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Research Article

Induced Muscle Fiber Regeneration in Permanent Skeletal Muscle Denervation: Implication for Functional Electrical Stimulation of Denervated Degenerated Muscles in Spinal Cord Injury

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Abstract

The differentiation of muscle fibers regenerating in the absence of the nerve is well documented in animal experiments and in muscle biopsies harvested from human patients suffering with permanent long-term denervation. During the last twenty years, clinical studies have employed long impulse biphasic electrical stimulation as a first step treatment for humans living with long-term denervated muscles subsequent to Spinal Cord Injury (SCI). Trophic and functional recovery from severe atrophy/degeneration occurred in long-term Denervated Degenerating Muscles (DDM) treated with two years of h-bFES beginning from 1 and 5 years from SCI but, using the commercial muscle stimulator and the large electrodes now available. On the other hand, the extent of recovery decreases with further years of muscle denervation. This is a fact that has sound foundations on muscle biopsy analyses and on Quantitative Muscle Color Computed Tomography (QMC-CT) of treated muscles. If myogenesis and induced-myogenesis in patients could be modulated during the many months needed to recover tetanic contractility of denervated muscles, the time-span needed to achieve functional recovery of permanent denervated human muscle by h-bFES started in between 1 and 5 years post SCI will be shortened and possibly attainable also when h-bFES will be started later than 6 years from SCI, i.e., in the vast majority of patients in need of them.

Keywords: Functional Electrical Stimulation (h-bFES); Home Based; Human denervated muscle; Induced aneural myogenesis; Muscle regeneration; Recovery of external-work contractility; SCI

Introduction

In recent years, basic research into the use of electro stimulation to provoke muscle plasticity after Spinal Cord Injury (SCI) has prompted clinical trials which have investigated the use of long impulse electrical stimulation as a treatment for long term permanently denervated human muscles [1,2]. These studies were aimed at improving muscle trophism and increasing external muscle power to a level sufficient to restore an impulse-assisted ability to perform "stand up" exercises [3-9]; that is, the muscle mass and strength would be increased to the point that individuals could actually stand up with assistance from direct electrical stimulation of the denervated muscles. These treatments usually were initiated relatively late after denervation (more than 8 month after SCI) due to clinical constraints and/or the beliefs that electrical stimulation of denervated muscles is ineffective and may actually interfere with myofiber reinnervation. These ideas are still maintained by many experts despite recent evidence that electro stimulation may indeed enhance nerve growth and appropriate muscle reinnervation [10-12]. Here we discuss the hypothesis of combining spontaneous or induced aneural myogenesis with long-term electrical stimulation of permanently denervated muscles.

Permanent denervation of skeletal muscle results in early loss of function and subsequent tissue wasting that is believed to lead to muscle fiber death and ultimately to substitution of contractile tissue with adipocytes and collagenous sheets [8,9]. More specifically, permanent muscle denervation in mammals produces a long-term period of denervation atrophy followed many months later by severe atrophy accompanied with lipodystrophy and fibrosis, and additionally by non-compensatory myogenic events (regeneration of muscle fibers). In the rodent life span (3-4 years), these events begin two-four months after permanent denervation and persist the entire lifetime; however differences between species can be significant and at this point are poorly outlined (see below). Nonetheless, all of these events, regardless of time frame, constitute the concept of muscle plasticity, that is, the wide range of adaptive responses of muscle tissue to increased or diminished use [13].

The mechanisms leading to cell death in rodent skeletal muscle

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undergoing post-denervation atrophy have been described in detail [14-16]. Briefly, as time elapses from the denervation event, ultrastructural characteristics very similar to those considered as markers of apoptosis are noted along with clear morphological manifestations of muscle cell death, starting from progressive destabilization of the differentiated phenotype of muscle cells, as evidenced by spatial disorganization of myofibrils and the formation of myofibril-free zones. Dead muscle fibers are observed, those being typically surrounded by a folded intact basal lamina and having an intact sarcolemma and highly condensed nuclear chromatin and sarcoplasm. The numbers of nuclei displaying abnormal morphology typical of necrosis exceed the numbers of nuclei positive for apoptosis by 30-40 fold.

