Plantaris muscle adaptation to atrophy generated by disuse: an ultrastructural study

F.M. Giordano,[1]* E. Vizziello,[1]* J.G. Tidball,[2] E. Falcieri,[1,3] D. Curzi[1]

[1]*Department of Earth, Life and Environmental Sciences, University of Urbino "Carlo Bo", Urbino, Italy;*
[2]*Department of Integrative Biology and Physiology, University of California, Los Angeles, CA, USA;*
[3]*Institute of Molecular Genetics and Rizzoli Orthopaedic Institute, Bologna, Italy*

Contributions: FMG and EV contributed equally to the manuscript

Corresponding author: Davide Curzi
Dipartimento di Scienze della Terra, della Vita e dell'Ambiente, Via Ca' le Suore 2, 61029 Urbino, Italy
Tel. +39.0722.304311 - Fax: +39.0722/304244
E-mail: davide.curzi@uniurb.it

Summary
Muscle atrophy is a mechanism which induces muscle mass loss, through an increased protein degradation and a decreased synthesis. This alteration compromises the muscle contractile capacity and is implied in numerous physiological and pathological conditions (e.g. denervation, aging, bed rest, dystrophies and myopathies). The aim of this work is to analyze the ultrastructural changes generated by disuse on rat plantaris muscle, mainly composed by fast fibers. Eight animals were equally assigned to control and unloaded group, and the treated rats were tail-suspended for 10 days. After suspension, the animals of both groups were euthanized and the hind-limb muscles were processed for electron microscopy. Transmission electron microscopy observations show morphological modifications in the unloaded group respect to the control group, such as sarcomere disorganization, continuity lack among myofilaments and Z-line thickening. In the fiber cytosol, swollen mitochondria and dilated triads can be observed, as well as a growing amount of glycogen, which suggests a possible fiber shift. Therefore our findings reveal macroscopic and microscopic modifications induced by disuse, which concern both contractile and energetic system.

Key words: Skeletal muscle, ultrastructure, suspension, atrophy, disuse.

Introduction

Muscle atrophy is a mechanism which involves the loss of contractile proteins, implying a decrease of fiber section size, strength and endurance (Rommel *et al.*, 2001). These modifications reduce the muscle mass, compromising its capacity to generate the contractile force (Snijders *et al.*, 2013).

The skeletal muscle maintenance depends on the balance between protein synthesis and degradation, both involved in the preservation of muscle mass. Atrophy and hypertrophy (the reverse condition which concerns a muscle mass increase) depend on the modifications of this balance. Therefore, muscle atrophy is characterized by an increased protein degradation, associated with a rapid contractile protein loss, and a protein synthesis inhibition (Lecker *et al.*, 1999; Sandri *et al.*, 2008). Atrophic mechanism is still under discussion, in particular the myonucleus role and the molecular pathways concerning its activation. Siu and Alway (2009), basing on the myonuclear domain theory, have hypothesized that during muscle atrophy there would be a numerical reduction of myonuclei, with a maintenance of myonuclear domain size. Regarding the molecular mechanisms, disuse atrophy may be related to Atrogin-1/MAFbx and MuRF1, IGF-1-AKT-mTOR pathway and myostatin pathway (Brooks *et al.*, 2014).

In the literature several works analyse the atrophy role in different conditions. The muscle mass loss may be due to particular disorders such as diabetes, muscular dystrophies and denervation. The last can be the consequence of numerous diseases, such as Spinal Muscular Atrophy (SMA), where the motorneuron loss at spinal cord level implies a clear weakness and hypotonia of affected muscles (Martínez-Hernández *et al.*, 2014).

Duchenne Muscular Dystrophy is a commonly studied disease in which dystrophin, a protein that links the muscle fiber cytoskeleton to extracellular matrix, through the cell membrane, appears absent. Cheng *et al.* (2013) investigated clinical and pathological features in children with progressive muscular dystrophy (PMD), showing, by means of EMG exam-

ination, that PMD manifests as atrophic signs in the early stage, accompanied by neurogenic damage in the late stage.

