*Electromagnetic Biology and Medicine*, 31(4): 320–332, 2012

ISSN: 1536-8378 print / 1536-8386 online
DOI: 10.3109/15368378.2011.641706

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# Evaluation of inflammatory biomarkers associated with oxidative stress and histological assessment of magnetic therapy on experimental myopathy in rats

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The effect of pulsed electromagnetic field (PEMF) therapy, also called magnetic therapy, upon inflammatory biomarkers associated with oxidative stress plasma fibrinogen, nitric oxide (NO), L-citrulline, carbonyl groups, and superoxide dismutase (SOD) was evaluated through histological assessment, in rats with experimental myopathy.
The groups studied were: (A) control (intact rats that received PEMF sham exposures); (B) rats with myopathy and sacrificed 24 h later; (C) rats with myopathy; (D) rats with myopathy and treated with PEMF; and (E) intact rats treated with PEMF. Groups A, C, D, and E were sacrificed 8 days later. Myopathy was induced by injecting 50 μl of 1% carrageenan λ (type IV) once subplantar. Treatment was carried out with PEMF emitting equipment with two flat solenoid disks for 8 consecutive days in groups D and E, at 20 mT and 50 Hz for 30 min/day/rat. The biomarkers were determined by spectrophotometry. The muscles (5/8) were stained with Hematoxylin-Eosin and examined by optic microscopy. Quantitative variables were statistically analyzed by the Fisher test, and categorical applying Pearson's Chi Squared test at $p < 0.05$ for all cases.
In Groups B and C, the biomarkers were significantly increased compared to A, D, and E groups: fibrinogen ($p < 0.001$); NO, L-citrulline and carbonyl groups ($p < 0.05$); SOD ($p < 0.01$) as well as the percentage of area with inflammatory infiltration ($p < 0.001$).
PEMF caused decreased levels of fibrinogen, L-citrulline, NO, SOD, and carbonyl groups and significant muscle recovery in rats with experimental myopathies.

**Keywords** Magnetotherapy, Experimental myopathy, PEMF, Oxidative stress, Inflammatory biomarkers, Pulsed electromagnetic field therapy

## INTRODUCTION

The term myopathy is defined as "muscle disease" affecting the muscle's structure, morphology, and biochemistry (Kumar et al., 2008).

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One of the agents that can induce inflammation is carrageenan, a polysaccharide and powerful irritant, widely used in experimental animals (Albertini et al., 2008). The vascular and cellular response to inflammation is mediated by chemical factors that increase the plasma concentration of acute phase proteins, including fibrinogen, the levels of which are not specific but are significant parameters related to the process of inflammation. This was demonstrated in previous studies from our laboratory in which injuries were induced in rats by different methods (Campana et al., 2004; Rubio et al., 2009; Servetto et al., 2010; Soriano et al., 2006).

Several of the cytokines, alarmins, and eicosanoids that predominate in muscle tissue may act both as catabolic or anabolic factors. The dual effect of these molecules — proinflammatory and anabolic/catabolic — are relevant for inflammatory diseases (Loell and Lundberg, 2011).

In inflammatory processes, nitric oxide (NO) acts as a possible modulator, being synthesized from L-arginine in an equimolar reaction with $O_2$, NADPH, and the production of L-citrulline. High levels of NO coproduct were found in serum of patients with inflammatory diseases (Ciurtin et al., 2006). NO plays an important role in normal physiological processes and pathological conditions (Pham et al., 2003); oxidative damage due to its overproduction and that of other reactive oxygen species (ROS) may be involved in inflammatory pathogenesis (Kanwar et al., 2009; Yudoh et al., 2005). When the increase in the intracellular content of ROS exceeds the antioxidant defenses of the cell, oxidative stress occurs, which alters the cell function and contributes to the development of inflammatory conditions (Sies, 2007).

Protein is one of the most important targets of ROS and its oxidation can lead to loss of protein function, as well as the conversion of protein forms that are more susceptible to degradation by proteinases. ROS and other radicals generated by products of cellular metabolism cause oxidation of amino acids such as arginine, lysine, proline and threonine, which leads to loss of protein function and/or enzyme activity and subsequent formation of carbonyl groups (Barreiro et al., 2005).

Most of the antioxidant activity in living organisms is due to the combined action of enzymes: catalase, glutathione peroxidase, and superoxide dismutase (SOD). SOD originates a series of reactions designed to remove excess ROS and prevent irreversible damage, so it is credited with antioxidant and anti-inflammatory effects (Zhang et al., 2002).

Conventional edema treatment in inflammation includes the use of anti-inflammatory agents as well as complementary medicine such as pulsed electromagnetic field (PEMF) therapy. Magnetotherapy is known also to be beneficial in treatment of chronic pain associated with connective tissue (cartilage, tendon, ligament, and bone) injury and associated joint soft tissue injury (Barnes, 2007; Gourdarzi et al., 2010; Harden et al., 2007; Rumbaut and Mirkovic, 2008; Thomas et al., 2007). Although this therapy is widely used in the rehabilitation clinic with positive results in inflammatory conditions, there is not enough scientific literature to support its therapeutic effect, so we decided to investigate it in an experimental model of myopathy, identifying inflammatory biomarkers associated with oxidative stress: plasma fibrinogen, NO, L-citrulline, carbonyl groups, and SOD, and analyzing the possible anatomopathological changes.

