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Session JP3
Room 3
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Gravitational Biology: The Rat Model
Morphology of brain, pituitary and thyroid in the rats exposed to altered gravity


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Introduction

The presented data are results of two studies which have been carried out to elucidate the mechanism of altered gravity influence on animals. The first — morphological study of brain in the rats exposed to microgravity (μG) in space flight aboard SLS-2. The second — morphological study of pituitary and thyroid in the rats exposed to single and repeated hypergravity (HG).

Electron microscopic studies of brain cortex ultrastructure in the rats exposed to μG in 7-, 9- and 14-day space flights aboard Cosmos biosatellites and SLS-1 revealed the dynamics of adaptive changes of cortex ultrastructure in space flight. However, in these experiments the rats for 3—11 h after landing were in conditions of Earth gravity the influence of which on animals leaded to sharply increase in extero- and proprioceptive afferent flow entering the brain cortex. Therefore the aim of the first of presented studies was morphological investigation of brain somatosensory cortex in the rats exposed to μG and dissected during space flight aboard SLS-2 on purpose to avoid postflight Earth gravity influence on animals.

The results of vestibular studies in cosmonauts, repeatedly flown in space, and the data of ground-based experiments with animals, repeatedly rotated on centrifuge, point to decrease in response to micro- and hypergravity in each subsequent spaceflight or centrifuge rotation. These data are indicative of speeding up adaptation of vestibular apparatus, brain and hormonal system to functioning in conditions of repeated influence of altered gravity. However, the mechanism of accelerating these adaptive reactions remains unclear. In the second of presented studies for understanding the mechanism of speeding up adaptation of mammals to repeated prolonged alteration of gravity the pituitary and thyroid in the rats exposed to single and repeated hypergravity (HG) were studied by means of morphological methods.

Methods

The rats were dissected in flight aboard SLS-2 on day 13 of the mission. Brains from 5 rats were excised after decapitation, sagitally sectioned and halves from each brain were fixed by immersion in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, precooled at 4 °C. The fixed brains were kept during flight at 4 °C. After landing fixed brains were transferred to Ames Research Center where fragments of somatosensory cortex were excised, post-osmicated, dehydrated in ethanol, acetone and embedded in araldite. Embedded in araldite fragments of cortex and fixed in glutaraldehyde halves of brains were transferred to Moscow for morphological study. Brains of 5 ground control rats were processed with the same protocol. The structures of somatosensory cortex were studied by means of electron microscopy and morphometric analysis.

In experiment with the rats exposed to single and repeated HG the rotation of animals in peripheral cages of centrifuge, having a radius of 1.41 m, was used for prolonged 2 G influence. For estimation of Coriolis acceleration influence, the rats, rotated in the cage on the axis of centrifuge, with 1.1 G level, was studied also. Centrifuge was run constantly and stopped for 20 min each day to service. Vistar male rats (197 ± 2 g) were subdivided into 5 groups with 5 rats in each. The rats exposed to repeated 1.1 G and 2 G were rotated on centrifuge for 19 days and repeatedly — after a 30-day interruption — 5 days. The animals exposed to single 1.1 and 2 G were rotated on centrifuge for 5 days simultaneously with the rats repeatedly exposed. The rats were decapitated by guillotine. The pituitary and thyroid were fixed in Buen's liquid and embedded into histoplast. Pituitary sections were stained with paraldehyde-fuxin and Halmy's mixture aiming the development of somatotrophs and thyrotrophs. In each pituitary in 100 somatotrophs the cell and nucleus volume and in 100 thyrotrophs the cell and nucleus cross section area were measured with the help of image analysing system. Thyroid sections were stained by Marais method to develop iodated thyroglobulines and by hematoxylin-eosin. In 100 follicles of each thyroid the thyrocyte nucleus volume, follicle epithelium height and follicle cross section area were measured by means of image analysing system.
Results

Electron microscopic analysis of brain somatosensory cortex in rats exposed to μG aboard SLS-2 revealed in cortex layers II—IV the next changes of ultrastructure: 1) appearance of "light" presynaptic axonal terminals, which are characterized by low electron density of matrix and significant decrease in the number of synaptic vesicles; 2) decrease in electron density of pre- and postsynaptic membranes in axo-dendritic synapses formed by "light" axonal terminals; 3) vacuolization of dendritic microtubules and destruction of spine apparatus in dendritic spines; 4) autophagocytosis in the middle and wide dendrites; 5) decrease in amount of axo-dendrite synapses; 6) increase in area occupied by glial cell processes. These data — on the basis of morpho-functional correlations — indicate the decrease in afferent flow entering the cortex in μG. It was found only single "light" and "dark" degenerating axonal terminals, described earlier in brain cortex of the rats exposed to μG in space flight and dissected postflight under Earth gravity in a few hours after landing. In some part of stellate and pyramidal neurons of layers II—IV the increase in amount of ribosomes and Golgi apparatus elements was found indicating the increase in functional activity of these cells. The state of ultrastructure in large majority of large pyramidal neurons in layer V point out the hypofunction of these neurons. The results of morphometric study conform these obtained data.

