Chitosan-Coated Gold Nanoparticles as Promising Nanosystem for Directional Drug Delivery to Target Organs

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Abstract: The present research under the application of a chitosan-coated gold nanoparticles nanosystem was made on experimental animals into the chitosan transportation property of orally administered nanostructured gold delivery into the bloodstream and its further distribution through the animals' organs. Transportation properties are dependent on the molecular weight of chitosan: in case the molecular weight is equal or more than 30'000 an accumulation of nanoparticles is found in kidney, liver, lung and spleen as opposed to the oligomer of chitosan with the molecular weight of ~ (5-10)* 10^3 when the nanostructured gold is detected in kidney and liver only. The adaptogenic activity of the chitosan-coated gold nanoparticles nanosystem was revealed in conditions of hypoxia simulation. A significant feature of the nanosystem is that the nanostructured gold is completely egested from an organism within a month.

Keywords: Chitosan-coated gold nanoparticles, carrier properties, organ distribution and egestion under oral alimentation.

1. INTRODUCTION

Nowadays an application of nanotechnologies in medicine and pharmaceutics finds its way in the development of new directional effect drugs, targeted bioactive substance delivery systems and highly efficient early-stage cancer diagnostic systems [1-3]. Nanoparticles used as carries to transport drug may vary in forms (nanotubes, nanocapsules & etc.) and nature (polymeric and inorganic).

One of the most important classes of nanocarriers comprises nanostructured inorganic particles-silicon compounds, various metals (gold, silver, platinum), fullerenes and carbon nanotubes. The use of noble metals and gold nanoparticles (NPs) in particular enables the development of delivery means that are clearly distinguished by a certain number of unique qualities: the inertness, antioxidant activity, controlled release of a therapeutic agent as well as the development of such preparations as thermal-sensitizers [3-6].

To keep the dispersion high nanoparticles are stabilized with natural and synthetic compounds: proteins, polyethylene glycol and its derivatives, polyamides, maltodextrins and gum-arabic [7-10]. In this case their biodistribution in organs is considerably affected by the surface functionalization of nanoparticles due to stabilizer. However, these stabilizers have some lacks. Thus, macromolecules of maltodextrins as well as PEG and derivatives by virtue of their electrical neutrality cause nonspecific delivery of NPs in all cell structures of the organism; moreover the latter are strong oxidants. It is inappropriate to use polyamides inasmuch as products of their utilization in the organism exhibit neurotoxic effect. As for proteins they are decayed by proteases what will occur of the NPs agglomeration. In this connection the use of semi-synthetic polymer chitosan to stabilize the noble metals for their application in biotechnology, medicine or pharmaceutics is quite promising. In addition the polymer behaves as a metal ions reductant due to its structural reactive functional groups. The interest in this polymer relates to its unique physiological and eco-logical properties such as biocompatibility, biological degradation, physiological activity and availability of raw deposits [11-13]. The polymer is nontoxic and easily re-adsorbed. It can be gelatinized and displays antacid and antiulcer activities. Furthermore chitosan can function as an adsorbent and a carrier of drugs. It is also capable of penetrating into the intercellular substance [14]. The experiments previously carried out by us on animals exhibited distinct antioxidant properties and an adaptogenic effect of chitosan that were revealed in the therapy course of the X-ray sickness caused by an exposure to the injurious 5 Gy γ-radiation (a decrease in free-radical oxidation activity, a recovery facilitation of haematogenic functions of the damaged marrow) [15]. The research [16] displayed high effectiveness of the chitosan-coated gold nanoparticles being used as insulin delivery means still maintaining high biological activity.

