

Long-Term Denervation in Humans Causes Degeneration of Both Contractile and Excitation-Contraction Coupling Apparatus, Which Is Reversible by Functional Electrical Stimulation (FES): A Role for Myofiber Regeneration?

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Abstract. Over the last 30 years there has been considerable interest in the use of functional electrical stimulation (FES) to restore movement to the limbs of paralyzed patients. Spinal cord injury causes a rapid loss in both muscle mass and contractile force. The atrophy is especially severe when the injury involves lower motoneurons because many months after spinal cord injury, atrophy is complicated by fibrosis and fat substitution. In this study we describe the effects of long-term lower motoneuron denervation of human muscle and present the structural results of muscle trained using FES. By means of an antibody for embryonic myosin, we demonstrate that many regenerative events continue to spontaneously occur in human long-term denervated and degenerated muscle (DDM). In addition, using electron microscopy, we describe i) the overall structure of fibers and myofibrils in long-term denervated and degenerated muscle, including the effects of FES, and ii) the structure and localization of calcium release units, or triads; the structures reputed to activate muscle contraction during excitation-contraction coupling (ECC). Both apparatus undergo disarrangement and re-organization following long-term denervation and FES, respectively. The poor excitability of human long-term DDM fibers, which extends to the first periods of FES training, may be explained in terms of the spatial disorder of the ECC apparatus. Its disorganization and re-organization following long-term denervation and FES, respectively, may play a key role in the parallel disarrangement and re-organization of the myofibrils that characterize denervation and FES training. The present structural studies demonstrate that the protocol used during FES training is effective in reverting long-term denervation atrophy and dystrophy. The mean fiber diameter in FES biopsies is 42.2 ± 14.8 SD ($p < 0.0001$ vs DDM 14.9 ± 6.0 SD); the mean percentile of myofiber area of the biopsy is 94.3 ± 5.7 SD ($p < 0.0001$ vs DDM 25.7 ± 23.7 SD); the mean percentile fat area is 2.1 ± 2.4 SD ($p < 0.001$ vs DDM 12.8 ± 12.1 SD); and the mean percentile connective tissue area is 3.6 ± 4.6 SD ($p < 0.001$ vs DDM 61.6 ± 20.1 SD). In DDM biopsies more than 50% of myofibers have diameter smaller than $10 \mu\text{m}$, while the FES-trained subjects have more than 50% of myofibers larger than $30 \mu\text{m}$. The recovery of muscle mass seems to be the result of both a size increase of the surviving fibers and the regeneration of new myofibers.

Key Words: Excitation-contraction coupling; Functional electrical stimulation; Human, long-term denervation; Lower motor neuron lesion; Myofiber regeneration; Spinal cord injury.

INTRODUCTION

Over the last 30 years there has been considerable interest in the use of functional electrical stimulation (FES)

to restore movement to the limbs of patients paralyzed by upper motor neuron lesions in the spinal cord (1–7). There is, however, another group of patients that, due to the marked atrophy of the denervated muscle, presents severe secondary medical problems of bone and skin and is therefore more difficult to treat. In these patients, the spinal cord injury (SCI) results in irreversible loss of the nerve supply to some or all of the muscles of the affected limbs (conus cauda lesion syndrome). The absence of functional motor nerve makes it more difficult for surface electrodes to recruit a sufficient population of myofibers needed to regain functional movements. Indeed, using clinically approved stimulation devices the long-term denervated degenerated muscle (DDM) is difficult to stimulate in subjects who have been injured for more than 6 months.

Despite these difficulties, pilot studies on FES of human long-term DDM have been published (2, 8, 9). Especially encouraging are some of our previous results (2), which have shown that electrical stimulation by long impulses can be used to restore muscle mass and force production even after long-lasting complete denervation of patients paralyzed by lower motor neuron lesions (10).

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TABLE 1
Intervals between SCI and Harvest of Muscle Biopsy, SCI and FES, and FES and Biopsy

Subject	Age/sex	Time intervals between:		
		SCI and muscle biopsy	SCI and FES	FES and biopsy
Den1	37/female	9-month denervation	—	—
Den2	20/male	9-month denervation	—	—
Den3	37/male	15-month denervation	—	—
Den4	45/female	8.0-year denervation	—	—
Den5	54/female	8.7-year denervation	—	—
Den6	33/male	19.0-year denervation	—	—
Den7	53/male	37.5-year denervation	—	—
FES1	50/male	4-year denervation	1.8-year	2.2-year FES
FES2	55/male	5-year denervation	2.9-year	2.2-year FES
FES3	47/male	3.6-year denervation	1.2-year	1.3-year FES
FES4	44/male	26-year denervation	23.3-year	2.8-year FES
FES5	54/male	4.3-year denervation	0.8-year	3.4-year FES
FES6	41/male	6.3-year denervation	2-year	4.3-year FES
FES7	35/male	13.6-year denervation	8.7-year	4.8-year FES
FES8	45/male	29.8-year denervation	24-year	5.8-year FES
FES9	34/male	7.5-year denervation	0.7-year	6.9-year FES
FES10	58/male	9.6-year denervation	1.8-year	7.8-year FES
FES11	29/male	10.6-year denervation	1.2-year	9.4-year FES

This clinical work strongly supports the idea that FES is a powerful tool for functional restoration of long-term DDM, a fact that has only been recently recognized. The main reason for the delay in use of FES in the restoration of muscle function was the lack of customized technology that must meet demands completely different from those of FES equipment for motor nerve stimulation (1). A stimulator for activation of DDM via surface electrodes must deliver biphasic long-duration impulses with a pulse width between 10 and 200 ms and amplitudes of up to ± 80 V, and ± 200 mA (11, 12). This stimulation protocol effectively elicits twitches in DDM and then (i.e. within a few months of training), tetanic contractions (10). Finally, muscle function in the lower extremities is restored sufficiently to allow for unsupported standing (13).

Whereas the mechanical and electrical properties of mammalian denervated muscle, together with the structural alterations of the contractile apparatus, are well described in the literature (14–22, 24, 25), not much is known about the effect of long-term denervation on the excitation-contraction coupling (ECC) apparatus. This information is much needed since the limitation of any rehabilitation protocol of DDM is its poor (if any) excitability.

The ECC apparatus is constituted by the close apposition of exterior (transverse tubules (TTs)) and interior membranes (sarcoplasmic reticulum (SR)) that form specialized junctions called Ca^{2+} release units (CRUs) or triads (26–30). Takekura and Kasuga (31) have described in rats the effect of early and late postnatal denervation on both TT and SR, however, the denervation period in their experiments was limited to 1 month.

In this paper, using light and electron microscopy, we present a detailed analysis of human long-term DDM before and after FES. Analyses of biopsies from both DDM- and FES-trained patients suggest that recovery of muscle mass following FES training is the product of both size increases in the surviving fibers and regeneration of new myofibers. In addition, we describe the effect of long-term denervation on the ECC apparatus. The disorganization of the triads together with the disarrangement of the contractile apparatus that follows long-term denervation could explain the unusually long impulses needed to activate the long-term DDM.