In ground-based experiment with the rats exposed to single and repeated HG the following results were received after pituitary and thyroid morphological study. In pituitary somatotrophs after single 2 G influence the content of hormonal product in cytoplasm, zones of its degranulation and volume of cells did not differ from those of vivarium control animals and only volume of nuclei exhibited a tendency to increase. After repeated 2 G in somatotrophs it was found the sharply decreased hormonal product content in cytoplasm together with large zones of product degranulation and increased dimensions of nuclei and nucleoli pointing out the intensification of growth hormone production and secretion. In pituitary thyrotrophs the single 2 G did not induce any significant morphological changes. After repeated 2 G the cytoplasm of thyrotrophs was filled with the hormonal product, the zones of product degranulation were hardly noticeable and dimension of cells were sharply increased, while the dimension of nuclei had a tendency to decrease. These data indicate the inhibition of thyrotropic hormone secretion in the rats exposed to repeated HG. The results of morphological study of somatotrophs and thyrotrophs after single and repeated 1.1 G point to the decrease in cell functional activity. In thyroid of rats exposed to single 2 G influence it was revealed the diminution of cross-sectional area of follicles (by 38%), the signs of parenchyma proliferation and the increase in amount of perifollicular capillaries. In spite of unchanged content of iodinated thyroglobulines and thyrocyte nuclear volume the state of thyroid structure point to the increased functional activity of thyroid. After repeated 2 G the cross-sectional area of follicles was reduced by 51%, amount of perifollicular capillaries was increased still more in comparison with single 2 G, thyrocyte nuclei had sharply expressed tendency to increase in volume and the content of iodinated thyroglobulins in colloid was significantly rose. In total these data indicate higher thyroid activity after repeated HG than after single one. However, processes of parenchyma proliferation were less pronounced and thyroid structure was more "in order".

Conclusion

The results of morphological study of brain somatosensory cortex in rats exposed to μG in space flight aboard SLS-2 and dissected during space flight on 13 day of the mission allow to conclude — on the basis of morpho-functional correlations — about decrease in afferent flow entering the somatosensory cortex in μG and point out the developed in laG hypofunction of large pyramidal neurons. The comparison of results of thyrotroph and thyroid morphological study in the rats exposed to repeated 2 G with the data of other authors about functional connection of thyrotrophs and thyrocytes allows to suppose than inhibition of thyrotropic hormone secretion following the repeated HG is conditioned by increase in activity of thyrocytes the influence of which on thyrotrophs is realized by feedback mechanism via thyroid hormones. The obtained data indicate the acceleration of pituitary somatotroph and thyroid cell reactions in response to repeated prolonged HG and point out the animal capability for memorizing of gravity alteration. The work devoted to morphological study of the rats exposed to single and repeated HG was partly supported by contract NAS 15-10110. * — SLS-2 experiment. ** — HG experiment.
INTRODUCTION

To complete our previous studies on the sympathetic nervous system (SNS) adaptation to microgravity, β adrenoceptor biochemical characteristics were determined in rats. This work was performed in two major peripheral organs involved in blood pressure regulation i.e. heart (atria and ventricles were separated) and kidneys of rats flown for 18 days onboard the NASA space shuttle and in 14-day-tail suspended rats to compare actual to simulated microgravity influences on this system.

METHODS

In each experiment, animals were divided into 2 groups: for spaceflight mission, 6 flight rats and 12 ground control rats (6 vivarium and 6 Animal Enclosure Module groups) were considered; for hindlimb suspension study, 10 suspended rats (Morey's model) and 20 control animals (10 horizontally attached and 10 isolated rats) were used. In both investigations, the biochemical properties of β adrenoceptors were assessed using 125I-pindolol binding followed by a Scatchard analysis to calculate the dissociation constant (Kd) and the maximal binding capacity (Bmax) of these receptors in both organs considered.

RESULTS

A 18-day-spaceflight did not significantly change any biochemical characteristics of cardiac and renal β adrenoceptors since Kd and Bmax values in flight rats were similar to those in ground control groups.

The 14-day-tail suspension induced no significant alterations of β adrenoceptors Kd and Bmax values in heart and kidneys comparing to both control groups.

