The Effect of a Nanostructured Chitosan-Bee Venom-Gold Nanoparticle System on Free Radical Process Activity, Blood System Adaptation, and Tumor Growth in Rats with Transplanted Cancer PC-1

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The aim of the investigation was to estimate by blood system indices adaptogenic and antioxidant properties, as well as anti-tumor efficiency of nanostructured chitosan–bee venom–gold nanoparticles on laboratory animals with transplanted PC-1 strain.

Materials and Methods. We studied the impact of a nanopreparation — chitosan–bee venom–gold nanoparticles — on antioxidant, adaptogenic, antitumor effects when injected to animals (35 white rats) with transplanted liver cancer PC-1 (alveolar hepatic cancer). In time course of the experiment we analyzed physiological and biochemical blood values. On day 28 following preparation injection we determined the area of external tumor surface of the study animals and controls.

Results. We conducted a comparative assessment of free radical oxidation by the number of lipid peroxidation end products and antioxidant system activity in blood plasma, as well as a stress level by white cell count and leukocyte ratio, and the area of tumor external surface in laboratory animals after the course of treatment with the preparation. We revealed antitumor, antioxidant and adaptogenic activity of a chitosan–bee venom–gold nanoparticles preparation.

Conclusion. A nanostructured preparation (chitosan–bee venom–gold nanoparticles) in therapeutic doses (one order less than toxic ones) effectively inhibits transplanted tumor PC-1 growth (alveolar hepatic cancer) exhibiting significant antioxidant and adaptogenic activity.

Key words: nanostructured preparation; chitosan–bee venom–gold nanoparticles; nanostructured system; PC-1 tumor strain.

The development of malignant tumors is accompanied by disturbance of quite a number of body systems. Tumor primarily affects the immune status of the body. In the development of malignant tumors an imbalance in the system of free radical oxidation of lipids–antioxidant defence of the body occurs, which is accompanied by the onset of so-called oxidative stress.

Drugs used for chemotherapy have a very narrow therapeutic range. The doses that are necessary to achieve an antitumor effect do not differ much from those causing a toxic effect. Therefore, the search of therapeutic agents with multifunctional properties, which together with low toxicity would exhibit antitumor, antioxidant, adaptogenic, immunotropic effects becomes quite urgent.

Recently the antioxidant and adaptogenic effects of chitosan, a chitosan–gold nanoparticle system have been widely used under the action of extreme factors of various origin, and a positive effect of a succinate-ascorbate chitosan oligosaccharide complex on the activity of free radical processes, stimulation of tumor tissue destruction in an experimental model of malignant growth was determined [1].

The researchers at Washington University School of Medicine have successfully encapsulated melittin (the main toxin in bee venom) and demonstrated its use in cancer therapy in mice [2]. The biological efficacy of bee venom, when used as a complex multi-component system is known to be much higher than the efficacy of its separate components [3]. No data on the effect of whole bee venom on tumor processes has been found in the available scientific literature.

Due to its protein origin, bee venom can be broken up by various body proteases. This limits the time of its presence and functioning in tissues [4, 5]. In this regard, at the Department of High Molecular Compounds and Colloid Chemistry, the Faculty of Chemistry, Lobachevsky State University of Nizhni Novgorod, we synthesized a complex nanostructured preparation chitosan–bee venom–gold nanoparticles, in which chitosan functions

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as a stabilizer of gold nanoparticles, provides drug permeation into the extracellular space and screens the protein component of bee venom from the destructive action of proteases, increasing the lifetime of the complex drug, and gold serves as a nanocarrier [6, 7].

The blood system is among the first to respond and reflect in full the condition and metabolic character of the whole body. The white blood cell count and leukocyte ratio are used as the main indices characterizing the body's functional condition (stress, activation response, exercise) [8].

The aim of the investigation was to study the effect of the nanostructured chitosan–bee venom–gold nanoparticle complex on the activity of free radical processes and body adaptation in rats with transplanted PC-1 strain, assessed by blood system indices, as well as the effect of the nanocomplex on tumor growth.

Materials and Methods. The experiment was carried out on white nonlinear female rats of three months old weighing 150–200 g (n=20). All the procedures on the laboratory animals were carried out in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 18 March 1986); International Guiding Principles for Biomedical Research Involving Animals (Geneva, 1993); the Guidelines for Laboratory Practice in the Russian Federation (Ministry of Health Order No.267 of 19 June, 2003) and the Rules of Works Using Experimental Animals (Ministry of Health Order No.755 of 12 March, 1977).