## MATERIALS AND METHODS

### Experimental Groups

Fifty Wistar strain female rats weighing 200 ± 20 g were used, distributed in 5 groups (n = 10): (A) control (intact rats that received PEMF sham exposures, 30 min/day for 8 consecutive days prior to being sacrificed); (B) rats with myopathy and sacrificed

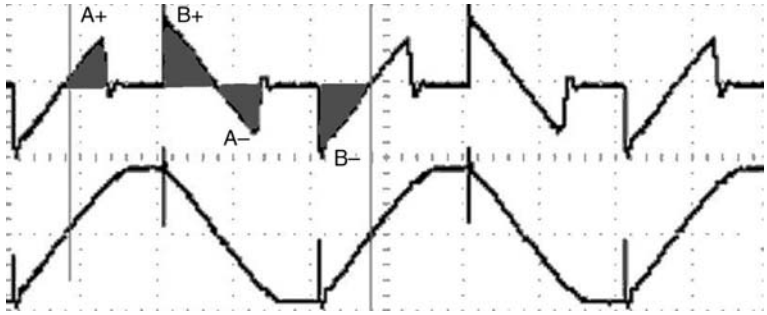


**FIGURE 1** Carrier signal: biphasic truncated sinewave waveform.



24 h later; (C) rats with myopathy and sacrificed 8 days later; (D) rats with myopathy and treated with PEMF; (E) intact rats treated with PEMF. A larger number of animals was not used per group because of the low dispersion showed by the variables studied in previous work (Campana et al., 2004; Rubio et al., 2009; Servetto et al., 2010; Soriano et al., 2006).

### Experimental Model of Myopathy

Fifty μl of 1% carrageenan λ (type IV), dissolved in distilled water, was injected subplantar once into the left hind limb of the rat in groups B, C, and D, in order to produce muscle injury, inducing an inflammatory process (Albertini et al., 2008; Bortone et al., 2008; Di Rosa, 1972; Winter et al., 1962).

Myopathy groups C and D were sacrificed 8 days after induction of myopathy, and group B was sacrificed 24 h after injection with carrageenan. The purpose of this group was to know the degree of injury and inflammation generated, prior to starting treatment with PEMF. The investigation was conducted according to the guide for the care and use of laboratory animals published by the U.S. National Institutes of Health, NIH publication (No. 85-23, revised 1996).

### Treatment with PEMF

It was carried out with field electromagnetic emitting equipment (Magnetherp 200 - Meditea; Buenos Aires, Argentina). Modulated output signal ASK (Amplitude Shift Keying) with the following characteristics: carrier frequency, 50 Hz; modulating frequency, 0.78 Hz; amplitude RMS, 18.6 V; peak to peak voltage, 68.8 V and carrier signal: biphasic truncated sinewave waveform (Fig. 1). Modulating signal: square waveform (Fig. 2). The PEMF has two circular magnetic plates (each 12 cm in diameter and 1.8 cm thick), separated by 15 cm, placed inside a transparent vinyl cylinder to keep the rat calm and resting its plantar surface on the electrode (Fig. 3).

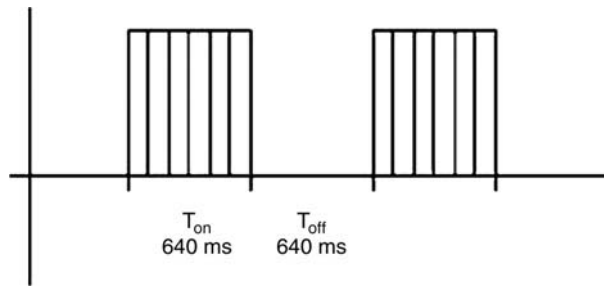


**FIGURE 2** Modulating signal: square waveform.

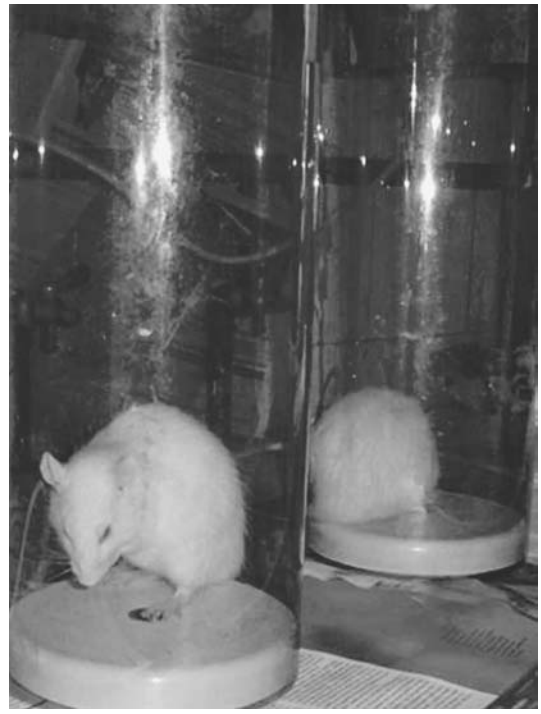

**FIGURE 3** Treatment with PEMF.



A daily session was conducted for 8 consecutive days in both group D (starting sessions 24 h after the injection of carrageenan) and group E, using PEMF of 20 mT and 50 Hz for 30 min/day/rat, the same intensity used in the treatment of inflammatory processes in humans.

