Original Article

Toxicity assessment of the selenium nanoparticles *in vitro*

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ABSTRACT

The negative effects of nanoparticles include cytotoxicity, the development of nonspecific inflammatory reactions and oxidative stress, and oncogenicity. In this regard, the assessment of the toxicity of nanoparticles is becoming increasingly relevant. Selenium nanoparticles were selected for the experiment. Acceptable ranges of nanoparticle concentrations for various research methods have been determined in experiments. In the MTT test, the average lethal concentration of selenium nanoparticles for cells of the A549 line and the FL subline practically coincided and amounted to 1.3 and 1 mg/ml, respectively, which is an order of magnitude higher than the average lethal concentration for human lymphocytes. In an experiment on rat cardiomyocytes, the LC50 of selenium nanoparticles was determined at the level of 8 μg/ml. In an experiment on cells of the A549 line, the suitability of trypan blue staining was evaluated to determine the number of dead (with impaired membrane permeability) cells. The number of dead cells, determined by the number of stained cells, turned out to be somewhat overestimated compared to the results of calculating the difference between living cells in the control and the experiment after flushing rounded and detached cells. When studying the effect of nanomaterials on the integrity of the cell membrane, it was found that the activity of LDH at maximum concentrations of nanomaterials increased by 1.5 times.

Keywords: Toxicity, Selenium, Nanoparticles, Cytotoxicity

Introduction

The number of names of nanomaterials and the volume of their application in various fields of science, medicine, energy, and industry is growing rapidly. The speed of nanotechnology development is ahead of the development of methods for assessing their safety and regulatory documents [1, 2]. Currently, there are practically no studies on the potential toxic properties of nanomaterials.

The small size of nanoparticles leads to multiple increases in the specific surface area of materials, which contributes to the transit of a variety of substances by increasing the adsorption capacity. The chemical reactivity and catalytic properties of the substance increase. These parameters are also directly affected by physicochemical properties, including shape, surface structure, and polarity [2, 3]. Therefore, the probability of the development of various processes within individual cellular structures increases organelles, biological membranes, penetration, and contact with the cell nucleus and DNA. To a large extent, the cytotoxic properties of nanoparticles are explained by their ability to aggregate inside cells [4-8].

Most of the studies on the assessment of the biological effect of nanoparticles were conducted on mammals and were aimed at studying the effects on the respiratory system [9, 10]. However, there are other ways of exposure, such as skin, gastrointestinal tract [11]. It should be taken into account that the protection mechanisms specific to a particular route of entry, capable of protecting the body from harmful chemicals, are not always consistent with respect to nanoparticles [12]. It should also be taken into account that nanoparticles can enter the circulatory

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system both from the environment and during therapeutic interventions, as well as a result of entering the body by any other means [13, 14].

Regardless of the route of entry, after entering the circulatory system, nanoparticles with blood are distributed throughout the body and can interfere with signal transmission processes. Nanoparticles with a diameter of > 40 nm are comparable in size to large proteins and can form various complexes with them depending on the properties of the surface. Complexes may have different biokinetics, activity, and even functions [15, 16]. If the particle does not disintegrate and its size exceeds 5 nm, the nanoparticle cannot be excreted through the kidneys and, thus, can cause more pronounced damaging effects in the body [17]. The release of inflammatory mediators due to the accumulation of nanoparticles in the lungs can cause hypercoagulation of blood and increase cardiovascular risk [18]. The development of atherosclerosis is also associated with exposure to nanoparticles [19, 20]. Nanoparticles can overcome the blood-brain barrier and penetrate the central nervous system [21, 22].

The main route of exposure to nanoparticles is inhalation. It is believed that the size of nanoparticles allows them to stay in the air for a long time, get into the lower respiratory tract, and penetrate the intercellular space, which prevents excretion from the body [23]. Nanoparticles, even if they are not toxic in chemical composition, can cause oxidative stress [24], release inflammatory mediators, and contribute to the occurrence of lung diseases and other systemic effects [25].

The impact on a person can be professional, directly in the production process, or indirectly through contamination of the air of the working area or office space, as well as accidental, as a result of atmospheric air pollution, by-product emissions [26].

Thus, the assessment of the toxic effect of nanoparticles of various chemical compositions and structures is relevant and timely. The establishment of testing principles and procedures to ensure the safe production and application of nanomaterials on the market is necessary and achievable. Studies aimed at developing adequate approaches to predicting the risk of nanoparticles affecting human health, taking into account the chemical structure, size, shape, physico-chemical properties, production technology, and scope of application at the initial stage, it is advisable to conduct in vitro. Acute toxicity was studied in white mice and guinea pigs. The initial weight of laboratory animals ranged from: for mice -

20-25g of guinea pigs - 350-400g, respectively. It seen that in the "green" synthesis, salts of different metals (Ag, Se, Au, Zn) can be used and the method is an eco-friendly one based on the direct reaction between the components mentioned above, while the presence of other elements not being necessary.

