Cardiomyocyte Count in Rat Myocardium under the Effect of Antitumor Agents Cyclophosphamide and Triterpenoids

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Structural reorganization of the myocardium in response to antitumor agents (cyclophosphamide, betulonic acid and its amide) was studied. Cardiotoxic effects of these chemicals manifested in cardiomyocyte contracture and lytic injuries and by significant hemodynamic disorders. The most pronounced lytic and necrobiotic changes in cardiomyocytes were detected after injection of cyclophosphamide followed by betulonic amide; this led to a more pronounced decrease in heart weight as a result of a decrease in total cardiomyocyte count. Antitumor drugs differently changed the ratio of mono- to binuclear cardiomyocytes, which differ by their regeneratory and compensatory adaptive potential.

Key Words: *cardiotoxicity; cyclophosphamide; betulonic acid and its amide; cardiomyocyte count*

Cardiotoxic effect of drugs and chemicals with antitumor activity is one of the most serious problems of modern oncology and cardiology [4]. Pronounced cytotoxicity of many cytostatics, which is universal, necessitates the development of new protocols for their use, delivery to the focus, and search for approaches at correction of their side effects.

Cyclophosphamide (CP) used in antitumor therapy is characterized by alkylating effect on DNA molecules, which causes the death of tumor cells [1]. Some cardiotoxic effects of CP were described [11-14]. Other side effects of CP and its metabolites include damage to the urinary organs, fetal cells, and erythroid cells [10,13].

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Side effects of cytostatics necessitate creation of preparation for combined treatment reducing the organotoxic effects of chemotherapy. One of these compounds is betulonic acid (BA), a lupane series triterpenoid extracted from betulin, a component of birch bark [8]. Betulonic acid and its derivatives (*e.g.* betulonic amide) are characterized by a wide spectrum of biological effects, which prompts their clinical application [5,7]. These compounds were first obtained at Laboratory of Medical Chemistry, N. N. Vorozhtsov Institute of Organic Biochemistry, and are now studied experimentally [6]. Betulonic acid and its amide used in combination with cytostatics reduce the severity of toxic damage to the liver and kidneys without reducing the specific effects of antitumor drugs [2,3]. On the other hand, biological effects of these compounds are not yet fully studied.

We compared structural reorganization of the myocardium in experimental animals treated with

antitumor drugs (CP, BA and its amide) and evaluated cardiomyocyte (CMC) count in the heart as an integral index of the intensity of regeneratory and compensatory adaptive reactions.

MATERIALS AND METHODS

The study was carried out on 57 male Wistar rats divided into 6 groups. Animals of groups 1-3 (n=28) received a single intraperitoneal injection of CP (Biokhimik) in a dose of 125 mg/kg; 24 h after CP group 2 animals received daily intragastric water-Twin BA solution (50 mg/kg), group 3 animals received water-Twin solution of BA amide (50 mg/kg). Group 4 animals (n=13) were injected with water-Twin solution of BA alone in the same dose, group 5 animals (n=10) received BA amide alone. Controls (group 6; n=6) simultaneously with the experimental animals were intraperitoneally injected with saline in the volume corresponding to their body weight and then daily received intragastric water. All animals were decapitated under ether narcosis 3 and 14 days after CP injection.

The heart was separated from the adjacent tissues and rapidly weighed. Heart specimens were fixed in 10% neutral formalin and 4% paraformaldehyde. The material was treated routinely for preparing semithin sections and embedded in eponaraldite mixture. Paraffin sections were stained with hematoxylin and eosin, Perls and van-Gieson reactions were performed. Semithin sections were stained with 1% Azur II. The study was carried out under a universal Leica DM 4000B microscope. Microphotographs were made with a Leica DFC 320 digital camera and Leica QWin software.

Total CMC population in the heart was quantitatively evaluated by the method of alkaline dissociation of fixed tissues [4]. In addition to CMC evaluation, quantitative ratio of mono-, bi-, and multinuclear (3 and more nuclei) CMC in the heart was evaluated.

The results were processed statistically using Student's t test.

RESULTS

The most pronounced changes in the heart weight after injection of antitumor agents were detected on day 3 of the experiment. Heart weight decreased by 11% after injection of CP or BA alone, by 15% after injection of CP followed by BA and after BA amide, and by 19% after CP followed by BA amide. By day 14 of the experiment, the heart weight was restored in groups 1, 2, 4, and 5. In group 3, the heart weight remained 8% reduced. It is noteworthy that in group 5, heart weight increased by 10.5% by the end of the experiment.

