Contractile Proteins Content of Long Term Permanent Denervated Human Muscle after Functional Electrical Stimulation

Julita Bielewicz[(1)], Maria Elena Zanin, Abdulaje Galbani and Ugo Carraro

C.N.R. Institute of Neuroscience, Neuromuscular Section, Laboratory of Applied Myology, Department of Biomedical Science, University of Padova, Italy; (1) Department of Medical Biochemistry, Wroclaw Medical University, Poland

Abstract
Severe atrophy of skeletal muscles occurs during long-term permanent denervation (LT-PD). We studied 14 human muscle biopsies taken from patients suffering lower motoneuron denervation from 2 to 30 years. Patients were treated with Functional Electrical Stimulation (FES) for different periods of time (0.5 to 6 years). We describe new gel electrophoresis analyses, which allow to quantify protein markers of damage/degeneration and recovery/regeneration of muscle tissue. Electrical stimulation of denervated muscles increases size of the myofibres, maintains sarcomeres preventing/reversing apoptosis/necrosis and secondary degeneration of the long-term denervated tissue. In this work we quantify myosin content in long-term permanent denervated muscles without and with FES. Results confirm the effectiveness of the FES training.
Key words: FES, LT-PD, myosin heavy chains, myosin isoforms, SDS PAGE.

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The main goal of FES is providing muscular contraction and producing a functionally useful movement, but it could be used just to obtain recovery of myofiber size [4]. The electrical stimulation is believed to promote muscle growth, neuron sprouting, and increased sensation. Previous published results [6, 8, 11] shown worthwhile restitution of muscle trophism using FES. In this work we analyze and quantify muscle re-growth due to electrical stimulation by measuring myosin content in biopsies of muscle tissue after long-term permanent denervation before and after FES.

Material and Methods

Biopsies

Subjects were either LT-PD (4 - 8.7 years), or LT-PD after FES training (2.3 - 7 years). Needle biopsies of human muscles were taken from right and left leg.

Molecular markers and quantification of total protein in cryostatic sections of these human biopsies were as described in [11].

4% Stacking Gel – SDS PAGE to determine total protein in cryosections of human biopsies

The total protein quantities is determied using a short run in 4% stacking gel SDS PAGE, prepared according to Laemmli [2,9] in 0.75x80x130 mm (thick; high; large) polyacrylamide slab. The Separating gel (bottom gel) is made of 35 mm 10% T acrylamide-Bis (36.5:1), 375 mM Tris HCl (pH 8.8) and 0.1% SDS, only to allow easier transfer of the slab during staining and destaining procedures.

Stacking gel (upper gel: cm 2.0 from the bottom of the well and the interface of stacking and separating gels) is prepared of 45 mm 4% T acrylamide-Bis (36.5:1), 120 mM Tris HCl (pH 6.8) and 0.1% SDS. The electrophoresis buffer is made of 25 mM Tris, 192 mM glycine pH 8.3 and 0.1% SDS. The washing buffer for shoulders consists of 125 mM Tris HCl and 0.1% SDS.

To determine the content of protein in sample we load in each slab known quantities of albumin and purified myosin markers (0.25-2µg) [2]. Electrophoresis runned under constant current mode at 20mA per slab. Under this amplitude initial voltage was 80V. The run was stopped after 60 min, when the dye front was ~1 cm from the stacking and separating gel interface. Proteins were stained in 200 ml of 0.1% Coomasie Brilliant Blue in 5% acetic acid during 30 min, and destained by 3 washings, 15 min each in 20 ml 40% methanol and 17% acetic acid. Slabs were saved in 1% acetic acid.

5% Stacking Gel – SDS PAGE to determine Myosin/Total protein ratio in cryosections of human biopsies

When stacking gel concentration was increased from 4% to 5% in the stacking gel, in the running condition above described myosin heavy chains separated from protein front during the stacking period of gel electrophoresis. After staining and destaining, as above described, MHC/Total protein ratio was determined by gel densitometry.

10% Separating Gel – SDS PAGE to determine Myosin/Actin ratio

SDS polyacrylamide gel electrophoresis was performed according to Laemmli [3,4,9]. Separating gel is made of 10% acrylamide and stacking gel of 5% acrylamide. The run was under constant current mode at 20mA per slab, with initial voltage 80V. Run was ended after 3 hours, when the dye front was lefting slab lower surface, at which time voltage raised to 210 V. Protein were detected by either Coomasie Brilliant Blue, as previously described. For better quality there is also made Silver Stain [10]. One washing, 30 min in 50% methanol and 12% acetic acid; two washings (10min each) in 10% ethanol and 5% acetic acid; 10 min washing in 3.4mM K2Cr2O7 and 3.2 mM HNO3; 4 washings (30 sec each) in distilled H2O; 30 min in 12mM AgNO3 under lamp illumination; washing in distilled H2O; very fast washing in 0.28mM Na2CO3 and 1% formaldehyde; washing in distilled water and store developed gel in 1% acetic acid.

Myosin/Actin ratio was determined by gel densitometry.

7% SDS PAGE to determine MHC isoforms

Myosin heavy chains (MHC) isoforms were separated using analytical SDS-PAGE on 7% polyacrylamide gel [1,6,7,14].

