Ultrastructural Signs of Cyclophosphamide-Induced Damage to Cardiomyocytes

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Ultrastructural changes in rat cardiomyocytes were studied after single administration of cyclophosphamide in the therapeutic dose. The major signs of cyclophosphamide-induced damage to cardiomyocytes included moderate lysis of myofibrils, dilation of vesicles in the granular and agranular sarcoplasmic reticulum, and destruction of mitochondria with the formation of myelin-like residual bodies. Ultrastructural changes in cardiomyocyte nuclei primarily manifested in variations of the shape, deep invaginations of the nuclear membrane, and translocation into the subsarcolemmal region.

Key Words: *cyclophosphamide-induced myocardial injury; cardiomyocytes; ultrastructur*

Ultrastructural changes in cardiomyocytes (CMC) induced by cytotoxic drugs reflect the molecular and supramolecular events that occur during the interaction of these substances and/or their metabolites with various structures of the cell [4]. Studying the intracellular reorganization of CMC under cytotoxic exposure is important to evaluate the type and severity of damage and to determine the mechanism of injury and regeneration. Among a variety of cytotoxic drugs with cardiotoxic activity, cyclophosphamide (CP) is of particular interest. CP is used as a cytostatic in chemotherapy for malignant neoplasms. CP serves as an immunosuppressive drug in transplantation of organs, progenitor cells, and stem cells and during autoimmune diseases. Moreover, CP has antiangiogenic and antivasculogenic properties [7,8,14].

Cardiotoxic activity of CP manifests in a decrease in contractile function of the heart, decompensation, and development of congestive cardiomyopathy. These effects of CP are dose-dependent. They occur in patients receiving CP in high doses (120-270 mg/kg) [9,11]. CP in low doses has an immunostimulatory effect [10] and increases proliferative activity of epithelial cells [1]. Diffuse hemorrhagic necrosis of the myocardium, syndrome of acute myopericarditis and, more rarely, congestive heart failure are observed in the fatal outcome of CP chemotherapy. However, the mechanisms for heart sensitivity to this drug and development of fatal heart failure in some patients remain unknown. CP belongs to a group of prodrugs. Prodrug metabolism by cytochromes P450 results in the formation of bioactive substances with an unknown range of activity. Therefore, studying cardiotoxic activity of CP metabolites should include experimental evaluation of structural changes [3]. Little is known about the type of CP-induced ultrastructural changes in various cells (*e.g.*, CMC).

Here we studied the type of CP-induced ultrastructural changes in CMC. The major ultrastructural signs for CP-induced injury were evaluated.

MATERIALS AND METHODS

CP-induced damage to CMC was studied in 20 male Wistar rats. The animals received intraperito-

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neal injection of CP (Biokhimik) in a single dose of 125 mg/kg. An equivalent volume of physiological saline was injected intraperitoneally to control rats (n=12). The animals of both groups were decapitated under ether anesthesia on days 3 and 14 after CP treatment.

Samples of the left ventricular myocardium were fixed in 4% paraformaldehyde and postfixed in 1% OsO_4. They were treated by the standard method, impregnated, and embedded into a mixture of Epon and araldite. Semithin and ultrathin sections were obtained on a LKB III ultratome. Semithin sections were stained with azure II. Ultrathin sections were contrasted by uranyl acetate and lead citrate. Semithin sections were subjected to a morphometric study. The diameter of CMC was measured under a Leica DM 4000B universal microscope using Leica QWin software (not less than 100 CMC from each animal). Ultrathin sections were examined under a JEM 1010 electron microscope (accelerating voltage 60 V).

RESULTS

Five rats died on days 7-10 after injection of CP in a dose of 125 mg/kg. The weight of the heart decreased by 11% on day 3 day after CP injection. By the 14th day, the weight of the heart in treated rats did not differ from than in control animals. Myocardial architectonics remained practically unchanged in all periods of the study (except for venous and capillary plethora and plasmorrhage). CP causes a variety of circulatory disorders that are manifested in plethora and hemorrhage, occur in various organs and tissues (particularly in the urinary bladder), and serve as one the major pathogenetic stages in CP-induced injury [13].