The relationship between diabetes and atrophy is still under discussion, although in literature it is well known that the disease inhibits muscle regrowth. Kataoka *et al.* (2014) have caused disuse atrophy in diabetic and non-diabetic rats, through immobilization. In the following recovery period, muscle IGF-1 levels increased significantly in the non-diabetic rats, but not in the diabetic ones. In conclusion, the decreased level of IGF-1 and the impairment of angiogenesis associated with the disease, are probably responsible for the inhibition of regrowth and the consequent atrophy. There are also other conditions which lead to atrophy such as aging, forced resting and microgravity (Sinha *et al.*, 2014). Aging is a physiological condition characterized by the loss of skeletal muscle mass and the decline in satellite cell content (Verdijk *et al.*, 2014). These age-related modifications were observed particularly in type II muscle fibers and twelve weeks of resistance exercise training, significantly increased muscle fiber section and satellite cell content (Verdijk *et al.*, 2009). However, Snijders *et al.* (2014) suggest that muscle disuse atrophy is not accompanied by changes in skeletal muscle satellite cell content.

Resting may be considered similar to the microgravity condition (Linderman *et al.*, 1994) because of the mechanical loading absence. In fact, muscle unloading, caused by the absence of movement or gravity, leads to atrophy (Brooks *et al.*, 2014). Lloyd *et al.* (2014) confirmed the loss of skeletal muscle mass in hindlimb suspended mice. This condition causes atrophy and is also related to trabecular bone loss, which occurs both in elderly subjects and astronauts.

Ferreira *et al.* (2008) studied mice muscle atrophy after 48 hours of tail suspension and reported a significant decrease of muscle weight and protein concentration. Tagliavini *et al.* (2014), investigated muscle adaptations in patients with collagen VI-related myopathies. Their observations showed swollen mitochondria, hypodense matrix, dilated sarcoplasmic reticulum and apoptotic features.

The aim of our research is to investigate, at ultrastructural level, morphological and morphometrical changes due to disuse atrophy in rat plantaris muscle, which is mainly composed by type II muscle fibers and then particularly sensitive to the atrophic condition.

Materials and Methods

Animal model

Eight male albino Sprague-Dawley rats, aged eight weeks, were placed in individual suspension cages on a standard 12:12-h dark-light cycle in a room maintained at 24±1°C and fed a standard diet without limitations. After 1 week of quarantine, 4 rats were randomly chosen for the unloading protocol (unloaded group). These animals were tail-suspended for 10 days and the tail was wrapped in a piece of Fast-Trac adhesive tape, covered with a stockinette and secured with fiber tape (Curzi *et al.*, 2013). The Fast-Trac tape was passed through a wire hook, which was then suspended from a fishing swivel. The swivel was, in turn, suspended from the overhead track system. This arrangement allowed the rats to move freely about the cage on their forelimbs. The suspension tracks were blocked, so that the rats were unable to touch the sides of the cage with their hindlimbs (Grindeland *et al.*, 1994; Linderman *et al.*, 1994). The other four rats were euthanized under general anesthesia with i.v. injection of sodium pentobarbital (50 mg/kg body wt) after 10 days (CTRL group). At the end of the unloading protocol, under general anesthesia also the suspended rats were sacrificed. Animal care and use were in accord with the "Ames Research Center Animal Users Guide" (AHB 7180).

Transmission electron microscopy (TEM)

From the sacrificed animals, the right plantaris muscles were taken, quickly weighed on a precision scale, tied to an applicator stick at physiological length and immediately fixed with 1.4% glutaraldehyde in a 0.2 M sodium cacodylate buffer at pH 7.2 for 1 h at 4°C. The samples were then rinsed in cacodylate buffer and minced into small bundles (<1 mm^3) of muscle fibers that were fixed in the same solution for an additional hour. After washing, samples were post-fixed with 1% osmium tetroxide for 1 h in the same buffer, rinsed in cacodylate buffer and dehydrated in a graded series of ethanols. They were embedded in epoxy resin and longitudinally sectioned (Law *et al.*, 1995). Semithin sections were stained with 1% toluidine blue in distilled water at 60°C and thin sections, stained with uranyl acetate and lead citrate, were then observed with a Philips CM10 electron microscope (Curzi *et al.*, 2012; Salucci *et al.*, 2013).

Morphometric analysis

Measurements were made only on longitudinal muscle sections and one hundred images were evaluated for each group and twenty-five for each rat. Hundred squared areas (Area= 50 μm^2), one for each image, were analyzed. The number of swollen mitochondria and the sarcomere diameters were measured in semiautomatic mode, using *Image J* analysis software. All data were expressed as mean values ± SEM and the results were compared using Student's t-test. Significance was set at $p \leq 0.01$.