CONCLUSION

The spaceflight study evidenced the lack of modification in β adrenoceptor characteristics in the 2 peripheral organs considered after the mission. All these results do not allow us to conclude about the SNS adaptation pattern neither to actual microgravity nor to simulated microgravity. We have to take into account the necessity to develop inflight sacrifice protocols to avoid possible effects of reentry into Earth gravity which could mask the influence of microgravity on the SNS. That is why the international space station will be very useful to provide further information about the biological system adaptation to actual microgravity in animals.
INFLUENCE OF HYPERGRAVITY ON THE DEVELOPMENT OF MONOAMINERGIC SYSTEMS IN THE RAT SPINAL CORD

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INTRODUCTION
The development of the Central Nervous System depends on the interaction of a genetic program with the environment. Gravity constitutes a major element of the environment and its influence is exerted on motor, sensory and autonomous functions. These functions are integrated in the spinal cord. Our aim was to study the influence of gravity on the development of a modulatory system of these functions -the monoaminergic medullary projections- by using an hypergravity model. We evaluated whether the structure and function of these descending systems are modified by this physical condition.

METHODS
Pregnant Sprague-Dawley rats from embryonic day 15 and their litter were submitted to hypergravity (1.8 g) until postnatal day 15. Animals simultaneously localized in the same room, but not submitted to hypergravity were used as controls. Two groups of animals were studied: at postnatal day 1 and at postnatal day 15.

Morphological evaluation: After intracardiac perfusion of animals with 5% glutaraldehyde in 50mM cacodylate buffer, spinal cords were removed and postfixed in the same fixative for 24h. Transverse vibratome sections (50µm) at thoracic and lumbar level were processed for serotonin (polyclonal antibody, 1:10000) or tyrosine hydroxylase (polyclonal antibody, 1:5000) immunodetection.

RESULTS
Postnatal day 1. Serotonergic system.
Control animals: Thin and varicose immunoreactives serotonergic fibers were observed invading the ventral horn, whereas a discrete and scarce innervation is observed in the intermediolateral columns.
Animals in hypergravity showed only an incipient serotonergic innervation in ventral funiculi.

Postnatal day 15. Serotonergic system.
Control animals: Serotonergic fibers were observed in the ventral horn concentrated around motoneuron somata, in the intermediolateral columns, and in the dorsal horn. This innervation pattern is reminiscent of the well defined pattern of the adult animal.
Animals in hypergravity showed a similar pattern of serotonergic fibres, but its distribution in the target areas was disorganized with sharp changes of orientation and many fibers appeared dystrophics.
Similar results were observed for tyrosine hydroxylase immunodetection, at both time intervals.

CONCLUSION
The influence of hypergravity on the motor, sensory and autonomous functions during development, leads to a delay in the developmental schedule of monoaminergic projections regulating these functions in the spinal cord. Moreover, an anarchic, disorganized and dystrophic innervation is observed in the target regions, which correspond to the precise levels of integration of sensory-motor and autonomous functions in the spinal cord.
A Vestibular Evoked Potentials (VsEPs) Study of the Function of the Otolith Organs in Different Head Orientations with respect to Earth Gravity Vector in the Rat
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Recently we have successfully recorded short latency (t<12.7 msec) VsEPs in response to linear acceleration impulses, using a new technique that was developed in our laboratory. The stimulating apparatus consisted of a horizontal sliding device (to which the rat was attached) that was linearly accelerated (nose forward) by force impulse generated by a solenoid. Stimulus intensity varied between 1-10g' (risetime: 1msec, displacement: 30μm). The electrical activity was recorded by needle electrodes and was analyzed (filtered: 300-1500 Hz, amplified and averaged: N=128) by a standard evoked potential system. Various control experiments suggested that the recorded response is initiated in the otolith organs. In this study we investigated the effect on the VsEPs of holding the head in different orientations with respect to the direction of earth gravity vector (EGV) while delivering the linear acceleration impulses. The amplitude of the first wave of the VsEPs to linear accelerations (4g') in 6 rats was significantly increased when the rat was in the upside down position than when in the prone position. In 9 different rats VsEPs (4g' linear accelerations) were recorded in 7 different head orientations with respect to the EGV, all with bilateral symmetry with respect to the EGV. These included head perpendicular to the EGV (standard prone position); head parallel to the EGV, nose up; head parallel to the EGV, nose down; and 4 additional intermediate head orientations: 26° head down and up and 45° head down and up. The amplitude of the first wave of the VsEPs varied consistently among rats in the different head orientations, having a maximum at 45° head down and head up positions. The results of these experiments suggest that the amplitude of the first wave is proportional to the instantaneous superposition of the contributions of gravity and of the induced linear force impulse. These results provide further evidence that the VsEPs to linear acceleration are initiated in the otolith organs. Therefore the VsEPs may serve as an electrophysiological tool for understanding vestibular function in different gravitoinertial environments.
INTRODUCTION