Light microscopy revealed the appearance of CMC with lysis of the sarcoplasm and vacuole-like dilations. The number of these cells increased by the end of study. It should be emphasized that the diameter of CM remained practically unchanged in CP-receiving rats. The diameter of CM in rats of the treatment group was 16.14±0.58 and 16.05± 0.24 μ on days 3 and 14 after CP injection, respectively (*vs*. 15.80±0.13 μ in the control). These data indicate that administration of CP is not followed by inhibition of intracellular regeneration in CMC. A decrease in the weight of the heart over the first 3 days after treatment is mainly related to the reduction of CMC [3].

Electron microscopy of rat myocardium was performed on day 3 after CP injection. We revealed the presence of CMC with severe lytic and destructive changes in major organelles. CMC with a well-preserved intracellular organization were also found. CP had a strong effect on myofibrillar bundles, Golgi complex, mitochondrial compartment, and agranular sarcoplasmic reticulum (ASR). Various CMC were characterized by isolated or combined ultrastructural changes.

A specific feature of CMC with insignificant ultrastructural changes was the presence of considerable amounts of glycogen granules in the sarcoplasm. Tinctorial properties of these granules differed in treated and control animals. Glycogen granules were located not only in the interfibrillar and subsarcolemmal region and perinuclear space, but also between myofilaments in myofibrillar bundles. The increase in the content of glycogen in CMC was accompanied by its sequestration (formation of light rings around glycogen granules). These changes were particularly pronounced in the subsarcolemmal and perinuclear regions (Fig. 1, *a*). High content of glycogen in CMC of CP-receiving rats reflects the impairment or changes in energy supply to cells.

The ultrastructure of CMC on day 3 after CP injection did not differ from the control. However, deep invaginations of the nuclear membrane were often found. The elongated nuclear processes were sometimes observed. The nuclei were translocated into the subsarcolemmal region. The nucleoli were large and looped. They mainly included the fibrillar component of the nucleolonema. Several nucleoli were dispersed. The amount of marginal chromatin significantly increased in nuclei with deep invaginations of the nuclear membrane. Changes in the perinuclear region of CMC were more significant. The Golgi complex with a well-developed vacuolar apparatus was often identified. The bordered vesicles were present. Sometimes we observed an irregular widening of dictyosomes and disintegration of their membranes (Fig. 1, *b*). Autophagosomes (residual bodies) and lipid inclusions were located near the Golgi complex in some cells. Glycogen sequestration was often observed. Fragments of the Golgi complex were also found in other regions of well-preserved CMC.

Change in myofibrillar bundles manifested in focal or diffuse lysis of myofibrils (Fig. 1, *c*). Numerous polysomes and chaotic newly-formed myofilaments were always revealed in the site of myofibrillar lysis on day 3 after CP injection. These changes reflect activation of intracellular regeneration. The dilated vesicles of ASR were often present in the myofibrillar region of CMC with severe lytic changes of myofibrils. They looked like vacuoles. However, dilation of ASR after CP injection was less pronounced than that in doxorubicin-induced damage to CMC [2].