Muscle degeneration (we will not use the word dystrophy to avoid confusion with genetic muscle diseases) is a later effect of denervation during which a significant portion of the muscle mass is substituted by different cells (mainly fibroblasts) and collagen sheets which surround myofibers (endomysial fibrosis) followed by degeneration or lipodystrophy [17]. These later alterations appear to occur in 5 to 10 month-denervated muscle of rat after a heavy reduction in the number of capillaries per myofiber [18].

When the fully differentiated pattern of fast and slow myosins becomes established in normal adult skeletal muscles, acute denervation has very little influence on the type of contractile proteins synthesized in the early (one to four months) atrophying muscle fibers. Nonetheless, a small net change in fiber type appears to be a typical feature of the early phases of denervation. This relative imbalance in fiber type is attributed to the preferential atrophy of fast fibers followed by atrophy of slow fibers [19,20]. However, after more than six months of permanent denervation in rats, there is an almost complete transformation of mixed muscles into nearly pure fast muscles [21-23], with only a small amount of residual slow myosin present with the fast myosin. Analyses of denervated and aneurally regenerated muscles suggest that in long-term denervated rat soleus the slow-to-fast transformation is mainly the result of repeated cycles of cell death and regeneration [24-26]. Such a slow myosin disappearance is less pronounced in other species [27], but it is not known if this means that post-denervation myofiber regeneration is less pronounced.

Spontaneous Myogenesis in Denervated Skeletal Muscles

There is a general consensus that in mature mammalian skeletal muscles "satellite cells" are the source of myonuclei of new myotubes and young muscle fibers, in particular in regenerative processes after trauma or myotoxic injuries [28,29]. Despite some doubt [30], in mammalian skeletal muscle there is also some evidence suggesting that satellite cell proliferation is necessary to support the process of compensatory hypertrophy. Specifically, when elevated radiation doses are used in rodents to prevent satellite cell proliferation prior to initiation of skeletal muscle functional overloading, the hypertrophy response is nearly absent [31].

Evidence of myogenic events is also observed in atrophying denervated skeletal muscles of rodents. In contrast with older reports, light and electron microscopy studies reveal that long term denervated muscle maintains a steady-state severe atrophy for the animal life span. Furthermore, some morphological and molecular features indicate that events of aneural regeneration occur continuously [21,32]. New muscle fibers are present as early as one month after nerve section and reach a maximum between 2 and 4 months in rat leg muscles following denervation. Myogenesis gradually decreases with progressive post-denervation degeneration, although myogenic events continue to occur and are not secondary to mandatory muscle reinnervation [33]. Muscle partial denervation or reinnervation is a major technical problem in the study of long-term denervated muscle in rodents. The small size of rat legs and the high capacity for peripheral nerve regeneration necessitate the development and execution of careful surgical approaches in establishing the experimental model [18]. Furthermore, minimal residual innervations are the most common event in clinical cases. Research on this "disturbing" variable in long-term denervated human muscle management is needed.

The myogenic response in long-term denervated rat muscle is biphasic and includes two distinct processes. The first process, which dominates during the first two months post denervation, resembles the formation of secondary and tertiary generations of myotubes which occurs during normal muscle development. The activation of this type of myogenic response (myofiber generation, which truly increases the number of myofibers present in an anatomically defined muscle, i.e., hyperplasia) does not depend on cell death and degenerative processes [17].

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 [18]. Myofibers of varying sizes are vulnerable to degeneration and death, which indicates that cell death does not correlate with levels of muscle cell atrophy in denervated muscle [17]. These regenerative processes frequently result in the development of abnormal muscle cells that branch or form small clusters surrounded by two layers of basal lamina (the old layer and the new one which was secreted by the new myofiber) [17,21,32,33]. Spontaneous myofiber regeneration in long-term denervation has been quantified and shown to be non-compensatory and to result in the reduction of satellite cell pools [17,24,25,34-39]. The turnover of myonuclei (but not necessarily of regenerating myofibers) in adult rats, studied by continuous infusion of 5-Bromo-2-Deoxyuridine (BRDU), occurs at a rate of 1 to 2% per week at most [38,40]. Evidence of satellite cell depletion in denervated muscle raises some questions about the long-term potential effects of regenerative myogenesis [35,36]; however, myotoxin-induced myogenesis suggests that a long lasting effect could occur (see below).