The purpose of this work was to develop methodological approaches to determining the toxicity of selenium nanoparticles for hygienic assessment of their safety. Selenium nanoparticles were chosen as an object of research due to the growing interest in the scientific community and the high dynamics of their industrial production for various purposes [27-29].

Materials and Methods

Selenium nanoparticles manufactured by Aldrich (USA) were used for the research: the content of selenium nanoparticles is 99.5%, and the particle size is <100 nm. The particles had an organic coating for dispersion in a polar solvent.

Cell cultures used for in vitro toxicity studies

Human lung carcinoma cells (A549) were cultured in DMEM medium with the addition of 10% inactivated calf embryonic serum (FBS), 45 units/ml of penicillin, and 45 mg/ml of streptomycin in 5% CO2 medium at 37 °C in a CO2 incubator **(Figure 1)**.

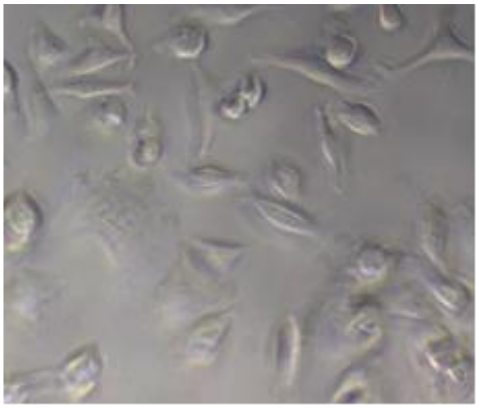

Figure 1. A549 cell culture

Human amnion cells (subline FL) have an epithelial-like morphology. Cultivation method: monolayer. Cultivation conditions: medium - 199 or EMEM, serum - cattle 10% (199); embryonic bovine 10% (EMEM). The procedure of re-sowing is the removal of cells using 0.02% versen with 0.1 mg/ml chymopsin, the multiplicity of sieving is 1:4 - 1:10, and the optimal density is 0.5-1.0x105 cells /ml. Karyology: 2n=46, the limits of variation in the number of chromosomes are 47-66, the modal number of chromosomes is 60, and the number of markers is 9, of which 3 are specific to NONLA cells (N 1,2,3, G discs). G6FDG isoenzymes, a.16. Sensitivity to human interferon. Field of application: virology, carcinogenesis, cell biology.

Human lymphocyte culture is one of the mandatory test systems for assessing the influence of mutagenic environmental factors. One of the advantages of this test system is that by the observed types of aberrations, the type of mutagenic effect can be quite definitely identified [30].

Blood was taken from the ulnar vein, and placed in a sterile tube containing a heparin solution (200 U/ml of blood). Under sterile conditions, blood samples were transfused 0.8 ml into Carrel vials with a culture medium prepared for cultivation. The composition of the culture medium: 6.16 ml of MEM medium; 1.6 ml of inactivated veal serum; 0.08 ml of L-glutamine; 0.08 ml of antibiotic solution; 0.15 ml of phytohemagglutinin. The vials were placed in a thermostat at 37 ° C to incubate the cells for 48 hours. To block mitosis at the metaphase stage, a solution of demecolcin in a concentration of 0.2 μg/ml of medium is

added to the vials 2 hours before incubation. After incubation, the cell culture was poured into centrifuge tubes and centrifuged at 10000 rpm for 15 minutes to precipitate the cells. The filler fluid was removed using a water jet pump, a hypotonic solution preheated to 37℃ (0.75 M KCl) was added and the precipitate was resuspended in it. Next, the tubes with cell culture were kept in a water bath (37℃) for 10-12 minutes. Upon reaching hypotonization, the sample was centrifuged again under the same conditions, followed by the removal of the supraventricular fluid. To fix the cells, the precipitate was resuspended in 1-1.5 ml of a freshly prepared fixative (a mixture of methyl alcohol and glacial acetic acid in a proportion of 3:1) on a shaker and brought its volume to 10 ml. The retainer was changed with subsequent centrifugation three times.

Culture of cardiomyocytes of rats of the SHR line. The starting material for the primary culture of rat cardiomyocytes is obtained by disaggregation with 0.25% trypsin solution at 37℃ for 30 minutes of the cardiac tissue of 12-14-day-old rat embryos. After the removal of trypsin, the precipitate is suspended in a growth medium (90% of the Needle medium, 10% of the embryonic serum of calves with the addition of antibiotics). The suspension is passed through a nylon filter (0.3 x 0.3 mm cell). After counting in the Goryaev chamber, the cells are seeded into 96-well plates and cultured in a DMEM growth medium.

