The effects of prolonged oral administration of gold nanoparticles on the morphology of hematopoietic and lymphoid organs

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ABSTRACT

Currently, the usage of gold nanoparticles as photosensitizers and immunomodulators for plasmonic photothermal therapy has attracted a great attention of researches and end-users. In our work, the influence of prolonged peroral administration of gold nanoparticles (GNPs) with different sizes on the morphological changes of hematopoietic and lymphoid organs was investigated. The 24 white outbred male rats weighing 180-220 g were randomly divided into groups and administered orally for 30 days the suspension of gold nanospheres with diameters of 2, 15 and 50 nm at a dosage of 190 μg/kg of animal body weight. To prevent GNPs aggregation in a tissue and enhance biocompatibility, they were functionalized with thiolated polyethylene glycol. The withdrawal of the animals from the experiment and sampling of spleen, lymph nodes and bone marrow tissues for morphological study were performed a day after the last administration. In the spleen the boundary between the red and white pulp was not clearly differ in all experimental groups, lymphoid follicles were significantly increased in size, containing bright germinative centers represented by large blast cells. The stimulation of lymphocyte and myelocytic series of hematopoiesis was recorded at morphological study of the bone marrow. The number of immunoblasts and large lymphocytes was increased in all structural zones of lymph nodes. The more pronounced changes were found in the group with administration of 15 nm nanoparticles. Thus, the morphological changes of cellular components of hematopoietic organs have size-dependent character and indicate the activation of the migration, proliferation and differentiation of immune cells after prolonged oral administration of GNPs.

Keywords: gold nanoparticles, functionalization, hematopoietic and lymphoid organs, prolonged oral administration, rats

1 INTRODUCTION

The accelerated development of nanotechnologies has led to their wide use in various industrial and health care applications. As the gold nanoparticles (GNPs) exhibit unique properties, they have been used in various medical applications, including detection of cancerous cells and photothermal plasmonic antitumor therapy.[1,2] Such applications require extensive toxicological studies to investigate the safety of nanoparticles and their effects on organs and systems and on the body as a whole.

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Nanotoxicological studies should be organized in compliance with certain requirements, which include the establishment of the characteristics of nanoparticles, their biokinetics and availability of validated methods of assessing the effects of nanoparticle exposure. Thus, it was found that the size-dependent effects of GNPs depend on the biodistribution of the GNPs in animal body. In 2001, Hillyer and Albrecht first noted size-dependent nature of the gold biodistribution upon oral administration,[3] when 4, 10, 28 and 58 nm GNPs of 200 mg/ml-concentration were administered perorally for seven days with drinking water. The 4 nm-GNPs passed through the gastrointestinal tract freely, absorbed by enterocytes in the villi and have been degraded and then were distributed through the bloodstream to organs. The penetration through gastrointestinal tract into blood stream was decreased with increasing of particle size.

Furthermore, the dose selection remains an important issue in nanotoxicology, because the effects of NPs are largely due to the concentration of nanoparticles in tissues. Currently, most of *in vivo* data obtained after intravenous administration of the nanoparticles,[4,5,6] whereas data about other routes of administration is rather limited.

In work of Zhang et al.,[7] the comparative toxicological study of different administration routes of 13.5 nm GNPs in mice was presented. Animal survival, weight, hematology, morphology and organ index were characterized at different concentrations (137.5–2200 μg/kg) over 14–28 days. The results show that only high concentrations of GNPs induced decrease in body weight, red blood cell (RBC) count and hematocrit. Among three administration routes, the oral and intraperitoneal routes showed the highest toxicity, manifested in significant decrease in body weight, spleen index and RBC count.

Despite numerous studies focused on the biodistribution and toxicity of GNPs, their effects on the immune system were insufficiently studied. In pioneering review of Pacheco[8] the effect of GNPs on the nonspecific immune response was described. It was noted that in blood of rabbits the total leukocyte count increased with small reduction of mononuclear forms and a significant increase of segmented neutrophils after intravenous administration of 5ml of GNPs.

Chen et al.[9] found severe sickness in mice after intraperitoneal administration of naked GNPs ranging from 8 to 37 nm in dosage of 8 mg/kg/week. Pathological examination of the major organs of mice in experimental groups indicated an increase of Kupffer cells in the liver, loss of structural integrity in the lungs, and diffusion of white pulp in the spleen. Modifying the surface of the GNPs by incorporating immunogenic peptides ameliorated their toxicity. This reduction in the toxicity is associated with an increase in the ability to induce antibody response.

