Effects of Magnetic Stimulation on Oxidative Stress and Skeletal Muscle Regeneration Induced by Mepivacaine in Rat

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Abstract: We investigated the effect of magnetic field stimulation (MS) on oxidative damage and skeletal muscle injury prompted by mepivacaine injection in the anterior tibial muscle of Wistar rats. The effects of mepivacaine and MS on oxidative stress were evaluated by lipid peroxidation, GSH levels and catalase activity. Muscle regeneration was analyzed by haematoxylin-eosin stained, NADH-TR histochemical reaction, desmin immunostaining as well as by morphometric parameters such as fibers density and fiber area were evaluated. Our data revealed that mepivacaine induced oxidative stress, that MS prevents the harmful effects induced by mepivacaine and that it facilitates the regeneration process of skeletal muscle. In conclusion, the results show the ability of MS to modify skeletal muscle response to mepivacaine.

Key Words: Magnetic stimulation, total nitrite, muscle fiber regeneration, reactive oxygen species.

INTRODUCTION

Skeletal muscle fibers have considerable ability to regenerate after injury or trauma. The regeneration process is characterized by activation and proliferation of satellite cells followed by differentiation of their progeny into myoblats and fusion of myotubes. Newly formed or regenerating myofibers are characterized by many large central nuclei [1].

In humans, the efficacy of muscle repair declines from the age of 40 to a point where at the age of 80 40% of the muscle mass and more than 50% of the ability to generate power is lost [2]. This phenomenon is associated with decreased reduced glutathione/oxidized glutathione (GSH/GSSG) ratio in aged muscles together with increases in markers of oxidative damage such as malondialdehyde, protein carbonyl groups and 8-hydroxy-2-deoxyguanosine [3]. Thus, Hansen *et al*. report that oxidative intracellular environments impair myoblast differentiation, while reducing environments favor myogenesis [4]. The "study" did not find anything, change to: Oxidative stress was reported to play a crucial role in the modulation of myogenic differentiation and skeletal muscle regeneration after hindlimb ischemia, where reactive oxygen species (ROS) slow and inhibit myogenic differentiation and myotube formation, while nitric oxide (NO) may contribute to faster regeneration [5]. Additionally, nitric oxide synthase (NOS) and NO have been associated with neuromuscular transmission and muscle contractility [6].

Enhancing the repair of injured muscles is of great clinical interest [7,8]. A new method in which magnetic microparticles were introduced into the cytoplasm of cultured myoblasts that were placed in a magnetic field was reported to accelerat myoblast differentiation and fusion. Recently, static magnetic field has been reported to be beneficial for enhancing muscle differentiation *in vitro* by improving myoblast alignment, increasing fusion index and myotube hypertrophy [9]. In addition, Sakuraba *et al*. reported that magnetic stimulation (MS) prevented muscle atrophy in an experimental model of atrophy caused by suspension [10,11]. Based on these data we hypothesized that application of MS may facilitate skeletal muscle regeneration.

The main object of this study was to evaluate the effects of MS on nitric oxide levels, oxidative damage and degeneration-regeneration in an experimental model of acute necrosis induced by mepivacaine.

RESULTS AND DISCUSSION

The most important finding of the present study is that MS can reverse the deleterious effects of injury and oxidative damage triggered in muscle by mepivacaine injection, by inducing antioxidative actions and facilitating muscle regeneration.

Effects of MS on Free Radical Production and Nitrites Levels Induced by Mepivacaine

Adequate amounts of reactive oxygen species (ROS) are essential for cell homeostasis, as they affect processes of cell death by apoptosis and necrosis [12,13] as well as cell modulate proliferation and differentiation [12, 13]. Four days of mepivacaine injection induced a high degree of oxidative stress characterized by increases in lipid peroxidation product levels ($P< 0.01$) and decreased GSH content ($P< 0.05$) in skeletal muscle tissue (Table **1**).

