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Ultrastructure and Stereology of Cardiomyocytes in the Development of Regenerative and Plastic Myocardial Insufficiency during Ontogeny

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The age-related features of ultrastructural reorganization of cardiomyocytes were studied in rats with anthracycline-induced injury. The development of regenerative and plastic insufficiency of cardiomyocytes in animals of various age groups was accompanied by stereotypic ultrastructural reorganization. The major changes concerned the nucleus, myofibrillar compartment, and rough sarcoplasmic reticulum. Intracellular reorganization of cardiomyocytes in young animals was observed in the same period, but included a greater decrease in the volume density of myofibrils as compared to that in old rats. The recovery of cardiomyocyte ultrastructure in young animals occurred in the earlier period. Cardiomyocytes of old rats were characterized by greater structural modification of mitochondria and considerable area of lytic changes in myofibrillar bundles. Cardiotoxicity of doxorubicin in young animals was manifested in severe destruction of the capillary endothelium, which occurred in the early period after treatment.

Key Words: *ontogenetic periods; cardiomyocytes; cardiotoxicity; doxorubicin; ultrastructure; stereology*

The use of anthracycline antibiotics (*e.g.*, doxorubicin) in chemotherapy of oncology patients is often accompanied by the development of cardiomyopathies and heart failure during various periods after treatment. Some authors reported that the risk of heart failure after treatment with anthracycline antibiotics in children is much higher than in adults [6-8,12]. Other researchers believe that the risk of heart failure after chemotherapeutic treatment increases significantly with age, since oncology patient of the old age group have a variety of cardiovascular diseases [5,6]. Therefore, various schemes of chemotherapy are limited or forbidden in elderly patients. It should be emphasized that the risk of cardiomyopathy and heart failure varies significantly in patients of all age groups and depends on exogenous and endogenous factors (genetic and epigenetic factors).

Much attention was paid to studying the cardiotoxicity of anthracycline antibiotics and other cytostatics [9,10]. However, little is known about the mechanisms and structural basis for drug-induced myocardial injury in various age periods. Ultrastructural changes in cardiomyocytes (CMC) after cytostatic treatment

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were not compared during various periods of ontogeny. It is difficult to perform clinical studies of the myocardium. Hence, the data on structural changes and biomolecular reactions of CMC after cytostatic treatment can be obtained only under experimental conditions.

Here we studied the intracellular reorganization of CMC in rats that were exposed to a cardiotoxic effect of doxorubicin (DOX) in the period of progressive or regressive growth.

MATERIALS AND METHODS

Ultrastructure of the myocardium was studied in male Wistar rats during progressive (1 month, *n*=40) and regressive growth (24 months, *n*=18). Anthracycline cardiomyopathy in animals was induced by an intraperitoneal injection of doxorubicin hydrochloride (FERANE) in a single dose of 5 mg/kg. The samples were obtained 5, 14, and 21 days after treatment. Age-matched controls (3 specimens in each age group) received an intraperitoneal injection of physiological saline. The volume of physiological saline was estimated from body weight of animals. Before and during the experiment, all animals were maintained in a vivarium and received the standard diet and water *ad libitum*.

Myocardial samples were fixed in 4% paraformaldehyde, postfixed in 1% OsO_4, treated by the standard method, and embedded into a mixture of Epon and araldite. Ultrathin sections were prepared on an Ultracut EM UC7 ultratome (Leica), contrasted with uranyl acetate and lead citrate, and examined under a JEM 1400 electron microscope (Jeol, accelerating potential 80 kV). The images were obtained using a Veleta digital camera and iTEM software (Olympus). Ultrastructural stereological study was performed with iTEM software (final magnification ×23,500; initial magnification ×20,000).

RESULTS

The intracellular structure of CMC was shown to differ in young and old intact Wistar rats. CMC of young animals included a considerable number of glycogen granules (Fig. 1, *a*). The Golgi complex (particularly the vesicular part of this complex) was well developed. Myofibrillar bundles of CMC were thickened in old rats. The size of mitochondria in old rats was greater than in young animals. The Golgi complex was rarely found in old specimens. A specific ultrastructural sign of CMC in old rats was the presence of giant secondary lysosomes with osmiophilic lipofuscin granules in the perinuclear sarcoplasm.

