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PECULIARITY OF ULTRASTRUCTURE AND ^{45}Ca METHABOLISM OF OSTEOCLASTS IN CONDITIONS OF HIND LIMB UNLOADING AND MICROGRAVITY

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Peculiarity of Ultrastructure and ^{45}Ca Methabolism of Osteoclasts in Conditions of Hind Limb Unloading and Microgravity. Rodionova N. V., Oganov V. S. — Using methods of electron microscopy, morphometry and cytochemistry the peculiarities of ultrastructure of osteoclasts and resorptive processes of the mineralized matrix in spongy bone of the rat femoral bone metaphyses following the experimental hind limb unloading model (28 days), as well as in rats exposed on American Space Station SLS–2 (2 weeks) were studied. The methods of light and electron microscopy radioautography ^{45}Ca were used in the experiment of hind limbs unloading. The results of investigations demonstrated that in zones of adaptive remodeling the resorption and destruction processes in the bone tissue increase under the supportive unloading. It takes place by increasing of functional activity of osteoclasts (in microgravity conditions we registered the «giant» osteoclasts). The dynamics of ^{45}Ca –incorporation into osteoclasts is an indicator of a direct involvement of cells in calcium transfer from the resorpting mineralized matrix to intercellular environment and intensification of this process at hind limb unloading model.

Key words: osteoclasts, resorption, electron microscopy radioautography, hind limb unloading model, microgravity.

Особенности ультраструктуры и метаболизма ^{45}Ca остеокластов в условиях разгрузки задних конечностей и микрогравитации. Родионова Н. В., Оганов В. С. — С использованием методов электронной микроскопии, морфометрии и цитохимии изучены особенности ультраструктуры остеокластов и процессов резорбции минерализированного матрикса бедренной кости крыс в условиях модели опорной разгрузки задних конечностей (28 суток), а также у крыс после пребывания на американской космической станции SLS–2 (2 недели). Применен также метод световой и электронно-микроскопической радиоавтографии с ^{45}Ca. Результаты исследования показали, что в зонах адаптивного ремоделирования процессы резорбции и деструкции костного матрикса усиливаются при опорной разгрузке. Это происходит путем интенсификации функциональной активности остеокластов (в условиях микрогравитации нами зарегистрированы «гигантские» остеокласты). Динамика включения ^{45}Ca в остеокласты свидетельствует о прямом участии клеток в переносе кальция из резорбированного костного матрикса в межклеточное пространство и интенсификацию этого процесса при снятии опорной нагрузки.

Ключевые слова: остеокласты, резорбция, электронная микроскопия, радиоавтография, модель опорной разгрузки задних конечностей, микрогравитация.

Introduction

The investigations carried out on space stations and biosatellites, model experiments have shown that microgravity and hind limb unloading conditions lead to the bone mass loss (clinically significant osteopenia), sometimes to osteoporosis (Grigoriev et al., 1998; Oganov et al., 2003).

The majority of works have stated a decrease of bone trabecules volume in long bones (Vico et al., 1988; Doty et al., 1992; Földes et al., 1990; Durnova, Kaplansky, 2003). Nevertheless, the mechanisms of bone tissue decrease remain in much obscure. An urgent problem is an existence of enhancement of osteoblastic resorption and bone destruction processes under microgravity and long-term hind limb unloading

model and what is their specific. The results of the light-optical researches on this problem are contradictory. So, the histomorphological analysis of rat bones exposed on SLS−1 has revealed a significant enlargement of bone surface occupied by osteoclasts and an increase of their number (Durnova et al., 1994). Some authors (Weinreb, 1989; Sinaki, 1995) suppose that resorption in microgravity is enhanced due to functional activation of osteoclasts. According to results of other experiments (Cosmos 1129; Cosmos 2044), enhancement of osteoclastic resorption was not observed (Vico et al., 1988; Carmeliet, 2001).

It should be noted that electron microscopic researches showed that osteoclasts are not a homogenous population (Triftandidi, Yagodovsky, 1982) revealed inactive osteoclasts being in a «preparation» stage and active osteoclasts that possess a typical structure. Our studies of osteoclasts ultrastructure showed that osteoclasts significantly differ in shape, size, nuclei number, degree of the development of cell organelles, «brush border», as well as by the level of a biosynthetic activity registered by the intensive 3H-uridine and 3H-methionine uptake. Basing on this, we defined young, functionally active, functionally inactive osteoclasts and osteoclasts undergoing destruction. (Takahashi et al., 1988) distinguished active, inactive and resting osteoclasts.

