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Ultrastructurural Picture of Cyclophosphamide-Induced Damage to the Liver

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We studied the ultrastructural reorganization of hepatocytes and other populations of liver cells after cyclophosphamide treatment. Single administration of cyclophosphamide was followed by significant ultrastructural changes in two major populations of liver cells (hepatocytes and sinusoidal endothelial cells). This treatment was also accompanied by reactive changes in Kupffer cells, lymphocytes, and plasma cells migrating into the spaces of Disse. Cyclophosphamide-induced spatial reorganization of hepatocytes was associated with a progressive decrease in the structural density of mitochondria, significant reduction of the smooth endoplasmic reticulum, and increase in autophagocytosis (*e.g.*, sequestration of glycogen). Intracellular reorganization of endothelial cells was related to the following two factors: damage (necrobiosis) of some cells; and increase in metabolic processes, phagocytosis, and regenerative reactions in other cells.

Key Words: *hepatotoxicity; hepatic lobule; cyclophosphamide; ultrastructure; stereology*

Antitumor drug cyclophosphamide (CP) produces an alkylating effect on DNA and induces tolerance during transplantation [1,4,9,12]. Non-selective activity of CP and urotoxic, nephrotoxic, and cardiotoxic side-effects of this drug and its metabolites as well as reproductive disturbances caused by CP treatment seriously limit its use of clinical practice [2,10]. The hepatotoxic effects of antitumor products can be studied during experimental modeling of these processes [1,2,8].

Analysis of ultrastructural changes in cells, estimation of the degree of intracellular regenerative processes, and evaluation of the general and specific features of liver remodeling under these conditions are required to determine the mechanisms of CP-induced damage to hepatocytes and other populations of liver cells. This approach is necessary to develop new methods for the correction of side effects of pharmaceutical therapy.

Here we studied the ultrastructural changes and intracellular regeneration of hepatocytes and other populations of liver cells after single treatment with CP.

MATERIALS AND METHODS

CP-induced ultrastructural reorganization of the hepatic lobule was studied on 4-month-old male Wistar rats (n=20). The animals received an intraperitoneal injection of CP (Biokhimik) in a single dose of 125 mg/kg. The control group consisted of 12 rats receiving single intraperitoneal injection of physiological saline (equivalent volume). All animals of the

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treatment and control groups were maintained at room temperature and had free access to water and food. They were decapitated under ether anesthesia on days 3 and 14 after CP injection.

The liver was isolated from adjacent tissues and rapidly weighted. Liver samples were fixed in 4% paraformaldehyde, postfixed in 1% OsO_4, and processed routinely. Ultrathin sections were contrasted with uranyl acetate and lead citrate and ultrastructural and stereological analysis was performed. The study was conducted under a JEM 1400 electron microscope (Jeol). Microphotographs were made with a Veleta digital camera and iTEM software (Olympus).

An ultrastructural stereological study of hepatocytes was performed using iTEM software (initial magnification ×20,000; final magnification ×23,500). We evaluated the volume density of mitochondria, rough endoplasmic reticulum, liposomes, lysosomes, and cytoplasm (with inclusions); surface density (relative density of the surface) of mitochondria, rough endoplasmic reticulum, liposomes, and lysosomes were also determined. The secondary stereological parameters (quantitative relationships between the major components of the hepatocyte cytoplasm) were calculated from the primary stereological parameters.

The statistical analysis included the calculation of the means, variance, and errors of the means. The significance of differences was evaluated by Student's *t* test.

RESULTS

Hepatocytes of the periportal and pericentral regions of the hepatic lobule in intact rats had specific ultrastructural characteristics. The majority of periportal hepatocytes contained a considerable amount of glycogen granules. They formed so-called "fields", which were located between morphofunctional complexes of mitochondria and cisternae of the rough endoplasmic reticulum. The content of glycogen granules was lower in pericentral hepatocytes. Rough endoplasmic reticulum was well defined. Profiles of the rough endoplasmic reticulum formed typical morphofunctional complexes that were regularly distributed in the cell. Most hepatocytes in both regions had a well-developed smooth endoplasmic reticulum, whose vesicles were mainly located in the region of glycogen granules.

