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From Cell Biology to Tissue Engineering

Muscular hypertrophy and atrophy in normal rats provoked by the administration of normal and denervated muscle extracts

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Summary. This study was conducted to determine the effects of extracts obtained from both normal and denervated muscles on different muscle types. Wistar rats were used and were divided into a control group and four experimental groups. Each experimental group was treated intraperitoneally during 10 consecutive days with a different extract. These extracts were obtained from normal soleus muscle, denervated soleus, normal *extensor digitorum longus*, and denervated *extensor digitorum longus*. Following treatment, the soleus and *extensor digitorum longus* muscles were obtained for study under optic and transmission electron microscope; morphometric parameters and myogenic responses were also analyzed. The results demonstrated that the treatment with normal soleus muscle and denervated soleus muscle extracts provoked hypertrophy and increased myogenic activity. In contrast, treatment with extracts from the normal and denervated EDL had a different effect depending on the muscle analyzed. In the soleus muscle it provoked hypertrophy of type I fibers and increased myogenic activity, while in the *extensor digitorum longus* atrophy of the type II fibers was observed without changes in myogenic activity. This suggests that the muscular responses of atrophy and hypertrophy may depend on different factors related to the muscle type which could be related to innervation.

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Introduction

Skeletal muscle properties and maturation are modulated by both neural and non-neural factors, such as action potentials, contractile activity, and the mediation of known and unknown neurotrophic substances (Hyatt et al., 2003, 2006). Changes occur following denervation, either as a result of altered metabolic demands or related to the establishment of optimal conditions for the reinnervation of muscular fibers (Magnusson et al., 2005). Therefore, a muscle with loss of motor innervation may, at least in the early stages, be a rich source of signals that can affect both muscle fibers and satellite cells. In fact, following adult skeletal muscle denervation myogenesis is reactivated (Borisov et al., 2001) affecting satellite cell activity (Bornemann et al., 1999; Yoshimura and Harii, 1999), possibly via factors released by the muscle fibers that diffuse through the tissue (Schmalbruch and Lewis, 2000; Ehrhardt and Morgan, 2005). Therefore, extracts obtained from denervated soleus muscle have a myotrophic effect *in vivo* on the soleus muscle of normal rats manifest by muscular fiber hypertrophy and formation of new muscle fibers (Jimena et al., 1993, 1998; Pena et al., 1995). Furthermore, it has been found that skeletal muscle extracts contain neurotrophic factors that promote the survival of motorneurons *in vitro*

(Valmier et al., 1990; Comella et al., 1994).

Nevertheless, the disruption of nerve motor and trophic control also appears to provoke different responses depending on the muscle type. For example, it is known that denervation atrophy is more pronounced in fast-twitch muscle than in slow-twitch muscle (Dedkov et al., 2003) and while muscles composed exclusively of white fibers atrophy, red muscles initially hypertrophy (Bakou et al., 2014). Furthermore, although the reason is unknown, it appears that the neural influence on the differentiation of satellite cells is greater in red muscle than in white muscle (Hyatt et al., 2006). Based on these differences between red and white muscle, and specifically their reaction to loss of innervation, we postulated that the effects of muscle extracts obtained from red or white muscles as well as their responses could differ.

In the present study, we analyze whether extracts obtained from both normal and denervated soleus muscle (typically red muscle with 85-100% slow-twitch fibers) and normal and denervated extensor digitorum longus (EDL) (typically white muscle with around 95% fast-twitch fibers) have similar effects on the soleus and EDL muscles in normal rats. Surprisingly, while the extracts obtained from the soleus muscle provoked hypertrophy in both muscles, the extracts from the EDL induced atrophy in EDL muscles but not in soleus muscles.

Materials and methods

Animals and research design

In this study a total of 80 Wistar rats (250-300 g body weight) were used. The rats were housed in a temperature controlled cage (24°C), maintained in a light-dark cycle of 12-12 hours and had *ad libitum* access to water and food. All procedures described in this study regarding animal care and experimentation were approved by the Bioethics Committee of the University of Cordoba.

Twenty rats were divided in five groups: four experimental groups and one control. Each experimental group (n=4) was treated with a different type of muscle extract obtained from normal soleus muscle (n-SOL-ex), denervated soleus muscle (d-SOL-ex), normal EDL (n-EDL-ex), or denervated EDL (d-EDL-ex). Normal rats (n=4) without any type of intervention were used as a control group.

Muscle extracts and treatment of rats

Sixty rats were used to obtain muscle extracts. To obtain extracts from denervated muscle, the rats underwent a complete bilateral transection of the sciatic nerves (from which a 10 mm long segment was extracted). The soleus and EDL muscles were excised 4 days post-denervation, as previous studies have found that extracts obtained during this time period had a greater effect (Jimena et al., 1998). The rats in each experimental group were injected intraperitoneally (1 ml/day during 10 consecutive days) with the corresponding type of extract. The method used to obtain the extracts has been previously described (Jimena et al., 1993). Briefly, the muscles were minced, homogenized in PBS (4°C), centrifuged at 4,000 rpm (5 min) and filtered successively through 5 μm and 3 μm Millipore filters; the resulting material was centrifuged at 8,000 rpm (3 min) and the supernatant successively filtered through 0.8 μm, 0.45 μm and 0.22 μm Millipore filters.

The rats were sacrificed at the end of the experimental protocol; each rat was first anaesthetized with an intraperitoneal injection of 75 mg/kg of Ketamine (Imalgene® 100 mg/ml, Merial Laboratories, Lyon, France) and killed by decapitation. The soleus and EDL muscles of both posterior limbs were excised and the muscle belly obtained. These were sectioned, mounted transversely on cork disks and frozen in isopentane cooled by liquid nitrogen. A series of transverse sections 8 µm thick were obtained in a cryostat (Leica CM1850 UV).

The sections were stained following standardized procedures (Dubowitz and Sewry, 2007) with hematoxylin-eosin (H-E), modified Gomori trichrome for general morphological analysis, and acridine orange (AO) in order to identify abundant RNA in the muscle fibers. To identify fiber types, nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) and adenosine triphosphatase (ATPase, pH 9.6) histochemical methods were used. Immunostaining for desmin was performed using a monoclonal antibody (1:50, Desmin, DE-R-11, Dako, Denmark), incubated for 2 hours and visualized with the LSAB+System-HRP (K0979, Dako, Denmark) following the manufacturer's instructions. The expression of Desmin in cells associated with muscle fibers was used as a marker of satellite cell activation and to detect early activated myogenic precursor cells *in vivo* (Lawson-Smith and McGeachie, 1998).

Transmission electron microscopy

Small fragments were excised from each muscle and were fixed by 24 hours immersion in 2.5% glutaraldehyde in phosphate buffer at pH 7.4. Next, the fragments were washed in buffer and postfixed with 1% OsO_4. Following dehydration with acetones, the fragments were embedded in araldite. Ultrathin sections were obtained with an LKB 8800 Ultratome III microtome, collected on copper grids and stained with uranyl acetate and lead citrate. Sections were examined using a Philips CM10 Transmission electron microscope installed at the Central Research Support Service (SCAI, University of Córdoba, Spain).

Morphometric analysis

In each muscle, five fields (0.2 mm^2 each) were photographed at random at x200 magnification in order to measure parameters of muscle fiber size (fiber cross-sectional area and lesser diameter) and number of fibers/area. Myofibrillar ATPase pH 9.6 staining was used to measure the size of type I and II fibers in the soleus muscle and NADH-tr stain was used to measure the size of the type I, IIa and IIb fibers in the EDL. Sections were photographed using a Sony Exwaved HAD digital camera mounted on a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan). Morphometric analysis was performed using the image analysis program Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA).