### Experimental Material

The blood was obtained by decapitation of the animals, after anesthesia by Ketamine 10 mg/kg/rat, and was centrifuged at 3000 rpm to obtain the plasma. The plasmatic biomarkers were determined by spectrophotometry using techniques described by several authors: fibrinogen by Ratnoff and Menzie (1957), L-citrulline by Boyde and Rahmatullah (1980), NO as a Griess reaction (Choi, 2003), and SOD in red blood cell lysate using Randox Kit (Woolliams et al., 1983).

The muscles (5/10) were cut and one part placed in formaldehyde 10% (single-blinded), stained with Hematoxylin-Eosin (H-E) to see the amount of fibrous or connective tissue generated by inflammation and observed by optic microscopy. Another part of the same tissue was suspended in buffer composed of N-[2-hydroxyethyl] piperazine-N'- [2-ethanesulfonic acid] (HEPES), CLK, disodium ethylenediaminetetraacetate (EDTA), $SO_4Mg$ and phenylmethylsulfonyl fluoride (PMSF); homogenized and centrifuged for the determination of carbonyl groups by spectrophotometry, following the technique described by Levine et al. (1990). The protein concentration was measured by Bradford's method (1976).

### Statistical Analysis

The quantitative variables are expressed as mean $\pm$ SE and were statistically analyzed by ANOVA and the Fisher test, and the percentage area with inflammatory infiltrates was determined in 5 optical microscopy photographs (100X) for each group and analyzed with the Axiovision 4.8 program - Carl Zeiss Imaging

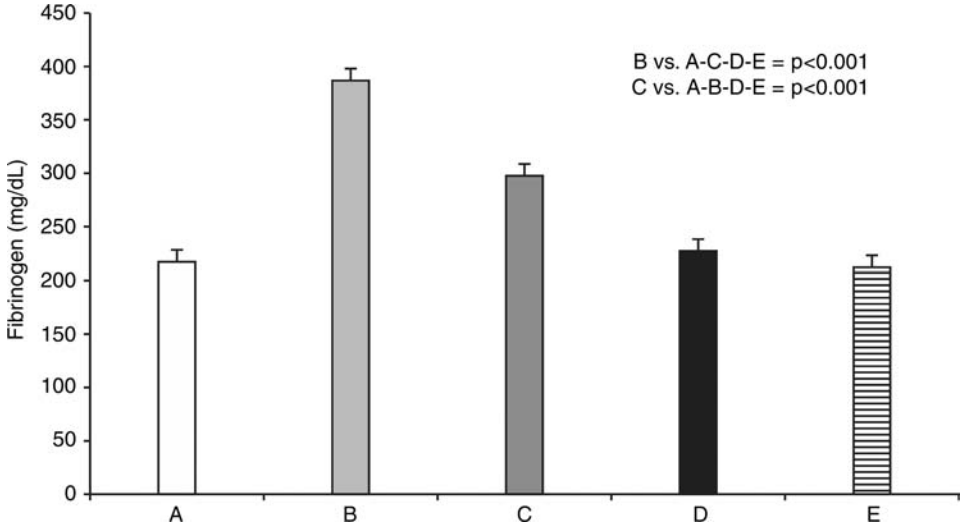


**FIGURE 4** Effect of PEMF on plasma fibrinogen levels in rats with myopathy from injection of carrageenan (n = 10). Means ± SE are presented.

Solutions GmbH (Hallbengmoos – Germany), referenced to a scale of 1 μm. For the quantification analysis, Pearson's Chi Squared test was applied, establishing significant difference when $p < 0.05$ for all cases.

## RESULTS

The effect of PEMF on fibrinogen levels in rats with myopathy can be seen in Fig. 4. Fibrinogen significantly increased in the myopathy group sacrificed 24 h later (387.46 ± 11.82 mg/dL) (B) and the myopathy group sacrificed 8 days later (298.38 ± 9.69 mg/dL) (C), compared with the groups: control (218.46 ± 9.37 mg/dL) (A), with myopathy and treated with PEMF (227.46 ± 7.52 mg/dL) (D), and the group treated with PEMF (212.86 ± 7.47 mg/dL) (E) ($p < 0.001$). There were significant differences between groups B and C ($p < 0.001$). There were no significant differences between groups A, D, and E.