It would be necessary to elucidate which morpho-functional forms of osteoclasts dominate in zones of adaptive remodeling of bone structures at supportive unloading and what changes are in their structure and function to understand the mechanisms of bone tissue loss.

In this work with the use of electron microscopy we have studied the specifics of ultrustructure of resorptive processes in metaphyseal bone trabecules, which functional forms of osteoclasts prevail in these population and whether there are any differences in their states under hind limb unloading model and microgravity conditions in comparison with control.

The aim of our research also is to study the peculiarities of ultrastructure and ^{45}Ca metabolism in osteoclasts and resorption processes in spongy bone under hind limb unloading model and microgravity with the use of electron microscopy and radioautography with ^{45}Ca.

Material and methods

In this work also were processed spongy bone tissue biosamples from the femoral metaphyses of mature rats (males of a Spray-douly line, 180−200 g weight) from the experiment onboard the American Space station SLS−2. The flight group and synchronous control consisted of 5 rats each. The duration of flight was 2 weeks. Time interval between landing and sacrifice of rats was 3−4 hours. The biomaterial was supplied by V. S. Oganov, the SSC RF Institute of Biomedical Problems (Moscow, Russia).

Also the investigations were carried out on spongy bone tissue, biosamples taken from the proximal femoral metaphyses of 6-month-old albino rats (males of a Wistar line, 200 g weight) after 28 days of hind limb unloading model. Hind limb unloading model was made by the «tail suspension» method (angle 35є). 5 rats were used in experiment and 5 rats were used in control.

For an electron microscopic study, the bone pieces (1 mm^3) were fixed in a 2.5% glutaraldehyde solution on a phosphate or cacodilate buffer (pH 7.4), postfixed in 1% OsO_4, dehydrated and embedded in araldite (reagents supplied by the «Sigma» Firm, Germany). Ultra-thin and semi-thin sections of bone tissue (with ^{45}Ca) were made electron-microscopic level. Ultra-thin sections were contrasted by lead citrate (Venable, Coggeshall, 1965).

^{45}Ca (Firm «Isotope», Russia) was administered intraperitoneally in a dose of 1 μCi/g to experimental and control rats. After an experiment with modeled hind limbs unloading the pieces of spongy bone from femoral proximal metaphyses were isolated 15 min, 1 hr and 6 hr, 48 hr following the isotope injection. The animals were anaesthetized with ether before sacrifice in all experiments.

Activity of acid phosphomonoesterase was exposed in the method of Gomori in modification (Buchvalov, 1982).

For preparing ^{45}Ca radioautographs the ultra-thin and semi-thin sections were covered with M-type photoemulsion (Moscow factory of technical photoplates, Russia). The semi-thin sections were stained with 1% methylene blue solution. ^{45}Ca labels (silver grains) were counted over osteoclasts and bone matrix in resorption zones per unit section area (field of microscope vision, ob. 100, oc. 12,5). The morphometric processing of electron micrographs (5 to 10 per each biosample) was performed by a dot count method (Avtandilov, 1990). Obtained values were processed statistically with the use of program «Microsoft Excel».

Results and discussion

We performed light-optical researches carried out on the same spongy bone samples from the femoral proximal metaphyses of rats exposed to hypokinesia and microgravity conditions, earlier. We have established a decreasing of a bone tissue specific volume in metaphyses, revealed a tendency for an increasing of the number of functionally active osteoclasts (Rodionova et al., 1996; 2004).