Results

In the CTRL group, the sarcomere arrangement along myofibril seemed well-organized and the myofibrils appeared parallelly oriented (Figure 1A). On the contrary, the treatment determined sarcomere and Z-line misalignment and the latter appeared thickened or completely absent (Figure 1B). After ten days of suspension, in fact, the ultrastructural images of plantaris muscles revealed a general tissue disorganization.

At high magnification, in the mitochondria of CTRL fibers, the typical double-membrane system and the cristae were observable (Figure 1C). Conversely, in the suspended rats, these organelles appeared swollen and characterized by a unique membrane and by the absence of cristae (Figure 1D). Furthermore, in the unloaded group, the Z-line correlated disposition of mitochondria and triads seemed to be lost and the terminal cisterns of sarcoplasmic reticulum appeared dilated and severely compromised (Figure 1E-F).

The classical sarcomere structure, where thin actin filaments appeared well bound to the Z-lines (Figure 2A), in treated group appeared frequently altered, and myofilaments were randomly oriented (Figure 2B). In the muscles of unloaded group, cytosol spread among myofilaments and glycogen amount increased respect to the CTRL group (Figure 2C-D).

Morphometric analysis confirmed the ultrastructural observations. In fact, the percentage of swollen mitochondria displayed a significant increase ($P<0.01$) in the unloaded rats (66.18%) respect to the CTRL (19.97%) (Figure 2E). On the contrary, comparing the sarcomere diameter means in the two groups, a significant decrease ($P<0.01$) in the suspended animals (0.62 μm) in comparison to the CTRLs (1.24 μm) (Figure 2F) was evident.

Discussion

Several pathological or physiological conditions are directly or indirectly characterized by muscle atrophy, the role of which is currently investigated by numerous researchers. The aim of this study was to describe the morphological features of atrophic plantaris muscle by means of electron microscopy. In the literature, the higher capacity of fast fibers to respond to muscle atrophy, respect to the slow fibers, is well known (Ciciliot *et al.*, 2013). Therefore, the plantaris muscle, mainly composed by fast fibers, has been chosen. After 10 days of suspension, rat plantaris muscles display numerous and deep ultrastructural modifications, which regard not only to the fiber structural system, but also to the related structures. Unloaded muscles show morphological changes such as a general sarcomere disorganization, myofilament misalignment, cytosol spreading among them and a significant sarcomere diameter decrease. These features suggest a structural impairment related to a protein amount decrease and to an altered capacity to transfer the contractile force generated from single sarcomeres. In fact, several works show that this condition induces strength and endurance decrease (Rommel *et al.*, 2001; Lloyd *et al.*, 2014). Whereas on one hand the unloading appears to structurally affect the contractile system, on the other hand it also influences the activation system of muscle contraction. In fact, the triad alterations revealed in the suspended rats, both concerning sarcoplasmic reticulum and transverse tubule system, suggest an impairment of excitation-contraction coupling. Our findings display also significant modifications in mitochondria, which appear to lose their physiological position and structure. These changes reveal that atrophic process generated by unloading may influence the energetic mechanism of muscle fibers, which may not have sufficient levels of available energy. Moreover, electron microscopy observations suggest an apparent glycogen increase in the cytosol, which may be explained with a progressive shift of muscle fibers to a glycolytic metabolism, as well as reported by Du *et al.* (2011).

In conclusion, the muscle atrophy generated by disuse reveals macroscopic and microscopic tissue alterations, which involve the impairment of organelles essential for function and viability of muscle fibers. These modifications, appeared just after ten days of suspension, display the strong and quick impact of this condition on the muscle function.

