CORRELATION BETWEEN ULTRASTRUCTURAL AND MOLECULAR CHANGES IN DENERVATED MUSCLE: A PARADIGM BASED ON FUNCTIONAL ELECTRICAL STIMULATION

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ABSTRACT
Soon after denervation, skeletal muscle undergoes ultrastructural and molecular changes that ultimately lead to muscle atrophy and cell death. Functional electrical stimulation (FES) is effective in preventing the atrophy of denervated muscles, but it is unclear whether FES can prevent the progression of ultrastructural changes in the sarcotubular system and cell death after denervation. In this work, we developed a model to study the effects of FES on the ultrastructural changes in the sarcotubular system and the cell death which follow skeletal muscle denervation. The right sciatic nerve of adult rats was sectioned and after 1 day, and 1, 2 and 3 weeks the tibialis anterioris muscle was electrically stimulated. FES was done for 1, 2, 3, 10 and 20 weeks after denervation. The stimuli (10 Hz) were given for 10 s followed by a 10 s rest for a total of 30 min/day. Transmission electron microscopy showed that progressive dilatation of the sarcotubular system, as well as signs of cell death, were prevented when stimulation was started one day or one week after denervation. This model could be useful for understanding the correlation between the structural changes and the molecules involved in the dilatation of the sarcotubular system or cell death.

Key words: Atrophy, cell death, denervation, electrical stimulation, skeletal muscle

INTRODUCTION
Soon after skeletal muscle denervation, ultrastructural changes occur which include focal dilatation of the sarcotubular reticulum and T system followed by changes in the width of the Z discs [8,9]. Concomitantly, there is an increase in the levels of calsequestrin, a muscle-specific sarcotubular system calcium-binding protein, and of other calcium-binding proteins, such as ryanodine receptors, SERCA-1 and IP3R, in response to the muscle atrophy that follows denervation [10,11]. In long term denervation, muscle cell death occurs and is accompanied by over-expression of the Bax and caspase-8 genes, two well known proapoptotic proteins [5,6,11,14-16].

Functional electrical stimulation (FES) is effective in preventing the atrophy of denervated muscles in clinical and experimental situations [1]. Although a few studies have shown that FES can modulate the expression of some molecules [7,17] it is unclear whether FES can prevent the ultrastructural changes in the sarcotubular system and cell death after denervation [1,4].

In this work, we developed a model to study the effects of FES on the ultrastructural changes in the sarcotubular system and the cell death which follow skeletal muscle denervation.

MATERIAL AND METHODS
Muscle denervation
Adult Wistar rats (250-350 g) of either sex obtained from the Central Animal Home at UNICAMP were used. The animals (n = 120) were anesthetized with a mixture (1:1) of ketamine hydrochloride (Francotar®) and thiazine hydrochloride (Virbaxyl®) in sterile water (2.5 ml/kg body weight) injected intraperitoneally. The right sciatic nerve of adult rats was sectioned and after 1 day, and 1, 2 and 3 weeks the tibialis anterioris muscle was electrically stimulated. FES was done for 1, 2, 3, 10 and 20 weeks after denervation. The stimuli (10 Hz) were given for 10 s followed by a 10 s rest for a total of 30 min/day. Transmission electron microscopy showed that progressive dilatation of the sarcotubular system, as well as signs of cell death, were prevented when stimulation was started one day or one week after denervation. This model could be useful for understanding the correlation between the structural changes and the molecules involved in the dilatation of the sarcotubular system or cell death.

Key words: Atrophy, cell death, denervation, electrical stimulation, skeletal muscle

This paper is dedicated to the memory of our colleague Prof. Gregorio Santiago Montes.
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denervation. Each group of rats was further divided into five subgroups (n = 3 each), according to the duration of the treatment with electrical stimulation (1, 2, 3, 10 and 20 weeks). The rats that did not receive electrical stimulation (n = 60) were also divided as described for the stimulated rats and served as age- and time-matched denervated controls.

For electrical stimulation, the rats were slightly anesthetized with ether and placed in a tubular device, in which the *tibialis anterioris* muscle could be held in a neutral position. Electrical stimuli were generated by a programmable microcontroller and were delivered by an active skin electrode placed on the anterolateral aspect of the thigh under the proximal insertion of the *tibialis anterioris* muscle. The indifferent electrode was placed under the distal insertion of *tibialis anterioris* muscle. Electrical stimuli (10 Hz) were delivered for 10 s followed by 10 s of rest, for a total of 30 min daily.