Proprioceptive systems of vertebrates are important for control of posture and movement. On Earth those activities inherently encounter the influence of gravity, and, of course, proprioceptors have developed in the presence of Earth gravity. Yet, it has not been demonstrated what role, if any, gravity has in the "proximate" and "ultimate" causation - borrowing from concepts espoused by Ernst Mayr - for the existence of proprioceptors. We examined a proprioceptor in skeletal muscle - the muscle spindle - as well as the vestibular nuclei in the brains of rats that underwent part of their development in a near-zero gravity state. Thus, this is an initial analysis into whether gravity affects the ontogeny of these sensory systems.

METHODS

Female (F) and male (M) offspring of 10 pregnant rats that spent days 9 to 20 of gestation (where conception is day 1) in microgravity ($10^{-3}g$) were the experimental or Flight group. They were a payload (NIH.R1) aboard the space shuttle Atlantis launched November 3, 1994 (STS-66). This experimental group was compared with age-matched, ground-based offspring of Synchronous (n=10) and Vivarium (n=25) control dams. The rats reported on here were sacrificed when they were 100 days old; all were born and grew postnatally in Earth gravity (1g).

The animals were anesthetized and perfused with saline followed by 4% paraformaldehyde. The right soleus muscles were dissected from the hind limbs and immersed in either Bouin's fixative (light microscopy) or 4% paraformaldehyde (electron microscopy); brains were placed in aldehyde fixative.

Muscles examined by light microscopy were serially cross-sectioned in their entirety at 20μm thickness in a cryostat, picked up onto coverslips, stained with a modified van Giesen method, and permanently mounted onto slides. Those sections were examined for the presence of encapsulated muscle receptors. For each muscle spindle found, its position within the muscle was charted. Muscles examined by electron microscopy were embedded in epoxy resin. They were skip-serially cut in cross section. When a spindle was identified on 2μm thick sections stained with Stevenel's blue, then thin sections of it were cut, picked up on coated slot grids, contrasted with salts of heavy metals, and examined with a transmission electron microscope before continuing to section farther along the remaining muscle. Spindles were assessed ultrastructurally for the integrity of intrafusal muscle fibers and the presence of nerve endings.

Brain stems were cut in cross section at 50μm thickness from midbrain through medulla. Thionin stained, serial sections were examined by light microscopy to identify the vestibular nuclear complex (i.e. superior, lateral, medial, and inferior nuclei). The composite was traced from each section at constant magnification. The area bounded by that outline was measured using a digitizing tablet coupled to a computer running morphometry software. The volume of each vestibular nuclear complex in a rat was calculated separately by summing the individual section values ($\sum (area \times section \ thickness)$). Then values for the left and right sides were averaged for each animal. Comparisons of the overall average volume were made among groups and between sexes.

RESULTS

Muscle spindles were present in all Flight and control soleus muscles that have been examined. The average ±standard deviation ($X \pm SD$) number of spindles per soleus muscle in Flight rats (4F, 1M) was 18.6 ±0.9. That was not significantly different from the values of 17.3 ±2.0 for Synchronous controls (2F, 1M) and 18.0 ±2.0 for Vivarium controls (4F, 2M). The distribution of spindles along the soleus muscle was also very similar among the groups. For example, Flight females (n=4) had 79% of their spindles distal to the nerve entry zone, while the combined control female rats (n=6) had 77% of that receptor type in the equivalent length of muscle. Nor were
there statistically significant differences between Flight and Synchronous control rats' soleus muscles embedded in plastic which were analyzed in even more detail (n=1 muscle each; both were F). In 80% of spindles for each of those muscles, four intrafusal fibers were present, and the remaining 20% had three fibers. Similarly, the average equivalent diameter of those intrafusal fibers was 12.3 ±2.1μm for the Flight (n=15 spindles) versus 11.5 ±2.4μm for the control (n=15 spindles). The average equivalent diameter bounded by the outer capsule of the spindle was also very close in the Flight and Synchronous control muscles, being 46.3 ±21.8μm and 44.1 ±13.7μm, respectively. Finally, sensory and motor nerve terminals were identified abutting intrafusal fibers of spindles in the Flight as well as Synchronous control muscles. In summary, by every measure that we used for muscle spindles, there was no notable difference between the experimental and control adult rats.