Our electron microscopic observations showed that in the population of osteoclasts, both in controls and experiment under hind limb unloading model and micro-

gravity, the osteoclasts of different morpho-functional states (young, functionally-active, functionally-inactive and degenerating) are encountered. Young osteoclasts have 2 to 3 nuclei in an ultra thin section, compact cytoplasm and relatively high nucleoplasmic ratio. «Brush-border» is not developed. Young osteoclasts are characterized by a great number of mitochondria and free polysomes in a cytoplasm. Mature functionally active osteoclasts have 5 to 8 nuclei, well-developed cytoplasm with phagolysosomes and «brush-border». A decreasing of the intensity of resorption processes in one or another region of the growing bone leads to transfer of osteoclasts into a functionally inactive state. The cytoplasm of these cells becomes compact and osmophylic; a febrile layer and the «brush border» are reduced. In thouse bone regions where resorption is ceased, the osteoclasts show signs of destruction. The nuclei of perished osteoclasts undergo picnosis, the cytoplasm becomes destroyed, these cells are undergoing to apoptosis. The above-described morphofunctional states of osteoclasts represent successive stages of their living cycle typical for normal morphogenetic processes in bone (Rodionova, 1983, 1989, 2006).

Comparing with controls were mature osteoclasts are located singularly on bone trabecules, in experiments as in the hind limb unloading model as in the conditions of microgravity here are regions of intensive resorption in which the osteoclasts can form groups of 2 to 3 cells. An electron microscopic study showed that such osteoclasts could be considered as functionally active cells. The presence of 3–5 nuclei in a section, well-developed cytoplasm and the “brush border” are typical for these osteoclasts (fig. 1).

According to the quantity and distribution of organelles the cytoplasm of active osteoclasts is not homogeneous and is conditionally divided into two zones (our point

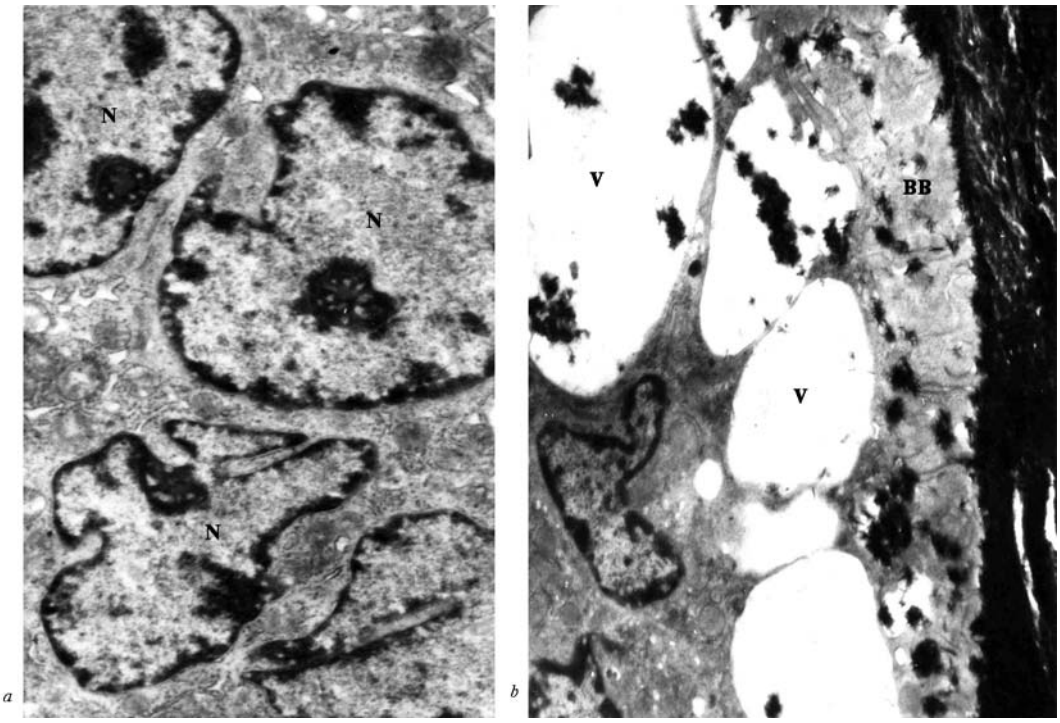

Fig. 1. The fragments of the cytoplasm of the functionally active osteoclast: *a* — nucleous (N) of osteoclast (x 16 000); *b* — zone II with large vacuoles (V), containing a fragments of mineralised bone matrix (x 8 000) and developed "brush border" (BB). Femoral bone of 6-month old rat. Control. Electron micrographs

