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Mechanisms of spinal motoneurons survival in rats under simulated hypogravity on earth

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ABSTRACT

It was previously shown that different cell types in vivo and in vitro may die via apoptosis under weightlessness conditions in space as well as in simulated hypogravity on the Earth. We assessed survivability of spinal motoneurons of rats after 35-day antiorthostatic hind limb suspension. Following weight bearing, unloading the total protein content in lumbar spinal cord is dropped by 21%. The electrophysiological studies of m. gastrocnemius revealed an elevated motoneurons' reflex excitability and conduction disturbances in the sciatic nerve axons. The number of myelinated fibers in the ventral root of experimental animals was insignificantly increased by 35-day of antiorthostatic hind limb suspension, although the retrograde axonal transport was significantly decreased during the first week of simulated hypogravity. The results of the immunohistochemical assay with antibodies against proapoptotic protein caspase 9 and cytotoxicity marker neuron specific nitric oxide synthase (nNOS) and the TUNEL staining did not reveal any signs of apoptosis in motoneurons of suspended and control animals. To examine the possible adaptation mechanisms activated in motoneurons in response to simulated hypogravity we investigated immunoexpression of Hsp25 and Hsp70 in lumbar spinal cord of the rats after 35-day antiorthostatic hind limb suspension. Comparative analysis of the immunohistochemical reaction with anti-Hsp25 antibodies revealed differential staining of motoneurons in intact and experimental animals. The density of immunoprecipitate with anti-Hsp25 antibodies was substantially higher in motoneurons of the 35-day suspended than control rats and the more intensive precipitate in this reaction was observed in motoneuron neuritis. Quantitative analysis of Hsp25 expression demonstrated an increase in the Hsp25 level by 95% in experimental rats compared to the control. The immunoexpression of Hsp70 found no qualitative and quantitative differences in control and experimental lumbar spinal cords. Taken together our results show that (1) rat motoneurons survived after 35-day antiorthostatic hind limb suspension and the changes in neurons had a mostly functional character, and (2) the increased immunoexpression of Hsp25 can be considered as the anti-apoptotic factor.

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1. Introduction

Muscle weakness, atonia and atrophy of skeletal muscle fibers constitute the hypogravity motor syndrome that develops in response to microgravity [1]. It is interesting to note that similar pathological disorders occur in the skeletal musculature in the event of degeneration of spinal motoneurons [2]. However, it remains an open issue what is the primary trigger of these changes in skeletal muscle tissue in microgravity. There was an assumption that the factor triggering the development of the hypogravity mFotor syndrome is linked to the nervous system. Neuromorphological studies on laboratory animals under microgravity demonstrated a reduced functional activity of spinal motoneurons [3]. After a space flight, a low activity of succinate dehydrogenase [4] and significantly reduced protein content were recorded in the cytoplasm of motoneurons in the ventral horns of the rat spinal cord [5]. In addition, analysis of the protein profile of the hippocampus in mice after a seven-day antiorthostatic hind limb suspension also showed a decrease in the total protein content (in particular, the concentrations of tubulin, β-synuclein, and pyruvate dehydrogenase) [6]. In vitro studies under simulated microgravity in a system of three-dimensional randomized positioning showed that neurons and glial cells underwent apoptosis as a result of abnormal cytoskeleton organization and mitochondrion damage [7]. In space, apoptotic cells were detected in cultures of lymphoblasts [8] and osteoblasts [9,10]. After antiorthostatic suspension in vivo, apoptosis was also observed in skeletal muscle fibers of the rat hind limb [11] and in mouse osteocytes of the lumbar vertebra [12]. We previously reported that, after a 35-day antiorthostatic suspension of rats, the expression of choline acetyltransferase (ChAT) was 54% reduced in motoneurons of the lumbar spinal cord. In addition, atrophy of the hind limb skeletal muscles, a 10 mV drop in the resting membrane potential, and an increase in the excitability of the muscle fiber membrane were recorded [13]. Analysis of these data suggests reactive changes in the spinal cord (primarily, in motoneurons) in response to antiorthostatic hind limb suspension. Thus, under microgravity or conditions that simulate microgravity both in vitro and in vivo, the probability of motoneuronal apoptosis exists. In the present investigation we studied the viability of spinal motoneurons in rats after 35-day antiorthostatic suspension. Apoptosis markers were used to look for possible death of motoneurons, and markers of neuron viability were used to study mechanisms of neuroprotection.

2. Materials and methods

For microgravity simulation, mature male Wistar rats weighing 260–280 g were used in the Morey–Holton model of antiorthostatic hind limb suspension [14], which is widely used for studying the effects of microgravity on laboratory animals on the Earth. Animal protocol was approved by the Kazan State Medical University Animal Care and Use Committee. Experimental and control euthanized mice and rats were sacrificed for the following investigations.