MATERIALS AND METHODS

Patient Characteristics

All 22 subjects (2 females and 20 males, ages 20–56 years) had experienced traumatic conus cauda lesion. Eleven were biopsied on both legs after long-term denervation only, while the remaining eleven conus cauda patients were biopsied after FES training. Two biopsies (one in each group) were not analyzed. None of the patients presented any other neurological disorders or muscle diseases. Table 1 shows the intervals between i) SCI and biopsy, ii) SCI and beginning of FES, and iii) beginning of FES and harvest of the biopsy.

Stimulation Device, Parameters, and Protocols

Functional response of denervated muscle to electrical stimulation depends on the stage of atrophy/degeneration of the post-denervation muscle, which in turn depends on the length of time between the denervation event and the start of stimulation treatment. In subjects who had been injured for 1 to 3 years, biphasic stimulation impulses with very long duration and high intensity were applied at the beginning of treatment.

During the several months of muscle training, a muscle twitch was only elicited by impulses lasting 150 to 200 ms with amplitudes up to ± 200 mA. The duration of these impulses was approximately 1,500 times longer than those used in patients with upper motor neuron lesions, in which the lower motor neurons were preserved. With an inter-pulse interval of about 500 ms, the resulting stimulation frequency was slightly less than 2 Hz (single twitches elicited every half second). There were no commercially available stimulators that can deliver such a high current intensity; therefore, a generator capable of long and high-strength stimuli was developed (11).

For surface stimulation, electrodes made of conductive silicone rubber (Fa. Schuhfried, Vienna, Austria) were applied directly to the skin using a wet sponge cloth at the beginning of training or, later, a gel was used when skin trophism improved. It is important to use flexible electrodes that keep contact to the uneven and moving skin surface. These electrodes guarantee homogeneous electrical current distribution and provide a homogeneous distribution of the electrical field in the stimulated thigh (10).

The first effect of the electrical training was the progressive increase in excitability of the muscle fibers. In order to achieve a higher stimulation frequency, the pulse duration and the inter-pulse intervals were shortened to 50 to 40 ms after 3 to 6 months of the electrical stimulation regimen in order to achieve a higher stimulation frequency. Based on inter-pulse intervals of 10 ms (Kern's Current), an impulse frequency between 17 and 20 Hz was achieved, resulting in fused tetanic muscle contractions (10).

Overall Progressive Regime of FES for Restitution of DDM

Phase 1: At least 9 months after SCI, patients commenced a training program, which, after appropriate instruction, they performed in their home environment. Anatomically shaped electrodes of large surface area were strapped to the anterior surface of the thighs in proximal and distal positions. Due to their reduced excitability, long-term denervated myofibers were stimulated by very long biphasic rectangular impulses to test "complete denervation" and to attain single twitch contractions of thigh muscles. Twitch contractions were elicited by biphasic rectangular current pulses lasting 150 to 200 ms and up to ± 200 mA amplitude, representing an impulse energy up to 4 Joules, to recruit fibers throughout the quadriceps femoris muscles. Training was initiated at 2 Hz, delivered for 15 min/day (series of 4 s "on," 2 s "off"), 5 days/week. During the first/second month, the progressively increasing muscle excitability permitted an increase of 2 Hz stimulation to series of 5 s "on," 1 s "off," 3 to 5 min stimulation / 1 to 2 min rest, twice a day.

Phase 2: As training proceeded, excitability of the muscle continued to increase, and after 3 to 4 months, training pulses of shorter duration (80 to 100 ms) were used.

Phase 3: Burst stimulation for denervated muscles, Kern's Current. After 4 to 6 months, the protocol was changed to a tetanic pattern (consisting of 40 ms pulse and 10 ms pause) delivered at 20 Hz for 2 s "on," 2 s "off," 3 to 5 min stimulation / 1 min rest (i.e. 3 to 5 times a session), twice a day, 5 days/week.

Phase 4: The number of repeats was increased to 15 to 30 repetitions per set, 2 min rest between each set, 6 to 8 sets, twice a day (force/endurance stimulation). Finally, "force training" sessions were introduced by tetanic contractions against 70% to 80% of maximum load, 8 to 12 repetitions, 4 to 6 sets with 2 min rest, once a day. At first, the leg contracted to full knee extension without any ankle weight, and later with ankle weight of up to 5 kg.

Analyses of Human Muscle Biopsy

After a small skin biopsy was taken (6-mm-diameter), needle muscle biopsies (UNIMED, Lausanne, Switzerland, 5 \times 100 mm article no. 23.601) were harvested from both the right and left *vastus lateralis* muscle. The resulting specimens were then prepared for light and electron microscopy.

Hematoxylin and Eosin, Oil red O, and Mallory Trichrome Stains

Cryosections (10- μ m-thick) of frozen biopsies were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Milan, Italy), oil red O stain (Merck, Darmstadt, Germany) for lipid displaying, and the trichrome stain (Mallory, Bio-Optica, Milan, Italy) for demonstration of distribution of collagen, using conventional techniques.

Immunohistochemistry

Cryo-sections were labeled with anti-MHCemb antibody (NCL-MHCd diluted 1:20, from Novocastra, Newcastle-upon-Tyne, UK) for 1 h at room temperature. The slides were then washed twice with TBS (5 min each) and incubated with FITC-conjugated anti-mouse Ig F-2266 diluted 1:200 (Sigma-Aldrich) for 1 h at room temperature. This was followed by a second 5-min washing of the slides with TBS and nuclei counter-staining by Hoechst 33258 (from Sigma-Aldrich, Milan, Italy). In the negative controls the primary antibody was omitted.

Electron Microscopy and Size Distribution Spectrum of Total Myofibers

The biopsy samples were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2 h on ice followed by a buffer rinse and 1 h fixation in 1% osmium tetroxide. The specimens were dehydrated in a graded series of ethanol solutions and embedded in epoxy resin. Semi-thin sections (1 μ m) were stained using conventional techniques with toluidine blue (Merck) and used to plot fiber size distribution. The minimum transverse diameter of each myofiber was measured against a reference ruler. The myofibers were grouped and relative percentages plotted in 10- μ m steps (32).

Ultrathin sections (~ 40 nm) were cut in Leica Ultracut R (Leica Microsystems, Vienna, Austria) using a Diatome diamond knife (Diatome Ltd., Biel, Switzerland) and stained in 4% uranyl acetate and lead citrate. Sections were examined using a Philips M 301 or a FP 505 Morgagni Series 268D electron microscope (Philips Int., Rome, Italy), equipped with Megaview III digital camera and Soft Imaging System (Philips Int.).

Morphometric Analysis

Images were acquired using a Zeiss microscope connected to a Leica DC 300F camera at low magnitude under the same

conditions used to acquire a reference ruler (Leica Microsystems). Morphometric analysis was performed with Scion Image for Windows version Beta 4.0.2 (Scion Corp, Frederick, MD) free software downloaded from the web site: www.scioncorp.com. The figures were mounted and labeled using Adobe Photoshop version 6.0 (Adobe System, Milan, Italy).

RESULTS

Atrophy and Dystrophy of Human Long-Term Denervated Muscle

Denervation of skeletal muscle causes rapid loss in both mass and contractile force, which is followed long-term by other severe structural changes (lipodystrophy). Figure 1 shows 3 images of a representative 8.7-year denervated human muscle after staining with H&E (Fig. 1A), oil red O stain for lipid distribution (Fig. 1B), and the Mallory trichrome stain for outlining the distribution of collagen (Fig. 1C). This is a typical long-term denervated human muscle in which atrophic and severely atrophic myofibers have been substituted by adipocytes and collagen.