The size of the vestibular nuclear complex was measured in six Flight (3F, 3M), six Synchronous control (3F, 3M) and four Vivarium control (2F, 2M) rats. There were no statistically significant differences when comparing the composite volume (X ±SD) of Flight (4.92 ±0.50mm^3) versus Synchronous control (4.81 ±0.62mm^3) versus Vivarium control (4.83 ±0.14mm^3) groups. In contrast, the volume of the vestibular nuclear complex was significantly smaller for the eight female rats (4.56 ±0.31mm^3) as compared to the eight males (5.15 ±0.41mm^3). Thus, the sex of the rat had more of a bearing on the size of the adult vestibular nuclear complex than did a limited prenatal experience in microgravity.

CONCLUSION
Exposure to a space flight environment through most of the latter half of gestation does not preclude the existence of normal proprioceptive structure when those rats subsequently grow to adulthood on Earth.

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EFFECTS OF A NINE-DAY SHUTTLE MISSION ON THE DEVELOPMENT OF THE NEONATAL RAT NERVOUS SYSTEM: A BEHAVIORAL STUDY

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INTRODUCTION

We are interested in the adaptability of the developing nervous system to environmental influences and the mechanisms underlying this plasticity. Previous studies using the tail-suspension model of weightlessness identified a sensitive period, postnatal day (P)8-P13, during which hindlimb unloading leads to marked changes in motor performance. When tail suspension was continued until P31, the altered motor function was prolonged (Walton et al., Neurosci. 51: 763, 1992). We report here on the post-flight motor performance of the first neonatal rats flown in space. The animals were flown on the middeck of Endeavour (STS-72, NIH-R3) in January, 1996. Animals were launched at the ages proposed for the upcoming Neurolab mission. The flight animals (P7-16 and P15-24 during the mission) were housed in modified RAHF cages fitted within animal enclosure modules (AEM). Age-matched AEM-housed animals were used as ground controls for the behavioral studies.

METHODS

Righting reflexes, swimming and walking were videotaped at 60 fps (swimming and walking) or 200 fps (righting and walking) from landing day (R+0) through R+14. Free walking was also taped on R+52, R+67, R+215, R+234, R+279 and treadmill walking on R+80 and R+207. Surface righting was analyzed by measuring the speed with which the head, forelimbs (FL) and hindlimbs (HL) righted. Swimming stroke duration was measured using frame-by-frame analysis. The step cycle was analyzed using the Peak 5 Motion Measurement System (Peak Performance Technologies, Inc).

RESULTS

Differences between AEM and flight animals were seen in all three evaluations of motor function. Surface righting was significantly slower in flight compared to AEM control animals. On R+0 the righting reflex was slow in both groups of flight animals. The P7-16 flight animals righted their heads in 0.24±0.01 sec compared to 0.17±0.01 sec in the control animals. The values for the FL were 0.27±0.01 sec for flight and 0.21±0.01 sec for controls. The values for the HL were 0.35±0.01 sec for flight compared to 0.29±0.01 sec for control animals. For the P15-24 animals the values were; head, flight 0.18±0.01 sec, control 0.15±0.004 sec; FL, flight 0.21±0.01 sec, control 0.18±0.004 sec; HL, flight 0.26±0.01 sec, control 0.21±0.004 sec. The differences were significant in both groups at the p<0.0001 level. Righting reflexes recovered rapidly; the P15-24 animals recovered by R+1 and the younger animals recovered by R+3.

Nine days in microgravity did not seem to effect swimming speed. However, the flight and AEM animals could easily be distinguished on the basis of their swimming style. Most marked was a hyperextension of the hindlimbs as reported in the tail-suspended animals. This is being analyzed using the Peak system. We found that the P15-24 AEM control animals swam faster than the age-matched flight and other control animals.

Analysis of free walking showed differences in both hindlimb and forelimb joint angles during locomotion. The most marked effect was an extension of the hindlimbs that was seen during both the swing and stance phases of the step cycle. For example, in the P7-16 rats, the mean stance maximum angle for the ankle was 118.24±2.43° in the flight compared to 94.86±2.01° in the control animals (p<0.0001). In the P15-24 group, the value was 124.33±2.50° for the flight and 99.18±2.61° for the control animals (p<0.0001). The swing minimum angle was 42.19±2.50° for the P7-16 flight animals compared to 27.37±1.13° for the age-matched controls (p<0.0001). No significant difference was seen in the swing minimum angle in the P15-24 animals. The animals recovered slowly, with some differences remaining after 6 months of re-adaptation to 1G.