To study the viability of lumbar motoneurons innervating the hind limb skeletal muscles the lumbar spinal cord from experimental and control animals was processed for immunohistochemical assay, immunoblotting and transferase mediated dUTP nick end labeling (TUNEL) assay of apoptosis. The ventral roots of the L5 level were used for morphometric analysis of myelinated axons. Morphological investigations at specified time points were preceded by measurement of the electrophysiological activity of m. gastrocnemius. To evaluate retrograde axonal transport under simulated hypogravity condition mature male mice were employed.

2.1. Electrophysiological methods

Threshold and peak amplitudes of m. gastrocnemius reflex (H) and motor (M) responses were determined in experimental and control rats on completion of the 35-day period of weight-bearing deprivation [15]. Two hours prior to the electrophysiological studies the animals were given general ether anesthesia and underwent spinalization between the second and third thoracic vertebrae to get rid of supraspinal influence without affecting the breathing activity. Electrical responses to sciatic nerve stimulation were recorded with the help of needle electrodes. Stimulation was produced by single rectangular pulses with the duration of 0.5 ms and frequency of 0.5 pulse/min. Stimuli intensity varied from 0.3 to 50 V to evaluate maximal amplitude in dynamic. Stimulation, amplification and response recording were performed using the electromyograph MG-42 (Medicor).

2.2. Retrograde labeling of lumbar motoneurons

Retrograde labeling of motoneurons was performed to evaluate the efficiency of axonal transport after antiorthostatic hind limb suspension. Mice three and six days after antiorthostatic hind limb suspension were euthanized, the right sciatic nerve was cut in the midthigh, and a 7 mm silastic tube with a 1.47 mm inner diameter was applied to the central nerve stump as described previously [16]. For retrograde labeling of motoneurons, 5% fluorescent dye fluorogold (fluorochrom, Denver, Co.) in vehicle was administered into the tube (7 μl total volume). The lower end of the tube was sealed with petroleum jelly (Vaseline); the tube was glued to the surrounding skeletal muscles with tissue adhesive (3 M Vetbond), and the incision was closed with wound clips. After application of Fluorogold for 24 h, mice were sacrificed. In every experiment, we verified that the tube was still in place and that Fluorogold did not stain the adjacent tissue. Only the mice, which had the yellowish proximal nerve stump sited inside the tube (~90% of animals) were selected for study. Twenty-four hours postsurgery, mice were euthanized and perfused with cold PBS, followed by cold 4% paraformaldehyde in PBS (pH 7.4). Lumbar spinal cords were removed and post-fixed in 4% paraformaldehyde, cryoprotected in 30%

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sucrose, and embedded in Tris-buffered saline tissue-freezing medium (Triangle Biomedical science, Durham, NC). Cryostat serial coronal 30 μm sections were analyzed with fluorescence microscopy using a wideband ultraviolet filter. Fluorogold positive motoneurons in the ipsilateral side were recognized by their size, shape, and location and counted in every section without allowing for split nucleoli.

2.3. Semi-thin sections

After the 35-day suspension, four millimeter fragments of ventral roots, distal from spinal cord, were obtained at the L5 level from euthanized rats and fixed in 2.5% solution of glutaraldehyde for 4 h. This was followed by 24 h incubation in 1% solution of osmium tetroxide. Before embedding into an epoxy resin mixture (Epon/Araldit), the ventral roots were bisected crosswise, i.e. cross semi-thin sections were prepared at a distance of 2 mm from the ventral root exit from the lumbar spinal cord. The semi-thin sections were stained with methylene-blue according to the standard procedure. Digitized images of semi-thin sections were analyzed using the ImageJ software (NIH). The total number of myelinated fibers in the cross-sections of the ventral root was determined.

2.4. Immunohistochemistry

At the end of 35-day suspension, the rats were euthanized and perfused, through the systemic circulation, first with a cold phosphate–saline buffer (pH=7.4) and then by a cold 4% paraformaldehyde solution (pH=7.4). The lumbar part of the spinal cord was post-fixated in paraformaldehyde, cryoprotected in 30% sucrose, and embedded in Tris-buffered saline tissue-freezing medium (Triangle Biomedical Science, Dirham, NC). Frozen free-floating coronal sections (25 μm) of the lumbar the spinal cord were prepared as described previously [17]. For immunohistochemical reaction the following antibodies (AB) were used: anti-caspase 9 (1:50, Sigma), anti-Hsp25 (1:1000, Stressgen), anti-Hsp70 (1:500, Stressgen), and anti-nNOS (1:200, BD Bioscience). The streptavidine–biotin complex (Elite ABC Kit; Vector Laboratories) was used for the immune reaction with primary antibodies. Immunoprecipitate was visualized using diamine benzidine (DAB Substrate Kit for Peroxidase; Vector Laboratories).