Table 2 shows the relative percentages of myofiber area, adipocytes, and collagen in the biopsies from long-term denervated subjects. Adipocytes and collagen, which were scarce in humans for up to a year of lower motoneuron denervation, increased thereafter in spite of the fact that the mean fiber diameter of the measurable myofibers was less than 20 μm from 0.7 to 19.0 years of denervation (mean diameter 14.9 ± 6.0 in 20 biopsies). Adipocytes began to accumulate 1 to 2 years after denervation, constituting up to 30% of muscle biopsy area after 8 to 10 years of continued denervation (denervated, degenerated muscle, DDM).

Severely Atrophic Myofibers: Nuclear Clumping and Disorganization of the Myofibrillar Components

Long-term denervated fibers underwent a progressive disorganization of both the contractile and ECC coupling apparatus. Figure 2 shows severely atrophic myofibers of muscle (denervated for 15 months) that displayed several peculiar features of long-term denervation. One characteristic was the presence of clumps of myonuclei (Fig. 2A, B) alternating with long stretches of anucleated sarcoplasm in which myofibrils were absent. Figures 2C shows a cross-section of a severely atrophic myofiber displaying an unusual triangular shape. In addition to the angulated aspect, the presence of overabundant folded layers of the original basal lamina (arrow) was also a peculiar feature of severe atrophy. The majority of the myofibers (either round or angulated) with central or peripheral clumps of myonuclei were also often surrounded by increased numbers of collagen sheets and exhibited either a disorganization or a full loss of myofibrils. These myofibers also tended to have lipid bodies, folds of the basement membrane, and sparse mitochondria.

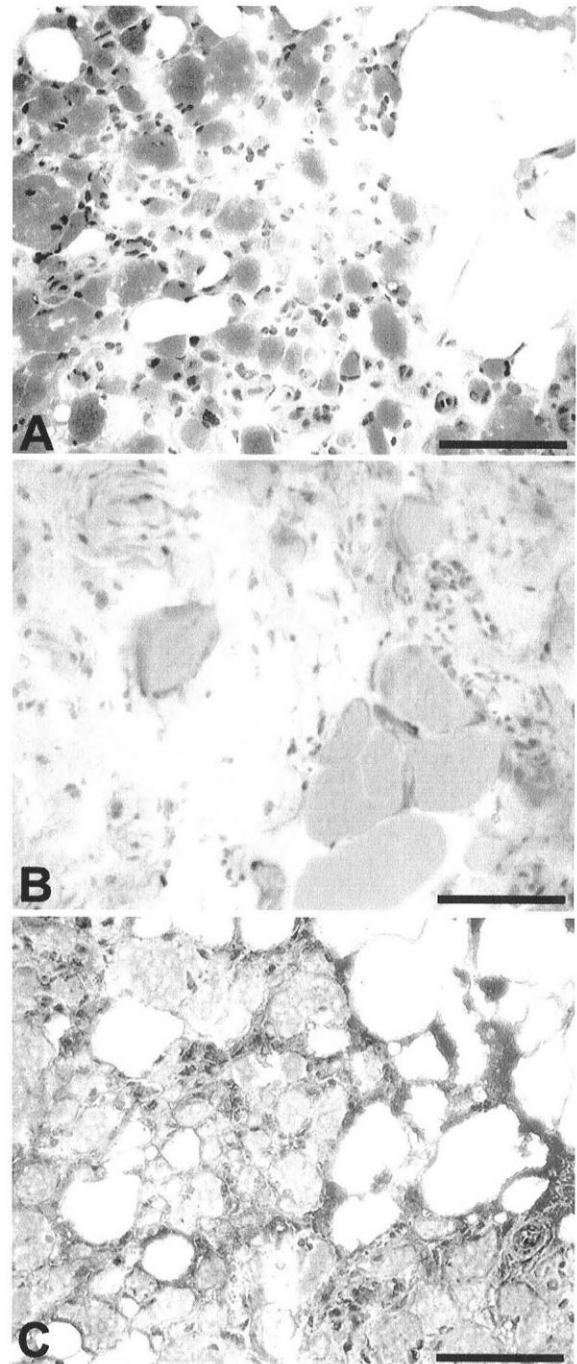


Fig. 1. Human long-term denervated quadriceps femoris. Light microscopy. **A:** H&E stain. **B:** Oil red O stain for lipid distribution. **C:** The Mallory trichrome stain for demonstration of distribution of collagen. Scale bar: 100 μm .

Morphological Changes of Contractile and ECC Apparatus following Long-Term Denervation

Another characteristic of long-term denervated myofibers was the almost complete disorganization and disarrangement of the contractile apparatus (Fig. 2), which usually began at the periphery of the fibers (Fig. 3). Figure 3A shows a longitudinal section of a large size fiber

TABLE 2
Atrophy and Dystrophy of Human Long-Term Denervated Muscle

Time		Minimum diameter μm	Percent Area covered by			
Denervation	FES		Myofibers	Adipocytes	Collagen	Loose connective tissue
0.7	—	18.1 ± 9.9	49.3	2.7	19.5	28.5
0.7	—	18.6 ± 7.4	52.6	2.5	6.3	38.6
0.7	—	20.7 ± 16.8	64.0	6.3	2.3	27.4
0.7	—	21.1 ± 13.2	75.1	5.2	3.1	16.6
1.2	—	16.2 ± 10.0	72.4	3.1	1.4	23.1
2.9	—	15.9 ± 10.3	26.4	12.6	54.2	6.8
2.9	—	22.3 ± 18.0	11.6	3.1	18.2	67.1
3.3	—	8.12 ± 4.5	5.4	44.8	19.1	30.7
3.5	—	20.8 ± 19.4	16.0	14.8	38.9	30.3
3.5	—	20.4 ± 14.0	12.1	6.2	9.0	72.6
4.0	—	9.0 ± 10.5	14.4	9.2	8.1	68.2
4.0	—	8.8 ± 11.0	13.9	16.7	14.2	55.2
7.5	—	7.7 ± 13.9	10.8	3.6	15.1	70.6
7.5	—	7.6 ± 6.9	14.5	6.3	7.0	72.2
8.6	—	22.2 ± 10.5	18.1	10.4	4.2	67.3
8.6	—	8.4 ± 3.7	2.0	14.6	3.3	80.1
8.7	—	4.5 ± 1.5	1.2	30.5	65.3	3.0
8.7	—	7.7 ± 3.7	2.4	35.0	50.0	12.6
19.0	—	15.3 ± 15.3	28.4	2.6	15.0	54.0
19.0	—	21.6 ± 25.3	22.5	25.5	13.5	38.5
Mean		14.9	25.7	12.8	18.4	43.2
\pm SD		6.0	23.7	12.1	18.7	24.8

in which the contractile apparatus was completely missing at the fiber periphery (between large arrows) and quite disorganized in the fiber interior (arrows pointing at the Z lines). In muscle that had been denervated for longer times, the sarcomeric apparatus became much more disorganized (data not shown): in those patients M lines were sometimes found but A bands tended to be randomly oriented while no evidence of Z lines was found. Also, the spatial and structural arrangement of the ECC apparatus changed dramatically in long-term denervated muscle. As for the contractile apparatus, the ECC apparatus began degenerating from the fiber periphery. In fact, triads were generally completely missing in the cortical area of degenerating fibers, while junctions were rarely seen in the fiber interior, where a modest amount of myofiber structure could be still present (Fig. 3A, arrows pointing at Z lines). An association of triads with the myofibril A-I junction was rare, while the 4 to 1 ratio was never seen. Furthermore, triads in denervated muscle never appeared normal. In order to help further elucidate the differences between triads in normal and denervated muscle, Figure 3B contains a normal triad, formed by the close apposition of 2 SR vesicles to a central TT, in normally innervated muscle: the TT profile was flat, had a regular width, and the junctional gap between the 2 membranes contained ryanodine receptors (RyRs), or feet (indicated by the arrows). Feet were clearly visible as large electron densities that spanned the gap between the 2 membranes and were usually arranged in 2 rows.