In work of Zhang et al.,[10] it was found that intraperitoneal administration of 5, 10, 30 and 60 nm PEG-coated GNPs in mice at dosage of 4000 μg/kg over 28 days caused the increase in spleen index and thymus index that can be explained by GNPs effects on the immune system. Transmission electron microscopic observations showed that the 5, 10, 30 and 60 nm particles located in the blood and bone marrow cells, and that the 5 nm and 60 nm particles aggregated preferentially in the blood cells. The 10 nm GNPs induced an increase in white blood cell (WBC) concentration, while the 5 nm and 30 nm particles induced a decrease of concentration of WBCs and RBCs. Thus, the contradictory data exist on the immunological effects of GNPs, they may cause either pro-inflammatory[11] or anti-inflammatory effects,[12-14] depending on their size, conjugation and hydrophobicity.[15]

The aim of our study was to evaluate the influence of prolonged peroral administration of gold nanoparticles (GNPs) with different diameters on the morphological changes of hematopoietic and lymphoid organs.

2 MATERIAL AND METHODS

2.1 Preparation and characterization of GNRs

Spherical GNPs with average diameters of 2, 15 and 50 nm and a mass-volume concentration of 57 μg/mL were synthesized as previously described (Dykman L.A. et al., 2008). In particular, 15- and 50-nm GNPs were synthesized by the Frens method (Frens G., 1973), and the smallest 2-nm GN were obtained by the Daff method (Duff D.G. et al., 1993). The corresponding particle number concentrations are 8.8×10^{13}, 1.7×10^{12}, and 4.5×10^{10} particles/mL, respectively. The average size of the GNPs was determined from electron microscopic images obtained using a Libra-120 microscope (Carl Zeiss, Jena, Germany) in Centre of Collective Use of Institute of Biochemistry and Physiology of Plants and Microorganisms RAS. For increased biocompatibility, 15- and 50-nm GN were conjugated with thiolated polyethylene glycol mPEG-SH (MW = 5000, Nektar Therapeutics) as described previously (Dykman L.A. et al., 2008). The smallest 2-nm GNPs were used as prepared. The GNP-mPEG conjugates were formed by the covalent binding of

thiol groups to the surface of GNPs. The resulting conjugates were washed twice by centrifugation to remove excess reaction products and resuspended in 0.9% sodium chloride solution.

2.2. In vivo experiments

The experimental study was conducted in Centre of Collective Use of Saratov State Medical University (Russia). The experiments were conducted according to the University's Animal Ethics Committee and the relevant national agency regulating experiments in animals. The international guiding principles for biomedical research involving animals of the Council for the International Organization of Medical Sciences and International Council for Laboratory Animal Science (CIOMS&ICLAS, 2012) were followed during the animal experiments.

The experiment was performed using 24 healthy adult albino male rats weighing 180-220 g. The rats were kept under a constant 12-h light-dark cycle at 22-25°C with standard food and water provided *ad libitum*. The study of morphological changes in the immunogenetic organs was conducted after a 30-day oral administration of GNPs of different sizes. The animals were randomly divided into three experimental groups and a control group (6 animals in each group). Rats in experimental groups were orally administered 1 ml of gold nanoparticles once a day for 30 days at a dosage of 190 μg/kg of body weight as follows: 1st group - 2 nm GNPs, 2nd group - 15 nm GNPs, 3rd group - 50 nm GNPs. Rats in control group were administered orally 1 ml of saline once a day for 15 days. The withdraw of animals and sampling were performed a day after the last administration. Bone marrow sampling was conducted by the standard procedure from the femur. For morphological examination, bone marrow smears stained with May-Grunwald. Morphological analysis of smears performed under high magnification (×1000) with differential counts of 200 cells. For morphological examination, the organ specimens of rats (spleen, lymph nodes) were fixed in 10% formalin solution, then subjected to a standard alcohol and acetone wiring and embedded in paraffin. After sample dewaxing, 5-7 micron thick sections were stained with hematoxylin and eosin. Morphometric analysis of the spleen preparations was conducted using the digital image analysis system Mikrovizor medical μVizo-103 (LOMO, Russia).