We used aqueous dispersion of gold nanoparticles stabilized by bee venom and chitosan with the molecular weight of $1.3 \cdot 10^5$ kDa and the deacetylation degree of 0.80–0.82. The gold nanoparticles were obtained in chitosan solution (Bioprogress, Russia) by UV-induced reduction of chloroauric acid ($HAuCl_4$). The average size of the gold nanoparticles in the biopreparation ranged from 5 to 10 nm, because there is evidence that the gold nanoparticles are practically non-toxic in the size range from 3 to 20 nm [9, 10]. The bee venom was obtained on the apiaries in Bor District, Nizhny Novgorod region by electrical stimulation. The poison DL_{50} was 8–10 mg/kg.

Tumor inoculation (0.5 ml of a 30% tumor cell suspension in Hanks solution) was performed subcutaneously in the right inguinal region. The alveolar liver cancer PC-1 strain was obtained from the tumor strains bank in N.N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Science. The animals were divided into 4 groups of 5 animals each: intact (the relative standard); control 1 (tumor-bearing animals without treatment); control 2 (tumor-bearing animals treated with a chitosan–gold nanoparticles preparation; chitosan — 100 mg/kg; gold — 0.25 mg/kg); experimental group (tumor-bearing animals treated with a chitosan–bee venom–gold nanoparticles preparation; chitosan — 100 mg/kg, bee venom — 0.5 mg/kg, gold — 0.25 mg/kg). This preparation was administered a week after tumor inoculation by injecting it five-fold every other day in the amount of 0.25 ml per animal.

Blood sampling was performed from the sublingual vein on the 1, 14 and 28 days following the completion of preparation administration.

The blood was tested for a number of white blood cells with the help of the hematology analyzer Abacus Junior 30 (Diatron, Austria), blood indices — lymphocytes, segmented neutrophils in blood smears by the conventional method of coloring smears by Romanovsky–Giemsa [11]. Additionally, the leukocyte ratio was calculated (the ratio between the number of lymphocytes to the relative content of segmented neutrophils), the value of which decreases with stress and increases with the adaptive response to sustained activation [8]. The blood plasma was tested for the content of one of the end products of lipid peroxidation (LPO) — Schiff bases by Volchegorsky (1989); intensity of free radical oxidation and antioxidant system activity by biochemiluminescence [12]. The effect of the nanostructured system on tumor growth was evaluated for tumor weight in the control and experimental animals, which was determined by weighing them on day 28 following the completion of preparation administration.

The results of the studies were treated statistically using the BioStat software. The independent samples were compared using the univariate analysis, the Student t-test and the nonparametric Kruskal–Wallis test. When calculating the Student t-test Bonferonni adjustment was used which allows to establish type 1 error that arises when comparing more than two samples by this method [13].

Results. On day 1 following preparation administration no significant differences in the Imax and 1/S values, characterizing the maximum activity of free radical processes and capacity (activity) of the antioxidant system respectively in the animals of all the groups were observed, except for the control group 2 (chitosan–gold nanoparticles), where the 1/S value was higher than that in the other groups of animals (Table 1).

The content of the end products of LPO (Schiff bases) in the experimental group (chitosan–bee venom–gold nanoparticles) was significantly higher than that in the other groups (Figure 1).

The values of the main indices (a number of segmented neutrophils, lymphocytes and leukocyte ratio) in the control groups 1 and 2 indicated that the tumor-bearing animals were in a state of persistent activation close to normal, and the values in the experimental group showed that the animals were under stress (Table 2).

On day 14 in the control group 2 the Imax value, which characterizes the ability of the system to free radical oxidation, did not differ from the values in the intact animals, while in the control group 1, on the contrary, it was significantly higher. In the experimental

Table 1

Imax and 1/S indices of blood plasma in the tumor-bearing animals on day 1 following preparation administration (chitosan — 100 mg/kg; bee venom — 0.5 mg/kg; gold — 0.25 mg/kg) (M±m)

Groups	Imax (mV)	1/S (relative units), $\times 10^{-3}$
Intact animals	234.50±18.76	1.06±0.02
Control 1 (tumor-bearing animals)	184.50±11.36	1.04±0.03
Control 2 (chitosan–gold nanoparticles)	180.50±20.27	1.27±0.02*+
Experimental (chitosan–bee venom–gold nanoparticles)	244.40±20.96	1.04±0.02#

Note: * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$); # control 2 ($p<0.05$).