In DDM the residual ECC apparatus showed the following features and alterations: i) changes in the dispositions and orientation; ii) changes in both the TT and SR profiles (Fig. 3C, D); iii) the appearance of dyspedic junctions (i.e. junctions lacking feet, the cytoplasmic domain of RyRs representing the Ca^{2+} release channel of the SR (Fig. 3C)); and iv) the appearance of pentads (i.e. close apposition of 2 TTs with 3 elements of the SR terminal cisternae (not shown)).

In denervated muscle, a triad with normal features such as the one described in Figure 3B was never found. Usually the appearance of the triads was extremely different (compare Fig. 3B with 3C and 3D): both the SR and TT profiles were altered and also the molecular complement seems to be defective since RyRs feet were lacking.

Triads lacking feet have been defined as dyspedic in $\text{RyR1}^{-/-}$ mice (33–35) and can be identified based on 2 peculiar features: lack of feet in the junctional gap and a reduced junctional gap between TT and SR membranes. Dyspedic triads were unable to perform ECC since the RyR Ca^{2+} release channels (the feet) were missing (33–39). While, we cannot exclude the possibility that few RyRs were still present in these junctions (Fig. 3C, arrow), it was clear that normal arrays of RyRs were missing from triads of denervated muscle. This observation suggests that not only the frequency of triads in denervated fibers was very low, but also that the few triads that were present were functionally impaired. In short, a

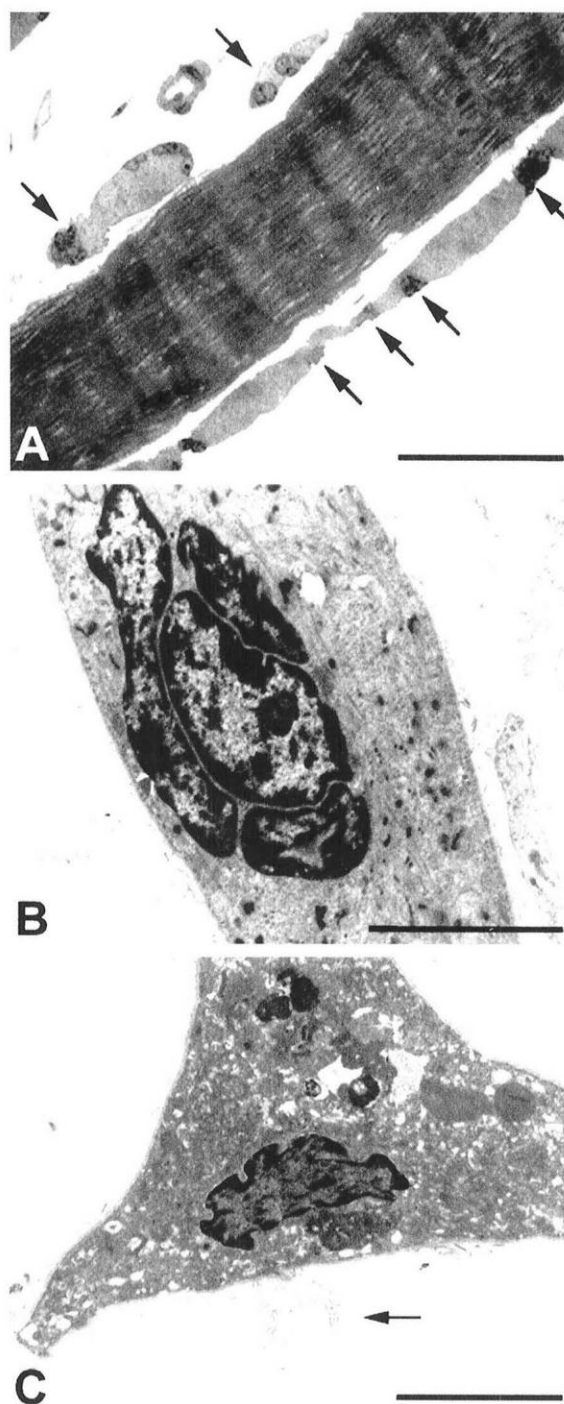


Fig. 2. Ultrastructural feature of severe atrophy in long-term denervated myofibers. **A:** Longitudinal semi-thin section of severely atrophic myofibers: arrows point to clumps of myonuclei, which alternate with long stretches of anucleated, myofibrillar sarcoplasm. **B:** Electron micrograph of a longitudinal very small myofiber showing a clump of 4 myonuclei: myofibrillar structure and mitochondria are scarce or absent. **C:** Cross-sections of a very small myofiber: the angulated profile and the overabundant folded layers of the original basal lamina (arrow) are also peculiar features of severe atrophy. Scale bars: A = 50 μ m; B, C = 10 μ m.

functionally ECC apparatus was practically missing in the human long-term denervated muscle.

Myofibers Regeneration in Long-Term Denervated Muscle

In long-term denervated human muscles, contrary to the general expectation, myofiber regeneration was frequently observed, even if at a low rate. In Figure 4A, B, transverse sections of long-term denervated human muscle biopsies are shown. The round, green areas represent cross-sections of myofibers positive for the anti-MHCemb antibody. These were newly regenerated myofibers that had developed during the last couple of weeks before biopsy. From 9-month to 19.0-year denervation, the recently regenerated myofibers represented $1.1\% \pm 1.2\%$ of total myofiber population, with any time-dependent variability. Electron microscopy confirmed the presence of myotubes in long-term denervated human muscle. Figure 4C shows that a double-layer of basement membrane (black and white arrowheads) delimited a very early regenerating myotube that contained a few thick myofilaments. The basal lamina double-layered fibers were rare, supporting the fact that anti-MHCemb antibodies stain about 1% of the myofibers in long-term denervated and degenerated human muscles.

Effects of the Electrostimulation Training (FES) on Long-Term Denervated Muscle

Figure 5 shows the effects of 4.3-year electrostimulation training after 2-year DDM. Serial cryosections are stained with H&E (Fig. 5A), oil red O stain for lipid distribution (Fig. 5B), and the Mallory trichrome stain for demonstration of distribution of collagen (Fig. 5C). The biopsy shows round myofibers of heterogeneous size, but with large fibers clearly prevailing (Fig. 5A). Adipocytes were almost absent (Fig. 5B) and collagen was present in normal amounts (Fig. 5C). Table 3 confirms that in all the studied biopsies from FES-trained subjects, adipocytes were absent and collagen was present in normal amounts. Note that a residual population of small myofibers was present in the electro-stimulated muscles, as also shown by fiber size spectra (Fig. 6). Figure 4D and 4E confirms that a few of them, which stained positive by anti-MHCemb antibodies, were the result of recent regeneration events.