Moreover, in a recent study it was found that high intracellular redox potential of skeletal muscle impaired myoblast differentiation, while reducing environments favored myo-

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1573-4064/09 $55.00+.00

Table 1. Changes in the Levels of Lipid Peroxidation Products (MDA+4-HDA), Reduced Glutathione (GSH) and Caspase-3 Activity in Anterior Tibial Muscles Treated with Mepivacaine Alone or in Combination with Magnetic Stimulation

Groups	Lipid Peroxidation Products nmol/mg Protein	GSH nmol/mg Protein	Caspase-3 Activity O.D. Arbitrary Units/mg Protein
Cont	0.48 ± 0.10	15.12 ± 2.31	0.0550 ± 0.021
4d MI	1.95 ± 0.41[a]	7.54 ± 2.12[b]	0.0150 ± 0.002
4d MI-MS	1.56 ± 0.18[a]	10.36 ± 4.01	0.0350 ± 0.001
7d MI	1.07 ± 0.05[c]	15.02 ± 1.18	0.0300 ± 0.001
7d MI-MS	0.98 ± 0.46	13.28 ± 1.02	0.0375 ± 0.002

Values are mean ± SD; n = 4 animals per group. [a]$P < 0.01$ vs Cont; [b]$P < 0.05$ vs A; [c]$P < 0.01$ vs 4dMI.

genesis. Together, these findings suggest that reactive oxygen species are involved in muscle degeneration [4]. Zaccagnini *et al*. (2007) studied the role played by oxidative stress on myogenic differentiation and skeletal muscle regeneration in mice with alterations in p66 isoform of the adaptor protein ShcA (129 Sv-Ev $p66^{ShcA}$ a wild-type and knock-out inbred mice). They showed that oxidative stress and $p66^{SchA}$ play a crucial role in regenerative pathways [5].

Our study also revealed that three days of MS treatment triggered a 20% reduction in lipid peroxidation levels, whereas GSH content increased dramatically (37.4%) compared with the non-treated group (Table **1**). Nevertheless, it have not differences between group 7d MI-MS and 7d MI group, which showed similar recovery respect control group characterized by reduction of oxidative stress biomarkers compared with 4d MI and 4d MI-MS group, respectively (Table **1**). Taken together, this seems to indicate that ROS play an important role in the pathogenesis of degeneration of skeletal muscle fibers. Thus, our data would indicate that skeletal muscle response effectiveness after seven days, which is harnesses by MS.

Nitric oxide (NO) is a short-living molecule synthesized by nitric oxide synthase (NOS). It is a cell-signalling molecule expressed ubiquitously in normal and pathological conditions and is involved in the regulation of numerous processes. NO influences contraction, blood flow, and metabolism, as well as myogenesis [14,15]. The levels of total nitrites (NOx) in the anterior tibial muscle were reduced by 17.92% in 4d MI compared with Cont, whereas MS (4d MI+MS) triggered an enhancement of 34.10% with respect to 4d MI (Fig. **1**). The most evident effects of MS on NOx levels occurred after six days of MS treatment (26.26 ± 7.14 μmol/mg protein in the 7d MI+MS group *vs* 13.33 ± 0.96 μmol/mg protein in the Cont group; $P < 0.05$) (Fig. **1**). These data coincided with those reported in other studies which indicate that NO is involved in myogenesis [14,15].

MS Effects on Cell Damage and Regeneration Triggered by Mepivacaine

Some studies have shown that single intramuscular injection of mepivacaine leads to a myotoxic injury, which ultimately results in the degeneration of muscle fibers and cell

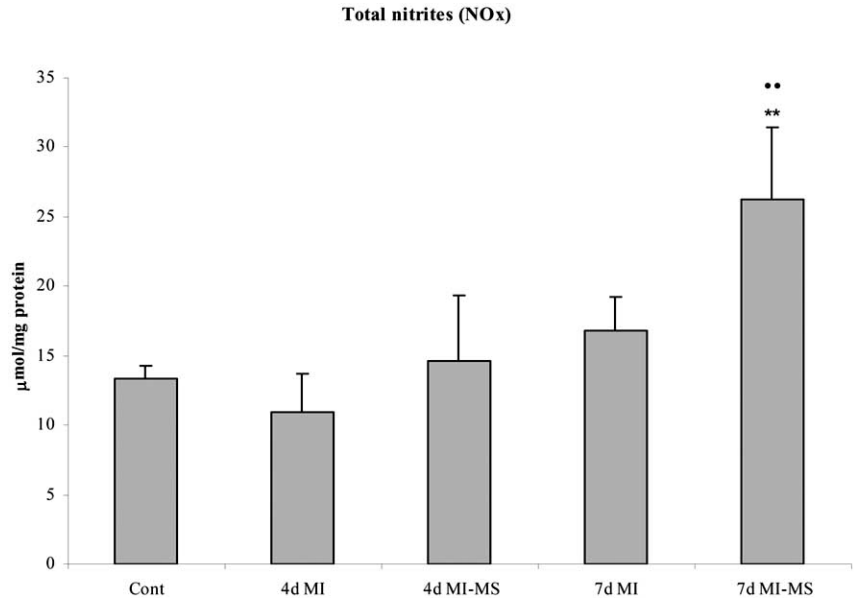

Fig. (1). Effects of mepivacaine injection and magnetic stimulation on total nitrites in anterior tibial muscles. Values are mean ± SD; n = 4 animals per group. $^{**}P < 0.05$ *vs* Cont; $^{\bullet\bullet}P < 0.05$ *vs* 7d MI.