In group 1 animals, normal architectonics of the myocardium was preserved on day 3 after injection of CP. Contractures of some CMC were noted, but signs of lytic changes in CMC were rare. A characteristic feature of CP damage to the myocardium was the appearance of CMC with vacuole-like dilatation of the cytoplasm (Fig. 1, *a*). Small foci of myocardial necrosis were noted. In parallel, hemodynamic disorders, primarily venous and capillary plethora, lymphostasis, perivascular and interstitial edema were observed in all animals of this group.

The absolute count of CMC 3 days after CP injection changed negligibly (Table 1), indicating that cellular form of CMC regeneration was preserved. At the same time, the percentage of mononuclear CMC decreased by 31% and that of binucleated cells increased by 10%. Cardiomyocytes with mitotically dividing nuclei appeared in rat myocardium during this period (Fig. 1, *b*). Small ("minor") CMC with closely located nuclei were often seen (Fig. 1, *c*), which was not characteristic of mature CMC.

The population of mononuclear CMC is regarded as a regeneratory reserve of the myocardium [9]. Karyokinesis in mononuclear CMC leads to an increase in the binuclear CMC population, predominating in the myocardium of all adult mammals; these cells are not only the main structural and functional elements of the myocardium, but also its main compensatory adaptive reserve. Quantitative analysis of CMC population in control and experimental animals also showed that binuclear CMC are the terminal cell form, because the pool of polynuclear cells does not exceed 3%.

Hemodynamic disorders persisted after 14 days of the experiment. It is noteworthy that in addition to blood cells, blood vessels and capillaries in virtually all animals contained plasma; plasmorrhagia was observed. The count of CMC with lytic and vacuole-like changes increased in comparison with the previous term. Small foci of CMC necrosis and necrobiosis and their infiltration with mononuclears were observed (Fig. 2, *a*). CMC nuclei were polymorphic and often shifted to the cell periphery. "Minor" CMC with closely located nuclei were seen in the myocardium during this period of the experiment (Fig. 2, *b*), which can be considered as a morphological equivalent of completed karyokinesis. Moderate diffuse mononuclear infiltration of the myocardial stroma and more intense perivascular sclerosis are worthy to note. Intramural arteries were mainly in a state of spasm; the vascular me-

