One Minute of Daily Reloading and Electrostimulation of Unweighted Muscles Induces Minimal Muscle Damage, but Peculiar Regeneration in Suspended Rat Muscles

Katia Rossini[1], Corrado Rizzi[2], Adonella Bruson[1], Marco Sandri[1], Ugo Carraro[1,3], Damien Leterme[4] and Maurice Falempin[4]

1) Department of Biomedical Sciences, 2) Institute of Plastic and Reconstructive Surgery, 3) CNR Unit for Muscle Biology and Physiopathology, University of Padova, Italy, and 4) Laboratory of Physiology of Contractile Structures, University of Sciences and Techniques of Lille, France

Abstract

From the observations that space flights have same effects on astronaut muscle which have clinical expression in fatigue, weakness and incoordination, almost two decades ago, muscle unweighting became a popular experimental animal model that will permit the determination of the role played by gravity in muscle physiology. Characterisation of the muscle adaptation and degeneration processes in response to gravity absence and reload is a prerequisite for developing rational measures to enable human experiences in the space. We here present morphologic and molecular analyses of serial sections of Soleus and Extensor Digitorum Longus muscles of unloaded rat legs which were reloaded and electrostimulated sixty seconds per day during the first week of leg suspension. Besides demonstrating differences in total protein and collagen content, isomyosin patterns and MHC/Actin ratio, which are known components of the adaptation process of the muscle to gravity/activity changes, our analyses show that myofiber necrosis/regeneration occurs in the experimental muscles. Small splitted myofibers, whose branching and fusion is shown in adjacent serial sections, ought to be interpreted as regeneration events since they are stained with an anti-embryonic myosin heavy chain monoclonal antibody. The result adds an additional evidence of exercise-induced damage and regeneration which may occur in experimental models of muscle unweighting/reloading; while their contribution to suspension-induced isomyosin changes during actual unweighting ought to be neglected, relevance in unloading-reloading conditions is undisputable. The peculiar features of the regeneration process also ask further study.

Key words: Atrophy, collagen, electrostimulation, exercise-induced damage, isomyosins, MHC, molecular markers of damage, regeneration, repair, muscle suspension, rat.

BAM 5 (2) 181-188, 1995

Rat hindlimb unweighting is a common model to investigate the microgravity induced atrophy of slow muscles [33]. Several experimental models of functional unload and reload are reported in literature for Soleus and Gastrocnemius muscles. In the tail-suspension model, introduced by Morey in 1979 [25], the hindlimbs are prevented from bearing weight by tail traction procedure in which approximately one-third of the tail remains uncovered, thereby allowing normal thermoregulatory processes to occur.

We studied several morphologic (myofiber type, size, number and shape) and molecular markers (protein and collagen contents, myosin/actin ratio and myosin heavy chain isoforms) in serial cryostat sections of Soleus and EDL muscles during one week of unloading and very short daily reload and electrostimulation of suspended rat leg muscles. Even if the main aim of the experiment was not achieved (i.e., the short daily regimen of reloading accompanied by a strenuous exercise does not contrast the suspension atrophy) results are interesting and worth to be corroborated by further research, in particular as the disturbed myogenesis of damaged/regenerated myofibers is concerned.

Materials and Methods

Experimental design

Male rats (267+/-9 g body weight) were used for the study. All animals were housed in large cages, at room temperature, without light-dark cycle and provided food and water ad libitum. After a period of animal room acclimatisation, four rats were suspended by using the Morey-Holton and Wronski [26] tail-suspension, model, the height of the hook was adjusted and the size of the cage was such that the animals could easily reach food and water. In the same room, the other three rats were housed without restraint in individual standard vivarium cages, these animals were used as control (N). Rat chow and water were available ad libitum.

Daily, the suspended rats were reloaded for one minute restraining and electrostimulating the animals on the floor of the cage (S-R). The left leg was electrostimulated during the reload period (E/S-R) by electrodes implanted on the sciatic nerve at 80 Hz, 500 msec on, 500 msec off; each impulse had a duration of 0.2 msec and an intensity 0.14-0.2 mA [21]. After a week the animals were weighed and killed. The Soleus and EDL were removed, weighed, and frozen at rest length in liquid Nitrogen. Muscles were stored at -80°C in sealed plastic bags until use. Serial sections were cut at middle bell and processed for histological, histochemical and molecular analyses as follows.

