It can be tentatively suggested that it is these cells of the vessel wall and its immediate vicinity that are the main source of origin of regenerating bone through the successive transformation of a vascular cell of pericyte type into a fibroblast-like cell, which could be observed in the early stages of formation of the regenerating tissue, and later into an osteoblast. Bone formation can perhaps also take place in other ways, but even so the proliferative activity of the vascular cells must be an important factor in osteogenesis.

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STEREOLOGIC ANALYSIS OF CARDIOMYOCYTE ULTRASTRUCTURAL ORGANIZATION IN RED-CHEEKED SUSLIKS IN DIFFERENT SEASONS

L. M. Nepomnyashchikh, L. V. Kolesnikova, G. I. Nepomnyashchikh, and V. P. Tumanov

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KEY WORDS: heterothermic animals; hibernation; myocardium of red-cheeked susliks; cardiomyocyte ultrastructure; morphometry and stereology.

Hibernation of heterothermic animals is a unique adaptation to unfavorable external environmental conditions [2]. The use of characteristic ecological and physiological features distinguishing hibernating mammals for practical purposes is possible through careful investigation of the organs and systems of these animals and, in particular, of their cardiovascular system. However, the cellular mechanisms lying at the basis of the abrupt seasonal fluctuations in cardiac function in heterothermic animals have by no means been completely elucidated. Among the morphological studies of this problem [6, 7, 10, 13] there have been few attempts to undertake a quantitative study of the tissue components of the myocardium [3]. Quantitative investigations of ultrastructure of the cardiomyocytes of hibernating mammals have yielded contradictory results and have been restricted to a narrow range of morphometric parameters [8, 9, 12].

The aim of the present investigation was a stereologic analysis of the ultrastructural organization of the myocardium of red-cheeked susliks in different seasons of the year.

EXPERIMENTAL METHOD

Altogether 20 hearts of red-cheeked susliks *Citellus erythrogenys* Brandt were studied. These mammals are distinguished by prolonged (7-8 months) hibernation; the body temperature of the active animals is 37°C and of hibernating animals 8°C. Five groups of animals were used: 1) active animals in the fall before hibernation (six susliks), studied at the end of

Department of Pathomorphology and Morphometry, Institute of Clinical and Experimental Medicine, Siberian Branch, Academy of Medical Sciences of the USSR, Novosibirsk. Department of Pathological Anatomy, A. V. Vishnevskii Institute of Surgery, Academy of Medical Sciences of the USSR, Moscow.

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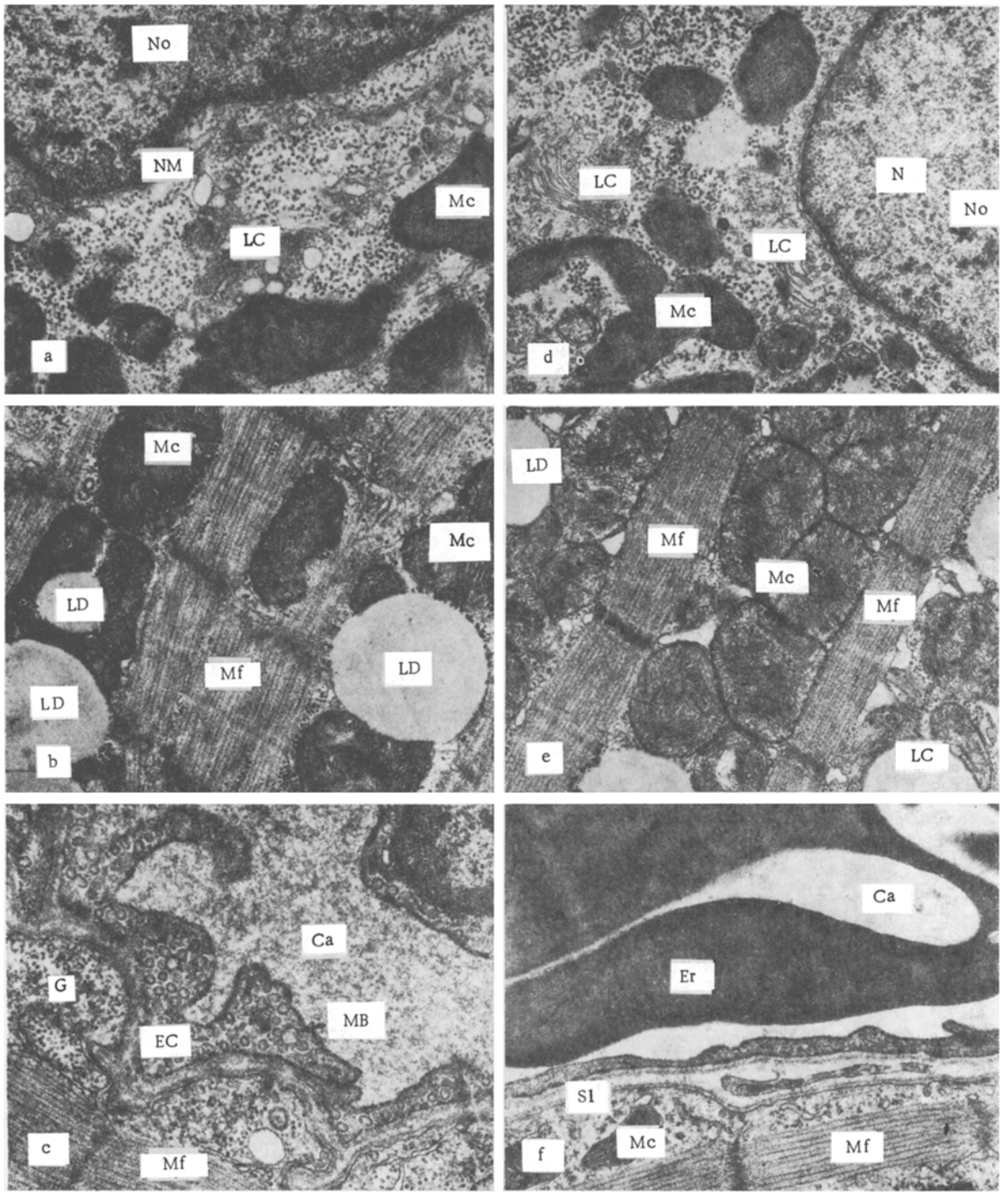