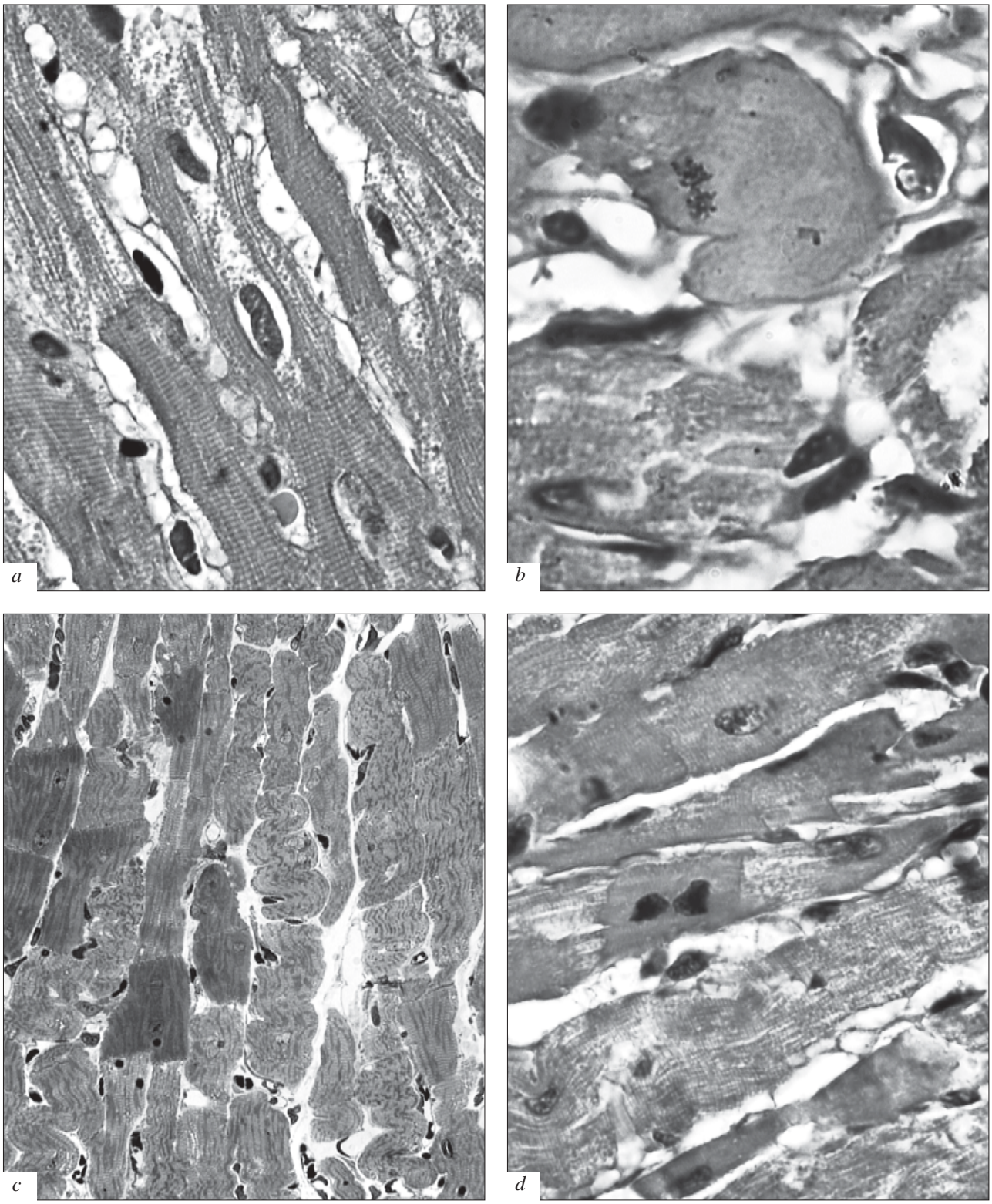

Fig. 1. Morphological changes in rat myocardium 3 days after injection of CP and BA. *a*) pronounced lytic changes in the CMC with formation of vacuolated dilatation round the nuclei after CP injection, ×1000; *b*) mitotic division of nucleus in CMC, ×1600; *c*) small CMC with closely located nuclei, ×500; *d*) mosaic changes in CMC after BA injection, ×1000. *a*, *b*, *d*: hematoxylin and eosin staining; *c*) semithin section, Azur II staining.

