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APOPTOSIS IN LONG-TERM DENERVATED RAT SKELETAL MUSCLE

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ABSTRACT

Apoptosis occurs in normal tissues and in many pathological conditions, and is regulated by a variety of genes. In this work, we used electron microscopy and the comet assay to study the morphological and biochemical (DNA cleavage) changes associated with apoptosis in long-term denervated rat skeletal muscle. Male Wistar rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and the sciatic nerve was cut at mid-thigh level followed by the excision of a 5-10 mm long segment of the nerve. Rats that were studied more than four months after the operation were re-operated at least every three months. Samples were obtained from red (*soleus*) and white (*extensor digitorum longus*, EDL) striated skeletal muscle at different times after denervation. The ultrastructural changes associated with fiber atrophy appeared earlier and were more evident in red than in white muscle. Degenerated fibers frequently contained normal and altered (ghost-like) nuclei, which suggested repair by satellite cells. Apoptotic nuclei were seen in both muscles. There were no differences in the ultrastructural alterations associated with apoptosis in red and white muscles at any stage after denervation. Apoptosis was also confirmed by the comet assay, which showed the presence of many apoptotic cells, but revealed no significant difference between the two muscle types. Overall, these results suggest that the long-term denervation of red and white skeletal muscles causes atrophy and apoptosis, with the latter probably being responsible for the muscle fiber loss after long-term denervation.

Key words: Apoptosis, denervation, rat, skeletal muscle

INTRODUCTION

Apoptosis is a particular type of cell death that occurs in various physiological and pathological conditions [12,13]. Because apoptotic cell death requires RNA and protein synthesis, this process has been described as self-directed cellular "suicide". Several genes that control apoptosis have been identified and provide a molecular basis for analyzing the key steps in this phenomenon [9,20]. Apoptosis occurs during normal embryogenic development and metamorphosis, e.g., in retinal development and palatal fusion, where it acts in opposition to mitotic proliferation to sustain normal tissue homeostasis [7]. Apoptosis is also responsible for cell deletion in normal adult tissues [16], a phenomenon known as "programmed cell death". Finally, apoptosis is triggered in many pathological conditions by a variety of chemical, physical and viral agents [15].

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From a strictly morphological viewpoint, the distinction between apoptosis and necrosis can be appreciated by electron microscopy [12,14]. Necrosis typically involves foci containing many cells that swell (especially the mitochondria and endoplasmic reticulum) because they lose the ability to maintain their fluid and electrolyte balance. In addition, nuclear chromatin forms irregular clumps (pyknosis) or dissolves (karyolysis). Eventually, the cell membrane loses its integrity and the cell is disrupted. This process elicits inflammation, may require many hours to complete, and is not dependent on energy.

In contrast, apoptosis typically involves individual cells or small clusters of cells. The cells shrink, become more electron-dense, and lose intimate contact with adjacent cells. The mitochondrial morphology is usually normal, but the chromatin forms clumps at the nuclear periphery, and this results in a characteristic histological appearance known as karyorhexis. The cell eventually fragments into membrane-bound "apoptotic bodies" that contain mixtures of organelles

and nuclear fragments. Apoptotic bodies eventually undergo changes that ultrastructurally resemble classic necrosis, with mitochondrial swelling and complete membrane breakdown. The process does not elicit inflammation, may be completed in 1-4 h (except for apoptotic body digestion), and is energy-dependent. The early cellular events of apoptosis occur quickly, with only a few minutes elapsing between the onset of the process and the formation of a cluster of apoptotic bodies [12]. Apoptotic bodies are usually phagocytosed by resident or migratory macrophages. Since necrosis and apoptosis may occur simultaneously, and since necrosis is usually much more evident, the extent of cell degeneration attributable to apoptosis may be underestimated [14].

Apoptosis occurs in skeletal muscles after denervation [2,5,8,21]. Muscle atrophy after denervation is accompanied by a reduction in muscle cell number, possibly as a result of apoptosis, which may be underestimated [10]. Distinct isotypes of apoptosis may occur in animals of different taxonomic groups and in different cell lineages of the same organism [4]. In the present study, we used electron microscopy and the comet assay, which detects DNA strand breaks in individual cells [22], to investigate whether apoptosis contributes to the loss of cells in long-term denervated white and red muscles in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (150-200 g) obtained from Bauru School of Dentistry Laboratories were housed in plastic cages (5 rats/cage) on a 12 light/dark cycle at 21°C, with free access to food and water. All of the animal procedures deseribed here were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Electron microscopy

The rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and the sciatic nerve was cut at mid-thigh level followed by the excision of a 5-10 mm long segment of the nerve. The proximal nerve stump was ligated, bent backwards and sutured subcutaneously to the hamstring muscles in order to prevent reinnervation. The rats were subsequently returned to their cages and allowed to recover for 12, 16, 19, 30 and 38 weeks, after which they were perfused via the abdominal aorta with Ringer solution containing 1% procaine hydrochloride and 5,000 units of heparin/l followed by 2.5% glutaraldehyde in 0.1 M phosphate buffer. The denervated and contralateral *soleus* and *extensor digitorum longus* (EDL) muscles were excised, postfixed with 1% osmium tetroxide and embedded in epoxy resin for light and electron microscopy. Semithin cross-sections (1-3 µm thick) through the middle of each muscle were cut with dry glass knives, stained with paraphenylenediamine (PPD) and viewed with phase optics. All muscles showing signs of re-innervation such as myelinated axons or groups of unusually large fibres were discarded. For electron microscopy, thin sections were cut from the blocks, collected on 300-mesh grids and contrasted with uranyl acetate and lead citrate.

Comet assay for DNA cleavage

DNA cleavage in myonuclei was assessed using the comet assay [4] in *soleus* and EDL muscles of two adult rats denervated for 38 weeks, and in one control *soleus* muscle. Myofibers were dissociated from the muscles and placed in 1 ml of ice-cold Hank´s buffered saline solution (HBSS) containing 20 mM EDTA. The muscles were minced finely and allowed to sediment, after which a 5 µl aliquot of the cell suspension was added to 90 µl of 1.5% low melting point agarose at 37°C and layered onto a microscope slide. The slide was initially coated with a thin layer of 0.5% normal agarose to ensure firm attachment of the second agarose layer. A coverslip was placed over the cell suspension and the slides were incubated at 4°C for five minutes to allow the agarose to solidify. The slides were subsequently immersed in lysing solution for 1 h and placed on a horizontal gel electrophoresis unit. DNA damage was visualized after staining with ethidium bromide staining using a fluorescence microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. One hundred randomly selected cells were analyzed for each muscle sample. Tail lengths were scored on a scale from 0 to 4 (Fig. 3), and each cell was then assigned to one of the five classes (0-4). The total score for each muscle sample was obtained by summing the products of the class number x number of cells in that class. For example, for control muscle, the total score (54) was the sum of (84x0) + (1x1) + (2x2) + (3x3) + (10x4) = 54. The results of the comet assay are shown in Table 1.

Table 1. Results of the comet assay. One hundred randomly selected cells were analyzed for each muscle sample. Each cell was assigned to one of five classes (0-4). The total score for each muscle sample was calculated by multiplying the number of cells in each class by the score of that class, e.g., for control muscle the total score of 54 resulted from the sum of (84x0) + (1x1) + (2x2) + (3x3) + (10x4) = 54.

	Class 0	Class 1	Class 2	Class 3	Class 4	Total score
Control	84	1	2	3	10	54
EDL 1	53	4	8	3	32	157
S 1	42	0	10	0	48	212
EDL 2	43	8	2	11	32	173
S 2	59	0	3	4	34	154

EDL 1 and EDL 2 = EDL muscle from rats 1 and 2, S 1 and S 2 = soleus muscle from rats 1 and 2.