Ultrastructural stereological study showed that the intracellular reorganization of CMC in Wistar rats manifested in a decrease in the volume and surface density of mitochondria (by 5 and 16%, respectively; Table 1). The volume density of myofibrils slightly decreased with age. Significant decrease in the surface density of myofibrils was followed by reduction of the surface/volume ratio between these structures (by 39%; Table 2). These changes reflect thickening of myofibrillar bundles in the majority of CMC from old animals. Age-related changes were observed in the transport system of CMC (T system and smooth sarcoplasmic reticulum, SSR). The relative volume of these structures decreased by more than 2 times (Table 1). The volume ratio of study structures to myofibrils was also reduced. Microfocal lysis of myofibrils was followed by dilation of SSR vesicles and visualization of tubules in the T system. The interstitial space included a considerable number of monocytes and activated macrophages.

Moderate lysis of the sarcoplasmic matrix in CMC and disappearance of glycogen granules were found in 1-month-old rats by the 5th day after DOX injection (Fig. 1, *b*). Fragmentation of the nucleolus and segregation of the nucleolonema were observed in some CMC. Small myelin-like structures were present in the perinuclear and subsarcolemmal space or close to the intercalated discs, which reflects activation of autophagocytosis. The fine structure of mitochondria remained practically unchanged. We revealed an insignificant decrease in the volume and surface density of mitochondria (Table 1). Loosening of the matrix and decrease in the number of mitochondrial cristae were observed in some CMC. Ultrastructural changes in myofibrils were manifested in moderate lysis. The surface density of myofibrils decreased more significantly than the volume density (by 23 and 4%, respectively). Significant changes were found in SSR. The significant increase in the volume and surface density of SSR (by 80 and 45%, respectively) reflected dilation of SSR vesicles (Fig. 1, *b*).

Severe and spread necrobiotic changes in endothelial cells were revealed 5 days after administration of DOX into the myocardium of 1-month-old animals (Fig. 1, *c*). Necrotic endothelial cells were swollen and electron dense. They grew into the lumen of capillaries. Organelles were practically unidentified in the cytoplasm of these cells. Some endothelial cells included the centrioles, which reflected increased proliferative activity of these cells. Large residual bodies were found in the lumen of capillaries. Activated fibroblasts, adjacent collagen fibers and bundles, and reactive macrophages were present in the interstitial space.

CMC of 24-month rats were characterized by diffuse lysis of myofibrils 5 days after DOX administration (Fig. 2, *a*). These changes contributed to a decrease in the volume and surface density (by 6 and 4%,