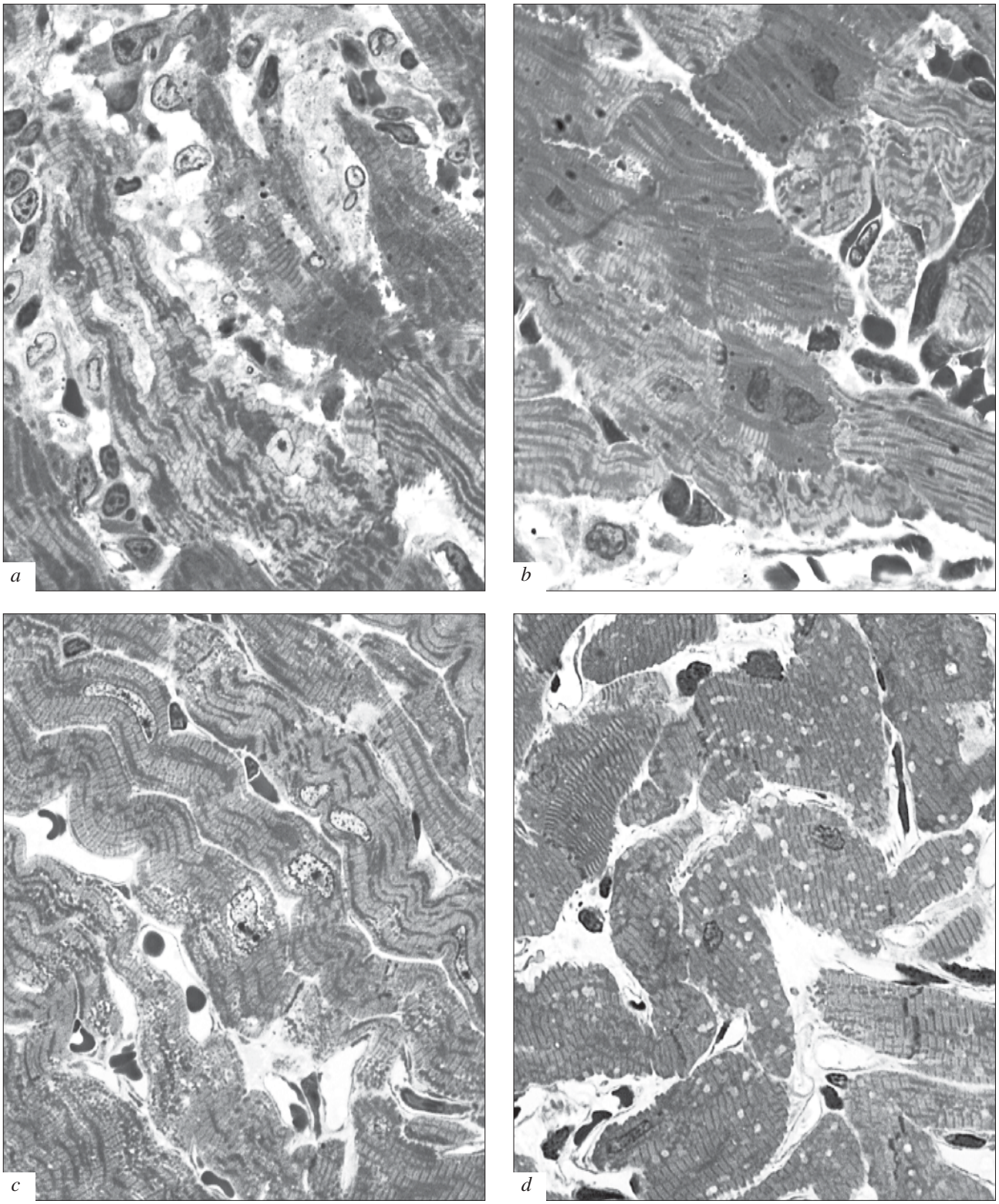

Fig. 2. Structural reorganization of rat myocardium after CP and BA amide treatment. Semithin sections. Azur II staining ×1000. *a*) formation of cardiosclerosis focus at the site of CMC death 14 days after CP injection; *b*) small CMC with closely located nuclei 14 days after CP injection; *c*) moderate lytic changes in CMC 3 days after injection of BA amide; *d*) CMC with vacuole-like dilatation of sarcoplasma 3 days after CP and BA amide treatment.

TABLE 1. Morphometric Parameters and Quantitative Characteristics of CMC Population in the Heart of Wistar Rats after Treatment with CP and Betuline Derivatives ($M \pm m$)

Parameter	Control	CP	CP+BA	CP+BA amide	BA	BA amide
Day 3						
Body weight, g	204.0±7.0	198.0±8.3	195.0±11.1	185.0±10.8	231.9±8.2	212.0±10.8
Heart weight, g	0.79±0.05	0.70±0.03	0.67±0.04	0.64±0.03	0.70±0.03	0.67±0.01
Heart weight share, mg/g	3.87±0.20	3.53±0.04	3.43±0.06	3.45±0.30	3.03±0.08	3.16±0.20
CMC concentration, 10^3/mg tissue	17.343±0.809	17.334±1.268	16.869±1.124	15.172±2.219	18.514±2.116	18.099±3.578
Absolute CMC count in heart, $\times 10^6$	12.316±0.252	11.607±1.199	11.276±1.085	9.145±1.296	12.236±1.302	11.918±2.591
Percent of cells						
mononuclear	21.5±5.3	14.9±0.4	14.7±1.7	25.9±8.1	29.3±7.8	41.3±2.8*
binuclear	75.1±5.6	82.6±0.7	82.8±1.0	73.2±8.0	69.6±7.4	57.7±2.6*
multinuclear (3 and more nuclei)	3.4±0.3	2.5±0.3	2.5±0.8	0.9±0.3*	1.1±0.6*	1.0±0.5*
Day 14						
Body weight, g	248.3±15.9	226.7±14.2	204.0±7.5	225.0±15.0	231.0±6.8	262.0±4.6
Heart weight, g	0.76±0.04	0.76±0.08	0.72±0.02	0.70±0.04	0.78±0.02	0.84±0.03
Heart weight share, mg/g	3.06±0.06	3.35±0.10	3.52±0.20	3.11±0.30	3.38±0.09	3.21±0.20
CMC concentration, 10^3/mg tissue	13.087±1.733	19.651±2.996	16.755±1.928	14.430±0.681	13.898±1.009	13.077±1.023
Absolute CMC count in heart, $\times 10^6$	9.237±1.411	13.716±2.792	11.808±1.625	10.024±0.857	10.376±0.765	10.370±0.718
Percent of cells						
mononuclear	30.5±2.7	26.5±7.9	17.9±3.2*	19.9±3.1*	18.5±2.2*	21.8±1.0*
binuclear	66.9±2.2	70.2±7.3	80.2±3.1*	79.4±3.8	80.2±2.2*	76.6±0.5*
multinuclear (3 and more nuclei)	2.5±0.5	3.2±0.8	1.8±0.9*	0.7±0.1*	1.3±0.5	1.6±0.7

