STRUCTURE AND FUNCTION OF DENERVATED TIBIALIS ANTERIORES ARE MAINTAINED BY ELECTRICAL STIMULATION IN RATS

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ABSTRACT

Objective: Electrical stimulation for treatment of denervated muscles should be implemented by selective treatment. This study evaluated the effect of selective electrical stimulation on the structure and function of denervated muscle. Methods: Fifty Wistar mice were allocated to control, stimulated denervated and non-stimulated denervated groups. Following an electrodiagnostic evaluation, the animals underwent complete unilateral denervation of the proximal anterior tibialis muscle. Weekly re-assessment was carried out in order to adjust the parameters of the selective treatment, applied three times a week. The animals were sacrificed at 7, 14, 28 and 56 days after undergoing the surgical procedures. Histochemical procedures and morphologic and morphometric studies were carried out. Results: The denervated stimulated animals did not present contracture of the ankle joint and self-mutilation was not found on the feet. Significant alterations around the type IIB (denervated stimulated at 7 days), type IIA and hybrid (denervated stimulated at 28 and 56 days) fibers showed less atrophy. The transition of muscle fibers types was significant, indicating the preservation of the functional pattern of the anterior tibialis muscle at 7 and 14 days. Conclusion: We found that the selective electrical stimulation was able to temporarily maintain the structure and function of the denervated anterior tibialis muscle.

Keywords: Electrical stimulation. Denervation. Muscle, skeletal. Atrophy. Electrodiagnosis. Rehabilitation.

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INTRODUCTION

The skeletal muscle is a specialized tissue formed by elongated cells, most of the time the same length as the muscle, denominated muscular fibers. These cells, when observed in cross section, have a polyhedral or hexagonal shape and peripheral and flattened multiple nuclei.[1] This tissue is capable of adapting when faced with a wide variety of stimuli. The interruption of the nerve supply is capable of making slow muscular fibers faster and fast muscular fibers slower due to an alteration of the concentration of MHC isoforms.[2,3] The fast and slow skeletal muscular fibers undergo atrophy after denervation. When the denervation of the muscle occurs through the complete section of its motor branch, all its fibers will atrophy.[4] The main direct effect of denervation atrophy is the reduction of the area and of the diameter of the muscular fiber and consequent reduction of its strength. There is also the conversion of type of fiber, from slow to fast, in denervated muscles.[5,6]

Physiotherapy is capable of helping patients with peripheral nerve lesions, having as its main objective the treatment of the consequences of this lesion. Several therapeutic resources can be used for this treatment, including passive and active kinesiotherapy, neuromuscular reeducation, massotherapy and electrotherapy.

The electrical stimulation of denervated muscles is intended to exercise this muscle electrically for it to remain as healthy as possible while the injured axons regenerate and innervate it once more. Accordingly, functional recuperation will be facilitated after reinnervation. The effects of electrical stimulation of the denervated muscle can be assessed under several aspects. There have already been analyses of the effects of electrical stimulation on the metabolism of muscular fibers, influences on reinnervation and on the maintenance of the contractile properties of the denervated muscle, conversion among the types of fibers, electrophysiological analyses and effects on denervation atrophy.[7-9]

The electrical stimulation of denervated muscles requires an adaptation of the stimulation parameters to the tissue excitability characteristics.[10] This is the role of Stimulus Electrodiagnosis, a test that determines the exact parameters that a denervated muscle requires to be stimulated at a given time, respecting its excitability

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alterations. It involves the construction of the intensity-duration curves using square and exponential pulses besides knowledge of the indicative points of electrodiagnosis (rheobase, chronaxia and accommodation).[11]

The aim of this study was to analyze the effect of transcutaneous electrical stimulation on the maintenance of the totally denervated skeletal muscle of the rat, through the use of a universal pulse generator and specific stimulation parameters acquired by means of the construction of an intensity-duration curve and of indicative points of electrodiagnosis.

MATERIALS AND METHODS

The experimental work was developed at the Bioengineering Laboratory of Faculdade de Medicina de Ribeirão Preto and approved by the Committee of Ethics in Animal Experimentation - CETEA, of Faculdade de Medicina da USP de Ribeirão Preto.