The review [17-20] based on available publications shows the detailed analysis of the organ distribution

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and toxicity of gold NPs versus their size, dose and the nature of stabilizing agent. The authors point out that gold NPs within the range in size from 3 to 20 nm hardly possess any toxic properties meanwhile clusters sized between 1-2 nm can build irreversible bonds with key biomolecules (DNA, RNA, ferments and etc.) and entail changes in functioning of the intracellular molecular processes. As proposed there won't be any pronounced injury noted that is going to be caused by gold nanoparticles to an organism on condition that within a short-term (for about a week) administration of the gold NPs its daily dose shall not exceed 0.5 mg/kg. It is also worth mentioning that in most cases the research works refer to administration of the gold NPs principally by way of injection in doses ranging between 0.1 and 10 mcg/g. Any information on per oral administration of the nanostructured gold, its penetration into organisms and its further biodistribution through targeted organs is limited. According to the authors [21] 4 nm in size dextrin-stabilized gold nanoparticles administered to mice orally in concentration of 200 mcg/ml is capable of penetration into the bloodstream and redistribution through 9 organs. The maximum concentration of gold NPs (Au NPs) was registered in kidney (app. 0.075 mcg/g) meanwhile other organs showed concentrations of about 0.035-0.02 mcg/g (small intestine, lung, stomach, spleen and liver) and even brain (4.7 ng/g). At the same time there is no data either on the distribution of orally administered nanostructured chitosan-stabilized gold and the kinetics of its egestion from the organism. The latter is extremely important in view of the possible cumulation of the NPs, and in some cases its toxicity [22].

In this account the objective of our study was to carry out research into the distribution of orally administered chitosan-coated gold nanoparticles through experimental animals' organs, the kinetics of their egestion from the organism and its bioactivity under model condition of hypoxia.

2. EXPERIMENTAL SECTION

2.1. Used Materials

The research was made with the use of chitosan with the number-average molecular weight of 1.3×10^5 and chitosan oligomer with the molecular weight of 4×10^3 and deacetylation degree of 80%. The AuNP was prepared by way of the UV-induced reduction of the hydrochloro-auric acid in chitosan solutions during 2 hours at 25 °C and further thermostatting at 75 °C during 1 hour. The complete $HAuCl_4$ conversion into Au NPs in the time of this experiment was proved by SAXS method. The kinetics of Au NPs formation was spectrophotometrically controlled by the appearance and increasing of the absorption band at wavelength range 510-550 nm corresponding to the Au NPs plasmon resonance (Figure **1a**). Figure **1b** shows the change of the Au NPs relative content (ω, %) in the chitosan solution during UV-irradiation and heat treatment. The dimensional characteristics of gold nanoparticles were obtained from the SAXS and TEM data. The TEM studies were performed on a Morgagni 268D (FEI) microscope with an optical intensification in 9×10^4. Sample objects were thin films obtained by evaporating solutions, in which the formation of gold nanoparticles was completed. The TEM images of gold nanoparticles formed in a solution of chitosan are shown in Figure **2**. One can see that the nanoparticles have a spherical shape, the nanoparticle system is low polydisperse. The average size of Au NPs in this biopreparation ranged from 3 to 10 nm. The histogram shown in Figure **2** (b) was plotted using the samples of more than 100 particles. Authors of [14, 16] indicate that the Au NPs with the size range from 3 to 20 nm are nontoxic.

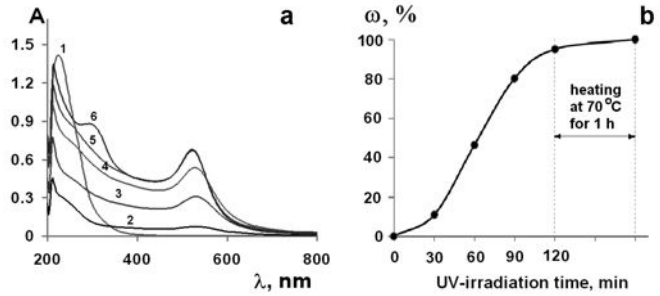

Figure 1: The kinetics of gold nanoparticles formation in 3 wt.% chitosan solutions containing 2 wt.% acetic acid with initial concentration of $HAuCl_4$ 3 wt.% by UV-irradiation: (**a**) the changes in absorption spectra of solutions; (**b**) the change of the Au NPs relative content (ω, %) in the chitosan solution during UV-irradiation and heat treatment.