To evaluate myogenic response, in sections stained with antidesmin the following was recorded: number of desmin+ activated satellite cells, mononuclear desmin+ cells, and desmin+ small muscle fibers. Two independent investigators blind to the sample identification performed all measurements and quantifications. At least 300 fibers per muscle were evaluated (2,400 fibers/group).

Statistical analysis

Data are presented as mean ± standard deviation (S.D.) of the mean for the 40 areas analyzed encompassing the two muscles of all the animals in each group. ANOVA analysis of variance was performed followed by the Holm-Sidak test if data passed the normality test, or the Dunn test for non-normal data.

Results

Control group

The organization and structure of the muscle fibers were completely normal in both soleus and EDL muscles in the normal rats, noting the considerable homogeneity in the size of the fibers in the soleus muscle, whereas in the EDL there were differences in size between fiber types (Fig. 1). In all cases the muscle fibers demonstrated polygonal profiles with peripheral nuclei. Ultrastructurally, the muscle fibers did not show any type of change and sporadic unactivated satellite cells were found.

Experimental groups

Effects of extract derived from the soleus muscle

The extracts obtained from both the normal and denervated soleus muscles provoked a myotrophic effect in both the soleus and EDL muscles. As shown by the increase in cross-sectional area, lesser diameter and the decrease in the number of fibers per area, both muscles responded with hypertrophy of muscle fibers and there were significant differences for these parameters relative to the control (Tables 1, 2). Additionally, a myogenic response consisting of muscle fiber neoformation and activation with differentiation in both satellite cells and cells located in the interstitial space was found with the antidesmin stain and quantitatively confirmed (Tables 3, 4). However, this response was generally greater when extract from denervated soleus was used compared with extract from normal muscle, and the response was always greater in the soleus muscle compared with the EDL.

The histological changes were common to both types of muscle, but were always more pronounced in the soleus muscle. With H-E the muscle fibers appeared to have a normal appearance with the exception of a higher number of nuclei. In some cases, a few were observed to be surrounded by a basophilic cytoplasmic halo protruding over the muscle fiber, which was very suggestive of activated satellite cells (Fig. 2). Between

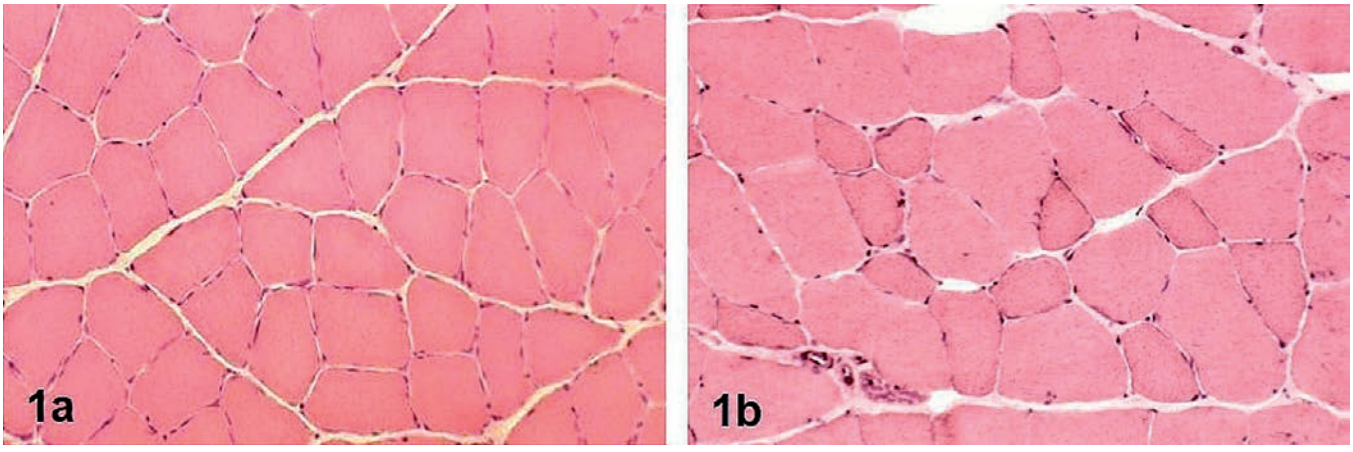

Fig. 1. Control group. Normal histology of soleus **(a)** and EDL **(b)** muscles. H&E. x 20

Table 1. Effects of extracts derived from soleus muscles: histomorphometric changes in soleus muscles.

	csfa type I (μm^2)	csfa type II (μm^2)	md type I (μm)	md type II (μm)	fibers/area
Control SOL	2744.60±606.73	2498.43±259.34	45.17±6.25	41.73±2.73	63.70±6.68
n-SOL-ex	3617.73±1059.49[a]	3176.17±572.48[a]	50.33±9.10[a]	50.92±6.52[a]	50.40±7.01[a]
d-SOL-ex	4024.52±983.62[a,b]	3681.21±589.86[a,b]	53.06±6.45[a,b]	53.38±6.25[a]	44.82±3.81[a,b]

csfa, cross-sectional fiber area; md, minor diameter; fibers/area, number of fibers/area. Values are mean ± SD. [a]: significant differences vs control (P<0.05); [b]: significant differences vs n-SOL-ex (P<0.05).

Table 2. Effects of extracts derived from soleus muscles: histomorphometric changes in EDL muscles.

	csfa type I (μm^2)	csfa type IIa (μm^2)	csfa type IIb (μm^2)	md type I (μm)	md type IIa (μm)	md tipo IIb (μm)	fibers/area
Control EDL	1100.56±185.77	1973.51±330.58	3425.97±484.57	28.41±3.77	38.52±6.15	52.99±5.47	69.20±5.76
n-SOL-ex	1469.03±242.08[a]	2499.94±385.62[a]	4107.26±758.72[a]	33.86±4.42[a]	44.26±4.26[a]	58.26±8.07[a]	60.04±3.00[a]
d-SOL-ex	1396.22±230.90[a]	2321.07±288.12[a]	3771.51±316.74[a,b]	31.72±4.17[a]	43.19±2.59[a]	55.51±4.69[a,b]	61.00±2.34[a]

csfa, cross-sectional fiber area; md, minor diameter; fibers/area, number of fibers/area. Values are mean ± SD. [a]: significant differences vs control (P<0.05); [b]: significant differences vs n-SOL-ex (P<0.05).

Table 3. Effects of extracts derived from soleus muscles: myogenic response in soleus muscles.

	Nuclei/fiber	Desmin+ SC	Desmin+ IC	Desmin+ SF
Control SOL	1.54±0.14	0.16±0.06	0.16±0.10	0.23±0.09
n-SOL-ex	2.88±0.27[a]	3.29±0.40[a]	1.12±0.30[a]	0.91±0.15[a]
d-SOL-ex	2.99±0.20[a]	3.56±0.37[a]	1.90±0.45[a,b]	1.20±0.26[a]

Nuclei/fiber, number of nuclei / fiber; Desmin+ SC, positive satellite cells for desmin; Desmin+ IC, positive interstitial cells for desmin; Desmin+ SF, positive small fibers for desmin. Values are mean ± SD. [a]: significant differences vs control (P<0.05); [b]: significant differences vs n-SOL-ex (P<0.05).

Table 4. Effects of extracts derived from soleus muscles: myogenic response in EDL muscles.

	Nuclei/fiber	Desmin + SC	Desmin+ IC	Desmin+ SF
Control EDL	1.58±0.14	0.20±0.07	0.13±0.07	0.10±0.05
n-SOL-ex	2.11±0.15[a]	0.72±0.11[a]	0.32±0.10[a]	0.59±0.09[a]
d-SOL-ex	2.38±0.29[a]	0.82±0.17[a]	0.35±0.11[a]	0.42±0.18[a]

Nuclei/fiber, number of nuclei / fiber; Desmin+ SC, positive satellite cells for desmin; Desmin+. IC, positive interstitial cells for desmin; Desmin+ SF, positive small fibers for desmin. Values are mean ± SD. [a]: significant differences vs control (P<0.05); [b]: significant differences vs n-SOL-ex (P<0.05).

