

Original article

Denervated muscle extract promotes recovery of muscle atrophy through activation of satellite cells. An experimental study

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Abstract

Purpose: The objective of the present study was to determine whether a denervated muscle extract (DmEx) could stimulate satellite cell response in denervated muscle.

Methods: Wistar rats were divided into 4 groups: normal rats, normal rats treated with DmEx, denervated rats, and denervated rats treated with DmEx. The soleus muscles were examined using immunohistochemical techniques for proliferating cell nuclear antigen, desmin, and myogenic differentiation antigen (MyoD), and electron microscopy was used for analysis of the satellite cells.

Results: The results indicate that while denervation causes activation of satellite cells, DmEx also induces myogenic differentiation of cells localized in the interstitial space and the formation of new muscle fibers. Although DmEx had a similar effect in nature on innervated and denervated muscles, this response was of greater magnitude in denervated vs. intact muscles.

Conclusion: Our study shows that treatment of denervated rats with DmEx potentiates the myogenic response in atrophic denervated muscles.

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Keywords: Muscle atrophy; Muscle denervation; Muscle extract; Myogenic response; Satellite cell; Skeletal muscle

1. Introduction

Achieving the stimulation of myogenesis in patients with neurogenic disorders, both intrinsic and traumatic, remains an important challenge.¹ Satellite cells are considered to be primarily responsible for the myogenic response in postnatal growth, hypertrophy, and post injury regeneration.² For this reason efforts are directed to stimulate these cells to, for example, delay sarcopenia and enhance, in various clinical situations, muscle regeneration or recovery of muscle atrophy. Therefore, to know the behavior and interaction of these cells with their environment in these situations is

essential to the design of therapies for injuries and neuromuscular disorders.^{3–5}

It is known that one of the factors that controls satellite cell activity is innervation^{6–9} and that reactivation of myogenesis is initiated following denervation of adult skeletal muscle.^{10–12} This re-establishment of embryonic conditions potentially causes denervated muscle to be very active at both the cellular and molecular level and although the corresponding muscle mass decreases with time post-denervation, the muscles conserve a certain recovery capacity.¹³ In this context, a denervated muscle should be, at least initially, a rich source of signals to activate myogenic cell precursors, as it is also regenerative muscle.¹⁴ This hypothesis would explain the evidence for myogenesis observed in both human biopsies with neurogenic muscular atrophy¹⁵ and animal models of muscular atrophy due to denervation,^{11,13,16–18} where new muscular fibers are frequently found. It is not clear whether denervation transiently affects satellite cell activity directly via factors liberated from the muscle fibers or from the nerve injury itself.^{7,19–22} A deficit of these

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factors has been proposed as possibly being responsible, at least in part, for a limited availability of satellite cells^{23,24} or for their incapacity to fuse with muscle fibers, which contributes, in this way, to an abortive restorative response.^{25,26}

Therefore, it is clear that skeletal muscle maintains, at least for a certain period of time, its ability to restore after the loss of innervation. This finding is important to the search for strategies that take advantage of this situation to stimulate myogenic capacity and enhance the rehabilitation of affected muscles. Recently van der Meer et al.²⁷ reported that when conditions associated with denervation occur, it is advisable to commence treatment of atrophy as early as possible while the number of ribosomes and satellite cells remain elevated and full-scale atrophy has not yet been established. We have demonstrated in previous studies that an extract obtained from denervated soleus muscle has myotrophic effects on normal soleus muscles in rats,^{28,29} which promotes the activation of satellite cells.^{30,31} However, it remains to be established whether similar effects also occur in short-term denervated muscles.

The purpose of this study was, therefore, to examine the satellite cell response in short-term denervated rat soleus muscle when treated with extract from denervated soleus muscle. It was hypothesized that the combination of denervation and treatment with denervated muscle extract (DmEx) could enhance the response of satellite cells in these muscles. Our findings could be of clinical interest as they suggested therapeutic applications in various neuromuscular pathologies.

2. Materials and methods

2.1. Animals and experimental design

In this study a total of 46 male Wistar rats (approximately 60 days old and weighting 250–300 g) were used. The rats were housed in temperature controlled boxes (24°C) with a light-dark cycle of 12–12 h and fed *ad libitum*. All procedures described in this study were approved by the Bioethics Committee of the University of Cordoba.

Sixteen rats were divided into 4 groups of 4 rats each: (1) a control group of normal rats (NR); (2) a group of denervated rats (DR) subjected to bilateral transection of sciatic nerves; (3) a group of normal rats treated with DmEx (NR+DmEx); and (4) a group of denervated rats treated with DmEx (DR+DmEx). In the groups NR+DmEx and DR+DmEx, the rats were injected intraperitoneally with 1 mL of extract for 10 consecutive days; in the latter group, the treatment began immediately following denervation. This treatment period was chosen based on the fact that during this time the denervated muscle was in a more favorable biological situation to be recovered from atrophy.^{27,32} The rats into the 2 control groups (NR and DR) were injected intraperitoneally with 1 mL of physiological saline solution over the same experimental period.