Fig. 1. Ultrastructure of cardiomyocytes of red-cheeked susliks in a state of hibernation and after awakening (8300 ×). a) Perinuclear zone: increase in quantity of condensed chromatin on inner surface of nuclear membrane (NM) and in nucleoplasm; lamellar complex (LC) consists of concentrations of vesicles of varied diameter; b) circular arrangement of cristae and electron-dense inclusions in mitochondria (Mc); close contact of lipid drops (LD) with mitochondria; c) concentrations of glycogen granules in subsarcolemmal zone; multivesicular bodies (MB) in cytoplasm of endothelial cells (EC); d) perinuclear zone; lamellar complex consists of stacks of flattened cisterns; e) linear arrangement of cristae in mitochondria; f) thin layer of glycocalyx covering sarcolemma (Sl). G) Glycogen granules, Ca) capillary, Mf) myofibrils, Mc) mitochondria, Er) erythrocyte, N) nucleus, No) nucleolus. a, b, c) Hibernating animals; d, e, f) active animals.

TABLE 1. Results of Stereologic Ultrastructural Investigation of Cardiomyocytes of Red-Cheeked Susliks in Different Seasons (M ± m)

Parameter	Fall	Winter		Spring	
	active state	hibernation for 3 months	hibernation for 6 months	1st day after awakening	14th day after awakening
Relative volume ($V_{v_i}^{cyt}$), mm^3/cm^3, of:					
myofibrils	513,9±23,8	517,6±14,7	509,5±36,6	457,1±21,4	515,3±8,2
mitochondria	320,7±17,1	251,4±22,1	275,9±23,9	275,0±15,8	305,8±15,4
sarcoplasmic reticulum	13,3±0,8	30,5±3,3**	29,2±2,6**	22,9±2,7*	16,6±0,2*
T system	14,9±1,9	16,4±2,1	20,3±1,7	19,8±2,5	17,5±2,8
lipid drops	6,9±2,6	38,0±6,8*	21,7±10,2	25,2±9,7	26,3±3,1**
remaining structures of cytoplasm	130,3±7,4	146,1±5,8	143,4±5,7	200,0±20,8	118,5±5,8
Relative surface area ($S_{v_i}^{cyt}$), m^2/cm^3, of:					
myofibrils	0,825±0,010	0,722±0,040	0,926±0,031	0,843±0,116	0,700±0,028*
mitochondria	1,484±0,057	1,107±0,096*	1,389±0,089	1,349±0,098	1,345±0,037
sarcoplasmic reticulum	0,338±0,023	0,835±0,031***	0,760±0,063**	0,553±0,055*	0,515±0,044*
T system	0,270±0,023	0,253±0,022	0,434±0,021**	0,350±0,022	0,271±0,027
lipid drops	0,051±0,019	0,162±0,032*	0,115±0,053	0,160±0,053	0,202±0,020*
Surface-volume ratio (S_{v_i}/V_{v_i}), m^2/cm^3, of:					
myofibrils	1,612±0,077	1,399±0,099	1,847±0,202	1,871±0,318	1,359±0,056
mitochondria	4,641±0,182	4,412±0,146	5,079±0,395	4,903±0,178	4,426±0,291
sarcoplasmic reticulum	25,775±3,171	28,070±2,541	26,123±1,254	24,244±0,607	31,079±2,717
T system	18,200±0,709	16,365±2,622	21,475±0,738*	18,076±1,457	15,957±2,022
lipid drops	7,399±0,009	4,312±0,461**	5,867±0,743	7,179±1,080	7,736±0,332
Bulk density ratio ($V_{v_i}/V_{v_{Mf}}$), number of:					
mitochondria and myofibrils	0,630±0,061	0,490±0,053	0,554±0,088	0,604±0,039	0,595±0,038
sarcoplasmic reticulum and myofibrils	0,026±0,003	0,059±0,006*	0,058±0,005**	0,051±0,007*	0,032±0,0001
T system and myofibrils	0,029±0,004	0,032±0,003	0,040±0,001*	0,044±0,007	0,034±0,005
remaining cell structures and myofibrils	0,256±0,021	0,282±0,011	0,286±0,030	0,442±0,074	0,230±0,010