Interspecies differences raise additional doubts, since there are significant differences in post-denervation effects, even in rate of atrophy [41]. In humans, denervation atrophy progresses at a relatively slower rate in comparison to rat (years in humans vs months in rodents), but we have observed that the myogenic reactions to denervation in human muscle are very similar to those well described in rodents; this is indeed encouraging as to the potential application of our work in clinical rehabilitation.

Induced Myogenesis in Long-Term Denervation

In addition to traumatic events, regeneration of muscle fibers has been studied after induction of muscle damage and regeneration by vitamin E deprivation [42], autografting [17,18], and as induced by myotoxin treatment [43,44]. Studies demonstrate that permanent denervation does not prevent induced muscle regeneration [32] and a long-term retention of this capability has been demonstrated. For example, four-months after denervation, rats treated with bupivacaine develop massive and synchronous myofiber regeneration in both fast

and slow muscles within a few days of treatment [26,45]. Additionally, when rat muscles are denervated for seven months and then the rats are treated with marcaine or notexin, autografting of the muscles is followed by substitution of old fibers by new fibers [32,34,36,46,47]. Specifically, two weeks after auto-transplantation, the aneurally regenerated myofibers increased in size up to 25% of the normal size of innervated muscle fibers, and then they decreased in size to almost one tenth of their normal adult size [32]. Evidence that some of the proliferating satellite cells re-enter an undifferentiated, stem cell-like state, being capable of myogenesis after further injuries, was provided by a study consisting of a series of myotoxic exposures which provided time for regeneration to occur between each toxic injury. The results revealed that new muscle fibers form after insult for up to four treatments [48,49]. Further, satellite cell proliferation and myofiber regeneration is enhanced in long-term denervated muscles when they are treated with electrical stimulation [50]. This is not surprising because satellite cell proliferation is increased in innervated muscle by increased physical activity, in particular strength training [51], but also by massage [52].

