Intracellular Reorganization of Hepatocytes during Doxorubicin Treatment

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The dynamics of intracellular reorganization of rat hepatocytes was studied after a single injection of doxorubicin. The hepatocyte population is presented by three main ultrastructural phenotypes. Quantitative ratio between these phenotypes changed during the development of anthracycline hepatopathy. Ultrastructural changes in hepatocytes are determined by the development of the regeneratory plastic insufficiency characterized by segregation and annular reorganization of the nucleoli, destruction of the mitochondrial compartment, and the resultant lipid infiltration of the cytoplasm, as well as intensification of autophagocytosis, all these changes persisting for a long time. This was paralleled (in some hepatocytes) by signs of intracellular regeneration and compensatory adaptive restructuring manifesting in pronounced hyperplasia of the Golgi complex and remodeling of the biliary and sinusoidal hepatocyte poles reflecting activation of exo-, endo, and transcytosis. Disproportional changes in the volume density of the granular and agranular endoplasmic reticulum indicate transitory predominance of protein-producing processes over detoxifying ones in the hepatocyte population.

Key Words: *hepatocytes; doxorubicin; ultrastructure; stereology*

The hepatotoxic effects of drugs always remain in the focus of attention of scientists and clinicians as one of the most intricate problems of modern medicine. It is noteworthy that severe injuries to the liver and lethal outcomes are noted mainly after overdosage of just some drugs (for example, acetaminophene) and are very rare events for the majority of drugs [11,12]. On the other hand, the spectrum of hepatotoxic effects of many drugs which are often used is wide and variegated, particularly in children [7]. Among most widely used drugs producing hepatotoxic effects are nonsteroid antiinflammatory, antihypertensive, and antidiabetic agents, anticonvulsants, some psychotropic drugs, statins, *etc.*

Hepatotoxicity of cytostatics used in antitumor therapy is less studied, as it is assumed that damage to the hemopoietic and reproductive systems and the myocardium (development of cardiomyopathy) are the main side effects of antiblastoma therapy. However, studies of the liver morphology after injections of some cytostatics to animals showed that even a single injection of these drugs caused a significant structural reorganization of the organ and modified its regeneratory potential [2,4].

The pathogenesis of drug-induced hepatopathy is very intricate and in many cases unclear. Exogenous cytotoxic agents [9,10] and metabolites forming in hepatocytes as a result of drug biotransformation and sometimes exhibiting even more pronounced cytotoxic effects [13,14] play an important role in it. Liver ischemia resulting from chronic cardiac insufficiency or sinusoidal obstructive syndrome of toxic genesis [4,8] are also signi-

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ficant. In order to clear out the mechanisms of toxic damage to hepatocyte, one has to study, in addition to molecular biology, the ultrastructural changes in hepatocytes and evaluate the intensity of intracellular regeneratory reactions.

We studied the type and dynamics of intracellular reorganization of hepatocytes and distinguished their main structural and functional types (by the ultrastructural phenotypes) after a single injection of doxorubicin to experimental animals.

MATERIALS AND METHODS

Electron microscopic study of hepatocytes was carried out in 35 male Wistar rats aged 4 months receiving intraperitoneal injection of doxorubicin hydrochloride in a single dose of 10 mg/kg. The animals were kept at ambient temperature with free access to water and food and were decapitated on days 1, 3, 5, 7, 14, 21, and 30 postinjection. Controls were injected with saline in the same volume.

Preparations for electron microscopy were fixed in 4% paraformaldehyde, postfixed in 1% OsO_4, and processed routinely. Ultrathin sections sliced on an LKB-III ultratome were contrasted with uranyl acetate and lead citrate and examined under a JEM 1010 electron microscope.

Ultrastructural stereological analysis of hepatocytes was carried out on negatives at a final magnification of 20,000 (initial magnification 8000). The volume densities of mitochondria, agranular endoplasmic reticulum (AER), and granular endoplasmic reticulum (GER) were evaluated. Surface density was evaluated for the mitochondria and GER. Based on the primary stereological parameters, the secondary ones (volume and surface/volume) ratios between the main ultrastructures were calculated.