Legend. *P < 0.05, **P < 0.01, ***P < 0.001 compared with active animals before hibernation.

August; 2 and 3) after hibernation for 3 months (five animals) and 6 months (three susliks), studied in December and the end of March, respectively; 4 and 5) active animals during the 1st and 14th days after awakening (three susliks in each group), studied at the beginning of April. After removal from the thorax the hearts were placed in a cold chamber until they completely stopped beating, then weighed separately, and the relative weight of each heart and its left ventricle was calculated. Pieces of papillary muscle were fixed in 4% paraformaldehyde, post-fixed in 1% osmium tetroxide solution, dehydrated, and embedded in Epon and Araldite. Semithin (1 μ) and ultrathin sections were cut on the LKB III Ultramicrotome. The semithin sections were stained with azure II and examined in the Docuval universal biological microscope (Carl Zeiss, East Germany). The diameter of the cardiomyocytes was measured by means of an MOV-1-15× ocular micrometer in semithin longitudinal sections through the muscle fibers. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in the JEM-100B electron microscope. By means of the stereologic and statistical methods described previously [4] the bulk and surface density of the myofibrils, mitochondria, smooth sarcoplasmic reticulum (SSR), T system, and lipid drops and the relative volume of the remaining structures of the cardiomyocytes were determined. On the basis of the primary stereologic data, volume and surface–volume ratios were calculated for the principal intracellular organelles. Stereologic analysis was carried out on negative electron micrographs with a final magnification of 16,000.