The mean value of the minimum diameter in the FES-trained DDM is $42.2 \pm 14.8 \mu\text{m}$ (SD). The data compared well with the values of $54.0 \pm 7.2 \mu\text{m}$ for normal muscle in sedentary subjects and $14.9 \pm 6.0 \mu\text{m}$ (SD) for long-term denervated human muscles, respectively. In Figure 6, the cumulative myofiber spectrum of long-term DDM was compared to that after FES. Approximately 50% of myofibers have diameters smaller than 10 μm in DDM biopsies, while the FES-trained subjects have more than 50% of myofibers larger than 30 μm . Some FES

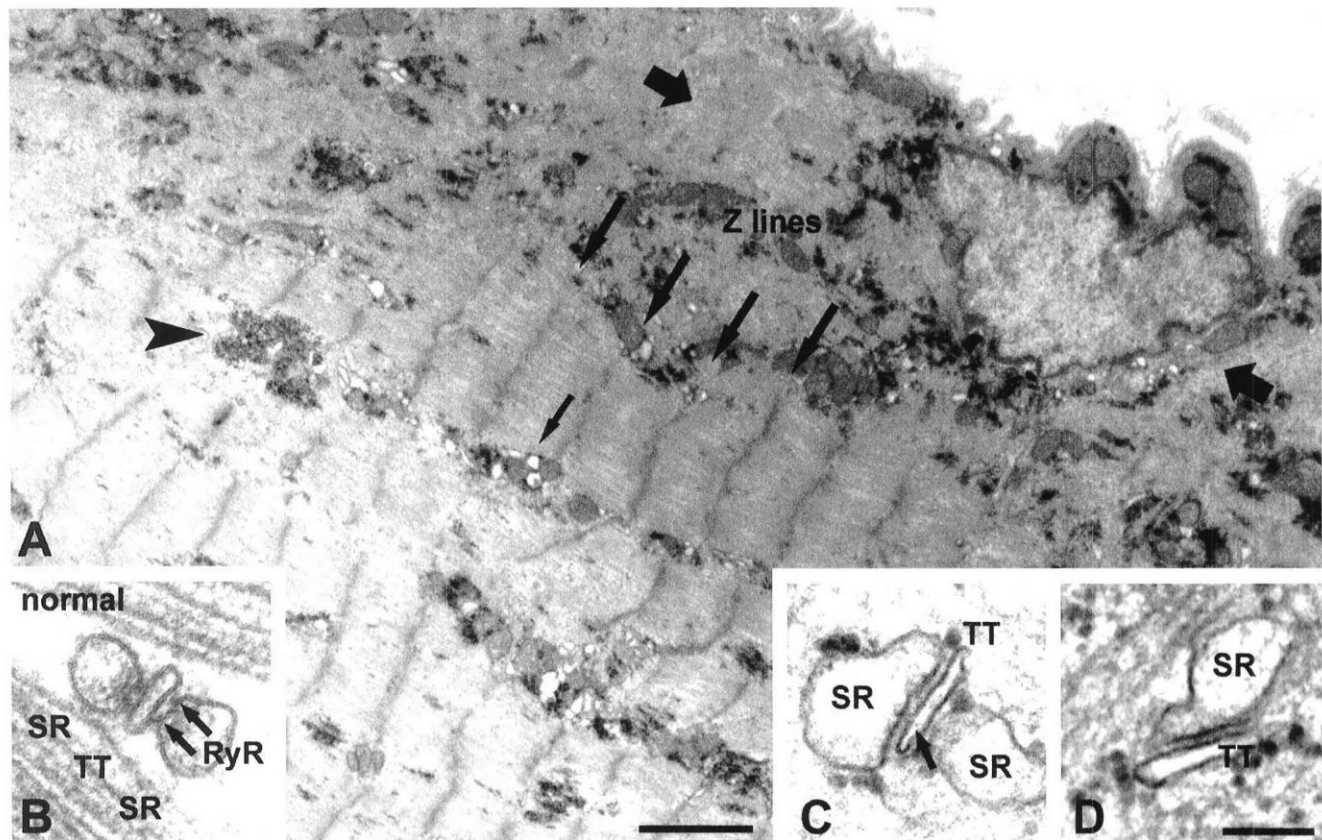


Fig. 3. Ultrastructural feature of ECC apparatus in long-term denervated myofibers: contractile and ECC apparatus. **A:** Muscle fiber displaying several peculiar features of long-term denervation: i) disarrangement of the contractile apparatus, which is usually more pronounced at the periphery of the fibers (between large black arrows); ii) large aggregates of glycogen granules (arrowheads). The remaining sarcomeric structure of myofibrils is still visible and indicated by arrows pointing at the Z lines. **B:** A triad in a normally innervated muscle: the TT profile is flat, has a regular width and forms 2 junctions, one on each side, with the 2 terminal cisternae of the SR. The junctional gap between the 2 membranes contains RyRs (arrows). **C, D:** Following long-term denervation the frequency of ECC units decreased drastically and the morphology of the few left junctions also changes dramatically: the TT and SR profiles are very variable with many junctions appearing to be dyspedic (i.e. they lack RyRs). Small densities are occasionally visible between the TT and SR membrane (**C**, arrow); these densities are usually randomly organized and smaller than the density representing RyRs described in normal junctions (**B**). Scale bars: **A** = 2.0 μm ; **B–D** = 0.1 μm .

myofibers appeared hypertrophic (diameter larger than 80 μm).

Table 3 shows that the mean percentile myofiber area in FES biopsies was 94.3 ± 5.7 SD ($p < 0.0001$ vs DDM 59.2 ± 37.8 SD), mean percentile fat area 2.1 ± 2.4 SD ($p < 0.001$ vs DDM 12.8 ± 12.1 SD), and mean percentile interstitial tissue area 3.6 ± 4.6 SD ($p < 0.001$ vs DDM 61.6 ± 20.1 SD). The mean percentile of collagen area in long-term denervated muscles was 18.4 ± 18.7 , while it was not easily recognizable in FES biopsies. These results were well within normal range and indicated that recovery of muscle trophism was indeed taking place.

Ultrastructure of Electrostimulated Muscle: Restoration of both Contractile and ECC Apparatus by FES Training

The effects of FES on the ultrastructure of long-term DDM were also striking. Figure 7 shows a fiber from a

patient that from 1.3 years after SCI was trained for 2.4 years with FES (3.7-year denervation at biopsy harvesting). After FES training, several features resembling those seen in normal muscle fibers were restored: i) myofibrils were reassembled and showed a normal complement of thin and thick filaments and, consequently, a normal assembly of I and A bands; ii) Z and M lines (arrows) of adjacent myofibrils were quite well aligned with one another; iii) ECC apparatus re-associated to the A-I junction of the sarcomere (see triads pointed by arrows); iv) triads, formed by 2 SR vesicle apposed to a central TT, now presented a normal profile (compare Fig. 7B, showing a junction in an FES-trained fiber with Fig. 3B, showing junction in normal human muscle); v) triad frequency was restored so that almost every sarcomere had 2 triads on each side (Fig. 7B, arrows); and vi) triads contained RyRs, the SR Ca^{2+} release channels, 2 on each side of the tubule as in normal skeletal muscle junctions (compare Fig. 7B with Fig. 3B).