death by necrosis. This process was shown to peak on the third and fourth day, followed by the regeneration of many fibers [16-20]. These data are in agreement with our results, which did not reveal any changes in the activity of caspase-3, a biomarker of apoptosis, whereas oxidative damage biomarkers indicated an intensive oxidative stress response (Table **1**); these findings could indicate the participation of necrosis in myodegeneration induced by mepivacaine injection.

In addition, no significant changes were observed in muscle regeneration and morphometric analysis at day 4 in the 4d MI and 4d MI-MS groups (data non-shown). Nevertheless, MS had a positive effect on muscle regeneration after six days of treatment. An increase in the density and size of regenerating muscle fibers was observed with respect to 7d MI group. Moreover, while the endomysial and perymisial space increased and numerous mononucleated cells were present 7 days post-injury in the 7d MI group, 7d MI-MS group displayed minor inflammatory mononuclear cells, the extracellular space was reduced and the general histoarchitecture of the muscle, divided into fascicles, was more evident (Fig. **2**).

The reasons for success with MS remain obscure but may have been related, at least partly, to the increase in NO observed here. NO plays an important role in muscle hypertrophy, muscle regeneration and improves muscle repair in *mdx* dystrophic mice [21, 22]. Moreover, it has been showed that NO release mediates satellite cell activation [21], which are responsible for the repair and the regeneration of muscle tissue [23]. In our study the number of satellite cells was not quantified; however, the increased density of regenerating muscle fibers were significantly higher in 7d MI-MS group (53.57 ± 6.88 fibers/area) than 7d MI group (34.83 ± 9.99 fibers/area) (Fig. **3**) provides morphological evidence that early enhancement of muscle regeneration was probably related with the increase of NO which mediates satellite cell activation. Experiments to investigate the expression of satellite cell markers after MS application are currently being performed. This data supported, at least partly, the important role played by NO in the regeneration of skeletal muscle stimulated by MS.

Some differences were also noted when comparing 7d MI group muscle and regenerating muscle treated with MS, suggesting that the growth and maturation of new muscle fibers was accelerated by MS application. Desmin staining provides a useful method for evaluation of muscle fiber regeneration [24]. Our observations demonstrate a strong positive immunostaining on all regenerating muscle fibers of 7 MI group, whereas desmin showed a marked reduction of immmunoreactivity in the 7d MI-MS group, which is coincident with the more advanced stage of maturity (Fig. **2**).

Another feature indicating accelerated regeneration was histochemical fiber type differentiation, suggesting subsequent innervation of regenerating muscle fibers. Reinnervation is an essential step for the normal progression of muscle regeneration; in regenerating rat skeletal muscles after cardiotoxin-induced myonecrosis, fiber-type discrimination in