2.5. Transferase mediated dUTP nick end labeling (TUNEL) staining

Lumbar spinal cords of rats were collected on days 7, 14, 21 and 35 after hind limb suspension and were processed similar to immunohistochemical assay for TUNEL diagnosing of apoptosis. Hybridization of biotin-conjugated 3′uracil was performed on free-floating cross-sections of lumbar spinal cord using terminal deoxynucleotidyl transferase (TdT) following the manufacture's protocol (Promega, DeadEnd^{TM} Colorimetric Tunel System). Biotin-conjugated nucleotide was bound afterwards to streptavidine conjugated with horse-radish peroxidase (streptavidine–biotin method). Histochemical reactions were visualized with diamine benzidine. Specificity of the TUNEL staining was verified by sections incubation in the presence of DNAase.

2.6. Immunoblotting

Spinal cord was isolated from the rats after 35-day suspension, frozen in liquid nitrogen and kept in a freezer at $-80\,^{\circ}$C. Denatured protein extract of the lumbar spinal cord was separated electrophoretically and transferred into PVDF membranes (Immobilon P, Millipore). The membranes were incubated with anti-Hsp25 (Stressgen) and anti-Hsp70 (Stressgen) antibodies diluted to 1:1000. Primary antibodies were detected using streptavidine–biotin complex (Elite ABC Kit, Vector Laboratories). Immunoprecipitate was visualized using diamine benzidine (DAB Substrate Kit for Peroxidase; Vector Laboratories). After membrane staining and scanning, densitometry of the obtained images was made using the Scion Image software. The intensity of the immune reaction was normalized for the β-actin signal, which was additionally detected when the membranes had been washed after the first immunoenzyme reaction. Antibodies against β-actin (Sigma) were used at 1:200 dilution. The ratio of signal from the target proteins to β-actin in untreated animal tissue was considered to be 100%. The amount of protein in the experimental samples were presented as percentage to control samples.

2.7. Statistical analysis

Student's *t*-test was performed using the prism (GraphPad Prism, Windows version 3.00; GraphPad Software, San Diego, CA). Results represent mean values $\pm$ SD ($M \pm m$). p-values < 0.05 was considered statistically significant.

3. Results

Analysis of the entire spinal cord demonstrated a less pronounced lumbar spinal cord enlargement in rats subjected to 35-day suspension. No difference was observed in total protein in denatured protein extracts from the cervical spinal cord enlargement of the control ($2.57 \pm 0.$ 28 mg/ml; n=8) and experimental animals (2.57 ± 0.30 mg/ml; n=5), respectively. As for the lumbar spinal cord, total protein is decreased by 21% in experimental animals when compared with control animals; in the experimental rats (n=4) it measured 2.79 ± 0.39 mg/ml, whereas in the control rats (n=8) it amounted to 3.54 ± 0.34 mg/ml (Fig. 1).

Electrophysiological studies of m.gastrocnemius in experimental animals after 35 days of weight-load deprivation demonstrated threshold decrease and amplitude increase of the H-response ($47 \pm 10\%$ and $155 \pm$ 15%, respectively), when compared with the controls.

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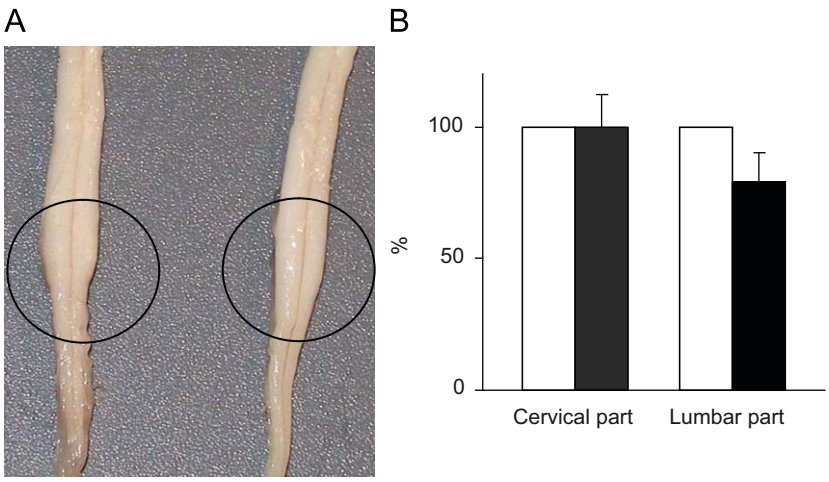

Fig. 1. Spinal cords of control and experimental (35-d weight-load deprivation) rats. (A) Spinal cords of control (left panel) and suspended (right panel) animals. The circle shows the lumbar spinal cord enlargement, and (B) total protein level in the lumber spinal cord in %. Control values are taken for 100%.