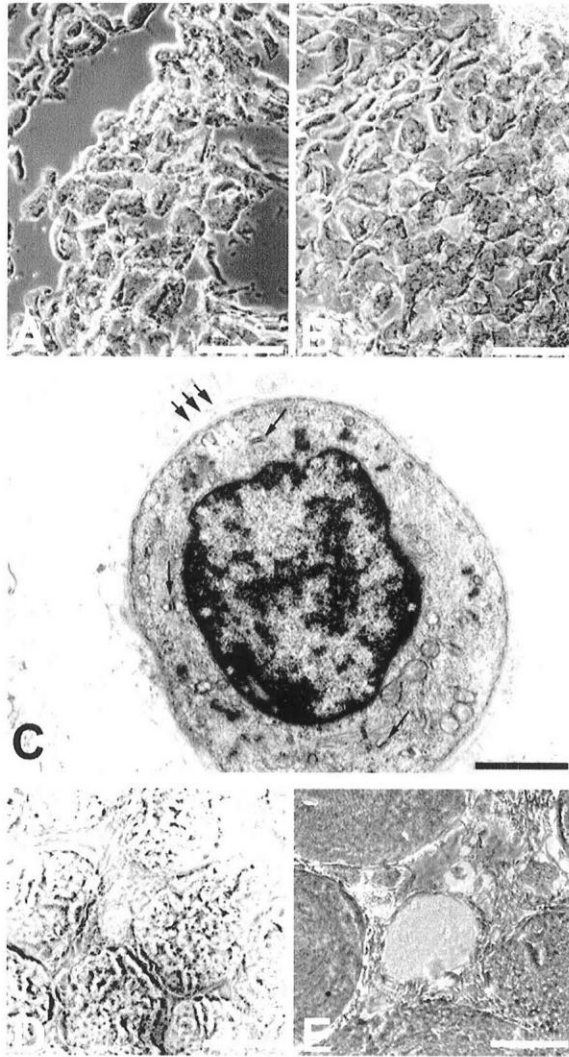


Fig. 4. Regenerative events in long-term denervated muscle with and without FES: immunohistochemistry and electron microscopy. **A:** 0.7-year denervation and **(B)** 8.7-year denervation. Early regenerated myofibers stained with antibody to embryonic myosin are in green. Double exposure anti-MHCemb/Hoechst 33258 shows that some blue nuclei are centrally located. **C:** Electron micrograph of a regenerating myotube in long-term denervated muscle surrounded by 2 layers of basal lamina: black and white arrows indicate old and new basal lamina, respectively. The small, round fiber presents a large central nucleus, a few thick filaments (white arrowheads), mitochondria, and well-developed triads (thin black arrows). **D:** 2-year denervation followed by 4.3-year FES training and **(E)** 23.3-year denervation followed by 2.7-year FES training. Early regenerated myofibers stained with antibody anti-embryonic myosin are in green. Double exposure anti-MHCemb/Hoechst 33258 shows that some blue nuclei are centrally located. Note that the myofibers are much larger than in denervated muscles seen in panels **(A)** and **(B)**. Scale bars: A, B = 100 μ m; C = 2.0 μ m; D, E = 50 μ m.

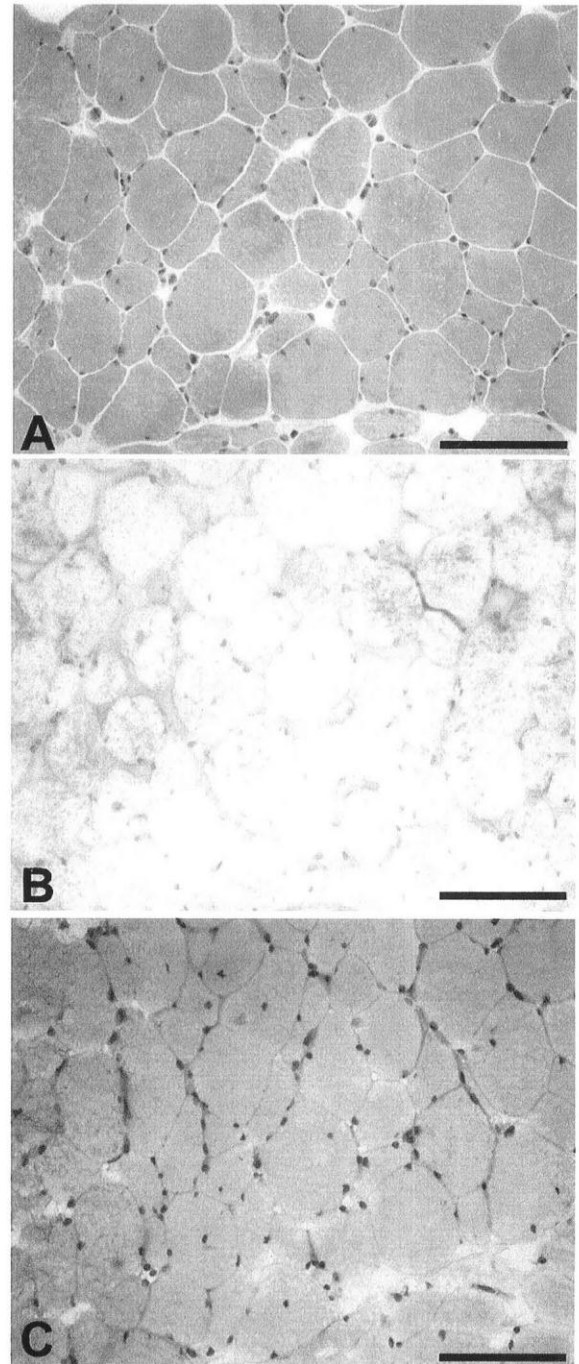


Fig. 5. Effects of FES training on histology of human long-term denervated quadriceps femoris. Light microscopy. **A:** H&E stain. **B:** Oil red O stain for lipid distribution. **C:** The Mallory trichrome stain for demonstration of distribution of collagen. The biopsy shows many large round fibers even if a residual population of small myofibers is still present among the large ones. The effect of FES training on long-term denervated muscle is striking: compare Figure 5 with Figure 1. Adipocytes are absent and collagen is of almost normal appearance (Table 3). Scale bar: 100 μ m.