2.2. DmEx

The remaining 30 rats in the study were used to obtain DmEx as previously described.²⁸ All the rats underwent

complete bilateral transection of the sciatic nerves (from which a 10-mm long segment was extracted). Both soleus muscles were excised 4 days post-denervation, as previous studies had found that extracts obtained during this time period had a greater myotrophic effect.²⁹ Briefly, the muscles were minced, homogenized in phosphate-buffered saline (PBS) (4°C), centrifuged at 4000 rpm (5 min), and filtered successively through 5 µm and 3 µm Millipore filters (White GSWP 25 mm, Bedford MA, USA); the resulting material was then centrifuged at 8000 rpm (3 min) and the supernatant successively filtered through 0.8 µm, 0.45 µm, and 0.22 µm Millipore filters.

2.3. Tissue preparation

One day after the last treatment dose, rats were anesthetized with an intraperitoneal injection of 75 mg of Ketamine (Imalgene 100 mg/mL; Merial Laboratories, Lyon, France) and killed by decapitation. Soleus muscles of both limbs were excised and the muscle belly was mounted transversely and rapidly frozen in isopentane cooled by liquid nitrogen. Transverse serial sections, 8 µm thick, were obtained in a cryostat at –20°C, and stained with hematoxylin-eosin for general histologic examination and acridine orange (AO) to identify regenerative or newly-formed fibers.³³ Additional sections were immunohistochemically stained with primary antibodies specific against desmin (1:50; Desmin, DE-R-11; Dako, Glostrup, Denmark), which are used as a marker of activated myogenic precursor cells in the early phases;^{34,35} myogenic differentiation antigen (MyoD) (1:50; Dako), which is used for labeling activated satellite cells;²⁰ and proliferating cell nuclear antigen (PCNA) (1:100; Dako), which is used as a marker for dividing cells.³⁵ The reaction product was visualized using the biotin-avidin peroxidase method (K0679; Dako). Negative controls were performed in parallel without primary antibodies. Nuclei counterstaining was performed with Mayer's hematoxylin.

Small muscle fragments were fixed in 2.5% glutaraldehyde during 24 h. Samples were post-fixed in 1% osmium tetroxide, dehydrated with acetones, and embedded in araldite. Ultrathin sections 60-nm thick were obtained in an ultramicrotome, stained with uranyl acetate and lead citrate, and examined using a Philips CM10 Transmission electron microscope (Central Research Support Service, SCAI, University of Cordoba, Cordoba, Spain).

2.4. Morphometric analysis

Histologic and immunohistochemical sections were photographed with a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) that incorporated a Sony DXC-990P color video camera (Sony, Tokyo, Japan) and images were transferred to a computer equipped with the image analysis software Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA). Five fields per section were randomly photographed at 200 × magnification (157,500 µm²) and designated for quantitative analyses.

An average of 150 muscle fibers per sample was manually traced in a blind fashion by the same experienced investigator and used for measurement of the following parameters: (1)

fiber cross-sectional area (μm^2) in sections stained with hematoxylin-eosin; (2) number of desmin + sections, desmin + interstitial cells, and desmin + muscle fibers per area in sections stained with desmin; (3) number of MyoD + nuclei per area in sections stained with MyoD; and (4) number of PCNA + nuclei per area in sections stained with PCNA.

Satellite cells were identified with electron microscopy and photographed at magnifications ranging between $4000\times$ and $6000\times$. Selection criteria of these cells were (1) nucleus with abundant heterochromatin and (2) a position between basal and plasma membranes of the muscle fiber. The percentage of satellite cells was determined by counting the number of satellite cells and myonuclei and dividing the number of satellite-cell nuclei by the total number of nuclei (satellite cell nuclei + myonuclei). The counts for each of 5 sections per muscle were analyzed and a mean percentage of satellite cells per muscle was calculated. Once identified and quantified, the following morphometric parameters were obtained: cell area (μm^2), nucleus area (μm^2), and percentage of activated satellite cells. Activated satellite cells were considered to be those with a ratio of nucleus area to cell area greater than that quantified for the satellite cell population in normal rats.

2.5. Statistical analysis

For the statistical study SigmaStat Version 3.1 software package (Systat Software Inc., Point Richmond, CA, USA) was used. For each experimental group, data are expressed as mean $\pm$ SE (of the 4 rats of each group). Statistical significance ($p < 0.05$) between groups was determined by ANOVA on ranks. In those samples that met normality criteria, the Student-Newmann-Keuls test was performed and in the rest the Dunn test was used.

3. Results

3.1. Light microscopy

In the AO staining, muscle sections of the NR group showed normal histology and no significant changes were evident in the denervated muscles of the DR group, with the exception of a smaller size and less rounded profiles (Figs. 1A, 1B). In contrast, the groups treated with the DmEx demonstrated histologic changes that included larger muscle fibers with an apparent increase in the number of nuclei, larger and rounded, together with the presence of scattered small fibers (Figs. 1C, 1D).