Single treatment with CP was followed by the development of similar ultrastructural changes in the hepatic lobule. However, the degree of these changes differed in various periods of the study. The specific features of ultrastructural organization of periportal and pericentral hepatocytes were shown to become more pronounced on day 3 after CP injection. Wide glycogen "fields" occupied a greater part of the cytoplasm in periportal hepatocytes. The mitochondria were characterized by moderate polymorphism. We revealed condensation of the matrix in some organelles and destruction of the cristae. Cisternae of the rough endoplasmic reticulum often formed sheaths around mitochondria. They were arranged in long and densely packed stacks (particularly near the nuclei). Some hepatocytes were characterized by irregular dilation and shortening of cisternae of the rough endoplasmic reticulum (Fig. 1, *a*). The volume and surface density of this structure increased moderately (Table 1). Small lipid droplets were found in the sinusoidal pole. They were characterized by irregular exhaustion and myelin-like transformation (Fig. 1, *b*). Numerous residual bodies were revealed in the sinusoidal poles and along the lateral membranes. They migrated into the spaces of Disse. The smooth endoplasmic reticulum was significantly reduced in all hepatocytes. The vesicles of this structure were nearly unidentified.

Pericentral hepatocytes contained a lower amount of glycogen. A greater part of the cytoplasm was occupied by mitochondria and numerous cisternae of the rough endoplasmic reticulum. Small lipid inclusions or vacuole-like residual bodies with flaky content were located in the subplasmalemmal region of some hepatocytes. The mitochondria of all hepatocytes had electron-dense matrix with poorly distinguishable small or dilated cristae. Destructive changes in the mitochondria contributed to a moderate decrease in the volume and surface density (Table 1).

Intracellular reorganization of hepatocytes in both regions was observed on day 3 after CP injection. It was manifested in the lysis and sequestration of glycogen granules. These changes were revealed in all cells, but differed in the degree. The intensity of these processes was maximum in periportal hepatocytes. These cells were characterized by the formation of a light band around glycogen granules, presence of small myelin-like (residual) bodies, and migration of residual bodies into the spaces of Disse (Fig. 1, *c*).

Secondary lysosomes were accumulated in the biliary poles of hepatocytes in both regions. A well-developed Golgi complex was presented by peripherally dilated dictyosomes, which contained the flaky substance.

Significant changes in the microcirculatory bed of the hepatic lobules were revealed on day 3 after CP injection. The heterogeneity of endothelial cells in sinusoidal capillaries was related to the presence of necrobiotic and activated cells in the endothelial lining. Activated endothelial cells were hypertrophic (Fig. 1, *d*) and contained a well-developed rough endoplasmic reticulum, mitochondria, numerous bordered vesicles, microtubules, individual small lipid droplets, and residual bodies with the flaky content (structurally similar to those in hepatocytes). Necro-

biotic endotheliocytes were electron transparent. Regional damage to the plasma membrane was identified. The spaces of Disse were filled by the flaky substance with submerged microvilli of hepatocytes. We revealed thickened bundles of collagen fibers, activated Kupffer cells, lymphocytes, and plasma cells. Stellate cells (lipid-containing fibroblasts) were rarely found.

The changes in some hepatocytes became more pronounced on day 14 after CP injection. The wide "fields" of glycogen were revealed in periportal hepatocytes. Heterogeneous lipid inclusions were present between these structures in some cells. A dense border of glycogen granules was formed in these regions (peripheral zone of lipid droplets). Glycogen granules were accumulated inside the lipid inclusions (Fig. 2, *a*). A fine structure of many mitochondria was strongly changed. Destruction of the cristae and condensation of the matrix were observed (Fig. 2, *b*). Some organelles were characterized by myelin-like and osmiophilic degeneration. A significant destruction of the mitochondria contributed to a decrease in the volume and surface density (by 24 and 21%, respectively, $p<0.05$; Table 1). Destruction of the mitochondria, sequestration of glycogen, and intensification of autophagocytosis in some hepatocytes were accompanied by the appearance of numerous sites of cytoplasmic degradation (partial necrosis; Fig. 2, *c*).

Numerous structures of the Golgi complex were identified in hepatocytes during this period of the study. The peripheral area of dictyosomes in the Golgi complex was mainly dilated and contained the flaky substance. Numerous bordered vesicles were well defined in the vesicular part of the Golgi complex. These signs illustrate the intensification of synthetic processes. Large agglomerates of secondary lysosomes were concentrated in the biliary pole of hepatocytes. The volume and surface density of these structures increased significantly (by 327 and 250%, respectively; Table 1).