CONCLUSIONS

These findings indicate that neonatal rats can nurse and survive 9 days of space flight. Further they show that development of the nervous system is altered for both weight-bearing (walking) and non-weight-bearing (swimming and surface righting) behaviors. However, the effect is more persistent for motor functions requiring the animals to bear their weight. Re-adaptation of righting reflexes to 1G was fast in both age groups, taking longer in the younger animals. The rapid recovery is probably due to the short flight duration. Persistent changes would be expected after longer flights such as Neurolab (16 days) when some of the animals will not return to 1G until after the end of their critical period of development. Due to the presence of sensitive and critical periods, neonatal rats offer a good model to study the effects of microgravity on the mammalian nervous system. Neonates are more sensitive to changes in the environment than adult animals, yet the cellular and molecular mechanisms underlying adaptation to microgravity and re-adaptation to 1G are likely to be the same in all mammalian species. Supported by NASA, the NINDS, and the NICHD.
MUSCLE ATROPHY ASSOCIATED TO MICROGRAVITY IN RAT: BASIC DATA FOR COUNTERMEASURES

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Many reports have shown that real or simulated (using the hindlimb suspension HS model) microgravity led, especially in slow antigravitational muscles such as soleus, to pronounced structural atrophy, loss of force and changes in fibre typing and myofibrillar protein isoform expression. Mechanical measurements of contractile speed indicated that the slow-twitch soleus muscle acquires faster contractile properties after HS.

Among all the mechanisms suggested as a trigger for the muscular changes, a modification in the neuromuscular activity can be considered since i) EMG patterns were modified during real or simulated microgravity and ii) suppression of the nervous command during HS either by selective inhibition of the electrical impulse by TTX or by total denervation avoided the slow to fast changes in the fibre composition and kinetic properties of the soleus muscle. Therefore, we investigate the possibility to counteract the transformations of the unweighted rat soleus muscle using protocols which might regulate the nervous command.

Sustained low-frequency electrostimulation which resembled the firing pattern of normal slow motor units was imposed during HS. It prevented the kinetic and histochemical changes associated to HS; however, neither the loss of mass nor the decrease in force output were prevented.

The role of the afferent message was controlled in deafferented + suspended rats. Similar results as those described above were obtained. Moreover, stimulation of the Ia afferents by tendinous vibrations associated to passive stretching of the muscle led to a partial recovery of all properties.

Therefore, the whole results underlined the role of the pattern of discharge of the motoneuron and its regulation by the afferent message in the control of the muscle properties in unweighting conditions.
SIMULATED WEIGHTLESSNESS BY UNLOADING IN THE RAT. RESULTS OF A TIME COURSE STUDY OF BIOCHEMICAL EVENTS OCCURRING DURING UNLOADING AND LACK OF EFFECT OF A rhBMP-2 TREATMENT ON BONE FORMATION AND BONE MINERAL CONTENT IN UNLOADED RATS.

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Mechanical function is known to be of crucial importance for the maintenance of bone tissue. Numerous experimental studies have shown that loss of total-body calcium, and marked skeletal changes occur in people who have flown in space. Infrequency and high financial cost of flights have created the need for ground models designed to mimic weightlessness effects. Anti-orthostatic suspension devices are now commonly used to obtain hindlimb unloading in rats, with skeletal effects similar to those observed after spaceflight. These effects have been demonstrated at the tissular level, by means of histomorphometric evaluations. They can be summarized in a decrease in bone volume and a reduction in bone formation activity beginning on the 5th day of unloading. But biochemical changes relative to bone and calcium homeostasis also affect blood during unloading. We have recently investigated this problem.

A time-course study of these bone and calcium biochemical events was performed during a 2-week unloading experiment in rats. We found significant changes in circulating levels of osteocalcin, total alkaline phosphatases (ALP), and parathyroid hormone (PTH). Indeed, as compared with values measured in age-matched control rats (i.e. normal loaded animals), we found higher levels of PTH on the 7th day of unloading, and lower circulating levels of osteocalcin at 2, 5, 7 and 14 days of unloading, and lower levels of total ALP on the 7th and the 14th day of unloading. These findings argue for a decrease in bone formation activity measurable as soon as the 2nd day of unloading.

In order to evaluate countermeasures against unloading bone loss, we used local factors, known to be important agents regulating differentiation and/or proliferation of bone forming cells. As part of these local growth factors, BMP-2 (bone morphogenetic protein 2) has been shown to be a potent inductor of bone formation when applied locally in vivo. We then investigated whether general administration of BMP-2 could counteract the effects of unloading on bone formation and on bone mass.

Recombinant human (rh) BMP-2 was continuously administrated at the dose of 2μg/kg bw/day to control and unloaded rats during a 14-day experiment. At the end of the 14-day period, animals were anesthetized, and tibiae and femurs were removed and processed for histomorphometric and bone mineral content analyses. Rat calvaria osteoblastic cells were used for cell-culture studies in order to verify rhBMP-2 activity. Cells were treated with rhBMP-2 for 24-120 h and cell proliferation and cell differentiation were tested by means of [³H]-Thymidine incorporation and alkaline phosphatase (ALP) activity evaluations.

