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Experimental Study of Structural, Functional, and Biochemical Changes in Immune Organs under Conditions of Antitumor Activity of Copper Nanoparticles

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The effects of copper nanoparticles on the structure and function of the immune system organs (thymus and spleen) and intensity of free radical processes in the spleens of rats with sarcoma 45 were studied. A relationship between morphological and biochemical changes and antitumor efficiency of copper nanoparticles was demonstrated.

Key Words: *copper nanoparticles; spleen; thymus; antitumor effect*

The biological effects of nanoparticles (NP) of various metals are now intensely studied due to their use in oncology [8]. The importance of studies of the effects of highly dispersed copper powder on the organism with a tumor can be explained by a great variety of functions of this essential trace element and with high penetration of NP into the cells and their incorporation in various metabolic chains [2]. The relationship between free radical reactions activation and malignization and metastasizing processes and the involvement of copper in free radical oxidation and antioxidant defense [6], and its significance for the immune system functioning [9] are well known. These facts attest to possible effects of copper NP on tumor development and antitumor resistance mechanisms.

We studied structural and functional changes in the immune organs and status of the antioxidant defense system in the spleens of rats with transplanted sarcoma 45 under conditions of antitumor effects of copper NP.

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MATERIALS AND METHODS

Physicochemical parameters of copper NP obtained by the plasmochemical method were studied. The size of particles was measured by optical methods, including atomic force microscopy, spectrophotometry, and fluorometry. Copper NP size was 40-100 nm, their mean diameters about 75.7 nm.

Experiments were carried out on outbred male rats (n=42; 180-220 g). Sarcoma 45 was transplanted to 34 animals. Suspension of copper NP (1.25 mg/kg) was injected locally into the tumor (12 animals) or intraperitoneally (12 animals). The NP concentration in the suspension was 1 g/liter (1 mg/ml). Control group consisted of 10 animals with sarcoma 45 intraperitoneally injected with saline (0.3 ml). Copper NP powder was suspended in saline directly before injection in a concentration of 1 mg/ml. The suspension (or saline) was injected on days 9, 10, 12, and

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13 after tumor transplantation. The injections were repeated after 5 days (on days 19, 20, 22, and 23 after transplantation). The animals were decapitated on day 29 of experiment. The reference group consisted of 8 intact animals. Copper NP were synthesized at the plasmochemical complex of Research Center of Chemistry and Technology of Element-Organic Compounds (Saratov).

The morphology and function of the immune organs was studied by histochemical methods (Brachet's staining in Simakova's modification) followed by morphometry. Morphometry was carried out using Avtandilov's standard grid. Activity of lymphoproliferative processes in the thymus was evaluated by measuring the width of the cortical matter and medulla and estimating the stromal-parenchymatous coefficient. In the spleen follicles with germinative centers were counted (per 10 follicles) and the mitotic activity of lymphoblast elements in them was evaluated by counting mitotic figures in 30 visual fields. Activity of cell-cell interactions was evaluated by changes in the number of thymocyte contacts with tissue basophils and number of complex-associated macrophages and splenocytes. The antioxidant status and LPO levels were evaluated by measuring activities of SOD, catalase, and glutathione-dependent enzymes (glutathione peroxidase, GPx; glutathione reductase, GR; glutathione transferase, GT) and the levels of reduced glutathione and MDA by routine spectrophotometric methods in 10% homogenates of the spleen (0.04 M Tris-HCl buffer, pH 7.4). The data were statistically processed by Student's *t* test and Mann–Whitney's *U* test.

RESULTS

A clear-cut antitumor effect was observed in the majority of animals with tumors injected with copper NP: mean tumor volume (Vmed) and weight (Mmed) decreased 6-10-fold up to complete regression. Complete regression of the tumor irrespective of the mode of administration was attained in 12 animals, partial regression (Vmed=1.88±0.2 cm^3, Mmed=1.85±0.1 g) in 6 animals. Even in cases when the tumor did not regress (n=6) by volume and weight (Vmed=8.5±***0.4*** cm^3, Mmed=4.32±***0.3*** g), morphological signs of its destruction were seen. Intensive tumor growth was observed in all animals of the control group (Vmed=7.54±0.5 cm^3, Mmed=5.96±0.3 g).

The microscopic picture of the thymus and spleen in the control animals was characterized by pronounced signs of reduced functional activity. The lymphoid tissue hypoplasia was presented by reduced density of lymphoid elements and their focal destruction. Small lobules with a narrow cortical matter zone and focal destruction of the lymphoid elements predominated in the thymus; several atrophic lobules with thickened connective tissue septae and adipose tissue between the lobules were seen (Fig. 1, *a*). The number and size of follicles decreased in the spleens of control animals; the content of germinative centers in these cells and of dividing plasma lymphoblasts in them reduced (Fig. 1, *b*). The thymus-dependent zones in the spleens (periarterial cuffs) were mainly small.

