Morphogenesis and Histostereological Analysis of Hepatopathy Induced by Cyclophosphamide

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 149, No. 1, pp. 113-120, January, 2010
Original article submitted October 12, 2009

Characteristics and regularities of structural reorganization of the liver in experimental cyclophosphamide hepatopathy were studied. Single injection of the drug caused pronounced morphofunctional changes in the liver (small focal hepatocyte necroses mainly in the periportal zone, mononuclear cell infiltration developing in the presence of circulatory disorders). The key event in the spatial reorganization of the liver after cyclophosphamide injection is reduction of hepatocyte volume density, increase of their surface density and surface/volume ratio. The percentage of binuclear hepatocytes increases in the liver early after cyclophosphamide injection; later the percentage of mononuclear cells increases. Other important changes in the spatial reorganization of the liver under the effect of cyclophosphamide are the increase in the volume and surface densities of sinusoids. These changes develop as a result of augmenting vascular plethora and blood cell stasis with the development of thrombosis during later periods.

Key Words: *liver; hepatotoxicity; cyclophosphamide; morphometry; stereology*

Toxic lesions in the liver develop most often after chemotherapy and can reflect the hepatotoxic effects of drugs used in oncology [11]. On the other hand, evaluation of the possible hepatotoxic effects of this or that drug in patients should take into consideration the data on the initial status of the organ, development of diseases of any kind, presence of viral hepatitis and other infections, alimentary deficiency, *etc.* [4]. The most ample and reliable information on the hepatotoxic effects of antitumor drugs can be obtained on intact experimental animals.

Cyclophosphamide (CP), a DNA-alkylating agent is widely used in antitumor therapy [1,13] and for induction of tolerance in transplantation [15]. After injection, CP is metabolized in the liver with the formation of 7 major metabolites, of which aldophosphamide, nitric yperite, and yperite phosphamide are characterized by the cytotoxic effect. The resultant metabolites inhibit the growth of rapidly proliferating (primarily tumorous) cells. The absence of selective action and the uro-, nephro-, and cardiotoxicities of CP metabolites often become a serious problem in the clinical use of this drug [3].

The hepatotoxic effects of CP remain an object of discussions. Some scientists think that the liver is least sensitive to toxicity of CP metabolites [8], while others claim that this organ is highly sensitive to the cytotoxic effect of CP [2]. The conclusions on CP hepatotoxicity are based mainly on the results of experiments with repeated low-dose (up to 20 mg/kg) or single high-dose (150 mg/kg) injections of CP [7,8,14]. The morphogenetic processes induced by CP in the liver, the severity of cytotoxic effects towards hepatocytes and other cell populations, and the intensity of regeneratory reactions remain the least studied problems.

We studied morphological changes and tissue reorganization of the liver after a single injection of CP.

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0007-4888/10/1491-0104

MATERIALS AND METHODS

Structural reorganization of the liver after CP injection was analyzed in 4-month-old male Wistar rats (n=20). The animals received with CP (Biokhimik) intraperitoneally in a single dose of 125 mg/kg. Controls (n=12) were injected with saline in the volume corresponding to their body weight simultaneously with experimental rats. The animals were kept at ambient temperature with free access to water and food and were decapitated under ether narcosis 3 and 14 days after CP injection.

The liver was separated from the adjacent tissues and rapidly weighed. Liver specimens were fixed in 10% neutral formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin and PAS reaction was carried out. In order to prepare semithin sections, liver fragments were fixed in 4% paraformaldehyde, postfixed in 1% osmium tetroxide, and processed routinely. Semithin sections were stained with 1% azur II and used for morphological and stereological analysis under a Leica DM 4000B universal microscope. Microphotographs were made using Leica DFC 320 digital photocamera and Leica QWin V3 software.

Stereological analysis was carried out using common methods [6]. The following parameters were evaluated: volume density (volume percentage) of hepatocytes, hepatocyte nuclei, sinusoids, connective tissue cells and connective tissue main substance, and the surface density (surface percentage) of hepatocytes, hepatocyte nuclei, sinusoids, and connective tissue cells. Based on the primary stereological parameters, the secondary parameters were calculated, describing the quantitative interrelationships between various components of the stroma and parenchyma: surface/volume ratio of the structures, stroma/parenchyma volume ratio, nucleus/cytoplasm ratio, sinusoid/hepatocyte volume ratio.