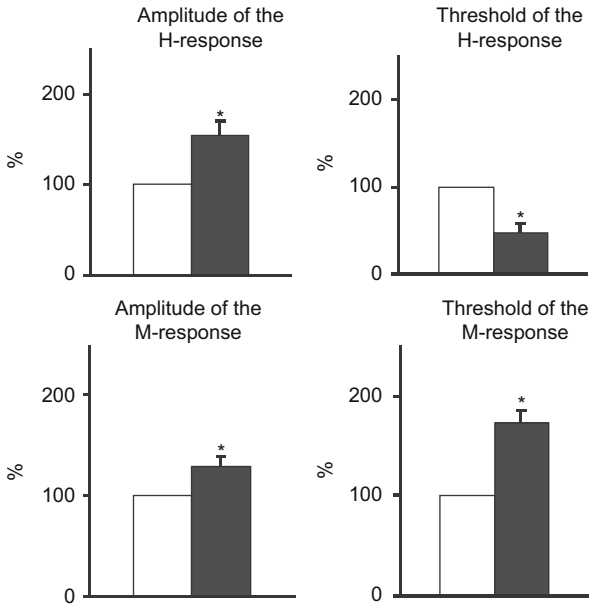

Fig. 2. Values of m. gastrocnemius motor (M) and reflex (H) reaction parameters in rats after 35-day weightbearing deprivation. *Y*-axis—M- and H-threshold and amplitudes in % to control values; $^{*}p < 0.05$.

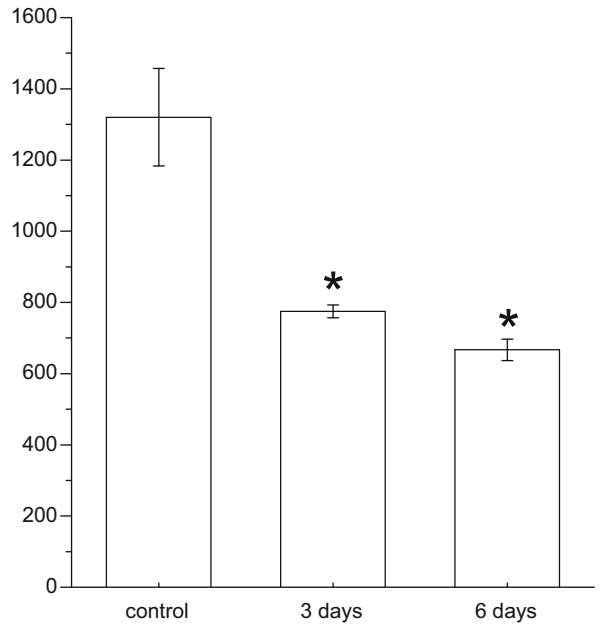

Fig. 3. The number of FG-labeled lumbar motoneurons in mice after 3 and 6 days of simulated hypogravity. $^{*}p < 0.05$.

Threshold and amplitude of the M-response in experimental rats (n=10) reached $172 \pm 13\%$ and $131 \pm 11\%$ relative to controls (n=10) (Fig. 2).

To investigate the efficiency of axonal transport after antiorthostatic hind limb suspension we used retrograde labeling of lumbar motoneurons in mice. After 24 h, the total number of Fluorogold-labeled motoneurons was significantly decreased in mice after 3 days (775 ± 18, n=4) and 6 days of simulated hypogravity (667 ± 30, n=6) when compared with control (1320 ± 137, n=6) (Fig. 3).

The morphometric assay of semi-thin sections of the ventral root at L5 level in the intact animals revealed 1980 ± 317 myelinated fibers (Fig. 4). Following the 35-day suspension, the relative myelinated fiber content in the ventral root of the experimental rats reached 2563 ± 374. No reliable differences were observed in the number of myelinated fibers of the experimental (n=4) and intact (n=4) animals ($p > 0.1$). Although insignificant increase in the myelinated fibers number indirectly may indicate on sprouting of myelinated fibers in regenerating motoneurons.

