Two years of FES training improves muscle fibers of thigh muscles in long-term thoracic level-complete spinal cord injury

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Abstract
To investigate the structural relationships and the progression of functional electrical stimulation (FES)-induced regression of muscle atrophy up to 20 years of spastic paraplegia, clinical follow-up and muscle biopsies were performed in Vienna, Austria. Tissue composition and fiber morphology were analyzed by light microscopy in Padua, Italy. Histology conﬁrms that: 1. the difference in average size of muscle ﬁbers between mid-term (2 to 5 years) and long-term (5 to 20 years) paralyzed leg muscles is actually very small; 2. They maintain the striated appearance characteristic of normal skeletal muscle ﬁbers even after 20 years of paralysis; 3. SDH histochemistry, a marker of mitochondrial content of muscle ﬁbers, shows a dramatic decrease that is more pronounced in long-term paralyzed leg muscles. 4. Myosin ATPase histochemistry demonstrates that there is a progressive relative increase of the fast-glycolytic muscle ﬁbers at the expenses of the oxidative muscle ﬁbers. Together with our previous evidence of ultrastructural alterations of the activating and metabolic machineries and of the presence of muscle ﬁbers with lower motor neuron denervation characteristics, these features explain the low-force output and the reduced endurance of paretic muscles; 5. After two-year of FES-training the deterioration process is reversed, taking the fast muscle fibers to almost normal size values for sedentary adults. The stable muscle atrophy that characterizes long-lasting spastic paraplegia and the evidence that extent of FES-training recovery does not correlate with time from SCI strongly suggest that there are no upper-time limits to begin a FES training program.

Introduction
There is numerous long-term spinal cord injury (SCI) patients (from 10 to 20 years of SCI) who could beneﬁt from Functional Electrical Stimulation (FES) treatments, but information on their muscle conditions was scarce [1-3], before we reported on the extent of muscle atrophy in long-term thoracic-level upper motor neuron (UMN) paraplegia [4]. The many reports published have mostly studied muscle properties up to 3–5 years post-SCI [5]. Indeed, after the ﬁrst few months, in which muscle mass decreases signiﬁcantly, [6–8] muscle atrophy reaches a steady state. Within the ﬁrst month, the thickness of the muscle bulk, measured by ultrasound, decreases up to 40% [8,9]. We will indicate the period up to 3 months post SCI as the “early phase” of paraplegic atrophy. Afterwards, a 50% stable atrophy in spastic incomplete or complete paralysis is well documented up to [10–12]. After SCI, human muscles seemed to reach and sustain a stable atrophy [4]. Histopathology conﬁrmed that the difference in average size of muscle fibers between long-term and mid-term paralyzed leg muscles was actually progressing, but very small. We add here more information on fiber type composition/transformation of quadriceps m. in long-term UMN-complete thoracic paraplegia by histochemical approaches and on the trophic effects observed after 2 years of FES training.

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Materials and methods

Patients

The study enrolled fifteen subjects who had experienced traumatic SCI affecting the upper motor neuron (from T4 to T12). Eight of them finished the study. All subjects enrolled in the project were volunteers, who had received detailed information and had signed an informed consent.

We certify that all applicable regulations concerning the ethical use of human volunteers were followed during the course of this research. (Approval: EK-03-035-0403).

Biopsies were obtained as pre-FES sampling from patients who were going to undergo a 2-year FES treatment. Patients were divided into two groups: a mid-term group composed of six patients paralyzed from two to up five years and a long-term group formed by four patients paralyzed from six up to twenty years. All subjects were classified as ASIA A. Age, sex, body weight, height, etiology and level of SCI are reported in Kern et al. 2008 [4]. Clinical and functional assessments, muscle biopsies as well as follow-up were performed at the Institute of Physical Medicine and Rehabilitation, Wilhelminenspital, Vienna, (Austria), while light microscopy of biopic specimens at the Department of Biomedical Science, University of Padova, Italy.

Muscle biopsies

Through a small skin incision (6mm in diameter), needle muscle biopsies were taken from the right and left vastus lateralis muscles before start and after 2 years of FES training for each patient, as described by Kern et al. 2004 [16]. The resulting specimens were then prepared either for light and/or electron microscopy.

