

Physical training is associated with changes in Nuclear magnetic resonance and morphometrical parameters of the skeletal muscle in senescent mice

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The effect of a three-month training period on T2 relaxation time as well as on myofibre size and type was investigated in the lower limbs of senescent mice. After training, T2 (which is a magnetic resonance imaging parameter known to increase during acute exercise) was significantly higher in trained mice (36.37 ± 1.27 vs 37.76 ± 2.06 ms, $p=0.003$, $n=8$), whereas no change was found in non-trained animals (36.35 ± 1.02 vs 36.24 ± 1.15 ms, $p=0.278$, $n=8$). The percentage of muscle limb area evaluated *in vivo* on magnetic resonance images before and after the experimental period was unchanged in trained mice (69.84 ± 2.50 vs 70.29 ± 2.29 , $p=0.896$, $n=3$) and decreased in non-trained animals (72.98 ± 1.68 vs 64.62 ± 2.34 , $p=0.006$, $n=3$). Cross-sectional area of fast and slow myofibres, evaluated on paraffin-embedded samples after immunolabelling for skeletal fast fibre myosin, was lower in non-trained than in trained mice in both gastrocnemius and quadriceps muscle, but no change in slow/fast fibre ratio nor in apoptotic rate was found. These data show that training can prevent sarcopenia in senescent mice by affecting muscle status and inducing myofibre hypertrophy in the absence of significant muscle damage.

Key words: training, ageing, skeletal muscle, magnetic resonance imaging, fibre type, sarcopenia.

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Ageing is associated with a progressive decline of muscle mass, strength and quality, a condition overall known as sarcopenia. These age-related changes have been reported even among healthy, physically active subjects and the rate of muscle loss in humans has been estimated to range between 1 and 2% per year, past the age of 50 (Hughes *et al.*, 2002). Therefore, sarcopenia represents a powerful risk factor for frailty, loss of independence and physical disability in elderly (Roubenoff *et al.*, 2000). In addition to the impairment of motor function, sarcopenia can involve a number of metabolic and physiological consequences which have been only partially investigated: changes in muscle mass can be associated with osteoporosis (Szulc *et al.*, 2005), altered thermoregulation (e.g., decreased thermogenic capacity of muscle due to reduced mass) (Wilson and Morley, 2003) as well as with a decrease in resting metabolic rate secondary to decreased fat-free mass and decreased physical activity, resulting in a higher prevalence of insulin resistance, type 2 diabetes mellitus, dyslipidemia, and hypertension (Karakelides and Sreekumaran Nair, 2005).

The contributing mechanism(s) leading to sarcopenia remain to be fully elucidated; they are probably multifactorial, including among others: denervation and reinnervation of motor units (Ansved and Larsson, 1989), alteration in the hormonal milieu in which anabolic hormone concentrations progressively decline (Tenover, 1997), elevated concentrations of inflammatory mediators (Bruunsgaard *et al.*, 2001), myonuclear loss through apoptotic mechanisms (Dirks and Leeuwenburgh, 2005), loss of satellite cells (Verdijk *et al.*, 2007), decrease in microvascular function and exercise tolerance (Payne and Bearden, 2006).

Understanding the mechanisms which induce to sarcopenia therefore represents an essential step towards the development of effective interventions to contrast muscle loss and physical disability at

advanced age. At present, physical exercise has been recognized as the most effective activity to prevent or treat age-related muscle wasting (Marcell, 2003).

In this study we investigated the effect of a programmed long-lasting aerobic physical exercise on the skeletal muscle of senescent mice by combining nuclear magnetic resonance (NMR), which provides *in vivo* information on muscle status, and light microscopy techniques (morphometry and immunohistochemistry) to obtain a quali-quantitative characterization of myofibres.

Materials and Methods

Sixteen 20 month-old male mice were used in this study: eight mice were trained by treadmill running (45 min at 9.5-14.2 m/min five days a week) for three months, while eight had only spontaneous activity in the cage. All animals had free access to chow and water. The muscles chosen for our study were the quadriceps and the gastrocnemius, mostly containing fast, type II fibres and therefore largely affected by sarcopenia (Larsson *et al.*, 1978; Lexell, 1995).