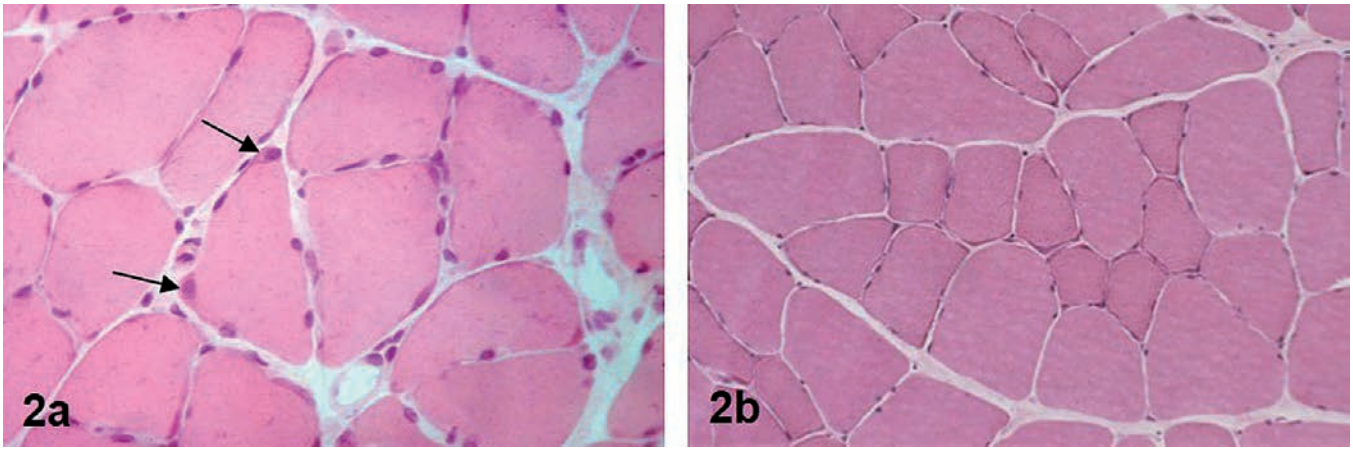

Fig. 2. d-SOL-ex group. **a.** Soleus muscle. Muscle fibers are large with an apparent increase in the number of nuclei; some of them are very large, have a rounded morphology and are surrounded by a basophilic ring (arrows). **b.** EDL muscle. Muscle fibers are increased in size with no apparent changes in the nuclei. H&E. x 40

the muscle fibers, small basophilic fibers were observed occupying the edges of the muscle bundles. With AO stain, the periphery of the muscle fibers was outlined with intense orange fluorescence and a higher number of nuclei were also evident (Fig. 3); the small fibers also showed an intense orange fluorescence. No cytoarchitectural changes were observed with histochemical stains, even in the percentage of fiber types (Figs. 4, 5).

The immunohistochemical stain with desmin revealed the presence of desmin+ peripheral profiles suggestive of activated satellite cells, desmin+

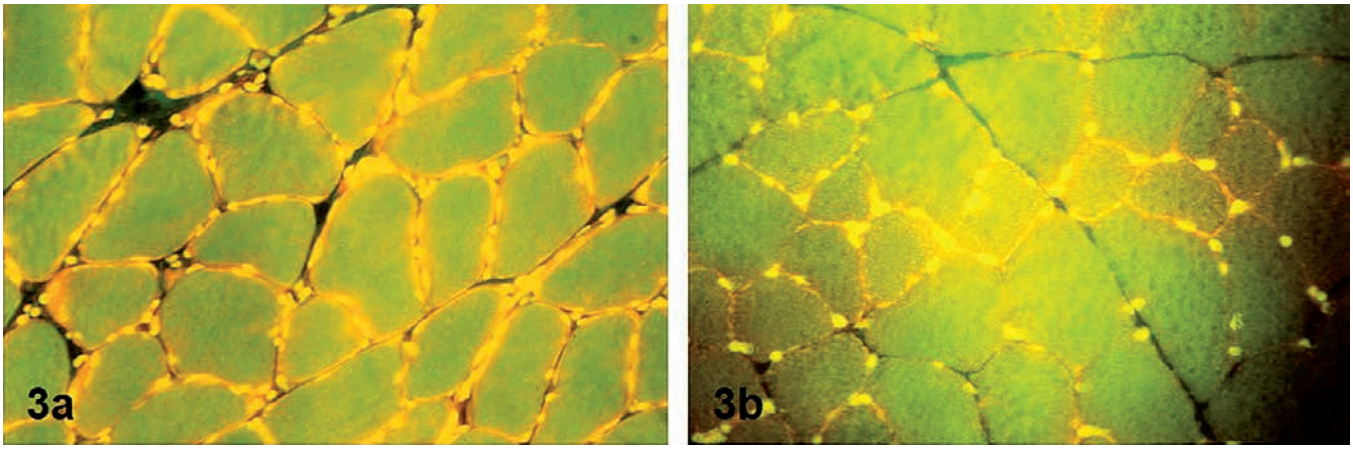

Fig. 3. d-SOL-ex group. **a.** Soleus muscle. Muscle fibers exhibit orange fluorescence at the periphery with an obvious greater number of nuclei (yellow fluorescence). **b.** EDL muscle. Note that the increase in the orange fluorescence and in the number of nuclei is smaller compared with the soleus muscle. Acridine orange. x 40

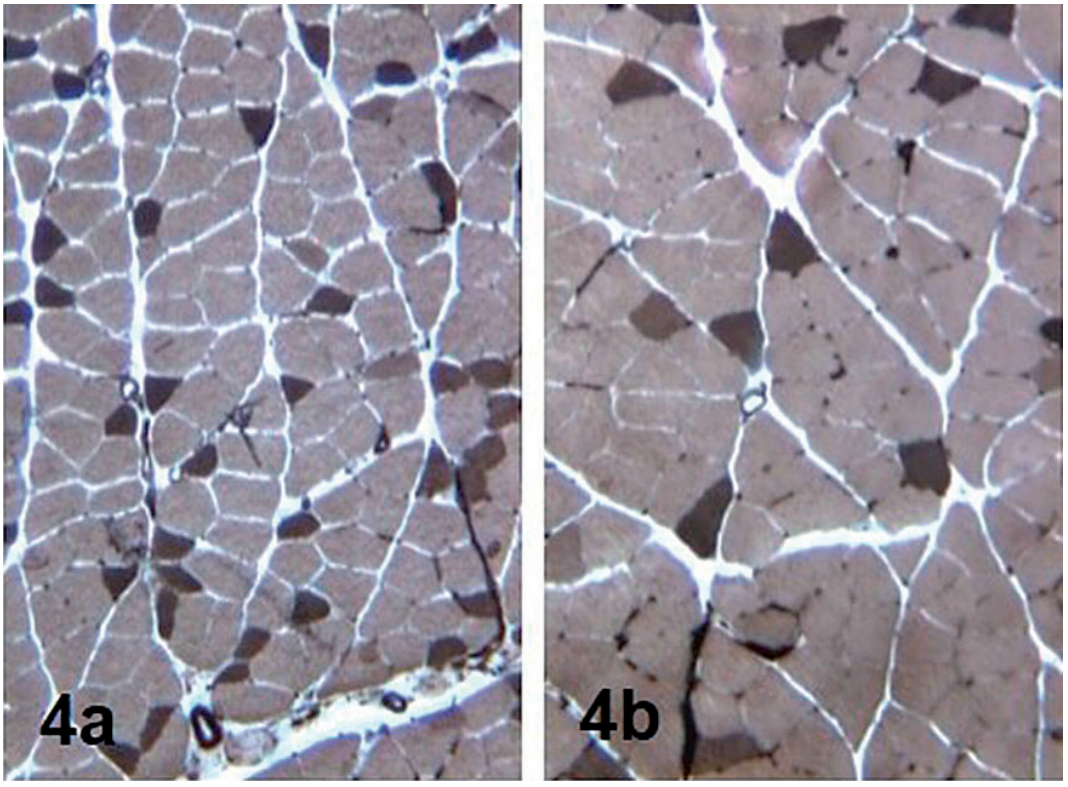

Fig. 4. a. Control group. Soleus muscle. Cross section stained with myosin ATPase at pH 9.6 showing the type I (light) and type II fibers (dark). **b.** d-SOL-ex group. Soleus muscle. Note that compared with the previous image, the muscle fibers are larger in size and apparently there is no change in fiber type. x 10

