Anticancer Activity of Multicomponent Nanostructured System «Gold Nanoparticles-Apitoxin-Chitosan»

A.E. Mochalova, A.S. Koryagin, E.V. Salomatina, V.N. Dydykina, Yu.D. Zotova and L.A. Smirnova*

N.I. Lobachevsky State university of Nizhniy Novgorod, 603950 Nizhniy Novgorod, Gagarin ave, bld. 23, korp. 5, Russia

Abstract: The anticancer activity of the multicomponent nanostructured drug "gold nanoparticles – apitoxin - chitosan" was revealed on white rats with implanted carcinoma under injecting encompassing of neoplasms. Drug at therapeutic doses when the apitoxin containing in it one degree lower than toxic (DL 50) effectively inhibits the growth of implanted tumor. On the 28th day after the course administration the external surface area of the tumor decreases in 5 times compared to control animals. Antitumor effect is also reflected as the normalization of free-radical processes. Indicators of biochemiluminescence (I_{max}) of animals reduces from 250 mV with implanted tumor in the control group to 150 mV of animals with implanted tumor which injected the drug. The value of leukocyte coefficient which characterizes the status of the animals organism (normal or stress), in experiments with the drug do not significantly differ from the normal values (5.36 ± 0.72; 6.73 ± 1.09 respectively) and appreciably higher than in control group of animals with implanted tumor that the drug is not administered (1.91 ± 0.15). Leukocyte ratio 1.91 shows that control animals are under stress.

Keywords: Nanostructured drug "gold nanoparticles-apitoxin-chitosan", animals, implanted tumor, inhibition of tumor growth, antioxidant properties.

INTRODUCTION

The neoplasms especially the malignant tumor unalterably take the second place in the structure of the disease incidence and human mortality [1]. The progression of malignant tumors is accompanied by the abnormality in various organ systems. In the first place the tumor negative influences on the immune status of the organism [2, 3]. It is obvious that the imbalances in the free radical lipid peroxidation (RLP), antioxidant protection [4], as well as decrease of the organism immune status take place during the malignant tumors development.

The drugs which are used for cancer chemotherapy have a very narrow therapeutic action spectrum. The necessary doses for achievement of antitumor effect do not differ greatly from the doses which are able to cause toxic effects [1]. In this regard it is important to search the therapeutic agents of a new generation with multifunctional properties which possessing the low toxicity will exhibit antitumor, antioxidant and immunotropic effects.

Scientists of Medical School of Washington University have shown antitumor activity of melittin - the main component of apitoxin - in experiments on mice [5]. It is common knowledge [6] that the strength of therapeutic effect caused by apitoxin is higher than melittin. However, the investigations of apitoxin as a complex of biologically active substances were not carried out under the tumor processes [6].

It should be noted that apitoxin possesses the protein nature and consequently is able to be broken down by the different organism proteases. This limits the time of its "life" and the time of manifestation of its physiological effect in tissues [7-9].

Minimization of mentioned disadvantages is able to be achieved by the formation of multicomponent systems consisting of nano-sized indifferent core with adsorbed the apitoxin components on it, which surface-coated by biocompatible, biodegradable polymer. It is perspective to use the gold nanoparticles as nano-sized core (Au NPs) because gold is inert and indifferent to living tissues, and its nanoparticles can penetrate into the blood stream. Authors of [10, 11] indicate that the Au NPs with the size range from 3 to 20 nm are nontoxic. It is appropriate to use the natural origin polymer chitosan - 1,4-poly(2-deoxy-2-amino-D-glucose) - as the shell due to its unique properties such as biocompatibility, biodegradability, hypoallergenic [12, 13]. The cationic nature of chitosan provides its ability to open out the tight junctions of the intestinal epithelium and penetrate into the intercellular space [14]. The creation of such drugs will allow to use it both for injection and for oral administration.

This article aimes at the investigation of the antioxidant and antitumor activity of nanostructured system "Au NPs - apitoxin - chitosan" under injection of

*Address correspondence to this author at N.I. Lobachevsky State university of Nizhniy Novgorod, 603950 Nizhniy Novgorod, Gagarin ave, bld. 23, korp. 5, Russia; Tel: 8(831)4657223; Fax: (831) 462 32 20;
E-mail: smirnova_la@mail.ru

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the aforementioned substance to the laboratory animals with the transplantable tumor of liver carcinoma strain RS-1.

MATERIALS AND METHODS

This work was carried out on white nonlinear rats-females with average weigh 150 - 200 g. All procedures with laboratory animals were implemented in compliance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 18 March 1985 g); "International guidelines for biomedical research involving animals" (1993); "Rules of laboratory practice in the Russian Federation" (order of the Ministry of Health RF № 267 from 19.06.2003) and "Rules of works using experimental animals" (Ministry of Health RF № 3755 from 03.12.1977).

