Modulation of Trophism and Fiber Type Expression of Denervated Muscle by Different Patterns of Electrical Stimulation

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Abstract
After fifty years of basic research on electrostimulation-induced muscle plasticity, in recent years a few studies have employed long impulse biphasic electrical stimulation as a treatment for human denervated muscle. Tissue trophism and muscle power are improved to a level sufficient to restore some functions by Functional Electrical Stimulation (FES). These treatments usually start late after denervation due to clinical constrains and the past wrong believe that electrical stimulation may interfere with eventual myofiber reinnervation. Effects of denervation *per se*, of spontaneous or induced aneural myogenesis, and of long-term electrical stimulation starting either early or late after denervation are here described. Interrelations of these aspects are remarkable, and relevant to trophic and functional recovery from severe atrophy/dystrophy of long-term denervated muscle. If myogenesis could be modulated, we should be able to substantially abbreviate the time needed to achieve functional recovery of long term denervated human muscle by FES.
Key words: FES, functional electrical stimulation, human denervated muscle, muscle regeneration, myogenesis.

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Fifty years of basic research on electrostimulation-driven muscle plasticity result in recent years in clinical trials testing long impulse biphasic electrical stimulation as a treatment for human denervated muscle to improve tissue trophism and in some cases muscle power to a level sufficient to restore function [25, 43, 44, 86]. These treatments usually start late after denervation due to clinical constrains and old believe that electrical stimulation is useless in these cases and may interfere with eventual myofiber reinnervation, while there is now evidence that it may accelerate nerve growth and muscle reinnervation [59, 70, 88].

Effects of long-term denervation *per se*, of spontaneous or induced aneural myogenesis, and of long-term electrical stimulation (starting early or late after denervation) are here described. Interrelations of these denervation effects are remarkable and influence trophic and functional recovery from severe atrophy/dystrophy of long-term denervated muscle. Their modulation by FES may shorten time to recovery and increase functional uses of long term denervated human muscle.

Long-Term Denervation of Skeletal Muscle

Peripheral denervation of skeletal muscle is followed by loss of function and tissue wasting, which was thought to end in death of myofibers and at last in substitution of contractile tissue with adipocytes and collagene sheets [34]. We now know that long-term muscle denervation is a long-lasting period of post denervation severe atrophy accompanied by lipodystrophy and fibrosis, but also by non-compensatory myogenic events (myofiber generation and regeneration). In the time scale of rodent life (3–4 years), these events start two-four months after permanent denervation and last the life long, but interspecies differences are significant and poorly known (see below). All these events are part of the wide range of adaptive responses of muscle tissue to increased or diminished use, which belongs to the concept of muscle plasticity. Table 1 lists adaptive mechanisms or consequences, and their relations.

Severe Atrophy. Denervation of myofibers early induces muscle atrophy, a decrease of tissue mass that could be attained by reduction in size of each myofiber (*hypotrophy*) and/or loss of myofibers (*hypoplasia*). We will use "*severe atrophy*" when hypotrophy reaches 90% reduction in myofiber size. It is accompanied by disarray of the sarcomeres up to disappearance. Chains of myonuclei mark severe atrophy, but hypernucleosis is also present in early new myofibers (myotubes). To distinguish between these two events an anti-embryonic

Table 1. Muscle plasticity.

MUSCLE PLASTICITY

TROPHISM & DEGENERATION

HYPOTROPHY **EUTROPHY**
HYPERTROPHY

SEVERE ATROPHY
HYPERPLASIA
MYOFIBER DEATH
LIPO/FIBRO/DYSTROPHY

MYOFIBER DEATH AND REGENERATION

Development
Denervation/Reinnervation } **GENERATIVE**
MYOGENESIS
Increased Function

NECROTIC DEATH → **REGENERATIVE**
MYOGENESIS

myosin stain is necessary and sufficient. Myonuclear loss and myofiber death could be early events following denervation, while long anucleated longitudinal sections with several-clumped myonuclei along the myofiber are markers of long-lasting severe atrophy [82]. Disruption of molecular mechanisms tethering nuclei to actin cytoskeleton are possibly responsible of these nuclear events [67, 78]. On the other hand, muscle clumps remind organization of nuclei at the synapsis, so they may represent an additional mechanism of the long-lasting capacity of aneural myofibers to survive being ready to be reinnervated even far from their original end plate.