Taking into consideration our previous findings [13], demonstrating decrease in ChAT immunoexpression in lumbar spinal cord, reduction of resting membrane potential and increased membrane excitability in skeletal muscle fibers, along with the results of this investigation,

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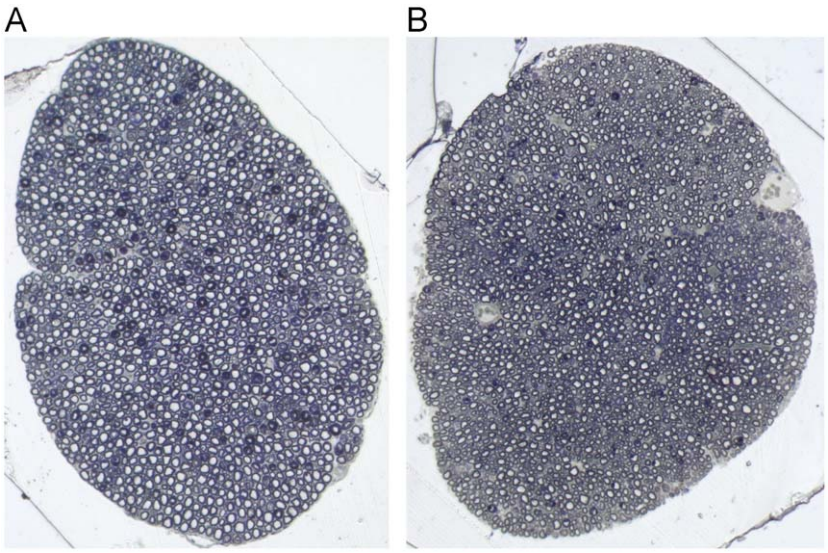

Fig. 4. Semi-thin sections of rat L5 ventral root. (A) control, and (B) 35-day suspended rat. In cross-section of the ventral roots of control and experimental animals there are normal myelinated fibers.

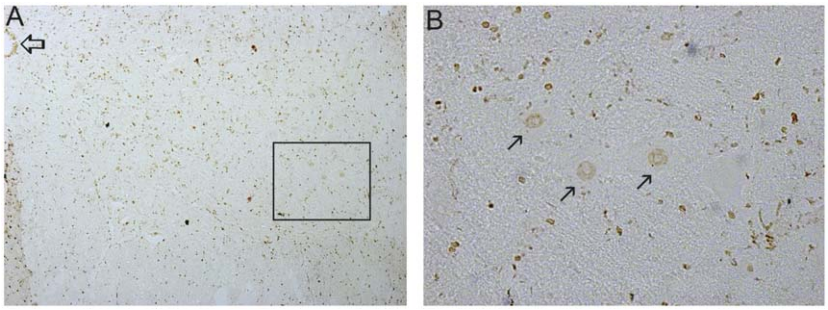

Fig. 5. TUNEL assay. (A) Cross-section of rat lumbar spinal cord at L4–L5 level after 35 days of antiorthostatic hind limb suspension. Specific immunoprecipitate in nuclei of all spinal anterior horns cells appeared after incubation of the cross-sections in the presence of DNAase. The contour arrow points to the central canal, and (B) selected fragment of (A) under a large magnification. Black arrows point to immune positive motoneuron nuclei.

we hypothesize that these changes may be due to death of lumbar motoneurons under the condition of simulated hypogravity.

To test this hypothesis we examined whether simulated hypogravity may induce apoptosis in lumbar motoneurons. No indications of apoptosis were observed in either control or experimental rats after 7, 14, 21 and 35 days of suspension using the TUNEL assay. The negative reaction was supported by the data from parallel incubation of control spinal cord sections in the presence of DNAase. Positive nuclei of neurons, glial and endothelial cells in control sections are presented in Fig. 5. In addition, there was no change in nNOS immunostaining in lumbar motoneurons, which is thought to trigger neuron apoptosis [18]. Comparative analysis of immunohistochem;cal staining with anti-nNOS antibodies of spinal cord sections of the control rats and animals subjected to 35-day suspension did not reveal any qualitative differences in the immune reaction (Fig. 6). Immunoprecipitate of equal intensity was localized in the perikary and motoneuron neuritis. The immunohistochemical staining with antibodies to proapoptotic protein caspase 9 showed nuclear localization of this enzyme in lumbar motoneurons both in experimental and control animals (Fig. 7). Thus, immunoexpression of nNOS and proapoptotic protein caspase 9 was also not indicative of motoneurons apoptosis. These evidences allowed us to conclude that motoneurons do not enter apoptosis when exposed to laboratory hypogravity on the Earth.

Further we have proposed that the survivability of lumbar motoneurons under condition of simulated hypogravity is due to activated unknown adaptation mechanisms to hypogravity. It is well known that heat-shock proteins (Hsp) serve as markers of intracellular stress or compensatory adaptation. In the present immunohistochemical study we used anti-Hsp70 and anti-Hsp25 antibodies for immunohistochemical staining control (n=3) and experimental (n=3) rat spinal cords. The immunohistochemical staining with anti-Hsp70 antibodies found no qualitative difference in the reaction of the control rats and rats after 35-day antiorthostatic hind limb suspension. Specific immunoprecipitate was