Statistical processing of the results included evaluation of the means, calculation of dispersion and errors in the means. The significance of differences was evaluated by Student's t test.

RESULTS

The mortality of experimental animals after single injection of CP was 25%. Common toxic effects of CP manifested in body weight loss (by 19 and 12% after 3 and 14 days, respectively, p<0.05; Table 1). Liver weight decreased by 17% after 3 days and returned to normal by day 14.

Tissue changes in the liver after a single injection of CP were stereotypical, with different intensity for each period. Three days after CP injection, the architectonics of the liver virtually did not differ from that of controls: the liver cord structure was intact. On the other hand, hemodynamic disorders were observed in all animals: unevenly plethoric sinusoids, central and portal veins (Fig. 1, *a*). A significant dilatation of the sinusoidal lumen was seen in some cases. The sinusoids contained numerous mononuclear cells (mainly Kupffer cells). The hepatotoxic effects of CP included the emergence of hepatocytes in a state of necrobiosis in the periportal zone (Fig. 1, *b*) and pronounced subplasmalemmal "vacuolation" of the hepatocyte cytoplasm (Fig. 1, *c*). Accumulation of lipid droplets on the sinusoidal poles was seen in many hepatocytes (Fig. 1, *d*). Kupffer cells accumulated around necrobiotic hepatocytes. Regeneratory reactions of the hepatocytes manifested in their more intense mitotic activity, particularly in the periportal zone (Fig. 1, *e*). The counts of binuclear hepatocytes also increased in these zones.

Fourteen days after CP injection, the hemodynamic disorders in the liver persisted. Venous and sinusoidal plethora were observed (Fig. 2, *a*), as a result of which the liver somewhere looked like a honeycomb. In some cases, the sinusoidal lumen was blocked with numerous hypertrophic cells (Kupffer cells, segmented leukocytes, erythrocytes; Fig. 2, *b*). Pronounced polymorphism of erythrocytes and modification of their shape are worthy of note; these shifts could be caused by the toxic effect of CP. Significant "vacuolation" of the cytoplasm persisted in many hepatocytes (Fig. 2, *c*). Numerous small sclerotic foci formed in the liver parenchyma in sites of hepatocyte death (Fig. 2, *d*). The hepatocytes exhibited virtually no mitotic activity. Solitary hepatocytes with pronounced marginal chromatin condensation and destroyed cytoplasm were seen in the periportal zone. These cells could be classified as apoptotic (Fig. 2, *e*). Pronounced portal sclerosis (Fig. 2, *f*) developed by this day of experiment and protruded (in the form of cords) into the adjacent parenchyma. The portal tract stroma was infiltrated with mononuclear cells, so that the infiltration formed extensive massive cuff-like accumulations along the portal tracts.

Spatial reorganization of the liver periportal and pericentral zones 3 days after CP injection manifested in reduction of the hepatocyte volume density (by 12% in both zones; Tables 1, 2). The hepatocyte surface density increased (by 10-11%) in both zones, this causing an increase of the parenchymatous cell surface/volume ratio (by 23-26%). These changes indirectly indicated a reduction of hepatocyte size during this period. The hepatocyte nucleus/cytoplasm ratio increased in both zones (by 15-27%), reflecting an increase in the count of binuclear hepatocytes (by 26% in the periportal and by 32% in the pericentral zones) and intensification of the biosynthetic processes in hepatocyte nuclei, their

TABLE 1. Body Weight and Stereological Analysis of the Liver Periportal Zone in Wistar Rats Injected with CP (*M*±*m*)