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

dia was thickened as a result of smooth muscle cell hypertrophy and hyperplasia. Total CMC count 14 days after CP injection increased by 50%; the percentage of mononuclear CMC increased, but remained below the control (by 13%).

CMC with intact morphology and tinctorial properties predominated in the myocardium after BA treatment; contractures and lytic changes in CMC were seen (Fig. 1, *d*), "minor" CMC with closely located nuclei were observed. Pronounced polymorphism of CMC nuclei was observed. Hemodynamic disorders manifested in vascular plethora, erythrocyte sludge; foci of hemo- and plasmorrhages were seen. Interstitial edema was moderate. The content of atrophic and necrobiotically modified CMC increased in the myocardium after 14 days of the experiment; this was paralleled by an increase in the content of hypertrophic CMC. Modified shape and tinctorial characteristics of CMC nuclei and their frequent shifting to the subsarcolemmal zone are worthy of note. Hemodynamic disorders were paralleled by moderate diffuse infiltration of myocardial stroma with mononuclears; small foci of cardiosclerosis were seen in some animals.

Treatment with BA caused no appreciable changes in the total CMC count or the mono/binuclear CMC ratio in rat hearts by day 3 of the experiment (Table 1). On the other hand, the percentage of multinuclear cells decreased significantly. By day 14 of the experiment, the total CMC count in the heart also did not change appreciably in comparison with the control, while the percentage of mononuclear CMC decreased by 39% and that of binuclear CMC increased by 20%.

Morphological changes in the myocardium after BA amide treatment during the first 3 days of the experiment virtually did not differ from those observed after BA treatment. Contractures in CMC were combined with lytic changes (Fig. 2, *c*). The zones of sarcoplasma and myofibril lysis in the CMC were small. After 14 days, injuries progressed in some CMC, the number of damaged cells increased; small foci of CMC necrosis with accumulation of mononuclears appeared. Diffuse and small focal cardiosclerosis developed. Treatment with BA amide caused no appreciable changes in the total CMC count in the heart at all terms of the experiment. Changes in the ratio of CMC with different numbers of nuclei were significant. Three days after treatment with BA amide, the percentage of mononuclear CMC increased by 92%, while the percentage of binuclear and mononuclear CMC decreased significantly (Table 1). After 14 days the changes were opposite, the percentage of mononuclear CMC decreased (by 28%) in comparison with the control, while the percent of binuclear cells increased (by 14%).

Injection of CP followed by BA produced no appreciable changes in the myocardial architectonics on day 3 of the experiment, but the mosaic pattern of CMC changes (more pronounced than after CP alone) was observed. The number of CMC with contractures increased. Enlargement of "minor" CMC was observed in the perivascular zones. Moderate interstitial edema and diffuse infiltration of the stroma with mononuclears were observed. Similarly as after CP, moderate hemodynamic disorders were noted. Mosaic pattern of myocardial injuries persisted after 14 days. The number of CMC with lytic changes of the cytoplasm increased and reached 30-40% in some animals. The phenotypical heterogeneity of CMC increased: atrophic cells were seen along with hypertrophic ones. This latter circumstance promoted recovery of heart weight. Moderate diffuse infiltration of myocardial stroma with mononuclears (diffuse cardiosclerosis) was observed; in addition, mononuclears accumulated in small foci of CMC death. Hemodynamic disorders persisted; interstitial and perivascular edema increased in some cases.

Quantitative changes in the CMC population 3 days after CP injection followed by BA treatment were the same as after CP alone (Table 1). Quantitative ratio of mono/binuclear CMC in the myocardium of these animals did not change after 14 days of the experiment, in contrast to animals injected with CP alone.