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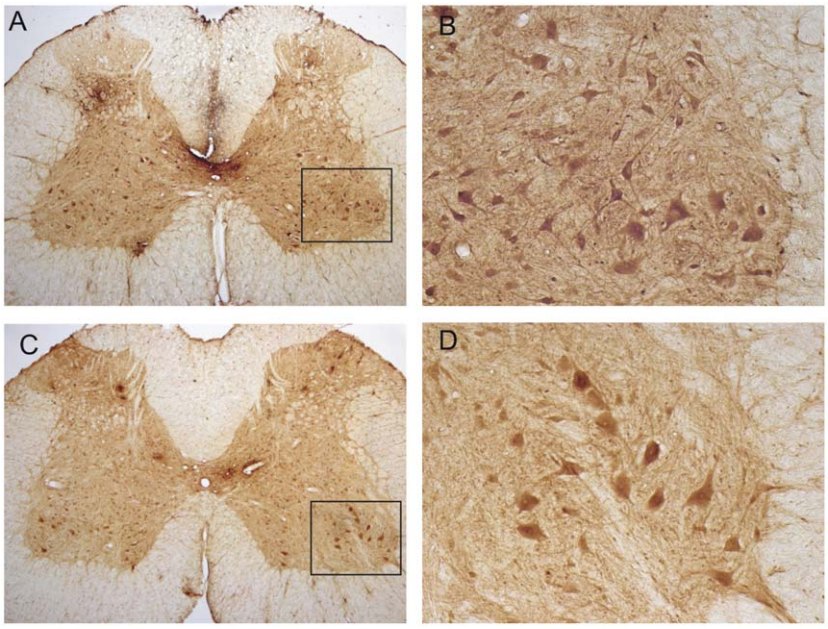

Fig. 6. Immunohistochemical reaction with anti-nNOS antibodies in rat lumbar spinal cord cross-sections at L4–L5 level. (A) and (B) Control animals, (C) and (D) 35-day suspended rats, and (B) and (D) selected fragments on (A) and (C) under a large magnification. Immunopositive motoneurons are seen in ventral horn of the lumbar spinal cord. The character of immunohistochemical reaction does not differ in control and experimental rats.

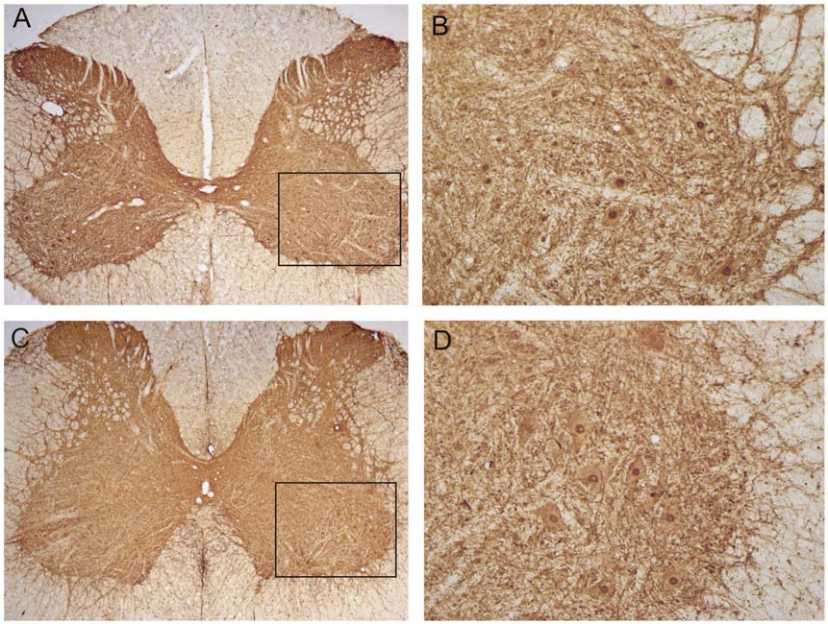

Fig. 7. Immunohistochemical reaction with anti-caspase 9 antibodies in rat lumbar spinal cord cross-sections at L4–L5 level. (A) and (B) control animals, (C) and (D) 35-day suspended rats, and (B) and (D) selected fragments on (A) and (C) under a large magnification. Immunohistochemical reaction demonstrates the nuclear staining of motoneurons in experimental and control lumbar spinal cord.

observed in motoneuron perikarya and neuritis (Fig. 8). Immunoblot demonstrated statistically insignificant increase in the Hsp70 level by 42% in the experimental lumbar spinal cord when compared to control. Comparative analysis of the immunohistochemical reaction with anti-Hsp25 antibodies revealed differential staining of motoneurons in intact and experimental animals. Under similar conditions of immunohistochemical reaction the density of immunoprecipitate with anti-Hsp25 antibodies was substantially higher in motoneurons of the 35-day suspended than control rats (Fig. 9). Additionally the more intensive precipitate in this reaction was observed in motoneuron neuritis of the experimental rats. Quantitative analysis of Hsp25