Statistical processing of the results included calculation of the means for parameters, dispersion, and errors of the means. The significance of differences was evaluated using Student's *t* test.

RESULTS

Ultrastructural phenotypes of hepatocytes after doxorubicin injection can be divided into 3 main types: hepatocytes with well-developed GER (packed in heaps predominantly at the sinusoidal pole) and mitochondrial compartment (orthodox phenotype); hepatocytes with modified topography of ultrastructures (massive depositions (fields) of glycogen and discomposition of the main organelles forming functional blocks) — mitochondria and GER; and hepatocytes with pronounced AER hyperplasia. The phenotypical heterogeneity of hepatocytes reflects their different functional specialization linked with predominantly secretory or detoxifying activity [1]. Functional specialization of cells with massive glycogen depositions seems to consist in creation of depots of this polysaccharide for its subsequent use in accordance with requirements of the body. Destructive changes in the organelles were detected in hepatocytes of all types, but differed by type and severity.

One day after doxorubicin injection, the hepatocytes with the orthodox phenotype and with modified topography of ultrastructures were equally incident. The most significant ultrastructural changes in the nuclei were detected in hepatocytes with massive deposition of glycogen and organelle discomposition. Euchromatin predominated in the hepatocyte nuclei, sometimes vacuole-like dilatations (optically empty or containing floccular substance) and lipid incorporations were seen. Segregation of the granular and fibrillar components was seen in many nucleoli (Fig. 1, *a*), annular nucleoli appeared (Fig. 1, *b*). Segregation of the nucleolonema and its annular reorganization are the ultrastructural markers of inhibited formation of ribosomes and hence, inhibition of the regeneratory plastic processes in cells [3]. Changes in the cytoplasmic compartment manifested mainly in small focal lysis and glycogen sequestration.

Large looped nucleoli, in which the nucleolonema sequestration processes were also observed, predominated in the nuclei of hepatocytes with the orthodox phenotype. Uneven dilatation of the GER cisterns, partial destruction of some mitochondria, and formation of myelin-like structures were seen at the sinusoidal pole of hepatocytes. The most significant changes were observed in the hepatocyte biliary pole, where hyperplastic Golgi complex and numerous secondary lysosomes with lipofuscsin incorporation were located (Fig. 2, *a*). Hyperplasia of Golgi complex was paralleled by even dilatation of the terminal compartments of the cisterns with floccular contents. An important aspect in reorganization of the hepatocyte biliary pole was significant extension of close contacts (*zonula occludens*) between the neighboring hepatocytes (Fig. 2, *a*). Numerous microvilli were seen in the biliary capillary lumens, osmiophilic myelin-like structures were sometimes detected.

Hepatocytes with pronounced AER hyperplasia were scanty (Fig. 1, *c*). Vesicles of AER predominated in these cells, which rendered them a "foamy" look. The mitochondria were small, GER tubules short, α-glycogen granules were evenly distributed between organelles in the cytoplasm.