Morphometric analysis showed a significant muscle fiber atrophy ($p < 0.05$) in the DR group ($1230.9 \pm 16.9 \mu\text{m}^2$) compared to the NR group ($2809.2 \pm 18.1 \mu\text{m}^2$), which represented a 67% reduction in size. In both groups treated with DmEx, muscle fiber areas increased significantly ($p < 0.05$), representing an upward trend of 28% for NR + DmEx rats ($3602.1 \pm 25.9 \mu\text{m}^2$) and a 59% for DR + DmEx rats ($1967.1 \pm 22.8 \mu\text{m}^2$) compared with NR and DR groups, respectively. Thus, the myotrophic effect of the DmEx was more extensive on denervated than on control muscle fibers based on the greater increase in the percentage.

While no positives were observed for any of the immuno-histochemical markers used in muscles of the NR group, in the

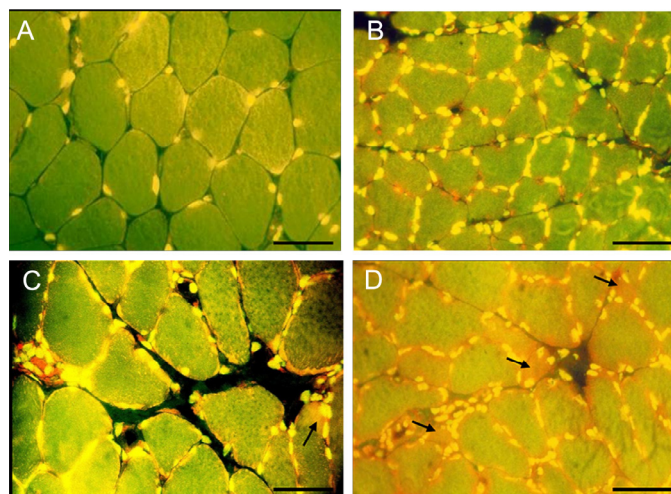


Fig. 1. Histochemistry of nuclei acids in muscle fibers. Comparison of soleus muscle sections stained by acridine orange of representative animals of (A) NR, (B) DR, (C) NR + DmEx, and (D) DR + DmEx groups. Normal muscle fibers exhibit green background staining of cytoplasm and yellow DNA of nuclei. The muscle fibers with areas with increased concentration of RNA show a bright orange fluorescence. Note differences in muscle fiber size and number of nuclei among groups. Small fibers with orange fluorescence (arrows) are observed only in the groups treated with DmEx (C, D). Scale bars = $40 \mu\text{m}$. DmEx = denervated muscle extract; DR = denervated rats; NR = normal rats.

groups DR, NR + DmEx, and DR + DmEx they were detected (Fig. 2). MyoD + nuclei were observed both in interstitium and inside muscle fibers (Fig. 2A), being significantly more abundant in the groups treated with DmEx than in the DR group (Fig. 2D). Nonetheless, this increase was 5 times higher in the DR + DmEx group than in the NR + DmEx (Fig. 2D). The majority of the PCNA + nuclei were located in the interstitium and were only occasionally visible in the muscle fiber periphery (Fig. 2B). The number of PCNA + nuclei was significantly higher ($p < 0.05$) in the groups treated with DmEx, particularly in the DR + DmEx group (Fig. 2D).

Anti-desmin staining revealed the existence of positive profiles in the periphery of some muscle fibers that contained nuclei (Fig. 2C) and the presence, preferentially in the perimysial space, of mononucleated cells and desmin-positive small muscle fibers (Figs. 2C, 3A, 3B). With the exception of the NR group, these profiles were observed in all 3 other groups. But while there were no significant differences between the 2 groups treated with DmEx, differences were found between DR and DR + DmEx groups and between DR and NR + DmEx groups (Fig. 2D). The small fibers were preferentially located on the edges of the muscle fascicles and many of them showed basophilia and orange fluorescence with AO (Fig. 1D). Their presence was limited to the 2 groups treated with extract and was significantly more frequent in the DR + DmEx group (Fig. 3C).

Therefore, it was evident that denervation evoked a myogenic response in the skeletal muscle of the DR group. A similar, but significantly different, response also occurred in the NR + DmEx group. Finally, with the exception of the number of desmin-positive profiles, this response was of much greater