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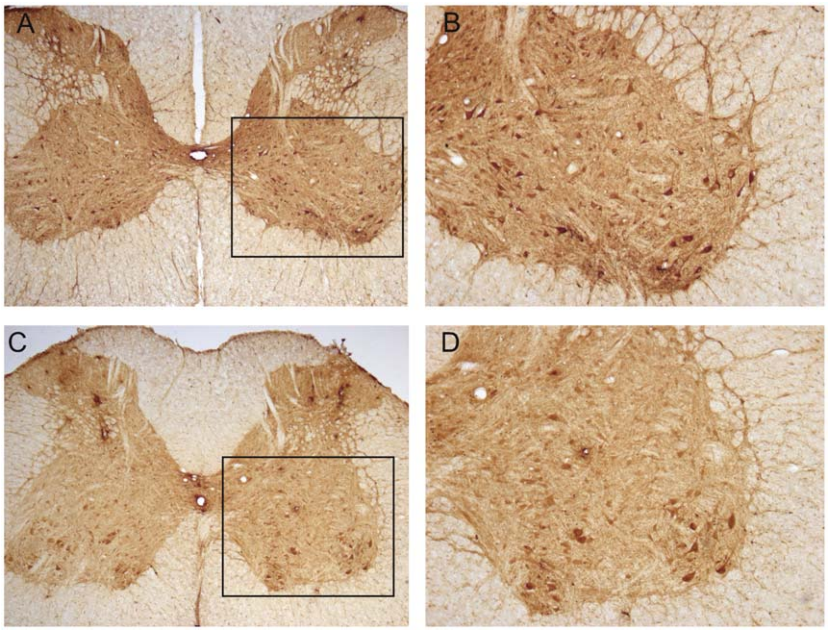

Fig. 8. Immunohistochemical reaction with anti-Hsp70 antibodies in rat lumbar spinal cord cross-sections at L4–L5 level. (A) and (B) Control animals, (C) and (D) 35-day suspended rats, and (B) and (D) selected fragments on (A) and (C) under a large magnification. Motoneurons have an equal immunostaining in lumbar spinal cords from control and experimental animals.

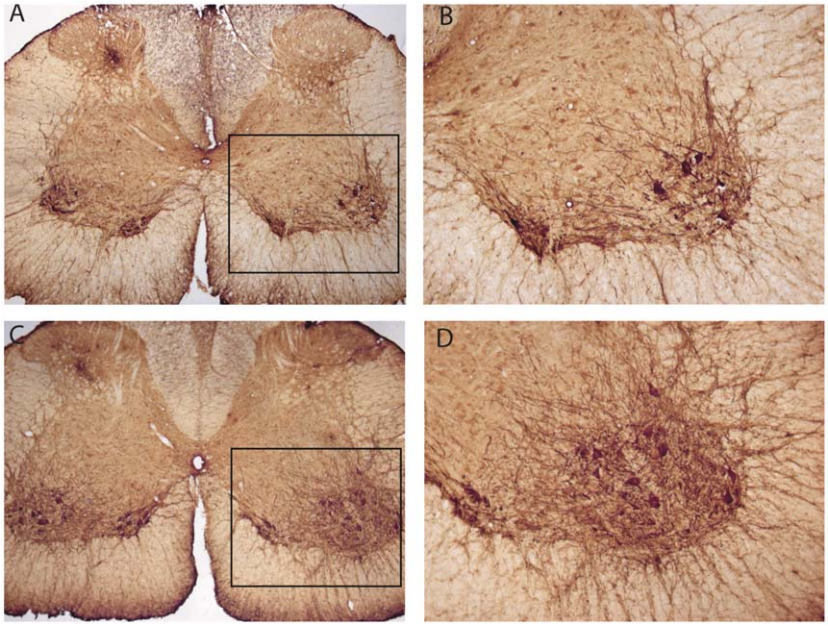

Fig. 9. Immunohistochemical reaction with anti-Hsp25 antibodies in rat lumbar spinal cord cross-sections at L4–L5 level. (A) and (B) Control animals, (C) and (D) 35-day suspended rats, and (B) and (D) selected fragments on (A) and (C) under a large magnification. In the lumbar spinal cord from experimental rats the intensity of immunohistochemical reaction is much higher than in control spinal cord. Specific immunoprecipitate has strong localization in neuritis of the affected motoneurons.

expression demonstrated a substantially different Hsp25 content in the lumbar part of the spinal cord of the control and 35-day suspended rats. The experimental rats had an increase in the Hsp25 level by 95% as compared to the controls (Fig. 10).