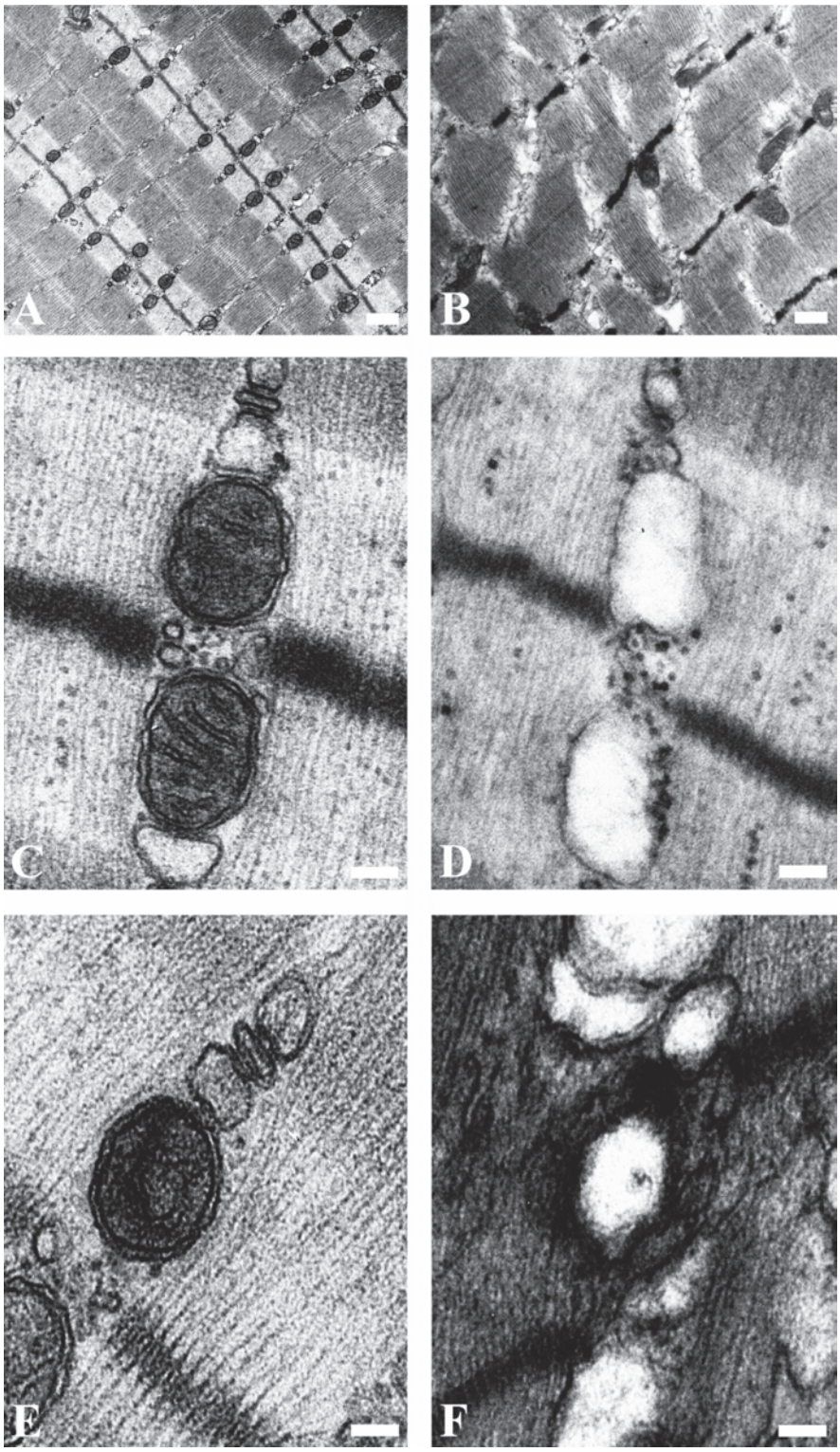

Figure 1. Longitudinal sections of CTRL (A) and unloaded (B) rat muscles. In CTRL rats, mitochondria with the characteristic double membrane and mitochondrial cristae, are observable (C), differently from suspended ones which show swollen mitochondria (D). Triads are perfectly intact in CTRL (E), in comparison to the suspended group, where they appear strongly dilated (F). Bars: A, B 0.5 µm; C, D, E, F 0.1 µm.