mononuclear cells isolated or grouped in the interstitial space, and also desmin+ small muscle fibers (Fig. 6). The latter demonstrated basophilia with H-E and fluorescent orange with AO staining.

Analysis with electron microscope confirmed the observations made with light microscopy. Many muscle fibers showed a subsarcolemmic space that was enlarged and occupied by numerous ribosomes. The myofibrillar organization was preserved with the exception of some small areas of myofibrillar disruption. Activated satellite cells were also found, correlating well with that observed with light microscopy; protruding over the muscle fiber surface and demonstrating increased cytoplasm with mitochondria and abundant ribosomes (Fig. 7). There was no morphological evidence that these cells crossed the basal lamina

In the vicinity of the muscle fibers, mononucleated cells similar to satellite cells were found, although they

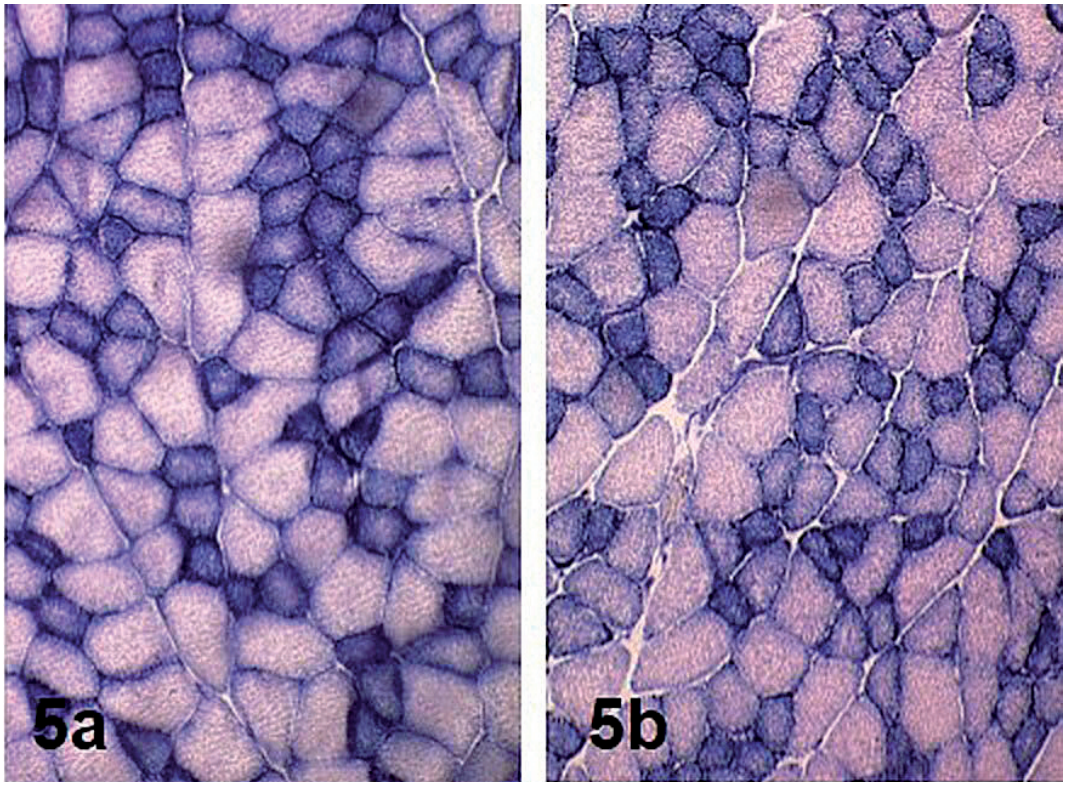

Fig. 5. a. Control group. EDL muscle. Different muscle fibre types can be identified by histochemical staining for NADH-tr type I (high oxidative) appear dark, type IIb fibers (low oxidative) appear light and type IIa appear intermediate. **b.** d-SOL-ex group. EDL muscle. Apparently the size and proportion of fiber types are very similar to the previous image. x 10

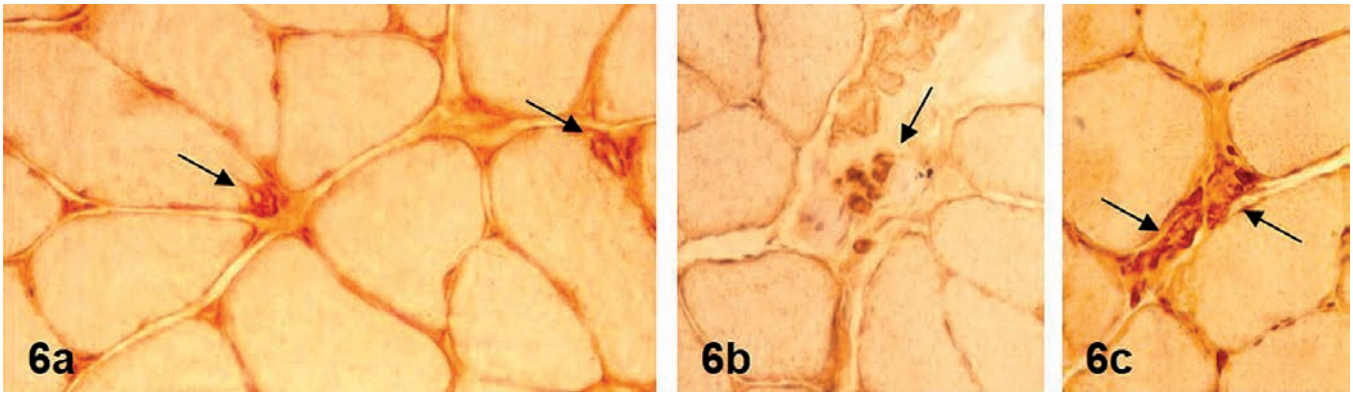

Fig. 6. d-SOL-ex group. Soleus muscle. Arrows indicate: peripheral increase of immunolabelling with desmin antibody, probably corresponding to activated satellite cells **(a)**, a group of several desmin + cells located in the perimisial space **(b)** and two desmin + small muscle fibers **(c)**. x 40

were completely surrounded by a basal lamina (Fig. 8); some of them had a more developed cytoplasm containing organelles (Fig. 8b). It was remarkable that many pericytes showed a developed cytoplasm, and were separated considerably from the capillary wall (Fig. 9). We also found, alongside capillaries, small muscle fibers with ultrastructural features of neoformation: euchromatic nucleus, ribosomes, mitochondria and