Cryostat section area and volume

After sectioning a normalized micromesh was mounted parallel to the transverse section of the muscle mounted on the cryostat block and photographed. Projection of the slides permits to determine the area of the cryostat section by superimposing the muscle area to the mesh. Since section thickness is known, volume is easily calculated.

Histology, histochemistry and immunohistochemistry

Serial sections (10 µm) were stained with hematoxilin and eosin (H-E) Van Gieson method, myofibrillar AT-Pases according to [3], and directed immunofluorescence as described [2] using anti-MHCemb (G6 clone) which is generous gift of Professor Stefano Schiaffino. Cryosections were examined in a Zeiss Axioplan fluorescent microscope. The alkaline and acid ATPase sections were used for size measurement of type 1 and type 2 myofibers. Morphometric analyses were performed on glass slides or photographs using an image analyser IBAS 2000 (Kontron, Zeiss). Measurements of the profile area were obtained by a television scanner and a grey level detector which allowed us to identify, select and measure single features [27]. Fibers were counted by identification of all the myofibers on H-E stained sections. Percentage content of type 1 myofibers was determined in random selected areas of ATPase stained sections.

Molecular microanalyses

Groups of serial cryostat section (20 µm each) were processed for total protein, collagen content and electrophoretic analyses, as follows:

Total protein: Two cryostat sections collected in cylindrical glass test tube were dissolved in 0.5% Deoxycholic acid by sonication at room temperature using Biosonik III (Bronwill scientific), and the protein determined by the method of Lowry [22].

Collagen: Two serial sections collected in screw caf Pyrex text tube were analysed for total collagen determination as described in [34], and [13] with the following modifications. The sections were hydrolyzed for 20 h in 1 ml of 6 N HCl at 110° C. After hydrolysis the samples were dried either in a rotating evaporator or in a multi-sample benchtop centrifuge connected to a water driven vacuum-pump (Electrofor, Rovigo, Italy). Dried samples were dissolved in 1 ml of distilled water. Aliquots of 0.4 ml were added to 0.5 ml aliquots of chloramine-T solution (sodium N-chloro-p-toluene sulfonamide 1.41 g, was dissolved in 10 ml of n-propanol, 10 ml of water and 80 ml of a pH 6 buffer. The pH 6 buffer was prepared by solving 25 g of citric acid $1.H_2O$, 60 g of sodium acetate $3.H_2O$, 17 g of NaOH, and 6 ml glacial acetic acid in a final volume of 500 ml of distilled water). The mixture of sample and chloramine-T was vigorously mixed and incubated at room temperature (20-25°C). After 20 min, 0.77 ml of a freshly made aldehyde/perchloric acid solution (composed by 15 g of p-dimethylaminobenzaldehyde suspended in 62 ml n-propanol to which were slowly added 26 ml of 60% perchloric acid and distilled water to 100 ml of total volume) was added. The samples were vortexed and incubated at 65°C for 20 min. Full colour development occurs in 20 min. Usually, the samples were immediately read at 550 nm, but this is not a necessary requirement since the colour is stable for several hours. Hydroxyproline content was determined by interpolation using a hydroxyproline standard curve we made every time. In the described conditions the curve is linear from 0.5 to 20 µg of hydroxyproline. Since the mean content of hydroxyproline in collagen is 13.4%, collagen is easily calculated taking into account the lower weight of the aminoacid in polypeptides due to peptide bonds.

Electrophoretic analyses: Two serial sections were solubilized in 2.3% SDS, 10% v:v glycerol, 0.5% 2-mercaptoethanol, 62.5 mM Tris HCl pH 6.8 at 100 µg/ml, using the protein content determined by the Lowry method. Test tubes were closed up with aluminium foil, and incubated 3 min in boiling water to denature proteins.

MHC/Actin ratio: Analytical SDS PAGE was performed essentially according to Laemmli [19] in a discontinuous gel gradient system made of 7% and 12.5% polyacrylamide (0.75 x 130 x 130 mm slabs, made of 90 mm of 12.5%, 30 mm of 7% separating gels and 10 mm of stacking gel, from the bottom of the wells). Major modification is that 37.5% v:v glycerol was present in the separating and in the stacking gels [5, 6]. Electrophoretic buffer

is: 25 mM Tris, 192 mM glycine pH 8.3, 0.1% SDS. Overnight separation was achieved using constant current mode at amperage of about 5 mA per slab, but setting the value to that which gave an initial voltage of 35 Volts. Gel electrophoresis started late in the afternoon and the run was finished after about 18 h when the front dye reached the end of the slab. Usually the voltage rose to 140-150 Volts. Usually 5 µg of total protein were loaded per well. MHC and Actin bands were easily detected after 1hr shaking with 200 ml of 0.1% Coomassie brilliant blue in 5% acetic acid, 40% methanol and destain with 6 changes of 200 ml of 40% methanol and 7% acetic acid every 15 min. To avoid variability due to stain-destain procedure the MHC/Actin ratio was checked against a standard muscle homogenate electrophorized every time in the same gel slab. Myosin heavy chains and actin content were determined by gel densitometry (see below).