The research was made with the use of chitosan with the number-average molecular weight of 1.3×10^5 and deacetylation degree of 0.80 - 0.82. The apitoxin was obtained in apiaries of Bor district of Nizhny Novgorod region by electrical stimulation. The AuNPs dispersions were prepared by way of the UV-induced reduction of the hydrochloro-auric acid in apitoxin aqueous solutions, and then chitosan aqueous solution was added in obtained substance consequently complexes AuNPs – apitoxin were coated by this polymer.

The dimensional characteristics of gold nano-particles were obtained from the 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 **1**.

One can see that the nanoparticles have a spherical shape, the nanoparticle system is polydisperse. The average size of Au NPs in this biopreparation ranged from 3 to 10 nm. The histogram shown in Figure **1b** was plotted using the samples of more than 100 particles.

The strain of alveolar liver cancer RS-1 was obtained from the State Bank of tumor strains of Federal State Institution of Russian Academy of Medical Sciences - "Russian Cancer Research Center named Blokhin". The inoculation of the tumor (0.5 ml of 30 % suspension of tumor cells in Hank's solution) was performed in the right inguinal region subcutaneously [15]. The animals were divided into 4 groups of 5 in each: 1 - intact (relative norm), 2 - control 1 - animals without tumor treatment, 3 - control 2 - animals with implanted tumor which was injected by the preparation "Au NPs-apitoxin-chitosan" (chitosan dose - 100 mg/kg; apitoxin - 0.5 mg/kg; Au NPs - 0.25 mg/kg). Introduction of the drug was carried out one and two weeks after the tumor inoculation by fivefold encompassing injection of preparation around the tumor in a volume of 0.25 ml per animal (treatment for 10 days) every other day.

The blood for hematological investigation was collected from the sublingual vein on the 28th day after the end of the course of the preparation administration.

It was determined in the blood: 1 - the number of leukocytes using the hematology analyzer «AbacusJunior 30»; 2 – the indicating characteristics – in the blood smears, lymphocyte, segmented neutrophils by the conventional Romanovsky-Giemsa method of blood smears staining [16]. Additionally we calculated the value of the leukocyte ratio (the ratio between the percentage of lymphocytes to the relative content of the segmented neutrophils), which

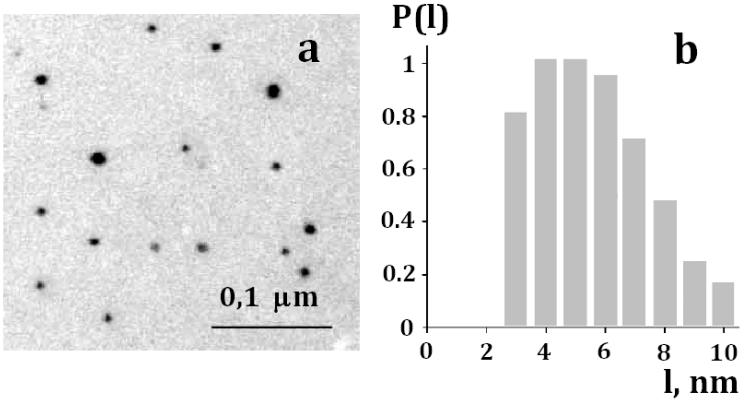


Figure 1: TEM images of gold nanoparticles (**a**). The nanoparticles size distribution according from to TEM (**b**).

decreases with increases in stress and adaptive response to sustained activation [10]. It was determined in the blood plasma: 1 - the content of the lipid peroxidation (LP) products (Schiff bases) by I.A. Volchegorsky method (1989) [17]; the intensity of free radical oxidation (FRO) and the activity of antioxidant system (AOS) by biohemilyuministsentnym method [18]. On the 28th day after the end of the preparation injection the measurement of tumors area was carried out.

The results of investigations are statistically processed through software BIOSTAT. The independent samples were compared using the univariate analysis, t - Student's test and the nonparametric Kruskal -Wallis test. The Student t-test was calculated with the Bonferroni correction allowing fix the error of the first kind arising in comparing more than two samples of this method [19].

