Use it or lose it: tonic activity of slow motoneurons promotes their survival and preferentially increases slow fiber-type groupings in muscles of old lifelong recreational sportsmen

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Abstract

Histochemistry, immuno-histochemistry, gel electrophoresis of single muscle fibers and electromyography of aging muscles and nerves suggest that: i) denervation contributes to muscle atrophy, ii) impaired mobility accelerates the process, and iii) lifelong running protects against loss of motor units. Recent corroborating results on the muscle effects of Functional Electrical Stimulation (FES) of aged muscles will be also mentioned, but we will in particular discuss how and why a lifelong increased physical activity sustains reinnervation of muscle fibers. By analyzing distribution and density of muscle fibers co-expressing fast and slow Myosin Heavy Chains (MHC) we are able to distinguish the transforming muscle fibers due to activity related plasticity, to those that adapt muscle fiber properties to denervation and reinnervation. In muscle biopsies from septuagenarians with a history of lifelong high-level recreational activity we recently observed in comparison to sedentary seniors: 1. decreased proportion of small-size angular myofibers (denervated muscle fibers); 2. considerable increase of fiber-type groupings of the slow type (reinnervated muscle fibers); 3. sparse presence of muscle fibers co-expressing fast and slow MHC. Immuno-histochemical characteristics fluctuate from those with scarce fiber-type modulation and groupings to almost complete transformed muscles, going through a process in which isolated fibers co-expressing fast and slow MHC fill the gaps among fiber groupings. Data suggest that lifelong high-level exercise allows the body to adapt to the consequences of the age-related denervation and that it preserves muscle structure and function by saving otherwise lost muscle fibers through recruitment to different slow motor units. This is an opposite behavior of that described in long term denervated or resting muscles. These effects of lifelong high level activity seems to act primarily on motor neurons, in particular on those always more active, i.e., on the slow motoneurons. The preferential reinnervation that follows along decades of increased activity maintains neuron and myofibers. All together the results open interesting perspectives for applications of FES and electroceuticals for rejuvenation of aged muscles to delay functional decline and loss of independence that are unavoidable burdens of advanced aging.

Trial Registration: ClinicalTrials.gov: NCT01679977

Key Words: aging, human skeletal muscle. lifelong physical exercise denervation and re-innervation, fiber type grouping; co-expression of fast and slow myosin heavy chains.

It is since long accepted that histological aspects of muscle suggest that denervation contributes to tissue atrophy in aging. The term "disseminated neurogenic atrophy" was used to describe the progressive accumulation and clustering of small, angular fibers.[1-4] Supporting evidence of a progressive loss of α-motoneurons has also been described,[5,6] and corroborating electrophysiological studies have confirmed a decrease with aging of number of motor units and an increase in their size, suggesting some reinnervation events.[7] Further evidence supporting rounds of denervation and reinnervation is based on the spatial distribution of fibers that in rodents becomes more clustered with advancing age.[5,8] In young humans

fiber types appear also randomly distributed across the muscle and become increasingly grouped together in aging.[9] It has therefore be proposed that apoptosis of α-motoneurons in the spinal cord (or axonal and neuromuscular junction impairments) with subsequent incomplete reinnervation of fibers by surviving motor neurons is a contributing factor to the loss of muscle strength and mass with age.[10] These rearrangement processes are accompanied by progressive relative increase of slow muscle fibers, though the literature provides contradictory results.[11] Some of this discrepancy has been dispelled by comparisons of muscle from normal and immobile patients: the inactive elderly have a shift toward fast isoform expression, as is common in "unloaded" muscle, e.g., during spaceflight,[12] or limb suspension, immobilization and paralysis,[13-15] whereas muscle wasting in "normal" elderly is accompanied by a shift toward a slow twitch phenotype. Thus the actual expression pattern of myosin isoforms in seniors is modulated by complex factors because it depends upon the conflicting influences of both aging and reduced activity tending to shift toward slow and fast isoforms, respectively.[16] To further complicate the situation, conflicting results regarding fast to slow myosin transition arise in endurance training studies using animal models and in clinical trials of humans involving either voluntary exercise or electrical stimulation (directly to denervated muscle or indirectly through nerve stimulation).[13,15,17-28] Whether in aging these shifts are under neural control, direct effect of use/disuse on muscle fibers or both mechanisms it remains to be clarified.