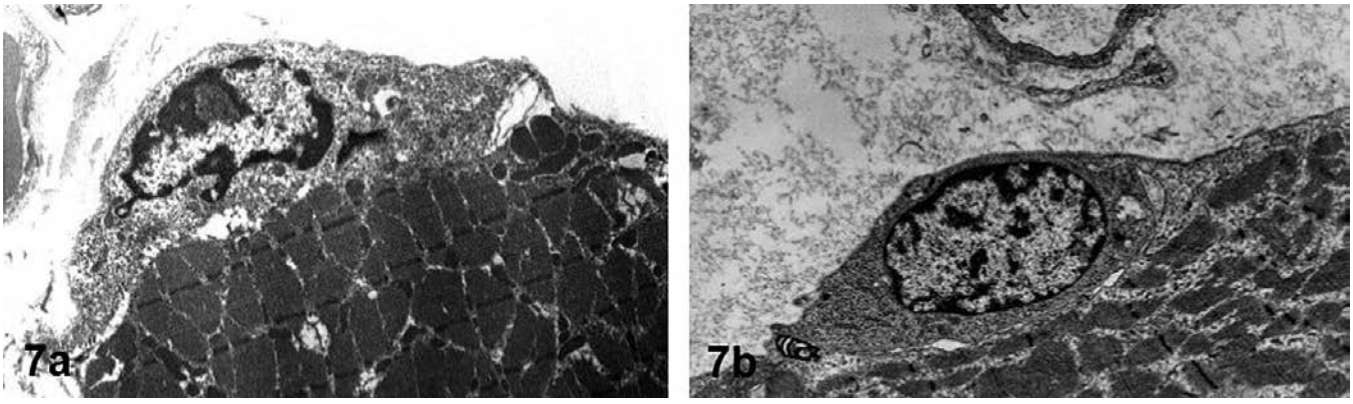

Fig. 7. d-SOL-ex group. **a.** Soleus muscle. A satellite cell activated with a large cytoplasm makes a protrusion at the surface of a muscle fiber. **b.** EDL muscle. Mildly activated satellite cell. a, x 5,200; b, x 3,900

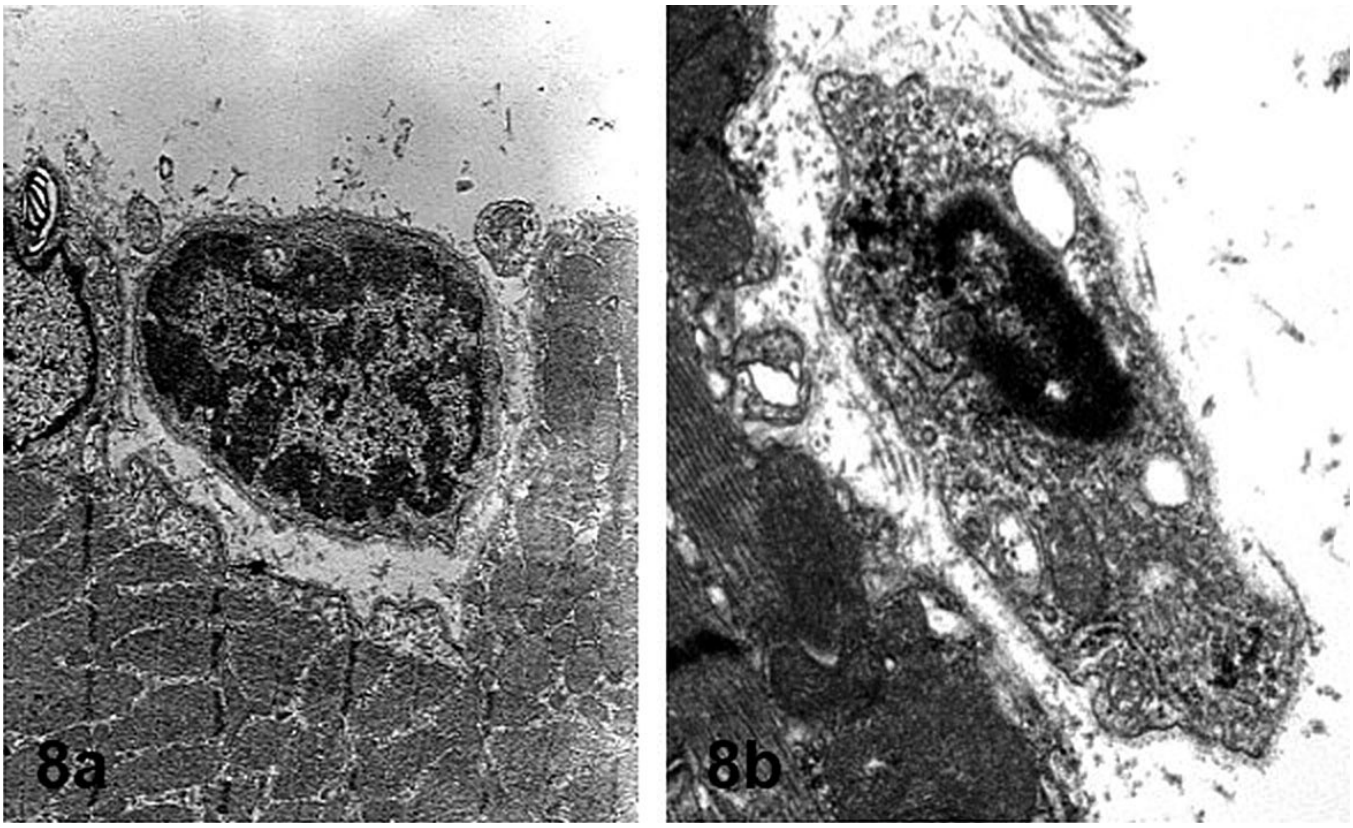

Fig. 8. d-SOL-ex group. **a.** Soleus muscle. Mononuclear cell with very little cytoplasm and completely surrounded by basal lamina. **b.** EDL muscle. Mononuclear cells with abundant cytoplasm containing ribosomes, mitochondria and rough endoplasmic reticulum; it is surrounded by a basal lamina. x 6,610

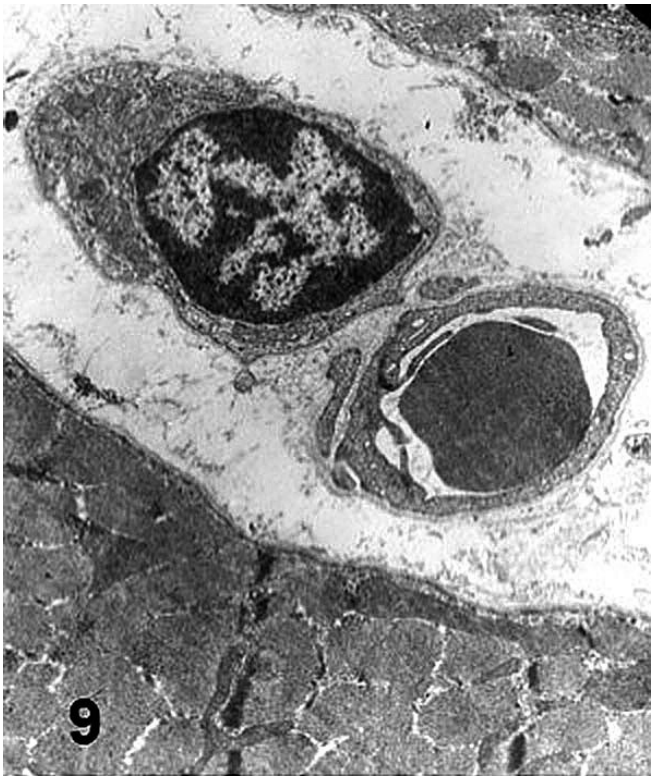

Fig. 9. d-SOL-ex group. Soleus muscle. Mononuclear cells surrounded by basal lamina next to a capillary, suggesting that it is a pericyte. Note that it contains a highly developed cytoplasm with mitochondria, ribosomes and rough endoplasmic reticulum. x 6,610

incipient myofibrils (Fig. 10).