SDS PAGE of MHC: High resolution SDS PAGE was performed on 7% polyacrylamide slabs (0.75 x 130 x 130 mm) as we recently described [29]. The gel was stained with the silver method [24] modified according to [1]. For Western blotting MHC were transferred in 25 mM Tris, 193 mM glycine, 20% methanol for 5 hrs at 4°C at constant voltage (80 Volts). After blotting, the amount of MHCs and their electrophoretic patterns were *a posteriori* controlled by Red Ponceau staining. The immunostaining procedure was performed according to [29] using an anti-MHCemb (G6 clone) which was generous gift of Professor Stefano Schiaffino.

Quantitative densitometry of SDS-PAGE: Densitometric scanning of gel slabs was performed using a GS 300 Transmittance-Reflectance Scanning Densitometer (Hoefer Scientific Instruments) connected to a Macintosh (Apple Computer). Data were processed using the GS-370 Data System for the Hoefer GS 300 Scanning Densitometer, Macintosh version. Slabs were scanned first along the electrophoretic direction and then along the axis perpendicular to the electrophoretic migration. Densitometric values were linear between 0.25-5 mg of protein after Coomassie blue stain [30].

Statistics

Student's test was used as a post hoc test to determine the significance between groups. The 0.05 level of probability was established for statistical significance. All results are presented as mean +/- SD.

Results and Discussion

Muscle atrophy

Body weights of the suspended rats demonstrated the expected small reduction [7, 8, 10, 11, 20]. Muscle mass also is significantly reduced comparing S-R soleus versus N soleus (table 1) with the expected atrophy of about 30%. On the other hand not significant change distinguishes E/S-R from S-R soleus. The suspended EDL muscles are also, but less atrophic (table 2), without significant change between E/S-R and S-R EDL. Muscle to rat weight ratios confirm the extra atrophy of slow, weight-bearing muscle,

*Table 1. Morphological properties of suspended-reloaded (S-R) and electrostimulated-suspended-reloaded (E/S-R) soleus. Δ%vsN, Percentage change of S-R soleus vs normal (N); Δ%vsS-R, Percentage change of E/S-R soleus vs S-R; *[a], Significant difference between normal and S-R or E/S-R groups. Values are mean ± SD.*

	N soleus	S-R soleus	Δ%vsN	E/S-R soleus	Δ%vsS-R
body weight	267 ± 9	238 ± 5*[a]	-10%		
muscle weight	121 ± 9	85.9 ± 5.8*[a]	-29%	81.8 ± 7.2*[a]	-5%
muscle/body weight ratio	0.45 ± 0.03	0.36 ± 0.03*[a]	-20%	0.34 ± 0.03*[a]	-5%
section area (mm^2)	7.6 ± 0.6	6.11 ± 0.8*[a]	-19%	5.6 ± 0.74*[a]	-8%
fiber number	2201 ± 256	2287 ± 352	+4%	2304 ± 201	+0.7%
Type1 fibers (%)	89.2 ± 2.7	92.3 ± 7.6	+3.5%	90.4 ± 5.7	-2%
Size type 1 fibers	56.1 ± 5.9	45 ± 1.3	-20%	41.5 ± 3.3*[a]	-8%
Size type 2 fibers	48.6 ± 9.6	33.9 ± 10.7	-30%	32.8 ± 5.7*	-3%

*Table 2. Morphological properties of suspended-reloaded (S-R) and electrostimulated-suspended-reloaded (E/S-R) EDL. Δ%vsN, Percentage change of S-R EDL vs normal (N); Δ%vsS-R, Percentage change of E/S-R EDL vs S-R; *[a], Significant difference between normal and S-R or E/S-R groups. Values are mean ± SD.*

