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PREPARATION OF SELENIUM NANOPARTICLES BY USING SILYMARIN AND STUDY OF THEIR CYTOTOXICITY TO TUMOR CELLS

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Abstract

The past few years have seen substantial progress in veterinary oncology: new methods have been developed for the diagnosis and treatment of oncological diseases in animals, and the range of possible therapeutic interventions has been broadened. Of interest, in particular, are the prospects for the creation of veterinary pharmaceuticals with the use of various nanoparticles, including colloidal selenium, on the surface of which are immobilized biologically active substances that have antitumor action. Selenium nanoparticles are cytotoxic to tumor cells and have also been considered as effective carriers for the in vivo targeted delivery of drugs, genetic materials, proteins, and so on. The well-tunable polyvalent structures of the selenium nanoparticle surface provide a convenient platform for integrating several therapeutic agents or biomacromolecules with covalent or noncovalent surface conjugation. We synthesized selenium nanoparticles in complex with silymarin, a flavonoid hepatoprotector extracted from the fruit of milk thistle [*Silybum marianum* (L.) Gaertn.], and we evaluated the cytotoxicity of the resultant preparation to normal and tumorous cells. By using electron microscopy and dynamic light scattering, it was found that the developed procedure ensured the preparation of stable suspensions of silymarin-conjugated selenium nanoparticles with sizes ranging from 20 to 40 nm. The obtained conjugate was shown to be markedly cytotoxic to the Hep-2 tumor cell line, suppressing cell respiration approximately 6.5-fold as compared to the control, whereas the respiration of SPEV-2 normal cells was inhibited approximately 2.3-fold. Initial colloidal selenium had much weaker effects on both cell types, and pure silymarin had no statistically significant influence on SPEV-2 cells (in contrast to Hep-2 cells). The results of this study could be used in developing next-generation anticancer agents and are of interest in the implementation of green chemistry-based approaches.

Keywords: *Silybum marianum*, flavonolignans, silymarin, selenium nanoparticles, conjugation, cytotoxic effects

Despite the fact that progress achieved in modern veterinary medicine enabled to reduce animal mortality caused by oncological diseases, cancer is often continues to be treated as one of the most sever diseases of our time. According to available data, over 50 % of all dogs and cats aged over 10 years die from cancer, while oncological diseases remain among the most encountered reasons for asking for the veterinary assistance [1].

The past few years have seen substantial progress in veterinary oncology: new methods have been developed for the diagnosis and treatment of oncological diseases in animals, and the range of possible therapeutic interventions has been broadened [2]. However, it should be noted that arsenal of the veterinary office practically lacks specific medication designated for treatment of the oncological diseases in animals. In particular, important perspectives are associated with novel veterinary pharmaceuticals of various nanoparticles, including colloidal selenium, on the surface of which biologically active substances having antitumor action are immobilized.

Selenium (Se) is microelement of great importance for health of humans and animals [3, 4]. Physiological role of selenium is mainly associated with its presence in selenomethionine and selenocysteine amino acids included in relatively small set (nearly 25) of selenoproteins possessing, in particular, oxidation-reduction and immunomodulating properties [5].

Of great interest is ability of selenium compounds to have cytotoxic effect on tumor cells due to potential of selenolate and hydrogen selenide to effectively react with oxygen and thiols resulting in non-stoichiometric absorption of thiols and NADPH, oxidative stress and, finally, in cell death due to apoptosis, necrosis or necroptosis [6, 7]. Oncologic inhibitory effect of selenium nanoparticles was illustrated, in particular, in a number of publications [8, 9].

Selenium nanoparticles are also considered to be effective carriers for the in vitro targeted delivery of drugs, genetic materials, proteins, and so on. The well-tunable polyvalent structures of the selenium nanoparticle surface provide a convenient platform for integrating of several therapeutic agents or biomacromolecules with covalent or non-covalent surface conjugation [10]. Amino acids [11, 12], fungal polysaccharides [13], vegetable extract from the leaves of *Terminalia arjuna* [14], folic acid [15], cell cultures of *Saccharomyces cerevisiae* [16], and so on are used at biosynthetic (“green”) production of selenium nanoparticles. In the study of the mechanisms of action of the modified selenium nanoparticles, some researchers indicate their trend towards activation of mitochondrial apoptosis in cell line MCF-7, thus causing oxidation stress and in furtherance dysfunction of mitochondrion and, thus, stunting growth of the oncological cells [15]. Moreover, they indicate reduction of the membrane potential of mitochondrion and over-production of the active oxygen forms in Hep-2 cells under the effect of selenium nanoparticles [17].

