Permanent denervation of rat Tibialis Anterior after bilateral sciatectomy: Determination of chronaxie by surface electrode stimulation during progression of atrophy up to one year

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Abstract

Permanent lack of motor innervation results in striking alterations of functional and structural properties of muscle. In SCI patients, direct Functional Electrical Stimulation of denervated muscles (FES of DDM) prevents or reverts muscle atrophy. FES is, however, believed to be effective when started early after SCI, since long term denervated muscle do not contract by commercial surface electrical stimulation. Whether this is the result of: 1. lack of muscle fibers; 2. their poor excitability, or 3. disarrangement of contractile machinery, is still an issue that deserves detailed time-course studies that are difficult in humans. In the present study, we investigated in a rat model, the progression of denervation-induced long term changes of Tibialis Anterior (TA). Muscle contraction elicited by external skin electrodes were studied up to 12 months after bilateral sciatectomy. Chronaxie analysis and histological structural findings showed that the sciatectomized TA undergoes: 1. a rapid atrophy process during the first month of denervation, accompanied by progressive increase of chronaxie that reached a stable increase during the second month after sciatectomy (early stage of rapid progressive atrophy); 2. a second phase of slow progressive atrophy up to 4 month-denervation, during which time no major changes in chronaxie or gross muscle mass occurred; 3. a third phase, during which the gross muscle mass remain stable, while the chronaxie either increased to infinite value (severe atrophy stage, from 4 to at least 12 months) or returned to the low values of reinnervated muscle. Histochemistry showed early disappearance of the slow type I fibers, followed by disappearance of the ATPase and of the SDH activities. On the other hand, the 9-month denervated muscle presents numerous "severely atrophic" muscle fibers. Our conclusion is that the lack of contractility of the long term denervated rat muscle is due to complete loss of contractile proteins long before muscle fibers decrease in number, a process that requires much more than 12 months in rat fast-twitch muscles.

Key words: rat muscle, long term denervation, electrical stimulation, rat tibialis anterior, FES, surface electrodes, chronaxie

The maintenance of internal structure of muscle fibers is strictly dependent upon innervation from their motor neurons and from a minimum level of activity [5, 12, 18]. When muscle fibers looses communication with their motor neuron either in spontaneous neuro-pathological conditions, or as the result of traumatic events such as spinal cord injury (SCI) or peripheral nerve damage, skeletal muscle undergoes paralysis and striking alteration of internal organization of the contractile cells, followed by long term severe loss of

mass [6, 7, 9, 15]. The consequences are particularly severe when the spinal cord injury is complete [25] and the lesion destroy the spinal motor neuron [4, 5, 11, 16].

Over the years the efficacy of Functional Electrical Stimulation (FES) to reverse muscle atrophy and restore movement in the limbs of paralyzed patients has been attempted and fiercely debated [13, 14, 17, 21, 22]. The dominant opinion was that FES training is not effective in all patients, in particular when treatment is started after extended periods from the injury event (months). For example, in the case of complete lower motoneuron denervation it is easy to elicit muscle contraction using standard FES devices very close to injury, but later on (few months in rats, but up to one year in humans) denervated muscle seems to become unexcitable by both surface and intramuscular electrodes, since the intensity of the current has to be increased to values over safety windows [16, 17], and finally the long term denervated muscle do not contract at all. Whether this is the result of: 1. lack of excitability, 2. lack of contractile machinery, or 3. lack of muscle fibers is still an issue that deserves detailed time-course studies that are difficult in humans. The present study in a rat model investigated the progression of denervation-induced changes in the Tibialis Anterior (TA) up to 12 months. Bilateral sciatectomy was performed to better mimic the unloading conditions, which are the main driving factors of muscle atrophy/degeneration occurring during flaccid paralysis. Muscle chronaxie had been determined using cutaneous surface electrodes in anaesthetized rat, and structural muscle characteristics by macroscopic and microscopic analyses after animal sacrifice. All together, the results here presented demonstrate that the lack of contractility of the long term denervated muscle is due to the complete loss of contractile proteins that occurs long before muscle fibers disappear, a process which in fast-twitch muscles of rodents takes place much later than 12-month after denervation [10].