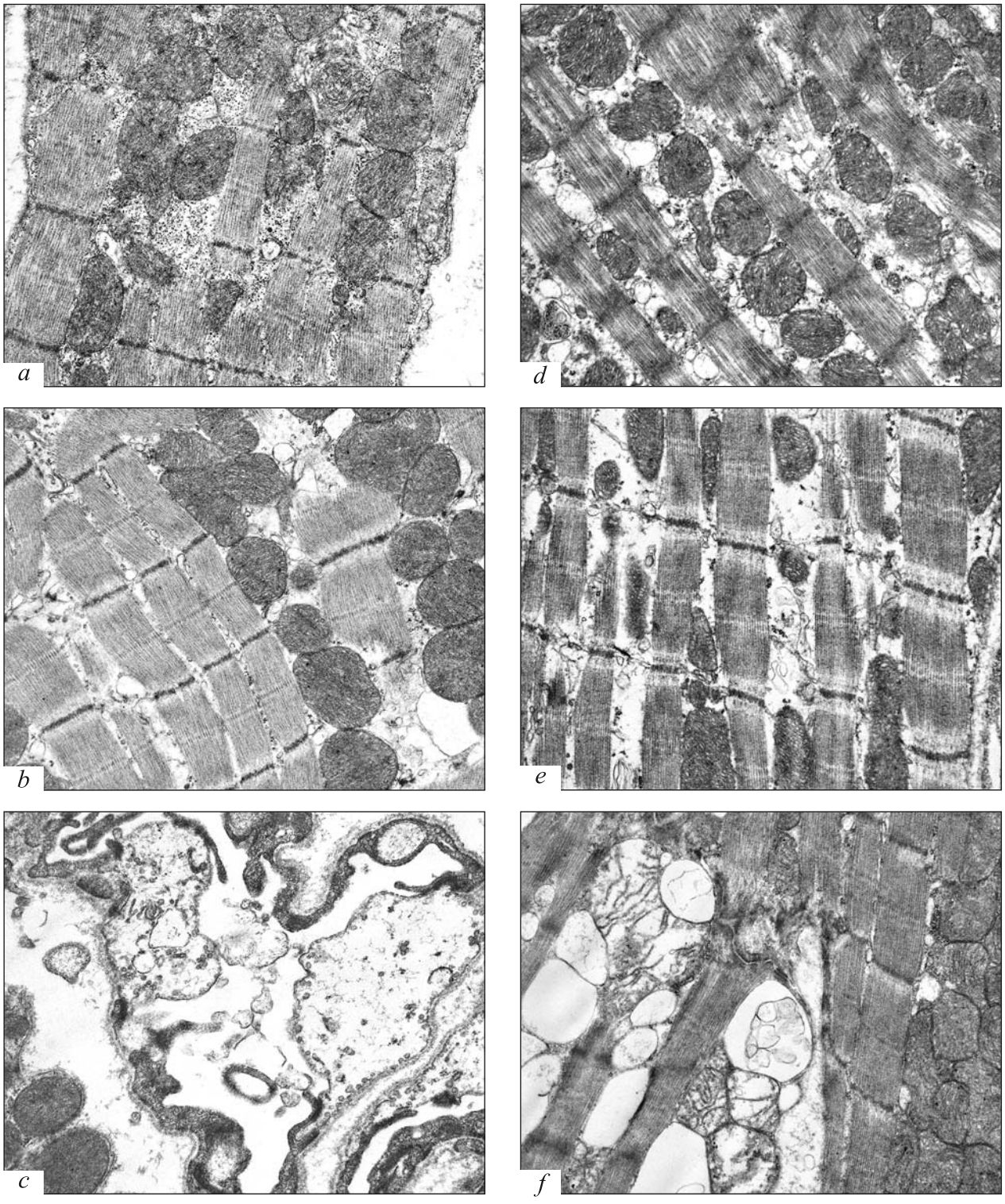

Fig. 1. Ultrastructural changes in CMC from 1-month-old Wistar rats after single treatment with DOX. (*a*) Numerous granules of glycogen in CMC from the intact rat; (*b*) focal lysis of myofibrils and pronounced dilation of RSS vesicles after 5 days; (*c*) necrosis of endothelial cells after 5 days; (*d*) diffuse lysis of myofibrils after 14 days; (*e*) lysis of myofibrillar bundles after 21 days; (*d*) local destruction of mitochondria after 21 days. ×20,000 (*a*, *b*, *d*, *f*); ×25,000 (*c*); ×15,000 (*e*).

respectively). The presence of numerous liposomes in the site of myofibrillar lysis illustrates the process of intracellular regeneration (Fig. 2, *b*). DOX-induced changes in the ultrastructure of CM mitochondria were more typical of old rats. They were manifested in irregular widening of the cristae, decrease in their num-

ber, and formation of residual bodies and myelin-like structures. Structural modification of mitochondrial cristae was manifested in fusion and increase in the electron density of these structures. The surface density and surface/volume ratio of mitochondria were shown to increase in this period (by 18 and 16%, respectively). These changes illustrate the prevalence of small organelles in CMC. The change in energy production and energy consumption are the major mechanisms of the cardiotoxic effect of DOX [11].

The volume and surface density of the T system increased more significantly in CMC of 24-month-old animals (by 220 and 35%, respectively). The increase in study parameters of SSR (by 163 and 63%, respectively) was related to significant dilation of vesicles. Dilated vesicles of RSS in some CMC were filled with the microgranular content (Fig. 2, *a*). The nucleoli were characterized by segregation of the fibrillar and granular components of the nucleolonema. Large clusters of secondary lysosomes were found in the perinuclear region of CMC. Severe injury to endothelial cells was not revealed in old rats (as differentiated from young specimens). Platelets were often identified in the capillary lumen of old animals.