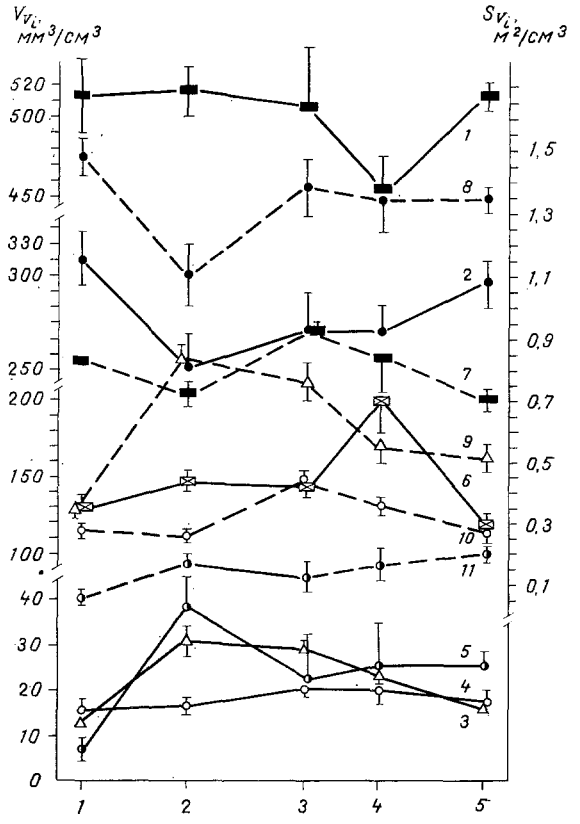

Fig. 2

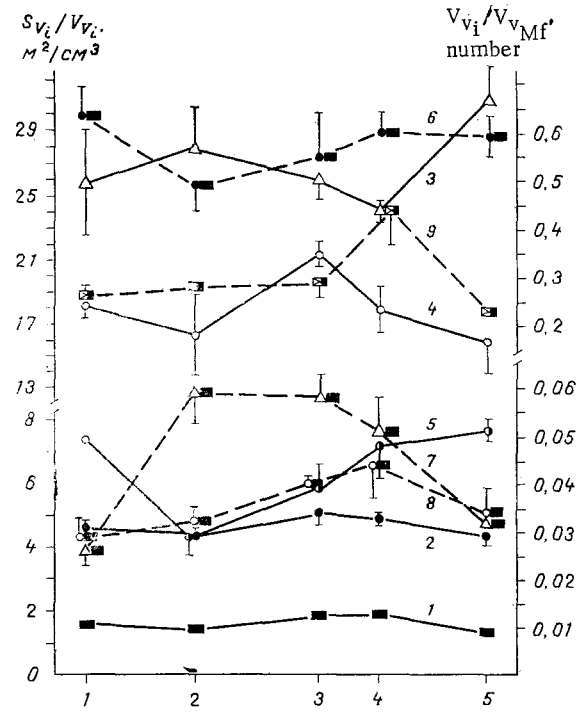

Fig. 3

Fig. 2. Results of measurements of primary stereologic parameters of cardiomyocyte ultrastructures of red-cheeked susliks in different seasons. Abscissa, groups of animals; ordinate: on left — bulk density, on right — surface density. 1, 7) Myofibrils, 2, 8) mitochondria, 3, 9) sarcoplasmic reticulum, 4, 10) T system, 5, 11) lipid drops, 6) remaining structures of cytoplasm.

Fig. 3. Results of calculation of secondary stereologic parameters of cardiomyocyte ultrastructures of red-cheeked susliks in different seasons. Abscissa, groups of animals; ordinate: on left — surface-volume ratio of organelles, on right — ratio between bulk densities of organelles and bulk density of myofibrils. 1) Myofibrils, 2) mitochondria, 3) sarcoplasmic reticulum, 4) T system, 5) lipid drops, 6) mitochondria/myofibrils, 7) sarcoplasmic reticulum/myofibrils, 8) T system/myofibrils, 9) remaining structures of cytoplasm/myofibrils.

The significance of differences between means was determined by Student's test (differences were considered to be significant when $P < 0.05$). To ensure the optimal size of the sample, dispersion analysis was used [5].

EXPERIMENTAL RESULTS

Before commencing hibernation, susliks had their greatest body weight, which decreased gradually in the course of hibernation. A decrease in absolute weight of the heart and left ventricle was observed toward the end of hibernation and on the first days after awakening. The smallest diameter of the cardiomyocyte (10.92 ± 0.04 μ) was observed in the myocardium of susliks in a torpid state and has been attributed to many factors, in particular, reduction of the blood flow [11] and also some dehydration of the myocardial tissue [1].

Cardiomyocytes of hibernating and active animals differed in their ultrastructural organizations. The fraction of heterochromatin was increased in the heart muscle cell nuclei of the hibernating animals. The lamellar complex was unique in structure: As a rule it consisted of several concentrations of vesicles of different diameters (Fig. 1a). The myofibrils in these cells were densely packed and the mitochondria were large and had a circular arrangement of their cristae. Electron-dense granules of different sizes could be seen frequently on mem-

branes of the cristae and in the matrix. An abundance of large lipid drops, located among myofibrils, and often in close contact with the outer mitochondrial membrane, was a noteworthy feature (Fig. 1b). Hyperplasia of SSR and dilatation of the tubules of the T system also were observed. Large concentrations of glycogen granules in different parts of the sarcoplasm and enlargement of the outer juxtamembranous layer of glycoproteins (glycocalyx) also were typical of the cardiomyocytes of hibernating animals (Fig. 1c).

The chief structural differences in the cardiomyocytes of the animals after awakening compared with hibernation were an increase in volume of the sarcoplasm and the appearance of many ribosomes and polysomes, which reflects intensification of protein synthesis. The lamellar complex of the active animals was organized from several stacks of flattened cisterns (Fig. 1d). The mitochondria became smaller, the cristae became linear in character, and the intramitochondrial electron-dense granules disappeared (Fig. 1e). The number of elements of the SSR was reduced, lipid drops were less frequently seen, and the number of lysosomes was increased. The glycocalyx layer became thinner (Fig. 1f).