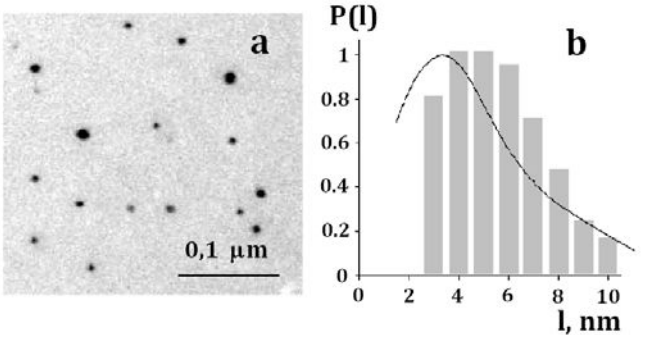

Figure 2: TEM images of gold nanoparticles (**a**). The nanoparticles size distribution according from to TEM (histogram) and SAXS (curve) (**b**).

We assume that the stabilization of nanoparticles by chitosan macromolecules is due to the formation of the polymer-particle complexes due to adsorption forces and OH- and NH_2-groups. On the one hand, it occurs due to the hydroxyl and unprotonated amino groups of chitosan (Figure **3**).

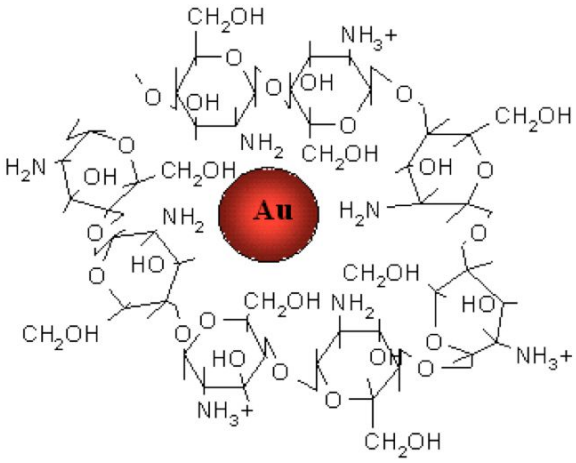

Figure 3: Stabilization of gold nanoparticles by chitosan macromolecules in its solution.

2.2. Biological Experiment

Experiments were carried out on white nonpedigreed male rats each being of about 200-250 g in weight and kept in vivarium. There were 35 animals used altogether divided into 7 groups: one intact and 6 test groups with 5 specimens in each group. Animals of the first three groups were administered the chitosan-coated AuNP while the oligochitosan-coated AuNP was administered to others of the rest three groups.

The preparation solutions were administered once a day for a period of 7 days orally through a feeding tube measured out in doses of 1 ml/day per animal which equals to the chitosan (oligochitosan) dose content of 100 mg/kg and the chitosan-coated AuNP dose content of 0.5 mg/kg. On the 1st, 5th, 10th, 15th and 20th day after the end of preparations administration the animals were decapitated under the ether anaesthesia, one specimen from each group respectively, and their organs (brain, heart, lung, spleen, kidney and liver) were eviscerated. To determine probes for gold the probe preparation and the method of autoclaving mineralization were performed in compliance with the methodological guidelines (MG 41.985-00).

Samples were determined for gold by means of the atomic emissive analysis. The samples first reduced into a stable analytical form were placed into the crater of graphite electrodes whereat the spectrum was excited by direct current arc generated by the Universal Generator (UG-4). To register spectrum a scanning dispersion spectrograph Diffraction Spectrograph (DS-1) was used.

The study of chitosan-coated AuNP antioxidant activity was carried out on scrub male rats with a mass of 250-300 g grown in a vivarium with free access to food and water and natural daily alternation of light. The rats were divided into four groups of seven animals. The first group consisted of intact animals (relative norm); the second (control) group consisted of the rats treated with a physiological solution; the third group consisted of the rats treated with a chitosan solution (dosage of 100 mg/kg); the fourth group consisted of the rats treated with a chitosan-coated AuNP dispersion (chitosan — 100 mg/kg; gold — 0.5 mg/kg). We previously showed that such doses of the medicine were effective [7, 11]. The medicine (1 mL per animal) was given orally by enteral feeding tube once a day for 7 days. It should be noted that there were no animals treated with gold nanoparticles because the nanoparticles cannot be in a colloidal solution without a stabilizer.

