Effect of physical exercise on the ultrastructural features of skeletal muscle mitochondria in old mice

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Summary
Sarcopenia is an age-related decline of muscle mass, strength and quality which represents a potent risk factor for frailty, loss of independence and physical disability in elderly. The mechanisms leading to sarcopenia are still largely unknown and no specific therapy is presently available to counteract its onset or progress. Several studies have stressed the importance of physical exercise as an effective approach to prevent/limit the sarcopenic process. In the present work we have investigated at transmission electron microscopy the effects of treadmill running on the mitochondrial structure in aged skeletal muscle by comparing exercised versus sedentary old (28 months) mice, and using adult (12 months) individuals as control. Our observations demonstrated that ageing induces an accumulation of mitochondria characterised by larger size and longer cristae than in adulthood, and by a frequent association with lipid droplets. The mitochondrial alterations are partially reversed in old mice after treadmill running, thus providing further evidence that an adapted physical exercise may represent a suitable non-pharmacologic approach to limit the negative effects of ageing on the skeletal muscle, even when applied at late age.

Key words: ageing, mitochondria, physical exercise, skeletal muscle.

Introduction
Ageing is associated with a progressive decline of muscle mass, strength and quality, a condition overall known as sarcopenia (Thompson, 2009); among healthy, physically active subjects the rate of muscle loss in humans has been estimated to range 1-2% per year, past the age of 50 (Hughes et al., 2002). In addition to motor function impairment, 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 the resting metabolic rate secondary to decreased fat-free mass and diminished physical activity, leading to a higher prevalence of insulin resistance, type 2 diabetes mellitus, dyslipidemia, and hypertension (Karakelides and Sreekumaran Nair, 2005). Therefore, sarcopenia represents a potent risk factor for frailty, loss of independence and physical disability in elderly (Roubenoff et al., 2000).

The mechanisms leading to sarcopenia are still largely unknown and no specific therapy is presently available to counteract its onset or progress. Studies performed on humans and other mammals have stressed the importance of physical exercise as an effective - although still debated - approach to prevent/limit the age-related muscle mass loss (see e.g., Yarasheski, 2002; Marcell, 2003; Zancanaro et al., 2007; Bautmans et al., 2009; Betik et al., 2008, 2009; Malatesta et al., 2011). In the attempt to elucidate the biological mechanisms responsible for the beneficial consequence of physical activity, many studies have been
addressed to several aspects such as inflammation (Murton and Greenhaff, 2010) and stress factors (Morton et al., 2009), protein metabolism (Koopman and van Loon, 2009; Kumar et al., 2009), mitochondrial (Peterson et al., 2012) or neuromuscular function (Aagaard et al., 2010), satellite cells’ effectiveness (Snijders et al., 2009; Kadi and Ponsot, 2010). However, most of these studies were aimed at investigating biochemical and molecular aspects, while the fine structural modifications of the myofibre organelles associated with physical exercise have been scarcely explored.

In the present work we have investigated at transmission electron microscopy the effects of physical training on mitochondrial structure in aged skeletal muscle by comparing exercised versus sedentary old mice, and using adult individuals as control. We focused on the hind limb as the most relevant to locomotion in mice, in particular we analyzed the quadriceps femoris muscle which is mainly composed by fast type II fibers that are especially prone to sarcopenia (Larsson et al., 1978; Lexell, 1995).

### Materials and Methods

**Animals and physical training**

Three adult (12 months) and six old (28 months) male mice from the INRCA breed (Ancona, Italy) were used in this study. The INRCA breed is a forty-year established Balb-c mice strain which has been widely used for studies on physiological ageing: these mice have a long life (mean life span 25 months; maximal life span 34 months; Mocchegiani et al., 2007), and a relatively low incidence of pathologies, in particular tumors (Staats, 1980; Bronson and Lipman, 1993). Animals were bred as a close colony, maintained under standard conditions (24±1°C ambient temperature, 60±15% relative humidity, and 12 h light/dark cycle), and fed *ad libitum* with a standard commercial chow diet. Three old mice were trained by treadmill running (30 min at 9 m/min belt speed, five days a week) for one month (old running group: OR); three old mice (old sedentary group: OS) and three adult animals (adult sedentary group: AS) had only spontaneous free-moving activity in the cage. Ultrastructural morphometry is a quite demanding method; therefore the number of investigated animals per group was kept to the minimum required for}

![Figure 1. Transmission electron microscope micrographs of quadriceps femoris myofibres from adult (a), old sedentary (b) and old running (c) mice. Note the large mitochondria aligned among the myofibrils (arrows) or arranged in clusters in the sub-sarcolemmal region (asterisks).](image-url)
statistical analysis. In order to avoid possible interference of acute with chronic effects of physical exercise, the animals were killed three days after the last treadmill session.

**Transmission electron microscopy**

The mice were deeply anaesthetized with pentobarbital (50 mg/Kg i.p.) and then perfused via the ascending aorta with a brief prewash of 0.09% NaCl solution followed by 300 mL of a fixative solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at 4°C. Quadriceps femoris muscles were quickly removed and placed in the same fixation solution for 2 hr at 4°C. After fixation, samples for ultrastructural morphology were rinsed with PBS, post-fixed with 1% OsO₄ for 2 hr at room temperature, dehydrated with acetone and embedded in Epon 812.

The muscles were cut longitudinally and the ultrathin (70-90 nm thick) sections were stained with uranyl acetate to be observed in a Philips Morgagni TEM operating at 80kV and equipped with a Megaview II camera for digital image acquisition.