Stereologic analysis revealed significant seasonal changes in the architectonics of the principal cytoplasmal organelles of cardiomyocytes of red-cheeked susliks (Table 1; Figs. 2 and 3). In animals hibernating for 3 months the surface density and the surface–volume ratio of the myofibrils were reduced only a little (by 12.5 and 13.2%, respectively) compared with these parameters in active animals before hibernation (group 1), but after 6 months of hibernation the relative surface area of the myofibrils was increased significantly by 12.2%. The small increase in the surface–volume ratio under these circumstances is evidence of a reduction in thickness of the myofibrils. On the first day after awakening (group 4) no significant changes were found in the surface–volume characteristics of the myofibrils, but a tendency was noted for the bulk density to decrease by 11%. On the 14th day of activity (group 5) the relative volume of the myofibrils was close to values characteristic of the animals of group 1, but the relative surface area was significantly less than the control.

The structural density of the mitochondria showed no significant change in the animals of the various groups studied. There was only a slight tendency for the bulk density of the mitochondria to fall in susliks hibernating for 3 and 6 months, and also on the first day after awakening. A significant decrease in surface density of the mitochondrial compartment was observed in susliks in a torpid state for 3 months, but under these circumstances there was no significant change in the surface–volume ratio. The ratio of bulk density of mitochondria to relative volume of myofibrils likewise did not change significantly.

The most marked seasonal changes took place in the membrane ion-transport apparatus. The bulk and surface density of SSR was increased by more than 120% in susliks hibernating for 3 and 6 months and it remained at a sufficiently high level during the first 14 days after awakening; this increase, moreover, developed without any change in shape of the tubules of SSR, as a result of which the surface–volume ratio was unchanged.

The bulk density of the T system did not change significantly at the times studied, and the small increase in this parameter after 6 months of hibernation to 20.3 ± 1.7 mm^3/cm^3 (14.9 ± 1.9 mm^3/cm^3 in the animals of group 1) was accompanied by a significant increase in the relative surface area by 60.7%. On the first day after awakening the surface–volume characteristics of the T system did not differ significantly from those for the susliks of group 1.

The significant increase in bulk and surface density of SSR and of the T system in animals hibernating for 3 and 6 months and the accompanying increase in the volume ratio of the SSR and T system to the myofibrils (Fig. 4), together with changes in the ultrastructure of the intercalated disk [13] and basement membrane [7, 10], were aimed at ensuring coordination between excitation and contraction of the myocardium at low body temperatures.

Information analysis of the stereologic parameters revealed an increase in entropy and relative entropy and a decrease in excess of the sarcoplasm of the cardiomyocytes of red-cheeked susliks in a state of hibernation for 3 and 6 months, and during the first day after awakening (Table 2), evidence of an increase in the indeterminacy of this system at these times.

The abrupt seasonal fluctuations in physiological activity of heterothermic animals (red-cheeked susliks) are thus accompanied by considerable modification of the three-dimensional ultrastructural organizations of the cardiomyocytes. The essence of this modification during hibernation is an increase in "saturation" of the unit volume of myofibrils with other cyto-

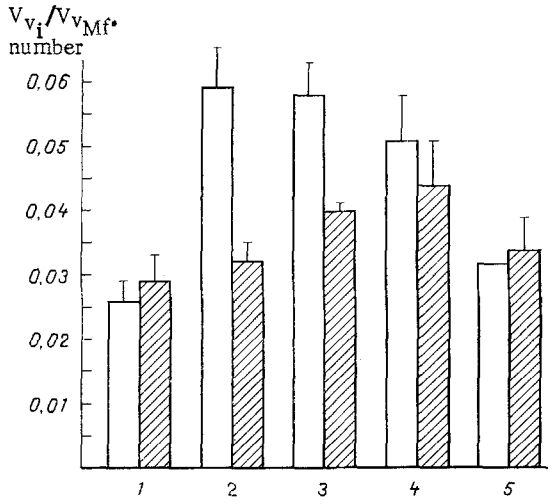

Fig. 4. Seasonal dynamics of ratio of bulk density of SSR and T system to relative volume of myofibrils in cardiomyocytes of red-cheeked susliks. Abscissa, groups of animals; ordinate, volume ratios. Unshaded column — SSR/myofibrils, shaded — T system/myofibrils.

TABLE 2. Parameters of Information Analysis of Sarcoplasm of Cardiomyocytes in Red-Cheeked Susliks in Different Seasons

Parameter	Fall	Winter		Spring	
	active state	hibernation for 3 month	hibernation for 6 month	1st day after awakening	14th day after awakening
Entropy, binary units	1,626	1,828	1,793	1,864	1,719
Relative entropy	0,629	0,707	0,694	0,721	0,665
Excess, %	37,1	29,3	30,6	27,9	33,5

plasmic organelles, providing a material basis for the rapid adaptive response of the heart of these animals during hibernation and on awakening.

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