Parameter		Control	Day after CP injection	
			3	14
Body weight, g		235.50±9.46	191.70±6.72*	206.40±16.33*
Liver weight, g		11.057±0.586	9.210±0.349*	11.458±0.968
Liver weight, mg/g body weight		46.75±1.34	48.07±0.77	55.62±1.96*
Hepatocyte %	mononuclear	90.3±1.8	89.0±1.8	93.7±1.2
	binuclear	8.7±1.8	11.0±1.8	6.3±1.2
Volume density (mm^3/cm^3) of	hepatocytes	676.7±103.7	595.0±25.3	640.0±15.3
	hepatocyte nuclei	51.7±3.1	55.0±5.0	50.000±0.001
	sinusoidal capillaries	173.3±21.4	197.5±16.5	193.3±6.7
	connective tissue cells	21.7±4.8	20.000±0.001	30.0±5.7*
	connective tissue fibers and main substance	128.3±20.9	187.5±10.3*	136.7±12.0
Surface density (m^2/cm^3)	hepatocytes	0.138±0.006	0.153±0.008	0.163±0.003*
	hepatocyte nuclei	0.037±0.003	0.040±0.004	0.037±0.003
	sinusoidal capillaries	0.118±0.011	0.138±0.009	0.140±0.025*
	connective tissue cells	0.025±0.005	0.023±0.003	0.037±0.007*
Surface/volume ratio (m^2/cm^3)	hepatocytes	0.210±0.021	0.258±0.013*	0.253±0.007*
	hepatocyte nuclei	0.708±0.041	0.730±0.038	0.733±0.067
	sinusoidal capillaries	0.707±0.054	0.710±0.067	0.717±0.044
	connective tissue cells	1.222±0.165	1.125±0.125	1.267±0.145
	sinusoidal capillaries/hepatocytes	0.185±0.029	0.233±0.024*	0.217±0.007*
Volume ratio (number)	nucleus/cytoplasm	0.085±0.013	0.108±0.013*	0.083±0.003*
	sinusoidal capillaries/hepatocytes	0.273±0.051	0.338±0.042*	0.303±0.015
	stroma/parenchyma	0.512±0.105	0.693±0.077*	0.567±0.037

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

possible polyploidy.

Three days after CP injection the sinusoidal volume density increased by 14-15% in both zones, surface density of the sinusoids increasing greater (by 17%) in the periportal zone (Tables 1, 2). Shifts of different direction in the volume densities of hepatocytes and sinusoids resulted in a significant increase of the sinusoid/hepatocyte volume ratio (by 24 and 29%, respectively, in the periportal and pericentral zones). The sinusoid/hepatocyte surface/volume ratio increased by 26% in the periportal and by 20% in the pericentral zone.

Quantitative characteristics of the connective tissue cells and main substance changed significantly. The volume density of connective tissue cells (with macrophages predominating) increased significantly (by 71%) in the pericentral zone, the volume density of the main substance increasing in both zones (by 46 and 38% in the periportal and pericentral zones, respectively). These changes and changes in the quantitative characteristics of the sinusoids resulted in an increase of the stroma/parenchyma volume ratio, most marked in the pericentral zone (by 42% *vs.* 22% in the periportal zone).

Fourteen days after CP injection the hepatocyte volume density was restored, while their surface density and surface/volume ratio increased, which could be due to emergence of the "small" hepatocyte population (Tables 1, 2). The nucleus/cytoplasm ratio normalized by this period of experiment. The percentage of binuclear hepatocytes decreased in both zones (by 28 and 49% in the periportal and pericentral zones, re-