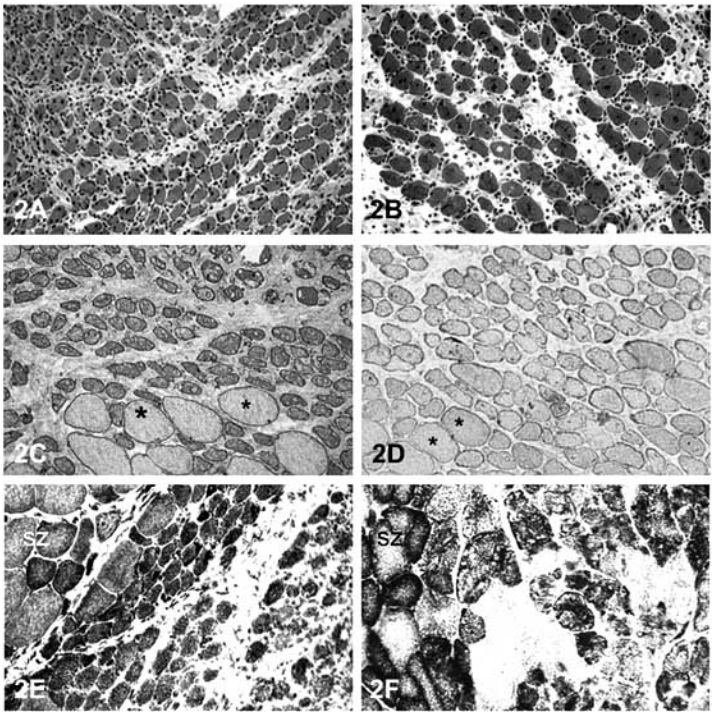

Fig. (2). Cross sections from muscles of 7d MI (left panel) and 7d MI+MS groups (right panel). a-b (H-E, 20x); c-d (anti-desmin, 20 x); e-f (NADH-tr, 20 x). (a) area of small regenerating muscle fibers with central nuclei and enlarged interstitial space with numerous mononucleated cells; (b) a similar section shows a greater number of regenerating muscle fibers with sparse central nuclei and absence of mononucleated cell infiltrate; (c) regenerating muscle fibers with strong desmin inmunoreactivity close to normal muscle fibers (asterisks) (d) muscle fibers show weak desmin inmunostaining close to normal muscle fibers (asterisks); (e) regenerating muscle fibers showing uniformity with oxidative enzyme stain; (f) early fiber type differentiation can be observed. (e,f) On the left side different fibers types are visible in surviving zone (SZ). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this paper).

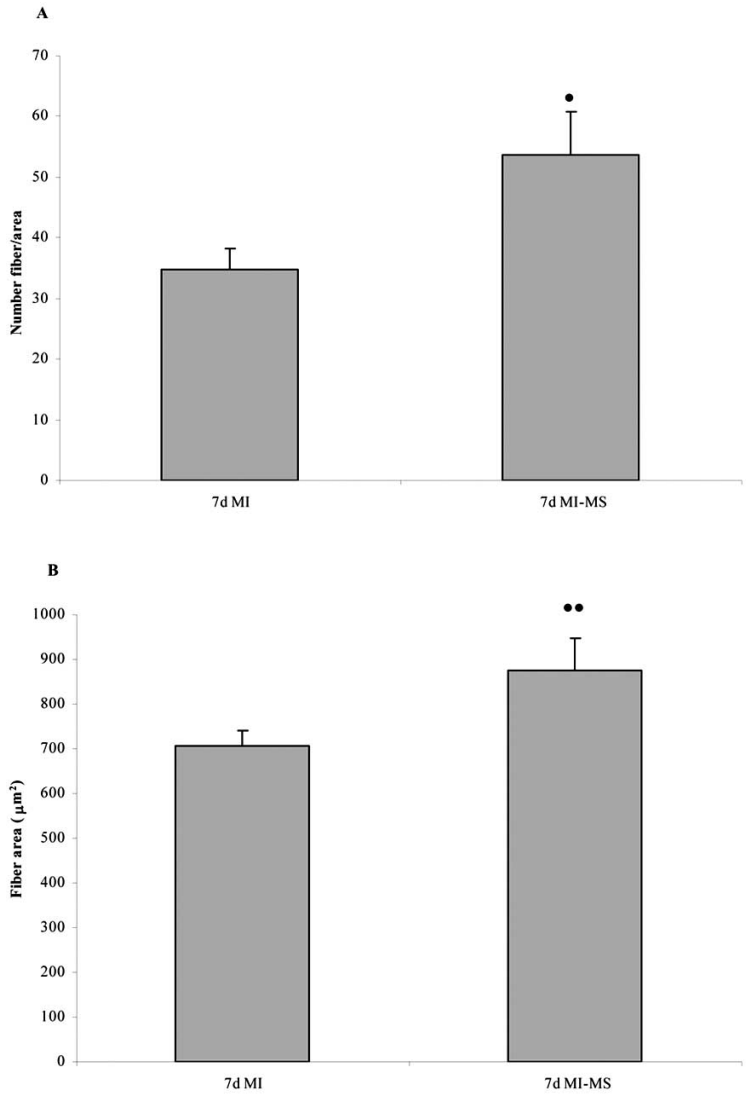

Fig. (3). Morphometric quantification of regenerating muscle fibers showing a significative increase in 7d MI-MS group compared to 7d MI group ($P< 0.01$) (panel A). Histogram of the mean fiber cross-sectional area, showing a significative difference between 7d MI and 7d MI-MS groups ($P< 0.05$) (panel B).

rat muscle regeneration is normally first noted from day 10 and becomes more obvious after day 14 [25]. In our study, the results suggested that muscle reinnervation in the 7d MI-MS group may have occurred earlier (Fig. **2**). While in the 7d MI group muscles the uniform staining of regenerating muscle fibers with NADH-tr reaction reflected their immaturity, the differentiation into type I and II in the 7d MI-MS group may be result of an accelerated process in the reinnervation of regenerating muscle fibers (Fig. **2**).