It was found that the development of a nonspecific adaptive reaction of the training appeared one week after introduction of the nanomedicines. Thus, simulation of hypoxia was performed 7 days after the end of the procedures. Animals of groups 2-4 were placed in a pressure chamber for 30 min, creating a vacuum corresponding to the height of 8000 m. Blood analysis was carried out the next day after exposure in the pressure chamber.

Blood samples were taken from the sublingual vein of anesthetized animals. In the blood plasma, the content of lactate dehydrogenase activity [21] were determined. Research data were statistically processed by the BIOSTAT software. Independent data samples were compared using a one-way ANOVA test and nonparametric Kruskal-Wallis and Newman-Keuls tests.

3. RESULTS AND DISCUSSIONS

Aminoglucans and chitosan in particular due to the whole complex of unique properties can be used as highly effective stabilizers of metal nanoparticles and transporters of medical preparations with a prolonged effusion effect. One of the most significant properties of chitosan that makes interest from the view point drug

uptake in pharmaceutics and medicine is its muco-adhesiveness [23]. Cationic chitosan polysaccharide reacts with sialic acids of the digestive tract from stomach to colon. Due to a prolonged interaction of the polysaccharide with the wall epithelium of a digestive tract the bioadhesion of chitosan extends time of the medications residence in the track.

A research [24] made on a cell culture shows that apart from the above mentioned properties macromolecules of chitosan can work way through the cells' cytoplasmic membranes. In this regard the ability of chitosan to open intercellular structures is not dependent on its deacetylation degree so long as it remains above 60 % whereas it is the molecular weight that is more important. Optimum molecular weights of the polysaccharide stay around ~ 34'000.

Thus the issue of penetration of orally administered nanostructured chitosan-stabilized gold into an internal environment of an organism and its further distribution through organs remains open.

Peroral administration of preparations deems to be much more preferable due to its simplicity, non-invasiveness, patency and analgia. In this connection we have investigated the distribution of the AuNP stabilized with chitosan of various molecular weights (~10^{5}-chitosan and ~3×10^{3}-oligochitosan) through experimental animals' organs subject to a protracted administration of the preparation. Supporting information is presented in Table **1**.

The comparison of data obtained from the article [16] and that of the Table as above provides enough evidence that the distribution of AuNP through organs is significantly influenced by the nature of a stabilizer. The chitosan-coated AuNP does not penetrate the hematoencephalic barrier and elude detection in brain as contrasted to such systems as polyethylene oxide-coated AuNP and dextrin-coated AuNP. However the distribution of nanoparticles through organs is significantly affected by the molecular weight of a polysaccharide. On condition that doses of administered preparations are equal the application of chitosan with molecular weights of ~ 10^{5} provides for a higher (almost twice as high) gold content in organs. It struck the eye that in case a preparation of the oligochitosan-stabilized AuNP was administered the excrements of experimental animals took a pink-lilac dye which is indicative of an insignificant absorption and its unobstructed flow through the intestines as contrasted to a preparation of the macromolecular chitosan-coated AuNP.

24 hours since the end of administration of chitosan with the molecular weight of 4'000 the gold content was detected in two organs only: kidney that is an excretion organ in itself and spleen a function of which among others is the phagocytosis of non-indigenous cells and particles as well as accumulation of antigens with a subsequent activation of lymphocytes. There was no gold detected in other tissues. On the 5^{th} day the gold content continued to decrease. By the 10^{th} day there was no gold practically detected in any organs. Chitosan with the molecular weight of ~ 10^{5} used to stabilize AuNP allowed its delivery into the tissues of kidney, liver, lung, spleen and heart.