The volume density of mitochondria increased significantly (by 23%, $p<0.05$), while the surface/volume ratio decreased (by 14%) on day 14 after administration of DOX into CMC of 1-month-old rats. The majority of mitochondria had vesicular cristae. Some organelles were shown to contain the lysed matrix and individual cristae. Progressive lysis of myofilaments (Fig. 1, *d*) was followed by a significant decrease in the volume and surface density of myofibrillar bundles (by 15 and 27%, respectively). As differentiated from the previous stage, we revealed a decrease in the volume and surface density of the T system (by 35 and 42%, respectively). These parameters for SSR were elevated by 54 and 35%, respectively, due to significant dilation of vesicles. Many nuclei of CMC contained the annular and dispersed nucleoli. Segregation of the

TABLE 1. Ultrastructural Stereological Study of Cardiomyocytes from Wistar Rats after Single Treatment with DOX (*M*±*m*)

Parameter		Control	Administration of DOX		
			5 days	14 days	21 days
1 month					
Volume density, mm^3/cm^3	mitochondria	311.5±14.6	303.7±14.9	383.1±18.8*	340.2±6.8
	myofibrils	547.5±12.3	525.4±22.1	463.8±16.8*	482.5±4.0*
	T system	2.0±0.2	1.9±0.1	1.3±0.2*	2.0±0.4
	sarcoplasmic reticulum	19.0±3.4	34.2±3.1*	29.2±3.0*	28.3±3.2
	sarcoplasmic matrix	120.0±8.6	134.9±19.1	122.6±13.3	146.9±14.6
Surface density, m^2/cm^3	mitochondria	1.766±0.075	1.699±0.111	1.870±0.057	1.901±0.092
	myofibrils	2.351±0.157	1.813±0.372	1.718±0.401	1.606±0.208*
	T system	0.104±0.023	0.094±0.007	0.060±0.014	0.133±0.057
	sarcoplasmic reticulum	0.537±0.131	0.780±0.121	0.725±0.073	0.691±0.040
24 months					
Volume density, mm^3/cm^3	mitochondria	296.9±11.8	297.8±11.4	307.5±14.5	328.3±19.1
	myofibrils	537.3±14.8	503.8±19.3	509.6±13.9	502.4±12.7
	T system	1.0±0.3	3.2±0.4*	5.4±0.7*	3.0±0.8
	sarcoplasmic reticulum	8.9±0.7	23.4±1.8*	12.2±1.1*	16.0±2.3*
	sarcoplasmic matrix	155.9±15.5	171.8±39.7	165.3±11.6	150.4±17.4
Surface density, m^2/cm^3	mitochondria	1.483±0.058	1.744±0.084*	1.640±0.120	1.922±0.158
	myofibrils	1.466±0.146	1.405±0.121	1.312±0.172	2.042±0.168*
	T system	0.111±0.050	0.150±0.061	0.177±0.046	0.124±0.035
	sarcoplasmic reticulum	0.333±0.024	0.543±0.099	0.335±0.122	0.504±0.113

Note. Here and in Table 2: *$p<0.05$ compared to the control.