The effect of PEMF on L-citrulline levels in rats with myopathy can be seen in Fig. 5. L-citrulline increased significantly in the group with myopathy sacrificed 24 h later (4.49 ± 0.22 mM) (B) and the group with myopathy sacrificed 8 days later (3.98 ± 0.12 mM) (C), compared with the groups: control (3.38 ± 0.04 mM) (A), with myopathy and treated with PEMF (3.54 ± 0.07 mM) (D), and the group treated with PEMF (3.52 ± 0.17 mM) (E) ($p < 0.05$). There were significant differences between groups B and C ($p < 0.05$). There were no significant differences between groups A, D, and E.

The effect of PEMF on NO levels in rats with experimental myopathy is shown in Fig. 6. NO increased significantly in the group with myopathy sacrificed 24 h later (14.09 ± 0.67 μM) (B) and the group with myopathy sacrificed 8 days later (11.47 ± 0.51 μM) (C), compared with the groups: control (7.39 ± 0.77 μM) (A), with myopathy and treated with PEMF (9.46 ± 0.68 μM) (D), and the group treated with PEMF (8.12 ± 0.61 μM) (E) ($p < 0.05$). There were significant differences between groups B and C ($p < 0.05$).

The effect of PEMF on carbonyl group levels in rats with experimental myopathy is shown in Fig. 7. Carbonyl groups increased significantly in the group with myopathy sacrificed 24 h later (3.25 ± 0.41 nmol/mg) (B) and the group with myopathy

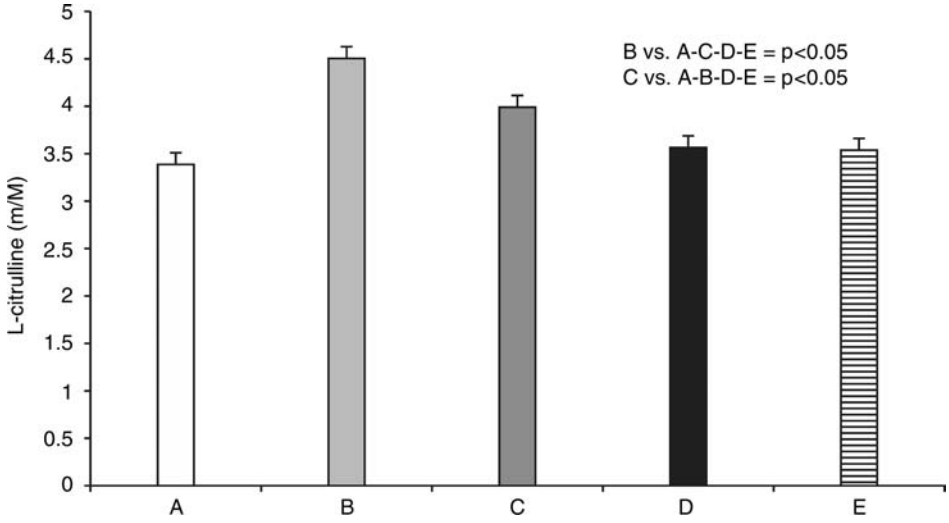


**FIGURE 5** Effect of PEMF on plasma L-citrulline levels in rats with myopathy from injection of carrageenan (n = 10). Means ± SE are presented.

sacrificed 8 days later (2.34 ± 0.19 nmol/mg) (C), compared with the groups: control (1.04 ± 0.08 nmol/mg) (A), with myopathy and treated with PEMF (1.57 ± 0.23 nmol/mg) (D), and the group treated with PEMF (1.18 ± 0.15 nmol/mg) (E) ($p < 0.05$). There were significant differences between groups B and C ($p < 0.05$). There were no significant differences between groups A, D, and E.

The effect of PEMF on SOD levels in rats with experimental myopathy is shown in Fig. 8. SOD increased significantly in the group with myopathy sacrificed 24 h later (154.50 ± 8.70 U/ml) (B) and the group with myopathy sacrificed 8 days later (153.81 ± 3.36 U/ml) (C), compared with the groups: control (134.00 ± 2.10 U/ml) (A), with myopathy and treated with PEMF (127.14 ± 3.99 U/ml) (D), and the group treated with PEMF (130.79 ± 2.44 U/ml) (E) ($p < 0.01$). There were no significant differences between groups B and C, nor between groups A, D, and E.

