strongly suggest that the appearance of early regenerated muscle fibers in injected plus mobilized group is not related to more muscle damage caused by exercise treatment, but on the contrary, is due to a more advanced regeneration process promoted by the exercise.

All animals used in this study were male rats with the same age at the beginning of the experiment. It is an important detail, since it is well-defined that age and sex influence the regeneration process (Grounds, 1987; Carlson and Faulkner, 1989). The older the animal the worse the regeneration, not due to satellite cell ability to proliferate and being activated, but because of the old hosts (Carlson and Faulkner, 1989). Related to sex, the phagocytosis of necrotic skeletal muscle fiber is impaired in male animals and testosterone levels might account for this impairment (Grounds, 1987). In addition, it is important to note that maybe the positive results we did observe with the exercise treatment was possible because this study was performed with young animals. Different results could be noted with older animals, since the capacity of muscle regeneration is impaired with aging due to the muscle tissue milieu. This impairment can be due to hormonal and other cellular factors, but also due to impaired ability of old motor nerves to reinnervate muscles (Carlson and Faulkner, 1989).

Although this experiment was conducted with small animals that present high regeneration capacity, the basic principles of these findings can probably be applied to muscular lesions in humans, in which the biological processes are similar, although slower (Lieber, 2002).

In summary, this study has demonstrated that different mobilization protocols applied to previously injured muscle resulted in different muscular regeneration responses, being the beginning of mobilization and its session duration determinant in accelerating the regeneration process. It was concluded that, for better muscle regeneration, induced mobilization was superior to free mobilization; early

mobilization was better than late mobilization, and 45 minutes of session duration was more favorable to regeneration than 15 minute sessions. Since the aim of this study was simply to analyze the morphological aspect of the muscle, further studies are necessary to determine whether the morphological alteration observed among the groups has an impact on the muscle function.

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