In the past years, nearly 30 of chemical matters with cancer-prevention effect, which could be useful in reduction of the oncological diseases in humans, have been described [18]. Among them, in particular, much attention was drawn to naturally-occurring polyphenolic antioxidants [19]. Flavonolignans extracted from the medical plant *Silybum marianum* (L.) Gaerth., having strong antioxidant properties, may inactivate both free radicals, as well as reactive oxygen species in a cell. Moreover, they block receptors and transport systems in the cell membrane, which ensure transfer of toxic substances into a cell, reduce activity of macrophage cells participating in antigen presentation, reduce production of γ-globulins, and block lipoxygenase and cyclooxygenase, thus, having anti-inflammatory, immunomodulating, and anticancerogenic effects [20].

In particular, it was shown that silibinin, one of the flavonolignans of *Silybum marianum,* which currently account for 70 % of the total number of flavonolignans [21], increase in vitro and in vivo H3 and H4 histone acetylation in heteroplastid cell line Huh-7 in nude mice [22, 23]. Silibinin inhibits HDAC activity in cells of non-small-cell lung cancer and reduction of their intra-cell composition [24]. Silymarin is a silibinin analogue also falling under the category of vegetable-based flavonolignan type hepatoprotectors extracted from seeds of

Silybum marianum.

We were the first to suggest the technique for production of the stabilized conjugate of selenium nanoparticles from the initially unstable suspensions with the use of silymarin and to study cytotoxicity of such conjugate for immortalized kidney cells of pig embryo (line SPEV-2) and cells of hepatocellular carcinoma of humans (line Hep-2).

The purpose of this study was to develop the method for stabilization of the selenium nanoparticles suspension upon formation of the complex with silymarin and to assess cytotoxic properties of the obtained product with regards to tumor cells.

Techniques. Selenium nanoparticles suspension was obtained by adding 40 ml L-cysteine suspension to 67 ml of selenous acid suspension. For production of the former suspension, 100 ml of distilled water was added to 0.128 g of selenous acid (Ural Plant of Industrial Chemistry — UZPH AO, Russia), for production of the later 100 ml of distilled water was added to 0.726 g of dry L-cysteine (Neolab OOO, Russia); pH of suspensions were brought up to 8.5 with 0.1 M NaOH. Obtained suspended matter (initial nanoselenium preparation) became red-brown.

Diameter (d) of synthesized nanoparticles was measured with the use of a transmission electronic microscope Libra 120 (Carl Zeiss, Germany) and by dynamic light scattering method (DLSM) at analyzer Zetasizer Nano-ZS (Malvern, Great Britain), as described [25].

Commercial silymarin (TEVA Czech Industries s.r.o., Czech Republic) was used for obtainment of the selenium nanoparticle conjugate. Silymarin concentration in final preparation was defined by high-efficiency liquid chromatography (HELC) methodology. Test was conducted at liquid chromatograph Stayer (Akvilon ZAO, Russia) with spectrophotometric detector A_{288} subject to the instructions attached. Column Onix Monolithic C 18 (made by Akvilon ZAO under license of Merck KGaA, Germany) was used for separation of the components.

Cytotoxic studies were conducted in cell lines SPEV-2 (immortalized kidney cells of pig embryo) and Hep-2 (tumoral cells of human hepatocellular carcinoma). Cells were grown in plastic flasks with Dulbecco modified Eagle's medium (DMEM) (Biological Industries, Israel) containing L-glutamin, 15 mm HEPES, 10 % fetal bovine serum and antibiotics (20 μg penicillin of 1000000 U and 25 μg gentamycin of 1000 U per 100 μg of medium). Culturing was conducted in a CO_2-temperature-regulated chamber at 37 °C in atmosphere containing 5 % of CO_2. Cells were detached from the plastic substrate by trypsin-versene solution at 37 °C within 10-15 minutes followed by precipitation in centrifuging at 900 g for 20 minutes. Precipitate was re-suspended in phosphate saline buffer at pH 7.2-7.4 with repeated centrifuging in the above mode. Following re-suspending of cells in full DMEM, they were placed in wells of cell culture plate (1×10^5 cells per well).