The study subjects were 50 adult male Wistar rats with body weight ranging from 250 - 300 grams, grouped and kept in standard plastic cages, under controlled environmental conditions (brightness: 12 hours of light/dark cycle) with free access to water and pelletized feed *ad libitum*. These animals were randomly allocated in three groups: one control group and two experimental groups, denervated group and denervated and stimulated group.

Before the denervation procedure, the animals from the experimental groups were anesthetized with an intramuscular injection of Ketamine (70mg/kg of body weight) and Xylazine (10mg/kg of body weight) and submitted to initial stimulus electrodiagnosis.

Complete proximal denervation procedure (Figure 1)

The animals from the experimental groups were anesthetized with an intramuscular injection of Ketamine (70mg/kg of body weight) and Xylazine (10mg/kg of body weight) and positioned in prone, with the lateral surface of the experimental limb duly trichotomized and fixed on the operating table. Antisepsis was performed with a solution of 20% iodized alcohol. The sciatic nerve was approached through a rectilinear longitudinal cutaneous incision, on the lateral surface of the thigh, which extended from the spinal column to the knee. The space between the femoral biceps and superficial gluteal muscles was developed by blunt dissection and the nerve was identified from the point of emergence from the column up to its trifurcation in the knee. The sciatic nerve was completely sectioned proximally at an approximate distance of 0.1 cm from its emergence in the column and its diverted proximal and distal stumps were sutured (Prolene 6/0, Ethicon®) in adjacent musculature, preventing the reinnervation of the tibialis anterior muscle. The musculature and the skin of the animals were subsequently sutured.

The tibialis anterior muscle was chosen to be denervated due to the easy localization and visualization of muscular contraction during the evaluation and the treatment, besides its accessibility for insertion of the surface electrode at these moments of the experiment as well.

Treatment protocol through electrical stimulation

The treatment of the denervated tibialis anterior muscle was performed 3 days a week, alternately, having an indefinite time for each session, depending on muscular fatigue (at the first signs of fatigue, the session was terminated). A reduction in the vigor of muscular contraction was considered a sign of fatigue.

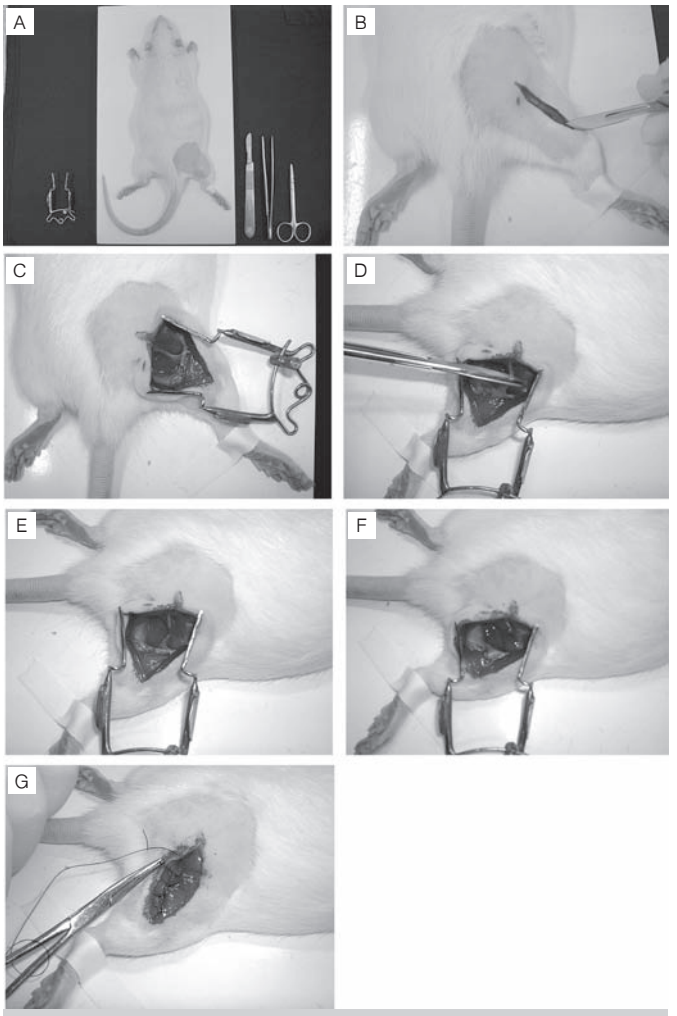

Figure 1 – *Surgical procedure. A) positioning of the animal; B) cutaneous incision; C) exposure of the sciatic nerve; D) section of the sciatic nerve; E) sectioned sciatic nerve; F) sciatic nerve sutured in contralateral musculature and G) final suture.*

At the beginning of each week of treatment, the indicative points of electrodiagnosis (rheobase, chronaxia and accommodation) were quantified so that electrical stimulation parameters were always adapted to the current state of excitability of the muscle.