The antitumor effect of copper NP was associated with activation of the spleen and thymus; the microscopic picture of these organs showed signs of their moderate and intense functioning. The results in the groups injected with copper NP via different routes virtually did not differ (Table 1). Medium-sized and large lobules predominated in animals with complete and partial regression of the tumor. The cortical matter area in them increased 1.8-1.9 times in comparison with controls, while the stromal/parenchymatous coefficient was 3-fold reduced; the lymphocyte population

TABLE 1. The Morphology and Function of the Thymus in Rats with Sarcoma 45 under Conditions of Antitumor Treatment with Copper NP (M±m)

Organ	Structural elements	Control (n=10)	Injections of copper NP	
			into tumor	intraperitoneally
Thymus	CM	39.1±2.8	71.5±1.9*	74.7±1.6*
	S/P×100 (%)	7.6±0.9	2.7±0.4*	2.3±0.3*
Spleen	GC	2.7±0.5	7.0±0.5*	7.3±0.4*
	MF	3.8±0.7	13.5±1.2*	16.5±1.4*
	MP+LP	1.0±0.3	2.9±0.3*	3.0±0.3*

Note. *p<0.05-0.001 in comparison with the control. CM: area of thymic lobule cortical matter; S/P: stroma/parenchyma index; GC: number of germinative centers; MF: mitotic figures; MP+LP: number of complexes of activated macrophages with lymphocytes in the red pulp.

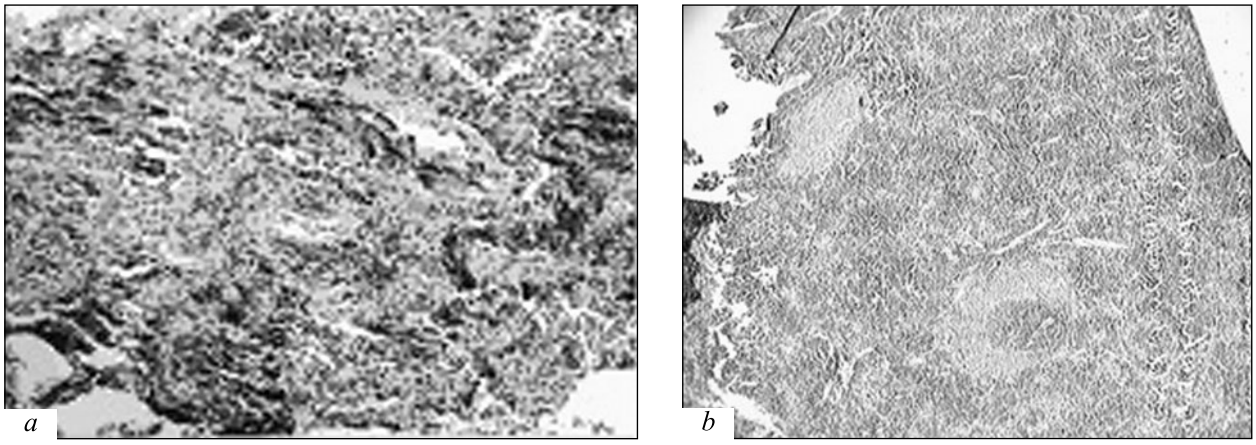

Fig. 1. Immune organs of control rats with tumors: thymus (*a*), spleen (*b*). Pronounced hypoplasia of lymphoid tissue: *a*) no lobular pattern, focal degeneration of lymphoid cells; *b*) reduced number of cells and follicles, no germinative centers and dividing lymphoblasts. Brachet's staining in Simakova's modification, ×10.

density increased (Fig. 2, *a*). The number of tissue basophil contacts with lymphocytes increased (Fig. 2, *b*). Lymphoid tissue hyperplasia was seen in the spleen as well (Fig. 2, *c*). The number (2.6-2.7 times) and size of the white pulp follicles with germinative centers increased, the counts of lymphoblasts in them increased more than 3.5 times (Table 1). The periarterial cuffs increased in size. The number of rosette-like macrophage and lymphocyte complexes in the red pulp increased almost 3-fold (Fig. 2, *d*). Increased lymphoproliferative activity, number of thymocyte contacts with tissue basophils and splenocyte-macrophage com-