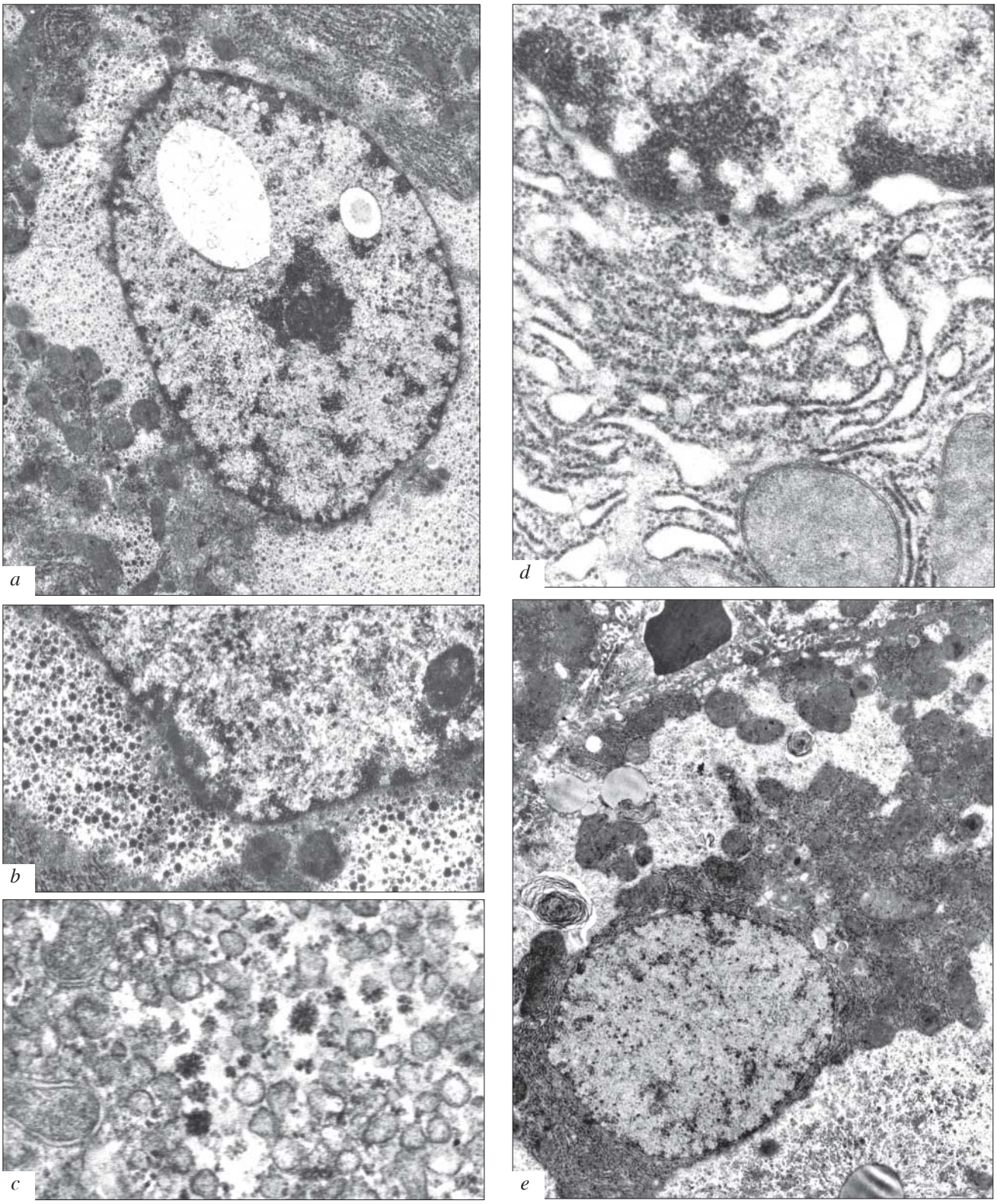

Fig. 1. Rat hepatocyte ultrastructure after a single injection of doxorubicin. *a*) segregation of nucleolar fibrillar and granular compartments in hepatocyte nucleus with massive glycogen deposition 1 day after injection, ×5000; *b*) annular nucleolus 1 day postinjection, ×8000; *c*) hyperplasia of agranular endoplasmic reticulum 1 day postinjection, ×20,000; *d*) significant dilatation of granular endoplasmic reticulum cisterns after 5 days, ×20,000; *e*) perinuclear location of organelles in a hepatocyte with massive glycogen deposition after 7 days, ×4000.

Three days after doxorubicin injection, the nuclei shifted to the biliary area in many hepatocytes; this shift reflected damage to hepatocyte cytoskeleton characteristic of anthracycline antibiotics [3]. Lythic processes were intensified in virtually all hepatocytes. Total swelling of mitochondria, ruptures of the external membrane, focal lysis of the matrix, and myelin-like transformation were observed in some cells. Degranulation and dilatation of GER cisterns, development of diffuse lysis of glycogen with the formation of small and large focal sequestra, sometimes separated by a fine membrane, were seen in hepatocytes with ultrastructure decomposition. Autophagocytosis processes were intensified; myelin-like structures and giant autophagosomes released into Disse's space appeared at the sinusoidal pole. Hyperplasia of the sinusoidal pole microvilli and activation of hepatocyte exo- and endocytosis were noted (Fig. 2, *b*). The majority of bile capillaries were dilated and contained floccular substance and osmiophilic structures; hyperplastic Golgi complex, secretory granules, primary lysosomes (Fig. 3, *a*), and numerous secondary lysosomes with lipofuscin granules were located peribiliarly.