	N EDL	S-R EDL	Δ%vsN	E/S-R EDL	Δ%vsS-R
body weight	267 ± 9	238 ± 5*[a]	-11%		
muscle weight	132.9 ± 8	121.4 ± 4.7*[a]	-9%	109.1 ± 9.6*[a]	-10%
muscle/body weight ratio	0.49 ± 0.03	0.51 ± 0.03	+4%	0.45 ± 0.05	-12%
section area (mm^2)	7.4 ± 1.2	7.5 ± 1.3	+1%	6.6 ± 0.9	-12%
fiber number	3602 ± 728	3146 ± 144	-12%	2863 ± 927	-9%
Type1 fibers (%)	3.3 ± 1	4.6 ± 1.5	+39%	4.1 ± 1.6	-11%
Size type 1 fibers	36.9 ± 2.5	35.5 ± 4.3	-4%	32.3 ± 6.6	-9%
Size type 2 fibers	46.7 ± 3.1	38.5 ± 12.3	-17%	39.6 ± 4.2	+3%

which is related to disuse, while the smaller atrophy of the EDL muscles is probably consequent to the initial stress present in the experimental model (note the small additional atrophy of the E/S-R EDL).

Histopathology and immunohistochemystry

When muscles are quenched in liquid nitrogen at resting length and sectioned near the muscle bell, area of the section is in good agreement with muscle weight and can be used as an index of muscle trophism. On the other hand, area of cryostat section determined on glass slide does not represent the actual cross-section of the muscle bell *in vivo*, since the sections shrink during adhesion to glass and drying. If in experiments or diseases the overall shape of the muscle remains constant, the section area to muscle weight ratio may be used has an index of muscle hypo- or hypertrophy, oedema or adiposis. Of course, if quenching of the full muscle at resting length and standardized sectioning can not be performed (as is the case of muscle biopsies) the parameter loses value as an index of muscle trophism, but section area remains worth determining, since it allows to compute absolute concentrations of any relevant muscle component (see below). Note in tables the good correlation of changes in weight and area of the muscle bell section of the experimental muscles. On the other hand no statistically significant changes in myofiber number occur in the experimental muscles. Minimal changes occur in percentage content of type 1 myofibers in the experimental soleus, while their size decreases as expected. Soleus type 2 myofibers are also similarly atrophic. Smaller changes in fiber size are present in both fast and slow myofibers of EDL, the smaller size being surprisingly found in the E/S-R EDL.

Besides atrophy, light microscopy allows to recognize in the experimental muscles the presence of inflammatory cells and groups of small myofibers with internalized nuclei in the experimental soleus (but not EDL) muscles. Tridimensional reconstruction of the groups of small myofibers in serial sections shows that they are branched, possibly regenerated myofibers which form complex sincitia (Fig 1). It is well known that mammalian striated muscles regenerate in response to injury, and that this secondary myogenesis occurs, as does during fetal development, by fusion of mononucleated cells. In adults, the cells responsible for the regenerative response are satellite cells, mononucleated cells found between basal lamina and sarcolemma of intact muscle fibers [23]. As firstly described by Ontell [28], the regenerative myogenesis in mouse is peculiar, since the process of myoblast fusion to adult myofiber is incomplete. In this species a high percentage of the myotubes formed by secondary myogenesis undergo complex branching and recombination, resulting in cytoplasmic continuity of myotubes that appear to be independent myotubes for extensive regions along their length. This phenomenon is a useful morphologic marker of mouse regeneration since it can be observed in serial sections. Similar features are present in the present experimental rat soleus. Figure 1 shows an example: a group of small fibers change size and shape in serial sections. Therefore they are recently regenerated myofibers since they are also positive when stained with an anti MHCemb