Materials and methods

Denervation of rat leg muscles

In the present article we describe the post-denervation changes of *Tibialis Anterior rat muscle* (TA) after bilateral sciatectomy. Denervation was performed according to Carraro et al. [8]. Briefly, male Wistar rats weighing 150 to 200 g were anesthetized using an intraperitoneal cocktail (ketamine hydrochloride 40 µg plus xylazine 20 µg / 100 mg of body weight). The posterior thight were shaven and aseptically prepared using povidone-iodine (Betadine) and 70% alcohol solution. Both right and left posterior legs were denervated by removal of a 2-cm section of the sciatic nerve and ligation of the proximal stump near the trochanter muscles. Very seldom (in 3 muscle out 36) after such an operation, muscles underwent long-term spontaneous reinnervation. At stated time (from 1 to 75 weeks post-sciatectomy) rats were

Figure 1. Atrophy of rat muscle after four-month bilateral sciatectomy. Note that under the detached TA, the EDL is hardly recognizable.

sacrificed with an overdose of anesthetic, and leg muscles immediately dissected (Fig. 1). Soleus, (TA), Extensor digitorum longus (EDL) and Gastrocnemius were weighted and prepared for light and electron microscopy. Typically, samples of each muscles were immediately fixed and embedded for electron microscopy, while the bulk muscles were frozen in liquid nitrogen.

In vivo electrophysiological recordings

To analyze the state of denervation, in rat anaesthetized using an intraperitoneal cocktail of ketamine hydrochloride 40 µg plus xylazine 20 µg/100 mg of body weight, muscle rheobase and chronaxie were determined by surface electrical stimulation and palpation of leg muscles [19]. Rheobase is the minimal electrical stimulus intensity necessary to produce a muscle contraction (rectangular phasic current; pulse interval, 1 s; inter-pulses interval, 2 s). Chronaxie is the minimal pulse duration necessary to induce a muscle contraction (rectangular phasic current; interpulses interval, 2 s; amplitude: twice the rheobase value).

Before fixing the electrodes, the skin was shaved and cleaned, and a gel conductor layer applied between the electrodes and the skin. Two types of electrodes were used: the indifferent and the electrode. The first was positioned around the thigh: its large area, (a 10 mm long x 2 mm large stripe) providing a decrease in concentration of the electrical charge on the skin, did not elicited thigh muscle contractions nor skin hyperemia during the electrical procedures. The active electrode was used to stimulate the TA and other distal leg muscles (Fig. 2). In selected cases, the distal electrode was small enough (2 x 2 mm square rubber electrode) to stimulate only the underneath muscle, achieving selective contraction of either TA or of one of

***Figure 2**. In vivo muscle electrophysiological analysis by skin electrical stimulation. Determination of rheobase and chronaxie. An indifferent electrode is positioned around the thigh: its large area, providing a decrease in concentration of the electrical charge on the skin, did not elicit thigh muscle contractions nor skin hyperemia during the electrical procedures. The second active electrode was used to stimulate the TA muscle and other distal leg muscles.*

the two *capita* of the Gastrocnemius. The electrical parameters were changed in sequence to provide rheobase and chronaxie values. At each setting the denervated TA was palpated manually to check a response.

Structural Analyses of Muscle Specimens

The specimens collected as described above, were prepared either for light and/or electron microscopy (EM).

Morphometric analysis. Cryosections (10 µm thick) of frozen biopsies were stained with hematoxylin-eosin (H&E), using conventional techniques. For the morphometric analysis, an unbiased sampling procedure was applied. The mean diameter of the muscle fibers were evaluated from the cross sections according to Rossini et al. [23]. Images were acquired using a Zeiss microscope connected to a Leica DC 300F camera at low magnification under the same conditions that were used to acquire a reference ruler. Morphometric analysis was performed using Scion Image software for Windows, version Beta 4.0.2, free software (by 2000 Scion Corporation, inc.) downloaded from the web site: www.scioncorp.com [23].