h-bFES of Long-Term Denervated Human Muscles

Over the last few decades there has been an increased interest in the use of home-based Functional Electrical Stimulation (h-bFES) to restore movement to the limbs of immobilized Spinal-Cord Injury (SCI) patients (i.e., upper motor neuron lesion, spastic paralysis) [1-9,53-55]. However, there is another group of SCI patients whose issues are more difficult to treat. Patients living with complete *Conus* and *cauda equina* syndrome present with paralysis and severe secondary medical problems because of the marked atrophy of denervated muscles and the associated loss of bone mass and skin dystrophy [1]. In these patients, injury also causes irreversible loss of the nerve supply to some or all the muscle fibers of the affected limbs, resulting in flaccid paralysis subsequent to lower motor neuron lesion. It is technically more difficult to treat these patients because the absence of functional nerve fibers makes it more difficult to recruit the population of myofibers necessary to regain functional movements at an acceptable force level using surface electrodes. Thus, the electrical energy required to stimulate these muscles directly is greater than that which can be delivered by commercially available stimulation devices used for innervated muscles. Despite these difficulties, pilot studies of the functional clinical application of FES to denervated muscles has been published. One study demonstrated that direct FES of the denervated Tibialis anterior muscle could result in gait correction [56]. Other papers [3-9,57], contrary to widely accepted opinion, have shown that electrical stimulation of even long-term denervated muscles (from 1 to 5 years) can produce muscle contractions strong enough (i.e., tetanic contractions) to restore muscle mass and force production. Indeed, through the successful EU Program: RISE [Use of electrical stimulation to restore standing in paraplegics with long-term denervated degenerated muscles (QLG5-CT-2001-02191)], we have demonstrated that h-bFES therapy improves the muscle condition of mobility impaired persons, even in extreme cases in which post-denervation muscle degeneration has occurred [3-9,57]. In this condition, affected muscles undergo sequential stages of loss of function and then, finally, complete loss of skeletal muscle tissue. We analyzed muscle biopsies from RISE patients at different time points after SCI and discovered that: a) within four to six months of injury the loss of stimulation-induced contractility produced ultrastructural disorganization; b) progressive atrophy persisted for up to 2-years after injury; and c) substantial loss of myofibers appeared more than 3 years after SCI. Importantly, we have shown that h-bFES of denervated muscles can inhibit muscle loss and also recover muscle from degeneration. However, even though h-bFES substantially improves muscle mass and strength, excitability of the treated muscles never reaches the level of normal innervated muscles. Indeed, we reported that long-term discontinuation of h-bFES resulted in loss of the improvements that came with the previous period of treatment [58,59]. Furthermore, with results produced by a 2-year longitudinal prospective study of 25 patients with complete *conus*/cauda equina lesions, we showed that the improvements produced by h-bFES can be maintained over time when treatment is continued [9]. Specifically, in this study, denervated leg muscles were stimulated by h-bFES using the prototype of a custom-designed stimulator and large surface electrodes for long-term denervated muscle [3-9,45,53,57] that are now commercially available (The Stimulette Den 2x , Dr. Schuhfried Medizintechnik GmbH, Vienna, Austria). Samples were harvested both before and after 2 years of h-bFES and muscle mass, force and structure were determined using: (a) computed tomography; (b) measurements of knee torque during stimulation; and (c) muscle biopsies analyzed by histology and electron microscopy. Twenty out of 25 patients completed the 2-year h-bFES program. The data demonstrated that treatment resulted in: (a) a 35% increase in cross-sectional area of the quadriceps ($P<0.001$); (b) a 75% increase in mean diameter of muscle fibers ($P<0.001$); and (c) improvements of the ultrastructural organization of contractile material. Further, an exciting 1187% increase in force output during electrical stimulation ($P<0.001$) was achieved. The recovery of quadriceps force was sufficient to allow 25% of the subjects to perform FES-assisted stand-up exercises, demonstrating that h-bFES of denervated muscle is an effective home therapy capable of rescuing tetanic contractility and muscle mass [9]. Important benefits for the patients included the improved cosmetic appearance of lower extremities and the enhanced cushioning effect for seating. Though demanding as to the daily hours committed to the training, the improvements of quality of life are enough to convince the patients to do not discontinue h-bFES even after many years.

Functional data highly correlate to histo-morphometric results [9]. Interestingly, immunohistochemistry for anti-embryonic MHC revealed that regenerating myofibers were present in all of the muscle biopsies. Some myofibers had central nuclei, a feature suggesting they had regenerated no more than 10-days before muscle biopsy harvesting. Frequency distribution of myofibers according to their minimum diameter in semi-thin sections showed that about 50% were severely atrophic (i.e., having a minimum diameter smaller than 10 μm), but a large proportion of myofibers were eutrophic (i.e., with a minimum diameter larger than 40 μm). The results were substantiated by structure to function correlations and by an advanced clinical muscle imaging technique, i.e., Quantitative Muscle Color-Computed Tomography (QMC-CT) [9].

Quantitative Muscle Color-Computed Tomography (QMC-CT)

Medical imaging, a vital field of research for diagnostic and investigative assessment, is of particular interest here as a tool to recapitulate and quantify internal and external tissue morphologies in a non-invasive manner. The most current research aspires to improve instrument design, image processing software, data acquisition methodology, and computational modeling. In particular, visually simplistic