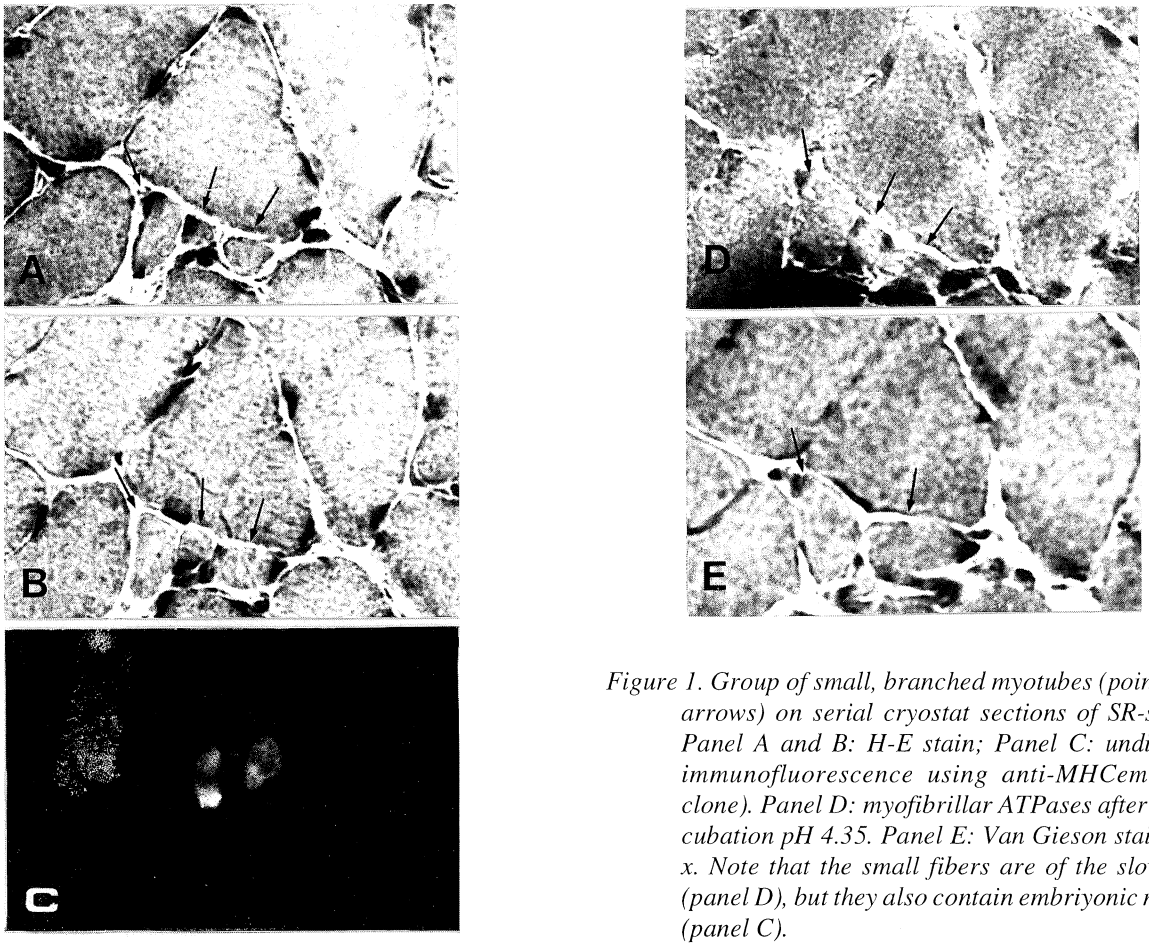

Figure 1. Group of small, branched myotubes (pointed by arrows) on serial cryostat sections of SR-soleus. Panel A and B: H-E stain; Panel C: undirected immunofluorescence using anti-MHCemb (G6 clone). Panel D: myofibrillar ATPases after preincubation pH 4.35. Panel E: Van Gieson stain. 640 x. Note that the small fibers are of the slow-type (panel D), but they also contain embriyonic myosin (panel C).

mAb. Less than ten groups of small fibers are consistently present in all the experimental soleus, without significant differences among the S-R and the E/S-R soleus. No regenerated myofibers were found in any of the normal soleus and in any of the normal and experimental EDL muscles. Since each group of small fibers represents the regeneration of one damaged myofiber, only less than 0.5% of myofibers appear to have been damaged in the experimental soleus. Though damage/regeneration seems to be a scanty event, the interesting open question remains if unloading is responsible of the incomplete fusion of the myoblasts in the regenerating myofibers of suspended-reloaded rat soleus.

Molecular markers of muscle plasticity, damage, regeneration and repair

Tables 3 and 4 confirm that the atrophy of the suspended muscles can be recognized using molecular markers. Total protein and collagen concentrations decrease in the experimental muscles, though the changes hardly show significance due to their paucity and small mean difference. Indeed the differences are better recognizable when two or more weeks of suspension are applied [12, 32, 33]. Note that the protein concentration of the electrostimulated/suspended-reloaded soleus attains the value of the normal muscle. This is the only value which is in favour of a trophic effect of the applied electrostimulation regimen.