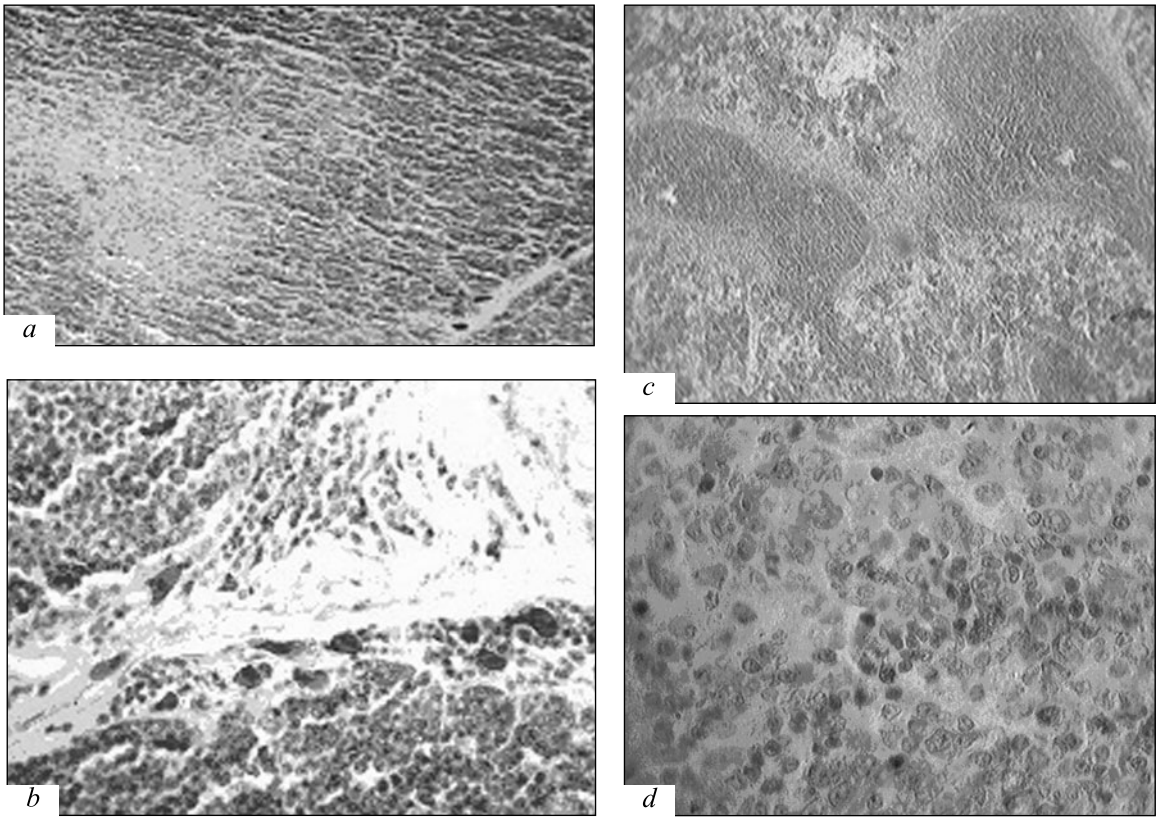

Fig. 2. Immune organs of rats with tumors injected with copper NP: thymus (*a*, *b*), spleen (*c*, *d*). *a*) the lobules and cortical matter area are enlarged, parenchyma predominates over stroma; *b*) increase in the number of tissue basophils, activation of their contacts with lobular cortical matter thymocytes; *c*) enlargement of white pulp follicles containing germinative centers; *d*) activation of cell-cell contacts – formation of rosette-like complexes (macrophages and lymphocytes) in the red pulp. Brachet staining after Simakova, ×20 (*a*), ×40 (*b*), ×10 (*c*), ×100 (*d*).

TABLE 2. Biochemistry of Free Radical Oxidation Processes in Splenic Tissue of Rats with Sarcoma 45 under Conditions of Antitumor Treatment with Copper NP (*M*±*m*)

Group	MDA, nmol/mg protein	SOD, arb. unit/ mg×Hb 10^{-3}	Catalase, μmol/mg protein	GPx, U/mg protein	GSH, μmol/mg protein	GT, U/mg protein	GR, U/mg protein
Intact	120±5	328±10	3643±79	413±18	16.8±0.7	55.6±2.6	13.4±0.9
Control	109±7	463±14**	3556±46	518±24**	31.1±1.9***	62.0±1.4*	11.4±0.7
Copper NP injections							
tumor growth	107±14	435±16**	3691±26	476±23*	22.1±1.9*^{oo}	70.8±1.2**^{oo}	12.3±1.3
partial regression of tumor	115±12	468±35**	3437±38***	483±35*	12.8±2.4^{ooo+}	47.4±0.1*$^{ooo+++}$	10.9±0.2*
complete regression of tumor	81±4**$^{o+xx}$	502±26*****$^{+}$	4098±43***$^{ooo++xxx}$	440±17^{o}	15.0±1.9^{ooo+}	62.0±1.3***^{xxx}	9.0±0.4***$^{ooo++x}$

Note. *p<0.05, **p<0.01, ***p<0.001 in comparison with intact group; ^{o}p<0.05, ^{oo}p<0.01, ^{ooo}p<0.001 in comparison with control group; ^{+}p<0.05, ^{++}p<0.01, ^{+++}p<0.001 in comparison with tumor growth; ^{x}p<0.05, ^{xx}p<0.01, ^{xxx}p<0.001 in comparison with partial regression. GSH: reduced glutathione.

plexes indicated more intense immune processes in the thymus and spleen under the effect of copper NP.