In the present review, we recapitulate results of analyses of muscle biopsies harvested from the *Vastus lateralis* of senior (65 to 79 years) recreational sportsmen, i.e., of subjects who from youth had routinely practiced sport activities usually more than three times a week, and up to the time of biopsy.[29] We would like to stress that this is an experimental model that is opposite to the more common studies of adaptation to muscle disuse. In agreement with previous studies of master athletes,[30-32] we demonstrated that lifelong high-level physical activity considerably increased the percentage of slow-type myofibers and the number of muscle fiber-type groupings.[9,11] The latter are direct evidence that lifelong cycles of muscle fibers denervation and reinnervation occurred. In other interim reports, we have demonstrated that muscle properties of these senior recreational sportsmen are more similar to those of active young men than to those of sedentary seniors.[33-35] Thus, our studies support the concept that lifelong high-level exercise has a beneficial effect on the motoneurons and, through them, on reinnervation of muscle fibers, resulting in preservation of muscle size, structure, ultrastructure[36] and function. New evidence in rodents are accumulating that volitional exercise have positive effects on cancer cachexia progression by modulating muscle fiber autophagy.[37] Further, preliminary results in rodents confirm that physical activity can also directly counteract tumor cell growth in skeletal muscle, a metabolically active tissue that is usually not a target for metastasis.[38]

Muscles harvested from three groups of persons were analyzed as detailed in Mosole et al.[29]

Study Subjects: Approval from the national committee for medical ethics was obtained before study onset (EK08-102-0608). All recruited subjects were male volunteers who received detailed information on the study and gave informed consent. Groups of young men (n=5; aged 22-33 years, 10 Vastus l. biopsies), seniors with normal life style (sedentary, n=6, aged 67-74 years, 10 Vastus l. biopsies) and seniors with a lifelong history of high-level recreational sport activities (n=7; aged 65 to 79 years, 10 Vastus l. biopsies) were enrolled. All subjects were apparently healthy and declared not to have any specific mobility impairment or disease.
Muscle biopsies: Upon enrollment, needle muscle biopsies were harvested through a small skin incision from the *Vastus lateralis* muscle and then frozen for light microscopy or fixed for electron microscopy.
Light microscopy and quantitative histological analyses: Serial cryosections (8 μm) from frozen muscle biopsies were mounted on polysine™ glass slides, air-dried and stained either with Hematoxylin and Eosin (H&E) or using conventional techniques for myofibrillar ATPases to evaluate muscle fiber types.[39] Immunohistochemistry of fast and slow Myosin Heavy Chains (MHC). Serial cryosections air-dried, washed and permeabilizated with 0.1% Triton (Sigma-Aldrich). The sections were incubated in primary monoclonal antibody, the anti MHC fast and slow (Novocastra) and then incubated with secondary antibody, anti-Mouse-Cy3 (Sigma-Aldrich 1:100) for MHC slow and anti-Mouse-FITC (Sigma-Aldrich 1:100) for MHC fast. The sections then mounted on glass slides using ProLong Gold antifade reagent with DAPI (Life Technologies). Other sections were treated with Co-immunofluorescence of anti-fast, anti-slow MHC and anti-Laminin antibodies in single sections. Morphometric analyses: Morphometric analyses of the fiber diameter and of the fiber type distribution were performed on cryosections using Scion Image for Windows version Beta 4.0.2 (2000 Scion Corporation) as previously described. [14,15,35,39-42]
Statistical analysis: ANOVA tests were performed with statistics algorithms of Origin™, OriginLab Corporation, USA. The level of statistical significance was set at p<0.05.

