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Histochemistry for investigating skeletal muscle under sarcopenic and dystrophic conditions

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Sarcopenia is an age-related condition characterized by the progressive loss of mass, strength and function of skeletal muscle, which affects also healthy subjects [1]. The mechanisms involved in the sarcopenic process are probably manifold, such as denervation and reinnervation of the motor units, alteration in the hormonal levels, elevated concentration of inflammatory mediators, decrease in microvascular function, and reduced regeneration capability of muscle tissue.

From a histological point of view, the aging skeletal muscle is characterized by reduction in fibre size and centrally located nuclei. Muscles rich in fast fibres (such as the *quadriceps femoris*, *gastrocnemius* and *biceps brachii*) are more compromised than muscles mainly containing slow fibres since the age-related atrophy specifically affects fast fibres. The number of satellite cells (SCs) was also observed to decrease with increasing age. SCs are undifferentiated mononuclear myogenic cells present in all skeletal muscles: they occur close to the muscle fibres, juxtaposed between the sarcolemma and the basal lamina, and are quiescent in the adult muscle, but can be activated by appropriate stimuli (such as an injury). After activation, SCs proliferate and fuse together to form new myofibres or fuse to existing muscle fibres: this is the basic mechanism responsible for the physiological renewing of muscle fibres and the maintenance of muscle mass and integrity in the adult.

In our studies we initially analyzed the differences from old to adult skeletal muscle focusing on myonuclei and SC nuclei [2,3]. We used animals of a Balb/c mice strain established 40 years ago, which has been widely used for studies on physiological aging because of their long life (mean life span 25 months) and the relatively low incidence of pathologies, in particular tumours. Two animal groups were considered: adult mice of 12 months and old mice of 28 months having only spontaneous free-moving activity in the cage. *Quadriceps femoris* and *biceps brachii* were isolated and used for fluorescence and transmission electron microscopy investigations: we analyzed the muscle fibre composition and measured fibre cross-section area by morphometry, demonstrating size variability and atrophy of fast fibres in old animals; moreover, we analysed some nuclear morphological and morphometrical features as well as the distribution and amount of transcriptional and post-transcriptional factors, showing quantitative modifications of some nuclear and nucleolar parameters and alterations in the amount of nucleoplasmic splicing and cleavage factors in old muscles.

We then investigated the effects of physical exercise in sarcopenic old mice [4,5]. In fact, several studies have stressed the importance of physical exercise as an effective - though still debated - approach to prevent/limit the sarcopenic process. Twenty-eight month-old mice were trained by treadmill running (30min at 9.5 m/min belt speed, 5 days a week) for one month. The same light and electron microscopy analyses described above were performed on *quadriceps femoris*, demonstrating that fast fibre atrophy may be counteracted by physical exercise even at advanced age, since the fibre size and the nuclear parameters of old trained mice approached the adult values. In addition, immunolabelling at light microscopy for Pax7 (a reliable marker of both quiescent and activated SCs) and MyoD (a suitable marker of activated SCs) allowed to demonstrate that the number of both quiescent and activated SCs decreases in the muscles of old mice in comparison to the adults, and that old trained mice revert to the adult values. We also studied the activation and differentiation in vitro of SCs isolated from skeletal muscles: SC-derived myoblasts were allowed to proliferate and differentiate into myotubes, and analysed at transmission electron microscopy. We found that myoblasts and myotubes from old trained mice show morphological features quite similar to adult subjects, whereas cells from sedentary old mice exhibit marked structural alterations.

On the basis of our results we may conclude that: i) in myonuclei and SC nuclei of old muscles alterations of transcriptional and post-transcriptional factors occur, but ii) physical exercise stimulates

pre-mRNA transcription, processing and export, thus increasing protein synthesis and SC activation in old muscles; moreover, iii) physical exercise improves SC capability to differentiate into structurally and functionally correct myotubes, increasing the regenerative potential of old muscles.

Interestingly, in human myotonic dystrophy (DM) the skeletal muscle shows structural and functional features reminiscent of sarcopenia, such as fibre atrophy, centrally located myonuclei and defective SCs. DMs are autosomal dominant disorders and two forms are presently known [6,7]: the more severe DM1-Steinert's disease (OMIM 160900) is caused by an expanded (CTG)n nucleotide sequence in the 3' untranslated region of the Dystrophia Myotonic Protein Kinase (DMPK) gene (OMIM 605377) on chromosome 19q13; the second form, DM2 (OMIM 602688) displays a milder clinical phenotype and is caused by the expansion of the tetranucleotidic repeat (CCTG)n in the first intron of the Zinc Finger Protein (ZNF)-9 gene (OMIM 116955) on chromosome 3q21. The basic mechanisms of both DMs reside in the nuclear sequestration of the expanded RNAs: CUG- and CCUG-containing transcripts accumulate in intranuclear foci in DM1 and DM2 cells respectively, and sequester the RNA-binding proteins CUGBP1 and MBLN [7]; in addition, we showed that snRNPs and hnRNPs splicing factors (which are essential for the physiological processing of pre-mRNA) are also sequestered into the foci [8]. This general impairment of mRNA pathways could explain the multisystemic pathological features typical of both DMs.

Our studies on DM muscles demonstrated that myonuclei from bioptic samples of *biceps brachii* undergo morphological alterations similar to those found in aged muscles and that an altered distribution of nuclear RNP-containing structures and molecular factors responsible for pre-mRNA transcription and maturation occurs [9,10]. Moreover, we investigated *in vitro* the structural and functional features of SC-derived myoblasts and we observed that DM myoblasts show cell-senescence alterations such as cytoplasmic vacuolisation, reduction of the proteosynthetic apparatus, accumulation of heterochromatin and impairment of the pre-mRNA maturation pathways earlier than myoblasts from healthy patients [11]. In addition, DM myoblasts generate myotubes characterised by structural defects similar to senescent healthy myotubes. The early occurrence of senescence-related features in SC-derived myoblasts would therefore reduce the regeneration capability of DM SCs, thus contributing to the muscular dystrophy in DM patients.

In conclusion, morphological and histochemical evidence shows that skeletal muscles undergo similar alterations under sarcopenic and dystrophic conditions, especially for the nuclear pre-mRNA pathways, suggesting a common involvement of the RNP nuclear components in the onset of muscle cell dysfunctions [12,13]. In this view, the positive effects of physical exercise observed on skeletal muscles of subjects affected by severe sarcopenic atrophy open interesting perspectives for studies aimed at its application to subjects affected by DM.

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