Fifty inter-myofibrillar and fifty sub-sarcolemmal mitochondria (×28,000) per animal were submitted to morphometrical analysis by using the software Image J (NIH, USA); mitochondrial area, the ratio between inner and outer membranes (estimating the extension of cristae independently of mitochondrial size) and the circularity factor (a value varying from 0 to 1, where 1 represents a perfect circle) were considered.

![Figure 2. Myofibre of an old sedentary mouse: many mitochondria occur in close proximity to lipid droplets (asterisks).](image)

![Figure 3. Inter-myofibrillar mitochondria in myofibrils of adult (a), old sedentary (b) and old running (c) mice.](image)
Figure 4. Mean±SE values of the variables measured in inter-myofibrillar mitochondria. Asterisks indicate values significantly different from each other.

Figure 5. Mean±SE values of the variables measured in sub-sarcolemmal mitochondria. Asterisks indicate values significantly different from each other.
Statistical analysis

For each analyzed variable, the Kolmogorov-Smirnov two-sample test was performed in order to verify the hypothesis of identical distributions among animals of each group. The data for each variable were then pooled according to the three experimental groups, i.e. AS, OS and OR, and the mean ± standard error of the mean (SE) was calculated. Statistical analysis of the results was performed by the Kruskal Wallis test; moreover, in order to determine which pairs of samples tended to differ, the Mann Whitney test was used. Statistical significance was set at \( P \leq 0.05 \).

Results

Ultrastructural observation of quadriceps femoris samples form A, OS and OR mice revealed a similar general organization of the myofibre in all animals (Figure 1). However, in OS and OR mice large mitochondria occurred both lined between the myofibrils and clustered in close proximity of myonuclei in the sub-sarcolemmal region (Figure 1 b,c); in addition, in OS animals the inter-myofibrillar mitochondria were frequently observed in association with lipid droplets which accumulate among the myofibrils (Figure 2), while in OR mice lipid droplets were quite scarce similarly to A animals. The morphological features of mitochondria underwent modifications with ageing: in A mice they were ovoid in shape with some lamellar transverse cristae, but in OS and OR animals mitochondria became larger and with longer cristae which sometimes showed tubular shapes (Figure 3).

Morphometrical analyses (Figures 4 and 5) confirmed a significant increase in mitochondrial size and in the inner/outer membrane ratio in OS mice in comparison to A mice, while mitochondria of OR mice showed intermediate values. These modifications occurred in both inter-myofibrillar and sub-sarcolemmal mitochondria. On the other hand, no significant change was found for the circularity factor, demonstrating that neither ageing nor physical exercise affect mitochondrial form.

Discussion

Our observations on mitochondria of quadriceps femoris muscle from A, OS and OR mice demonstrated that 1) ageing induces an accumulation of mitochondria characterised by larger size and longer cristae than in adulthood; 2) in OS mice the inter-myofibrillar mitochondria often occur in close proximity to the lipid droplets which accumulate in the myofibre; 3) physical exercise partially restores adult features in mitochondria of old muscles.

Our results confirm and extend previous studies reporting an increase in mitochondrial size and number in muscle cells during ageing (Ozawa, 1997). It has been hypothesised that these alterations could be related to unbalanced fission/fusion processes: in fact, these mechanisms are regulated by specific proteins such as Mfn2, Fis1 and Drp1 which undergo functional alterations during ageing, thus leading to an accumulation of abnormally large mitochondria (Peterson et al., 2012). These proteins are also involved in the development of the mitochondrial cristae (Mannella et al., 2006) and their dysregulation could affect also the inner membrane length. In addition, the well-known impairment of the degradation mechanisms occurring in ageing cells (Jameson, 2004) probably hampers the physiological elimination of defective mitochondria, thus contributing to their accumulation.

Our data demonstrate that the age-related structural alterations affect similarly inter-myofibrillar and sub-sarcolemmal mitochondria, according to a recent report showing similar features of the two mitochondrial sub-populations (Picard et al., 2013). The difference in the measured area between inter-myofibrillar and sub-sarcolemmal mitochondria essentially depends on their different orientation in the longitudinally sectioned myofibres.

It is known that during ageing inter- and intracellular lipids accumulate in skeletal muscles (Sakuma e Yamaguchi, 2013); this phenomenon could promote an increased utilization of fatty acids as the energy source for respiration, thus inducing enlargement of mitochondria and expand-
sion of their cristae (Halestrap and Dunlop, 1986; Malatesta et al., 2001). Under our experimental conditions, the mitochondrial alterations observed in old skeletal myofibres are significantly reduced after physical exercise, although the morphological and morphometrical features of these organelles in OR mice remain significantly different from those of A animals. This finding is consistent with the scientific literature, reporting that in elderly physical exercise may improve protein synthesis, gene expression and biogenesis in the mitochondria of skeletal muscle (Peterson et al., 2012). In addition, physical exercise was observed to markedly reduce the intracellular accumulation of lipid droplets, likely promoting a return to the preferential utilization of carbohydrates as metabolic substrate for respiration with a consequent decrease in mitochondrial size and cristae length (Halestrap and Dunlop, 1986; Malatesta et al., 2001).

In conclusion, the present study provides further evidence that an adapted physical exercise may represent a suitable non-pharmacologic approach to limit the negative effects of ageing on the skeletal muscle, even when applied at late age.

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**References**