Histochemistry. Serial cryosections (10 µm thick) were stained for Myofibrillar ATPase (pH 4.35 and 9.6, respectively) and SDH activity for mitochondrial oxidative capacity to evaluate type I (slow, fatigue-resistant), and type II (fast, fatigue-prone) muscle fibers, using conventional techniques. In normal rat muscles, type I fibers are dark following preincubation at pH
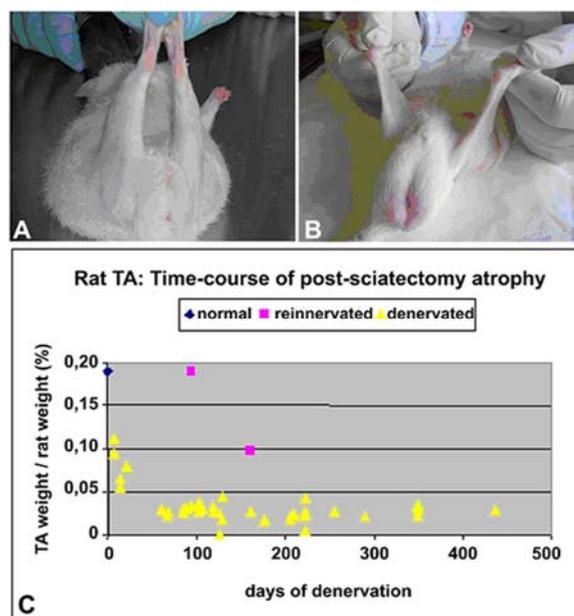
***Figure 3**. Macroscopic aspects and weights of long term denervated TA. In comparison to adult normal rat (A), the 12-month sciatectomized rat legs (B) appear to have completely lost their muscle mass. Panel C confirms the atrophy process started with a very rapid decrease of the muscle mass that reach a minimum at one-two months after denervation. Afterward, there was a progressive but quite slow loss of muscle from 3 months on.*

values of 4.3 and light after pH 9.6. In normal rat muscles, the reverse is true for type II fibers.

Preparation of muscle specimens for transmission EM. Part of the muscles were fixed in 3.5% glutaral-dehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2h followed by buffer washes and fixation for 1h in 1% osmium tetroxide. The specimens were rapidly dehydrated in graded ethanol and acetone, infiltrated with Epon-acetone (1:1) mixture for 2 h, and embedded in Epon. Semithin sections (100 nm) were cut in Leica Ultracut R (Leica Microsystem, Austria) using a Diatome diamond knife (DiatomeLtd. CH-2501 Biel, Switzerland) and stained with toluidine blue.

RESULTS and DISCUSSION

Macroscopic appearance and in vivo determination of chronaxie. The long term (12 months) legs of bilaterally sciatectomized rat (Fig. 3B) appeared to have completely lost their muscle mass (severe atrophy), when compared to those of an adult normal rat (Fig. 3A).

Figure 3C confirms that in our rodent model the atrophy process started with a rapid decrease: the wet muscle weight reached about 10% of control muscle mass within the first two months after denervation (Short-term denervation stage in the rat model: up to
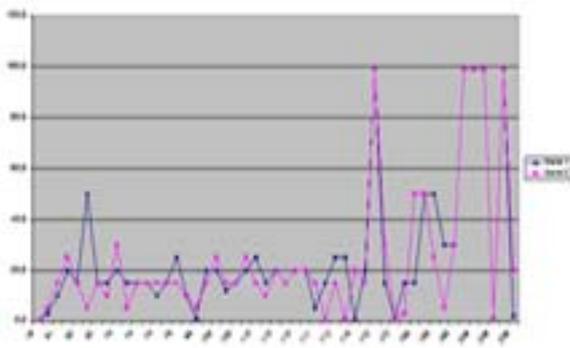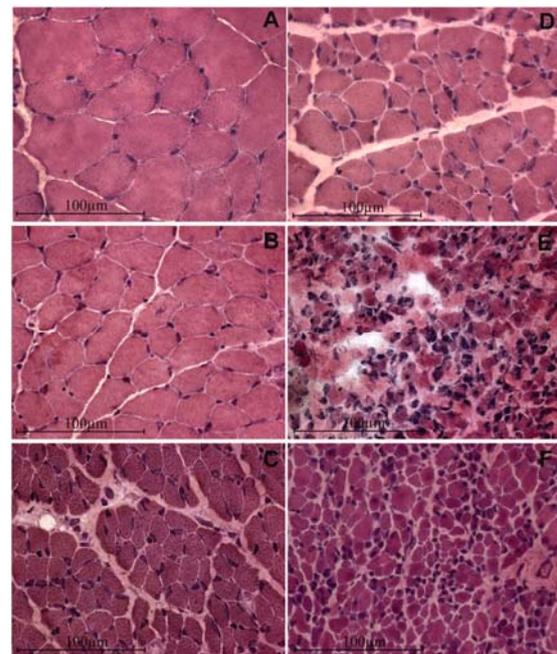
Figure 4. *Chronaxie of long term denervated rat TA muscle. The blue and the pink lines refer to two different experiments. The chronaxie measured by skin electrical stimulation increases early after denervation from 0.1-0.2 msec in innervated muscles to 0.5-1 ms one-two days after denervation (i.e., after nerve Wallerian degeneration). Then it progressively increased during the following two months to about 20 msec. Chronaxie stayed at this level up to about 6 months post-sciatectomy. Then the twitch contraction became questionably palpable and chronaxie increased to much longer values (from 30 to over 1000 msec). In 3 legs, out of 36, reinnervation occurred spontaneously and chronaxie shortened to values of normal innervated muscle, that is to 0.1-0.2 msec.*