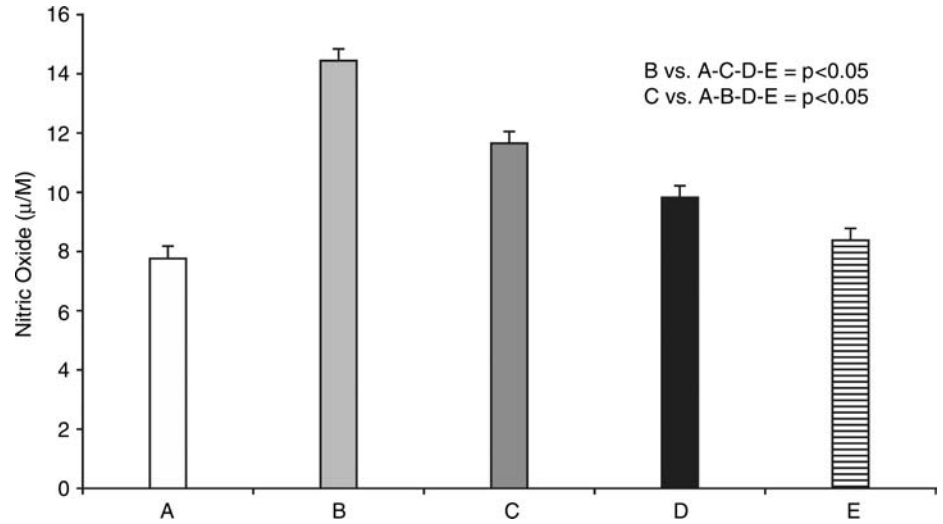


**FIGURE 6** Effect of PEMF on plasma Nitric Oxide levels in rats with myopathy from injection of carrageenan (n = 10). Means ± SE are presented.

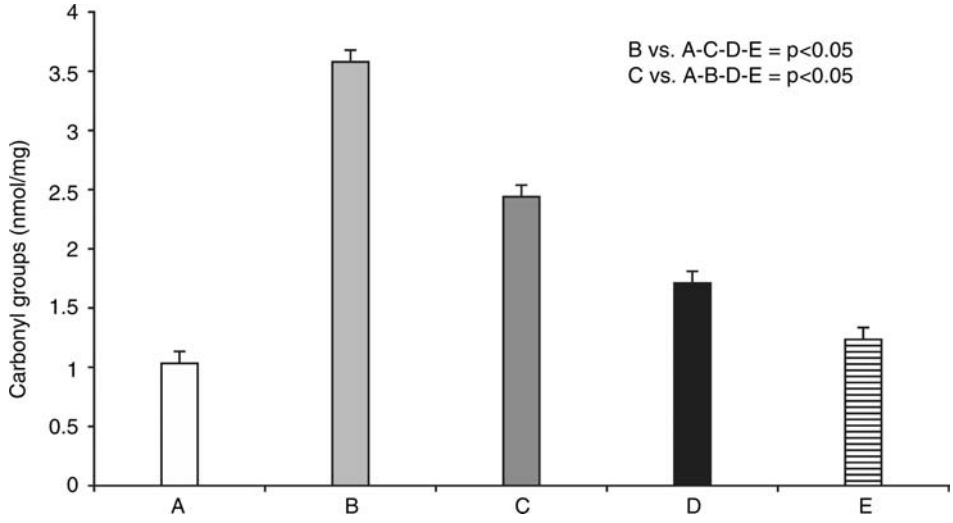


**FIGURE 7** Effect of PEMF on homogenate tissue carbonyl group levels in rats with myopathy from injection of carrageenan (n = 10). Means ± SE are presented.

Sections of skeletal muscle of the groups: control (A), with myopathy and sacrificed 24 h later (B), with myopathy and sacrificed 8 days later (C), and with myopathy and treated with PEMF (D), observed by optical microscopy and at 400X magnification, are shown in Figs. 9, 10, 11, and 12, respectively.

The percentages of area with inflammatory infiltrate in rats with induced myopathy and treated with PEMF can be seen in Table 1. The percentage of inflamed area was significantly increased in both groups with myopathy without treatment with PEMF (B) and (D), when compared with the groups: control (A), with myopathy and treated with PEMF (D) and intact rats treated with PEMF (E) ($p < 0.001$). There was no significant difference beween groups B and C.

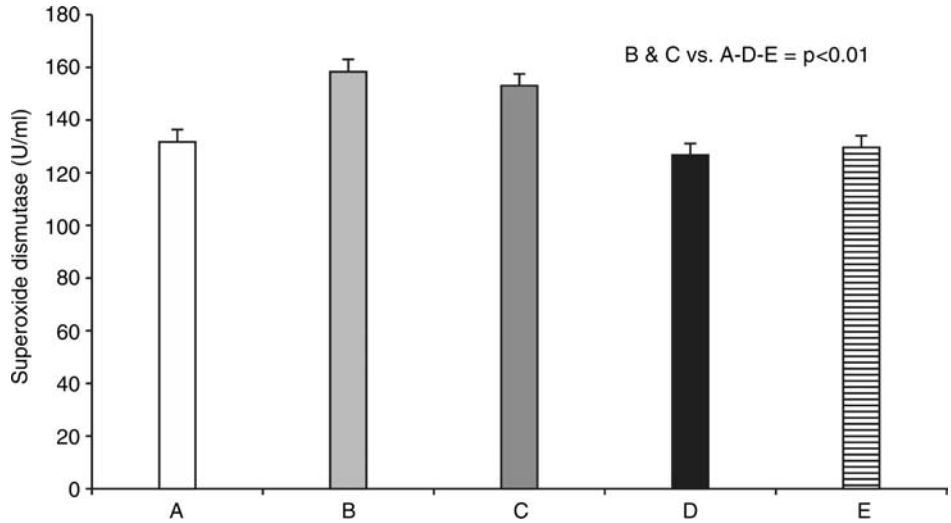


**FIGURE 8** Effect of PEMF on red blood cell lysate SOD levels in rats with myopathy from injection of carrageenan (n = 10). Means ± SE are presented.