**Electron microscopy**

At the end of 1, 2, 3, 10 and 20 weeks of daily electrical stimulation, treated and non-treated rats were anesthetized with a mixture of ketamine hydrochloride and thiazine hydrochloride, as described above, and then perfused intracardiacly with a modified Karnovsky solution (1% paraformaldehyde and 3% glutaraldehyde in 0.07 M cacodylate buffer, pH 7.3-7.4). The right *tibialis anterioris* muscle was removed and the middle region of the muscle belly was cut into small blocks and fixed in the same Karnovsky solution for 2 h. The specimens were washed several times in 0.1 M cacodylate buffer, pH 7.3 and postfixed for 2 h with 1% osmium tetroxide in cacodylate buffer followed by 0.5% uranyl acetate solution overnight then dehydrated in acetone and embedded in Epon. Ultrathin sections for transmission electron microscopy were obtained with a diamond knife and stained with 2% lead citrate prior to observation with a Zeiss EM-10 electron microscope.

**RESULTS**

In non-stimulated muscles, one week after denervation there was focal dilatation of the sarcotubular system (transverse tubules and sarcoplasmic reticulum). These focal dilatations became progressively more abundant two and three weeks after denervation (Fig 1A-C).

In muscles in which electrical stimulation was started one day after denervation, there were no ultrastructural changes in the sarcotubular system after one or two weeks of stimulation. Dilatation of the sarcotubular system was seen when stimulation was started one week after denervation and continued for one week, whereas two weeks of stimulation prevented these changes (Fig. 2). When electrical stimulation was started three weeks after denervation, ultrastructural changes occurred, even if the muscle was stimulated for three weeks.

In non-stimulated muscles, no signs of cell death were seen after short periods of denervation (1, 2 and 3 weeks), but occurred after longer periods of denervation in control rats matching the 10-week subgroup of electrical stimulation. In addition to the loss of a significant number of myofibrils and the formation of myofibril-free zones located mostly in
Muscle denervation and electrical stimulation

Peripheral areas of the sarcoplasm, the myonuclei frequently exhibited an unusual morphology which included nuclear membrane invaginations. The chromatin of control rats showed varying levels of apoptosis, which was much more frequent in the controls of the 20-week subgroup of electrical stimulation, i.e. almost 23 weeks after denervation. By this time, the nuclear material was homogeneously condensed and round, electron-lucent areas of small diameter were intermingled with areas filled with dense chromatin masses (Fig. 3).

Muscles stimulated for 10 weeks showed signs of cell death only when FES was started two or three weeks after denervation; when FES was started one day or one week after denervation, there were no signs of cell death (Fig. 4A-D). When FES was done for 20 weeks, cell death was less evident in muscles stimulated soon after denervation (one day or one week) than in rats that received FES two and three weeks after denervation (Fig. 4E-G).

DISCUSSION

We have developed an experimental model that effectively prevents ultrastructural changes in denervated skeletal muscle. The protocol for electrical stimulation is easy to perform, since any commercially available equipment for electrical stimulation can be used. The *tibialis anterioris* muscle was used because it can be accessed by superficial electrodes. This muscle has been extensively used in patients with spinal cord injury [20]. The reproducibility of this model make it particularly useful for studying morphological and physiological changes in denervated muscle.

Our observations on the sarcotubular system of denervated and non-stimulated muscles were similar to those described elsewhere [3,8,9]. The changes in the sarcotubular system seen in non-stimulated muscles did not occur when electrical stimulation was done soon (one day) after denervation. Longer periods of stimulation were necessary to prevent these changes when FES started after one week of denervation. These results show that the experimental design used was able to prevent early ultrastructural changes in the sarcotubular system caused by muscle denervation.