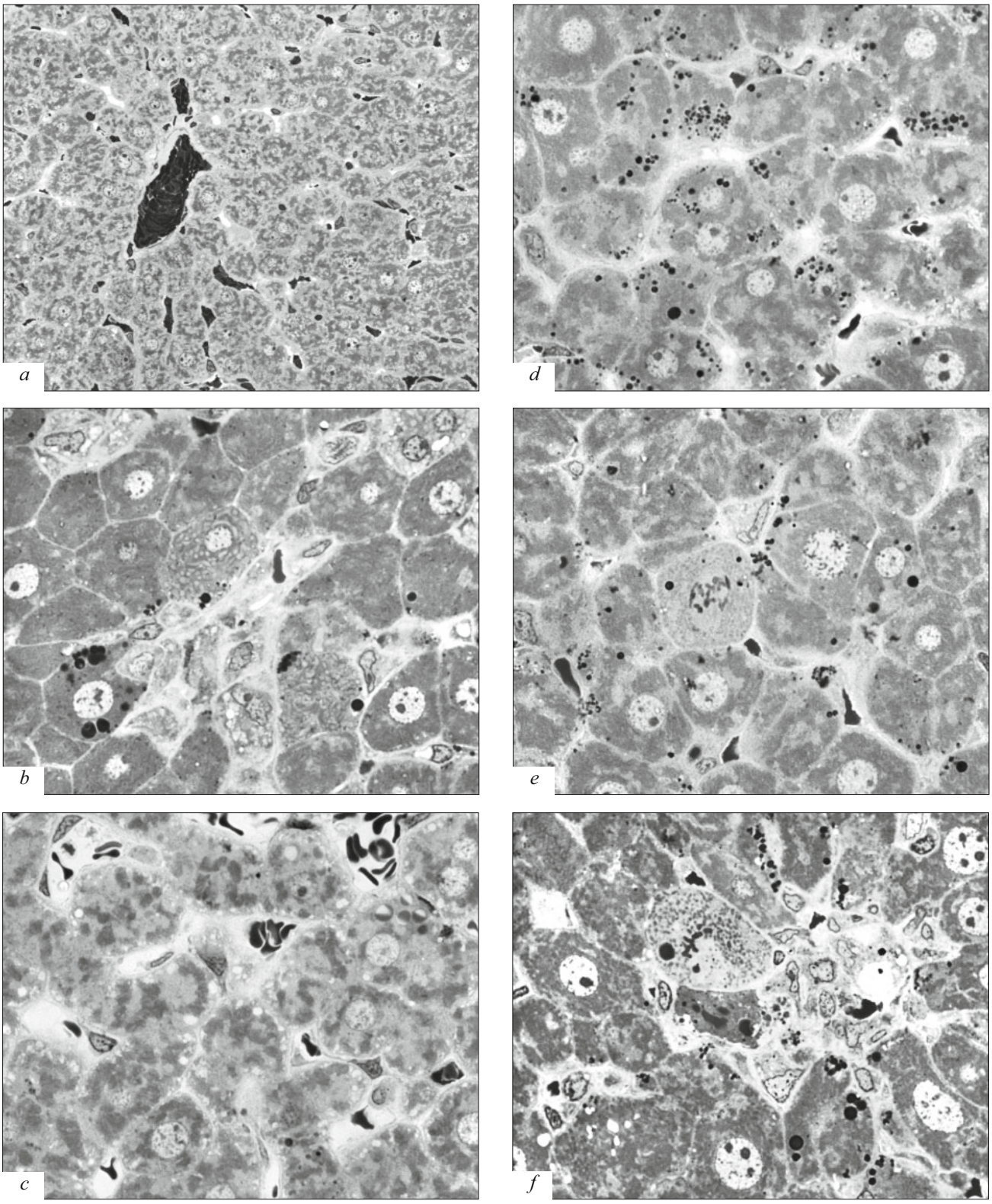

Fig. 1. Morphological changes in the liver of rats 3 days after a single injection of CP. *a*) plethoric central vein and sinusoids; *b*) hepatocyte necrobiosis in the periportal zone; *c*) subplasmalemmal "vacuolation" of the cytoplasm; *d*) lipid droplets in the sinusoidal hepatocyte poles; *e*) hepatocyte prophase and metaphase/anaphase; *f*) hepatocyte metaphase in the periportal zone. Here and in Fig. 2: Azur II staining, ×400 (*a*), ×1000 (*b-f*).

spectively). The quantitative reduction of the binuclear hepatocyte population could reflect the cytokinesis intensification with increase of the "small" mononuclear hepatocyte population. These changes were presumably aimed at restoration of the total hepatocyte count in the liver.

By day 14 after the treatment the sinusoidal volume densities remained high (12 and 20% increased in the periportal and pericentral zones, respectively). The sinusoidal surface density remained elevated (by 19%; $p<0.05$) only in the periportal zone. The sinusoid/hepatocyte volume ratio remained high in both zones (11 and 19% elevated, respectively), the sinusoid/hepatocyte surface/volume ratios were 17% increased in both zones.

The connective tissue cell volume density increased still higher during this period of experiment, particularly in the pericentral zone (by 127% *vs.* 38% in the periportal zone). The volume density of the connective tissue main substance did not change much. These changes in the stromal components led to an increase (by 11%) of the stroma/parenchyma volume ratio in the periportal zone. This parameter virtually did not change in the pericentral zone in comparison with the control.