Nuclear magnetic resonance

Magnetic resonance imaging was performed before and at the end of the three-month experimental period. Mice (n=8 for each experimental group) were anaesthetized with isoflurane and imaged in a Bruker Biospeck tomography equipped with an Oxford 33 cm bore magnet operating at 4.7 Tesla. Quantitative T2 maps of the lower limb muscles were taken (spin-echo multiecho sequence, FOV 4cm, slice thickness 2 mm, interslice distance 4 mm, TR 2000 ms, TE 10 ms, matrix size 128x128, in-plane resolution 0.312 mm²). In three animals of either experimental group, 3D imaging of the lower limbs was performed for morphometry: a spin-echo T1 weighted sequence was used (FOV 3,5 cm, slice thickness 40 mm, TR 200 ms TE 6 ms, matrix size 128x128x64, in-plane resolution 0,237 mm x 0,237 mm x 0,547 mm); eight coronal sections (Figure 1) per animal, taken at given distances from the coxo-femoral joint, (four acquired before and four at the end of the experimental period) were submitted to morphometrical analysis by using the software Image J (NIH): the total area of each leg section and the area occupied by the muscle tissue were measured, then the percentage of leg

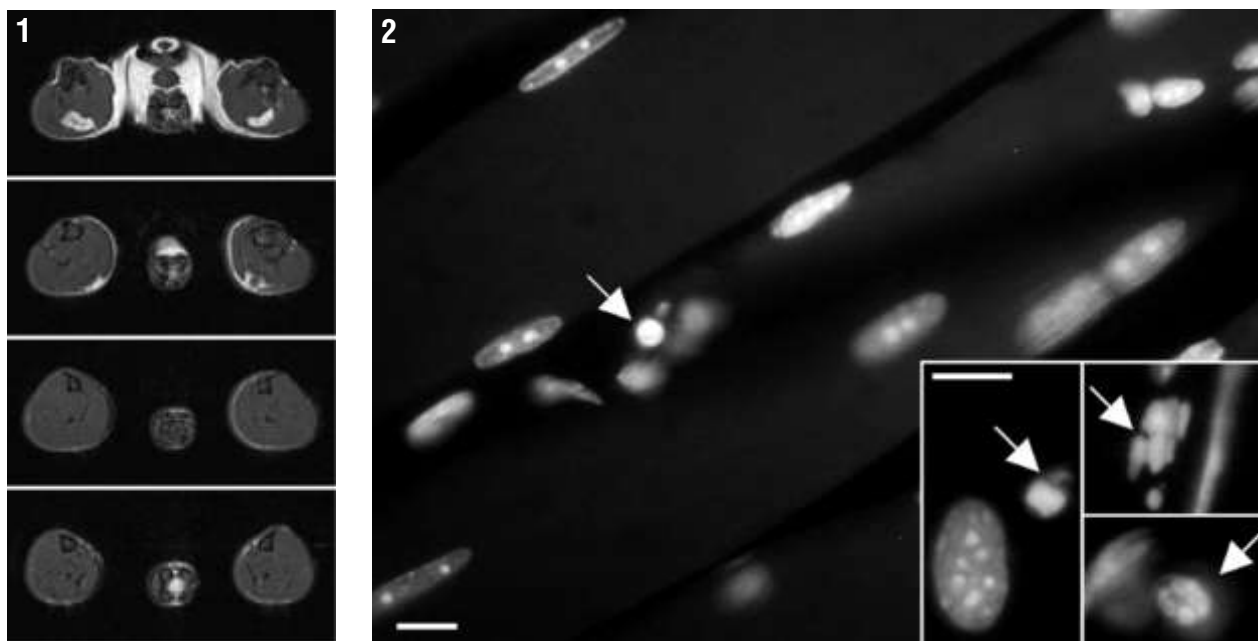


Figure 1. Magnetic resonance imaging of the coronal sections of mouse lower limbs; for quantitative analysis, the percentage of total limb area occupied by the muscle in the four sections was calculated and compared before and after the three-month experimental period.

Figure 2. Quadriceps from a trained mouse, DNA staining with Hoechst 33258: apoptotic nuclei (arrow) are quite scarce. B-D: Apoptotic nuclei (arrows) showing typical chromatin condensation and fragmentation. Bars: 20 μ m.

sectional surface occupied by muscle was calculated in order to estimate the muscle area independently from the leg dimension.