To study the cytological effect of the synthesized nanoparticles, cells were pre-cultured for 18-24 hours until formation of a layer occupying 80 % of the well surface. Selenium nanoparticles preparation conjugated with silymarine was lyophilically dried, re-dissolved in incubation medium (pH 7.2-7.4), and placed in wells of the plate with coontinous cell cultures (8 repeats at a dose of 8 μm of silymarin per well). For comparison, silymarin solution of the same concentration was used in the growth medium. In control wells no preparations were added. Viability of the cultured cells was assessed by their ability to reduction of Nitrotetrazolium blue (MTT) to formazan (MTT-test, MTT tetrazolium assay technology) [26] with determination of formazan concentration.

Statistical processing of obtained results was performed by standard

methods with the use of Student's *t*-test for assessment of the significance of differences between samples in trial and control tests. Based on calculation results of the arithmetic mean (*M*) and standard deviation (±SD), standard error mean (±SEM) and limits of a confident interval were identified for sample, accounting for the Student's *t*-test coefficient at 95 % confidence level (p = 0.05) and number of measurements *n* = 8.

Results. Based on data obtained by dynamic light scattering (DLS) method (Fig. 1), size of particles in the initial selenium preparation comprised d ≈ 43-110 nm. However, colloidal solution of non-conjugated nanoparticles has quite low stability with brick-red precipitation (allegedly amorphous selenium) occurred within 10-15 minutes. For the purpose identified, we have created the following original method of obtaining silymarin conjugate with selenium nanoparticles. Silymarin, 1.38 g, was dissolved in 100 ml of 0.1 M NaOH. Further, 67 ml of selenous acid solution was added to 100 ml of silymarin solution following with addition of 40 ml of L-cystein solution. By adding 0.1 M HCl to neutralize alkaline excess, pH was adjusted to 8.5. Based on DLS method (see Fig. 1), size of 97 % of all nanoparticles in the selenium-silymarin preparation was within the range of d ≈ 16-44 nm.

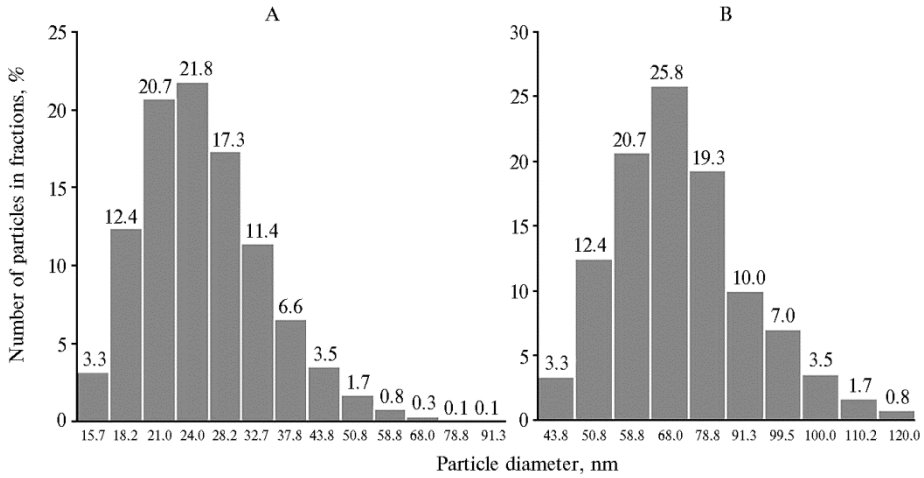

Fig. 1. Distribution of nanoparticles by size in the selenium preparation conjugated with silymarin (A)**, and without silymarin** (B) **based on dynamic light scattering methodology** (an analyzer Zetasizer Nano-ZS, Malvern, Great Britain).

Unlike the initial nanoselenium preparation, the obtained suspended matter of the conjugated selenium nanoparticles with silymarin became red-brown, transparent, visually not opalescent, and remaining stable for a long time. Thus, the achieved stability of the colloidal suspension of selenium-silymarin was accompanied by an increase in its dispersion (decrease of d values) typical for colloidal systems of such type, as was further confirmed by electron microscopic study (Fig. 2).

It was found that adding dried selenium conjugated with silymarin in the medium for cell incubation did not result in any changes in pH.

Cytotoxicity test of two produced preparations of selenium and native silymarin conducted on cell cultures SPEV-2 and Hep-2 found that pure silymarin did not cause statistically significant decrease in viability of SPEV-2 cells (Fig. 3, A). However, when this preparatioon was added to Hep-2 cell cultures, it resulted in decrease of formazan concentration by 10.3 % (see Fig. 3, B), as evidenced by the established inequalities $t_{actl.} = 2.45 > t_{estm.} = 2.14$; $p_{actl.} = 0.028 < 0.05$. Addition of the suspension of the initial nanoselenium resulted in reliable suppression

of SPEV-2 culture viability in MMT-test (by 7.3 %), whereas selenium conjugated with silymarin had resulted in a 55.6 % decrease of formazan concentration (see Fig. 3, A).