In our opinion there are certain factors that stipulate for the delivery of AuNP stabilised with the charged

Table 1: Gold Nanoparticles Distribution through Organs of Experimental Animals

Time elapsed after discontinuance oligochitosan-AuNP preparation administration to rats*, days	Gold Content in Organs (mg/kg)					
	Kidney	**Liver**	**Brain**	**Lung**	**Spleen**	**Heart**
1	0.12	Not detected	Not detected	Not detected	0.13	Not detected
5	0.10	Not detected	Not detected	Not detected	0.05	Not detected
10	0.06	Not detected	Not detected	Not detected	Not detected	Not detected
Time elapsed after discontinuance polychitosan-AuNP preparation administration to rats*, days	**Gold Content in Organs (mg/kg)**					
	Kidney	**Liver**	**Brain**	**Lung**	**Spleen**	**Heart**
1	0.25	0.16	Not detected	Not detected	0.06	Not detected
10	2.56	0.56	Not detected	0.06	0.13	Not detected
15	2.56	0.19	Not detected	0.10	0.16	Not detected
20	1.28	0.13	Not detected	0.06	Not detected	Not detected
28	0.05	Not detected	Not detected	Not detected	Not detected	Not detected

*Single dose AuNP-0.5 mg/kg, chitosan-100 mg/kg; cumulative dose AuNP-3.5 mg/kg, chitosan-700 mg/kg.

polycation-chitosan to various organs. The epithelial layer cells of the mucous lining of intestine are interconnected by tight junctions that exclude any gaps in between and do not allow any ions or molecules to penetrate through the intercellular space from the cavity of alimentary canal into the extracellular matrix. The adsorption and transportation of molecules of various substances take place in microvilli of enterocytes. Chitosan possesses a unique property to draw such tight junctions apart and allow for the transfer of nanoparticles between enterocytes to the basal membrane.

A basal membrane contains collagen, glycoprotein and negatively charged heparan sulfate protoglucans (mucopolysaccharides) that function as molecular sieves filtering molecules by size and charge. Neutrally and positively charged molecules with the radius of up to 4 nm (2.5 nm) easily sift such filter down to the endothelial cells of blood capillaries of the intestinal villi. The positive charge of chitosan molecules enables gold nanoparticles to pass even such a barrier successfully. The cells of endothelial capillaries have pores of diameters of up to 70 nm (fenestrated endothelium) and allow for the flow of almost any substance with the exception of oversized molecules. Nanoparticles penetrate through the capillary pores of fenestrated endothelium into the bloodstream which further transports them to organs. There are interspaces between the tissue capillary cells of the minimum width of ~ 4 nm. They are normally wider in post-capillary venules than in arterial capillaries. These peculiar features provide for the penetration of nanoparticles into the tissue of organs.

Meanwhile the way of nanoparticles penetration to cytoplasm through an apical membrane by means of pinocytosis and its further transportation through a basic-lateral membrane into the intercellular substance and finally into the bloodstream is not excluded.

It is worth taking notice of the fact that in our case contrary to results of the research as in [25,26] there was no nanostructured gold detected in the brain tissue. This apparent discrepancy seems to arise from the difference in the nature of the nanoparticles stabilizers: uncharged polyethylene glycol and dextrins as well as charged macromolecules of chitosan. There is no intercellular space in the walls of brain capillaries as they are alternately enlaced with the astrocytes processes thus creating a hematoencephalic barrier (HEB). It should be also noted that a HEB does not interfere with the diffusion of liophilic molecules and molecules of polyethylene glycol and dextrin in particular while charged molecules of the chitosan-stabilizer prevent nanoparticles from their penetration into brain tissues.

The difference revealed in the penetration ability and distribution of the chitosan-stabilized AuNP with molecular weights of chitosan of ~ 10^3 and ~ 10^5 through an organism may arise from two factors. On the one hand the number of NH_2- groups in a macromolecule of polysaccharide with molecular weight of ~ 10^5 is ~ 20-25 times higher than that in oligochitosan and these are NH_2- groups that determine the mucoadhesive properties of chitosan thus providing for a longer interaction with the walls epithelium and a more effective penetration into the intercellular junctions. On the other hand the survey [23] shows that a positive charge alone is not sufficient enough to open tight junctions between the epithelial cells and that a polymer must have a substantially high molecular weight. At the same time macromolecules with the molecular weight of ~ 34'000 are mostly fit for the effective opening of cytoplasmic membranes.