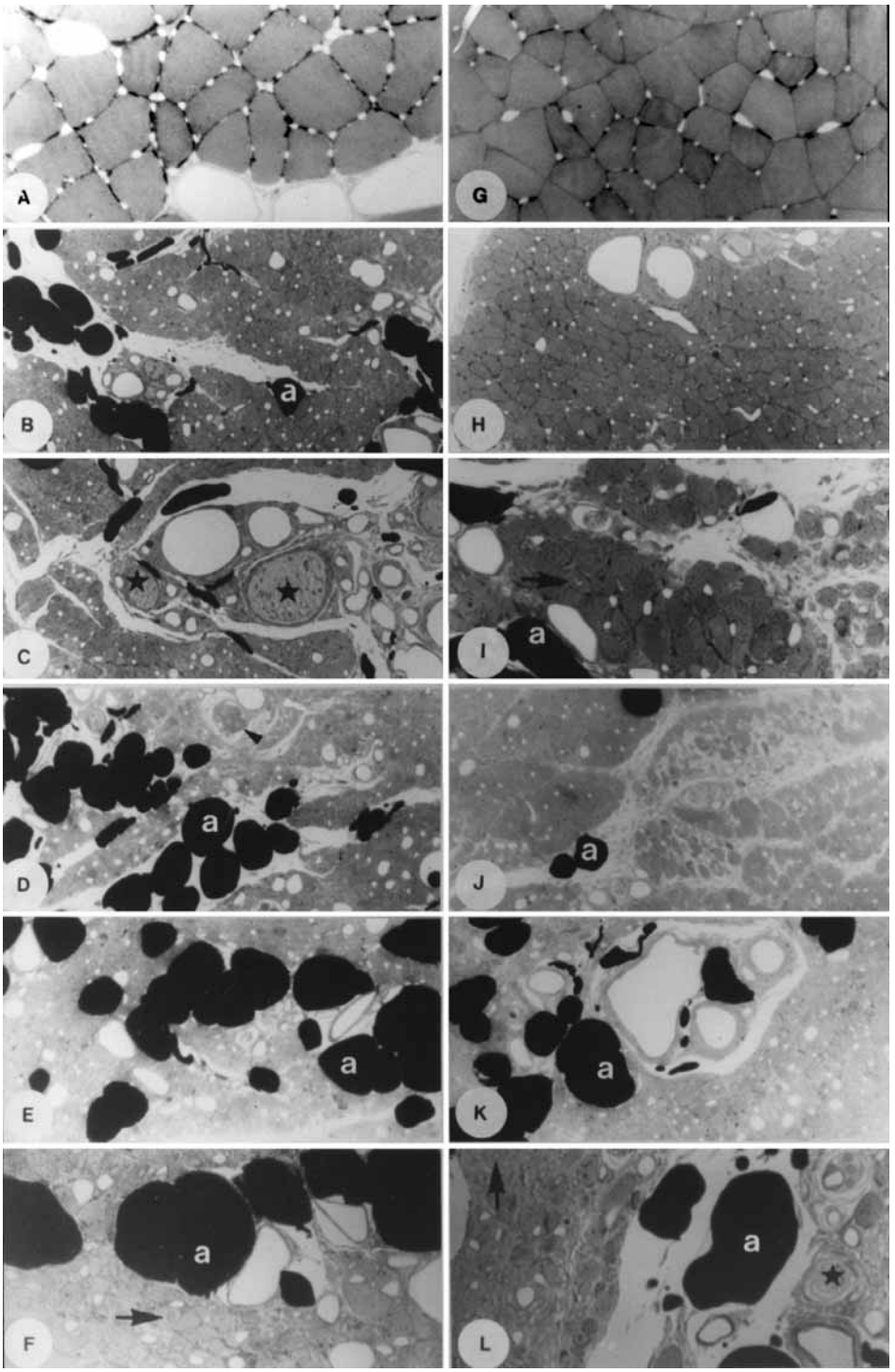

Figure 1. Photomicrographs of normal and denervated muscles. **A,** Normal soleus muscle. **B-F,** Soleus muscle 12 (**B**), 16 (**C**: asterisk indicates degenerated nerve fibers), 19 (**D**: arrowhead indicates the spindle), 30 (**E**) and 38 (**F**: arrow indicates a myofiber with a central nucleus) weeks after denervation. **G,** Normal EDL muscle. **H-L,** EDL muscle 12 (**H**), 16 (**I**), 19 (**J**), 30 (**K**) and 38 (**L**: the asterisk indicates degenerated nerve fibers) weeks after denervation. **(a)** - fat cells. Bar = 18 μm.