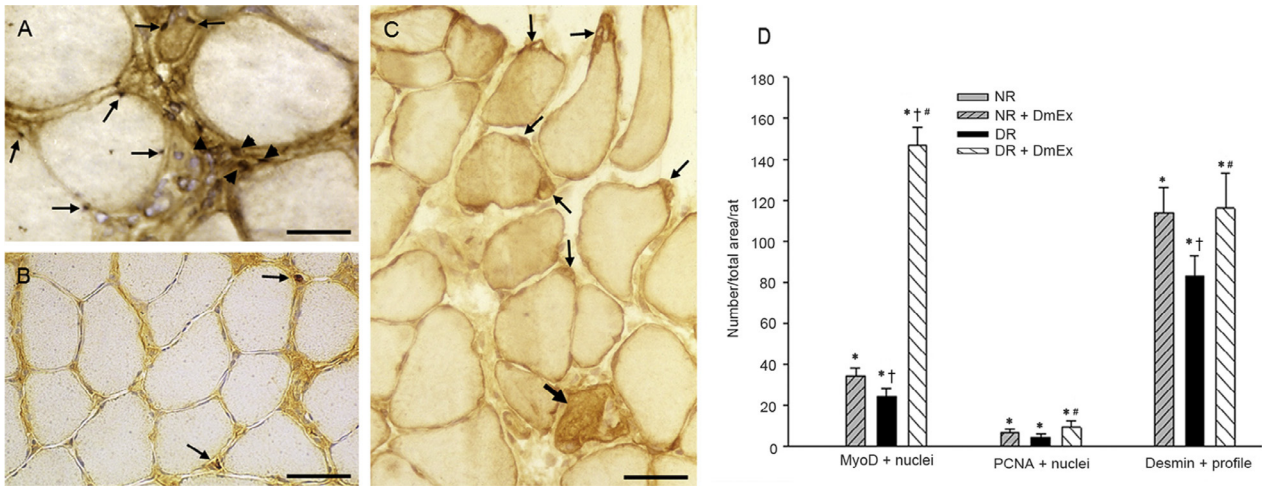


Fig. 2. Immunohistochemistry of the myogenic response. Transverse soleus muscle sections stained by immunohistochemistry against (A) MyoD, (B) PCNA, and (C) desmin in representative animals of the DR + DmEx group; hematoxylin counterstain. (A) MyoD labeled nuclei are observed in muscle fibers (arrows) and arranged in groups in the interstitial space (arrow heads). (B) Only 2 PCNA + nuclei are observed in the interstitium (arrows). (C) A few muscle fibers exhibit desmin immunoreactivity in peripheral areas, which contains nuclei that are suggestive of activated satellite cells (thin arrows) and other muscle fiber is heavily stained with desmin (thick arrow). Scale bars = (A) 18 μm , (B) 40 μm , (C) 25 μm . (D) Quantitative expression of markers MyoD, PCNA (nuclei), and desmin (satellite cell profile) in the 4 experimental groups tested in the study. Values are means $\pm$ SE, $n = 4$ rats/group. Values not detected in NR group. *Significantly different from NR group; †significantly different from NR + DmEx group; #significantly different from DR group. DmEx = denervated muscle extract; DR = denervated rats; MyoD = myogenic differentiation antigen; NR = normal rats; PCNA = proliferating cell nuclear antigen.

magnitude in the DR + DmEx group, but no significant difference was found between the 2 groups treated with DmEx.

3.2. Electron microscopy

Satellite cells in the NR group were always observed between the basal lamina and the plasma membrane of muscle fibers, but they never contacted with muscle fibers nor did they show surface protrusion. They were consistently found in a resting state and were characterized by a heterochromatic nucleus and scarce cytoplasm (Fig. 4A). In contrast, in the DR, NR + DmEx, and DR + DmEx groups, activated satellite cells were often observed (Figs. 4B–D), some of which demonstrated what appeared to be areas or points of fusion that corresponded with areas of continuity between the satellite cell cytoplasm and the sarcoplasm of muscle fibers (Fig. 4D). The cytoplasm was large and contained rough endoplasmic reticulum profiles, free ribosomes, polyribosomes, mitochondria, and occasionally contained Golgi apparatus and centrioles (Figs. 4B–D).

Activation of satellite cells, which could relate well with desmin-positive profiles, was confirmed morphometrically upon the basis of significant enlargements of the cellular area ($p < 0.05$) in groups NR + DmEx ($8.2 \pm 3.1 \mu\text{m}^2$), DR ($7.9 \pm 2.0 \mu\text{m}^2$), and DR + DmEx ($10.6 \pm 5.8 \mu\text{m}^2$) when compared to the NR group ($7.5 \pm 2.2 \mu\text{m}^2$). This variable was significantly higher in the DR + DmEx group than in both DR and NR + DmEx groups ($p < 0.05$). However, this increase occurred at the expense of the satellite-cell cytoplasm because the size of the nucleus did not show significant differences between the groups NR ($4.47 \pm 1.11 \mu\text{m}^2$), DR ($4.38 \pm 1.32 \mu\text{m}^2$), NR + DmEx ($4.39 \pm 1.27 \mu\text{m}^2$), and DR + DmEx ($4.53 \pm 1.80 \mu\text{m}^2$).