RESULTS AND DISCUSSIONS

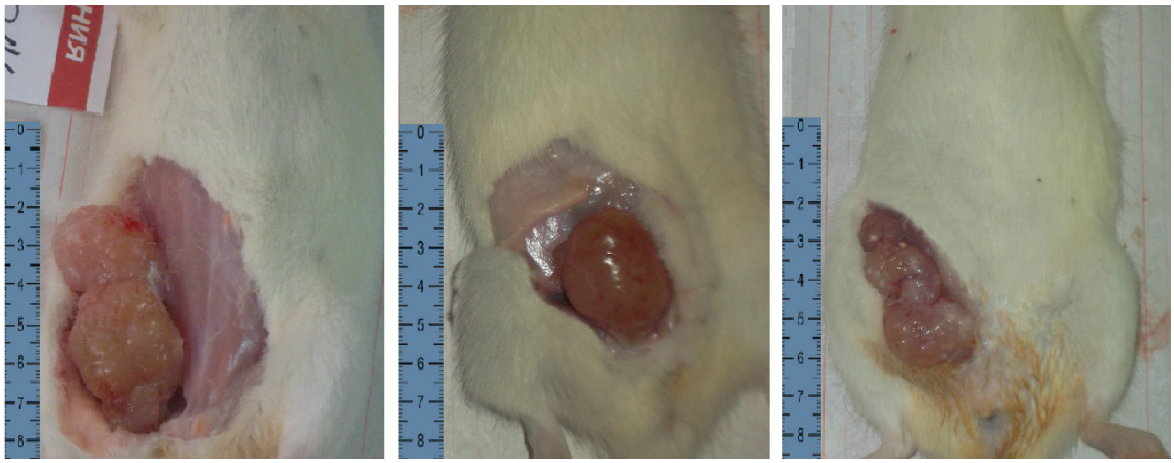
The inhibitory effect of the preparation on the tumor growth was assessed by sizing of the neoplasm area at control and experimental animals. The injection of the preparation has started after 7 or 14 days later the subinoculation of tumor. The response of the animals organisms with implanted tumor on drug administration has been also studied through the blood indices: the number of lymphocytes, white blood cells and segmented neutrophil, through the activity of free radical processes and antioxidant capacity of the system by the biochemiluminescence methods and the content of end-point lipid peroxidation products. All parameters were determined in the blood of animals at 28 days after injection of preparation. The results are shown in Figure **2** and Tables **1**-**3**.

The size of the tumors area from the experimental animals which treated by the preparation “Au NPs –

Table 1: The Surface Area of the Tumor on 28th Day after the End of the Preparation Injection for Different Groups of Animals with Implanted Tumor

The animals groups	The area (mm^2)	
	The therapy beginning after 7 days later tumor subinoculation	The therapy beginning after 14 days later tumor subinoculation
Control 1 without the preparation injection”	1582±116	1750±68
Control 2 Injection of the preparation “Au NPs -chitosan”	584±43**	812±51**
Experiment Injection of the preparation “Au NPs -apitoxin-chitosan”	302±24**	617±37**

The notices: probably significant distinctions: ** - p<0,05 towards control animals. The doses of preparation components: chitosan - 100 mg/kg, Au NP – 0.25 mg/kg, apitoxin – 0.5 mg/kg. The therapy was carried out during 10 days by the chipping of the tumor once in 2 days.



a b c

Figure 2. Photos of tumors on 28 day after the end of the therapy by preparation for different groups of animals with implanted tumor. The therapy was started after the 14 days later the tumor subinoculation:
a) Control 1 - without the preparation injection”
b) Control 2 - Injection of the preparation “Au NPs -chitosan”
c) Experiment - Injection of the preparation “Au NPs -apitoxin-chitosan”

apitoxin – chitosan" was 5 times less at the earlier dates of the therapy beginning (7 days after inoculation), and at a later dates (14 days after inoculation) ~ 3 times less (Figure **2b**) than in the control group 1 (Figure **2a**, animals with implanted tumor, $p < 0.05$). Table **1** and Figure **2a** show that tumor size is also ~ two times less in the control animals of group 2 treated with the preparation "Au NPs - chitosan" than untreated animals. These data indicate that the multicomponent preparation "Au NPs – apitoxin – chitosan" apparently inhibits the tumor growth. These effects of apitoxin were identified by other researchers, but on the other types of tumors and other delivery agents - perfluorocarbon [20]. It is remarkable that the inhibitory effect depends on the stage of tumor growth.

The positive antitumor effect of the preparation is also confirmed by indicating blood parameters characterizing immune status and the functional state of the organism (Table **2**).

The number of lymphocytes which provide the specific immunity from the animals injected by preparation in both cases of the tumor growth stage coincides with the value of intact substantially, while for the animals with implanted tumor in the control group 1 this characteristic was 1.5 times less. The leukocyte coefficient value from the animals which had treated by preparation was also close to intact group, while in the control group (animals without therapy) this parameter was several times lower. This indicates that the state of the experimental animals organism as well as animals treated with the preparation "Au NPs – chitosan" is close to normal, while the animals of control group 1 (without treatment) are under stress.