Рис. 1. Фрагменты функционально активного остеокласта: *a* — ядро (N) остеокласта (x 16 000); *b* — зона II с большими вакуолями (V), содержащими фрагменты минерализованного костного матрикса (x 8 000) и развитой "щеточной каемкой" (BB). Бедренная кость 6-месячной крысы. Контроль. Электронные микрофотографии.

of view): zone containing organelles for biosynthesis and energy supply (zone I) and a zone with a structures that provids specific functioning (zone II). Zone I includes nuclei, mitochondria, polysomes, endoplasmic reticulum and Golgi complex structures. In zone II in which the cells are directed towards the mineralized matrix, the filling of a cytoplasm with organelles decreases and it is prevailed here by lysosomes, a fine febrile component (actin), the “brush border” villi. Zone II contains phagolysosomes with fragments of the mineralized matrix, which is undergoing to disintegration. The majority of osteoclasts in control have a relatively weak-developed zone II, the “brush border” possesses small villi. A zone of contact with the matrix is not large in such osteoclasts.

Obviously it was shown at experiments, as in controls, nucleus of functional-active osteoclasts characterized by polymorphism and distinguished by condensation degree of chromatin aggregations localized everywhere in nucleus and on their periphery. Number of nuclei with low degree of heterochromatinization (“young nuclei”) increases in cells. Mitochondrion has more compact matrix and nearly disposition cristae. We have not seen clear differences between control and experiment in ultrastructure of nucleus and nucleoli. Mitochondria have more compact matrix and nearly disposition cristae.

Morphometric analysis of cell’s organelles in osteoclasts under hind limb unloading model showed reliable differences between degree develop in structures of Golgi complex of control and experiment: specific volume of Golgi complex are 0.180 ± 0.009 in control and 0.222 ± 0.011 in experiment ($P < 0.05$). Main differences concerned in degree development of structures which ensure specific functions of osteoclasts (zone II): febrile layer more developed in osteoclasts of experimental group, specific volume of phagolysosomes 0.238 ± 0.016 in control and 0.320 ± 0.017 in experiment ($P < 0.05$), “brush-border” more developed. Phagolysosomes have electron-light

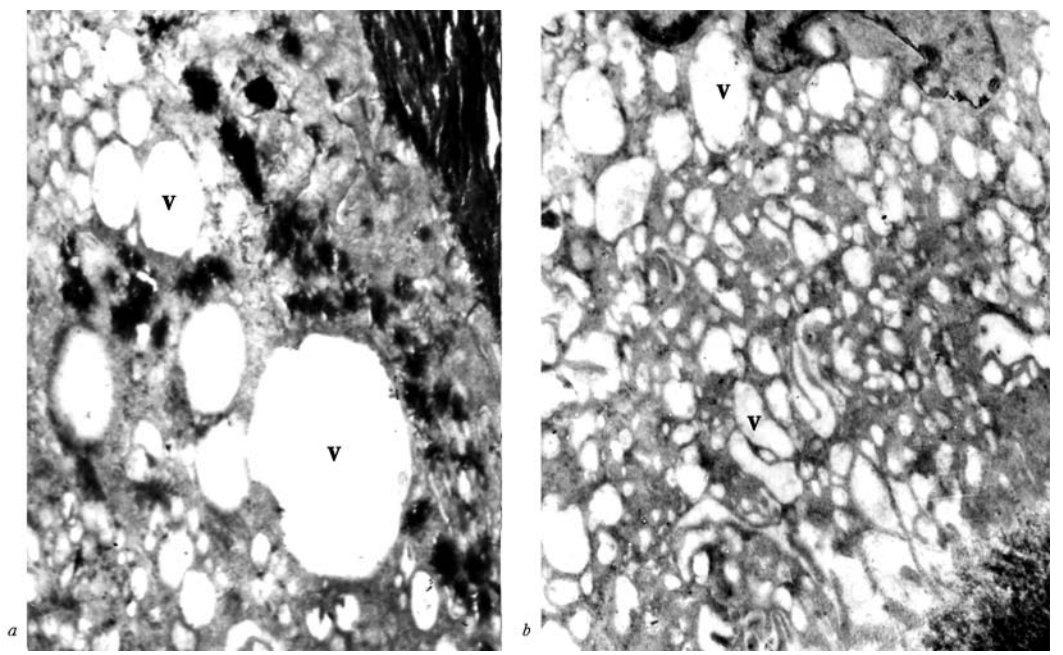

Fig. 2. A fragment of the zone II of functionally active osteoclasts at supportive unloading (*a*) and microgravity (*b*);. Are seen: vacuoles (V) and "brush-border" (BB). *a* — x 6 000; ; *b* — x 5 500. Electron microphotographs.