TABLE 3
Functional Electrical Stimulation of Long-Term Denervated Human Muscle: Reversion of Dystrophic Changes

Time		Minimum diameter μm	Percent area covered by		
Denervation	FES		Myofibers	Adipocytes	Collagen and loose connective tissue
1.8 +	2.2	43.3 \pm 12.7	95.1	2.7	2.2
1.8 +	2.2	35.8 \pm 17.0	96.8	1.6	1.6
2.9 +	2.2	31.4 \pm 16.7	98.5	1.1	0.4
2.9 +	2.2	34.7 \pm 16.1	97.6	1.0	1.4
1.3 +	2.4	30.7 \pm 23.7	97.5	1.3	1.2
1.3 +	2.4	26.7 \pm 22.9	96.9	3.0	0.1
23.0 +	2.8	36.7 \pm 42.3	79.9	11.0	9.1
23.0 +	2.8	24.9 \pm 29.0	94.3	1.8	3.9
0.9 +	3.4	36.3 \pm 29.1	93.3	3.7	3.0
0.9 +	3.4	25.4 \pm 27.0	95.7	3.1	1.2
2.0 +	4.3	45.7 \pm 18.3	97.5	0.2	2.3
2.0 +	4.3	46.5 \pm 14.2	98.0	0.5	1.5
8.6 +	4.8	58.2 \pm 24.1	96.3	1.2	2.5
24.0 +	5.8	69.2 \pm 30.5	79.3	1.9	18.8
24.0 +	5.8	81.8 \pm 31.4	87.1	1.2	11.7
0.4 +	6.9	32.6 \pm 16.0	95.2	1.8	3.0
1.8 +	7.8	57.8 \pm 25.1	99.0	0.5	0.5
1.8 +	7.8	39.5 \pm 23.0	92.9	3.9	3.2
1.2 +	9.4	48.1 \pm 12.2	97.7	0.5	1.8
1.2 +	9.4	39.2 \pm 18.0	97.4	0.5	2.1
Mean		42.2	94.3	2.1	3.6
\pm SD		14.8	5.7	2.4	4.6
p vs DDM		<0.0001	<0.0001	<0.001	<0.001

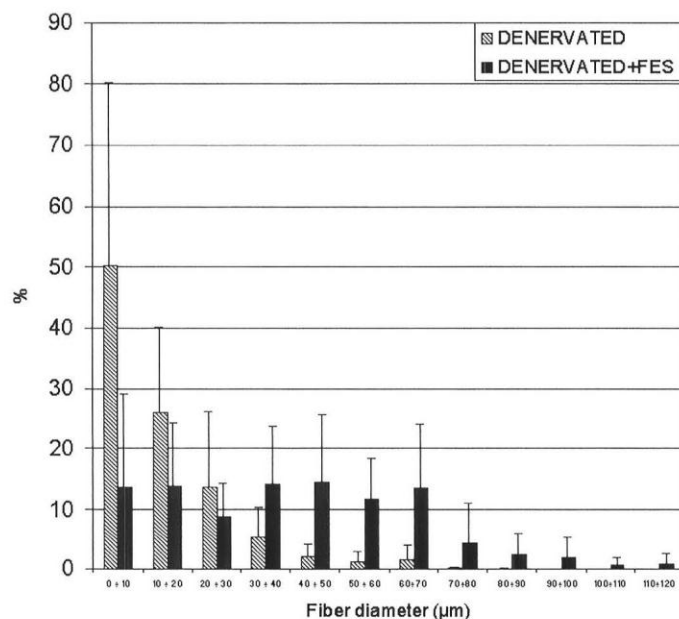


Fig. 6. Fiber size spectrum of long-term denervated muscle vs FES training. The cumulative value of fiber sizes in 20 muscle biopsies after either long-term denervation (grey bars) or FES treatment (black bars). More than 50% of myofibers have diameters smaller than 10 μm in DDM biopsies, while the FES-trained subjects have more than 50% of myofibers larger than 30 μm .

The presence of RyRs and the normal profile of triads suggested that functioning of these structures was restored. Since triads represent the structures that allow the release of Ca^{2+} from the SR in response to electrical stimulation, their structural restoration was an extremely important result of FES training.

Glycogen granules (Fig. 7, arrowheads), which are often intermitted between filaments, were the only abnormal features of these FES restored muscle fibers. These glycogen granules probably represent what was left of the large amount of glycogen that was found in denervated fibers (Fig. 3, arrowheads). The fact that the daily electrostimulation sessions were relatively short (lasting only 15 to 30 min) may explain why the FES denervated myofibers still lacked mitochondria.

DISCUSSION

The main finding of this work is that the degeneration of human muscle fibers that follows long-term denervation can be reversed using dedicated FES training (I, 11–13). More than 50% of myofibers have diameters smaller than 10 μm in long-term denervated biopsies, whereas the FES-trained subjects had more than 50% of myofibers larger than 30 μm (Fig. 6). Furthermore, we showed that 1) overall disarrangement of the contractile apparatus that follows human long-term denervation was accompanied by the nuclear changes previously recognized in rat experiments, i.e. the appearance of clumps of myonuclei