In our research the most effective delivery of nanostructured gold to organs was observed when the use was made of macromolecular chitosan. Chitosan is known to be first hydrolysed and then fermented and split in the alimentary tract into fragments of different molecular weights down to glucosamine. Therefore the change in the molecular weight and viscous properties of chitosan and those of a nanocomposite of the chitosan-coated AuNP were examined in vitro under the application of a multienzyme composition "pancreatine" (Figure **4**). The viscosity of the primary chitosan solution and the preparation of chitosan-coated AuNP twice as much differs on condition that the molecular weight of chitosan is identical. The latter is conditioned by a more compact structure of the nanocomposite as compared to a macromolecule of the native polysaccharide. Within the accuracy limits of viscosimetric method error the molecular weight of chitosan in both cases decreased equally (down to 30'000) which agrees with the most optimal value required for the effective penetration of preparation components into cells of an organism [26].

Thus molecules of chitosan with molecular weights of ~ 10^5 provide not only for the yield of stable through time gold nanoparticles but deliver them directly to targeted organs of an organism. This being the case the enzyme system of an organism splits chitosan macromolecules down to the most optimal value of

their molecular weight (~ 30'000-35'000) which is fit mostly for the effective opening of the intercellular junctions. The cationic nature of a polysaccharide-stabilizer prevents AuNP from their penetrating into brain tissues.

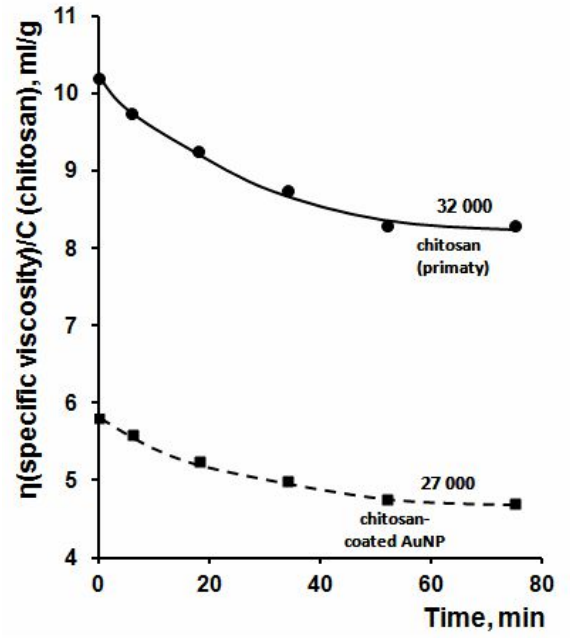

Figure 4: Variation with time in the reduced viscosity value of the solutions of chitosan and chitosan-coated AuNP treated with pancreatine.

In the after light of obtained evidence the biological activity of the chitosan-coated AuNP system deemed to be worth studying on experimental animals in conditions of peroral administration. One of indicators of the biological activity is the antioxidant property of a preparation. In this research the hypobaric hypoxia was regarded as an oxidative stress model. It is well known that an increase in the activity of free-radical processes induces activation of the stress-limiting antioxidant system in the organism. Schiff bases are final products of lipoperoxidation. They are toxic and exhibit mutagenic and membrane destabilizing properties. The results of biological activity investigations of chitosan-coated AuNP in condition of its peroral administration under hypoxia are presented in Table **2** and Figure **5**. It can be seen that the content of Schiff bases is significantly lower in the fourth group (threefold decrease) as compared to the control (physiological solution) and twofold lower as compared to the third group (chitosan) and does not differ from the values for intact animals.