With the increasing of the tumor size its resistance to the preparation which was used in mentioned concentrations and the regimes of "therapy" increases, although the therapeutic effect was observed in this case also. This indicates to necessity of the therapy corrective action according to the degree of tumor growth.

In Table **3** the parameters of the activity of free-radical processes for analyzed groups of animals are described.

For the comparative characteristic of the free-radical and antioxidant processes it is necessary to take into account simultaneously the I_{max} and 1/S values which characterize the ability of the system to the free-radical oxidation and system antioxidant capacity respectively. In both cases of treatment the I_{max} value in the control group 1 was significantly higher than in intact group. The I_{max} indicator of experimental animals is significantly lower than that of intact, i.e. their organisms are out of the oxidative stress state. The ability of apitoxin small doses to shift the organism from the stress state to the state of relative norm has been observed in hypoxic conditions [6]. It should be noted that the system "Au NPs – chitosan" also demonstrate the pronounced antioxidant action in the second series of experiments. The 1/S value in animals with previous therapy by preparation containing the apitoxin was

Table 2: The Value of Leucocytes and the Amount of Indicator Parameters of the Rats with Implanted Tumor at 28th Day after the End of the Preparation Injection

The animals group	The value of leucocytes, $\times 10^9$cell/l	The segmented neutrophils, %	Lymphocytes, %	Leukocytal coefficient
The beginning of therapy after 7 days later the tumor subinoculation				
Intact	11.27±1.45	8.70±0.82	51.70±4.40	6.73±1.06
Control 1	9.36±0.30*	19.14±0.88*	36.00±1.63*	1.91±0.15
Control 2	10.66±0.89	9.22±0.57**	48.22±3.44	5.22±0.81**
Experiment	8.64±1.01**	11.25±1.16**	54.25±3.43**	6.43±0.72**
The beginning of therapy after 14 days later the tumor subinoculation				
Intact	12.45 ± 1.87	9.17±0.57	67.20±1.03	7.4±1.03
Control 1	12.69 ± 1.13	22.93±1.03	48.14±2.5*	2.18±0.41
Control 2	11.21 ± 1.92	11.14±3.26**	55.04±5.89*	5.16±0.90**
Experiment	11.11 ± 1.45	10.08±1.48**	61.78±4.4**	6.36±0.87

The notices: probably significant distinctions: * - $p<0,05$ towards intact animals, ** - $p<0,05$ towards control animals. The animals groups and component doses are as in the Table **1**.

Table 3: The Value of Biochemiluminescence in the Blood Plasma of the Animals with Implanted Tumor at 28th Day after the End of Preparation Injection

The animals group	I_{max} (mv)	1/S (x10^{-3})
The beginning of therapy after 7 days later the tumor subinoculation		
Intact	190.20±9.34	1.00±0.01
Control 1	245.50±12.87*	1.06±0.02
Control 2	255.10±16.12*	1.00±0.02
Experiment	155.20±13.81**	1.18±0.03*,**
The beginning of therapy after 14 days later the tumor subinoculation		
Intact	171.25±21.627	0.85±0.01
Control 1	218.50±23.23*	0.87±0.02
Control 2	148.20±20.12***	1.51±0.08***
Experiment	131.5±17.81***	1.46±0.03*,**

The notices: probably significant distinctions: * - p<0,05 towards intact animals, ** - p<0,05 towards control animals of group 1. The animals groups and component doses are as in the Table **1**.

even higher than in intact group, i.e. the system is effectively inhibits the progression of free-radical processes. The high I_{max} values on the assumption of comparatively low 1/S values in animals with implanted tumor in the control group 1 indicate the shift of balance between anti- and pro-oxidant processes in favor of the latter.

The highly cytotoxic and antioxidant activity of the preparation “Au NPs-apitoxin-chitosan” is associated with the cooperative action of the main components of apitoxin - mellitin and phospholipase A_2. The phospholipase A_2 destructs the membranes lipid bilayer with formation mesophospholipids and free fatty acids. These processes are accompanied by the enchancement of free radical oxidation with subsequent induction of the organism’s antiradical system capacity. The suppression of the free radical oxidation is enhanced by the presence of the multi-preparation “Au NPs – chitosan” components. The ability to inhibit the development of free-radical processes by the system “AuNPs – chitosan” has shown *in vitro* by EPR spectroscopy [21].

CONCLUSION

In this study we demonstrated that nanostructured preparation “Au NPs – apitoxin – chitosan” in therapeutic doses (on one degree less then toxic) effectively inhibits the growth of transplanted tumor RS-1 (alveolar cancer of liver) and exhibit strong antioxidant and adaptogenic activity.

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