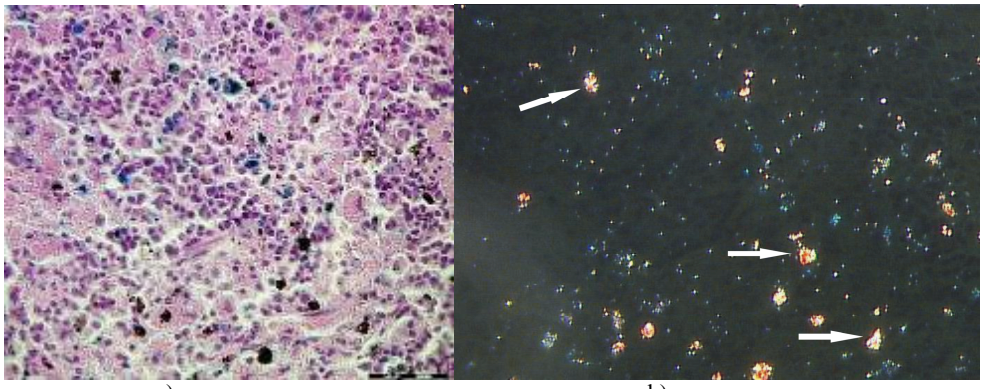
Counting of cell elements of mesenteric lymph nodes were carried out in the various functional areas of the lymph node by the standard method with increasing ×100, ×400, ×1000 using morphometric software Image Analyzer in 10 fields of view on the conventional unit area (6400 μm^2). Digital camera SCOPETEK DCM (Hangzhou Scopetek Opto-Electric Co., China) used to capture histopathological images. The classification of analyzed cell elements was conducted according to required criteria and report generation. The Perls reaction (Merkulov G.A., 1969) was used for the differential diagnosis of hemosiderin and GNP clusters. The reaction with methyl green pyronin by Brachet (Merkulov G.A., 1969) was used to identify and count the number of plasma cells in the lymph nodes.

The dark-field microscopy was used to identify clusters of GNPs in samples using a Leica DM 2500 microscope with a special attachment that permits lateral illumination ×630 and ×1500 magnification.

The experimental data were analyzed using a statistical software package, STATISTICA 10.0 (StatSoft Inc., USA). Preliminary statistical datum processing was performed by the Kolmogorov-Smirnov test to verify compliance of distributions to normal, and the equality of the population variance was determined using the Fisher F-test. The null hypothesis was rejected when $p<0.05$. The arithmetic mean (M) and the error of the arithmetic mean (m) were calculated for each index. The differences between the samples were assessed using Student's t-test.

3 RESULTS AND DISCUSSION

After 30-day of oral administration of GNPs, the morphological changes in the spleen were similar in all experimental groups, the boundary between the red and white pulp was not clearly distinct, lymphoid follicles were significantly increased in size, containing bright germinative centers represented by large blast cells. In all experimental groups of animals, the accumulation of black granules was noted in the red pulp of the spleen. Using the Perls reaction, the granules (hemosiderin) were stained in blue (see Fig. 1 a), while the other granules (GNPs) remained black. The unpainted granules showed a red color in dark-field microscopy of preparations stained by Perls (see Fig. 1 b).



a) b)

Figure 1. Spleen in experimental group with 50 nm GNP administration. Two types of particles in the spleen: granules of hemosiderin are blue, while conglomerates of GNPs are black, staining by Perls. ×630 (a). Glow of GNPs due to intense light scattering, the arrows demonstrate conglomerates of GNPs, dark-field microscopy, ×630 (b).

In the mesenteric lymph nodes, after 30-day administration of GNPs the changes in cellular composition were observed in all experimental groups. In groups with GNPs of 2 nm and 50 nm administration, the changes were found in all functional areas of lymph nodes: the increased zone of lymphoid follicles with germinative centers representing immunoblasts, macrophages and mitotic figures was noted in the cortex (Table 2); the zone of medulla cords was expanded due to increased number of plasma cells; the increased number of small, middle and large lymphocytes was revealed in the paracortical zone. In the group with GNPs of 15 nm administration, we found more pronounced changes in the paracortical area than in the areas of lymphoid follicles and medulla cords (Table 1).