The spaces of Disse were mainly narrowed. Small thick bundles of collagen fibers and residual bodies were found in these spaces. Sinusoidal endothelial cells were presented by activated cells. They formed the cytoplasmic processes and phagocytized the residual bodies that were excreted from hepatocytes. Hence, these cells were similar to macrophages. Total destruction of the endothelial lining in sinusoidal capillaries was related to death (degeneration) of endothelial cells and activation of Kupffer cells and neutrophils. Phagocytosis was typical of not only the cellular detritus and residual bodies, but also of large apoptotic bodies. By ultrastructural characteristics, they could be classified to dead hepatocytes. A specific feature of CP-induced damage to the liver in this period was the massive spread obstruction of sinusoidal

TABLE 1. Stereological Study of Hepatocytes from Wistar Rats after Single Treatment with CP (*M*±*m*)

Parameter		Control	Period	
			3 days	14 days
Volume density (mm^3/cm^3)	mitochondria	305.8±27.4	293.1±16.6	232.2±15.5*
	rough endoplasmic reticulum	68.9±4.8	80.2±18.1	70.0±12.4
	lipid droplets	18.2±10.2	3.3±2.1	15.1±12.9
	lysosomes	1.5±0.4	1.9±0.5	6.4±5.4
	cytoplasmic matrix	612.8±38.7	621.5±13.8	676.3±13.4
Surface density (m^2/cm^3)	mitochondria	1.857±0.168	1.779±0.114	1.474±0.050*
	rough endoplasmic reticulum	2.600±0.292	2.815±0.356	2.697±0.328
	lipid droplets	0.057±0.031	0.015±0.006	0.062±0.059
	lysosomes	0.020±0.006	0.020±0.006	0.070±0.056
Surface/volume ratio (m^2/cm^3)	mitochondria	6.1±0.1	6.1±0.5	6.4±0.5
	rough endoplasmic reticulum	39.0±6.8	37.1±2.9	39.5±2.8
	lipid droplets	2.3±0.8	4.5±2.3	4.0±1.3
	lysosomes	13.2±1.1	10.3±1.3	11.4±1.2
Volume ratio (number) of the rough endoplasmic reticulum to mitochondria		0.227±0.010	0.282±0.074	0.301±0.051