Starting from day 5, destruction and lysis of organelles increased and these changes persisted until the end of the experiment. Glycogen lysis in the cytoplasm of hepatocytes with pronounced discomposition of organelles resulted in the appearance of extensive electron-transparent spaces, in which destructured mitochondria, solitary AER vesicles, and few heaps of GER vesicles with local dilatations were seen. Significant dilatation of GER cisterns and perinuclear intermembrane spaces were seen in hepatocytes with the orthodox phenotype (Fig. 1, *d*). Hepatocyte nuclei formed significant invagination of the caryolemma, fan-like nuclei appeared. Hyperplastic Golgi complex was located in the perinuclear zone; secretory granules, primary lysosomes and peroxisomes were located near it (Fig. 3, *b*). The ultrastructure of the biliary pole did not change much in comparison with the previous period; enlarged *zonula occludens* was retained (Fig. 2, *c*). In parallel with this, normal ultrastructure and topography of organelles were observed in some hepatocytes, with numerous polysomes, bound and free ribosomes in the cytoplasm, indicating intracellular regeneration. A sharp reduction in the number of hepatocytes with predominating AER was noted starting from this period.

By day 7 of the experiment, the hepatocyte population was presented mainly by cells with massive glycogen deposition and organelle decomposition (Fig. 1, *e*). It is noteworthy that by this term the main organelles in these hepatocytes were located around the nuclei. Pronounced lysis of glycogen was observed in zones of its concentration, with the formation of large electron-transparent fields and sequestra. Lipid infiltration of the majority of hepatocytes manifested starting from this term of the experiment; lipid droplets varied in size, being sometimes comparable to the size of the nuclei. This infiltration was caused by damage to the mitochondrial compartment resultant from more intensive generation of free radicals under the effect of intoxication [6,11]. "Fusion" of lipid droplets with the mitochondria and osmiophilic transformation of the mitochondria were observed. A characteristic feature of this period was radial location of small glycogen granules around lipid droplets (Fig. 3, *c*). Autophagocytosis processes were activated in some hepatocytes in the presence of significant mitochondrial destruction (total lysis of the matrix, ruptures of external membrane, vesicular dilatation of cristae). It is noteworthy that destructive changes in the mitochondria were most pronounced in hepatocytes in the hyperplastic AER.

These changes persisted by day 14. Significant hyperplasia of Golgi complex is worthy of note for this period. Elements of the complex were located not only at the biliary, but also at the sinusoidal pole. Lipid infiltration of hepatocytes persisted. Giant myelin-like concentric structures appeared in the hepatocyte cytoplasm starting from this period (Fig. 3, *d*). More pronounced remodeling of the hepatocyte sinusoidal pole and Disse's space was noted: the number of microvilli increased, plasmalemma invaginations formed, with bundles of collagen fibers. Hepatocytes with manifest processes of intracellular regeneration and active protein synthesis (significantly increased number of polysomes, well-developed GER) were often seen.

By days 21-30 the number of hepatocytes with modified topography of ultrastructures (massive deposition of glycogen and organelle decomposition) increased; pronounced lysis of glycogen with the formation of extensive areas of sequestration (Fig. 2, *d*) and giant concentric myelin-like structures were observed. Hepatocyte nuclei contained mainly euchromatin and, as a rule, large looped nucleoli with segregated fibrillar and granular components (Fig. 3, *e*); dislocation of the nuclei persisted. Focal destruction of the mitochondria was noted in some hepatocytes, their giant forms appeared. The size and number of lipid droplets increased, radial location of glycogen around lipids was retained; membrane transformation of lipids with formation of giant myelin-like structures progressed. In some cases, giant lipid droplets com-