Small angular muscle fibers in young men and in septuagenarians have the size and the morphology of denervated muscle fibers

From our previous studies on skeletal muscle biopsies of paraplegic patients we know that muscle disuse resulting from decades of years of upper motor neuron

lesion (central denervation) induces at most a 50% decrease in size (i.e., from a myofiber diameter of approximately 70 μm to 35 μm),[14] while lower motor neuron denervated skeletal muscle, one year after denervation, shows muscle fibers with a diameter less than 30 μm.[15,40-42] Based upon these findings, we are confident in defining those muscle fibers having a diameter smaller than 30 μm as denervated. This is strengthened by the facts that half of them had diameters smaller than 25 μm and that several had angular aspects and were N-CAM positive.[29] In muscle biopsies of young subjects the muscle fibers with diameter less than 30 μm are seldom observed (0.4 %), while biopsies harvested from the sedentary seniors contain the highest percentage (6.5 %) of denervated muscle fibers. When denervated muscle fibers with diameter less than 30 μm are distributed according to their diameter, almost half of them have values less than 25 μm, and the sedentary seniors maintained the highest percentage (2.6%). The ANOVA tests confirmed that their higher amounts in sedentary seniors vs. both young men and senior recreational sportsmen were statistically significant. On the other hand, the differences between young men and septuagenarian amateur sportsmen did not reached statistical significance.[29]

Percentages of fast and slow myofibers in young men and in old sedentary or recreational sportsmen show significant shift toward slow fibers in the latter

Percentages of immunolabeled fast and slow muscle fibers (fibers positive after labeling with an either anti-MHC fast or an anti-MHC slow antibody) in young men vs. sedentary seniors were not statistically different, while in senior recreational sportsmen the slow fibers prevailed (68.5 %), the increase versus both young men (42 %) and sedentary seniors (46 %) being statistically significant.[29] It is worth noting that in sedentary seniors the fast fiber-type groupings prevailed, in agreement with previous data.[11]

Fiber-type groupings are almost absent in young men. Fast fiber type groupings are present in sedentary seniors, but in senior sportsmen there is a much higher content of slow type groupings

Fiber-type groupings were identified on the basis that one muscle fiber is completely surrounded by fibers of the same phenotype. Thus percentages of type-groupings are determined counting how many muscle fibers in the biopsy are surrounded by fibers of the same type. To avoid the problems related to the many different fast isoforms of fast MHC and the fact that our anti-fast MHC antibody do not discriminate among the fast isoforms, we focused our attention on the slow fiber-type clusters. We report also data of so called fast fiber-type groupings, but these are not true fiber-type clusters, since they contain at least three different types of muscle fibers.[13] The biopsies harvested from the

young subjects contained seldom areas in which the muscle fibers were grouped and they were of fast type (< 1 %). Some fast type groupings were present in the biopsies harvested from sedentary seniors: the central fibers characterizing fast type groupings being 3.0% of the total muscle fibers, while those of slow-type were around the 0.5%.[29] Even more evident was the fact that in the biopsies harvested from senior amateur sportsmen the slow type fibers were clustered in large areas (mean 7.9 %), reaching almost the 25% in the extreme case in which 93% of total myofibers were of the slow type.

Muscle fibers co-expressing fast and slow MHC in the three groups of muscle biopsies were infrequent and differences were not significant

Muscle fibers co-expressing fast and slow MHCs were seldom observed in all the analyzed biopsies and the ANOVA tests were not significant.[29] Serial sections from sedentary seniors presented co-expressing small-size angular (denervated) muscle fiber. Since those myofibers were angulated and very small (diameter less than 25 μm), we suggested that they were low myofibers co-expressing fast isoforms of MHC, as it is common in denervated and unloaded muscles, e.g., during spaceflight[12] or limb suspension, immobilization, spinal cord injury and peripheral denervation.[13-15,17,18,43,44] Muscle sections from senior recreational sportsmen were also immuno-stained with anti-fast MHC, anti-slow MHC, and with anti-laminin antibodies. The vast majority of those muscle fibers were positive to the anti-slow MHC (in green), some were positive to the anti-fast MHC (in red), while two of them were orange, since they were positive to both anti-MHCs.[29] It is of note that their size was similar to those of the pure fast or slow myofibers. Their content (very low) in the section was not in agreement with the concept that they were belonging to a motor unit undergoing exercise-driven slow-type transformation of myosin heavy chains. Thus, these normal-size co-expressing fibers were ex-denervated fast-type fibers co-expressing slow isoforms after reinnervation by sprouts from slow axons.[29]

Co-expressing muscle fibers are transforming myofibers, a direct evidence that some muscle plasticity is occurring in both sedentary seniors and senior recreational sportsmen.