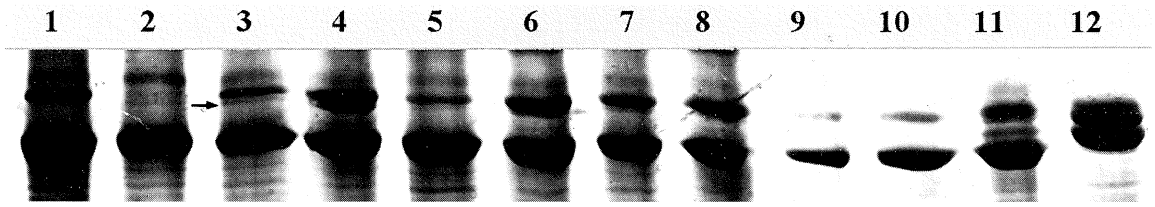

Figure 2. High resolution SDS-PAGE of MHC with Silver staining. Lane 1: 3 μg of N-soleus protein. Lane 2-4: 3 μg of S-R soleus protein. Lane 5-8: 3 μg of E/S-R soleus protein. Lane 9-10: 0.5 and 1 μg of soleus purified myosin. Lane 11: coelectrophoresis of 2 μg of soleus purified myosin and 0.5 g of EDL purified myosin. Lane 12: 1 μg of EDL purified myosin.

*Table 3. Molecular properties of suspended-reloaded (S-R) and electrostimulated-suspended-reloaded (E/S-R) soleus. Δ%vsN, Percentage change of S-R soleus vs normal (N); Δ%vsS-R, Percentage change of E/S-R soleus vs S-R; *[a], Significant difference between normal and S-R or E/S-R groups. *[b], Significant difference between S-R and E/S-R groups. Values are mean ± SD.*

	N soleus	S-R soleus	Δ%vsN	E/S-R soleus	Δ%vsS-R
protein (%)	17.3 ± 2.1	14.1 ± 1.2	-19%	17 ± 1.1	+20%*[b]
collagen (%)	1.5 ± 1.2	1.1 ± 0.1	-27%	0.9 ± 0.3	-18%
collagen/protein ratio x 100	0.06 ± 0.01	0.07 ± 0.01	+16%	0.06 ± 0.01	-16%
Myosin/Actin	2.3 ± 0.4	1.5 ± 0.3*[a]	-35%	1.6 ± 0.4*[a]	+7%
MHC2A+2X (%)	20.2 ± 5.9	20.2 ± 23.1	0%	22.9 ± 12.1	+13%
MHC1 (%)	79.8 ± 5.9	79.8 ± 23.1	0%	77.1 ± 12.1	-3%

*Table 4. Molecular properties of suspended-reloaded (S-R) and electrostimulated-suspended-reloaded (E/S-R) EDL. Δ%vsN, Percentage change of (S-R) EDL vs normal (N); Δ%vsSR, Percentage change of E/S-R EDL vs S-R; *[a], Significant difference between control and suspended or suspended-stimulated groups; n.r., not reported. Values are mean ± SD.*

	N EDL	S-R EDL	%vsN	E/S-R EDL	%vsS-R
protein (%)	19.7 ± 3.4	18 ± 2.2	-9%	17.8 ± 2.7	-1%
collagen (%)	1.4 ± 0.5	0.8 ± 0.3*[a]	-43%	0.8 ± 0.3*[a]	0%
collagen/protein ratio	0.08 ± 0.03	0.04 ± 0.01	-50%	0.05 ± 0.03	+25%
Myosin/Actin	1.8 ± 0.3	1.9 ± 0.5	+6%	1.9 ± 0.3	0%
MHC2A+2X (%)	35.5 ± 7.9	43.5 ± 6.9	+23%	38.8 ± 6.9	-14%
MHC2B (%)	64 ± 8.1	55.2 ± 6.7	-14%	60.4 ± 6.8	+7%
MHC1 (%)	0.5 ± 0.7	1.3 ± 1.6	n.r.	0.8 ± 0.9	n.r.

Unfortunately, the value of the suspended soleus is not statistically different from the normal value, even if decreased. A better marker of atrophy is the MHC/actin ratio [14, 15], which dramatically decrease in the suspended-reloaded soleus, but in both S-R and E/S-R groups. This change is very small or absent in the experimental EDL muscles.