Also, the increases in myofiber size suggested that these fibers had been reinnervated because this process is a necessary requirement for growth and maturation of regenerated muscle fibers [26]. Morphometrically, differences in average fiber area were observed between the two groups: the mean of the 7d MI-MS group ($876 \pm 46.75\ \mu m^2$) was significantly higher ($P< 0.05$) than of the 7d MI group ($707 \pm 41.08\ \mu m^2$) (Fig. **3**). Our findings coincide partly with those reported by De Pedro *et al*. [27], who showed that pulsed electromagnetic fields may accelerate muscular reinnervation processes [27], and a study that reported that MS is an effective method for reducing muscle atrophy after spinal cord injuries [11]. Thus, muscle reinnervation in the 7d MI-MS group may have occurred earlier, leading to precocious growth and differentiation of regenerating muscle fibers.

Conclusion

It is also important to bear in mind that according to our knowledge this is the first study: i) to research the parameters of indicative oxidative stress, nitric oxide production and regeneration in model of skeletal muscle injury induced by mepivacaine in the same animal; and ii) to evaluate the effect of MS on changes induced by mepivacaine injection in skeletal muscle.

In conclusion, the evidence obtained in this study suggests: i) the implication of oxidative stress in skeletal muscle damage induced by mepivacaine; and ii) the protective effect

of MS on muscle damage characterized by restoration of biomarkers of oxidative stress and increases in NO levels; and iii) that the early stages of regeneration can be facilitated by applying MS to injured skeletal muscle. These results suggest that MS could be used as a therapeutic method for the recovery of damaged skeletal muscle. However, further research must be performed in order to achieve a better understanding of the mechanisms involved in MS effects and its therapeutic use.

MATERIAL AND METHODS

Reagents and Drugs

Mepivacaine (Scandinibsa®, mepivacaine 2%) was purchased from Laboratorios Inibsa S.A. (Barcelona, Spain); all other reagents were purchased from Sigma S.A. (St. Louis, MO, USA).

Animals

Four male Wistar rats per cage, at the age of two-month were housed at a constant temperature (20 – 23º C), illumination (12-h light/12-h dark cycle, light on at 08:00 h) and were provided with food and water *ad libitum*. Animal welfare and procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ECC) and RD 223/1988, and were approved by the University of Cordoba's Bioethics Committee, Spain.

500 µl of mepivacaine was administered intramuscularly (i.m.) to the anterior tibial muscles in order to produce necrotic degeneration [14,15,23]. Magnetic stimulation (MS) was applied during three or six days as it is described elsewhere.

Twenty rats were divided into three groups as follows: i) Intact controls (Cont); ii) mepivacaine- injected (MI) only for 4 days (4d MI); iii) MI for 4 days and MS for the last 3 days (4d MI-MS); iv) MI only for 7 days (7d MI); and iii) MI for 7 days and MS for the last 6 days (7d MI-MS).

Magnetic Stimulation

To evaluated MS effect on regeneration and oxidative stress animals were placed in plastic cylindrical cages designed to keep them immobile while receiving magnetic stimulation. Each coil consisted of 1000 turns of enamelled cooper wire (7 cm diameter) contained in plastic boxes (10.5 x 10.5 x 3.5 cm). A pair of Helmholtz coils (selected to minimize electric field effects) generated the fields (Dham 1000(tm); Magnetoterapia S.A. de C.V., Mexico DF, Mexico). The stimulation consisted of an oscillatory magnetic field in the form of a sinusoidal wave with a frequency of 60 Hz and amplitude of 0.7 mT applied for 2 h. Previous studies of our group showed that animals showed no signs of discomfort (i.e. agitation) when exposed to MS [28,29]. The daily exposure time was selected based on our previous demonstrating *in vivo* neuronal regeneration and reduction of oxidative stress [28,29]. The two coils were initially located dorsally and ventrally to the near of lumbar and sacral vertebrae level for stimulating their legs. The distance between the two coils was approximately 12 cm. MS was applied for a period of six days. The length of *in vivo* exposure was determined on the basis of previous studies [30, 16-20]. MS was initiated on the next day after injury.