Myofiber death

Cell death and its mechanisms in rat skeletal muscle undergoing post-denervation atrophy are now known in details [9]. Clear morphological manifestations of muscle cell death, with ultrastructural characteristics very similar if not identical to those considered as nuclear and cytoplasmic markers of apoptosis are found. With increasing time of denervation, progressive destabilization of the differentiated phenotype of muscle cells is observed: spatial disorganization of myofibrils and formation of myofibril-free zones. These changes initially appear in subsarcolemmal areas near myonuclei, and by 4-month denervation they are spread throughout the sarcoplasm. Dead muscle fibers are usually surrounded by a folded intact basal lamina; they have an intact sarcolemma and highly condensed chromatin and sarcoplasm. Folds of the basal lamina around the dead cells result from significant shrinkage of cell volume. Clear manifestations of inflammation are absent in the denervated tissue, and macrophages are occasionally found in close proximity to dead myocytes.

Nuclear DNA fragmentation by the TUNEL method, a molecular marker of apoptosis, is present in only a very small number of cell nuclei in 2 and 4 month denervated muscle and to less extent in 7 month denervated muscle. The numbers of nuclei of abnormal morphology containing condensed and/or irregular patterns of chromatin distribution, as revealed by DNA staining and electron microscopy, exceed by 30-40 times the numbers of nuclei positive for the TUNEL reaction. In contrast to previous results [54, 80], a high discrepancy between frequency of morphological markers of apoptosis and DNA fragmentation seems a peculiar trait of myofiber death in rat denervated muscle.

Muscle *degeneration* (or *dystrophy*, as for genetic diseases) is an even later effect of denervation: a significant portion of the muscle mass is substituted by different cells, mainly adipocytes (fat degeneration or lipodystrophy) and/or fibroblasts and collagen sheets around myofibers (endomysial fibrosis). These last changes seem to occur in long-term denervated muscle of rat after heavy reduction of the number of capillary per myofiber [12].

Generation and regeneration of myofibers

Mature mammalian skeletal muscle fibers maintain a relatively finite, fiber type-specific relationship between the size of the myofiber and the number of myonuclei present in a given myofiber [2, 23, 35]. However, shortly after birth, mammalian myofibers are permanently differentiated and thus cannot undergo mitotic division or directly increase their myonuclear number (i.e., myonuclear division) [22]. Therefore, myofibers undergoing hypertrophy appear to require an external source of new nuclei to maintain or reestablish a relatively constant myonucleus-to-fiber size ratio. There is a significant body of evidence to suggest that satellite cells are the source of new myonuclei in mature mammalian skeletal muscles. There is also some compelling evidence indicating that satellite cell proliferation is required to support the process of compensatory hypertrophy in mammalian skeletal muscle: when radiation is used to prevent satellite cell proliferation before initiation of skeletal muscle functional overloading the hypertrophy response is absent [2, 69].

Surprisingly, evidence of myogenic events are also reported in denervated muscle. In contrasts with older reports, light and electron microscopy shows that the long term denervated muscle maintains a steady-state severe atrophy for the animal's life span, some morphological and molecular features indicating that events of aneural regeneration occur continuously [16, 18, 57]. New muscle fibers are present as early as one month after surgery and reach a maximum between 2 and 4 months following denervation of rat leg muscles. Then myogenesis gradually decreases with progressive post-denervation

atrophy and degeneration. These myogenic events are present in permanently long-term denervation, and are not related to muscle reinnervation [2].