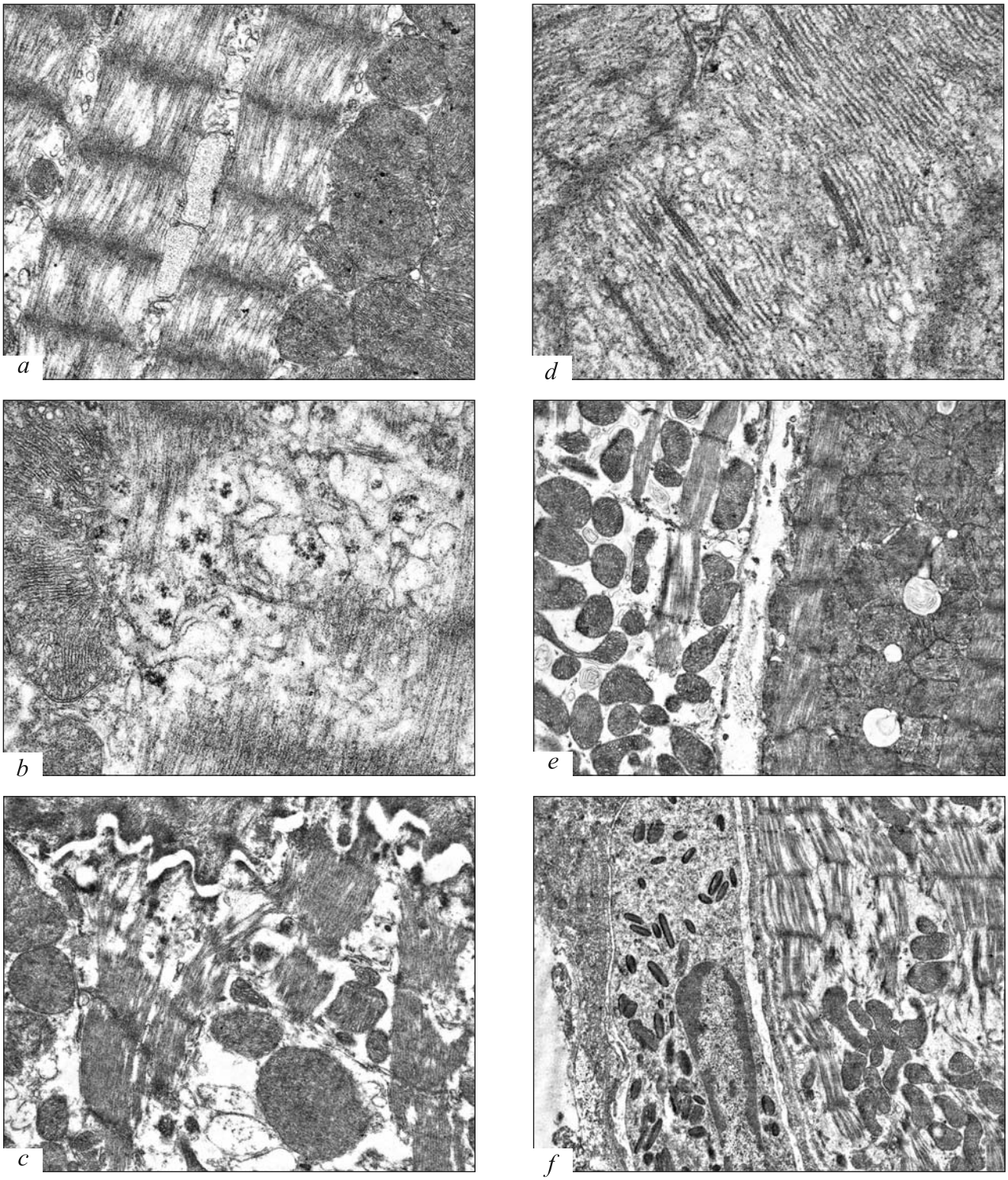

Fig. 2. Ultrastructural changes in CMC from 24-months-old Wistar rats after single treatment with DOX. (*a*) Microgranular substance in the dilated vesicles of RSS after 5 days, ×30,000; (*b*) clusters of polysomes in the region of myofilament lysis after 5 days, ×50,000; (*c*) structural modification of mitochondrial cristae after 14 days, ×100,000; (*d*) severe destruction of organelles near the intercalated disk after 14 days, ×25,000; (*e*) significant heterogeneity of CMC after 21 days, ×15,000; (*f*) diffuse lysis of myofibrils (21st day) and migration of the eosinophil, ×12,000.

fibrillar and granular components of the nucleolonema was identified. Autophagocytosis (accumulation of residual bodies and myelin-like structures) and focal lysis of the sarcoplasm were increased under these conditions. Activated monocytes/macrophages were found in the interstitial space. The processes of these

TABLE 2. Secondary Stereological Parameters of Major Organelles in Cardiomyocytes from Wistar Rats after Single Treatment with DOX (*M*±*m*)