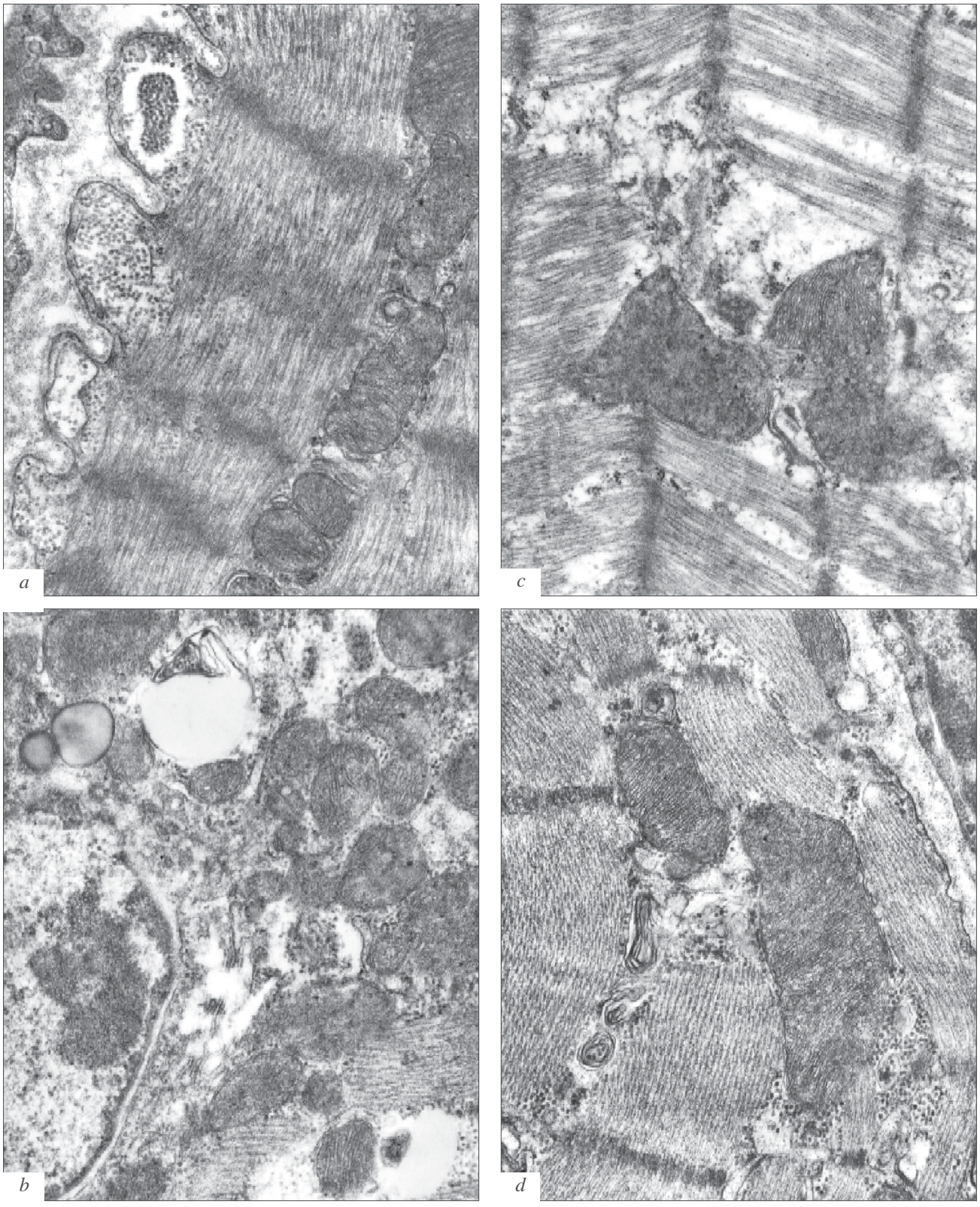

Fig. 1. Ultrastructure of rat CMC on day 3 after single injection of CP. Sequestration of glycogen in the subsarcolemmal festoons (×20,000, *a*); destructive changes in dictyosomes and formation of residual bodies in the perinuclear region (×20,000, *b*); lytic changes in myofibrillar bundles and appearance of polyribosomes in the site of lysis (×15,000, *c*); and formation of myelin-like structures near mitochondria (×20,000, *d*).