The time-course study in osteoblastic cell-culture showed that rhBMP-2 did not influence cell proliferation, but induced a dose-dependent increase in ALP-activity in cultured calvaria cells. However, treatment with rhBMP-2 in unloaded rats had no effect on parameters of skeletal growth and trabecular bone content. Moreover rh-BMP-2 treatment did not improve bone volume and static and dynamic bone formation parameters in the cancellous bone of tibial metaphyses in unloaded rats.

The present study shows that treatment with rh-BMP2 has not been effective in promoting skeletal growth, stimulating bone formation, and maintaining the metaphyseal bone content in this rat model of osteopenia. However, rhBMP-2 was effective in promoting osteoblastic cell differentiation in vitro. These new data could indicate that, in contrast to the efficiency of stimulating osteoblastic cell proliferation in preventing unloading induced bone loss, stimulating cell differentiation fails to prevent bone loss induced by unloading in rats.

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CYTOLOGICAL MECHANISM OF THE OSTEOGENESIS UNDER MICROGRAVITY CONDITIONS.

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INTRODUCTION
Decreasing of the bone growth rate and osteogenic processes, appearance of the osteoporosis and bone demineralization, diminution of the mechanical bone strength is characteristic alteration pattern for the weightlessness. The cytological mechanisms of microgravity-induced alteration and pattern of the adaptive and comprehensive reactions are needed in the further investigation. It is not clear the character of the osteogenic processes, the objective laws of the growth and differentiation pattern of the osteogenic and osteoclastic cells, peculiarities of their specific functions and of the cooperations during bone adaptive remodeling, the alterations of the bone matrix structure under microgravity conditions.

The aim of our work was to study the microgravity influence on the rat bone tissue cells during flight on the board of the american space laboratory SLS-2 during two weeks and to evaluate the readaptive processes in the bone after the space flight.

MATERIAL AND METHODS
As the material for the electron microscopy studying we used the fragments of the rat femoral epiphysis. Samples were fixed in 2,5% glutaraldehyde with edition 1,5% paraformol in phosphate buffer, pH7,2 during 24h. After 3 veces washing the samples were transported in 70% alcohol to the Kiev, when they postfixed in 1% osmium acid, dehydrated in alcohol and embedded in araldit. Ultrathin sections were stained according Reinolds and examined in electron microscope Tesla BS-500.

RESULTS
We can judge about the intensity and characteristic features of the osteogenesis in the weightlessness only considering to ultrastructural study of the morphofunctional cell alterations in the osteogenic zones. It is known, that the collagen and protein components of the proteoglycans are synthesized in the rough endoplasmic reticulum (RER) polysomes. Synthesis of the alcaline phosphatase occurs there too. Goldgi complex participate in the sulphated glycosaminoglycans synthesis. It plays first fiddle in the accumulation and transportation of the proteins and polysaccharide substances, alcaline phosphatase, Ca and P compounds. The accumulation P and Ca ions took place on the RER membranes. The degree of the development and state of this organoids are the important markers of the differentiation and specific function intensity in the bone cells.

Our investigation demonstrates that the normal osteogenic cell population is not uniform. Osteoblasts are distinguished on the shape, ultrastructure, biosynthetic activity and topographic relation with the mineralization zone. We are distinguishing 4 morphofunctional types (or stages). In the zones of the active osteogenesis there are the young osteoblasts (1st type) with narrow RER channels and well developed Goldgi complex, the mature functional active osteoblasts with enlarged RER channels and cisterns (2nd type). Osteoblasts with hyperlrophic RER are revealed too (3rd). They serve for the secret reserving. In the zones of the osteoplastic process fading (in the endost for example) osteoblasts turned into non-active state (4th type). This cells has narrow RER channels, many of the autotaglyosomas.

Using radionuclids we demonstrate, that proteoglycans and alcaline phosphatase biosynthesis, calcium compound accumulation and secretion are predominant in the 1st type osteoblasts. The synthesis of the collagen proteins predominants in the 2nd type osteoblasts. The 3rd type osteoblasts secrets proteins and glycosaminoglycans. These cells are characteristic for the intensive osteogenic zones.