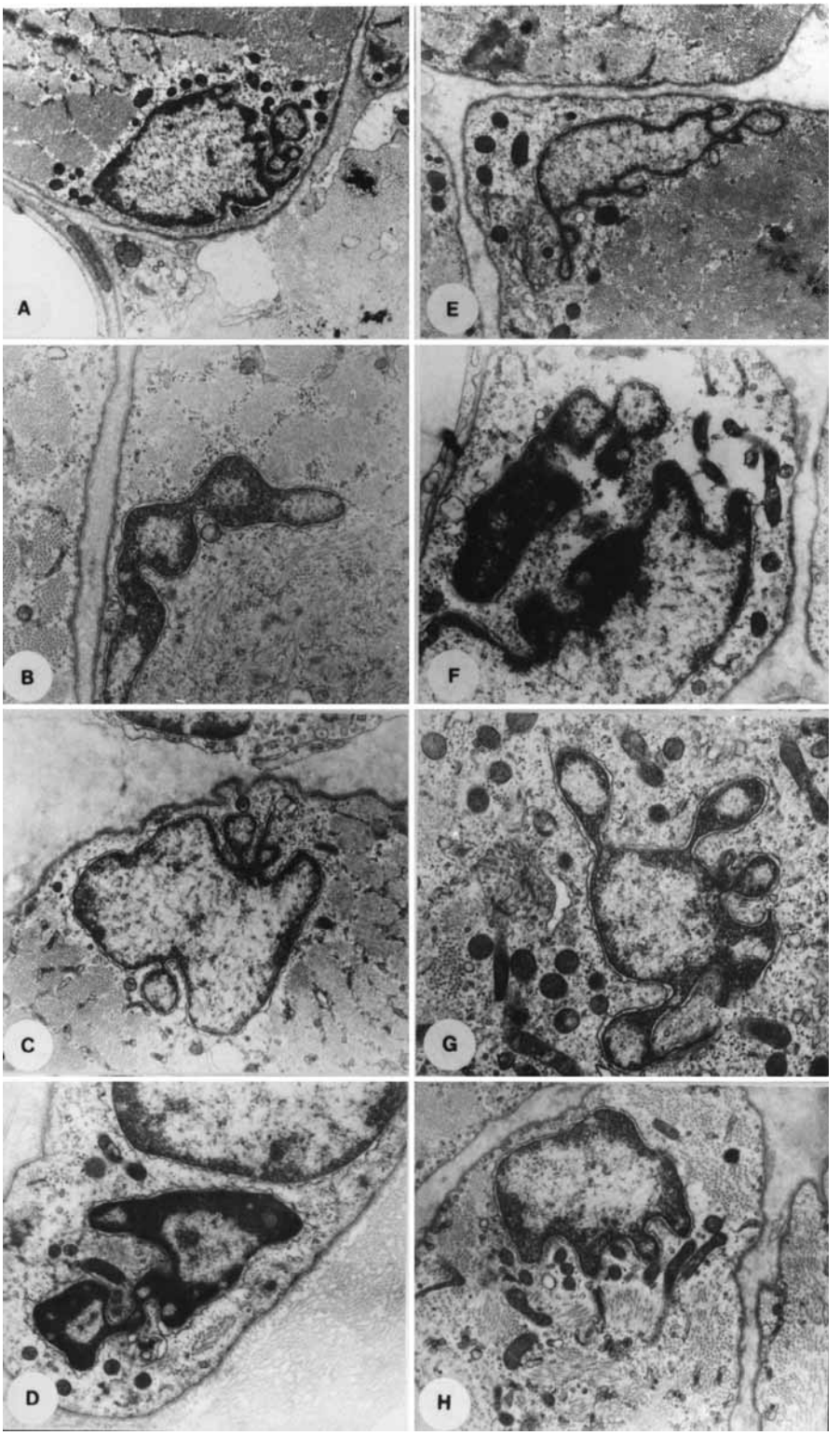

Figure 2. Electron micrographs of soleus (**A-D**) and EDL (**E-H**) muscles 12, 16, 19 and 38 weeks after denervation. (**A,C,E,G**) Bar = 0.38 μm; (**B,D,F,H**) Bar = 0.5 μm.

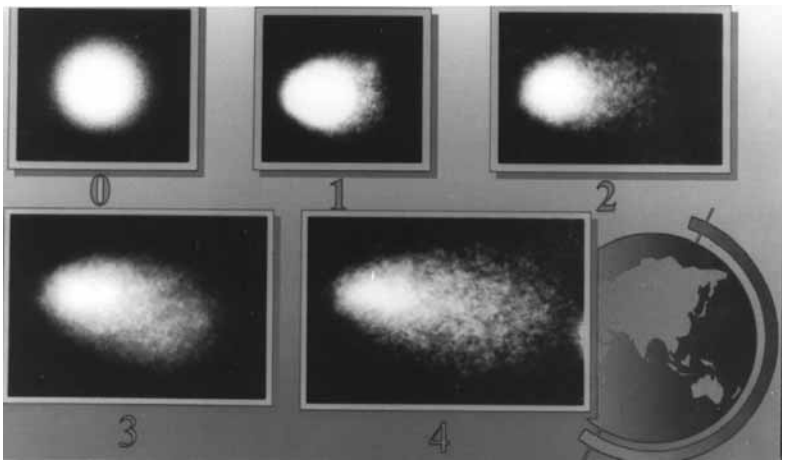

Figure 3. Single cell gel electrophoresis showing the comet classes. Class 3 and 4 cells (apoptotic cells) were seen in soleus and EDL muscles.