The entire electrical stimulation procedure was carried out with a Nemesys 941 universal pulse generator apparatus from Quark. (Figure 2) For performance of the pre- and post-denervation protocols and for the treatment of the denervated tibialis anterior muscle, the placement of the electrodes was standardized: a metal dispersive electrode (positive) with a sponge screen soaked in water and attached to the animal's back using sticky tape and active electrode (negative) in pen form, also with its tip soaked in water, of a sufficient size to stimulate just the region of the motor point of the electrically stimulated muscle.

The animals from each group were euthanized by means of cervical displacement after 07, 14, 28 and 52 postsurgical days and had their experimental tibialis anterior muscles removed. These muscles were weighed separately on a pair of precision scales and their distal and proximal extremities were excluded.

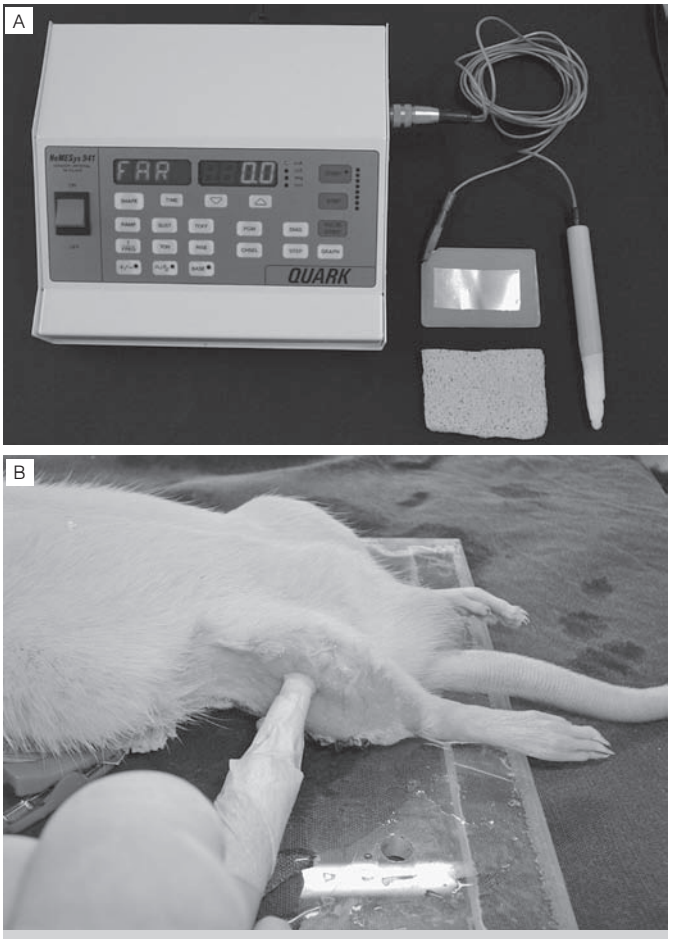

Figure 2 – *(A) electrical stimulation equipment, Nemesys 941, from Quark and pen type active electrodes, and sponge type dispersive electrode; (B) positioning of the animal for electrodiagnostic evaluation and electrotherapeutic treatment during experiment.*

Histochemical analysis of the muscles

The tibialis anterior muscle was exposed and removed through a distal incision on the right hind limb, close to the ankle joint. A fragment of the middle portion of the muscle belly was removed from each muscle. These fragments were covered with talcum and submitted to freezing in liquid nitrogen. After freezing, the fragments were stored in a freezer at -80ºC until the processing of the material.

Cross sections with a thickness of 5 μm were obtained from all the muscles through Micron HM 505 E Cryotome at a temperature of -25ºC. The sections were placed on 24x32 mm microslides and, afterwards, mounted on slides. The sections were processed for Hematoxylin-Eosin (HE) dyeing and hystoenzimological reactions for Myofibrillar Adenosine Triphosphatase (mATPases. E.C. 2.1.3.5.7.9.1) in acid and alkaline pH values.