It is worth to stress that the transforming myofibers participated to enlargement of slow-fiber type groupings.[29] Furthermore their densities in the sections of muscle of lifelong high level recreational sportsmen were not in agreement with the concept that they are belonging to a motor unit that is undergoing exercise-driven transformation. Our working hypothesis is that they are either denervated slow myofibers re-expressing fast isoforms (in sedentary seniors) or denervated fast fibers reinnervated by axon sprouting from slow motor neurons (in recreational sportsmen).[9,13,43-45]

Comparisons between sedentary seniors and same age recreational sportsmen makes obvious that the observed differences are not a function of age

It has long been recognized that denervation significantly contributes to aging muscle atrophy,[1-5] that muscle disuse accelerates the deterioration process,[16] while running activity performed for decades (as occurs in Master athletes)[46-48] protects against the age-related loss of motor units,[49-51] and of lean muscle mass.[52] However, the degree to which denervation causes in aging human muscle MHC transformation and loss of myofibers is an open issue, since reinnervation events may compensate in the muscle tissue, long-term or short-term, effects of motor neuron loss in spinal cord and/or of axonal abnormalities in peripheral nerves.[7-9,53]

In our study,[29] we used immunolabeling methods to analyze muscle biopsies harvested from septuagenarian recreational sportsmen and compared their relative amount of: 1. small angular myofibers (denervated muscle fibers), 2. fast and slow muscle fibers (muscle plasticity), and 3. central muscle fibers of fiber-type clusters (reinnervated muscle fibers) with those in muscle biopsies of sedentary septuagenarians and young men. The main results were: i) biopsies from young men seldom contain denervated, reinnervated or transforming muscle fibers; ii) biopsies from sedentary seniors contain both denervated and a few reinnervated clustered myofibers of the fast type; and iii) senior recreational sportsmen present with a larger percentage of healthy slow myofibers, that appear mainly clustered in slow fiber-type groupings.[29,33]

In further support, we proved that the actual range of age of recruited seniors is not correlated with percentages of slow fibers and slow fiber-type groupings in both sedentary and recreational sportsmen ($R^2 < 0.03$). On the other hand, the percentages of slow fibers and of slow fiber-type groupings were strongly correlated ($R^2 = 0.82$).[29] Further, the Gaussian distribution of the percentages of slow fibers in the biopsies harvested from *Vastus lateralis* of septuagenarian recreational sportsmen do not support a correlation between the kind of training and the percentages of slow type fibers: whether they performed mainly strength training, endurance training or a mixture of both strength and endurance trainings (mixed training) they were randomly distributed both among low or high transformed muscle biopsies. The important factor is not the age *per se*, or the kind of activity the senior recreational sportsmen performed, but the amount of activations/contractions during the previous many decades. Taking into account that slow motoneurons are active at least 20 times more often per day than the fast motoneurons,[54,55] in both everyday life and in sports activities, it is this higher-level of activity that is most likely to maintain motoneuron axons, muscle fibers and their MHC content.[17,20,29]

Our working hypothesis is that the muscle fibers co-expressing fast and slow MHCs contribute to the process of slow type transformation and clustering as random events that can't be interpreted as the result of the synchronous transformation of the whole fibers belonging to a motor unit, nothing to say to the large motor units in muscles of the cohort of senior recreational sportsmen, a mechanism that is well known to occur in cross-reinnervation models[13,45], while it is more presumed than demonstrated after volitional exercise.[11]

Our data suggest that slow-type transformation by reinnervation in senior recreational sportsmen is a clinically relevant mechanism despite the facts that: a) in agreement with interim results by histochemical ATPase staining,[33] muscle biopsies harvested from senior recreational sportsmen vary from those with scarce fiber-type transformation and groupings to those with almost fully transformed muscles, in which isolated fibers co-expressing fast and slow MHCs fill in the gaps; and b) there are many potential confounding factors such as the sampling of the *Vastus lateralis* (an heterogeneous muscle), individual genetic backgrounds, differences in the kind and extent of the high level physical activities. Despite these limitations, muscle properties of the group of senior recreational sportsmen are more similar to that of active young men than to those of sedentary seniors: specifically, relative to their sedentary cohorts, senior recreational sportsmen have greater muscle maximal isometric force, better mobility functions and better preserved muscle morphology and ultrastructure. [24-27,29,34,35] On the other hand we agree that our speculations need further study, in particular adding samples and subjects.