With the exception of the NR group, activation occurred in all the other groups, but never affected all satellite cells observed. While in the DR group, 40% of these cells showed

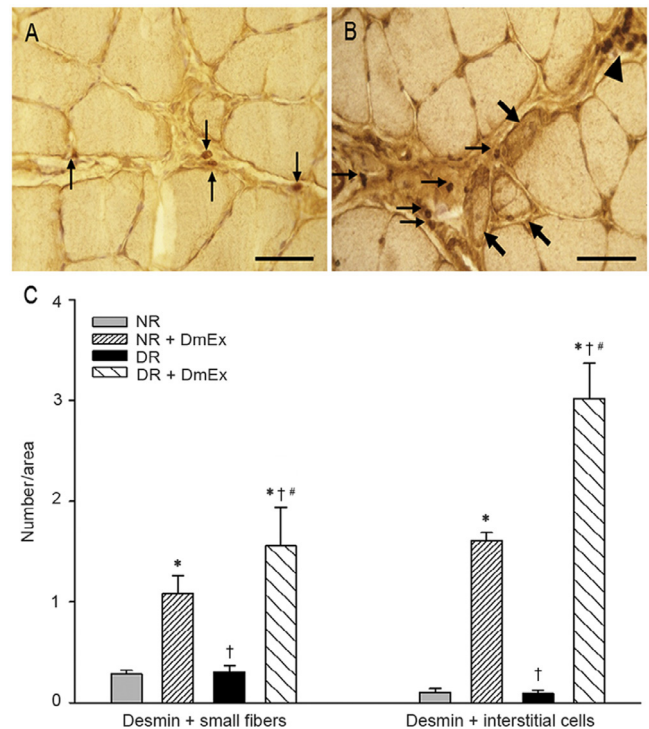


Fig. 3. Immunohistochemistry of the myogenic response into interstitial space. Transverse soleus sections stained by immunohistochemistry against desmin in representative animals of (A) NR + DmEx and (B) DR + DmEx groups; hematoxylin counterstain. (A) Arrows indicate desmin-positive cells in the perimysial space. Scale bars = 40 μm . (B) Several isolated (thin arrows) or in groups (arrow head) cells in the perimysium are desmin-positive; 2 small muscle fibers and a protrusion of another also are marked with desmin (thick arrows). Scale bars = 40 μm . (C) Desmin immunohistochemical staining showed positivity in small muscle fibers and interstitial cells. *Significantly different from NR group; †significantly different from NR + DmEx group; #significantly different from DR group. Values are means $\pm$ SE, $n = 4$ rats/group. DmEx = denervated muscle extract; DR = denervated rats; NR = normal rats.

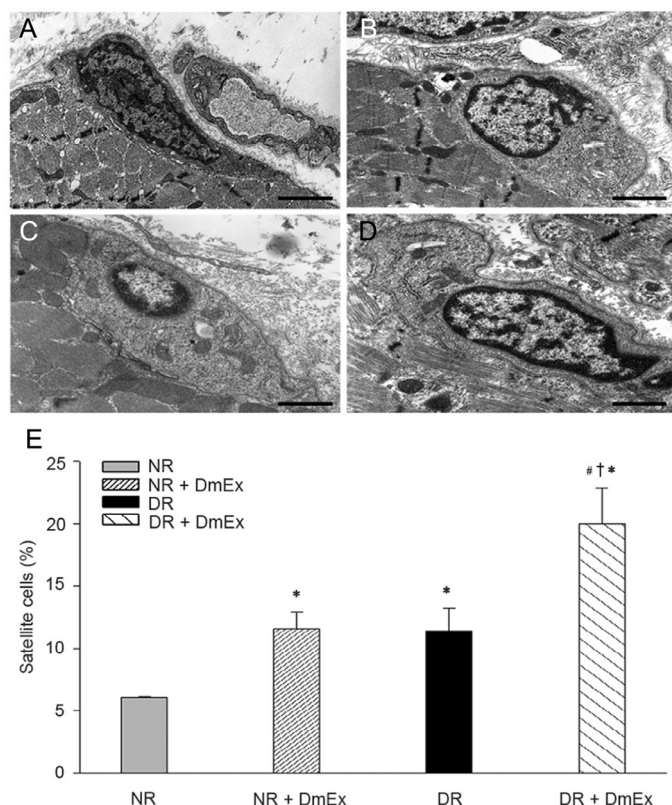


Fig. 4. Satellite cell activation and frequency. Electron micrographs of soleus muscle sections in representative animals of (A) NR, (B) DR, (C) NR + DmEx, and (D) DR + DmEx groups. (A) Quiescent satellite cells of NR soleus muscle. (B–D) Active satellite cells showing a high cytoplasm to nucleus ratio when compared to satellite cell in (A). Scale bars = (A, B, C) 2 μ m; (D) 1 μ m. (E) Satellite cell frequency in the 4 experimental groups tested in the study. The proportion of satellite cells is expressed as a percentage of the total number of nuclei identifiable in the muscle fibers. *Significantly different from NR group; †significantly different from NR + DmEx group; ‡significantly different from DR group. Values are means $\pm$ SE, n = 4 rats/group. DmEx = denervated muscle extract; DR = denervated rats; NR = normal rats.

ultrastructural features of activation, in the NR + DmEx and DR + DmEx groups, the percentage reached 58% and 61%, respectively. In the latter case, it is important to highlight that the satellite-cell population reaction within the same muscle was not homogenous. Thus, while some satellite cells showed clear morphologic features of activation, others had characteristics of inactivity.