Table 1

The morphological changes in mesenteric lymph nodes after 30-day oral administration of GNPs

Zones of lymphatic nodes	The number of cells (within area of 6400 μm^2)	Control group	Experimental groups with GNPs administration		
			2 nm	15 nm	50 nm
Lymph follicles	Small lymphocytes	64.2 ± 2.1	73 ± 1.1*	68.7 ± 1.4	76.6 ± 1.4 *
	Middle lymphocytes	18.2 ± 0.4	30.1 ± 1.8*	30.2 ± 0.6*	25.7 ± 1.3*
	Large lymphocytes	0	1.4 ± 0.2*	10.4±0.6*	10.3 ± 0.6
	Immunoblasts	2.5 ± 0.3	4.2 ± 0.9*	3.1 ± 0.6	2.7 ±0.2
	Plasmocytes	0	0	0	0
	Mastocytes	0	0	0	0
	Mitotic figures	0.2 ± 0.1	1.2 ± 0.1*	1.0 ± 0.4*	0.9 ±0.1
Paracortical zone	Small lymphocytes	60.1 ± 1.2	80.1 ± 1.6*	70.6 ± 2.1*	70.3 ±0.8*
	Middle lymphocytes	12.1 ± 1.1	19.6 ± 1.4*	20.4 ± 1.6*	26.7 ± 1.4*
	Large lymphocytes	0.8 ± 0.1	2.0 ± 0.4*	1.4 ± 0.7*	1.8 ± 0.2*
	Immunoblasts	0	0.8 ± 0.1	3.0 ± 0.4*	1.8 ± 0.5*
	Plasmocytes	0.7 ± 0.1	1.6 ± 0.2*	16.4±0.2*	1.0 ± 0.1
	Mastocytes	0	0	0	0
	Mitotic figures	0.8 ± 0.16	1.4 ± 0.1*	1.5±0.2*	1.0 ± 0.2
Medulla cords	Small lymphocytes	20.2 ± 1.3	28.6 ± 2.2	24.8 ± 1.9	24.7 ± 1.4
	Middle lymphocytes	10.2 ± 1.7	19.2 ± 0.8*	16.8 ± 0.9	16.2 ± 1.6*
	Large lymphocytes	10.2 ± 0.7	16.7 ± 0.8*	10.8 ± 0.7	8.2 ± 0.6*
	Immunoblasts	2.3 ± 0.3	2.5 ± 0.5	4.9 ±0.5*	4.0 ± 0.2*
	Plasmocytes	15.3 ± 1.6	21.8 ± 1.6*	33.5 ±0.6*	30.2 ±0.2*
	Mastocytes	0.3 ± 0.01	0.8 ± 0.1	1.1 ± 0.1*	1.0 ±0.1*
	Mitotic figures	0.7 ± 0.1	1.9 ± 0.2*	1.2 ± 0.4*	0.9 ± 0.1

Note: * — The significance of differences with control group.

Under dark-field microscopy, conjugates of GNPs were found in the cytoplasm of macrophages and lymphocytes predominantly in the mantle zone of the lymphoid follicles and in medulla cords in the groups administered 15- and 50-

nm GNPs. Extracellular accumulation of GNPs was detected in medulla sinuses. It should be noted that 2-nm GNPs were not recorded by available methods in any zones of the lymph nodes.

The signs of stimulation of leukocyte hemopoietic germ were observed at morphological analysis of the bone marrow after administration of all types of GNPs — the increase of the number of myelocytes, metamyelocytes and stab leukocytes was observed in all groups (Table 2). At the same time, the decrease of amount of the mature forms was noted after 30-day administration of GNPs, which causes the reduction of leukocytic-erythroblastic ratio.