Effects of extract derived from the EDL muscle

When EDL muscle extracts were utilized, the soleus and the EDL muscles responded differently. The soleus muscle, when treated with normal EDL extract, underwent hypertrophy of the type I fibers and showed a myogenic response (Tables 5, 7). However, treatment with denervated EDL extract resulted in atrophy of type II fibers and there was no myogenic response (Tables 5, 7).

The EDL muscle, when treated with normal EDL extract, suffered atrophy of the type IIa and IIb fibers and no myogenic response was observed. Treatment with denervated EDL extract resulted in atrophy of all fiber types and again no myogenic response was observed (Tables 6, 8).

The decrease in size appeared to affect the fibers as a whole, as no zones of varying size were found, the fibers exhibited a round profile, and no angulated atrophic fibers were seen. However, alteration of the staining pattern with NADH-tr was evident, with the presence of zones lacking enzymatic activity giving the fibers a *moth-eaten* aspect (Fig. 11). Ultrastructurally, in some fibers a distortion of the myofibrillar pattern (Fig. 12) and some pyknotic nuclei were observed.

Desmin immunostaining revealed no findings suggestive of myogenic response (Fig. 13). The satellite cells were observed to be inactive (Fig. 14) and no mononuclear cells surround by basal membrane, similar to those seen in the animal muscles treated with soleus and normal EDL extracts, were seen.

Table 5. Effects of extracts derived from EDL muscles: histomorphometric changes in soleus muscles.

	csfa type I (μm^2)	csfa type II (μm^2)	md type I (μm)	md type II (μm)	fibers/area
Control SOL	2744.60±606.73	2498.43±259.34	45.17±6.25	41.73±2.73	63.70±6.68
n-EDL-ex	3577.53±776.59[a]	2833.66±538.75	50.28±8.71[a]	44.00±6.42	49.26±3.99[a]
d-EDL-ex	2856.06±1195.61[b]	1766.66±375.67[a,b]	45.70±10.95[b]	35.55±5.73[a,b]	61.56±4.38[b]

csfa, cross-sectional fiber area; md, minor diameter; fibers/area, number of fibers/area. Values are mean ± SD. [a]: significant differences vs control ($P<0.05$); [b]: significant differences vs n-EDL-ex ($P< 0.05$).

Table 6. Effects of extracts derived from EDL muscles: histomorphometric changes in EDL muscles.

	csfa tipo I (μm^2)	csfa tipo IIa (μm^2)	csfa tipo IIb (μm^2)	md tipo I (μm)	md tipo IIa (μm)	md tipo IIb (μm)	fibers/area
Control EDL	1100.56±185.77	1973.51±330.58	3425.97±484.57	28.41±3.77	38.52±6.15	52.99±5.47	69.20±5.76
n-EDL-ex	1031.59±211.96	1740.57±355.37[a]	2526.59±367.55[a]	28.49±4.09	35.02±3.51[a]	45.86±4.34[a]	93.40±3.05[a]
d-EDL-ex	995.67±311.84[b]	1701.48±232.08[a,b]	2647.60±370.78[a,b]	27.26±4.64[b]	35.90±4.25[a,b]	45.80±5.86[a,b]	92.20±7.15[a,b]

csfa, cross-sectional fiber area; md, minor diameter; fibers/area, number of fibers/area. Values are mean ± SD. [a]: significant differences vs control ($P<0.05$); [b]: significant differences vs n-EDL-ex ($P<0.05$).

Table 7. Effects of extracts derived from EDL muscles: myogenic response in soleus muscles.

	Nuclei/ fiber	Desmin+ SC	Desmin+ IC	Desmin+ SF
Control SOL	1.54±0.14	0.16±0.06	0.16±0.10	0.23±0.09
n-EDL-ex	2.02±0.38[a]	3.27±0.51[a]	1.24±0.40[a]	0.78±0.21[a]
d-EDL-ex	1.52±0.15[b]	0.17±0.08[b]	0.18±0.09[b]	0.21±0.10[b]

Nuclei/fiber, number of nuclei / fiber; Desmin+ SC, positive satellite cells for desmin; Desmin+ IC, positive interstitial cells for desmin; Desmin+ SF, positive small fibers for desmin. Values are mean ± SD. [a]: significant differences vs control (P<0.05); [b]: significant differences vs n-EDL-ex (P<0.05).

Table 8. Effects of extracts derived from EDL muscles: myogenic response in EDL muscles.

	Nuclei/ fiber	Desmin + SC	Desmin+ IC	Desmin+ SF
Control EDL	1.58±0.14	0.20±0.07	0.13±0.07	0.10±0.05
n-EDL-ex	1.53±0.16	0.24±0.09	0.11±0.06	0.12±0.04
d-EDL-ex	1.49±0.18[b]	0.21±0.13[b]	0.14±0.11[b]	0.11±0.06[b]

Nuclei/fiber, number of nuclei / fiber; Desmin+ SC, positive satellite cells for desmin; Desmin+ IC, positive interstitial cells for desmin; Desmin+ SF, positive small fibers for desmin. Values are mean ± SD. [a]: significant differences vs control (P<0.05); [b]: significant differences vs n-EDL-ex (P<0.05).

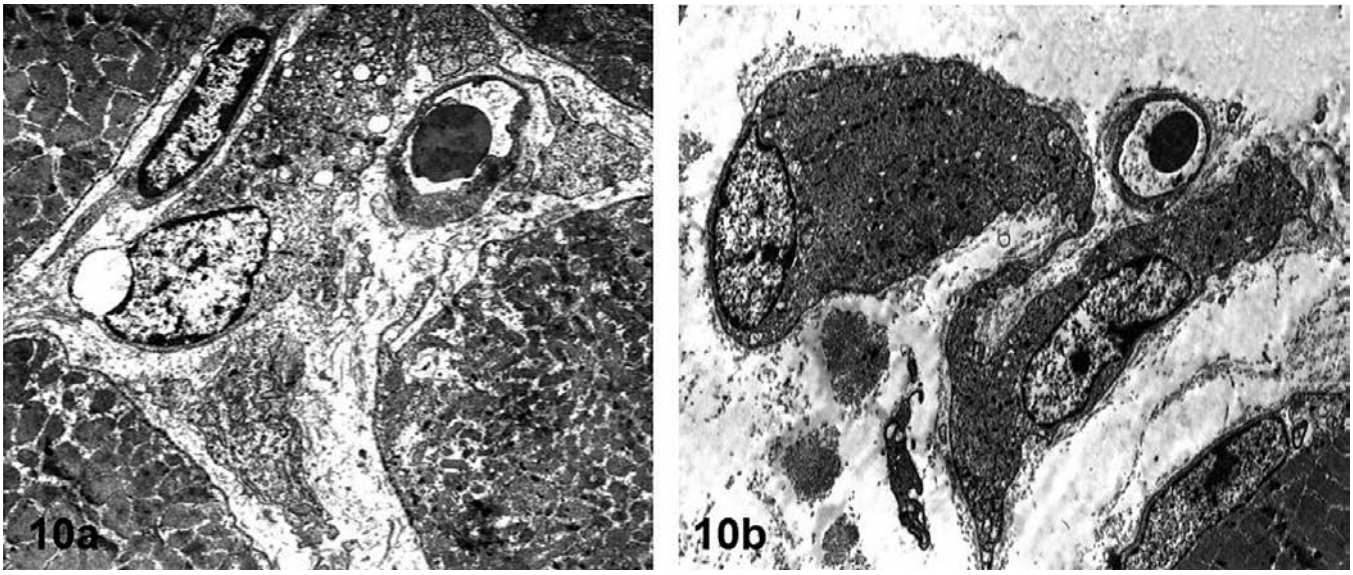

Fig. 10. d-SOL-ex group. Soleus muscle. **a, b.** Representative images of small muscle fibers located next to capillaries. These fibers contain incipient myofibrils and euchromatic nuclei. x 2,950