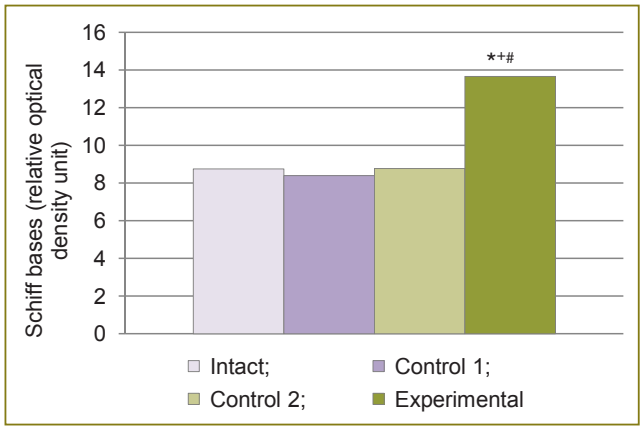

Figure 1. The content of Schiff bases in the blood plasma of the tumor-bearing animals on day 1 following preparation administration; * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$); # control 2 ($p<0.05$). Control 1 — tumor-bearing animals; control 2 — chitosan–gold nanoparticles; experimental group — chitosan–bee venom–gold nanoparticles

group it was significantly lower than in the control and intact groups. The 1/S values in the control animals of group 1 did not differ from those in the intact animals. In the control group 2 (chitosan–gold nanoparticles) the capacity of the antioxidant system was significantly lower than that in the intact animals and the control group 1. In the experimental group the 1/S values were significantly higher than in the rest of the groups ($p<0.05$) (Table 3).

The content of the end products of LPO (Schiff bases) in the blood plasma of the animals in the control groups 1 and 2 were significantly higher than in the intact rats; it was lower in the experimental rats than in the animals of the control groups and was not significantly different from that in the intact animals (Figure 2).

The number of leukocytes and lymphocytes in the control group 1 was not significantly different from their number in the intact animals, but there was a decrease in the number of segmented neutrophils and increase in the values of leukocyte ratio ($p<0.05$) (Table 4). In the control group 2 the number of segmented neutrophils was higher, and the values of leukocyte ratio and lymphocytes were lower than in the control group 1, but they did not differ from the same indices in the intact animals. In the experimental group the number of lymphocytes, the leukocyte ratio value were lower than the control group 1, at the same time the number of leukocytes, segmented neutrophils, the value of leukocyte ratio, except the number of lymphocytes, did not differ from the similar indices in the intact animals.

On day 28 following the completion of nanopreparation introduction the Imax value in the control animals of the both groups was significantly higher than in the intact ones. In the experimental animals this index was significantly lower than in the control groups, and it did not differ from the values in the intact animals ($p>0.05$). It is characteristic that the antioxidant capacity of the system (1/S) in the animals of the experimental group was also significantly higher than in the intact and control rats (Table 5).

Table 2

White blood cells and blood plasma indices in the tumor-bearing animals on day 1 following preparation administration (chitosan — 100 mg/kg; bee venom — 0.5 mg/kg; gold — 0.25 mg/kg) (M±m)

Groups	Number of leukocytes ($\times 10^9$/L)	Segmented neutrophils (%)	Lymphocytes (%)	Leukocyte ratio
Intact animals	8.69±0.60	10.82±1.42	45.60±2.83	5.21±0.90
Control 1 (tumor-bearing animals)	7.38±0.31	8.13±0.83	57.87±2.60	8.46±1.02*
Control 2 (chitosan–gold nanoparticles)	7.68±1.10	10.45±1.86	61.27±2.16*	7.60±1.77*
Experimental (chitosan–bee venom–gold nanoparticles)	10.70±0.80+	16.23±1.09*+	34.62±1.62*+	2.37±0.29*+

Note: * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$).

Table 3

Imax and 1/S indices in the plasma of the tumor-bearing animals on day 14 following preparation administration (chitosan — 100 mg/kg; bee venom — 0.5 mg/kg; gold — 0.25 mg/kg) (M±m)

Groups	Imax (mV)	1/S (relative units), $\times 10^{-3}$
Intact animals	212.40±12.06	1.04±0.02
Control 1 (tumor-bearing animals)	280.50±16.05*	1.12±0.02
Control 2 (chitosan–gold nanoparticles)	243.80±23.69	0.92±0.02*+
Experimental (chitosan–bee venom–gold nanoparticles)	153.20±14.35*+	1.15±0.02*#

Note: * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$); # control 2 ($p<0.05$).