Histochemistry

Fibre typing by succinate dehydrogenase (SDH) reaction: SDH staining was performed to distinguish between oxidative and non-oxidative (or “less” oxidative) muscle fibers. Serial cross-sections from SM muscles were incubated for 60 min at 37°C in SDH incubation solution (0.1% nitro blue tetrazolium in 0.1M phosphate buffer [pH 7.2-7.6] containing 0.1M sodium succinate) and then rinsed in distilled H2O (3 changes x 1 min). To remove unbound blue tetrazolium, the sections were incubated in 3 changes (1 min per change) of acetone in water solutions (30, 60, and 90% acetone) in first increasing and then decreasing order of acetone concentration. Sections were rinsed in distilled H2O for 3 changes (1 min each), dehydrated in graded ethanol solutions (i.e., 70, 90, and 100%), cleared in xylene, and finally mounted in permanent medium (Canada Balsam).

Fibre typing by myofibrillar actomyosin ATPase histochemistry: Two different procedures were used for staining myofibrillar actomyosin ATPase following the methods described by Brooke and Kaiser [18,19] and by Guth and Samaha [20,21].

Alkali preincubated myofibrillar ATPase activity: Sections were air dried for 10 min at room temperature and then fixed for 3 min in 5% (w/v) formaldehyde in a solution of 200 mM sodium cacodylate, 68 mM CaCl₂ and 340 mM sucrose (adjusted with HCl to pH 7.6 before formaldehyde was added). After incubation in wash solution (100 mM Tris and 18 mM CaCl₂; pH 7.8), an alkaline pre-incubation step was performed by maintaining the sections in 200 mM 2-amino-3-methyl-1 propanol (pH 10.4) for 15 min at room temperature. After two successive 1 min incubations in the wash solution, sections were incubated in ATP solution (2.7 mM ATP, 90 mM CaCl₂, 100 mM sodium barbital pH 9.4) for 45 min at 37°C.

Acid preincubated myofibrillar ATPase activity [22]: Sections were air dried for 10 min at room temperature and incubated in 100 mM sodium acetate (pH 4.35) for 10 min a room temperature. Sections were then washed (2 changes x 1 min each) in 18 mM CaCl₂ and 100 mM Tris HCl (pH 7.8) before incubation in the ATP solution (together with the serial sections undergoing the Alkali Preincubated Myofibrillar ATPase Activity technique). After washing in 2 changes (30 sec per change) of distilled H2O, sections were incubated for 5 min in 2% (w/v) CoCl₂, washed (2 changes x 30 sec each) in distilled H2O, incubated for 5 min in 2% (w/v) (NH₄)2S in water, and then washed in distilled H2O (2 changes x 30 sec each). Finally, the sections were dehydrated in graded ethanol solutions (i.e., 70-90-100%), cleared in xylene, and mounted in permanent medium (Canada Balsam).

Images were acquired using a Zeiss microscope connected to a Leica DC 300F camera, under the same conditions that were used to photograph a reference ruler. The minimum transverse diameter of each muscle fiber was measured against the reference ruler. Morphometric analyses were performed with Scion Image for Windows version Beta 4.0.2 (2000 Scion Corporation, Frederick, MD, USA), free software downloaded from the web site: www.scioncorp.com.

Results

Size and fiber type composition of normal and UMN paraplegic muscle fibers before and after two years of FES training

Generally, in UMN (spastic) paraplegia human muscles reach soon and maintain a stable atrophy (Table 1 and [4]). Equally clear is the fact that there is a substantial increase of the relative content of the fast type 2B fibers in the MT and LT SCI (Tables 2 and 3). Whether this is the results of muscle fiber transformation due to SCI-induced muscle disuse and unloading or the loss of slow type 1 and 2A muscle fiber types is still debated.