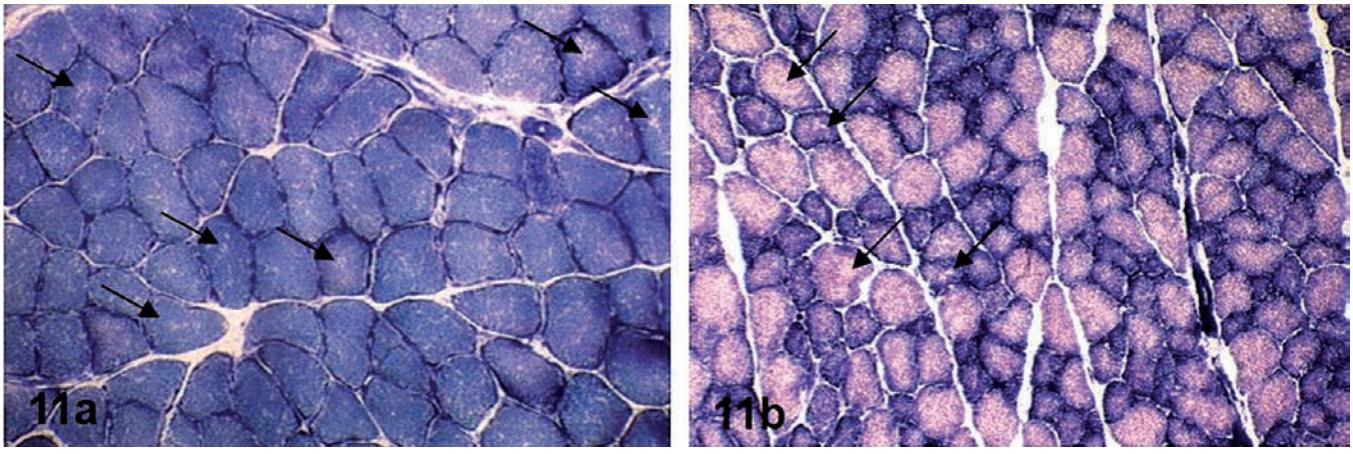

Fig. 11. d-EDL-ex group. **a.** Soleus muscle. Apparently normal muscle fibers, although some of them contain small areas with oxidative activity loss (arrows). **b.** EDL muscle. Compared with Figure 5a muscle fibers are smaller and some fibers show irregular absence of oxidative enzyme activity (arrows). NADH-tr. x 10

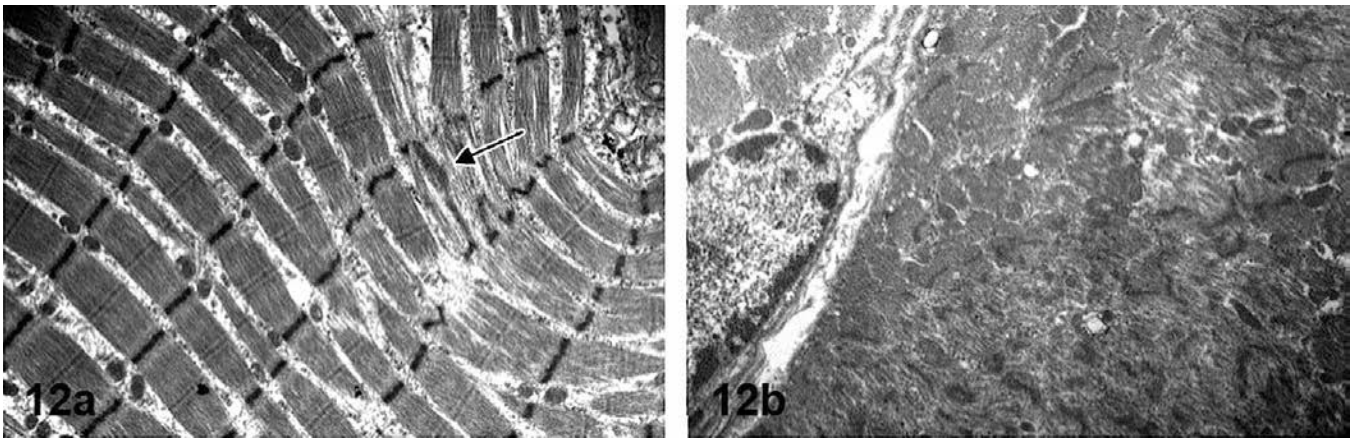

Fig. 12. d-EDL-ex group. **a.** Soleus muscle. Small area with myofibrils disruption (arrows). **b.** EDL muscle. Extensive Z disks streaming and disintegration of myofibrils. x 5,000

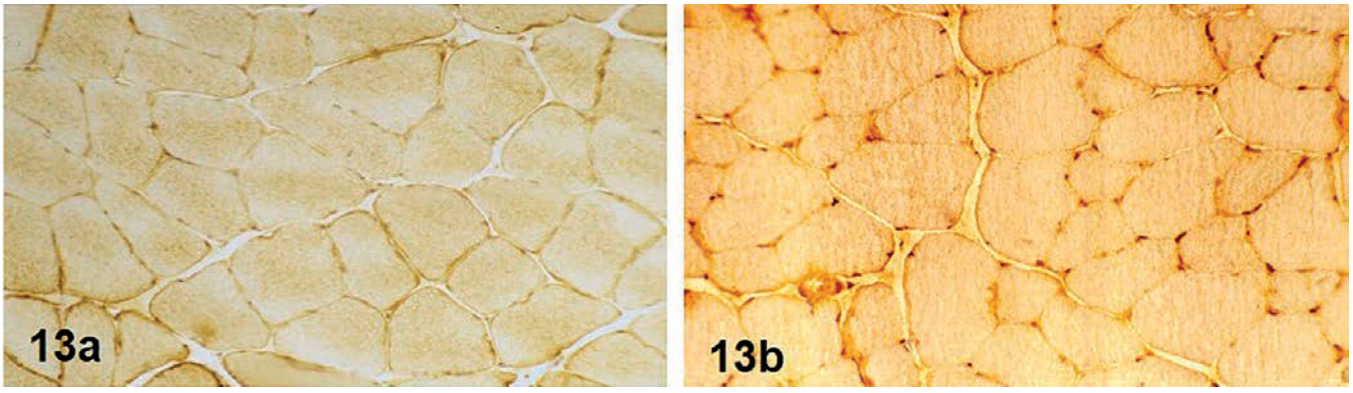

Fig. 13. d-EDL-ex group. Desmin immunostaining revealed no changes in soleus **(a)** or EDL muscle **(b)**. x 40