Statistical analysis

Student's *t*-test for independent samples was used for the statistical comparisons, with $p<0.05$ indicating significance. All statistical tests were done using the software "Statistica per Discipline Bio-mediche" (McGraw-Hill, Milan, Italy).

RESULTS

General morphology

Figures 1 and 2 show the morphological appearance of normal and denervated EDL and *soleus* muscles. In normal EDL muscle, the superficial part, which accounted for about two-thirds of the muscle, was dominated by large fibers poor in mitochondria, whereas the deep part of the muscle consisted mainly of small fibers rich in mitochondria. Regional differences were less marked in normal *soleus* muscle. After denervation, the regional differences in EDL muscle were initially more pronounced, but tended to disappear after 15-17 weeks. Spindles with more than four myofibers and fat invasion, characteristics indicative of muscle degeneration, were seen in denervated muscles (Fig. 1). Spindles with more than four myofibers were indicative of regenerating muscle, as were myofibers with central myonuclei. In *soleus* muscle, morphological changes were seen about 12 weeks after denervation, whereas the ultrastructure of EDL fibers changed only after about 16 weeks post-denervation. Myotube-like fibers with centrally-located nuclei became detectable in *soleus* and EDL muscles after about 12 and 16 weeks, respectively. After 38 weeks, most fibers in both muscle types had a central nucleus (myotube-like fibers). The interstitial spaces among the muscle fibers became wider as atrophy progressed and contained collagen fibers and fat cells.

Apoptosis

Ultrastructural examination of the *soleus* and EDL muscles 12, 16, 19, 30 and 38 weeks after denervation confirmed the presence of degenerating cells scattered throughout the tissue. Small, dense, round or oval bodies were observed within the compact, very dense sarcoplasm of some muscle cells. In many cases, the sarcoplasmic reticulum showed mild dilatation. The pyknotic nuclei were dense and the nuclear outline was usually abnormally convoluted. Residual bodies were also seen within the capillary walls or apparently lying free within the vascular lumen. Several myofibers contained ghost-like nuclei in addition to normal myonuclei (Fig. 2). There were no ultrastructural differences in the signs of apoptosis between red and white muscles at any stage after denervation.

One hundred randomly chosen cells from the *soleus* and EDL muscles from two rats were subjected to the comet assay and the results were compared to those from a control *soleus* muscle (Table 1). Tail lengths were classified from 0 to 4 (Fig. 3) and transformed into scores. Student's *t*-test revealed significant ($p<0.05$) differences between all of the denervated muscles and the control muscle, although the denervated muscles did not differ significantly from each other.

DISCUSSION

We have previously described the presence of non-apoptotic myofibers in long-term denervated *soleus* and EDL muscles, as confirmed by the *in situ* nick translation technique [21]. In the present study, we have shown that long-term denervated white and red muscles in rats also contain apoptotic cells and

that apoptosis may contribute to the loss of cells in such muscles.

In agreement with other studies, our ultrastructural analysis confirmed the occurrence of progressive muscle fiber atrophy after denervation [6,16,18,19], and the presence of many muscle cells with clear apoptotic features [2,4,11]. The TUNEL assay is frequently used to assess apoptosis, but can also stain necrotic cells. For this reason, the comet assay may be more reliable for studying apoptosis, although few studies have evaluated this possibility [8]. As shown here, the comet assay confirmed the occurrence of apoptosis and revealed a large number of cells in class 4. The occurrence and progression of apoptosis was independent of the muscle type.

Our data indicate a loss of function as an important factor in the initiation of apoptosis. This conclusion agrees with others [6], and with the lack of myofibers in long-term denervated frog muscles [3]. The comet assay showed that a significant number of cells fell into class 4, i.e., they are apoptotic, 38 weeks after denervation. Based on these observations, we conclude that long-term (>30 weeks) denervation of rat skeletal muscle causes apoptosis and that this could account for the eventual loss of fibers in such muscle. In this case, the likelihood of muscle regeneration is minimal, since the number of satellite cells decreases dramatically within 24 weeks of denervation [21]. Further investigations are required to determine precisely when apoptosis is activated after denervation.

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