Assay of Lipid Peroxidation Products

Measurement of malondialdehyde (MDA) + 4-hydroxyalkenals (4-HDA) has been used an indicator of lipid peroxidation. The levels of lipid peroxides were determined using reagents purchased from Oxis International (LPO-586 kit; Oxis International, Portland, OR, USA). The levels were expressed as nanomoles of MDA+4-HDA per milligram of protein (nmol/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603, Shimadzu) at a wavelength of 586 nm.

Assay of Reduced Glutathione Content

Reduced glutathione (GSH) plays a central role as coenzyme in different enzymes such as glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GST). Additionally, this tripeptide comprises an important part of the antioxidant system. The determination of the levels of GSH was carried out using reagents purchased from Oxis International, i.e. the GSH-420 kit. The content of GSH is expressed as GSH nanomoles per milligram of protein (nmol/mg protein), and the absorbance was evaluated in a spectrophotometer (UV-1603; Shimadzu) at a wavelength of 420 nm.

Assay of Total Nitrite as a Marker of Nitric Oxide Levels

Nitric oxide (NO) is produced endogenously by a variety of mammalian cells. This molecule induces vasodilatation; it inhibits platelet aggregation and adhesion on the vascular endothelium and elevates cyclic GMP intracellular levels.

Total nitrite (nitrite and nitrate; NOx) was used as a marker of NO levels and assayed following the Griess method [31] in homogenates of anterior tibial muscle. NO is transformed in nitrate and nitrite. Because a colorimetric reagent (the Griess reagent) exists for the determination of nitrite, it is common practice to use either enzymatic or chemical reduction to convert all nitrates to nitrite in a sample and measure total nitrite as indicator of NO production. Nitrate was reduced to nitrite by incubating a sample aliquot (150 µL) for 15 min at 37º C in the presence of 0.1 U/mL nitrate reductase, 50 µM NADPH and 5µM flavin-adenine dinucleo-tide in a final volume of 160 µL. When nitrate reduction is complete, total nitrite is then determined by means of spectrophotometry using the Griess reaction. Griess reagent is composed a mixture of sulfanilamide 2% (w/v) and N-(1-naphthyl)-ethylenediamine 0.2% (w/v). The reaction was monitored at 540 nm. The absorbance was evaluated in a spectrophotometer (UV-1603; Shimadzu). The values are presented in micromoles per milligram of protein (µmol/mg protein).

Caspase-3 Activity

The caspase-3 activity in the brain homogenates were measured using reagents purchased from BioVision Research Products (Mountain View, CA, USA). The activity is expressed as optical density arbitrary units per milligram of protein (O.D. arbitrary units/mg protein), and absorbance was evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at a 405 nm wavelength.

Protein Estimation

Protein concentration was determined by the Bradford method, using bovine serum albumin as a standard. Standard curve and samples were evaluated in a spectrophotometer (UV-1603; Shimadzu),

Tissue Processing and Histological Analysis

Serial transverse sections (8 μm thick) were cut using a cryostat at -20ºC and mounted on glass slides. The sections were air dried and stained.

The effect of MS on muscle regeneration was morphologically investigated by haematoxylin-eosin (H&E) stain and the histochemical reaction for nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR). Desmin has been used as a marker for regenerating muscle fibers; for desmin inmunostaining, a monoclonal antibody was used (1:50, Desmin, DE-R-11, Dako Denmark).

Morphometric analysis was performed using images of muscle sections stained with H&E examined at 200x; the images were acquired with a video camera mounted on a microscope attached to a personal microcomputer. The number of regenerating muscle fibers per area was referred to as "regenerating muscle fibers density" and cross-sectional area of these fibers was measured using an image-analysis program (IMAGE PRO PLUS). Regenerating fibers were identified by central nuclei, small size and, occasionally basophilia. At least 300 regenerating muscle fibers were measured in each muscle.

Statistical Analysis

Statistical analysis of data was accomplished by means of the SPSS® statistical software package (SPSS Iberica, Madrid, Spain). The Shapiro-Wilk test did not show a significant departure from normality in the distribution of variance values. To evaluate variations in data, a one-way analysis of variance (one-way ANOVA) was corrected with the Bonferroni test. The statistical significance of intra-groups for morphometric parameters was determined by t-student test. The level of statistical significance was set at $P<0.05$. All results are expressed as mean ± SD.

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Received:, 19 June, 2008 Revised:, 30 October, 2008 Accepted:, 13 November, 2008