Figure 5. *Long term denervation atrophy of the TA muscle in bilaterally sciatectomized rats. H-E stain. A, Normal; B, 1-month, C, 2-month, D, 4-month; E, 8-month; F, 12-month denervation. Innervated TA muscles are characterized by large, well packed myofiber profiles, and minimal intermyofiber spaces. The first stage of TA denervation is characterized by progressive reduction in muscle fiber diameter. In the mid-denervation stage there is a further reduction up to 4-month denervation, in which muscle fiber size is about 10% of normal. Then, the fiber size does not change significantly at least up to 1-year denervation by bilateral sciatectomy.*

eight weeks after sciatectomy). Afterwards, a progressive, but quite slow loss of muscle mass was reached and maintained at stable level from the third month on [15]. To assess contractility of the denervated leg muscles (Fig. 2), rheobase and chronaxie were determined in anaesthetized rat by surface electrical stimulation and palpation of the leg muscles. Figure 4 show the values of chronaxie of TA muscle measured up to 9-month after sciatectomy. As described before for the decrease in muscle mass, muscle excitability decreased early after denervation. In fact, chronaxie from 0.1-0.2 msec in innervated muscle changed to 0.5-1 ms within one-two days after denervation (i.e., after Wallerian degeneration of the nerve) and progressively increased to about 20 msec during the following month. Chronaxie remained at this level up to 6 months post-sciatectomy (Mid-term denervation stage in the rat model: from 2 to 6 months sciatectomy). Afterwards, the twitch contraction became questionably palpable and thus chronaxie increased to much longer values (from 50 msec to infinitum, i.e., the muscle twitch was not palpable). We define this third stage the "long-term denervation stage" of the rat model, i.e., denervation time longer than six months). In 3 out of 36 leg muscles, reinnervation occurred spontaneously and chronaxie

shortened to 0.1 msec, which is the value of normal innervated muscle (Fig. 4).

Histology. Innervated TA are characterized by large, well packed myofiber profiles, and minimal inter-myofiber spaces (Fig. 5A). The normal TA showed the peculiar checker-board ATPase staining at both acid and basic pH with a large prevalence of type II (fast) fibers, while SDH activity was present, and weak as expected, in the majority of TA fibers (not shown). The first stage of TA denervation was characterized by a progressive reduction of the fiber diameters (in 3 weeks denervated muscle the mean value of diameter was about the 50% of that of innervated muscle, Fig. 5B). Up to two-month denervation, muscle fibers showed a distinct ATPase and SDH enzymatic activities (Fig. 6A and B). In the mid-denervation stage there was a further reduction in the fiber diameters (in 4-month denervated muscle the mean diameter value was about 10% of that of innervated TA muscle, Fig. 5D). Afterwards, the mean
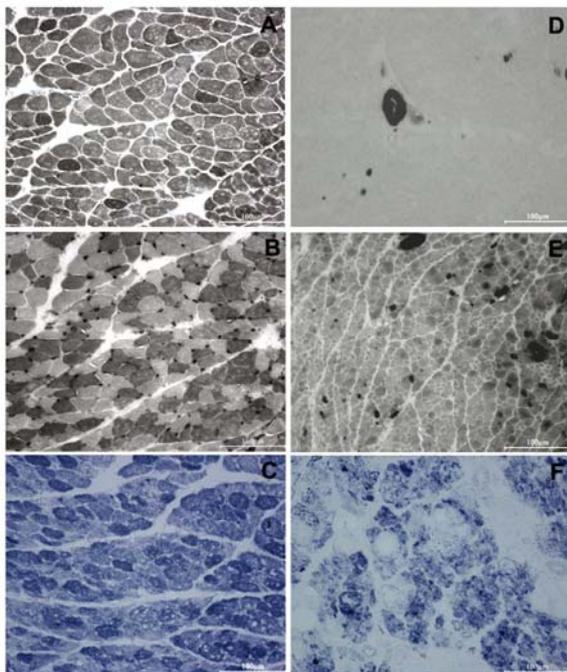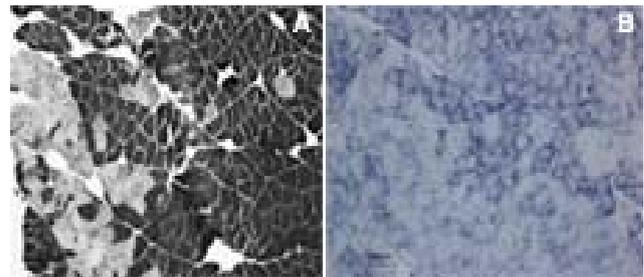
Figure 6. *Histochemistry of long term denervated rat muscle. A, B, C, 1-month denervation; D, E, F, 1-year denervation A, B, D, E, Myofibrillar ATPase, pH 4.35; C, F, SDH activity. The 1-month denervated muscle fibers show a distinct ATPase and SDH enzymatic activities. On the other hand, the severely atrophic myofibers of 1-year denervated muscle loose ATPase and SDH activities.*