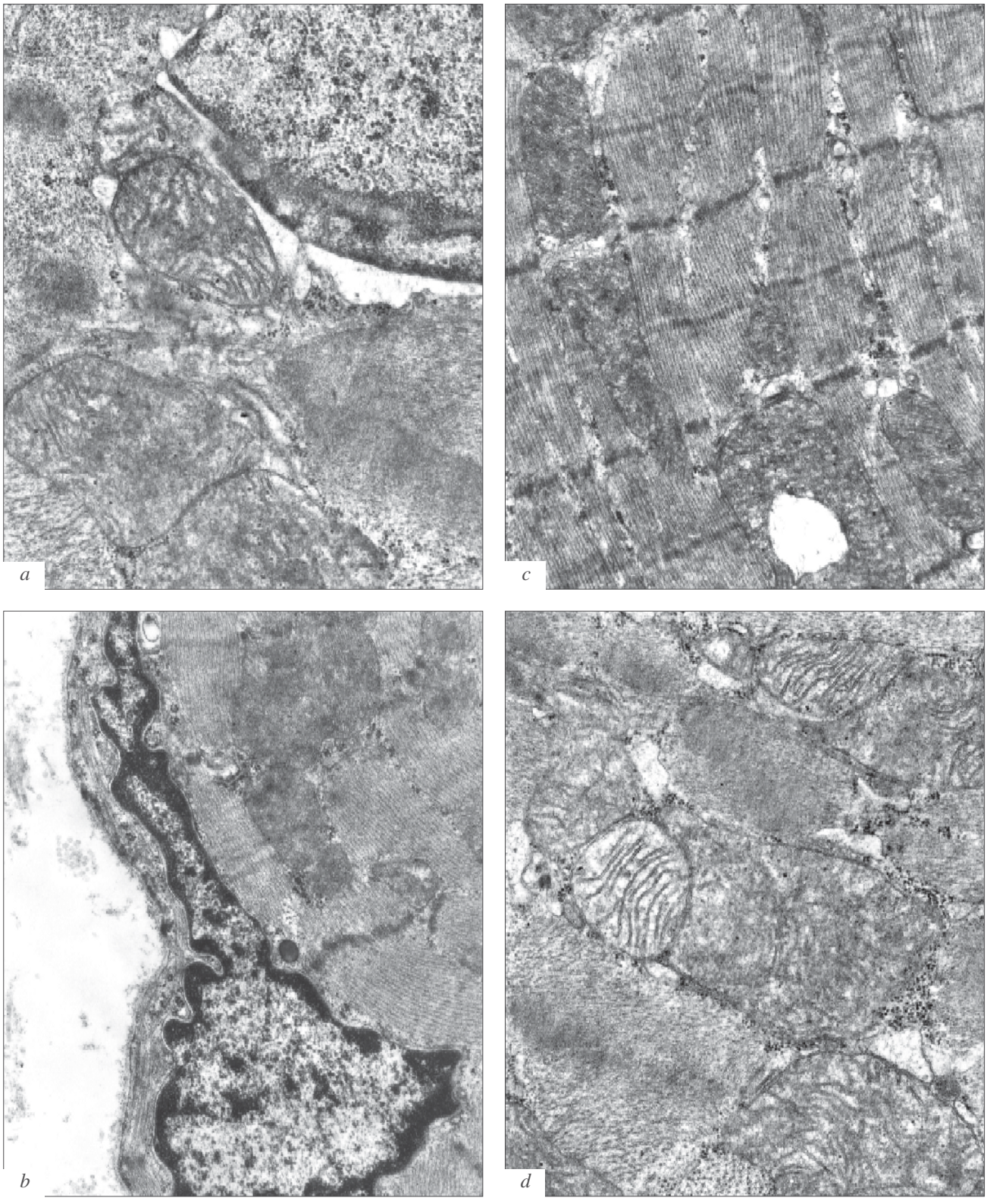

Fig. 2. Ultrastructure of rat CMC on day 14 after single injection of CP. Dilation of the intermembrane perinuclear space (×15,000, *a*); subsarcolemmal nucleus of unusual shape (×12,000, *b*); focal destruction of mitochondrial cristae with vacuole formation (×12,000, *c*); and mitochondria with reduced number of cristae and loosened matrix, dilated cisternae of the granular sarcoplasmic reticulum (×15,000, *d*).

We studied the type and severity of ultrastructural changes in mitochondria. Severe destruction or lysis of these organelles was not revealed. However, numerous myelin-like structures were always present in the site of their location (particularly, in the myofibrillar region; Fig. 1, *d*). These residual bodies were formed during degradation of mitochondria.

Lysis of myofibrillar bundles and dilation of ASR vesicles in CMC were revealed on day 14 after CP injection. The intermembrane perinuclear space was dilated in some CMC (Fig. 2, *a*). Their nuclei were translocated into the subsarcolemmal region. The presence of nuclei with unusual shape was probably related to damage to cytoskeletal proteins (Fig. 2, *b*). Lysis of myofibrillar bundles was rarely observed (primarily, in the region of intercalated discs). Numerous polysomes were located in the region of myofibrillar lysis (similarly to the previous stage).

Mitochondrial damage was more pronounced in this period. We often revealed focal lysis of the matrix, destruction of cristae, and appearance of vacuoles with the flake-like content in organelles (Fig. 2, *c*). In some CMC, nearly all mitochondria had lightened matrix and reduced number of cristae. They differed from organelles of normal CMC (Fig. 2, *d*). Dilated cisternae of the granular sarcoplasmic reticulum with the flake-like content were often found in the sarcoplasm. The sarcoplasm of CMC included a considerable number of glycogen granules. Residual bodies and autophagosomes were present in the myofibrillar region and near intercalated discs.

The type and severity of ultrastructural changes in CMC were studied after injection of CP in a single dose of 125 mg/kg. Under these conditions the severity of ultrastructural changes in CMC were less significant than after treatment with doxorubicin or rubomycin in cardiotoxic doses [2,4]. Our findings are consistent with the fact that CP has lower toxicity than doxorubicin. The patients receiving CP in high doses are characterized by milder signs of cardiac dysfunction and lower mortality rate from heart failure.