4. Discussion

It is well known that motoneurons, in addition to controlling the muscle endogenous program and maintaining the muscle fibers in differentiated state, are

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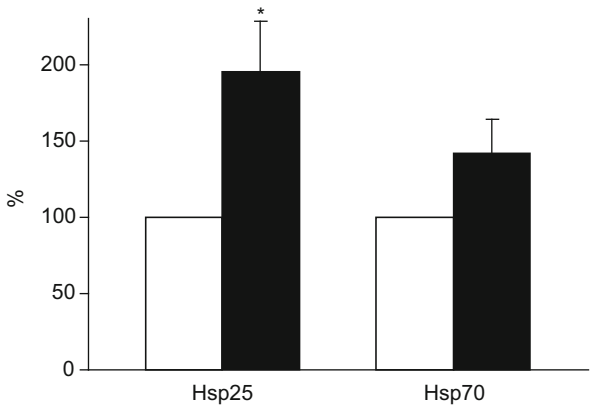

Fig. 10. Western blot. Densitometric analysis of the relative levels of Hsp25 and Hsp70 in lumbar spinal cord. Values of four independent immunoblots of intact ($n=5$, white column) and 35-day suspended ($n=7$, black column) animals are shown. Mean values of Hsp25 and Hsp70 immunoexpression are given relative to β-actin. Axis *Y*—Hsp25 and Hsp70 content in per cent; intact spinal cord values are assumed to be equal to 100%; $^{*}p < 0.05$.

responsible for modulation of myofiber phenotype within the muscle cell type. Deviations in the neurotrophic control as a result of axotomy or neurodegeneration give rise to a wide range of specific alterations in skeletal muscle (atrophy, changes in electrophysical properties of myofiber membrane, embryonic distribution of cholinergic receptors, etc.). Considering that motoneurons are directly involved in the morpho-functional control of skeletal muscle, we advanced the hypothesis that the hypogravity-induced alterations in skeletal muscles are partly caused by the changes in motoneurons functional properties or their death. Our observations in rat spinal cord after 35-day antiorthostatic hind limb suspension demonstrated a decrease in the total protein ratio by 21% and ChAT expression by 54% [13]. Elevated reflex excitability of motoneurons (lowered M-threshold and increased M-amplitude) suggests the possible neurodegenerative changes in lumbar spinal cord. Moreover, investigation of retrograde axonal transport with fluorescent probe FG found only half of the FG-positive motoneurons in experimental mice comparing to the control. This reduction is indicative of a considerable loss in the velocity of retrograde axon transport under the simulated hypogravity. The insignificant increase of myelinated fiber numbers in ventral root of the experimental rats could be considered as a result of neurites sprouting associated with regeneration of motoneurons. The analysis of these data infers that disorders in the neuromuscular system deprived of the weight-bearing loads can be the sequels of motoneuron death. Following this conclusion we studied possible proapoptotic and anti-apoptotic events in lumbar motoneurons. This was the confirmed demonstration of lumbar motoneuron viability under the condition of simulated hypogravity. It was also stated that changes in skeletal muscles were triggered neither by death of neurons nor degeneration of neuron processes. In antiorthostatic hind limb suspension condition immonoexpression of proapoptotic protein caspase 9 and cytotoxicity marker nNOS was very much alike in motoneurons of the control animals, and TUNEL staining did not detect apoptotic motoneurons in experimental rats at any point of the investigation.

Since we did not confirm the motoneurons death, the adaptive mechanisms preventing apoptosis were evaluated further. Heat-shock proteins are key players in compensatory adaptation under stress conditions. It has been thought that Hsp70 expression increases in hypoxic condition, whereas Hsp25 expression increases at the time of intracellular regeneration (e.g., after axotomy). Low expression of heat-shock proteins increases the probability of cell apoptosis [19,20]. It was also shown that microgravity decreases significantly the level of Hsp70 mRNA in osteoblast cultures [21]. Hence, activity of the genes encoding heat-shock proteins is an important factor in cell viability at microgravity condition. In our investigation we observed an increase in Hsp25 expression in lumbar motoneurons of the rats subjected to antiorthostatic hind limb suspension. Immunoblot showed a 95% increase in the Hsp25 level in the lumbar spinal cord of the experimental animals and immunohistochemicaly was shown the intensive precipitate localized in perikarya and, particularly, in motoneuron neuritis. Immunoexpression of Hsp70 in the lumbar spinal cord of experimental animals changed less noticeably as compared to Hsp25. No difference was found in the immunohistochemical reaction between control and experimental rats after 35 days of suspension; however, immunoblot indicated insignificant increase in Hsp70 level by 42% in the experimental spinal cords. Our data suggest that phenotypic alterations in motoneurons under conditions of weight-bearing deprivation are solely functional in nature; and increased Hsp25 expression is one of factors opposing to motoneurons apoptosis possible by inhibiting the NO synthase expression.

Acknowledgements

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