Muscle partial denervation or reinnervation is a major technical problem in the study of long-term denervated muscle in rodents. Small size of rat legs and high capacity of peripheral nerve regeneration need careful surgical approaches in establishing the experimental model [12]. On the other hand, minimal residual innervation is the most common event in clinical cases. Research on this "disturbing" variable and on its impact on FES of long-term denervated human muscles is needed.

The myogenic response in long-term denervated rat muscle is biphasic and includes two distinct processes. The first process resembles the formation of secondary and tertiary generations of myotubes during normal muscle development and dominates during the first two months of denervation. The activation of this type of myogenic response (*myofiber generation*, which truly increases the number of myofibers present in an anatomically defined muscle, *hyperplasia*) does not depend on cell death and degenerative processes [10].

A second type of myogenesis is a typical *regenerative* reaction that occurs mainly within the spaces surrounded by the basal lamina of dead muscle fibers [10, 18]. Myofibers of different sizes are susceptible to degeneration and death, which indicates that cell death in denervated muscle does not correlate with levels of muscle cell atrophy [10]. These regenerative processes frequently result in development of abnormal muscle cells that branch or form small clusters surrounded by two layers of basal lamina (the old and the new one, which is secreted by the new myofiber) [10, 18, 57]. Spontaneous myofiber regeneration in long-term denervation has been quantified and shown to be non-compensatory and to result in reduction of satellite cell pools [8, 10, 24, 42, 46, 47, 68, 74, 87]. The turnover of myonuclei (but not necessarily of regenerating myofibers) studied by means of continuous infusion of 5-bromo-2-deoxyuridine (BRDU) is at most 1 to 2% per week in adult rats [68, 75]. Evidence of satellite cell depletion in denervated muscle raises some problems on long-term potential of regenerative myogenesis [24, 42]. On the other hand myotoxic-induced myogenesis speaks in favor of a long lasting potential.

Interspecies differences raise additional doubts, since there are large differences in post-denervation effects, even of rate of atrophy [36]. In man denervation atrophy progresses at relatively slow rate in comparison to rat, but our observation that the myogenic reactions to denervation of the human muscle are very similar to those well described in rat, is heartening as to the potential application in patients' rehabilitation.

Post-Denervation Isogenes' Switchings (Fiber Typing)

After the fully differentiated pattern of fast and slow myosins has been established in normal adult skeletal muscles, acute denervation is of very little influence on the type of contractile proteins synthesized in early atrophying muscle fibers. Preferential atrophy of fast fibers followed by atrophy of slow fibers appears to be the typical feature of the early phases of denervation, producing only a small unbalance in fiber typing [41, 63, 79]. However during several months of permanent denervation there is an almost complete transformation of rat mixed muscles into almost pure fast muscles [13, 14, 17], the residual slow myosin being present with fast myosin in single myofibers [16, 79]. Analyses of denervated and aneurally regenerated muscles suggest that in long-term denervation of rat soleus the slow-to-fast transformation is mainly the consequence of repeated cycles of cell death and regeneration [46, 74]. Such a slow myosin disappearance is less pronounced in other species, but it is not known if this means that post-denervation myofiber regeneration is less pronounced [5].

Induced regenerative myogenesis in long-term denervation

Beside traumatic events, regeneration of muscle fibers is studied after induction of muscle damage and regeneration by vitamin E deprivation [3, 27], autografting [10] or by myotoxins' treatment [16]. Permanent denervation does not prevent induced muscle regeneration [57] and a long-term retention after denervation of this capability has been demonstrated: Bupivacaine induces in few days massive and synchronous myofiber regeneration of four-month denervated fast and slow rat muscles [15, 19, 20]. When the denervated muscles are treated with marcaine or notexin autografting of seven-month denervated rat muscles is followed by substitution of old fibers by new fibers [8, 32, 42, 51]. The aneurally regenerated myofibers grow in few days and maintain for two weeks one fourth of their normal adult fiber sizes, then they atrophy and reach a steady-state size at less than one tent of the normal fiber size [57].