In the rats from the flight group the metaphysis osteoblasts population is more uniform. It does not consist with the osteoblasts of the different functional stages, since such zones are characteristic for the normal osteogenesis in the control and synchronous control. Intensive osteogenesis take place in the some areas of the bone trabeculae only. It includes the aggregations of the 1st type osteoblasts. They have low nuclei-to-cytoplasm ratio and well developed RER with the narrow (0,1-0,2 mkm) channels. The state of the endoplasmatic reticulum suggested about relatively low level of the bone matrix biosynthesis. Mitochondria have the dense matrix and cristalline inclusions which demonstrate disturbance of the calcium metabolism. The nuclear chromatin is arranged on the perimeter and in the small agregations.
Osteoblasts like 4th type of the osteoblasts are predominant in the population. They have oval or elongated forms and lie parallel to the mineralization zone, beside the trabecula surface in the 1-2 layers or separately. The cytoplasmic borderline which attached to the bone matrix has strike contours. The thin or slightly extended RER channels lie in compact. In the Golgi apparatus vesicles and vacuoles are predominant. There are many autophagolysosomes with membrane RER fragments. This ultrastructure picture suggests about low biosynthetic activity in comparison with the 2nd and 3rd type osteoblasts. In the 4th type osteoblasts collagen proteins are transferring from the RER polysomes to the Golgi complex where they coupled with glycosaminoglycans and translocated in the intercellular space by vesicles. In all the types osteoblasts the alkaline phosphatase, phosphates and calcium compounds are excreted by exocytosis, by separation of the vesicles from the cytoplasm, and by destruction of the surface cell zones. The vesicles are registered in the mineralization zone as the mineralization centres.

In the flight group mineralization zones are more narrow than in the control. Many of the osteoblasts are similar to the fibroblasts on their ultrastructure. Functional active 2nd type osteoblasts are rare (1 to the 5-10 of the 4th type). Osteoblasts of the 3rd type with hyperplastic endoplasmic reticulum are a single cells.

This morphological pattern of the osteogenic cells suggested about the lower level of the growth and synthetic processes. This effect is depended upon the disturbance of the optimal balance of the bone matrix compounds biosynthesis. The relatively small number of the 2nd and 3rd type osteoblasts in the osteogenic cells population among the 1st and 4th type prevalence reflects decreasing of the collagen synthesis and secretion intensity in comparison with the control. It seems that there are no asynchrony of the specific biosynthesis, a part it is no the disconnection of the collagen proteins synthesis and glycosaminoglycans synthesis, which is characteristic for the intensive osteogenic zones, such as metaphysis.

We obtained the similar data during our investigation microgravity influence on the osteogenesis, which was conducted on the rat metaphysis from biosattelite "Cosmos - 2044". During the studying of the biochemical peculiarities of the osteoporosis development under the microgravity action it is established alteration in the collagen and glycosaminoglycans metabolism: deceleration of the synthesis and intensification of the catabolism. The comparison of this alteration with the bone structure damages (such as reduction of the Ca and P content and increasing their excretion with the biological fluids) permits to do the conclusion about interrelation between breach of the glycosaminoglycans synthesis and the mineralization state.

In the some osteogenic zones around the osteoblasts the large areas with collagen fibrills are revealed in the rats from the flight group. It suggests about the destruction of the osteogenic function in the cells and demonstrates the tendency to cells conversion into the fibroblastic ones. We can propose, that disappearance of the gravitation overload provoke osteoblasts to synthesise the collagen proteins what are characteristic for the fibroblastic phenotype. In the some studies the possibility of the new forms of the collagen synthesis in the bone tissue and of the specific gene expression inhibition (for example of the osteocalcin-producing gene) are postulated under microgravity condition. It conducts to the disruption of the mineralization osteoid processes and to the appearance of fibrosis zones. The decreasing of the bone calcium content under microgravity condition is characteristic for the spongiosa bones. Intensification of the resorptive processes in the bone spongiosa occurs by increasing number of the "gain" osteoblastic forms with well developed fibrillar zona and ruffled border.

During the readaptation period the structure and the cell composition of the osteogenic zones in the metaphysis rebuild. They approximate to the control.

CONCLUSION

Under microgravity condition decreasing of the osteopoietic activity and increasing of the resorptive processes in the bone were established. The osteoblasts population of the flight rats group is represented by the relatively low active for the specific biosynthesis forms (1st and 4th types). Osteoblasts of the different functional state are characteristic for the active osteogenic zones in the normal bones and in the synchronous control, but in the flight group rat bone they are extremly rare. In the some areas osteoblasts demonstrate the fibroblastic phenotype features. In such areas we discover the extended zones with the collagen fibrills on the trabecula surface. During the readaptation period (14 days after the space flight) the cell composition of the osteoblast population restores. After this time population is represented by the typical forms. The alterations, which was described above, may be considered as the adaptive reactions of the bone tissue cells on the microgravity effect.