Quantitative analysis showed a significant increase ($p < 0.05$) in the number of nuclei and myonuclei per fiber similar in the NR + DmEx (2.41 ± 0.39 nuclei/fiber and 2.15 ± 0.27 myonuclei/fiber), DR (2.92 ± 0.59 nuclei/fiber and 2.54 ± 0.40 myonuclei/fiber), and DR + DmEx (2.87 ± 0.43 nuclei/fiber and 2.30 ± 0.38 myonuclei/fiber) groups, in comparison with the NR group (1.32 ± 0.29 nuclei/fiber and 1.24 ± 0.17 myonuclei/fiber). The frequency of satellite cells was also increased in the 3 groups compared to the control NR, but muscles in the DR + DmEx group showed the greatest increase (Fig. 4E).

Only in the groups treated with DmEx, but not in the 2 untreated groups, satellite cells (or cells in a “satellite” position), showed some morphologic traits outstanding. Thus, they

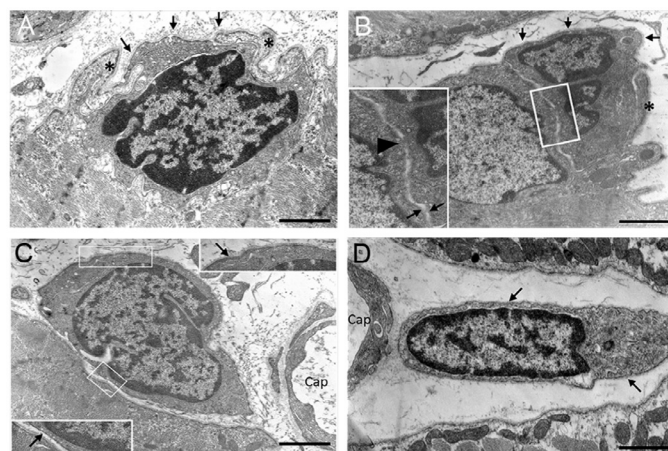


Fig. 5. Satellite cells and pericytes-like cells. Electron micrographs (DR + DmEx group) showing (A and B) a papillary projection (asterisks) of the parent muscle fiber partially covering a satellite cell externally. In both cases, the satellite cells are externally covered by basal lamina (arrows). (B) Inset: note that basal lamina seems to be between muscle fiber and satellite cell (arrows). The arrowhead marks an apparent contact patch containing pinocytotic vesicles. (C) Electron micrograph (DR + DmEx group) showing a cell very close to the surface of a muscle fiber and in the vicinity of a capillary (Cap) cell. Note that the outer side of the cell is covered by basal lamina (upper panel) but not on the side facing the muscle fiber (lower panel), which presents its basal lamina. (D) Electron micrograph (NR + DmEx group), which shows a mononuclear cell, surrounded by basal lamina (arrows) and located in the proximity of a capillary (Cap), which could be identified as a likely pericyte. Scale bars = 1 μ m. DmEx = denervated muscle extract; DR = denervated rats; NR = normal rats.

were detected cells “embraced” by papillary projections of the adjacent muscle fiber (Figs. 5A, 5B) or very close to the muscle fiber surface (Fig. 5C). In all of these cases, morphologic variations of the basal lamina were also observed, which included the presence of a basal lamina between the satellite cell and the muscle fiber (Figs. 5A–C).

In the interstitial space, pericytes-like cells that had ample cytoplasm with organelles and maintained their basal lamina were only observed in the 2 groups treated with DmEx and were found clearly apart from the capillary (Fig. 5D). Mononucleated cells were also found in the interstitium, and they were reminiscent of satellite cells, with heterochromatic nuclei and scarce cytoplasm with pinocytotic vesicles. Nevertheless, although close to the muscle fiber, they were isolated and completely enveloped by basal lamina.

4. Discussion

It is known that different types of muscle extracts contain factors capable of stimulating different cell types in muscle both *in vitro* and *in vivo*.^{36–38} We consider that the extrinsic factors contained in DmEx, and involved in the myogenic response in our case, must be proteins that act on the regulating factors of the myogenesis. In support of this idea, it has been explained that a reactivation of myogenesis results when a muscle is denervated.³⁹

Altogether, the results obtained in our study are consistent with those of previous studies^{28–31} that clearly support the concept that the liberation of trophic factors during denervation is

implicated in the activation response of satellite cells. Nevertheless, it does not appear that the muscles of the NR + DmEx group have been exposed to the same factors that are found in denervated muscle. In our study, the myogenic response was of greater magnitude in the NR + DmEx vs. the DR group. These differences could be explained by the fact that DR-group rats were sacrificed at 10 days, whereas the DmEx used were obtained at 4 days post-denervation. We have previously demonstrated that the time between denervation and obtaining the muscular extract has an impact on the extract's myotrophic effect.²⁹ Furthermore, the recovery of skeletal muscle by reinnervation following denervation is impaired when this exceeds 7 days.³²