Tables 3 and 4 show that the collagen percentage content decreases in all experimental muscles (both soleus and EDL) suggesting that collagen biosynthesis adapts to reduced muscle activity as described in experimental models of immobilisation [18]. Even this effect of suspension is not contrasted by the regimen of short daily reloading without or with electrostimulation.

No significant differences are present in MHC pattern of normal and experimental EDL, though the decreased percentage content of MHC2B is reminiscent of the effect of denervation, but not tenotomy or immobilization of fast rat muscle [16, 17, 31]. The MHC isoforms of soleus are maintained in the range of the normal ratio, that is both

MHC1 and MHC2A decrease, in keeping with the known effects of immobilization and tenotomy. It is worth remembering that in denervated soleus a preferential suppression of MHC1 has been clearly demonstrated [16, 17, 31]. On the other hand, Figure 2 shows that a minor MHC 2X band is present in experimental soleus muscles. Western blot analyses exclude the presence of MHCemb, though embryonic myosin was expected on the light of immunohistochemical analysis. Possibly, the MHCemb content is lower than the detection limit of our Western blot analysis. Significance of the MHC2X band in the experimental soleus remains to be established, though usually related to the significant increase in myofibrillar ATPase activity in the silenced soleus. Besides being present in regenerating myofibers of a permanently denervated soleus [4], we recently observed high level of expression of MHC2X in regenerating myofibers of rat soleus after reinnervation with a TTX-treated nerve (Midrio et al, manuscript in preparation). Its presence has been also recently confirmed in two- and four-week suspended rat soleus [9]. We have here demonstrated its presence as early as one-week of suspension. An additional open question remains, that is why only a subset of slow antigravity myofibers shows major changes in isomyosin expression.

In conclusion, even if the main aim of the experiment was not achieved (i.e., to test the trophic effect of a short daily regimen of reloading without and with a short but strenuous exercise) the shown data are interesting and worth to be corroborated by further research, in particular as the disturbed myogenesis is concerned.

Acknowledgements

Supported by funds of Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica to U.C., and by funds from the Italian C.N.R. to the Unit for Muscle Biology and Physiopathology.

Address correspondence to:

Ugo Carraro, Department of Biomedical Sciences, via Trieste 75, I-35131 Padova, Italy, fax +39 49 8286576.

References

[1] Betto R: Rivelazione delle proteine con Argento-Silver Staining, in Carraro U, Dalla Libera L (eds): *Elettroforesi ed cromatografia di biopolimeri e loro frammenti.* Padova, Unipress Padova, 1993, pp. 136-137.

[2] Biral D, Damiani E, Margreth A, Scarpini E, Scarlato G: Slow myosin heavy chain isozyme in nemaline myopathy. *Neurology* 1985; 35: 1360-1365.

[3] Brooke MH, Kaiser KK: Muscle fiber type: How many and what kind? *Arch Neur* 1970; 23: 369-379.

[4] Carraro U, Catani C, Degani A, Rizzi C: Myosin expression in denervated fast- and slow-twitch muscles: fiber modulation and substitution, in Pette D (ed): *The dynamic state of muscle fibers.* Berlin, Walter de Gruyer, 1990, pp 247-262.

[5] Carraro U, Catani C: A sensitive SDS-PAGE method separating myosin heavy chain isoforms of rat skeletal muscles reveals the heterogeneous nature of the embryonic myosin. *Biochem Biophys Res Commun* 1983; 116: 793-802.

[6] Danieli-Betto D, Zerbato E, Betto R: Type 1, 2A, 2B myosin heavy chain electrophoretic analysis of rat muscle fibers. *Biochem Biophys Res Commun* 1986; 138: 981-987.

[7] Desplanches D, Mayet MH, Sempore B, Flandrois R: Structural and functional responses to prolonged hindlimb suspension in rat muscle. *J Appl Physiol* 1987; 63: 558-563.

[8] Elder GCB, McComas AJ: Development of rat muscle during short- and long-term hindlimb suspension. *J Appl Physiol* 1987; 62: 1917-1923.

[9] Fauteck SP, Kandarian SC: Sensitive detection of myosin heavy chain composition in skeletal muscle under different loading conditions. *Am J Physiol* 1995; 268 (*Cell Physiol* 37): C419-C424.

[10] Flynn DE, Max SR: Effects of suspension hypokinesia hypodynamia on rat skeletal muscle. *Aviat Space Environ Med* 1985; 56: 1065-1069.