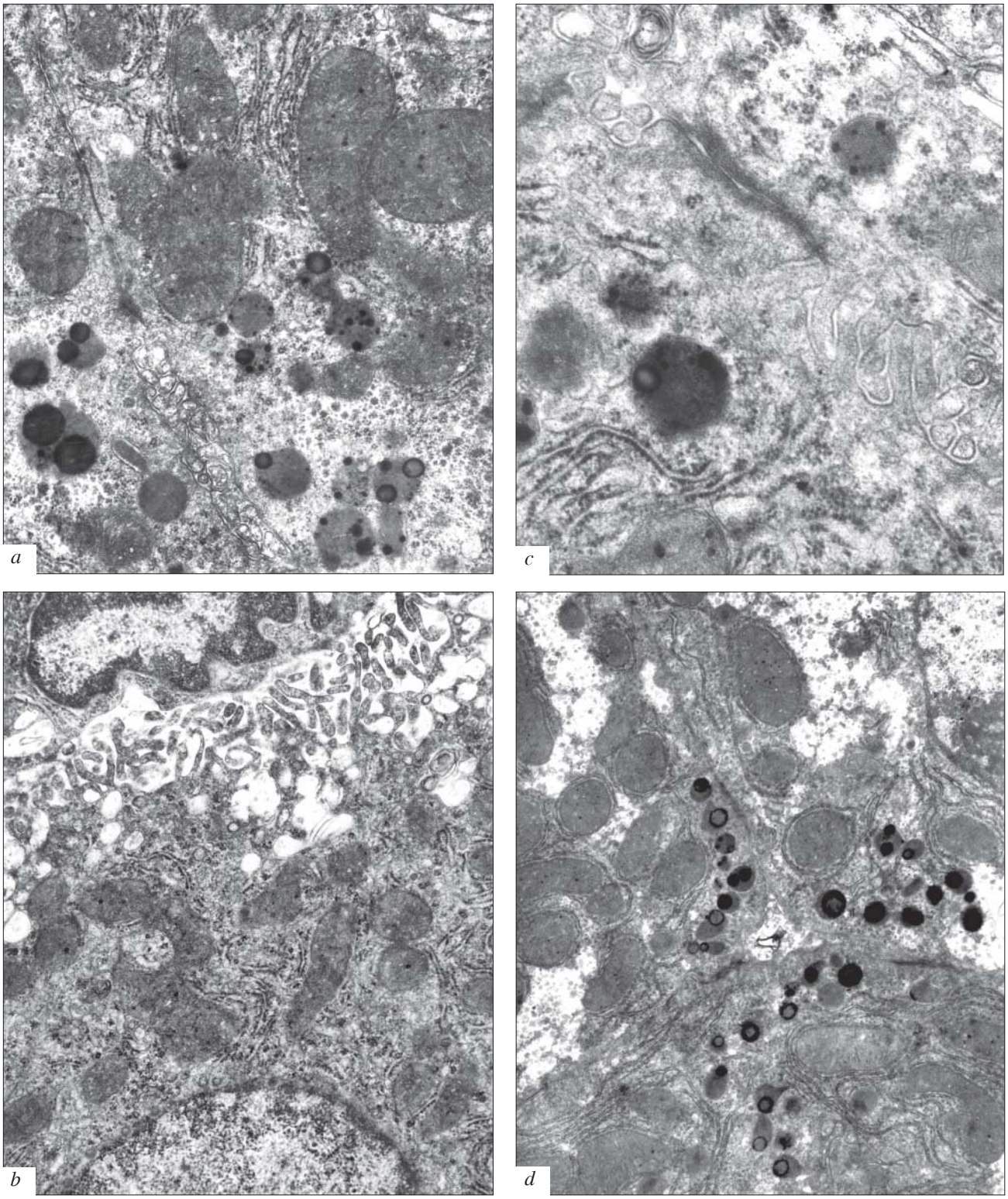

Fig. 2. Ultrastructural changes in the sinusoidal and biliary poles of the rat hepatocyte after a single injection of doxorubicin. *a*) secondary lysosomes with lipofuscin incorporation at the biliary pole and extension of the *zonula occludens* 1 day after injection, ×12,000; *b*) hepatocyte exo- and endocytosis activation, hyperplasia of microvilli at the sinusoidal pole after 3 days, ×8000; *c*) increased extension of the *zonula occludens* after 5 days, ×20,000; *d*) glycogen lysis in hepatocytes with discomposition of organelles, abundant secondary lysosomes at the biliary pole after 21 days, ×6000.