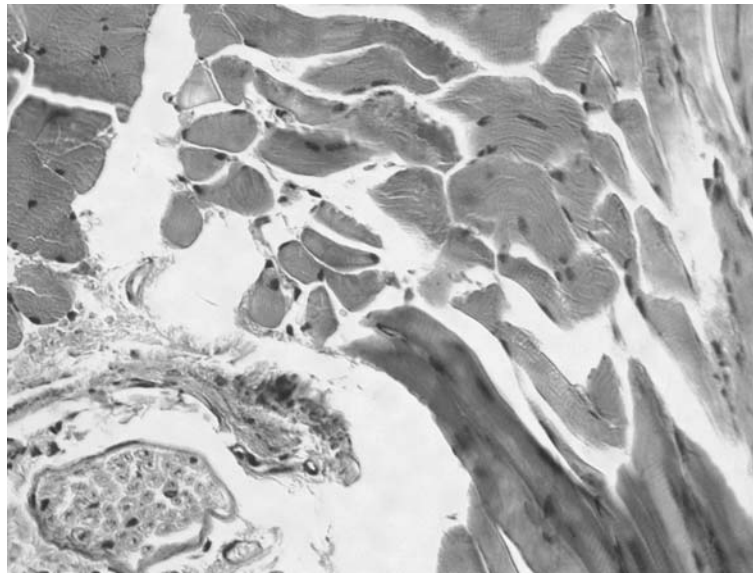

**FIGURE 9** Control group (intact rats that received PEMF sham exposures) (A). Striated muscle fibers are preserved and connective tissue and a transversal section of a nerve can be observed. No signs of myositis or fibrosis are seen. H-E (400X).

## DISCUSSION

The experimental model of myopathy was successfully reproduced. Significant edema and erythema of the limb was observed macroscopically, in addition to significant changes in concentration of biomarkers and histological structures.

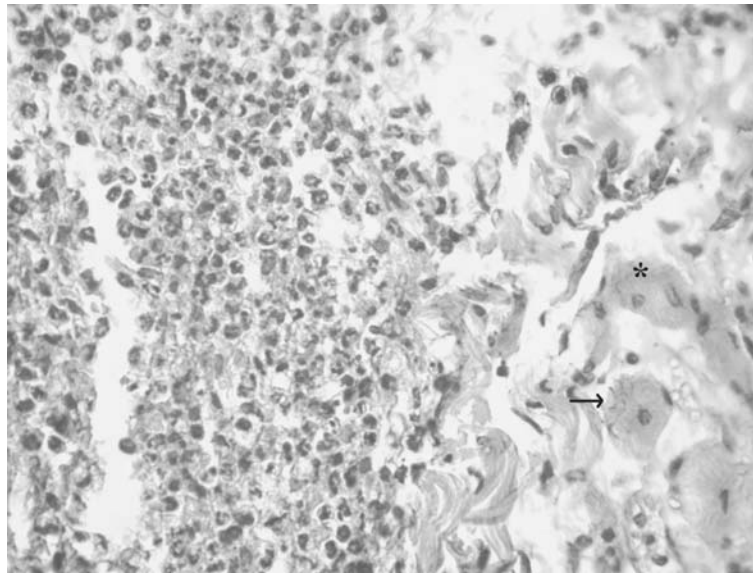


**FIGURE 10** Group with myopathy and sacrificed 24 h later (B). Intense acute inflammatory infiltrate, rich in polymorphonuclear neutrophils, macrophages and plasmacytes. The inflammation and edema dissociate the fibers. The muscle fibers show structural changes such as vacuolization of the sarcoplasmic tubules at the periphery of the fibers (arrow) and central disposition of the nuclei, with evident nucleoles (asterisk). H-E (400X).