Our results indicate that CP caused significant morphofunctional changes in the liver, similar to those caused by other antitumor drugs [5], despite the reports about slight damage inflicted to the liver by high-dose CP [8]. The major structural changes in the liver are small focal necroses of the parenchyma, disseminating from the portal tracts, paralleled by hypertrophy and hyperplasia of macrophageal cells. The inflammatory cell infiltration progressed with prolongation of the experiment; this was paralleled by thrombosis development and obstruction (particularly by Kupffer cells) of the sinusoidal, central and portal vein lumen. The regeneratory reactions of hepatocytes manifested in intensification of mitoses and increase in the percentage of binuclear cells during the early periods and by an increase in the population of "small" mononuclear hepatocytes (aimed at recovery of total hepatocyte count) during later periods. It is noteworthy that necrobiotic changes in hepatocytes, similarly as

TABLE 2. Stereological Analysis of Pericentral Liver Zone in Wistar Rats after CP Injection (*M*±*m*)

Parameter		Control	Day after CP injection	
			3	14
Hepatocyte %	mononuclear	89.2±1.4	85.8±2.4	94.6±0.7*
	binuclear	10.8±1.4	14.2±2.5	5.4±0.7*
Volume density (mm^3/cm^3) of	hepatocytes	701.7±83.3	567.5±35.7	636.7±28.5
	hepatocyte nuclei	51.7±3.2	52.5±2.5	50.000±0.001
	sinusoidal capillaries	158.3±17.2	182.5±13.2	190.0±20.8
	connective tissue cells	13.2±3.4	22.5±4.8	30.0±5.8*
	connective tissue fibers and main substance	128.8±15.2	175.0±20.6*	93.3±16.7*
Surface density (m^2/cm^3)	hepatocytes	0.127±0.008	0.140±0.014	0.153±0.007*
	hepatocyte nuclei	0.035±0.002	0.0400±0.0001	0.033±0.003
	sinusoidal capillaries	0.110±0.012	0.118±0.015	0.130±0.006*
	connective tissue cells	0.017±0.004	0.018±0.003	0.030±0.006*
Surface/volume ratio (m^2/cm^3)	hepatocytes	0.185±0.021	0.233±0.038	0.223±0.015*
	hepatocyte nuclei	0.681±0.033	0.768±0.033*	0.667±0.067
	sinusoidal capillaries	0.697±0.026	0.643±0.057	0.697±0.061
	connective tissue cells	1.333±0.334	0.835±0.093	1.0000±0.0001
	sinusoidal capillaries/hepatocytes	0.163±0.028	0.195±0.038	0.190±0.012*
Volume ratio (number)	nucleus/cytoplasm	0.083±0.010	0.095±0.012	0.080±0.006
	sinusoidal capillaries/hepatocytes	0.233±0.039	0.300±0.040	0.277±0.043
	stroma/parenchyma	0.443±0.076	0.630±0.094	0.463±0.064