A qualitative analysis of the slides was carried out using a Leica DM 2500 light microscope. The general aspects of the skeletal muscle tissue were evaluated in the HE dyeing processes and the different kinds of skeletal muscle fibers were determined by the mATPase reaction.

Using UTHSCSA ImageTool software, the morphometric analysis was conducted after the capturing of the images from the Leica DM 2500 optical microscope by the Leica DFC 300FX digital video camera, connected to a microcomputer.

Analysis of the proportion

For the tibialis anterior muscle they used only the slides pre-incubated at pH 4.6, as at this pH it is possible to identify three kinds of fiber that form this muscle such as fibers type I (FT I), FT IIA, FT IIB and FT Hybrid. The measurements were taken from the obtainment of images gathered in 3 random fields of the slides of the tibialis anterior muscle.

Analysis of the area of the muscular fibers

The area of 100 muscle fibers was measured in each histological cut. These were chosen randomly in the central region of the histological section.

The statistical data was properly handled and analyzed through the Mann-Whitney U-test with 5% of significance.

RESULTS

On the day of death, the mean weight of the animals was 248g in G1, 247g in GI, 307.5g in G2, 312g in GII, 402.5g in G3, 365g in GIII, 508g in G4 and 439g in GIV.

Macroscopic Alterations

The behavior of self-mutilation of the toes of the rear paws that had undergone denervation was observed during the experimental period. The animals gnawed toes and paws due to the absence of sensitivity and the non-recognition of that extremity as an integral part of their own bodies. This did not happen with the animals submitted to the treatment with electrical stimulation.

We also observed contracture in flexion of the ankle joint of the denervated and non-stimulated animals in the groups of 28 and 56 days. This joint was stiff, not yielding to manipulation. The animals submitted to stimulation did not suffer this type of impairment.

Electrical Stimulation

The animals from the denervated and stimulated groups were submitted to the electrodiagnosis exam and, after the determination of the specific parameters for transcutaneous electrical stimulation of the muscles, were submitted to the respective selective treatments. In the first week after denervation, all the stimulated animals had their muscles faradizable, that is, responding to a Faradic current. After the first week, the electrical stimulation parameters (T = time of stimulus-breadth of pulse and chronaxia) were gradually increased, evidencing the need for the denervated muscle to respond to stimuli of high intensity and duration. These alterations show the changes in excitability undergone by the skeletal muscle after its denervation.

The stimulation frequencies, directly related to the parameters of breadth and duration of pulse, also varied during the experiment, initially, on day 7 of the experiment, the denervated muscles responded to a stimulation frequency of 50 Hz that gradually dropped and reached the minimum values of 10 Hz at 14 days, 3 Hz at 28 days and 1 Hz at 56 days of denervation.

The chronaxia values, unlike the stimulation frequency, increased gradually, with their initial values at 0.1 ms, compatible with values of normally innervated muscles, and reaching 5 ms at the end of the experiment.

The motor point, region of greatest excitability of the muscle, was denatured in the denervated and stimulated groups in the period of 7 day and 14 days. In these periods, such points were more distal

to the muscle belly, close to the tendon of insertion of the tibialis anterior muscle. In the denervated and stimulated groups in the period of 28 days and 56 days there was a return of the motor point to the muscle belly region.

Morphology

The cross sections cuts of the tibialis anterior muscle in the control group presented polyhedral or hexagonal fibers, multinucleated, with peripheral and flat nuclei. The progression of the denervation evidenced more defined muscular fascicles, more irregular outlines of the muscular fibers with a rounded appearance besides rounded and centralized nuclei, with dischromic alterations inside them. Such alterations could be observed from group GII (14 days after denervation). We also observed that the denervated and stimulated muscles underwent similar changes to the group of just denervated muscles, but that these took longer to appear. These were observed as of group G3 (28 days denervated and stimulated).

Morphometry

Number of muscle fibers

The quantity of the different types of muscular fibers in the tibialis anterior muscle was reduced in all the four study groups when comparing the groups that were just denervated with the denervated and stimulated groups. Such modifications can be observed in the graph depicted in figure 3. There was a significant difference in the 7-day period in FT IIB (p=0.01855) and FT hybrid (p=0.001528), in the 14-day period in FT hybrid (p=0.01771), in the 28-day period in FT IIA (p=0.01097) and FT hybrid (0.03808) and in the 56-day period in FT IIB (p=0.001122).