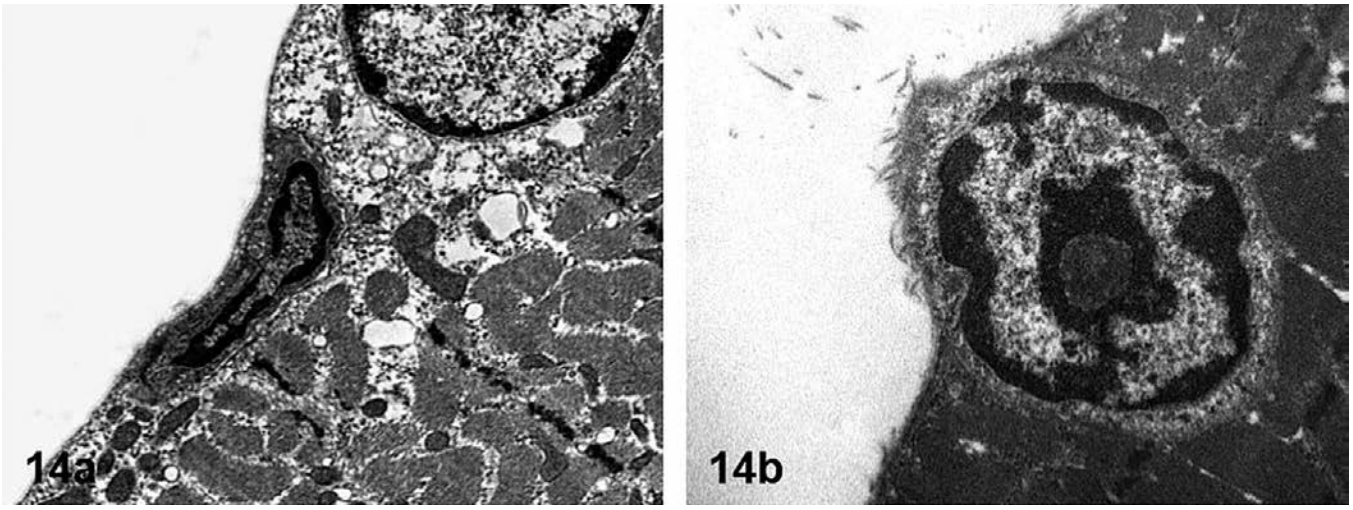

Fig. 14. d-EDL-ex group. Representative images of innactive satellite cells in soleus **(a)** and EDL muscles **(b)**. a, x 5,200; b, x 6,610

Discussion

The heterogeneity of muscle fibers and the satellite cells of red and white muscles is well established (Gibson and Schultz, 1982; Armstrong and Phelps, 1984; Baranska et al., 1997; Lagord et al., 1998) as are the different responses to diverse physiologic and pathologic situations (Darr and Schultz 1987; Bassaglia and Gautron, 1995; Lagord et al., 1998; Carter et al., 2010; Luque et al., 2015). In our study we demonstrated that extracts obtained from denervated muscles induced *in vivo* a hypertrophic or atrophic response in normal skeletal muscle, depending on whether the extract was obtained from the soleus or EDL respectively. This suggests that after nerve section the different types of muscles modify their tissue microenvironment accumulating the substances contained in the extracts. These results are consistent with the existence of neural factors that, independent of electrical activity, play a role in the myogenic and skeletal muscle growth processes and that appear to differ between fast- and slow-twitch muscles (Hyatt et al., 2003, 2006).

Given the hypertrophic and greater myogenic responses in both the soleus and the EDL muscles when treated with extract obtained from the soleus, it is evident that the factors contained in this muscle are those responsible for this response. It is interesting to note that extracts from the *anterior latissimus dorsi* ALD (red muscle) but not the *pectoralis major* PM (white muscle) have a stimulatory effect on the myogenesis of embryonic myoblasts of both muscles in cell cultures (Matsuda et al., 1983). Our conclusion is further strengthened by the fact that the response to denervation differs between muscles. It has been shown that the *anterior latissimus dorsi* and the *posterior latissimus dorsi* PLD (white muscle) undergo different morphologic modifications when deprived of their innervation, whereas the PLD muscle progressively atrophies post denervation, and the ALD muscle hypertrophies (Bakou et al., 2014).

To our surprise, the EDL muscle, but not the soleus, responded with muscular atrophy when the rats were treated with EDL muscle extract. In this case, the differences must be related to the characteristics specific to the muscle type. This could explain the differences found in the responses of the soleus and EDL muscles to the extracts. It has been proposed that the myotrophic or atrophic effects of certain substances, such as growth factors, may depend on the metabolic characteristics of skeletal muscle (Martin et al., 1996). Our study reveals a greater susceptibility of type II fibers. Generally, atrophy processes affect white muscle more; for example, the atrophy of type II fibers is one of the most frequent changes observed in human muscle pathology in a large number of different diseases and disorders (Carpenter and Karpati, 2001). Similarly, white muscle undergoes greater atrophy than red muscle following denervation (Bobinac et al., 2000; Dedkov et al., 2003) or in response to starvation or hepatic disease (Weber et al., 1992). It is striking that the motorneurons that innervate white muscles are preferentially affected in amyotrophic lateral sclerosis, whereas this does not occur in the soleus (Gordon et al., 2007).

Given that both the hypertrophic and atrophic effects of the extracts were greater when they were obtained from denervated muscle, it is clear that innervation must control the expression of the factors present in the extract. It is known that certain characteristics of muscle protein metabolism are under neural control, with differences between fast- and slow-twitch muscles (Guth and Watson, 1967), and that the denervation of a skeletal muscle leads to significant molecular and biochemical changes (Magnusson et al., 2005). Following denervation, a marked increase occurs in the mRNAs of the myogenesis regulatory factors and their corresponding proteins that play a key role in skeletal muscle myogenic processes (Buonanno et al., 1992; Hyatt et al., 2003).

In our study, satellite cells appear to have responded to the soleus muscle extract, but not to the EDL muscle extract. Furthermore, as the response was greater for the soleus muscle and for the denervated soleus muscle extracts, this suggests that red muscle must have greater myogenic potency. It has been shown that the myogenic response differs between both muscles following denervation, which appears to be related to a greater reliance on factors linked to neural control in the soleus muscle (Hyatt et al., 2006). However, it is not clear why the activity-dependent neural influence on the differentiation of satellite cells is greater in slow-twitch than in fast-twitch muscles (Hyatt et al., 2006) and our results may support this fact. At least in our model, in an intact and functional satellite cell population, factors derived from denervated soleus muscle provoked *in vivo*, in the two muscles studied, an increase in the size of the muscle fibers and a greater myogenic response in the soleus muscle compared with the EDL. However, we should not exclude that the increased response could also be facilitated due to the greater number of satellite cells in the soleus muscle (Gibson and Schultz, 1982) and their capability to differentiate earlier than those derived from EDL muscle (Ono et al., 2010).

If the signals contained in the extract activate satellite cells, they may also have activated the differentiation of other additional populations with myogenic potential existing in skeletal muscle. The observation of desmin+ cells, isolated or in small groups, in the interstitial space suggests that this occurred in our experiment, as all interstitial cells in normal muscle are negative for desmin (Zhang and McLennan, 1994). Given that the soleus muscle demonstrated a higher number of these cells compared with the EDL, this suggests that the soleus muscle may have an additional population of myogenic cells larger than that of the EDL. This possibility should be considered, as there is evidence that demonstrates that pericytes represent a population in skeletal muscle with myogenic capacity (Dellavalle et al., 2007, 2011). Furthermore, it is known

that the soleus muscle has a higher capillary density than EDL (Kubinova et al., 2001). Ultrastructural analysis showed morphological characteristics that correspond with pericytes, such as their cytological features, being completely surrounded by basal lamina and their location near capillaries. Cells ultrastructurally similar to these have been observed in denervated muscle (de Maruenda and Franzini-Armstrong, 1978), in muscle grafts regrafted into their own beds (Mong, 1988), in biopsies of malnourished children (Hansen-Smith et al., 1979), and in Werdnig-Hoffman's disease (van Haelst, 1970). Currently, emphasis is placed on the yuxtavascular position of the satellite cells (Christov et al., 2007; Mounier et al., 2011) and as a source of supply of new satellite cells (Dhawan and Rando, 2005).

In conclusion, the present study suggests that muscular atrophic and hypertrophic responses depend, at least in part, on the specific factors contained in the muscle type which appear to be controlled by its innervation. We believe that these results may be important to try to stimulate myogenesis in pathological conditions that affect skeletal muscle. Additionally, our model may also be useful to study the myogenic activation of other cells, different from satellite cells, as it does not alter the muscle structure as in other models of injury.

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