The major ultrastructural signs of CP-induced damage to CMC include moderate lysis of myofibrils, dilation of vesicles in the granular sarcoplasmic reticulum and ASR, and destruction of mitochondria with the formation of myelin-like residual bodies. Dilation of cisternae in the granular sarcoplasmic reticulum and destruction of mitochondria were also found in thyrocytes of CP-receiving animals [6]. These data illustrate the general mechanism of damage to various types of cells under the influence of CP. Significant dilation of vesicles in ASR of CMC serves as one of the major signs for doxorubicin-induced damage to these cells, which is detected by a light optical study. Reorganization of the sarcoplasmic reticulum is probably associated with abnormalities of the cytoskeleton in CMC and/or transport and metabolic dysfunction of ASR due to macromolecular injury.

The cardiotoxic effect of CP is related to low selectivity for proliferating cells. Genotoxic activity of CP and its metabolites is associated with the ability to alkylate structural elements of DNA (purines and pyrimidines). Cytotoxic properties of these compounds result from activation of free radical processes [5] due to accumulation of a cytotoxic CP metabolite phosphoramide in the cytoplasm [11] and induction of apoptosis [12]. Activation of lipid peroxidation determines mitochondrial alteration and impairment of energy metabolism in the cell. This hypothesis is confirmed by fine structural changes in mitochondria (lysis of the matrix, decrease in the number of cristae, and formation of vacuoles in organelles).

Changes in the nuclear apparatus during CP-induced damage to CMC are of particular importance. They include a change in the shape of nuclei (formation of elongated processes and deep invaginations of the nuclear membrane) and translocation into the subsarcolemmal region. Subsarcolemmal translocation of CMC nuclei is usually observed during the impairment of plastic processes of different genesis (*e.g.*, administration of anthracycline antibiotics, starvation, and genetically determined structural defects of cytoskeletal proteins). Nuclear translocation into the subsarcolemmal region was revealed in CMC with insignificant lytic changes. Otherwise, this sign was observed in cells with normal ultrastructure. The significance and mechanisms of nuclear reorganization in CMC during cytotoxic exposure and other types of cardiac dysfunction require further investigations.

REFERENCES

1. E. A. Iseeva and V. L. Bykov, *Morfologiya*, **130**, No. 6, 62-67 (2006).
2. E. L. Lushnikova, M. G. Klinnikova, O. P. Molodykh, and L. M. Nepomnyashchikh, *Byull. Eksp. Biol. Med.*, **139**, No. 4, 470-475 (2005).
3. E. L. Lushnikova, T. G. Tolstikova, L. M. Nepomnyashchikh, *et al.*, *Ibid.*, **144**, No. 9, 331-337 (2007).
4. L. M. Nepomnyashchikh, *Arkh. Patol.*, No. 1, 3-12 (2007).
5. A. V. Oleinik, *Vopr. Onkol.*, **31**, No. 7, 97-101 (1985).
6. G. Yu. Yukina, A. I. Nevorotin, and V. L. Bykov, *Morfologiya*, **125**, No. 1, 66-71 (2004).
7. R. L. Basser, R. Abraham, L. B. To, *et al.*, *Ann. Oncol.*, **10**, No. 1, 53-58 (1999).

8. A. C. Braverman, J. H. Antin, M. T. Plappert, *et al*., *J. Clin. Oncol*., **9**, No. 7, 1215-1223 (1991).
9. H. S. Friedman, O. M. Colvin, K. Aisaka, *et al*., *Cancer Res*., **50**, No. 8, 2455-2462 (1990).
10. M. Loeffler, J. A. Kruger, and R. A. Reisfeld, *Ibid*., **65**, No. 12, 5027-5030 (2005).
11. K. J. Schimmel, D. J. Richel, R. B. van den Brink, and H. J. Guchelaar, *Cancer Treat. Rev*., **30**, No. 2, 181-191 (2004).
12. P. S. Schwartz and D. J. Waxman, *Mol. Pharmacol*., **60**, No. 6, 1268-1279 (2001).
13. T. M. Wong, W. Yeo, L. W. Chan, and T. S. Mok, *Gynecol. Oncol*., **76**, No. 2, 223-225 (2000).
14. S. Zver, V. Zadnik, M. Bunc, *et al*., *Int. J. Hematol*., **85**, No. 5, 408-414 (2007).