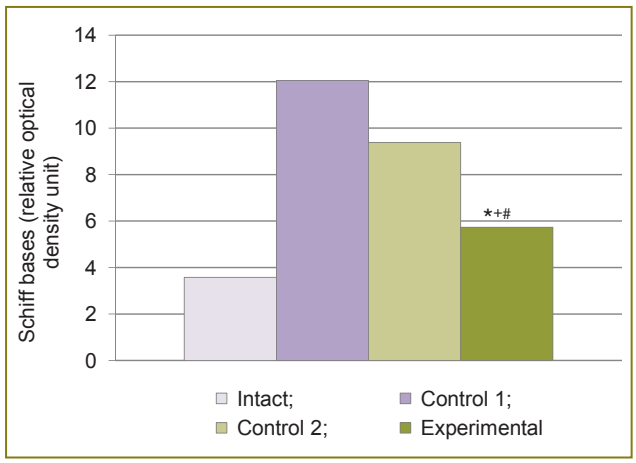

Figure 2. The content of Schiff bases in the blood plasma of the tumor-bearing animals on day 14 following the completion of preparation administration; * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$); # control 2 ($p<0.05$). Control 1 — tumor-bearing animals; control 2 — chitosan–gold nanoparticles; experimental group — chitosan–bee venom–gold nanoparticles

Table 4

The number of white blood cells and the values of blood indices in the tumor-bearing animals on day 14 following the completion of preparation administration (chitosan — 100 mg/kg; bee venom — 0.5 mg/kg; gold — 0.25 mg/kg) (M±m)

Groups	Number of leucocytes ($\times 10^9$/L)	Segmented neutrophils (%)	Lymphocites (%)	Leukocyte ratio
Intact animals	10.13±1.59	8.80±1.02	58.40±1.97	7.75±1.22
Control 1 (tumor-bearing animals)	8.43±1.09	5.44±0.69*	60.00±1.57	13.33±2.43*
Control 2 (chitosan–gold nanoparticles)	11.60±1.45	10.70±1.12+	52.60±1.90+	5.34±0.52+
Experimental (chitosan–bee venom–gold nanoparticles)	8.88±0.73	7.63±0.96	46.00±2.94*+	6.90±1.20+

Note: * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$).

Table 5

Imax and 1/S indices of the blood plasma in the tumor-bearing animals on day 28 following the completion of preparation administration (chitosan — 100 mg/kg; bee venom — 0.5 mg/kg; gold — 0.25 mg/kg) (M±m)

Groups	Imax (mV)	1/S (relative units), $\times 10^{-3}$
Intact animals	190.20±9.34	1.00±0.01
Control 1 (tumor-bearing animals)	245.50±12.87*	1.06±0.02
Control 2 (chitosan–gold nanoparticles)	255.10±16.12*	1.00±0.02
Experimental (chitosan–bee venom–gold nanoparticles)	155.20±13.81+#	1.18±0.03*+#

Note: * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$); # control 2 ($p<0.05$).

Table 6

The number of white blood cells and the values of blood indices in the tumor-bearing animals on day 28 following the completion of the preparation administration (chitosan — 100 mg/kg; bee venom — 0.5 mg/kg; gold — 0.25 mg/kg) (M±m)

Groups	Number of leucocytes ($\times 10^9$/L)	Segmented neutrophils (%)	Lymphocites (%)	Leukocyte ratio
Intact animals	11.27±1.45	8.70±0.82	51.70±4.40	6.73±1.06
Control 1 (tumor-bearing animals)	9.36±0.30*	19.14±0.88*	36.00±1.63*	1.91±0.15*
Control 2 (chitosan–gold nanoparticles)	10.66±0.89	9.22±0.57+	48.22±3.44	6.01±0.81+
Experimental (chitosan–bee venom–gold nanoparticles)	8.64±1.01*	11.25±1.16+	54.25±3.43	5.36±0.72+

Note: * statistically significant differences in the values compared to the group of the intact animals ($p<0.05$); + control 1 ($p<0.05$).

No differences in the content of Schiff bases between all the groups were observed.

The values of the main indices (number of segmented neutrophils, lymphocytes and leukocyte ratio) in the animals receiving nanopreparations (control 2, experimental) did not have a statistically significant difference compared to the values in the intact animals (Table 6).