[11] Graham SC, Roy RR, West SP, Thomason D, Baldwin KM: Exercise effects on the size and metabolic properties of Soleus fibers in hindlimb-suspended rats. *Aviat Space Environ Med* 1989; 60: 226-234.

[12] Herbert ME, Roy RR, Edgerton VR: Influence of one-week hindlimb suspension and intermittent high load exercise on rat muscles. *Exp Neurol* 1988; 102: 190-198.

[13] Huszar G, Maiocco J, Naftolin F: Monitoring of collagen and collagen fragments in chromatography of protein mixtures. *Anal Biochem* 1980; 105: 424-429.

[14] Jakubiec-Puka A, Kulesza-Lipka D, Krajewski K: The contractile apparatus of striated muscle in the course of atrophy and regeneration. I. Myosin and actin filaments in the denervated rat soleus. *Cell Tissue Res* 1981; 220: 651-663.

[15] Jakubiec-Puka A, Kulesza-Lipka D, Kordowska J: the contractile apparatus of striated muscle in the course of atrophy and regeneration. II. Myosin and Actin filaments in mature rat soleus muscle regenerating after reinnervation. *Cell Tissue Res* 1982; 227: 641-650.

[16] Jakubiec-Puka A, Kordowska J, Catani C, Carraro U: Myosin heavy chain isoform composition in

striated muscle after denervation and self-reinnervation. *Eur J Biochem* 1990; 193: 623-628.

[17] Jakubiec-Puka A, Catani C, Carraro U: Myosin heavy-chain composition in striated muscle after tenotomy. *Biochem J* 1992; 282: 237-242.

[18] Karpakka J, Virtanen P, Vaananen K, Orava S, Takala TES: Collagen synthesis in rat skeletal muscle during immobilisation and remobilisation. *J Appl Physiol* 1991; 70 (4): 1775-1780.

[19] Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage. *Nature* 1970; 227: 680-685.

[20] LeBlanc A, Marsh C, Evans H, Jonson P, Schneider V, Jhingran S: Bone and muscle atrophy with suspension of the rat. *J Appl Physiol* 1985; 58: 1669-1675.

[21] Leterme D, Falempin M: Compensatory effects of chronic electrostimulation on unweighted rat soleus muscle. *Pflügers Arch* 1994; 426: 155-160.

[22] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-277.

[23] Mauro A: Satellite cells of skeletal muscle fibers. *J Biophys Biochem Cytol* 1961; 9: 493-495.

[24] Merril R, Goldman Sedman SA, Ebert MH: Ultra sensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 1981; 211: 1437-1438.

[25] Morey ER: Spaceflight and bone turnover: correlation with a new rat model of weightlessness. *Bioscience* 1979; 29: 168-172.

[26] Morey-Holton ER, Wronski TJ: Animal models for simulating weightlessness. *Physiologist* 1981; 24, Suppl: S45-S48.

[27] Mussini I, Favaro G, Carraro U: Maturation, dystrophic changes and the continuous production of fibers in skeletal muscle regenerating in the absence of nerve. *J Neuropathol Exp Neurol* 1987; 46: 315-331.

[28] Ontell M, Hughes D, Bourke D: Secondary myogenesis of normal muscle produces abnormal myotubes. *Anat Rec* 1982; 204: 199-207.

[29] Rossini K, Rizzi C, Sandri M, Bruson A, Carraro U: High-resolution sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunochemical identification of the 2X and embryonic myosin heavy chains in complex mixtures of isomyosins. *Electrophoresis* 1995; 16: 101-104.

[30] Sandri M, Rizzi C, Catani C, Carraro U: Small and large scale preparative purification of myosin light and heavy chains by selective KDS precipitation of myosin subunits: Yield by SDS PAGE and quantitative orthogonal densitometry. *Basic Appl Myol* 1992; 2: 107-114.

[31] Szczepanowska J, Jakubiec-Puka A: Myosin heavy chains in striated muscle after immobilisation. *Basic Appl Myol* 1992; 2(2): 97-106.

[32] Thomason DB, Herrik RE, Surdyka D, Baldwin KM: Time course of soleus muscle myosin expression during hindlimb suspension and recovery. *J Appl Physiol* 1987; 63: 130-137.

[33] Thomason DB, Booth FW: Atrophy of soleus muscle by hindlimb unweighting. *J Appl Physiol* 1990; 68 (1): 1-12.

[34] Woessner JE: The determination of Hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch Biochem Biophys* 1961; 93: 40-447.