Table 1: Diameter of normal and UMN paralyzed human muscle fibers Pre and Post 2 years of FES training

<table>
<thead>
<tr>
<th>Type</th>
<th>µm +/- SD</th>
<th>Type 2B</th>
<th>Type 2A</th>
<th>Type 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal muscle</td>
<td>54,00 +/-14,58</td>
<td>43,91 +/-13,88</td>
<td>45,79 +/-13,56</td>
<td></td>
</tr>
<tr>
<td>PRE-FES</td>
<td>44,17 +/-4,88</td>
<td>42,81 +/-4,33</td>
<td>41,42 +/-4,28</td>
<td></td>
</tr>
<tr>
<td>POST-FES</td>
<td>60,53 +/-3,44</td>
<td>not present</td>
<td>42,29 +/-8,97</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Percentage of fiber types in normal and UMN paralyzed human muscles

<table>
<thead>
<tr>
<th>Type</th>
<th>Type 2B</th>
<th>Type 2A</th>
<th>Type 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal muscle</td>
<td>49</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>PRE-FES</td>
<td>68</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>POST-FES</td>
<td>85</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 3: Percentage of fiber types in UMN paralyzed human muscles in MT and LT SCI

<table>
<thead>
<tr>
<th>From SCI (Years)</th>
<th>Type 2B</th>
<th>Type 2A</th>
<th>Type 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal muscle</td>
<td>49</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>PRE-FES</td>
<td>&lt;5</td>
<td>59</td>
<td>15</td>
</tr>
<tr>
<td>POST-FES</td>
<td>&gt;5</td>
<td>76</td>
<td>0</td>
</tr>
<tr>
<td>&gt;5</td>
<td>80</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>&gt;5</td>
<td>91</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>
SDH staining

SDH is an oxidative enzyme localized in the mitochondria. The rodents type 1 fibers that have an oxidative metabolism stain darker than the type 2 fibers that have prevalently a glycolytic metabolism. These differences are less pronounced in human adult muscle. Nonetheless, the stain intensity of this mitochondrial enzyme decreases substantially in UMN paraplegic patient muscles (results not shown), suggesting a transition of the muscle fibers properties from those of the oxidative to those of the glycolytic muscle fibers.

Myosin ATPase, pH 4.35

Type 1 fibers stain dark, type 2A stain light, while the type 2B fibers are unstrained. Decrease of the slow type of myosin is evident in UMN paraplegic muscles at mid-term, while it almost disappeared in the long-term UMN muscles, that are prevalently composed of fast type 2B muscle fibers.

Myosin ATPase, pH 9.4

After incubation at pH 9.4, myosin enzymatic activity of the fast 2B fiber type is relatively stable, whereas the slow myosin of Type 1 fiber is lable. Thus, the fast fibers stain dark, while oxidative fiber types stain intermediate (Type 2A) or very light (Type 1). These features allow to identify and to determine size and relative content of the 3 types of muscle fibers present in human Quadriceps m. (Tables 1 and 2, respectively). In normal muscle, the fast type 2B muscle fibers have a larger size (around 55 µm of muscle fiber diameter), while the oxidative muscle fibers (Types 2A and 1) have around 45 µm of muscle fiber diameter. Pooling all MT and LT SCI samples all the three types of muscle fibers decrease in diameter, while only the type 2B muscle fibers increase in diameter after two years of FES. On the other hand, Post-FES muscle fibers generally increase of about 50% in size.

The possible transformation of oxidative 2A muscle fibers to fast glycolytic muscle fibers is strongly suggested when the percentual content of the three different fiber types was determined, Table 2 shows that in the pooled results the percentual content increase from the normal value of 50% to 70% in UMN SCI biopsies before FES training and to 85% after two years of FES, mainly at the expense of the 2A fiber types that are absent in this group of muscle biopsies. This trend is confirmed and more evident comparing the separated groups of mid-term versus long-term UMN SCI muscle biopsies before and after 2 years of FES.

On the other hand, some muscle biopsies contain variable amount of lower motoneuron-denervated muscle fibers (that, is severely atrophic muscle fibers positive with the anti-NCAM [4]).