When a series of myotoxic injuries are made leaving time for regeneration to occur between each injury, new muscle fibers form after up to four treatments. This provides evidence that some of the myoblasts reenter an undifferentiated, stem cell-like state being capable of myogenesis after further injuries to the muscle [29].

Satellite cell proliferation and myofiber regeneration is enhanced in long-term denervated muscles subjected to electrical stimulation [28, 31, 56, 66].

Electrical stimulation of denervated muscles

Early after denervation

Fiber type switching

Mammalian skeletal muscle is primarily composed of twitch fibres, which are grouped into two major physiological classes based on their speeds of contraction and relaxation [72]. Although these general categories can be broadly applied, there is a spectrum of fibre types, with a wide variety of fibre types including specialized

extremes as well as intermediate types present in various muscles. The fast and slow fibres express different isoforms, and frequently different concentrations, of most of the myofibrillar proteins, of the proteins underlying activation and relaxation and of many metabolic enzymes. Transitional fibres are frequently seen in electrostimulated muscles and reflect the dynamic remodelling of a muscle based on its in vivo physiological use and the plasticity of fiber type [64].

It is well established that changes in muscle stimulation patterns in animals result in changes in levels of fibre type-specific isoforms of most muscle proteins, but such changes in protein composition take weeks to occur due to the relatively slow turnover of most muscle proteins [65]. The time course of changes in muscle gene expression, monitored as transcript levels [83], is more rapid than that of changes in protein levels during chronic *in vivo* muscle stimulation in animals. An increase in the slow fibre isoform of myosin heavy chain mRNA has been demonstrated in whole muscle stimulated in living animals [11] or in culture [6], and on single muscle fibers electrostimulated in vitro [48]. The last two studies are definitive evidence that not only muscle development is independent from the direct contact with the motoneurons, but also that the influence of motoneurons activity on muscle plasticity could be at least in part mimicked by different patterns of electrical stimulation (for review see [33]).

Contractile phenotype of muscle fibers is under control of hormones, stretch and influences from the motoneurons. Motoneurons can affect muscle fibers by releasing neurotrophic substances and by evoking electrical activity in the muscle. For regulating contractile properties such as speed, strength and endurance it has been demonstrated that the signal to change is coded in the pattern of electrical activity. Thus, high amounts of activity lead to slow shortening velocity and myosin heavy chains, while low amounts of activity lead to a fast phenotype [33, 65, 72, 71]. Even slow myosin heavy chains could be expressed in denervated fast myofibers by slow-like continuous electrostimulation [14, 52, 84].

On the other hand, some restrictions seem to exist on extent of transformation of myofiber characteristics by electrical stimulation of denervated muscle. In a typical study, the slow soleus muscle and the fast EDL were denervated and stimulated directly with implanted electrodes for up to 82 days. Four different stimulation patterns were used in order to mimic the natural motor-unit activity in these muscles. Native stimulation patterns maintain normal contractile speed, but in the EDL, normal isotonic shortening velocity was maintained only by a stimulation pattern consisting of very brief trains with an initial short interspike interval (doublet), and not by the other native high-frequency patterns. The results indicate that for the control of contractile properties instantaneous frequency, total amount of stimulation, train length, interval between trains and presence of an initial doublet ought to be taken into account. Changes in contractile speed induced by a foreign stimulation pattern were quantitatively similar to the effects of cross-innervation both in the EDL and the soleus. Thus the change in activity pattern is the mechanism behind most of the changes induced by cross- innervation [26].

Others report negative results in guinea pig [45]. When guinea-pig soleus muscles were denervated and electrically stimulated for periods up to two months by stimuli consisting in 1 s bursts of 40 Hz pulses, repeated every 5 min (a chronic phasic stimulation that produces fastening of contractile properties in denervated soleus rat muscle) the fast conversion is not attained. Since myotoxic injury of this slow muscle produces new myofibers as fast as those seen in rat experiments, the data are compatible with the hypothesis that slow-to-fast transformation of denervated rat soleus is not directly brought about by chronic stimulation but by de-novo formation of fast-contracting regenerated fibres [46, 74]. It remains to be explained why myofibers of guinea pig soleus rarely undergo denervation death and regeneration in comparison to rat soleus. The persistence of fibrillation in guinea-pig but not rat after denervation may account for the species difference [45].