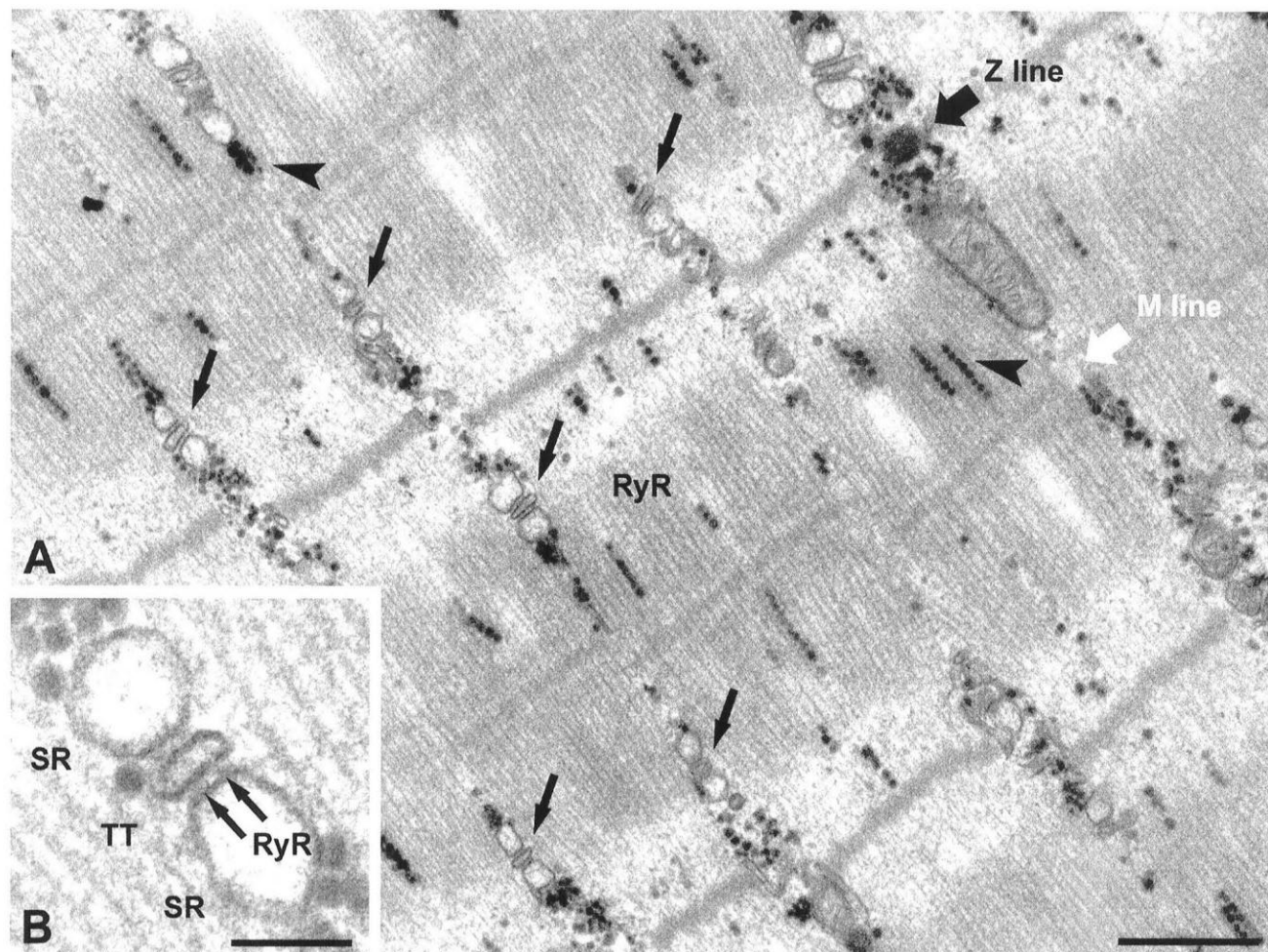


Fig. 7. Effects of FES on the ultrastructure of long-term DDM. **A:** After FES, several fiber features that resemble normal muscle fibers are restored: i) myofibrils are reassembled and show a normal complement of thin and thick filaments and, consequently, a normal assembly of I and A bands; ii) Z and M lines (large black and white arrows, respectively) of adjacent myofibrils are quite well aligned with one another; iii) ECC apparatus re-associate to the A-I junction of the sarcomere (small black arrows); iv) the frequency of triads, or CRUs, is also restored in a way that almost every sarcomere has 2 triads on each side (small black arrows). **B:** Triads now exhibit a normal profile (compare with Fig. 3B, showing a junction in normal human muscle). The triads in the long-term DDM now contain RyRs (arrows), the Ca^{2+} release channel of the SR, 2 rows on each side of the tubule, as in normal skeletal muscle junctions. Scale bars: A = 0.5 μm ; B = 0.1 μm .

alternating with long stretches of anucleated sarcoplasm (21). 2) Approximately 1% of the myofibers stained positive by the antibody to MHCemb, indicating that they had regenerated during the last few weeks before biopsy (40–43). 3) Electron microscopy analysis of DDM biopsies confirmed the immunocytochemistry findings: double-layered basement membranes delimited some myotubes and regenerated myofibers. During muscle regeneration, transient expression of laminin isoforms associated with early myogenesis was established (44, 45). 4) In addition, electron microscopy showed not only the disorganization of contractile apparatus, but also the disarrangement of the ECC apparatus, thus explaining, in terms of poor Ca^{2+} handling capacity, the low excitability of long-term denervated human muscle.

These observations are novel and never before published, most likely because long-term effects of denervation in human muscles have attracted very limited attention, since there is a general belief (mainly based on electrophysiological analyses) that all myofibers disappear within 12 months of SCI (14–17).

On the other hand, late denervation has been widely studied in animal models. In rats it has been shown that for the first 7 months after denervation, myofibers exhibit a net loss of nuclear domains followed by nuclear groupings (21). In rodents, permanent lower motor neuron denervation is accompanied by a continuous production of new myofibers, even after 1-year permanent denervation in both rat hemi-diaphragm and leg muscles (18–23). Furthermore, in permanent absence of motor innervation,

a completely regenerated muscle responds to repeated myotoxic injuries with muscle regeneration (21).

Embryonic myosin is expressed in myotubes and young myofibers and represents the soundest molecular marker of early myogenic events in both developing and regenerating adult muscle (40–43). If not re-innervated, the regenerating myofibers (after expressing adult type fast myosin) undergo atrophy and degeneration (19). In the present work we have shown that in FES-trained human muscles, some regenerated myofibers (i.e. MHCemb-positive fibers) are larger than those found in DDM. The extent of the contribution of regenerated myofibers in explaining the effects of FES training remains to be determined.

While it was common knowledge that denervation causes disarrangement of the myofibrils, not much was known until now about the effect of long-term denervation on the ECC apparatus. Denervation of the tibialis anterior in rats causes several obvious structural changes in the disposition of the triads in relation to myofiber striation (31). As little as 2 to 3 weeks of postnatal denervation causes a striking increase in the longitudinal segments of the TT network and a remarkable change in the disposition of the triads. Similar changes have been described in developing or mutant muscle (33–39). In the present work, we have extended the studies on alterations of the ECC apparatus in human fibers denervated for very long periods of time: the junctions between TT and SR exhibit a drastic decrease in number. In addition, the junctions lose the Ca^{2+} release channels, suggesting that there is also a functional impairment. As to the time course of changes, no final conclusions can be drawn on our results since we studied only 20 biopsies from 11 subjects at only 5 time-points (i.e. 1-, 3-, 4-, 8-, and 20-year denervation). The trend suggests that the 1:4 ratio (1 “defective” triad per Z line over the expected 4 normal triads) is present from the earliest time-point studied by electron microscopy (0.7 year). This lasted as long as studied (7.5 years), at least in those myofibers that have sarcomeres. The SR capacity to release Ca^{2+} is altered, providing a possible explanation for the needs of such long impulse duration and high intensity to be activated.

The very low excitability of long-term denervated human muscle is possibly the consequence of the segregation of the SR from the sarcolemma. The last conclusion is inferred from a pilot rat experiment. In vitro testing of the isolated muscles showed that the 8-month denervated rat muscle, which does not contract by direct electrostimulation, shortens when exposed to caffeine, a powerful releaser of Ca^{2+} from SR. This observation strongly complements the results of experiments designed to test in the rat model the residual functional activity of muscle membranes (sarcolemma and SR) in the long-term denervated muscles. Indeed, the RyR capacity, measured as 3H-ryanodine binding in purified SR membranes, was

drastically altered and capable of significantly modifying the activity of Ca-pumps. Conversely, sarcolemma fractions purified from the same muscles showed a greater resistance to the effect of long-term denervation as indicated by both the Na-K ATPase activity and the presence of voltage-dependent Ca-channels (measured as labeled DHPR binding), which were not statistically different with respect to the controls (unpublished observations).

Abnormalities up to the complete absence of organized myofibrillar structures are the obvious consequences of long-term denervation and absence of contractility. However, DDM human muscle can still respond with single twitches if very high electrical stimulation is applied (1, 2, 10–13). The most important finding of our work is that the FES protocol used during training of our patients reverses the massive structural alteration of both contractile and ECC apparatus in human DDM fibers (Figs. 5–7). Electrostimulation effectively elicited sustained muscle contractions, and CT scan measurements taken during the first few years of functional electrical stimulation revealed an increase in the tight muscle cross-sectional area and tissue density (10, 13).

Electron microscopy analysis shows that the structural effect of FES training on DDM fibers is striking: myofibrils that were completely disarranged without training (Fig. 3) appear structurally normal after patient training (Fig. 7). The ECC apparatus is reorganized and its association with the myofibrils appears to be normal. This coordinated reorganization provides the structural basis for the improved capability of the FES-trained muscle to respond to electrical impulses. Furthermore, they provide the structural basis for the recovered function in the lower extremities of the treated patients that allows unsupported standing (13).

Over the next years, using experiments in animals and clinical research, the goals of the EU-supported RISE project will be to identify safer stimulation protocols needed to induce early effects with, hopefully, a reduced stimulation burden for the patients. If all myofibers in the thigh could consistently attain endurance, the increased muscle mass and fatigue resistance will “rise” patients and their quality of life.

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