Discussion

Complete spinal cord injury results often in a significant loss of muscle mass, or atrophy, in the affected areas of the body, the severity and extent of which depends on the level of lesion and on the survival of the motor neurons. Muscle atrophy has been studied in SCI patients from a variety of points of view. However, most studies in literature are focused on the early post-injury phase and did not follow the progression of atrophy that occurs in the long run. In the last twenty years some of us have extensively studied long-term atrophy progression in lower motor neuron lesion (flaccid) patients and the perspectives of training these very difficult cases with a purpose-developed FES training (Vienna FES strategy for denervated degenerating muscles) [23-25]. We demonstrated that: 1. human skeletal muscles survive permanent denervation longer (years) than generally accepted; 2. FES started within the first year post-SCI provides the best results, but recovery of muscle mass and contractile function occur also in mid- (3 years) and long-term (up to 6 years) subjects, if purpose developed protocols, electrodes and stimulator (now commercially available) are used [26-36].

Over the years, the benefit of the use of functional electrical stimulation (FES) to restore movement of the limbs of paralyzed patients has been largely discussed. For this reason information on the long-term progression of atrophy would be actually extremely important for the treatment of patients since it could give important clues to physiotherapist on how and when starting FES rehabilitation procedures and when the results will be poor or very poor. To date, however, not much is known about the long-term progression of muscle atrophy in upper motor neuron lesion (or spastic) patients. Indeed, in 2008 we published the first and only study reporting results of UMN spastic paraplegia up to twenty years from SCI [4]. In that study we investigated the long-term progression of atrophy in spastic patients using a multidisciplinary approach that compared a mid-term group to a long-term group of patients, suffering with complete UMN lesion of the spinal cord (T3-T11), enrolled in a 2-year FES rehabilitation program. Those results confirmed that the complete UMN SCI paraplegics lose 50% of muscle mass during the first few (3 to 6) months but indicated very little progression of the muscle atrophy long-term (at least up to 20 years post injury), providing information that is of significant importance for the rehabilitation of long-term spastic patients. Furthermore, in the last 5 years we studied also elderly persons either performing high level amateur and competitive sports in their seventies or sedentary old septuagenarians before and after 3 months of FES training [37-45]. In all these cases we added our evidence to an extensive literature [46], demonstrating the negative effects of poor physical activity and the trophic recovery after an increased level of voluntary [47,48] and/or FES-induced muscle contractile activity maintained for weeks, months or years [36-46].

Here we added further evidence that the stable atrophy of long-term UMN spastic paraplegia is accompanied by changes in the population of unloaded muscle fibers that explain, together with the ultrastructural modification we previously described [4], the poor or very poor fatigue resistance of these heavily unloaded skeletal muscles. We show here once again that FES training may reverse the atrophy process, though the amount of contractile activity inducible in clinical settings do not allow to reach the level needed to substantially increase the oxidative metabolism and thus endurance of parietal muscles. Nonetheless, other positive effects of the Vienna FES training is a beneficial hyperaemia of the stimulated muscles and of the skin with aesthetic modification of the legs and a valuable anti-decubitus ulcer effect [24,25].

Conclusions

The analyses of type of fibers in muscles of UMN paraplegic patient before and after two-years of FES training demonstrate a remarkable and significant size increase, restricted to fast type muscle fibers, that increases in percentage, possibly as the result of muscle fiber transformation related to the dramatic decrease of load that is determined by paraplegia and a wheel-chair life-style.

Indeed, there is a decrease of oxidative muscle fibers, both the slow type 1 muscle fiber up to the absence of 2A fiber type in POST-training patients. An increase in relative content of the type 2B fibers is evident and progresses with time of SCI. The diameter of the slow type 1 muscle fibers slightly decrease with UMN, but do not change during SCI time progression or after FES-training.
Our conclusion is that muscle fibers adapt to unloading and to the decrease of contractile activity with fiber-type transformations. However, patent degeneration of the muscle tissue, as in the case of permanent long-term lower motor neuron denervation, was never observed. Furthermore, the fact that extent of training-induced results do not decreased with time from UMN SCI, is a strong evidence that there are no upper-time limits to begin FES.

Conflicts of interest
The authors declare none conflict of interest regarding the publication of this paper.

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