Рис. 2. Фрагменты зоны II функционально активных остеокластов при опорной разгрузке (*a*) и микрогравитации (*b*). Видны: вакуоли (V) и "щёточная каемка" (ВВ). *a* — x 6 000; *b* — x 5 500. Электронные микрофотографии.

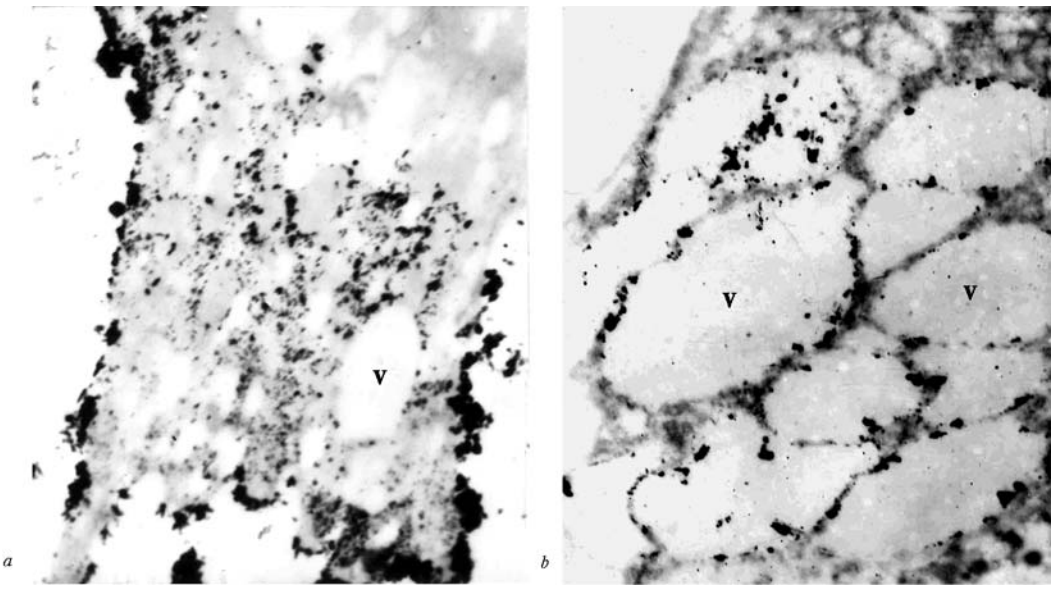

Fig. 3. The reaction on acid phosphomonesterase in the region of vacuoles (V) and "brush-border" (BB) of osteoclasts: *a* — supportive unloading (x 2 500); *b* — control (x 5 000). Electron microphotographs.

Рис. 3. Реакция на кислую фосфомоноэстеразу в зоне вакуолей (V) и "щеточной каемки"(BB) остеокластов: *a* — опорная разгрузка (x 2 500); *b* — контроль (x 5 000). Электронные микрофотографии.

contain with aggregations of mineralized bone matrix which are resorbing, forming large phagolysosomes (1−5 μm) (fig. 2, *a*). Acid phosphatase is found in the vacuoles and brush border region at the conditions of supportive unloading (fig. 3). Electron-microscopic data make possible to suppose that in conditions of hind limb unloading functional activities of osteoclasts increases in resorption zones.

Under microgravity and hind limb unloading model in biosamples we found the “giant” osteoclasts with more developed zones I and II (particularly zone II). In the “giant” osteoclasts zone II occupies an area (in sections) significantly larger than zone II in typical active osteoclasts. It includes a “light” febrile zone and numerous vacuoles with some fragments of mineralized matrix. The analysis of electron micrographs obtained in our studies showed that the development of a fibrillar layer precedes the formation of the “brush border”. Its regions directed towards the cytoplasm contain polysomes, endoplasmic reticulum channels, mitichondria. This is related probably with the involvement of these organelles in biosynthesis of contractile proteins which form microfilaments of the febrile layer. The movement of the “brush border” villi, their active penetration into the mineralized matrix, formation and transport of vacuoles inside the cytoplasm are performed due to activity of contractile structures of the febrile layer and the “brush border”. We found such «giant» osteoclasts in remodeling regions of bone trabecules in monkeys, which have been on the biosatelitte “BION−11” (Rodionova et al., 2002; Rodionova, Polkovenko, 2002).