fiber diameters did not changed up to 1-year of denervation (Fig. 5E and F).

On the other hand, the severely atrophic myofibers loose ATPase and SDH activities (Fig. 6D-F). Indeed, during long-term denervation stage, the surviving myofibers showed steady-state severe atrophy, and the normal fascicular architecture of the muscle was lost. Interstitial tissue that includes adipocytes and collagen sheets increased among the myofibers. The acid and basic myofibrillar ATPase demonstrated a fast-like transition of the few percentage of type I (slow) myofibers present in the TA, extending to TA our previous observation in hemi-diaphragm and gastrocnemius rat muscles [8-10]. After the sixth month denervation the histochemical SDH activity, a mitochondrial marker of type I (oxidative fatigue-resistant) muscle fibers, decreased and finally disappeared (Fig. 6F, and manuscript in preparation). On the other hands, while in normal TA muscles a low percentage of type I fiber were sparsely distributed among type II fibers (Fig. 6C), in the few reinnervated muscles the type II and Type I fibers were remarkably grouped (Fig. 7).

These results of the histochemical analyses might suggest that the severely atrophic muscle fibers are

Figure 7. *Histochemistry of reinnervated muscle in bilateral sciatectomy of rat legs. A, Myofibrillar ATPase, pH 10.4; B, SDH activity. While in short term denervated TA muscles a low percentage of type I fiber are sparsely distributed among type II fibers (see the checker-board appearance of 1-month denervated muscle in Fig. 6 A-C), in the reinnervated muscles the two fiber types are clearly grouped.*

dying muscle fibers. On the other hands, the semi-thin section analysis of a severely atrophic muscle 9-month after sciatectomy (see Figure 8) suggested that the large majority of the original muscle fibers were still present and alive. At low magnification (Fig. 8A), few adipocytes were observed (grey round areas), while many severely atrophic muscle fibers and patent capillaries and small vessels were detected (Fig. 8B). At high magnification (Fig. 7C), the arrow points to a severely denervated myofiber void of any contractile sarcomeric proteins, but showing nuclear grouping. In longitudinally sectioned myofibers, these clumps of nuclei alternated with long stretches of amyofibrillar sarcoplasm [16, 23].

These features distinguish these "severely" atrophic muscle fibers from newly regenerated myofibers that are present at low rate, but continuously during long term denervation in all muscle we studied so far, human included [11, 12, 20]. Thus muscle fiber can survive to the absence of innervation much longer than generally expected, in particular they remain void of any sarcomeric contractile proteins at least up to one-year of denervation in the rat [6, 7]. This is a considerable time, when considering that the life-span of the rat is 2-3 years.