The comparable percentage of activated satellite cells quantified in both groups treated with the DmEx (around 60%) suggests 2 important observations concerning the trophic factor (or factors) contained in this extract. First, this extract does not act in a similar way on the satellite cell population in a normal muscle and in a denervated muscle. In both cases 60% of activated satellite cells are reached; however, in the denervated muscle we started from 40% and in the normal muscle from 0. Thus, the effect of the extract appears to be greater on normal muscle; however, no reference range has been determined for normal rats, which might be a limitation in the interpretation of our results. Second, this extract could have activated a specific population of satellite cells. This suggestion may be justified by the fact that the total number of satellite cells capable of doing so have been activated in both cases and the existence of 2 subpopulations or compartments of satellite cells, termed proliferative and differentiative, is well known,^{19,34} and they appear to respond to signals differently.⁴⁰ Because PCNA + nuclei were seen in the interstitial space, but not in a satellite position with muscle fibers, and there was not ultrastructural evidence of satellite cell mitosis, it was possible that the DmEx only activated the differentiative compartment. In a recent experimental model of electrical stimulation to attenuate muscle disuse atrophy, it has been suggested that the requirements for satellite cells to engage in different cellular activities (quiescence-activation or proliferation-differentiation) seem to be different.⁴¹

The ultrastructural observation of fusion processes in the DR, NR + DmEx, and DR + DmEx groups, would indicate the incorporation of the satellite cell in the muscle fiber with which it is associated. It has been reported that rat DmEx contains specific factors that significantly increase the fusion of donor myoblasts with host muscle fibers.⁴² Because satellite cells serve as a source of myonuclei in both postnatal growth and muscle fiber hypertrophy,⁴³ its activation response must be linked to the myotrophic effect of the DmEx. In the NR + DmEx group, the fusion processes of satellite cells would contribute to the DmEx-induced muscle fiber hypertrophy.^{28,30} In the DR and DR + DmEx groups, however, this can be interpreted as a mechanism to compensate the myonuclear loss in atrophic muscle fibers.^{21,44–46} In our study, MyoD + nuclei were observed both associated with muscle fibers and in the interstitium; unfortunately, we cannot confirm the exact location of MyoD + nuclei, which requires additional markers such as dystrophin. This association is consistent with

the increase observed in the MyoD expression in both myonuclei and satellite cells during the first week post-denervation, which was interpreted as an attempt to prevent muscle atrophy.⁴⁷ Other authors have noted that, at least in the short term, there is no loss of nuclei during atrophy in mice skeletal muscle.⁴⁸ However, in the present study the number of myonuclei per fiber volume in muscles of the DR group increased significantly with respect to the NR group. This discrepancy could be explained by the decreased fiber size observed in the DR group that at 10 days post-denervation represents 43% of the normal muscle fiber. Another possible explanation could be related to the significant increase observed in the frequency of satellite cells at 10 days post-denervation (11%) in comparison with the 6% of satellite cells that were found in normal muscle fibers. These data are consistent with the rise, from 4% to 8%, in the satellite cell population reported at 3–4 days post-denervation.²⁵

It is important to point out that while in the DR group the myogenic response was limited to satellite cells, in the DR + DmEx group this response was comparatively greater and included the appearance of myogenic cells and myofiber neof ormation. The ability of denervated muscle microenvironment to be an optimal environment for myogenesis is supported by many studies, although this ability seems not to be the same for all muscles.³¹ It is known that short-term denervation provokes tissue disruption that results in a rise in the number of both satellite and interstitial cells, probably through release of a mitogen.^{44,49} Furthermore, in experimental studies of muscle denervation^{12,50} or in muscle biopsies from patients with neurogenic disorders¹⁵ small-sized muscle fibers are often observed with staining features of immaturity. These fibers, which are different from atrophic fibers, are suggestive of an evident myogenic response. Therefore, the initiation of myogenesis could be a consequence of changes in the tissue microenvironment. Allen and Rankin⁵¹ have already pointed out that variations in the concentration of growth factors in the microenvironment play an important role in the stimulation or inhibition of satellite cells. They also revealed that another possible mechanism might be related to the different sensitivity in their response to growth factors. In our opinion, systemic extract injections could induce variations in the microenvironment of skeletal muscle tissue that could act with some myogenic potential on satellite cells or other cell types.