imaging methods with high-resolution for assessing diseased or damaged tissues are a strategic priority in translational myology research. The follow-up of muscle atrophy/degeneration in neuro-muscularly traumatized people is difficult because of the lack of adequate imaging analyses and also the practical and ethical constraints on harvesting the muscle biopsies necessary to monitor the efficacy of the therapy/rehabilitation strategies. More sensitive methods for quantitative clinical imaging of skeletal muscle are needed. False Color Computed Tomography is popular in cardiology [60], but it is still a novelty for quantitative analyses of total volume and quality of anatomically defined skeletal muscles. QMC-CT is a highly sensitive quantitative imaging analysis recently developed, as a by-product of the Project RISE, to monitor skeletal muscle and perform follow-up examinations of muscle affected by wasting conditions [61-63]. QMC-CT uses CT numbers, i.e., Hounsfield Units (HU), for tissue characterization. It allows for discrimination of soft tissues as follows: subcutaneous fat, intramuscular fat, low density muscle, normal muscle, and fibrous-dense connective tissue. To evaluate this data, pixels within the defined interval of HU values (or, more generally, gray values when these data are not from CT scans) are selected and highlighted, while others with HU values outside the threshold remain black. Specific soft tissues areas are colored as follows: subcutaneous fat (yellow: -200 to -10 HU), intramuscular fat (orange: -200 to -10 HU), low density muscle (cyan: -9 to 40 HU), normal muscle (red: 41 to 70 HU) and fibrous-dense connective tissue (gray: 71 to 150 HU). Thus, soft tissue discrimination and its quantitation can be achieved. The Hounsfield values for the entirety of the lower limbs can be plotted on a histogram to display the tissues profiles [58,59,61-63].

Perspectives

After many years of basic research concerning electro stimulation-induced muscle plasticity, we conclude that functional electrical stimulation using long biphasic impulses and large surface electrodes is able to restore muscle mass, force production and movement in humans even after years of complete irreversible denervation. Patients suffering from flaccid paraplegia (denervation of lower extremity muscles, e.g., *conus* and *cauda equina* syndrome) are especially good candidates for these approaches if the h-bFES managements start from 1 to 5 years from SCI, since both functional and psychological improvements represent an important goal for SCI suffering patients [9].

In the long term, we may be hopeful of development and application of implantable devices as alternatives to the approaches based on surface electrodes. However, because electrical stimulation can elicit pain in patients for whom residual innervation is functional, a better knowledge and control of stimulation-induced muscle trophism must be achieved. Once this control is obtained, artificial synapses (i.e., a pool of miniaturized electrodes which contact each of the surviving or regenerated myofibers in the denervated muscle) would have to be designed and developed. Considering the powerful angiogenesis of regenerating muscle [52], one might consider that the sacrifice of some of the new vascular branches could be worth the development of the ability to deliver sufficient current to new myofibers by means of nano-fabricated intravascular electrodes.

Whether procedures based on *in vivo* protocols such as the induction of muscle damage/regeneration by injection of anesthetics in local anoxic conditions [26,32,34,45,46,52,55] or *ex-vivo* techniques such as the proliferation of autologous myoblasts derived from patient's muscle biopsies (i.e., from their own "satellite cells" or from conversion of autologous fibroblasts to myogenic cells) will develop into applied methods is still open to pre-clinical and clinical research. A promising aim would be to replenish the degenerated skeletal muscles of long-term (more than 5 years) SCI persons with these methods before or at the same time h-bFES for denervated muscles is applied. This option could give SCI patients the opportunity to train muscles which have been denervated for more than 10 years, the vast majority of the existing pools of patients suffering with irreversible, transverse-complete SCI.

Standing on our evidence, [9] we implemented also trials for the rejuvenation of skeletal muscles in elderly and the results were heartening [64-68]. For now, based upon pilot human studies and application of existing experimental knowledge, it can be anticipated that, h-bFES of long-term denervated muscles, using surface electrodes and purpose developed electrical stimulators [9] or, in case of severe muscle atrophy in elderly [64-68], commercially available neuromuscular electrical stimulators may improve mobility with substantial reductions in the risk and the severity of secondary medical problems, resulting in less frequent hospitalizations and a reduced burden on public health services. Integration of h-bFES with good nutrition and exercise where possible (e.g., volitional In-Bed Gym [68,69]) will allow SCI patients and older olds to look forward to improved health, increased autonomy and quality of life, and the prospect of better professional and social integration.

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