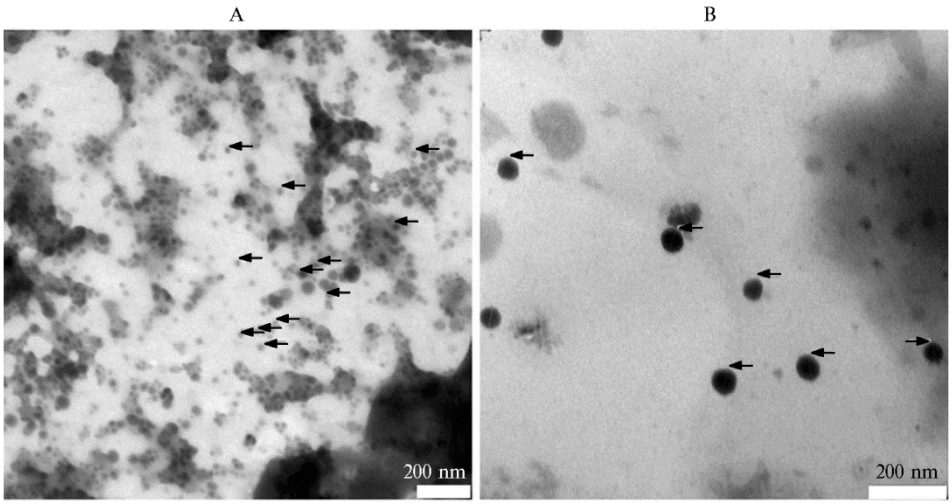

Fig. 2. Electron microscopy of nanoparticles (marked by arrows) **in selenium-silymarin conjugate** (A) **and of initial selenium nanoparticles** (B) (nanoparticles are marked by arrows).

More apparent response to added conjugated preparation had been observed in tumor cell line Hep-2 (see Fig. 3, B): selenium conjugated with silymarin caused a decrease in the number of viable cells, as per concentration of formazan, by 84.6 % (initial colloidal selenium solution decreased this value for the same line only by 34.6 %).

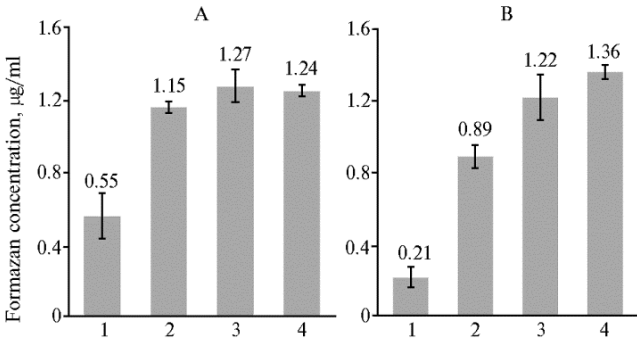

Fig. 3. Viability of cell cultures SPEV-2 (A) **and Hep-2** (B)**, expressed via formazan concentration, when influenced by selenium nanoparticles conjugated with silymarin** (1)**, initial selenium nanoparticles** (2)**, and pure silymarin** (3) **as compared to control** (no preparations added) (4). Vertical bars indicate confidence intervals for mean values at 95 % significance level.

Accounting for the results of this test and the properties of the initial substances described in publications we considered above, the enhanced cytological effect of selenium nanoparticles with silymarin could be caused by the synergy due to conjugation of the components. It is assumed that observed effect of the produced conjugate could be used for development of approaches to creation of next-generation anticancer agents. It appears that the advanced biosynthesis of selenium nanoparticles with the use of silymarin is of interest in terms of implementation of the principles of "green" chemistry [27].

Therefore, addition of silymarin in reaction mixture upon reduction of the solenoid acid with L-cystein results in formation of the stable suspension with organoleptic properties (color and transparency) typical for colloidal suspended matters of selenium at average more probable diameter of particles of 25 nm, which is triple less than in the initial less stable colloidal selenium solution. The produced conjugate of selenium nanoparticles with solymarin has clear cytologi-

cal effect on the line of tumor cells Hep-2 with approximately 6.5 times decrease in the number of viable cells as compared to the control, and approximately 2.3-times decrease of such value in cell lines SPEV-2.

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