Area of the muscle fibers

The areas of the different types of fiber underwent differentiated modifications in the evaluated periods. An increase of the area of FT I and FT hybrid was observed while FT IIA and FT IIB underwent reduction in this measurement. The values of the areas of such muscle fibers can be observed in Table 1. There was significant difference in the values of the areas of FT IIB (p=0.0191) in the 7-day period, of FT IIA (p=0.000001) and FT hybrid (p=0.0008) in the 28-day period and of FT IIA (p=0.00001) and FT hybrid (p=0.0000001) in the 56-day period.

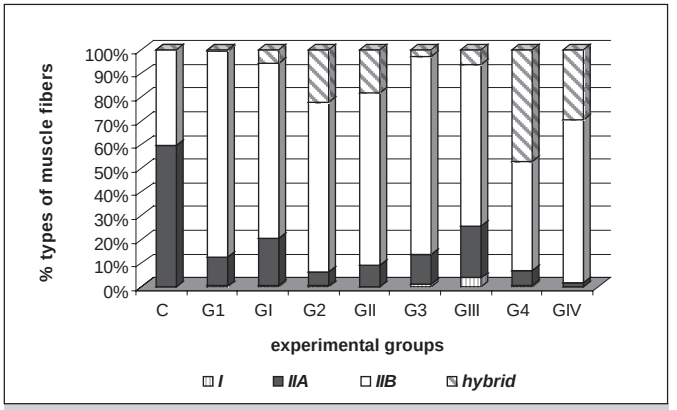

Figure 3 – *Percentage of the different types of muscle fiber (type I, IIA, IIB and hybrid) found in the tibiales anteriores muscles in the different experimental groups: C – control, G1 – denervated and stimulated 7 days, GI – denervated 7 days, G2 – denervated and stimulated 14 days, GII – denervated 14 days, G3 – denervated and stimulated 28 days, GIII – denervated 28 days, G4 – denervated and stimulated 56 days, GIV – denervated 56 days.*

DISCUSSION

Macroscopically, the absence of ankle joint contracture in the animals whose tibiales anteriores muscles were denervated and stimulated exemplified one of the beneficial actions of this treatment. The denervation of the tibialis anterior muscle provoked an imbalance among the muscles that cover the ankle joint and the maintenance of this situation throughout the experiment period probably contributed to the installation of a picture of fibrosis and permanent shortening of the joint capsule, leaving the joint stiff.[12] The electrical stimulation of the denervated muscle through a regime of constant treatment (three times a week) promoted the contraction of the tibialis anterior muscle, mobilizing this joint, and preventing contracture. The influence of electrical stimulation could also be observed in the absence of self-mutilation attitudes in the denervated and stimulated animals.

The change of location of the motor point, point of greatest excitability due to enhanced sensitivity to acetylcholine, observed in the denervated muscles, was compatible with other studies.[5,13] A normal skeletal muscle exhibits greater sensitivity to acetylcholine in the region of its motor plate, which in the majority of cases is located

Table 1 – *Numerical values of the areas (mean and standard deviation) of the different types of fiber in the different experimental groups: C – control, G1 – denervated and stimulated 7 days, GI – denervated 7 days, G2 – denervated and stimulated 14 days, GII – denervated 14 days, G3 – denervated and stimulated 28 days, GIII – denervated 28 days, G4 – denervated and stimulated 56 days, GIV – denervated 56 days. The values in bold followed by an asterisk are those with statistical significance (p < 0.05).*

		C	G1	GI	G2	GII	G3	GIII	G4	GIV
			d + ee	d	d + ee	d	d + ee	d	d + ee	d
			7 days		14 days		28 days		56 days	
I	mean		42.9946	41.97	34.481	31.387	52.7924	57.792	66.4467	37.16
	SD		20.1683	12.2719	12.4606	13.6287	37.1504	23.6115	52.1862	27.577
IIA	mean	71. 4178	30.023	30.645	29.4594	29.267	***34.0108****	***24.5424****	***51.4803****	***11.5069****
	SD		7.64311	7.98806	7.6566	8.3142	18.5174	9.8668	24.3084	3.7089
IIB	mean	66.5205	***45.2475****	***42.7195****	31.3497	31.9325	33.5214	34.5381	50.8485	45.7298
	SD	28.23	17.5955	14.6703	8.0781	9.5325	24.2925	17.1058	33.7254	27.0552
Hybrid	mean		44.1006	41.4598	28.4427	28.7653	***25.9649****	***36.2871****	***52.8664****	10.5344*
	SD		11.8472	15.5151	7.3053	8.534	16.9234	17.5918	29.9089	3.3818