The control tumor-bearing animals showed these indices to differ significantly from the values in both the intact animals and those receiving preparations (See Table 6).

On day 28 the tumor mass was determined. In the animals of the control group 1 it averaged 32.34±12.90 g, group 2 — 17.92±4.35 g, the experimental group — 4.62±1.58 g ($p<0.05$ when compared to the both control groups).

The tumor mass in the animals of the control group 2 was lower than in the animals of group 1, though the difference was not statistically significant. This indicated a certain though weak inhibitory effect of the chitosan–gold nanoparticle complex on tumor growth. The tumor mass in the experimental animals receiving the chitosan–bee venom–gold nanoparticles preparation was several times lower than in the control 1 and 2 animals ($p<0.05$).

Discussion. No statistically significant differences between the indices of free radical oxidation intensity, the functional state of the intact and control animals on the following day after therapy completion may be related to a small size and initial stages of tumor growth, which do not yet cause stress susceptibility in the body. The higher values of the antioxidant system capacity in the control group 2 than in the other groups seem to be conditioned by the anti-radical activity of gold that is not shielded by bee venom [14]. In the experimental animals which received the nanostructured gold nanoparticle–bee venom complex the number of Schiff bases was higher than in the other groups. Bee venom covering gold nanoparticles is supposed to reduce its antiradical activity. Finding the experimental animals under stress may be related to the action of bee venom, as this zootoxin is known to be a stress factor [7].

On day 14 following preparation administration the intensity of free radical oxidation in the control group 1 was significantly higher than in the other groups, and the activity of the antioxidant system was virtually the same as that in the intact animals. This may explain a significantly higher amount of Schiff bases in the plasma of these animals (See Figure 3 and Table 5). The increased Imax value in the control group 1 could be explained by tumor growth and its toxic effects on the body. In the experimental group the intensity of free radical activity was lower and the antioxidant capacity of the system was higher than in the other groups. Zootoxin introduction and tumor growth were accompanied by activation of the stress inducing sympathetic-adrenal, hypothalamic-pituitary-adrenal systems and, as a consequence, a subsequent increase in the capacity of the stress limiting systems, including the antioxidant one and, consequently, the reduced activity of LPO [15]. The increased activity of the antioxidant system in the experimental group provides, eventually, a lower number of Schiff bases in them than in the control groups 1 and 2 ($p<0.05$), that did not differ from the values in the intact group. The indices values showed that the animals of the experimental group started to go out of stress. The studied parameters in the control group 2 took an intermediate position between the corresponding values of the control group 1 and the experimental group. That indicated a certain positive effect of the nanostructured chitosan–gold nanoparticle system on the body involved in the tumor process.

On day 28 the free radicals level (Imax) in the group receiving the chitosan–bee venom–gold nanoparticles reduced, the antioxidant system (1/S) capacity also decreased. Therefore, eventually, the number of Schiff bases became the same in all the four groups by that time. At the same time the animals of the control group 1 were under stress while the animals in the experimental group and control group 2 were coming

out of stress (See Table 6). This is most likely due to the adaptogenic and antiradical effects of the nanostructured preparations that were also indicated in case of exposure to other extreme factors, such as ionizing radiation and hypoxia [15]. A very important fact is that of tumor growth inhibition by a chitosan–bee venom–gold nanoparticles preparation. The main components of bee venom — melittin and A_2 phospholipase — in the complex exhibit high membranolitic and cytotoxic activity [3]. These two effects of bee venom as an active ingredient of the nanocomplex when injected in the tumor, synergizing, cause a dysfunction of tumor cell membranes thus inhibiting its growth. The antitumor effects of the major component of bee venom — melittin — were exhibited in other types of tumors and other means of delivery [2]. Gold, as a carrier of biologically active substances, has quite a number of advantages: being indifferent, it exhibits high antiradical activity, which is particularly important in malignant tumors. Poison shielding by chitosan from the destructive action of the proteases provides a dosed release of the apitoxin components into the tissues and prolongation of their action.

Conclusion. The nanostructured chitosan–bee venom–gold nanoparticle complex in therapeutic doses (one order of magnitude less toxic) effectively inhibits the growth of transplanted tumor PC-1 (alveolar liver cancer), exhibiting a strong antioxidant and adaptogenic activity.

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Conflict of Interests. The authors have no conflicts of interest to declare.

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