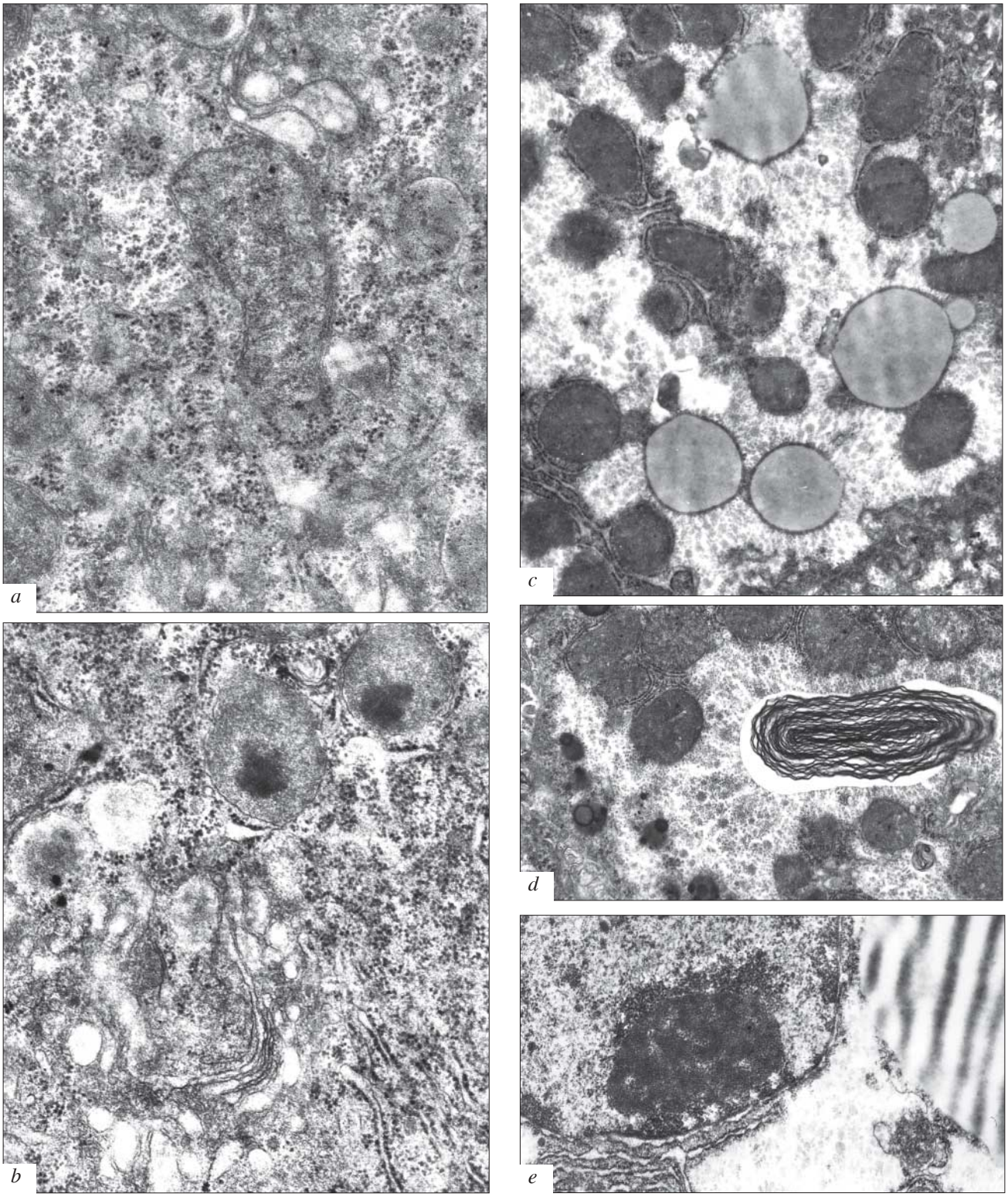

Fig. 3. Ultrastructural changes in the rat hepatocyte nucleus and cytoplasm after a single injection of doxorubicin. *a*) dilatation of terminal portions of Golgi complex 3 days after injection, ×20,000; *b*) hyperplastic Golgi complex, secretory granules and peroxisomes after 5 days, ×20,000; *c*) lipid droplets with glycogen granules located radially around them after 7 days, ×10,000; *d*) large myelin-like structure after 14 days, ×8000; *e*) segregation of fibrillar and granular components of the nucleolus after 30 days, ×10,000.

TABLE 1. Results of Ultrastructural Stereological Analysis of Rat Hepatocytes after Doxorubicin Injection (*M*±*m*)

Parameter	Control	Day after doxorubicin injection						
		1	3	5	7	14	21	30
Volume density of, mm^3/cm^3								
mitochondria	177.8±31.4	187.6±6.8	243.6±14.3	275.4±34.2	204.0±19.5	200.0±21.6	175.4±21.3	186.9±46.5
AER	241.3±53.7	117.9±30.6	76.2±14.4*	70.6±33.0	89.7±48.8	157.9±49.2	220.7±72.6	91.7±22.7
GER	72.6±21.3	81.9±6.4	111.9±13.1	112.7±25.4	100.8±12.0	74.6±5.7	62.3±18.4	73.8±35.8
lipid droplets	9.1±3.2	3.0±2.1	12.3±6.9	2.4±2.3	39.7±25.8	18.6±11.2	45.5±23.3	42.9±19.1