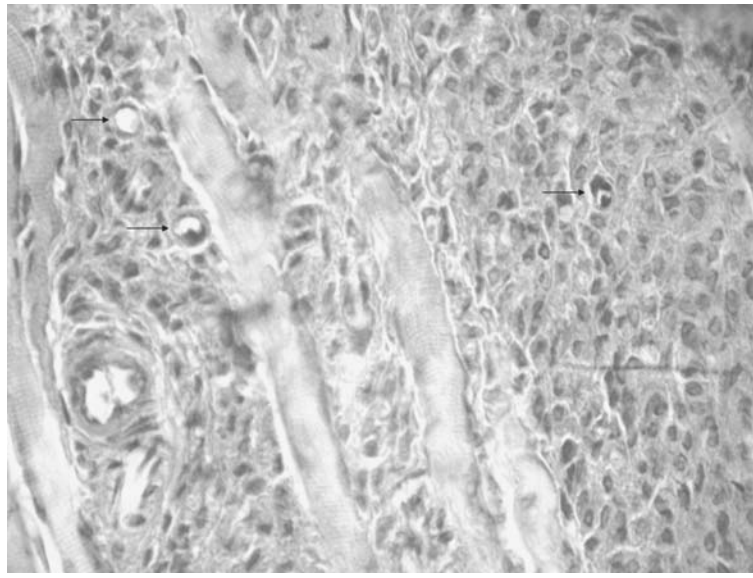

**FIGURE 11** With myopathy and sacrificed 8 days later group (C). Chronic moderate infiltration, with mononuclear and fibroblast proliferation. The interstitial connective tissue begins to organize itself and shows newly formed capillaries (arrows). The process involves the muscle and dissociates the fibers. H-E (400X).

The results of recent work from our laboratory showed no significant plasma inflammatory indicator (Rubio et al., 2009) and histological structure (Rubio et al., 2010) changes between a group of intact rats and a group of rats injected with saline, which also confirms that the induction of the inflammatory process is attributable only to carrageenan and not to the possible stress caused by the puncture.

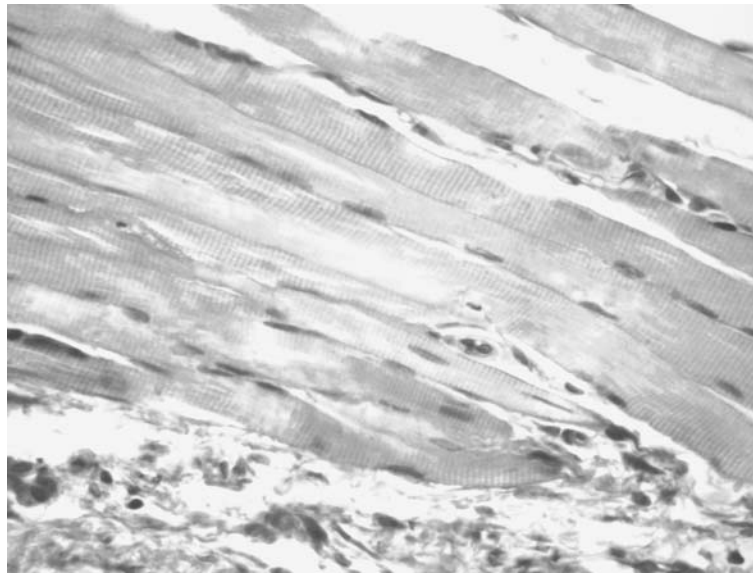

**FIGURE 12** With myopathy and treated with PEMF group (D). The muscle fibers are preserved. Some bundles have mild interstitial inflammation. The inflammation was slight, predominantly of histiocytes and mononuclear cells, and fibroblasts were abundant. No myofibrillar and sarcolemmal damage is seen and the nuclei retain their size and peripheral arrangement, H-E (400X).

TABLE 1 Percentage of area with inflammatory infiltrates from 5 optical microscopy photographs (100X) for each group.

| Groups | Treatment | Inflammatory infiltrate (%) |
|---|---|---|
| A | Control, intact rats that received PEMF sham exposures | 0 |
| B | Rats with myopathy and sacrificed 24 h later | 58.672 |
| C | Rats with myopathy and sacrificed 8 days later | 55.516 |
| D | Rats with myopathy and treated with PEMF | 33.358 |
| E | Intact rats treated with PEMF | 0 |

B vs. C = NS; C vs. D and E = $p < 0.001$; B vs. D and E = $p < 0.001$

The increase in fibrinogen (Fig. 4) matched results of research conducted with various injurious agents inductive of inflammatory and rheumatic diseases (Campana et al., 2004; Chicu and Badescu, 2005; Rubio et al., 2009; Servetto et al., 2010; Soriano et al., 2006; Traikov et al., 2009), which demonstrated the damaging effect of carrageenan.

The significant increase of L-citrulline (Fig. 5) in the groups with myopathy compared with the control group is correlated with clinical findings considering this an early marker of rheumatic diseases (Marini et al., 2004; Wanchu et al., 1999). It also behaved similarly to fibrinogen in the same groups studied, decreasing the concentrations of both variables after PEMF, which contributes to reinforce the anti-inflammatory effect of this treatment.

In the groups with myopathy, the concentration of NO (Fig. 6) was significantly higher than in all other groups, which correlates with the results for L-citrulline, generated as a coproduct in an equimolar equation with NO (Valdez et al., 2006). These results contrast with those obtained in another experimental myopathy model performed in our laboratory (Servetto et al., 2010), in which NO fell below that of the control group, so they presumably follow different routes.