Injection of CP with subsequent treatment with BA amide led to manifestation of contractures and lytic injuries to CMC 3 days after the treatment. In parallel, CMC with numerous vacuole-like dilatations in the sarcoplasma were seen (Fig. 2, *d*). Hemodynamic disorders manifested by venous and capillary plethora; pronounced lymphostasis and interstitial edema (sometimes significant) were seen. After 14 days, the manifestations of necrobiosis and degeneration of some CMC progressed; on the other hand, CMC hypertrophy was observed, and mosaic pattern of damage to the muscle segments was retained. Hemodynamic disorders were manifest, the severity of stromal edema varied. Diffuse infiltration of the stroma with mononuclears was observed, the number of these cells increased in the adventitia of intramural arteries.

Quantitative analysis of CMC population revealed a 26% decrease in the total count of parenchymatous cells after 3 days of the experiment, the mono/binuclear CMC ratio remained unchanged. By day 14 of the experiment, the total CMC count

in the heart was restored, with the percent of mononuclear CMC decreased by 35% and that of binuclear CMC increased by 19% in comparison with the control.

The results indicate that the substances with antitumor effects used in this study cause similar morphofunctional changes in the myocardium of experimental animals. The main structural changes in CMC (lytic and necrobiotic injuries) were caused by the cytotoxic effects of these antitumor agents. These changes were most pronounced after CP injection followed by treatment with BA amide. It is noteworthy that successive treatment with these substances resulted in the most pronounced decrease in heart weight on day 3 of the experiment and most marked reduction of total CMC count in the heart. On the other hand, treatment with BA amide alone led to an increase in heart weight by day 14 of the experiment and to most pronounced increase of the mononuclear CMC pool. These data indicate that BA amide potentiates the cytotoxic effects of CP and induces regeneratory reactions in the myocardium. Hence, special attention should be paid to the development of treatment protocols and selection of optimal doses of this agent for attaining the desired biological effect, protection or potentiation of cytotoxicity.

REFERENCES

1. E. B. Vladimirskaya, *Biological Bases of Antitumor Therapy* [in Russian], Moscow (2001).
2. O. R. Grek, S. V. Pozdnyakova, A. P. Nadev, *et al.*, *Eksp. Klin. Farmakol.*, **68**, No. 6, 49-51 (2005).
3. N. A. Zhukova, D. E. Semyonov, I. V. Sorokina, *et al.*, *Byull. Eksp. Biol. Med.*, **140**, No. 9, 348-350 (2005).
4. E. L. Lushnikova, M. G. Klinnikova, O. P. Molodykh, and L. M. Nepomnyashchikh, *Ibid.*, **139**, No. 4, 470-475 (2005).
5. N. I. Petrenko, N. V. Yelantseva, and V. Z. Petukhova, *Khimiya Prirodnykh Soedinenii*, No. 4, 276-283 (2002).
6. I. V. Sorokina, T. G. Tolstikova, N. A. Zhukova, *et al.*, *Dokl. Rossiisk. Akad. Nauk*, **399**, 1-4 (2004).
7. T. G. Tolstikova, I. V. Sorokina, G. A. Tolstikov, *et al.*, *Bioorgan. Khimiya*, **32**, No. 3, 291-307 (2006).
8. O. B. Flekhter, E. I. Boreko, L. R. Nigmatullina, *et al.*, *Ibid.*, **30**, No. 1, 89-98 (2004).
9. X. Chen, R. Wilson, H. Kubo, *et al.*, *Circ. Res.*, **100**, No. 4, 536-544 (2007).
10. B. M. Francis, J. M. Rogers, K. K. Sulik, *et al.*, *Teratology*, **42**, No. 5, 473-482 (1990).
11. W. H. Frishman, H. M. Sung, H. C. Yee, *et al.*, *Curr. Probl. Cancer*, **21**, No. 6, 301-360 (1997).
12. C. K. Lee, G. S. Harman, R. J. Hohl, and R. D. Gingrich, *Bone Marrow Transplant.*, **18**, No. 3, 573-577 (1996).
13. M. Paparella, P. Catalani, C. Pellizzer, *et al.*, *Altern. Lab. Anim.*, **27**, 295 (1999).
14. P. S. Schwartz and D. J. Waxman, *Cancer Res.*, **60**, 1268-1279 (2001).