Light microscopy

At the end of the experimental period three trained and three non-trained mice were anaesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The gastrocnemius and quadriceps muscles were further fixed by immersion for several hours at 4°C in the same fixative solution, then dehydrated with ethanol and embedded in paraffin wax. Five- μm -thick transversally sectioned muscle samples were either stained with hematoxylin-eosin or immunolabelled for fibre typing: sections were incubated overnight at 4°C with a mouse monoclonal antibody recognizing the heavy chain of the skeletal fast fibre myosin (clone MY-32, Sigma-Aldrich, Buchs, Switzerland), then revealed with an Alexa 488 conjugated antibody against mouse IgG (Molecular Probes, Invitrogen, Milan). The sections were finally counterstained for DNA with 0.1 mg/mL Hoechst 33258, to detect apoptotic nuclei based on chromatin morphology. Micrographs were taken with an Olympus BX51 microscope equipped with a 100 W mercury lamp under the following conditions: 330- to 385-nm excitation filter (excf), 400-nm dichroic mirror (dm), and 420-nm barrier filter (bf), for Hoechst 33258; 450- to 480-nm excf, 500-nm dm, and 515 nm bf, for Alexa 488. Images were taken with an Olympus Camedia C-5050 digital camera and stored on a PC by the Olympus software, for processing and printing. Morphometrical evaluation of fibre size was performed on samples immunolabelled for fast myosin (to assess fast and slow fibre size separately): the cross-sectional area of 100 muscle fibres per sample was measured at 40X magnification by using the software Image J (NIH). Moreover, the percentage of fast and slow muscle fibres was calculated on a total of 400 fibres per sample. Finally, the percentage of apoptotic nuclei was evaluated on a total of 1000 cell nuclei per muscle sample.

Statistics

Results for each variable were pooled according to the experimental groups and the mean \pm standard error of the mean (SE) values calculated. Statistical comparisons were performed by one-way ANOVA test (the statistical significance was set at $p < 0.05$).

Results

Nuclear magnetic resonance

In lower limb skeletal muscles from trained mice, T2 relaxation time increased significantly (36.37 ± 1.27 vs 37.76 ± 2.06 ms, $p = 0.003$) at the end of the experimental period, whereas in non-trained animals no change was found (36.35 ± 1.02 vs 36.24 ± 1.15 ms, $p = 0.278$).

Morphometrical evaluation of coronal sections of lower limbs revealed similar values for the percentage of muscle area in trained animals before and after the experimental period (69.84 ± 2.50 vs 70.29 ± 2.29 , respectively, $p = 0.896$), whereas muscle area significantly decreased in non-trained animals (72.98 ± 1.68 vs 64.62 ± 2.34 , $p = 0.006$). The percentage of the muscle area was similar in the two groups before the experimental period ($p = 0.303$).

Light microscopy

Morphological observation of muscle samples did not reveal necrotic features in trained nor in non-trained mice. Apoptotic nuclei (Figure 2) were quite scarce and their percentage did not differ in the two groups: $0.20 \pm 0.05\%$ vs $0.25 \pm 0.04\%$ ($p = 0.487$) and $0.25 \pm 0.06\%$ vs $0.21 \pm 0.05\%$ ($p = 0.783$) in the quadriceps and in the gastrocnemius muscle, respectively.

Morphometrical analyses demonstrated a significantly smaller myofibre cross-sectional area in non-trained than in trained mice in both gastrocnemius and quadriceps muscle; such a difference was associated with smaller fast and slow fibres, although the size difference was much more evident in the fast fibres (Figure 3A, B). No change in the percentage of slow and fast myofibres was found in either muscle of the two groups (Figure 3C).

Discussion

Sarcopenia consists in the reduction of skeletal muscle mass, strength and resistance, with consequent impairment of performance in older individuals. It is known that sarcopenia shows a loss of muscle fibres as well as a decrease in total cross-sectional area of the remaining myofibres (Lexell, 1995; Brooks and Faulkner, 1994), especially in muscle which mostly contain type II fibres (Larsson *et al.*, 1978; Lexell, 1995). Accordingly, we observed a significant reduction in both muscle mass and myofibre size in non-trained senescent

mice; in particular, although both fast type II and slow type I myofibres were significantly smaller than those of trained mice, fast fibres showed a higher sensitivity (30-50% of size reduction in comparison to 16-17% in slow fibres) to the sarcopenic process, consistent with previous findings (Vandervoort, 2002; Alway *et al.*, 2003). In the muscles analysed in this study, there was no evidence for the conversion from fast type II fibres to slow type I fibres nor for a clustering of slow fibres; however, we cannot exclude that this phenomenon may occur after longer ageing periods.