The level of MDA (LPO second product) in the spleen during tumor growth in control animals was virtually the same as the parameter in intact rats (Table 2). Activities of all the studied antioxidant enzymes (except catalase) under these conditions increased by 11-42%. Hence, the absence of MDA accumulation during tumor growth (usually paralleled by intensification of free radical processes) was mainly due to activation of the second line antioxidant defense enzymes (GPx, GT). Despite the increase in activities of GPx and GT utilizing reduced glutathione as the substrate, the level of glutathione was significantly (85%) higher than in intact rats. Activity of GR (enzyme involved in oxidized glutathione reduction) did not differ from the normal level.

This increase of glutathione level was presumably caused by its intense *de novo* synthesis, a mechanism of cell defense from oxidative stress, though an excessive level of this antioxidant could cause disorders in erythrocyte sequestration in the spleen [3-5]. Hence, our data indicated a greater role of the glutathione antiperoxide system in inactivation of reactive oxygen metabolites in the spleen under conditions of sarcoma 45 growth.

Activities of SOD, catalase, GPx, and GR in the spleens of animals with growing and partially regressing sarcoma 45 were at the levels of the controls after injection of copper NP. The main differences between these groups were detected for activity of GT reducing the hydroperoxides of various biomolecules, including lipids. Enzyme activity in rats with tumor growth increased by 14% of control, while in partial regression of the tumor it decreased by 23.5% vs. the normal level. The level of reduced glutathione decreased in comparison with the control in animals of both groups, more so in animals with tumor regression (29 and 59%, respectively), reaching the normal level. We think that these results, together with the absence of data on differences in MDA levels in the studied groups, indicate a different pattern of changes in the glutathione-dependent peroxidase system in animals with tumor growth and regression. In the former they indicate desynchronization in the work of the system, while in the latter they indicate optimization of its functioning.

The most significant changes in the studied parameters were found in the spleens of rats with complete regression of sarcoma 45. Activity of catalase increased by 10-16% in comparison with all other groups, while GPx activity returned to normal. A trend to elevation of SOD activity was observed in rats with complete regression of the tumor, in contrast to rats with tumors from other groups. A significant (32%) reduction of MDA level in comparison

with the normal level in rats with sarcoma 45 regression was presumably due to activation of the first-line antioxidant defense enzymes (SOD and catalase). Other shifts included a 33% reduction of GR activity in comparison with intact animals and normalization of reduced glutathione level. Hence, activity of the first-line antioxidant defense enzymes was restored and glutathione content and LPO intensity decreased in rats with complete regression of the tumor. These shifts reflected normalization of metabolic processes in the spleen. Elevation of SOD activity could be due to increase of copper content in the splenocytes (copper is a Cu/Zn-SOD cofactor [9]). On the other hand, SOD and catalase activation could result from the modulatory effect of copper on the immune system [6,9]. Activation of SOD can develop in response to stimulation of phagocytic NADPH oxidase by copper ions, while catalase activation can result from increase of proliferation and functional activity of T-lymphocytes producing catalase for defense and maintenance of proliferative activity [5,9].

Glutathione binds and transports copper ions, regulating copper homeostasis in the cells [5,7]. Some authors think that the Cu^{+}-glutathione complex is the main intracellular metal donor for Cu/Zn-SOD and for proteins carrying and depositing copper [7]. Our results indicate that glutathione level in the spleen decreases in response to copper NP in all animals. On the other hand, the intensity of this decrease does not depend on the route of administration and differs significantly in animals with different antitumor efficiency of copper NP. The reduction of glutathione level was less significant (29%) when the treatment was ineffective. In animals with tumor regression (partial or complete) it reduced at least 2-fold, reaching the values characteristic of intact animals (Table 2). Presumably, the reduction of glutathione concentration in these animals was due to not only a decrease of GR concentration, but largely to effective formation of the copper-glutathione complex. This led to reduction of the toxic effect of copper and normalization of its metabolism. In addition to copper transfer in complexes with proteins and amino acids formed as a result of gradual dissolving and transition of injected copper NP into ionic form, the particles react and are actively captured by lymphocytes and reticular cells of the spleen due to the size and hydrodynamic effects, this, in turn, promoting stimulation of cellular immunity [1].

Hence, injection of copper NP stimulates the proliferation and functional activity of immunocompetent cells in the spleen, including that in animals with tumor growth, though to a lesser degree.

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