in the muscle belly. Secondary to this region of greater excitability, a portion close to the myotendon junction also exhibited greater sensitivity to acetylcholine, consequently of greater excitability, intermediate between the motor plate and the rest of the muscle fiber. After denervation, the entire region of the skeletal muscular fiber becomes sensitive to acetylcholine and consequently, more excitable, as a form of stimulus to reinnervation.[5,13] The greatest post-denervation excitability in the distal region of the muscle observed in this experiment during the first two weeks is compatible with the second region of greatest excitability of the muscle, since the first region, the motor plate, was denatured due to the denervation. The return of the motor point to the central region of the muscle belly after this period was probably due to the influence of the external electrical stimulation. Therefore the treatment through electrical stimulation served as a stimulus for the muscular fibers to adapt to reinnervation, resuming their original characteristics in expectation of this event.

The morphological alterations undergone by the denervated muscles observed in this study were compatible with others found in literature. Guth[13] characterized evident morphological alterations after 14 days from denervation similar to those observed here, like the shape of the nuclei of the muscle fibers that became rounder and more centralized, besides the presence of central coloration changes. Such characteristics were suggestive of nuclear proliferation, thus indicating an adaptation of the muscle to denervation.[14]

The main alteration undergone by the denervated skeletal muscle was disuse atrophy.[15] It was one of the main justifications for the use of electrical stimulation in these cases, for its prevention.[16-18]

The reduction in the area of the muscle fibers after denervation was observed in this study only in the denervated groups and was compatible with the findings in literature. Several studies reported a significant reduction of the cross section area of the muscle fibers in the first two weeks after denervation.[15] They considered that at the end of the three weeks after this procedure, there was a reduction of 80% of the area of the muscle fibers when compared with normal fibers.[19]

The role of electrical stimulation in the delay and/or prevention of atrophy of the denervated muscle was the subject of countless publications. In this study, the stimulation frequency varied throughout the entire experiment period according to the excitability of the denervated muscle. Accordingly, there was no preprogrammed pattern of stimulation of these muscles. This electrical stimulation proved efficient in the maintenance of the area of type IIB muscle fibers at day 7 of the experiment. The frequency remained at 50 Hz, and in type IIA fibers at 28 days (f between 50 Hz and 3 Hz) and at 56 days (f between 50 Hz and 1 Hz). Besides these fibers of fast contraction, characteristic of the tibialis anterior muscle studied, the hybrid fibers, which appeared after the first week of denervation, also had their area maintained significantly through electrical stimulation provided on day 56 of the experiment, with frequency ranging from 50 Hz to 1 Hz.

The transition of the types of fiber could also be observed in the denervation process of the tibialis anterior muscle and underwent changes in comparison with the treatment with electrical stimulation. The findings of this experiment are in accordance with some results already presented in other studies.[2,3] The denervated and electrically stimulated muscles, at 7 days, presented maintenance of the pattern of the types of fiber corresponding to the tibialis anterior with predominance of type IIB fibers and significant increase of hybrid fibers in the muscles that were only denervated in the same period. From this period on, electrical stimulation was incapable of maintaining the pattern of types of fiber of the denervated tibialis anterior muscle. At 14 days, the denervated and stimulated muscles underwent transition from type IIB fibers to hybrid fibers, which significantly increased. After this period, no significant difference in the denervated and stimulated muscles was observed. The role of electrical stimulation in the maintenance of the characteristics of the denervated muscle was restricted to the initial periods of stimulation.

CONCLUSION

Electrical stimulation was capable of:

1 – Maintaining the range of movement of the ankle, without signs of self-mutilation in the animals whose muscles underwent denervation;
2 – Extending the morphologic alterations characteristic of denervation for a period of 28 days;
3 – Allowing the maintenance of the significant quantity of type IIB muscular fibers performed on the first 7 days of denervation, maintaining the fast contraction muscle characteristics of the tibialis anterior.

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