Co-expression of fast and slow MCHs is detected in muscle biopsies of any subject group, but in muscle fibers of different size

When they are found in the sedentary senior samples, the mixed fast and slow fibers are small and angular, suggesting that they are denervated slow type myofibers re-expressing fast MHC. They are commonly found in muscle atrophying as a result of denervation or unloading.[12-16] On the other hand, when the muscle fibers co-expressing fast and slow MHC are detected in skeletal muscle of senior recreational sportsmen, they are similar in size to the other muscle fibers and, therefore, are not lacking innervation. These normal-size muscle fibers co-expressing fast and slow MHC are likely denervated fast-type fibers co-expressing slow isoforms after reinnervation by sprouts from slow axons, a direct evidence that some muscle plasticity is still occurring in both sedentary seniors and in particular in senior recreational sportsmen. It is also noteworthy that the transforming myofibers participate in the enlargement of slow-fiber type groupings.[29]

Lifelong high-level exercise seems to allow the body to adapt to the consequences of age-related denervation and to preserve muscle structure and function by saving

otherwise lost muscle fibers through recruitment to different, mainly slow, motor units.

Taken together our results suggest that, beyond the direct effects of aging and of a lifelong history of high-level recreational sport activities on structure and function of the muscle fibers, changes occurring in the muscle tissue appear to be the result of sparse incremental denervation and reinnervation. In senior recreational sportsmen the increase in percentage of clustered slow fiber is conceivably the result of the positive effect of lifelong physical activity on the motoneuron pool, which has mainly spared the slow motoneurons from age related apoptosis/death, increasing the chance that peripheral reinnervation occurs for the sprouting of slow axons. Thus, regular physical activity is a good strategy to attenuate muscle functional decline and structural abnormalities associated with aging. Other mechanisms contribute to lifelong muscle health; however, our data support the concept that lifelong high-level exercise has a beneficial effect on motoneurons and/or their axons, mainly of the slow type, and, through them, on muscle fibers, maintaining their size, structure and functions.

Ongoing experiments are investigating a clinically translatable daily muscle stimulation paradigm in rats following nerve injury. Results show that reinnervation of muscle and functional behavioral metrics are enhanced with daily stimulation, with upregulation of intramuscular neurotrophic factors as a potential mechanism.[56]

Stimulation over a three-month period maintain elevated muscle-derived GDNF but not BDNF mRNA. Electrical stimulation elevates intramuscular trophic factor mRNA levels which may explain how electrical stimulation enhances neural regeneration following nerve injury.[57]

In addition, the impact of stimulation on terminal sprouting, a mentioned negative aspect of electrical muscle stimulation, was a minor contributor to long term functional reinnervation of stimulated muscles in that study.[56]

Conclusions

Sound evidence of efficacy in sedentary seniors of induced contractile activity are provided, beside those of physical exercise,[11] by the beneficial effects of Functional Electrical Stimulation (FES) in sedentary seniors.[23-27,35,36,40,42,48,51,52,58-63] Equally strong are the structural, functional and clinical proofs that home-base FES counteracts and reverses severe muscle atrophy and degeneration in the extreme case of irreversible muscle denervation of leg muscles due to complete spinal cord injury at the level of the *Conus* and *Cauda Equina*.[14,15,39-42,48,58,64-70]

Altogether these results open interesting perspectives for further applications of FES and electroceuticals for rejuvenation of aged muscles,[24-28,35] to counteract age-related mobility decline and loss of independence that are the heavy burdens of advanced aging.

Conflict of Interest

All authors declare no conflict of interests.

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