The electrophoresis gel was prepared with 50% glycerol in both separating and stacking gel [4]. The stacking gel was made of 29.6% glycerol, 5% T acrylamide-Bis (36.5:1), 125 mM Tris HCl (pH 6.8) and 0.1% SDS. The running (separating) gel is prepared of 25.7% glycerol, 7% T acrylamide-Bis (36.5:1), 375 mM Tris HCl (pH 8.8) and 0.1% SDS. The running buffer is made of 50 mM Tris, 384 mM glycine (pH 8.3) and 0.2% SDS. Under current mode at 4mA per slab, the initial voltage was 40 V. After 12-14 hours buffer was changed and electrophoresis was re-started under the same conditions. Electrophoresis was ended after 24 hours, at which time the voltage rose to 180-200 V. Protein markers were loaded in each gel (0.5-2µg). After run gels were stained with Coomasie Brilliant Blue. When protein were 10 time less then usual, slabs were stained by the Silver Stain method [10].

Figure 1. Total protein by stacking gel 4%

Figure 2. Electrophoretic separation of human MHC from total protein in 5% stacking gel.

Gel Densitometry

Protein contents in slab gels were quantitated by densitrometry using an Epson Perfection 1650 scanner connected to a PC. Data were computed using Scion Image for Windows version 4.0.2. by Scion Image software (PC version) of Scion Corporation.

Results and discussion

Protein content of skeletal muscle biopsies

Figures 1 to 4 show results of the molecular analyses by SDS-PAGE of protein from LT-PD skeletal muscle biopsies. Figure 1 shows total protein content in 6 representative samples. Figure 2 shows separation of the human myosin heavy chains from all other muscle protein. By the new 5% stacking-gel SDS PAGE we determined total protein, myosin/total protein ratio, myosin/actin ratio, and myosin isoforms. In accordance to previous data, skeletal muscle contains about 200 mg of total protein and 100 mg of myosin per gram of fresh muscle weight, i.e., myosin heavy chains represent 50 percent of total muscle proteins. Table 1 shows that the 4 to 8.7 year denervated muscle have a mean percentual of MHC of 13.6±5.6, while after FES the percent increases to 29.4±12.7.

Myosin/actin ratio

Myosin/actin ratio is known to change during muscle atrophy, e.g., after muscle denervation. Figure 3 shows examples of the myosin/actin ratio by 10% SDS-PAGE. In normal adult muscle myosin/actin ratio is about 2.2 [12,13]. After FES stimulation in LT-PD human muscles yhe rate is 1.81.

MHC isoforms

Figure 4 shows some examples of MHC isoform analysis by 7% SDS-PAGE. All the biopsies present adult fast-type myosin heavy chains (MHC 2), while the slow isoforms are seldom present (MHC 1) in cryostat sections of LT-PD FES-trained muscle.

The low content of MHC 1 is probably the result of the fact that FES training is no longer than 60 min per day.

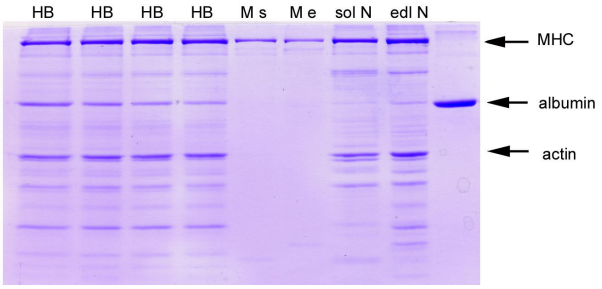

Figure 3. Electrophoretic separation of human actin and myosin in 10% separating gel. HB, human biopsy; Ms, soleo Myosin, Me, EDL Myosin, solN, criostatic section of normal rat soleus, edlN, criostatic section of normal rat EDL.

Figure 4. Electrophoretic separation of Human MHCs in 10% separating gel. MHC1- slow isoform; MHC2 fast isoform.

Table1. Percentual content of MHC in Total Protein. D(1-7), Denervated muscle; FES(1-7), denervated and stimulated muscle.

Biopsy	Denervation time (years)	FES Time (years)	MHC in total protein (%)
D1	4	---	16.9
D2	4	---	15.7
D3	7.5	---	18.7
D4	7.5	---	18.1
D5	7.7	---	2.6
D6	8.7	---	12.1
D7	8.7	---	11.2
Mean ± st dev			13.6 ± 5.6
FES 1	4	2.3	20.4
FES 2	4	2.3	17.8
FES 3	5	2.3	26.6
FES 4	6.3	4.3	19.7
FES 5	6.3	4.3	26.6
FES 6	7.5	7	48.1
FES 7	7.5	7	46.5
Mean ± st dev			29.4 ± 12.7

We here studied influence of functional electrical stimulation training on long- term paralyzed human muscle. There is still controversy about the use of electrical stimulation of denervated muscle fibers [6,8].

During the next years, the goals of the EU supported RISE project will be to identify, using experiments in animals and clinical research, safer stimulation protocols needed to induce early effects with, hopefully, a reduced stimulation burden for the patients. If all myofibers in the thigh could be consistently reached, the increased muscle mass and fatigue resistance will rise patients and their quality of life.

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Address correspondence to:

Prof. Ugo Carraro, Department of Biomedical Science, University of Padova. Via G.Colombo, 3; 35121 Padova (Italy). Tel +39 049 8276030; fax +39 049 8276040. e-mail: ugo.carraro@unipd.it.

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