lysosomes	9.3±4.7	7.2±5.4	5.4±4.5	5.1±3.4	4.2±2.8	20.1±9.4*	28.7±22.6**	4.2±3.0
cytoplasm	489.9±103.4	602.4±25.7	550.6±18.7	533.8±74.6	561.6±52.0	528.8±24.0	467.4±65.5	600.5±81.7
Surface density, m^2/cm^3								
mitochondria	1.25±0.16	1.31±0.10	1.48±0.14	1.50±0.17	1.27±0.19	1.27±0.09	1.09±0.11	1.11±0.33
GER	2.19±0.50	2.51±0.21	3.93±0.20*	3.54±0.67	3.90±0.23*	2.72±0.11	2.09±0.69	2.58±0.55
Surface/volume ratio of, m^2/cm^3								
mitochondria	7.13±0.32	7.07±0.82	6.10±0.61	5.47±0.24*	6.20±0.44	6.40±0.38	6.27±0.38	5.85±0.25
GER	31.63±3.64	30.83±2.70	35.63±2.37	32.30±2.23	39.23±2.20	37.07±3.90	35.00±11.02	40.90±12.44
Volume ratio of organelles								
GER/AER	0.31±0.06	0.89±0.38	1.62±0.41*	2.12±0.56*	3.44±2.62	0.58±0.18	0.32±0.07	0.96±0.63
GER/mitochondria	0.39±0.05	0.44±0.04	0.47±0.07	0.43±0.13	0.49±0.04	0.38±0.03	0.34±0.06	0.37±0.10
AER/mitochondria	1.35±0.17	0.63±0.17*	0.31±0.04*	0.25±0.09*	0.49±0.29	0.87±0.38	1.21±0.36	0.56±0.26

Note. *$p<0.05$, **$p<0.01$ compared to the control.

pressed and squeezed the nuclei to the periphery. Abundant secondary lysosomes with lipofuscin granules were detected peribiliarly; osmiophilic myelin-like structures were seen in the lumen of some bile capillaries (Fig. 2, *d*).