In the space condition (SLS−2) functional-active osteoclasts were recorded in proximal metaphyses on the bone tissue samples of white rats femoral bone from flight group and synchronal control group.

In the experiment the most essential distinctions of osteoclast ultrastucture in control and experimental groups concern to level of development of zone II.

Thus, in the most osteoclasts of flight group this zone includes higher developed febrile layer, wider part involving phagolysosomes and “brush border”.

In the experiment specific volume of phagolysosomes is reliable increasing: in control it is 0.235 ± 0.012, but in experiment it is 0.274 ± 0.013, $p < 0.05$.

But in microgravity conditions dimensions of phagolysosomes are decreasing, their diameter on the medium is varying from 0.5 to 1 μm.

In population of "giant" functional-active osteoclasts with highly developed cell periphery occur in remodeling zones at microgravity condition the changes are as follows: considerable surface of mineralized bone matrix is enveloped in febrile layer, phagolysosomes and "brush border", they form more volumetric resorption zone than in typical osteoclasts. In "giant" osteoclasts of experimental animals zone II that ensure specific resorption functions of mineralized bone matrix by the size (on the ultrathin section) embracing an area 1−2.5 more than zone II of typical functional-active osteoclasts. In this case in the zone II small vacuoles (0,5−1 μm) prevail, big phagolysosomes do not reveal, "brush border" is formed by short numerous villi (fig. 2, *b*).

One of the characteristic features of osteoclast activity is a degree of the development of zone II, namely of a specific volume of phagolysosomal vacuoles, which in functionally active osteoclasts equals to: under microgravity 0.452 ± 0.022; in synchronous control 0. 383 ± 0.019. The degree of the development of zone II in osteoclasts under microgravity is more pronounced. We suppose that the appearance and functioning of such osteoclasts in a population is conditioned by the need for development of fast adaptive resorption of the mineralized matrix in the most “vulnerable” bone zones under microgravity.

The mechanisms of bone matrix resorption have not been studied enough and are being intensely discussed (Chai et al., 1994; Nesbitt, Horton, 1997; Baron, 2002). It has been established that the osteoclasts synthesize lysosomal enzymes (tartrate-resistant acid phosphatase — TRAP, catepsin etc), which are present in endoplasmic reticulum, Golgi complex and numerous transport vesicles. The enzymes are secreted through the “brush border” into the extracellular space where the resorption occurs. The resorption takes place by oxydation and proteolysis of bone matrix and hydroxyapatite crystals. The first stage is a mobilization of hydroxyapatite crystals by means of «digestion» of their links with collagen and dissolution of hydroxyapatite crystals at low pH. Subsequently, the collagen fibers undergo destruction by catepsins or collagenases (Doty, 1981; Baron, 2002).

The analysis of the obtained electron micrographs of functionally active osteoclasts (particularly in hind limb unloading model and microgravity) permits to suppose that depolimerization of the organic matrix results in releasing of mineral component whose crystals enter the cell by endocytosis with the help of the “brush border” villi. At the stage of union of the “brush border” outgrowths the endocytose vacuoles have small size; subsequently their size increases as they accumulate enzymes and oxyacids. They can fuse; being displaced to the superficial zones of the cytoplasm and release their contents, including Ca, into the intercellular space.