The mechanism that causes sarcotubular dilatation in denervated muscle is unknown, although perturbations of intracellular calcium homeostasis are involved. The atrophy seen in unloaded muscles is related to increases in free cytosolic calcium concentrations and calcium linkage [2,5,11,15]. Resident proteins of the sarcoplasmic reticulum and T tubules, such as calsequestrin, ryanodine receptors,
SERCA1 and IP3R, have roles in maintaining the intracellular calcium homeostasis and are upregulated in response to muscle atrophy [10,11]. If dilatation of the sarcotubular system is related to the overexpression of these molecules, then our observations suggest that electrical stimulation can inhibit the expression of these molecules. This idea is supported by the finding that electrical stimulation of non-denervated muscles greatly suppresses the expression of DHPR and ryanodine receptors [17]. When used 1-3 weeks after denervation, the FES protocol described here could be helpful in clarifying the role of various proteins in the genesis of the ultrastructural changes seen in the sarcotubular system.

Denervated muscle fibers and their satellite cells become more susceptible to death after long term denervation [18,19]. Apoptosis is an evolutionarily conserved form of cell suicide and is possibly involved in the death of denervated muscle fibers, especially of satellite cells [3,4,13,14]. Caspases are the executioners of apoptosis, but their involvement in muscle cell death, along with other proteases such as calpains and cathepsins, is controversial. Bax is a pro-apoptotic protein found in many cells and Bcl-2 is the founding member of a family of proteins that plays a pivotal role in the regulation of cell survival by blocking apoptotic cell death in a variety of cells under various conditions. In general, when Bax is in excess, the cells execute a death command, whereas when Bcl-2 predominates, programmed cell death is inhibited and the cells survive. In skeletal muscle, however, Bax protein levels are unrelated to cell death and increased Bax expression is associated with muscle fiber regeneration. On the other hand, Akt/mTOR is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo [2,12,16].

In non-stimulated muscles, there were signs of cell death after longer periods of denervation in controls rats of the 10- and 20-week subgroups of stimulation. This finding agrees with other reports indicating that muscle fibers death, whether by necrosis or apoptosis, is mostly associated with long term denervation [3,8,9,18].

No signs of cell death were seen when FES was started one day or one week after denervation, but were present if the stimulation started later (two or three weeks after denervation). This suggests that apoptosis or necrosis possibly starts earlier than previously reported, for muscle fibers [3,13,18]. In agreement with this conclusion is the observation that when FES was done for up to 20 weeks, signs of cell death were less evident in muscles stimulated soon after denervation.

Figure 3. Ultrastructural signs of cell death after long term denervation in non-stimulated muscles. In the 10-week subgroup (A,B), the nucleus was intact but retracted, the nuclear membrane was invaginated (arrows) and chromatin with various degrees of condensation was seen. Asterisks-mitochondria dilatation. In the 20-week subgroup, the muscle cells showed a myofibril-free zone (asterisk - C), a higher content of heterochromatin compared to euchromatin and highly condensed, homogeneous nuclei (C). Bar: 1 μm for all panels.

after denervation when compared to those that received FES two and three weeks after denervation. Together, these results show that the experimental design used here was effective in preventing muscle cell death by apoptosis, and make it suitable for examining the influences of electrical stimulation on the expression of caspases, calpains, cathepsins, Bel-2, Bax and other molecules involved in cell death.

**Figure 4.** Muscles stimulated for 10 weeks (A-D). Irregular and slightly retracted myonuclei were seen when stimulation was started two (A) and three (B) weeks after denervation. Asterisk in B—normal myofibrillar arrangement. The myonuclei appeared normal when stimulation was started one day after denervation (C, D). Muscles stimulated for 20 weeks, beginning one day (E), and one week (F) and three weeks (G) after denervation. Panel G shows the early stages of degenerating myonuclei with invaginations of the nuclear membrane and chromatin dispersion. Scale bar: 0.5 μm, except for E (1 μm).
In conclusion, we have developed an experimental model that can be used to study the timecourse of the ultrastructural changes that occur after muscle denervation. This model could be useful for studying the correlation between structure and the molecules involved in the alterations of the sarcotubular system or the mechanisms involved in skeletal muscle death.

ACKNOWLEDGMENTS

The authors thank the Departamento de Anatomia Patológica, FCM, UNICAMP and the Centro de Microscopia Eletrônica, IB, UNICAMP for use of the transmission electron microscope and for technical assistance. M.J.M. and H.S.N. are supported by the Conselho Nacional de Pesquisa (CNPq). M.C.G.C.F.T. was supported by a fellowship from CAPES. This work was supported by CAPES, CNPq and FAPESP.

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Received: April 8, 2003
Accepted: June 26, 2003