Parameter		Control	Administration of DOX		
			5 days	14 days	21 days
1 month					
Surface/volume ratio, m^2/cm^3	mitochondria	5.7±0.2	5.6±0.2	4.9±0.2*	5.6±0.3
	myofibrils	4.4±0.6	3.5±0.8	3.7±0.9	3.3±0.6
	T-системы	53.8±13.5	51.8±6.5	49.2±14.1	61.8±12.2
	sarcoplasmic reticulum	27.9±3.1	23.8±4.2	28.9±7.4	25.4±3.6
Volume ratio of organelles, index	mitochondria/myofibrils	0.585±0.044	0.581±0.046	0.831±0.064*	0.705±0.052
	T system/myofibrils	0.004±0.0003	0.004±0.0002	0.003±0.0003	0.004±0.001
	sarcoplasmic reticulum/myofibrils	0.035±0.006	0.065±0.008*	0.063±0.007*	0.059±0.008*
24 months					
Surface/volume ratio, m^2/cm^3	mitochondria	5.1±0.5	5.9±0.2	5.4±0.5	5.8±0.5
	myofibrils	2.7±0.3	2.8±0.5	2.5±0.4	4.1±0.5
	T system	99.5±13.0	44.1±13.7*	37.4±9.2*	44.5±9.8*
	sarcoplasmic reticulum	38.2±4.7	25.9±4.0	26.3±3.4	34.2±6.3
Volume ratio of organelles, index	mitochondria/myofibrils	0.566±0.011	0.592±0.016	0.609±0.039	0.658±0.026*
	T system/myofibrils	0.002±0.0005	0.006±0.002	0.010±0.003	0.006±0.002
	sarcoplasmic reticulum/myofibrils	0.017±0.002	0.045±0.019	0.024±0.006	0.032±0.010

cells were shown to spread in the sarcolemmal invagination of CMC.

Changes in the fine structure of CMC mitochondria in 24-month-old rats were shown to persist on the 14th day after DOX injection (fusion, consolidation, and vesicular dilation of the cristae; Fig. 2, *c*). The mitochondria were polymorphic. We revealed the presence of giant mitochondria. The surface density of mitochondria was elevated by 11%, which reflects the prevalence of small organelles. The degree of focal and diffuse lysis in myofibrils was shown to increase under these conditions. Thinning of myofibrillar bundles was manifested in a decrease in their volume and surface density (by 5 and 11%, respectively). Clusters of polysomes were found in the site of myofibrillar lysis during this period. Individual myofilaments were adjacent to these clusters. The volume and surface density of the T system increased progressively (by 440 and 60%, respectively), which was probably associated with "denudation" of the tubules due to myofilament lysis. The 37% increase in the volume density of RSS was related to a significant dilation of its vesicles. Severe destructive changes were observed in the region of intercalated discs of some CMC (Fig. 2, *d*). Large secondary lysosomes and myelin-like structures were located in this region.

The volume and surface density of myofibrils in young rats remained reduced on day 21 after DOX injection (by 12 and 32%, respectively). Myofibrillar bundles were thinned due to lysis (Fig. 1, *e*). We revealed an increase in the number of CMC that were characterized by activation of intracellular regeneration and recovery of ultrastructure. The mitochondria remained polymorphic. Irregular dilation of the cristae was revealed in these specimens. Focal destruction of the mitochondria was found in some CMC. These mitochondria were displaced toward the intercalated discs (Fig. 1, *f*). Tight junctions in the region of intercalated discs were impaired in some specimens. CMC were dissociated (change in the structural integrity of muscle fibers). The volume and surface density of RSS remained high due to vesicular dilatation (particularly in the perinuclear and subsarcolemmal region). The signs of intensive regeneration were also revealed in other populations of myocardial cells from young rats. The amount of profiles of the rough endoplasmic reticulum and number of polysomes were increased in the capillary

endothelium. The plasmalemma formed numerous outgrowths in the lumen.