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

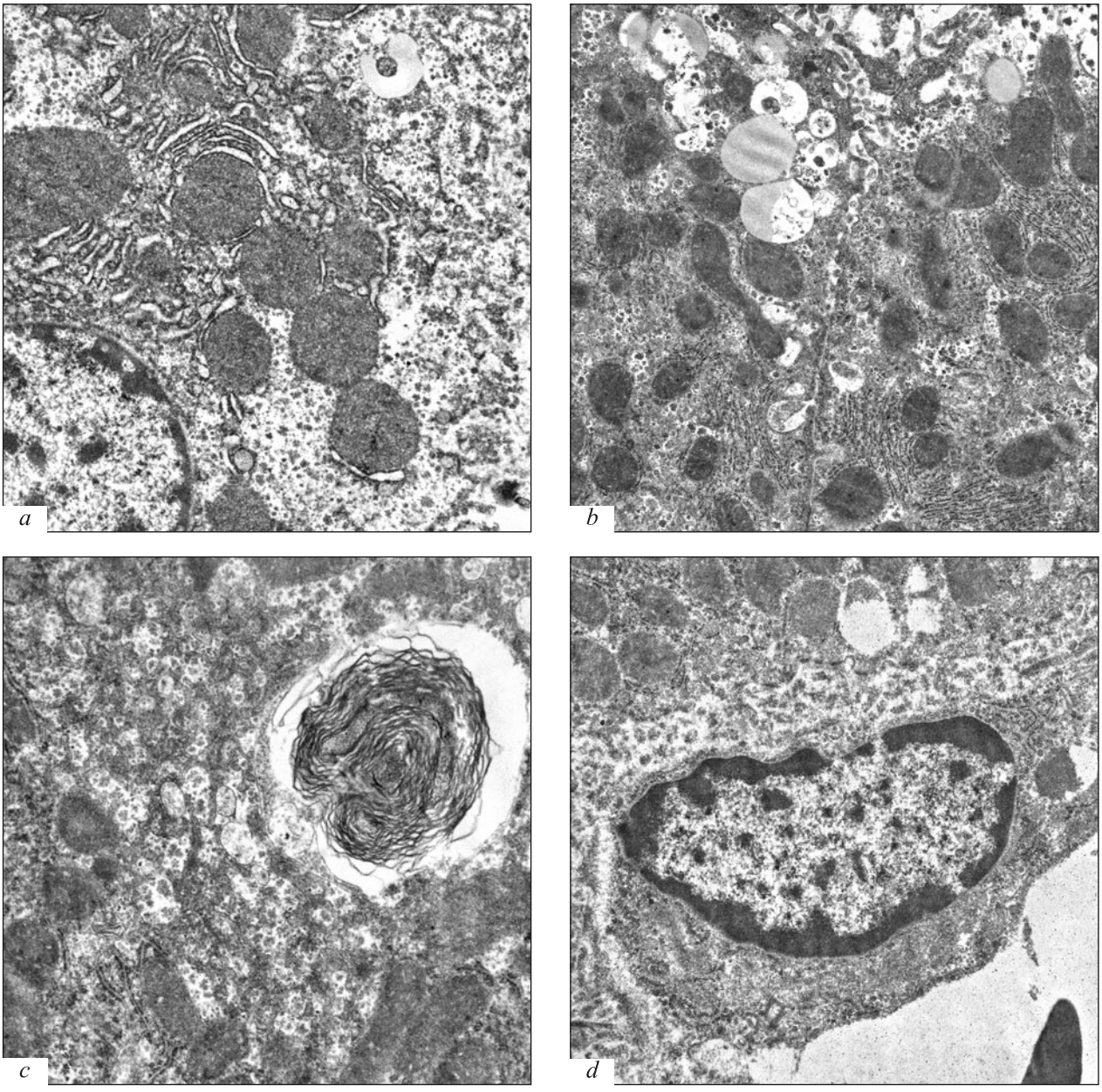

Fig. 1. Ultrastructural changes in rat hepatocytes 3 days after single treatment with CP. Dilation of profiles of the rough endoplasmic reticulum (×12,000, *a*); accumulation of lipid droplets and residual bodies in the sinusoidal pole and condensation of the mitochondrial matrix (×12,000, *b*); focal lysis of glycogen and formation of the myelin-like residual body (×20,000, *c*); active endothelial cell (×15,000, *d*).

capillaries by Kupffer cells, lymphocytes, and cellular detritus (Fig. 2, *d*).

Activated stellate cells were found in ruptures of the endothelial lining and formed contacts with hepatocyte microvilli and endothelium. The rough endoplasmic reticulum was well developed in the cytoplasm of stellate cells. Lipid inclusions were revealed. The degree of peripolesis was high (incorporation of lymphocytes between hepatocytes and damage to the intercellular contacts).

Our results indicate that CP causes significant ultrastructural changes in two major populations of liver cells, hepatocytes and sinusoidal endothelial cells. This treatment is also followed by the development of reactive changes in Kupffer cells, lymphocytes, and plasma cells that migrate into the spaces of Disse. The major structural changes in hepatocytes are related to pronounced changes in the fine structure of mitochondria, transitory changes in the rough endoplasmic reticulum, significant reduction of the smooth endoplasmic reticulum, and high-intensity autophagocytosis with the formation of numerous subplasmalemmal residual bodies and sequestration of glycogen. Hepatocytes were characterized by stimulation of

regenerative reactions and activation of transcytosis (hyperplasia and hypertrophy of structural elements in the Golgi complex). These signs reflect a complex action of CP and its metabolites on metabolic processes in hepatocytes. CP-induced spatial reorganization of hepatocytes was manifested in a decrease in the structural density of mitochondria and transitory change in the structural density of the rough endoplasmic reticulum. The same transitory destructive changes in mitochondria and rough endoplasmic reticulum were observed in Wistar rats after administration of CP in a single dose of 150 mg/kg [11].

The population of endothelial cells was presented by the following two phenotypes of cells: activated and necrobiotic (degenerated) cells. CP-induced intracellular reorganization of activated (intact) endothelial cells was associated with the intensification of metabolic processes and phagocytosis, induction of regenerative processes, and substitution of dead cells for newly formed endothelial cells. Severe damage to the vascular bed and thromboembolism after cytostatic treatment (*e.g.*, administration of CP) are the common complications of chemotherapy [7,12]. Damage to endothelial cells occurs under the influence of CP