Interestingly, we recently shown that in long term denervated human muscles the presence of atrophy-resistant muscle fibers does not the result from residual innervation or re-innervation [4], and similar results has been obtained in a rabbit model of selective denervation of the TA, which undergoes atrophy as a result of one year denervation [1-3]. Since in the latter cases denervation was carried out by selectively interrupting the motor nerve branches to the ankle dorsiflexors in one hind limb of the rabbit, the extended period of muscle trophism-excitability of the denervated rabbit TA could be related to the passive movements that the denervated myofibers undergo in association to the voluntary contraction of the full leg. Since these
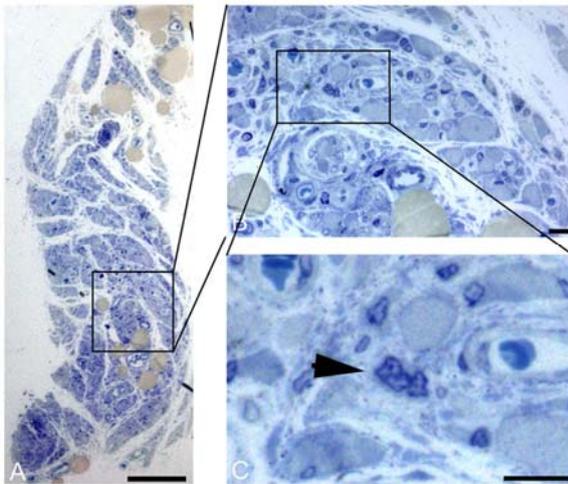
Figure 8*. Semi-thin section of 9-month denervated rat muscle. The three panels show increasing magnification of the same semi-thin section. Toluidine blue. Histochemical analyses seems to suggest that in the 1-year denervated muscle the small fibers are dying, but the semi-thin section of a severely atrophic muscle 9-month after sciatectomy (embedded for electron microscopy and stained with toluidine blue) demonstrates that the majority of the original muscle fibers are present. At low magnification (A), a few adipocytes are present (grey round areas) among numerous atrophic muscle fibers, and patent small vessels (B). At high magnification (Panel C), the arrow points to one of a severely atrophic myofiber, void of any contractile sarcomeric proteins, showing three grouped nuclei. In longitudinally sectioned myofibers, these clumps of nuclei alternate with long stretches of anucleated, amyofibrillar sarcoplasm [16, 23].*

criticisms in interpreting long term denervation muscle changes could be raised also in the case of rat long term hemidiaphragm denervation [9, 10], present results collected in bilaterally sciatectomized TA are very important, demonstrating that the long-term resistance to denervation atrophy is a constitutive characteristics of the skeletal muscle fibers, at least of the fast-twitch contraction type.

In a parallel work, we studied the progressive decay of excitability of rat Soleus to establish a correlation between electrophysiological behavior in single fibers *in vitro* and structural data in short- and long-term denervated muscles. The short-term denervated muscles, from 3 up to 13 weeks of denervation, were characterized by a drastic decrease of fiber diameter, alterations of the transverse-tubular network and a progressive disarrangement of the contractile and metabolic machineries. Functionally, there was a

parallel severe alteration of the passive properties of the sarcolemma and of L-type Ca2$^+$ current kinetics. In long-term denervated muscles, studied up to 1-year of denervation, the contractile machinery disarranged progressively up to a complete degeneration, whereas the above electrophysiological properties only showed further but small changes. Thus, in this parallel study we showed that even after one year of denervation, isolated muscle fibers retain a low resting membrane potential and that they are able to propagate an action potential, though the amount of applied current needed to excite these myofibers is much higher compared to normal muscle fibers [24]. Indeed, our findings clearly showed that the denervated muscle fibers maintain the resting membrane potential and the ability to propagate the action potential longer than they retain the myofibrillar apparatus. Taken together, all these studies in the rat demonstrate that the inability to contract of the long term denervated muscle, when subjected to a direct electrical stimulation, results from the loss of contractile machinery, possibly after it lost contact with the sarcolemma by the disruption of the excitation-contraction coupling apparatus, that is, of the T-tubules and the triads. On the other hands, 12 months after bilateral sciatectomy the majority of the original myofibers are present and able to propagate the action potentials along the sarcolemma.

Since persons affected by SCI may be treated with FES to maintain and/or improve muscle trophism/ function, the presence of excitable muscle fibers in long-term denervated muscle, could be extremely important for their treatment with FES. Of course, the pool of long term patients outnumbers new cases per year, the option suggested by our results (to start even long term after spinal cord injury, i.e., at a time at which mechanical muscle twitches could not be detected by direct electrical stimulation, either by surface or intramuscular electrodes) could strongly support the choice to start and the motivation to life-long perform FES exercise activity in these critical subjects.

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