Table 2

The morphological changes in bone marrow after 30-day oral administration of GNPs

Zones of lymphatic nodes The number of cells (within area of 6400 μm^2)	Control group	Experimental groups with GNPs administration		
		2 nm	15 nm	50 nm
Myeloblasts	1.0 ± 0.7	1.3 ± 0.7	0	1.0 ± 0.4 *
Neutrophil myelocytes	1.2 ± 0.3	6.0 ± 1.1*	3.8 ± 1.1	10.0 ± 1.4*
Neutrophil metamyelocytes	3.0 ± 0.7	12.5 ± 0.7*	11±0.4*	14.0 ± 2.0*
Neutrophil stab leukocytes	7.5 ± 1.8	16.5 ± 1.3*	18.3 ± 4.4*	14.5 ± 1.7*
Neutrophil segments	32.8 ± 8.3	21.5 ±2.4*	24.5 ± 1.8	25.0 ± 2.7
Eosinophils	1.7 ± 0.7	2 ±0.8	0.8 ± 0.4	0.8 ± 0.4
Basophils	0.3 ± 0.7	1.3 ± 0.5	1.0 ± 0.5	0.9 ±0.1
Monocytes	0.7 ± 0.1	2 ± 0.7*	3.0 ± 0.5*	0.5 ±0.5
Lymphocytes	16.0 ± 1.2	18.5 ± 2.3	20.3 ± 1.9	14.5 ± 2.1
Erythroblasts	0	0	0	0.3 ± 0.2*
Basophilic normocytes	1.0 ± 0.1	2.3 ± 0.7	3.0 ± 0.4*	2.3 ± 0.6*
Polychromatic normocytes	12.0 ± 0.2	14.5 ± 1.2	15.5±1.3	16.0 ± 1.4
Oxyphilic normocytes	3.0 ± 0.3	2.8 ± 0.5	2.3 ± 0.3	1.3 ± 0.5*
Leukocytic-erythroblastic ratio	2.6 ± 0.2	1.9 ± 0.1*	1.6±0.2*	2.0 ± 0.5

Note: * — The significance of differences with control group.

The changes in leukocyte ratio in the bone marrow and stimulation of the granulocytic lineage of hematopoiesis are probably related to the decrease in the number of leukocytes in peripheral blood after GNP_S administration. These results are consistent with the work of Zhang et al.,[10] where the size-dependent GNP biodistribution in the blood and bone marrow cells was noted at intraperitoneal injection and 5 nm and 30 nm particles induced a decrease of WBC population.

According to our previous results,[16] the signs of strengthening of the processes of cell proliferation and differentiation of lymphoid cells in lymph nodes were noted after prolonged administration of GNPs. The real-time photoacoustic mapping of lymphatics and detecting metastasis in sentinel lymph nodes using 30 nm spherical polyethylene glycol (PEG) coated magnetic nanoparticles and golden carbon nanotubes as contrast agents on melanoma-bearing mouse model were demonstrated.[17] The photothermal treatment of nanoparticled labeled non-pigmented breast cancer cells in sentinel lymph nodes was also shown in this paper. Previously we demonstrated that after single and repeated intraperitoneal administration of magnetite nanoparticles, their assemblages have been found at sites of injections and in lymph nodes of abdominal cavity.[18] Under continuous nanoparticle administration, their accumulation was so pronounced that lymph nodes were well contrasted at MRI.

In this study, we showed that prolonged peroral administration caused accumulation of GNPs in mesenteric lymph nodes. In the future study, it may be used for hyperthermia of mesenteric lymph nodes containing tumor cells (metastasis), and abdominal carcinomatosis.

4 CONCLUSION

In this work, we have studied the morphological changes in hematopoietic and lymphoid organs after a prolonged oral administration of differently sized gold nanoparticles. Finally, 2-, 15-, and 50-nm GNPs were administered for 1 mL of GNPs once a day for 30 days at a dosage of 190 μg/kg of body weight and the morphological changes was assessed after the end of administration of GNPs. The main results of the study are as follows.

After the prolonged administration of GNPs, the activation of proliferation and differentiation of immune cells and macrophages were observed in the spleen and lymph nodes. The 50-nm GNPs caused the most pronounced dystrophic and necrobiotic processes, and 2-nm GNPs caused proliferative changes.

The morphological examination of the bone marrow indicated stimulation of myelocytic germ of hematopoiesis under GNP administration. Specifically, we have recorded statistically significant increase in neutrophil metamyelocytes and neutrophil stab leukocytes for all GNP sizes and decrease in neutrophil segments which point to the stimulation of leukocyte hemopoietic germ.

The prolonged peroral administration caused accumulation of GNPs in spleen and mesenteric lymph nodes, which may be used further for hyperthermia of mesenteric lymph nodes with metastasis and abdominal carcinomatosis.

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