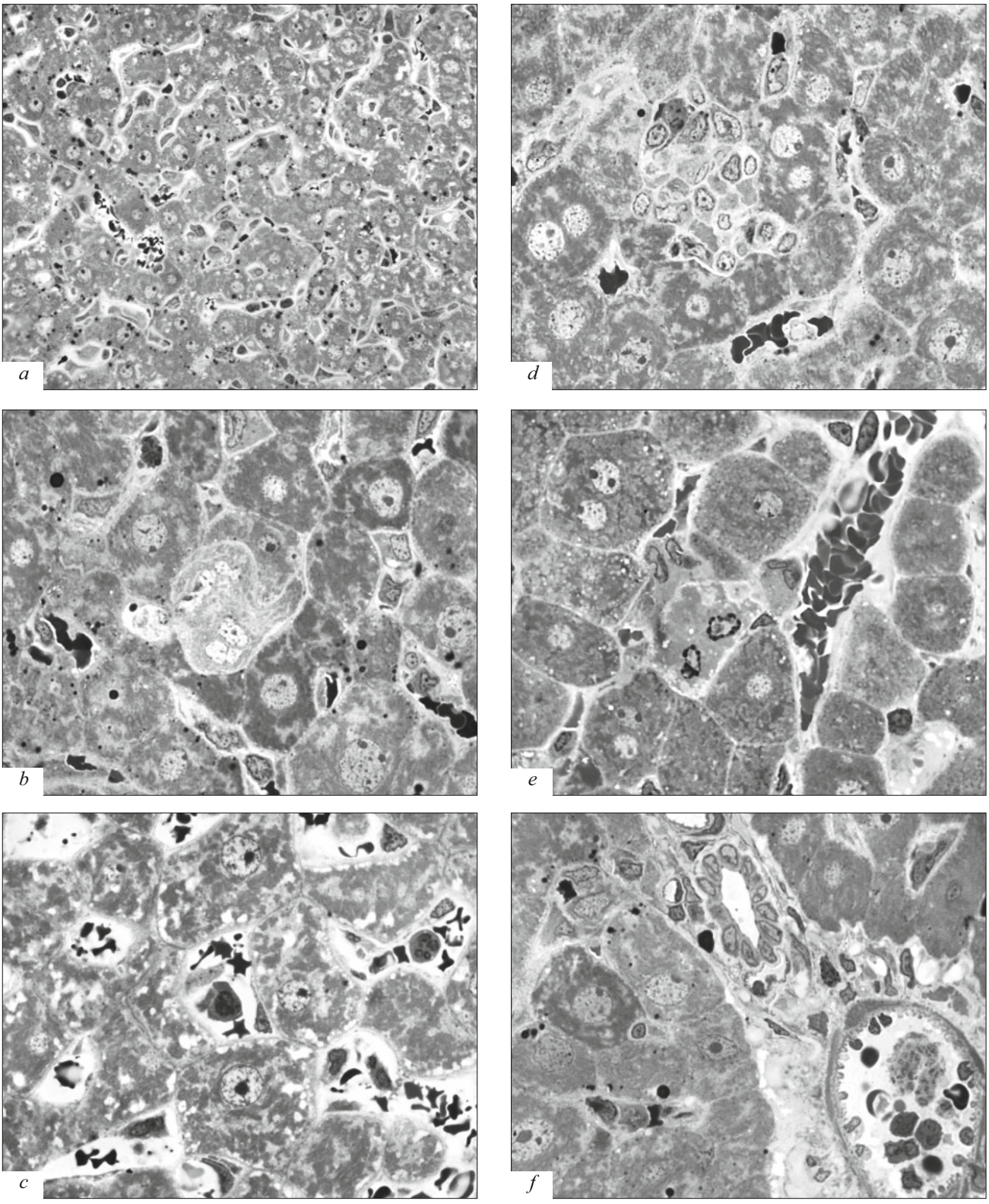

Fig. 2. Morphological changes in the liver of rats 14 days after a single injection of CP. *a*) plethoric sinusoids; *b*) obstruction of the sinusoid with Kupffer cells; *c*) subplasmalemmal "vacuolation" of the cytoplasm, polymorphic erythrocytes and segmented leukocytes in the sinusoidal lumen; *d*) focal accumulation of mononuclears at the site of hepatocyte death; *e*) binuclear hepatocyte apoptosis; *f*) portal tract sclerosis and portal artery thrombosis.

stimulation of their regeneratory reactions, were the most pronounced in the periportal zones of the liver. A significant portal and periportal sclerosis, detected during later period after CP injection, indicates more severe damage inflicted by the cytotoxic agents to the cell populations of the portal zones of the liver.

The decrease of the hepatocyte volume density and increase of their surface density and hence, of their surface/volume ratio under the effect of CP can be regarded as one of the regularities of structural reorganization of the liver under the effect of cytotoxic agents. Other expected shifts in the spatial reorganization of the liver after injection of cytotoxic drugs are the increase in the volume and surface densities of sinusoids. These changes develop as a result of augmenting vascular plethora and blood cell stasis during later periods of thrombosis.

A possible mechanism of lasting destructive effect of a single dose of CP can be failure of reparation of the main component of the hepatic vascular network, the sinusoids. CP actively destroys the liver sinusoidal endothelium [10,14]. Studies with repeated injections of low dose CP showed the pathogenetic role of exhaustion of the functional potentialities of the glutathione system, reduction of its intracellular reserves and subsequent impairment of the entire system of the hepatocyte antioxidant defense and stimulation of the free radical processes, this causing LPO intensification and damage to membrane structures [2,9]. By contrast, a single injection of high dose (150 mg/kg) CP leads to an increase of glutathione level and stimulation of phenylacetatesterase [8], which can be regarded as a defense reaction. Hence, the choice of the optimal doses and treatment protocol for this drug and other cytostatics is essential for minimization of the toxic effect and triggering of the defense mechanisms (glutathione system, *etc.*).

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