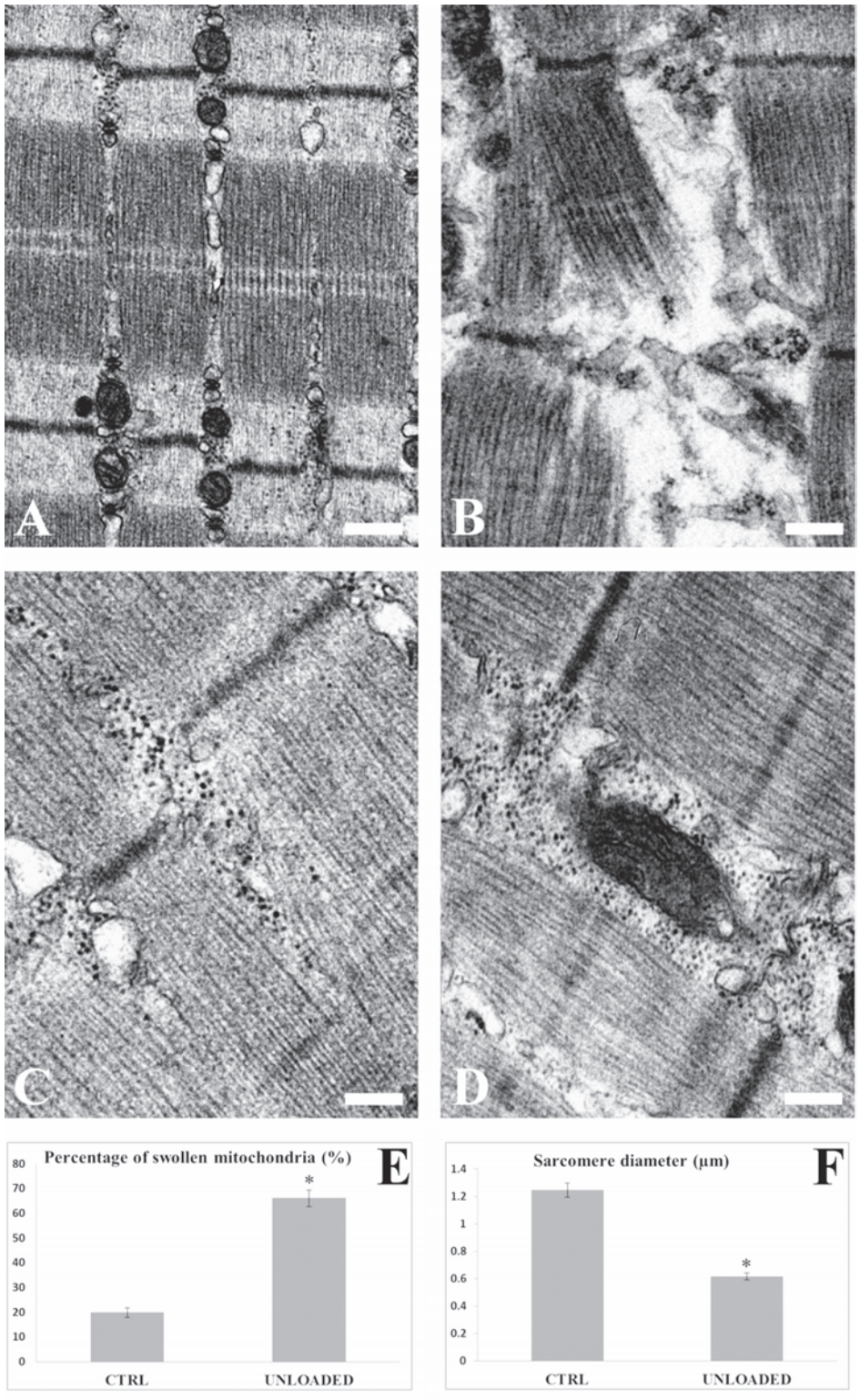

Figure 2. High magnification of CTRL (A) and unloaded (B) rat muscles. A lower amount of glycogen is observable in CTRL fiber cytoplasm (C), compared to the suspended group (D). Morphometric analyses reveal significant differences in the percentage of swollen mitochondria (E) and in the mean of sarcomere diameter between the two groups (F). Bars: A, B 0.4 µm; C, D 0.3 µm.

Acknowledgements

We would like to thank Mr. Oliviero Rusciadelli and Mr. Lorenzo Bedini of Urbino University for their technical help.

The study was supported by: Urbino University; Ministry of Education University and Research; NASA – Ames Research Center, Moffett Field, CA, USA; University of California, Los Angeles, CA, USA.

References

Brooks NE, Myburgh KH. Skeletal muscle wasting with disuse atrophy is multi-dimensional: the response and interaction of myonuclei, satellite cells and signaling pathways. Front Physiol 2014;5:99.

Cheng SQ, Qiang H, Cao YH, Li QL, Chen CP. Clinical and pathological features in children with progressive muscular dystrophy. Chin J Contemp Pediatrics 2013; 15:649-52.