The above-represented mechanisms of the mineralized matrix resorption were confirmed when studying the dynamics of ^{45}Ca incorporation into osteoclasts. We determined the concentration of ^{45}Ca label over osteoclasts in the metaphyseal zone of the femoral bone from 6-month-old rats (control and experimental group) during various intervals after a single radionuclide injection (fig. 4). The data of the light (semi-thin sections) and electron-microscopic radioautography demonstrate that osteoclasts intensely incorporate ^{45}Ca 15 min after its injection; the label is concentrated predominantly over mitichondria (fig. 5, *a*). By this time interval ^{45}Ca labeling is also observed in osteoid and superficial zones of mineralized bone matrix, where ^{45}Ca enters from the extravascular environment and also from osteoblasts. ^{45}Ca label in vacuoles of osteoclasts is registered at later intervals of the experiment, by 1 hr after injection. ^{45}Ca incorporations, registered over osteoclasts 15 min after its injection are indicative of ^{45}Ca absorption for the maintenance of the inner cell requirements and are not directly associated with the mineralized matrix resorption. By 1 hr and mainly by 6 hr after radionu-

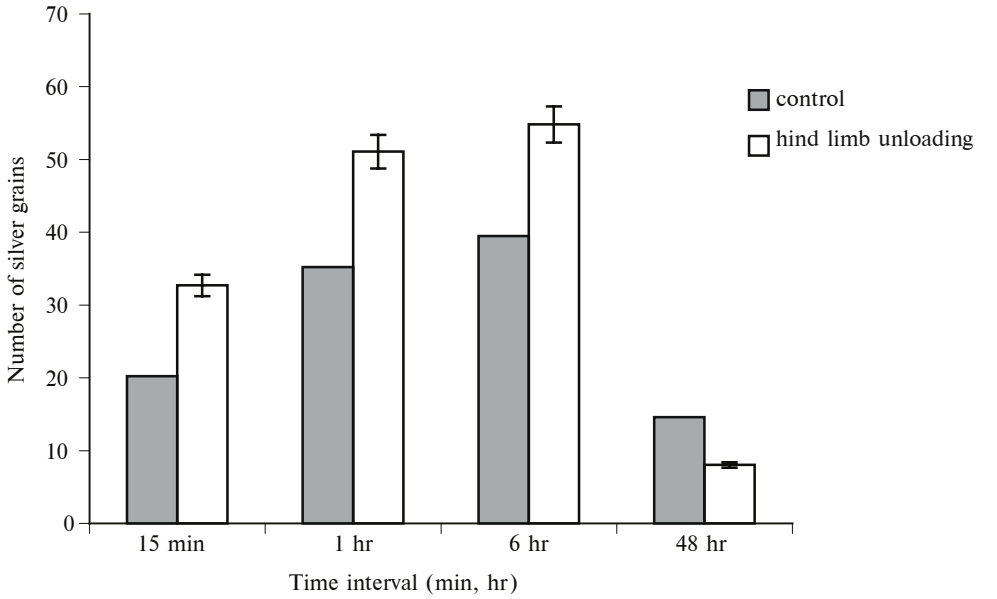

Fig. 4. Alteration of intensity ^{45}Ca incorporation into osteoclasts at different intervals after the radionuclide administration at hind limb unloading of rats.

Рис. 4. Изменения интенсивности включения ^{45}Ca в остеокластах в различные сроки после введения радионуклида при опорной разгрузке задних конечностей крыс.