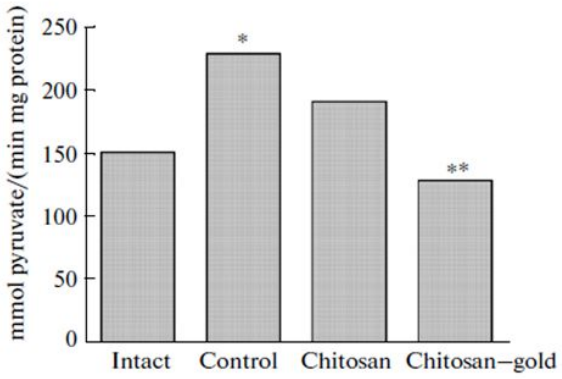

Figure 5: LDH activity in the plasma of the rats after the simulation of hypoxia upon prophylactic application of the medicine. Statistically significant differences: *- p ≤ 0.05 in relation to the intact animals; **- p ≤ 0.05 in relation to control (physiological solution).

It should be noted that in hypoxia conditions abnormality of energy metabolism take plase. Lactate dehydrogenase (LDH) is a key enzyme of energy metabolism appearing at the point between the aerobic and anaerobic carbohydrate oxidation path way. Activity of LDH in the blood plasma is significantly changed under hypoxia. A 48% increase in the LDH activity ($p < 0.05$) in the control group is an indicator of the abnormality of the bioenergetic processes, such as a sharp increase in anaerobic glycolysis, which leads to the accumulation of lactic acid and acidification of the environment, which have an adverse effect on the body up to the state that is incompatible with life (Figure **5**).

Table 2: Number of Leukocytes, Segmented Neutrophils, and Lymphocytes and Leukocyte Index the Next Day after a Simulation of Hypobaric Hypoxia upon Prophylactic Application of the Chitosan-Coated AuNP

No.	Group	Leukocyte number, 10^9	Segmented neutrophils, %	Lymphocytes, %	Leukocyte index
1	Intact	11.30 ± 1.90	28.20 ± 1.20	52.60 ± 3.20	1.90 ± 0.10
2	Control (physiological solution)	19.30 ± 4.30*	35.80 ± 1.48*	43.30 ± 1.40	1.30 ± 0.10*
3	Chitosan, MM = $1.3*10^5$, dose 100 mg/kg	12.76 ± 2.30	26.50 ± 0.90	56.80 ± 1.50**	2.20 ± 0.10**
4	Chitosan-coated AuNP, chitosan dose 100 mg/kg AuNP dose 0.5 mg/kg	9.46 ± 1.83**	23.40 ± 1.00	58.80 ± 2.40**	2.60 ± 0.20**

Note: Statistically significant differences: *-p ≤ 0.05 in relation to the intact animals; **-p ≤ 0.05 in relation to control (physiological solution).

LDH activity during hypobaric hypoxia in group 4 treated with the chitosan-coated AuNP was lower than in the control group and did not significantly differ from the values of the intact animals. These facts indicate that the cells exchange anaerobic oxidation of pyruvate to aerobic under the condition of oxygen deficiency. In the experimental group 3 treated with chitosan, LDH activity was lower than in the control group, but higher than in the animals treated with the chitosan-coated AuNP. The results of the investigation confirm the higher adaptogenic and antioxidant efficiency of the chitosan-coated AuNP system compared to chitosan.

4. CONCLUSIONS

The research carried out on experimental animals evinced the ability of the chitosan-coated AuNP nanosystem to facilitate penetrate into the bloodstream and further AuNP distribution through experimental animals' organs which is in its turn may encourage the development of directional drug delivery systems using gold nanoparticles as carriers. It should be also noted that the nanosystem is in itself biologically active and exhibits adaptogenic and antioxidant properties. The results of the Au NPs organ distribution are significant for local thermal-sensitizing cancer therapy. It is helpful for determine the optimal time of the therapy beginning, when concentration of Au NPs in animal's organs becomes maximal. The highest gold cumulation in the experimental animals organs is achieved in 7 days after the end of preparation administration. 28 days later the Au NPs in the animals' organs was not found.

5. ACKNOWLEGMENTS

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