Despite the high number of nuclei observed in the DR group, the occasional presence of desmin-positive interstitial cells and the absence of small neofibers indicates that denervation had induced little myogenic differentiation in the interstitial zone. This apparent discrepancy may be explained by the presence of new fibers or myotubes seen in long-term denervation,^{11,12} whereas the period of denervation in the DR group of the current study was only 10 days. In contrast, in the 2 groups treated with DmEx there was a significant presence of interstitial cells and desmin-positive small muscle fibers, which were more pronounced in the DR + DmEx group than in the NR + DmEx group. This finding confirms that this type of muscular extract has the capacity to induce new formation of muscle fibers.³⁰ Despite the fact that a control group consisting of rats injected with normal (intact) muscle extract was not included

in the present study, in previous studies a diminished myotrophic capacity in denervated vs. normal muscle extracts was shown.^{28,30}

In agreement with other experimental models^{52,53} the current study suggests that cells located in the interstitium participated in the neoformation of muscle fibers. Despite the inability to confirm the origin of these myogenic cells located in the extracellular space, different cell types have been implicated as potential muscle cell precursors, without excluding the consideration that interstitial myoblasts could derive from satellite cells.^{54–56} In any case, it appears that the DmEx assayed in the present study could have an intrinsic capacity to stimulate myogenic differentiation not only from satellite cells but also from other cells. It is noteworthy that stem cells derived from bone marrow have been induced to enter the myogenic lineage when cultivated with extracts from injured muscle, but not with extracts from normal muscles.⁵⁷

Among precursors having the capacity to postnatal growth, hypertrophy, and postinjury regeneration, pericytes associated with muscular capillaries appear to be a subpopulation of stem cells^{58–60} that can commit to the myogenic lineage as a consequence of the micro-environmental influence.^{14,61,62} Considering that in our model the maximal myogenic response was achieved when muscle denervation itself and DmEx were combined, this association could result in a “forced” and “unnatural” modification of the tissue microenvironment that could act as a signal recruiting other cell types to participate in the myogenic response seen in the DR + DmEx group. Although some of our observations support the notion that pericytes could play a role in the DmEx-induced myogenic response, further studies are still necessary to confirm or discard this hypothesis.

In focal lesions, pericytes can be released from the vascular position in response to chemotactic stimuli and function as stem cells and immunomodulators.⁶³ There is now ample evidence that these cells contribute not only to regenerative processes but also as a potential source of re-population of satellite cells.^{62,64} Some of our morphologic observations also suggest that the DmEx used could have stimulated the pericytes; however, our observations cannot confirm completely that pericytes are involved in the formation of new fibers. A high number of these cells were found activated, separated, and out of their habitual location along capillaries, while preserving their basal lamina. Small cells similar to satellite cells, completely separated from the muscle-fiber surface and completely surrounded by basal lamina, were also seen in the groups treated with DmEx. Comparable cells, both in morphologic appearance and location, have been reported and associated with pericytes in previous classical studies.^{65–67} It has also been observed that, in denervation, satellite cells can leave their associated fibers via either processes of sequestration by invagination of the basal lamina between the satellite cell and the muscle fiber or by migration mechanisms crossing the basal lamina and heading toward the endomysial space.^{26,68,69} However, we believe it does not make sense that while activated satellite cells are fusing with the muscle fiber, others are abandoning it. Moreover, there should be an interposition of basal lamina between satellite cells and the muscle

fiber if they were abandoning it, but these processes were not observed in the present study. Another possibility would be that they were incorporating with the muscle fiber.

The incorporation of myogenic precursors with an exogenous origin to the “satellite position” has been found in different experiments.^{70–72} The possibility has been raised that a subpopulation of satellite cells could be supplied by the migration of cells from either the interstitium or the circulation to occupy a sublamellar position.⁷³ Our observations of interstitial cells with partial loss of basal lamina and cytoplasmic projections of the muscular fiber “enveloping” mononucleated cells (Fig. 5) are compatible with the incorporation of exogenous myogenic precursors.

5. Conclusion

The results of the present study demonstrate that parenteral administration of an extract of denervated soleus muscle promotes in the short-term a myogenic response in denervated rat soleus muscle and could facilitate the recovery of muscle atrophy. These effects were based upon the activation of satellite cells, cells located in the interstitium, and the formation of new myofibers. It was noteworthy that while this response was of similar nature in control and denervated muscle, the size effect was of much greater magnitude in denervated vs. intact skeletal muscle. However, it should be noted that it is possible that the effect of extracts on muscle atrophy cannot be systemic because both the effect and the response of normal muscles seem to differ depending on the type of muscle.³¹ These findings may have practical implications in the rehabilitation of neuromuscular problems requiring an improvement in myogenesis.

Authors' contributions

EA conceived of and designed the study, performed the experiments, obtained and analyzed the data in light and electron microscopy, and drafted the manuscript; SC conceived of and designed the study, performed the experiments, obtained and analyzed the data in light and electron microscopy, and drafted the manuscript; EL conceived of and designed the study, performed the experiments, obtained and analyzed the data in electron microscopy, and drafted the manuscript; IJ performed the experiments, obtained and analyzed the data in light microscopy, and wrote the manuscript; IRC performed the experiments and obtained and analyzed the data in electron microscopy; FLC performed the experiments and obtained and analyzed the data in light microscopy; JP conceived of and designed the study, performed the experiments, obtained and analyzed the data in light and electron microscopy, and wrote the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

The authors declare that they have no competing interests.

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