Senescent mice submitted to a 3-month training programme did not show any alteration in muscle mass and myofibre size, indicating that a programmed long-lasting aerobic physical exercise can counteract the sarcopenic process. In addition, our data suggest that physical exercise would maintain the senescent muscle in a long-lasting *activated* condition: in fact, we found a slight but significant increase of T2 values in trained mice after an overnight rest; previously, increased T2 values in skeletal muscle were reported during acute exercise, representing an index of strong muscle recruitment, intense contraction or high metabolic state (Patten *et al.*, 2003). Although physical exercise increases oxidative stress on skeletal muscle, it appears to have a positive effect on mitochondrial

density and functions (Coggan *et al.*, 1992). It is possible that a moderate, daily physical exercise can improve mitochondrial activity without severely increasing free radical generation, thus having a positive effect on the whole muscle metabolism. Moreover, it has been recently reported (Melov *et al.*, 2007) that physical training induces in elderly skeletal muscles a gene transcription pattern quite similar to young individuals, thus demonstrating that physical activity not only affects the whole functional status of the muscles, but also reverts already established age-related changes.

Interestingly, neither necrotic features nor prominent apoptotic rate were observed in both trained and non-trained mice. Apoptotic rate in skeletal muscle has been shown to increase with ageing and disuse as well as with acute exercise, whereas it decreases with physical training; however, the role of apoptosis in sarcopenic muscle fibre loss is still debated (reviews in Dirks, 2005; Dupont-Versteegden, 2005). In this work the training protocol used did not induce cell damage or death in the presence of maintained muscle mass, thereby suggesting that moderate, constant physical activity is beneficial to sarcopenia; on the other hand, spontaneous motor activity could not prevent sarcopenia, albeit increase of necrotic or apoptotic processes in myofibres was not found.

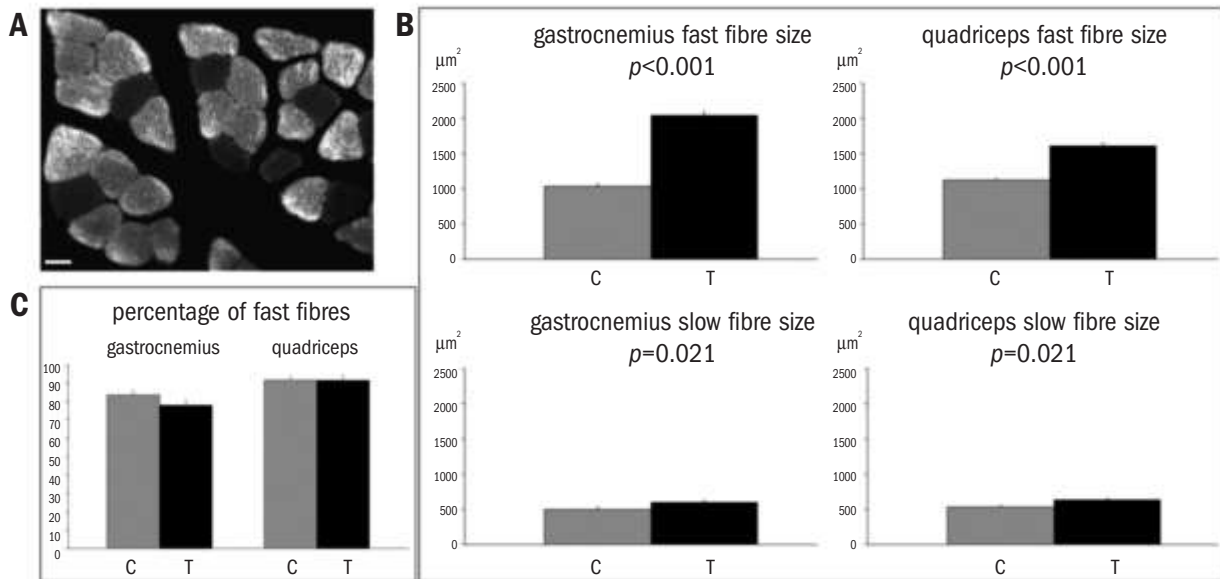


Figure 3. Immunolabelled fast type II fibres in the gastrocnemius of a trained mouse. Bar: 20 µm. **B.** Both fast and slow fibres of the gastrocnemius and the quadriceps show significantly smaller size in non-trained (control, C) than in trained (T) mice. **C.** The percentage of fast fibres does not change between non-trained (control, C) and trained (T) mice in both the gastrocnemius ($p=0.233$) and the quadriceps ($p=0.949$).

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