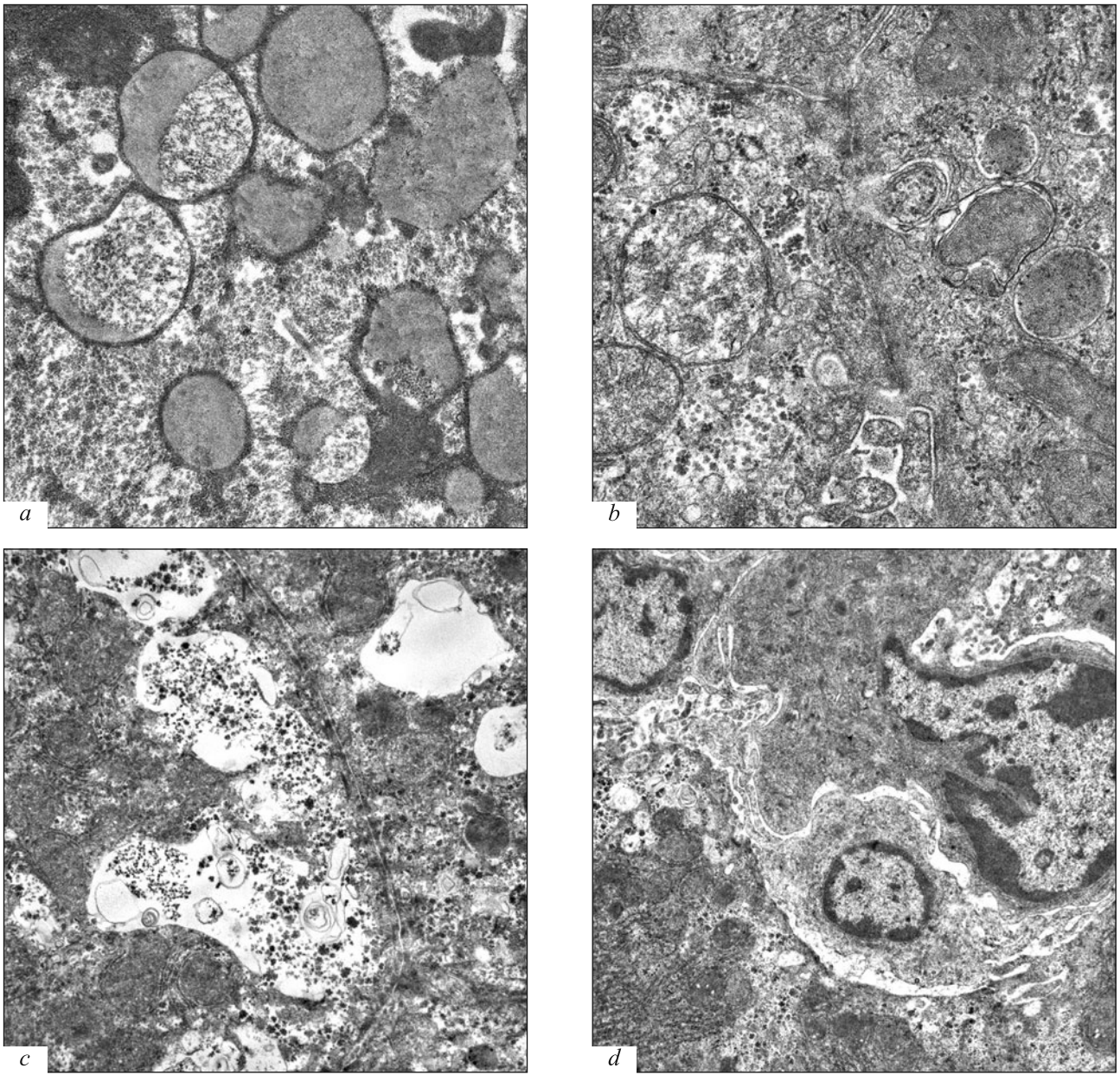

Fig. 2. Ultrastructural changes in rat hepatocytes 14 days after single treatment with CP. Heterogeneous lipid droplets with glycogen inclusions (×25,000, *a*); destructive changes in mitochondria adjacent to the biliary pole (×40,000, *b*); partial degradation of the cytoplasm in subplasmalemmal regions of hepatocytes (×15,000, *c*); obstruction of sinusoids with mononuclear cells (×10,000, *d*).

metabolites (4-hydroperoxycyclophosphamide and acrolein) in a concentration, which is 20-fold lower than that reported for hepatocytes. These substances cause the exhaustion of glutathione (by more than 95%) and induce cell death [3]. CP and its metabolites not only cause damage to sinusoidal endothelial cells [13], but also increase the procoagulant properties, decrease the anticoagulant activity of the blood, and induce venous occlusion [5].

The dynamics of damage to the major populations of liver cells is similar after treatment with CP in high and low doses. These changes are mainly transitory. Some authors reported that the ultrastructure of hepatocytes is recovered in the earlier period than that of stromal cells [6]. The doses and regimens of treatment with CP and other cytostatics should be optimized to minimize the toxic effect, activate the cytoprotective mechanisms, and decrease the risk of lethal complications.

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