When NO is found at high concentrations under conditions associated with inflammatory processes, in part it autoxidises, generating dinitrogen trioxide ($N_2O_3$), and it partly reacts with superoxide anion ($O_2^-$) forming peroxynitrite ($ONOO^-$). This reaction is characterized by being six times faster than that of $O_2^-$ with SOD and leads to lower availability of NO. This matches the increased levels of SOD in the group with myopathy, which suggests that there was an increase of $O_2^-$ in the group, since SOD is responsible for one of the mechanisms of elimination of $O_2^-$. The significant increase of this free radical and the possible presence of $ONOO^-$ may indicate the existence of oxidative stress (Sies, 2007). This is confirmed by the significant increase of carbonyl groups (Fig. 7) in animals with myopathy and sacrificed both at 24 h and after 8 days (groups B and C, respectively), highlighting the high degree of oxidative stress present in the muscle.

Raised concentrations of carbonyl groups have been found in various disorders, such as muscular inflammation, and is considered an excellent marker of protein oxidation mediated by oxidative stress (Andresen et al., 2008; Dalle-Donne et al., 2005).

In the groups with myopathy and treated with PEMF, the ON, L-citrulline, SOD, and carbonyl group values were similar to the control group, so we can attribute antioxidant activity to PEMF. These results may indicate that PEMF therapy regulates the levels of ROS, possibly acting at the level of the inner mitochondrial membrane, where not only does part of the synthesis of NO occur but there is also the greatest amount of $O_2^-$. These results partially coincide with Ciejka and Goraca (2009), who studied the influence of this therapy at the level of plasma antioxidant capacity. In addition, the studies of Kumar et al. (2011) reveal that oxidative stress is a major mechanism affecting health and that PEMF provides significant protection by controlling ROS production.

PEMF exposed in intact rats (E) does not cause disruption of normal tissue at morphological or systemic levels. Concentrations of biomarkers did not differ from control rats exposed to switched-off PEMF equipment (A), nor were histological changes observed, demonstrating that the sole manipulation of the animals did not produce stress and that magneto therapy is a low-risk treatment and does not affect cell viability at therapeutic doses, which agrees with other studies (Markov, 2009).

Another fact that seems to confirm the anti-inflammatory effect of PEMF was the macroscopic observation, in which a significant reduction in plantar diameter, rigidity, and limb edema was seen in animals with post-treatment myopathy.

Histological analysis of the group with myopathy revealed a significant percentage of area with mononuclear inflammatory infiltrate (Table 1), edema, destruction of muscle fibers, and their replacement by connective tissue and necrotic material, with few conserved muscle fibers. In the group with myopathy and sacrificed 24 h later (B), there was a severe inflammatory infiltrate characteristic of the action of carrageenan (Fig. 10). The predominance of macrophages in the inflamed muscle seems to account for the increased NO in plasma (Fig. 6), since it in these cells that it is produced, contributing to toxicity and cell damage. These results match those of Yudoh et al. (2005) and Rubio et al. (2009). In the group of rats with myopathy and sacrificed 8 days later (C), however, the inflammation became chronic (Fig. 11). The presence of neutrophils in groups B and C may explain the increased concentration of carbonyl groups in the inflamed tissue. In contrast, in the group with myopathy and treated with PEMF (D), there was a notable reduction in the area occupied by inflammatory infiltrate (Fig. 12).

In agreement with the view of other authors (Barnes, 2007; McKay et al., 2007; Morris and Skalak, 2005), we believe that these structural changes associated with systemic changes are due to stimulation of the microcirculation. These initial mechanistic studies may provide the basis for subsequent experiments aimed at defining additional cellular mechanisms (and the cell types involved) in the physiological effects of magnetic therapy on the microcirculation. Based on their prior studies, the above authors emphasized changes in vascular tone as a potential explanation for the physiological effects of magnets on edema. Attenuation of hyperpermeability is a plausible alternative explanation for the physiological effects of PEMF on edema reported in this study. We may assume that these results are in accordance with those of Barnes (2007), Goudarzi et al. (2010), McKay et al. (2007), Morris and Skalak (2005), and Rumbaut and Mirkovic (2008). Nevertheless, additional studies are necessary is order to confirm this assumption.

Numerous cellular studies have addressed effects of PEMF on signal transduction pathways. It is well accepted now that the cell membrane is a primary target for magnetic field action (Adey, 2004).

Kumar et al. (2011) reported that when a microware field penetrates a biological body, it induces endogenous physiological processes. The therapeutic effect is derived from the antioxidant role of the electromagnetic field of the applied pulsed field. The pulsed field contains a set of frequencies that may provide accumulative benefits. The biomarkers that were determined in this study are indicative of such processes.

In the present work, PEMF caused great changes in inflammatory biomarkers and oxidative stress: decreased levels of fibrinogen, L-citrulline, NO, SOD, and carbonyl groups in rats with experimental myopathies and significant muscle recovery.

## ACKNOWLEDGEMENTS

We acknowlege funding by Secretaría de Ciencia y Técnica (SECyT) de la Universidad Nacional de Córdoba and Consejo de Investigación Científica y Tecnológica (CICyT) de la Universidad Nacional de la Rioja.

***Declaration of interest***: The authors report no conflicts of interest. The authors alone are responsible for the content of and writing of the article.

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