Similar interspecies discrepancies are also known when normal muscle is indirectly electrostimulated [77].

Muscle trophism

For preventing atrophy in denervated muscles high frequency seems to be beneficial, particularly in fast muscles. [39, 53]. The results mimic in denervated muscle the well known efficacy of high frequency electrical stimulation in inducing skeletal muscle hypertrophy [4], of low frequency electrical stimulation protocol in inducing endurance-like adaptations when applied 5 days/wk for 3 wk [50, 61, 71].

Recent evidence shows that trophism and fiber type are regulated independently through different transduction signaling pathways [40, 58, 60, 76].

The role of putative neurotrophic substances remains unclear, with the exception of release of neuronal agrin, which is relevant in modulation of neuromuscular junctions [7].

Though still criticized by several members of the medical community (due to inexperience in the proper methodology), electrical stimulation applied early after nerve injury maintains eutrophy and function of denervated muscle [37, 55, 81].

Late after denervation

A few relevant publications concern restorative effects of electrical stimulation of long-term denervated muscles in animal models [1, 49, 73]. Intermittent electrical stimulation at 100 Hz (one train of 60 pulses every minute) either maintains (by early stimulation) or restores in two months the force output to nearly normal values when stimulation starts at two, four or even nine months after sciatectomy. Long-term stimulation causes similar

increases in muscle fiber cross sectional area in fast and slow muscles, and changes are identical when innervated muscles are stimulated in the same way [49].

Muscle residual innervation or reinnervation is a major technical problem in the study of long-term denervated muscle in rodents. Small size of rat legs and high capacity of peripheral nerve regeneration need careful surgical approaches in establishing the experimental model [12]. On the other hand, clinical minimal residual innervation is the most common case [43], and research on this "disturbing" effect is also needed. The innervated myofibers responding well to electrostimulation may discourage the use of current density needed to activate the aneural regenerated or atrophic myofibers intermixed to the innervated/reinnervated muscle fibers.

Functional Electrical Stimulation (FES) of Long-Term Denervated Human Muscles

Over the last 30 years there has been a good deal of interest in the use of FES to restore movement of the limbs of patients immobilized by spinal-cord injury (upper motor neuron lesion, spastic paralysis) [21, 28, 62]. There is, however, another group of patients whose problems are more difficult to treat due to marked atrophy of denervated muscles and the associated loss of bone mass and skin atrophy causing severe secondary medical problems. In these patients injury also resulted in irreversible loss of the nerve supply to some or all of the affected limbs (flaccid paralysis due to lower motor neuron lesion). It is technically more difficult to treat these patients because direct stimulation of muscles requires more electrical energy than commercially available stimulation devices can deliver. The absence of functional nerve fibers makes it more difficult to recruit a sufficient population of myofibers to regain functional movements at an acceptable force level by surface electrodes. Despite these difficulties pilot studies on functional clinical application of FES on denervated muscles have been published. One has demonstrated gait correction via direct FES of the denervated tibialis anterior muscle [81]. Two others [44, 86], contrary to widely accepted opinion, have shown that electrical stimulation in such patients can restore muscle mass, force production and movement after long-lasting complete denervation. In addition to this functional restoration, improvements were observed in the condition of associated tissues (cartilage, tendons, bones, joints and skin). In one study muscle function in the lower extremities was restored sufficiently to support standing up, standing, and even a few steps [44]. The associated stimulation equipment proofed to be effective for home-based training in a limited number of patients [38]. Recent analyses of biopsies from these patients confirm the presence of regenerating myofibers. In muscle biopsies of four-year denervated human subjects two-year after FES about 3% of myofibers are stained by anti-embryonic myosin antibody. Both small and large regenerated myofibers

Table 2. Stimulation protocols effective in inducing skeletal muscle hypertrophy or endurance in either innervated or long-term denervated muscles.