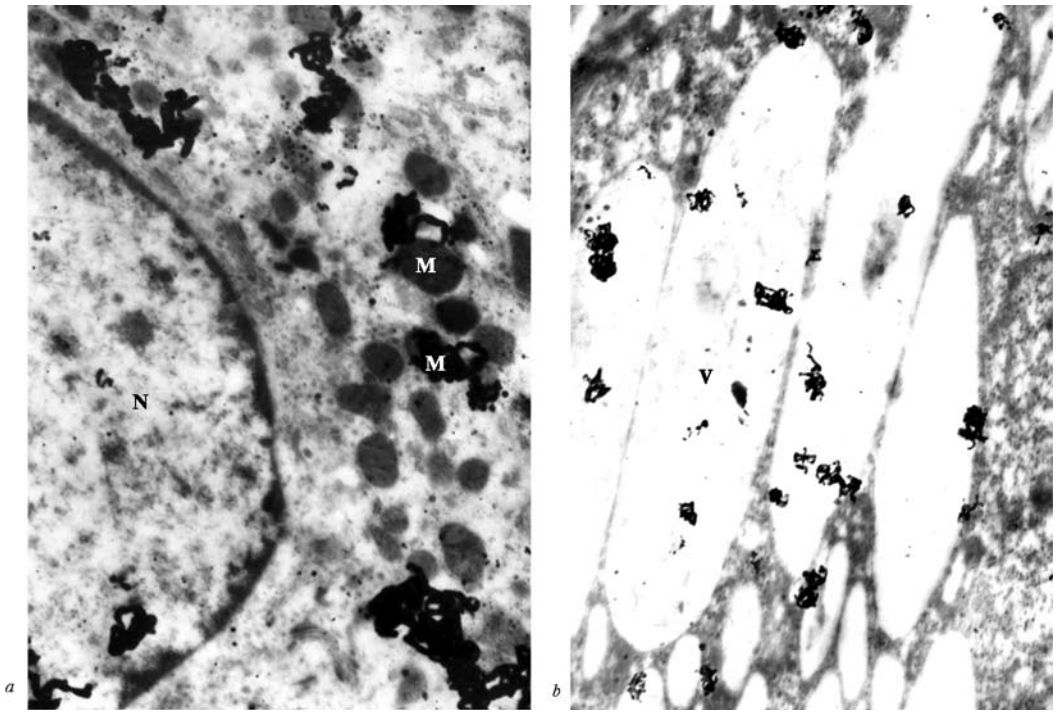

Fig. 5. Includings of ^{45}Ca in osteoclasts at the different stages after radionuclide injection: *a* — basic concentration of ^{45}Ca label over the mitochondria (M) 15 minutes after radionuclide injection (x 24 000); *b* — concentration of ^{45}Ca label in vacuoles (V) of osteoclasts 1 hour after radionuclide injection (x 12 000). Electron-microscopic radioautography

Рис. 5. Включение ^{45}Ca в остеокласты в различные сроки после введения радионуклида: *a* — преимущественная концентрация метки ^{45}Ca над митохондриями (M) через 15 мин после введения радионуклида (x 24 000); *b* — в вакуолях (V) остеокластов через 1 ч после введения радионуклида (x 12 000). Электронномикроскопическая радиоавтография.

clide injection the concentration of ^{45}Ca label over osteoclasts increases ($P < 0.05$) in experiment and control and reveal in vacuoles (fig. 5, *b*). In experiment intensity of including ^{45}Ca reliably higher than in control ($P < 0.05$). It happens possibly due to ^{45}Ca entry to the cells together with the mineralized matrix resorbed by osteoclasts. Entered ^{45}Ca is transported into the osteoclasts' cytoplasm, and then secreted over the openings of peripheral vacuoles and also probably by exocytosis. The gradual lowering of ^{45}Ca label over osteoclasts 48 hr after its injection in experiment and control testifies to some degree of Ca transfer from osteoclasts in the process of their functioning. A decrease in the number of silver grains over osteoclasts by this interval is associated also with the reduction of labeled Ca uptake in mineralized bone matrix.

So, the dynamics of ^{45}Ca incorporation into osteoclasts during various intervals after its injection testifies to a direct participation of osteoclasts in Ca transport from mineralized matrix into intercellular environment, from where it comes to the blood-vascular bed and other zones of mineralization and activation of these processes at hind limb unloading model.

Here, certainly one cannot exclude the systemic (extra-osseous) and local homeostasis regulating factors of Ca^{2+} etc., whose role under reduction of supporting load has been shown in majority of works (Stupakov, Volozhin, 1989; Grigoriev et al., 1998).

Conclusions

Using methods of electron microscopy, morphometry and cytochemistry we studied the peculiarities of ultrastructure of osteoclasts and resorptive processes of the mineralized matrix in spongy bone of the rat femoral bone metaphyses following the experimental hind limb unloading model (28 days), as well as in rats exposed on space station SLS−2 (2 weeks). The methods of light and electron microscopy radioautography ^{45}Ca were used. The results of investigations demonstrate that in zones of adaptive remodeling the resorption and destruction processes in the bone tissue increase under the mechanical unloading. It takes place by increasing of functional activity of osteoclasts (in microgravity conditions we registered the «giant» osteoclasts). The dynamics of ^{45}Ca-incorporation into osteoclasts is an indicator of a direct involvement of cells in ^{45}Ca transfer from the resorbing mineralized matrix to intercellular environment and intensification of this process at hind limb unloading model.

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