The degree of CMC heterogeneity in the myocardium of 24-month-old rats increased on day 21 after DOX injection (estimated from the severity of ultrastructural changes; Fig. 2, *e*, *f*). Nearly unchanged CMC had the densely packed myofibrils and mitochondria. Some CMC were characterized by severe lysis of myofibrillar bundles and sarcoplasmic matrix. The population of mitochondria in nearly unchanged CMC was presented by large organelles, many of which exhibited the fusion of cristae. The volume and surface density of these structures was shown to increase progressively (by 11 and 30%, respectively). The mitochondria were smaller in CMC with severe lytic changes of myofibrils. Vesicular dilation of the cristae was typical of these mitochondria. The area of diffuse lysis in myofibrillar bundles was greater in old rats. However, their number in CMC was shown to decrease insignificantly. The volume density of myofibrils decreased, while the surface density of these structures increased (by 39%). These changes were accompanied by a significant increase in the surface/volume ratio of myofibrils (by 52%). The volume and surface density of RSS remained elevated (by 80 and 51%, respectively), which illustrates the dilation of vesicles. The size of secondary lysosomes and residual bodies (*e.g.*, in the subsarcolemmal space) was increased in this period. The number of abnormal junctions between adjacent CMC was greater in the region of intercalated discs. A considerable number of myelin-like structures were released into the interstitial space through these junctions. Activated leukocytes were shown to migrate into CMC with severe lytic changes (Fig. 2, *f*).

The type of DOX-induced ultrastructural changes in CMC of young and old animals reflects the general features of regenerative and plastic insufficiency [1-3]. The major ultrastructural changes occur in the nucleus (segregation of the fibrillar and granular components of the nucleolonema), myofibrillar compartment (focal and diffuse lysis of myofilaments), and SSR (pronounced dilation of the vesicles and intermembrane perinuclear space). The degree of lytic and destructive changes in organelles and ultrastructures was compared in young and old animals. As differentiated from young animals, old specimens were characterized by greater area and progression of myofibrillar lysis. An important feature of DOX-induced ultrastructural changes in the myocardium of young animals is the development of severe, but transitory destruction of endothelial cells. The recovery of ultrastructure in the majority of CMC occurs earlier in young rats, which is related to higher intensity of intracellular regeneration [4].

REFERENCES

1. E. L. Lushnikova, M. G. Klinnikova, O. P. Molodykh, and L. M. Nepomnyashchikh, *Byull. Eksp. Biol. Med*., **139**, No. 4, 470-475 (2005).
2. L. M. Nepomnyashchikh, E. L. Lushnikova, P. M. Larionov, and M. G. Shurygin, *Vestnik Ros. Akad. Med. Nauk*, No. 5, 3-11 (2010).
3. L. M. Nepomnyashchikh, E. L. Lushnikova, and D. E. Semenov, *Regenerative and Plastic Insufficiency of the Heart: Morphological Bases and Molecular Mechanisms* [in Russian], Moscow (2003).
4. L. M. Nepomnyashchikh, O. P. Molodykh, E. L. Lushnikova, *et al*., *Byull. Eksp. Biol. Med.*, **148**, No. 12, 693-699 (2009).
5. A. Albini, G. Pennesi, F. Donatelli, *et al*., *J. Nat. Cancer Inst*., **102**, No. 1, 14-25 (2010).
6. E. G. Lakatta and D. Levy, *Circulation*, **107**, No. 2, 346-354 (2003).
7. S. E. Lipshultz and M. J. Adams, *J. Clin. Oncol*., **28**, No. 8, 1276-1281 (2010).
8. S. E. Lipshultz, S. R. Lipsitz, S. E. Sallan, *et al*., *J. Clin. Oncol*., **20**, No. 23, 4517-4522 (2002).
9. G. Minotti, P. Menna, E. Salvatorelli, *et al*., *Pharmacol. Rev*., **56**, No. 2, 185-229 (2004).
10. S. M. Swain, F. S. Whaley, and M. S. Ewer, *Cancer*, **97**, No. 11, 2869-2879 (2003).
11. M. Tokarska-Schlattner, M. Zaugg, C. Zuppinger, *et al*., *J. Mol. Cell. Cardiol*., **41**, No. 3, 389-405 (2006).
12. H. J. van der Pal, E. C. van Dalen, M. Hauptmann, *et al*., *Arch. Intern. Med*., **170**, No. 14, 1247-1255 (2010).