Protocol 1
Innervated muscles stimulated through electrodes implanted near the motor nerve. The high frequency electrical stimulation protocol induces skeletal muscle hypertrophy. Electrodes implanted near the sciatic nerve. Tetanic contractions are delivered at a frequency of 100 Hz, 6-12 V, 1-ms duration, 9-ms delay, for 10 sets of 6 repetitions, with each repetition lasting 3 s. A 10-s recovery is given between repetitions and 1 min between sets, with the stimulation protocol lasting a total time of 20 min This model takes advantage of the anatomic distribution of the hindlimb muscles of the rat. During each stimulation, all hindlimb muscles are recruited, and the dorsiflexor muscles are stimulated to contract against forces that are three times larger than the ones generated by the antagonistic plantar flexors. This type of stimulus, when adequately repeated, is sufficient to induce a hypertrophic response in the overloaded dorsiflexors [4, 86]. The low frequency electrical stimulation protocol has been shown to be effective in inducing endurance-like adaptations when applied 5 days/wk for 3 wk [61]. Stimulation is delivered at a frequency of 10 Hz, 5 V, 10-ms duration, 90-ms delay, for a total time of 30 min.
Protocol 2
Human long-term denervated leg muscles stimulated by means of surface electrodes Due to their reduced excitability long-term denervated myofibers are stimulated by long impulses [43, 44]. The duration of stimulation impulses has to be about 10-100 times longer in denervated than in innervated muscles. Impulses of 100-200 ms duration (0,5 Hz) and of 60-100 Volt with large surface electrodes are delivered at the beginning to attain forceful single twitch contractions of thigh muscles. As training proceeded and near normalisation of membrane-excitability occurs, impulse duration is reduced, reaching higher frequencies from 20 to 25 Hz, which elicit tetanic contractions.
Protocol 3
Burst stimulation for denervated muscles, Kern's Current at the beginning 40 msec impulse and 10 msec rest , 20 Hz 2 min on /2 min off 3 - 5 minutes, 1-2 minutes pause, 3 - 5 times a session, twice a day later on 15 - 30 repetitions per set 2 min rest between each set, 6 - 8 sets, twice a day first no weight, later on 1-5 kg on the ankle

are present. A few myofibers have central nuclei, a feature suggesting they are no more than 10-day old. Frequency distribution of myofibers according to their minimum diameter in semi-thin sections shows that about 50% are severely atrophic (minimum diameter smaller than 10 μm), but a large proportion of myofibers are eutrophic or hypertrophic, i.e. with a minimum diameter larger than 40 μm.

Perspectives

After fifty years of basic research on electrostimulation-induced muscle plasticity, Functional Electrical Stimulation by means of long biphasic impulses is able to restore muscle mass, force production and movement after long-lasting complete denervation [50]. Patients suffering from flaccid paraplegia (denervation of lower extremity muscles, *conus cauda* syndrome) are especially good candidates for these approaches. In the long-term we may consider the development and application of implantable solutions alternatively to the actual approaches based on surface electrodes.

Before this could be taken into consideration for the patients, whose residual innervation elicits painful sensation, a better knowledge and control of stimulation-induced muscle trophism and of body movements' control ought to be achieved. Then, artificial synapses, i.e. a pool of miniaturized electrodes, which contact each of the surviving or regenerated myofibers in the denervated muscle, have to be designed and developed. Taking advantage of the powerful angiogenesis of regenerating muscle [57], one may dream to sacrifice some of the new vascular branches to deliver sufficient current to new myofibers by means of nanofabricated electrodes.

For now, on the basis of pilot human studies and application of existing experimental knowledge, it can be anticipated that FES of long-term denervated muscles by surface electrodes will improve mobility with substantial reductions in the risk and the severity of secondary medical problems, resulting in less frequent hospitalization and a reduced burden on public health services. The patients could thus look forward to improved health, independence and quality of life, and the prospect of better professional and social integration.

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