Ciciliot S, Rossi AC, Dyar KA, Blaauw B, Schiaffino S. Muscle type and fiber type specificity in muscle wasting. Int J Biochem Cell Biol 2013;45:2191-9.

Curzi D, Lattanzi D, Ciuffoli S, Burattini S, Grindeland RE, Edgerton VR, et al. Growth hormone plus resistance exercise attenuate structural changes in rat myotendinous junctions resulting from chronic unloading. Eur J Histochem 2013;57:e37.

Curzi D, Salucci S, Marini M, Esposito F, Agnello L, Veicsteinas A, et al. How physical exercise changes rat myotendinous junctions: an ultrastructural study. Eur J Histochem 2012;56:e19.

Du F, Wang J, Gao Y, Wang H, Wang Q, Jiang S, et al. A hindlimb disuse model inducing extensor digitorum longus atrophy in rats: tail suspension-immobilization. Aviat Space Environ Med 2011;82:689-93.

Ferreira R, Neuparth MJ, Vitorino R, Appell HJ, Amado F, Duarte JA. Evidences of apoptosis during the early phases of soleus muscle atrophy in hindlimb suspended mice. Physiol Res 2008;57:601-11.

Grindeland RE, Roy RR, Edgerton VR, Grossman EJ, Mukku VR, Jiang B, et al. Interactive effects of growth hormone and exercise on muscle mass in suspended rats. Am J Physiol 1994;267:R316-22.

Kataoka H, NakanoJ, Morimoto Y, Honda Y, Sakamoto J, Origuchi T, et al . Hyperglycemia inhibits recovery from disuse-induced skeletal muscle atrophy in rats. Physiol Res 2014; [Epub ahead of print].

Law DJ, Caputo A, Tidball JG. Site and mechanics of failure in normal and dystrophin-deficient skeletal muscle. Muscle Nerve 1995;18:216-23.

Lecker SH, Solomon V, Mitch WE, Goldberg AL. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J Nutr 1999;129:227S-37S.

Linderman JK, Gosselink KL, Booth FW, Mukku VR, Grindeland RE. Resistance exercise and growth hormone as countermeasures for skeletal muscle atrophy in hindlimb-suspended rats. Am J Physiol 1994;267:R365-71.

Lloyd SA, Lang CH, Zhang Y, Paul EM, Laufenberg LJ, Lewis GS, et al. Interdependence of muscle atrophy and bone loss induced by mechanical unloading. J Bone Miner Res 2014;29:1118-30.

Martínez-Hernández R, Bernal S, Alias L, Tizzano EF. Abnormalities in early markers of muscle involvement support a delay in myogenesis in spinal muscular atrophy. J Neuropathol Exp Neurol 2014;73:559-67.

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nature Cell Biol 2001;3: 1009-13.

Salucci S, Burattini S, Baldassarri V, Battistelli M, Canonico B, Valmori A, et al. The peculiar apoptotic behavior of skeletal muscle cells. Histol Histopathol 2013;28:1073-87.

Sandri M. Signaling in muscle atrophy and hypertrophy. Physiology 2008; 23:160-70.

Sinha U, Csapo R, Malis V, Xue Y, Sinha S. Age-related differences in diffusion tensor indices and fiber architecture in the medial and lateral gastrocnemius. J Magn Reson Imaging 2014; [Epub ahead of print].

Siu PM, Alway SE. Response and adaptation of skeletal muscle to denervation stress: the role of apoptosis in muscle loss. Front Biosci (Landmark Ed) 2009;14: 432-52.

Snijders T, Wall BT, Dirks ML, Senden JM, Hartgens F, Dolmans J, et al. Muscle disuse atrophy is not accompanied by changes in skeletal muscle satellite cell content. Clin Sci 2013;126:557-66.

Tagliavini F, Sardone F, Squarzoni S, Maraldi NM, Merlini L, Faldini C, et al. Ultrastructural changes in muscle cells of patients with collagen VI-related myopathies. Muscles Ligaments Tendons J 2014;3:281-6.

Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH, van Loon LJ. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. Am J Physiol Endocrinol Metab 2007;292:151-7.

Verdijk LB, Gleeson BG, Jonkers RA, Meijer K, Savelberg HH, Dendale P, et al. Skeletal muscle hypertrophy following resistance training is accompanied by a fiber type-specific increase in satellite cell content in elderly men. J Gerontol A Biol Sci Med Sci 2009; 64:332-9.