Ultrastructural stereological analysis of hepatocytes after a single dose of doxorubicin detected a trend to an increase in the volume density of mitochondria on days 1-14 of the experiment (Table 1). Mitochondrial surface/volume ratio decreased by 11.5% by day 5 ($p<0.05$), indicating an increase in the size of these organelles. Volume densities of GER and AER changed most significantly: AER volume density decreased significantly from day 3 to day 7 (by 63-70%), while GER volume density increased (by 39-55%) during the same period. Starting from day 7, the volume density of lipid droplets increased significantly (4-5-fold); volume density of lysosomes increased significantly (2.2-5 times) on days 14-21 of the experiment. A characteristic feature of intracellular reorganization of hepatocytes was retention of the GER/mitochondria volume ratio and a significant increase of GER/AER volume ratio (5-11-fold 5-7 days after injection of doxorubicin). These changes can indicate a transitory reduction of the detoxifying function in the hepatocyte population (mainly at the expense of elimination of cells with well-developed AER) and predominance of protein production.

Hence, cells with modified topography of ultrastructures and massive deposition of glycogen predominate in doxorubicin intoxication of the liver, while hepatocytes with well-developed AER are scanty. At the ultrastructural level the cytotoxic effect of doxorubicin on hepatocytes manifests in segregation and annular reorganization of the nucleoli, destructive changes in the mitochondrial compartment, lipid infiltration of the cytoplasm, dilatation of cisterns of granular endoplasmic reticulum, and intensification of autophagocytosis. Pronounced hyperplasia of the Golgi complex and remodeling of the biliary and sinusoidal poles reflecting exo- and endocytosis activation can be regarded as a compensatory adaptive reorganization of hepatocytes aimed at transcytosis stimulation and elimination of secreted products [5]. An important feature of hepatocyte intracellular reorganization is increased GER/AER volume ratio indicating transitory reduction of the detoxification processes and predominance of protein synthesis in the hepatocyte population.

REFERENCES

1. A. F. Blyuger, V. K. Zaltsmane, and O. Ya. Kartashova, *Ultrastructural Pathology of the Liver* [in Russian], Riga (1989).
2. E. D. Gol'dberg, T. I. Fomina, T. V. Vetoshkina, *et al.*, *Byull. Eksp. Biol. Med.*, **126**, No. 11, 561-565 (1998).
3. E. L. Lushnikova, L. M. Nepomnyashchikh, M. G. Klinnikova, and O. P. Molodykh, *Morfologiya*, **128**, No. 4, 81-84 (2005).
4. O. P. Molodykh, E. L. Lushnikova, M. G. Klinnikova, and L. M. Nepomnyashchikh, *Byull. Eksp. Biol. Med.*, **141**, No. 5, 579-585 (2006).
5. E. S. Snigirevskaya, Yu. Ya. Sokolova, and Ya. Yu. Komissarchik, *Tsitologiya*, **48**, No. 4, 283-307 (2006).
6. A. P. Shiryaeva, E. V. Baidyuk, A. V. Arkadyeva, *et al.*, *Ibid.*, **49**, No. 2, 125-132 (2007).
7. S. Chitturi and J. George, *Semin. Liver Dis.*, **22**, No. 2, 169-183 (2002).
8. L. D. DeLeve, H. M. Shulman, and G. B. McDonald, *Ibid.*, **22**, No. 1, 27-42 (2002).
9. S. N. Hilmer, V. C. Cogger, M. Muller, and D. G. Le Couteur, *Drug Metab. Dispos.*, **32**, No. 8, 794-799 (2004).
10. H. Jaeschke, G. J. Gores, A. I. Cederbaum, *et al.*, *Toxicol. Sci.*, **65**, No. 2, 166-176 (2002).
11. W. M. Lee, *N. Engl. J. Med.*, **349**, No. 5, 474-485 (2003).
12. V. J. Navarro and J. R. Senior, *Ibid.*, **354**, No. 7, 731-739 (2006).
13. G. Minotti, M. Parlani, E. Salvatorelli, *et al.*, *Brit. J. Pharmacol.*, **134**, No. 6, 1271-1278 (2001).
14. B. K. Park, N. R. Kitteringham, J. L. Maggs, *et al.*, *Annu. Rev. Pharmacol. Toxicol.*, **45**, 177-202 (2005).