Methods for studying the cytotoxicity of nanomaterials in mammalian cell cultures

The MTT test (Methyltetrazolium test) is designed to detect metabolic disorders, namely mitochondrial dysfunction, reflecting the effect on cell viability. The cells were grown in a CO2 incubator (Herra Cell) at 37 °C, 5% CO2, and 80% relative humidity on 96-well plates (sowing concentration - 50-70 thousand cells/ml) [31]. For MTT, a set of CellTiter 96 AQuatic One Solution Cell Proliferation Assay (MTS), Promega was used. To measure the absorption of formazan, cells were incubated with MTS for 20 minutes in a thermostat, the absorption of formazan at λ = 492 nm was measured on an enzyme immunoassay device from Awareness, Microplate Rider Stat Fax 3200.

The evaluation result is statistically processed by Excel tools. The toxicity of nanoparticles was assessed by the IC50 indicator (the average inhibitory concentration is the concentration of a substance that suppresses this cellular function by 50%).

The method of assessing cell viability in culture by staining with trypan blue (methylene blue): assessing the integrity of the cell membrane is one of the most common ways to measure cell viability and cytotoxic effects [32]. Compounds with cytotoxicity often cause a violation of the integrity of the cell membrane, which can be established by vital staining with trypan blue (methylene blue), which freely penetrates through the damaged membrane and stains intracellular components, but is absent in healthy cells [33]. To assess viability, staining of cell suspensions is a more reliable method since when staining attached cells, they can detach from the substrate and be lost. The main disadvantage of this method is the inability to detect cells with impaired ability to reproduce [34, 35].

To experiment with different concentrations of selenium nanoparticles (100, 300 μg/ml), cells of the A549 line were sifted into 24-well plates. After the exposure window (8 and 24 hours), the cells were washed from the nanoparticles with saline solution, and stained with 0.4% dye solution. The number of dead colored cells was counted in five arbitrary fields of vision.

A method for assessing cell viability in culture by determining LDH activity: measurement of lactate dehydrogenase activity is used as one of the main tests for cytotoxicity [36]. The method is based on increasing the activity of the enzyme present in the cytoplasm of living cells in the culture medium, where it is released through damaged membranes of dead or dying cells. Small amounts of culture medium are taken at certain intervals after exposure to cells to measure the released lactate dehydrogenase and determine the dependence of the toxic effect on the time factor [37]. In this test, it is possible to assess the overall cytotoxicity in real time [38]. Lactate dehydrogenase is an oxidoreductase that catalyzes the conversion of pyruvate and lactate [39]. Cells secrete LDH into the bloodstream after damage or hemolysis of erythrocytes. Since LDH is a stable enzyme, it is widely used to assess the presence of damage or toxicity to tissues and cells. LDH also increases under certain pathological conditions, such as cancer. In the set used, LDH restores NAD+ to NADH+, which is specifically detected by the colorimetric method (450 nm).

The initial solutions of nanoparticles were prepared by dispersion in distilled water with the addition of albumin using four-fold ultrasonic treatment with a total duration of 1 h. Nanoselene concentration - 100, 300, 600, 800, 1000 μg/ml.

The MAK066 Lactate Dehydrogenase Activity Assay Kit, Promega, was used for the test. LDH content was determined in a cell-free medium by adding a substrate (NAD+, tetrazoline salt, sodium lactate, etc.). During the determination, the following reactions occur the first reaction - LDH reduces NAD+ to NADH by oxidizing lactate to pyruvate). In the second reaction, the catalyst (diaphorase) transfers H/H+ from NADH to the tetrazoline salt, forming formazan.

Serum samples were used for analysis. 2-50 μl of the sample was added to the wells of a 96-well tablet in two repetitions. The samples were brought to a window-particle volume of 50 μl with a buffer for LDH analysis. 50 μl of the basic reaction mixture was added to each well. Well mixed by pipetting. After 2-3 minutes, the initial T was determined. The optical density (A450) was measured at the wavelength λ = 450 nm at the initial time. The tablets were incubated at 37 °C, taking measurements (A450) every 5 minutes. The time of the penultimate measurement is Finite. To measure the absorption of formazan, the samples were incubated for 5 minutes in a thermostat, the absorption of formazan was measured on an enzyme immunoassay device from Awareness, Microplate Rider Stat Fax 3200.

The concentration of the reaction product of the conversion of 7-ethoxy resorufin to 7-hydroxy resorufin was determined based on calibration based on experimental data. According to the

calibration, the relative unit of optical density corresponded to the LDH content of 0.430 nmol/min/ml (iU/ml).

List of test equipment

All work with cell cultures was carried out in a sterile laminar box, cell culture was carried out in a CO_2 incubator. The following equipment was used in the work: Laboratory centrifuge OPN-3,02, Axioskop40 microscope, TS 80M-2 electric dry-air thermostat, SNOL-67/350 drying cabinet, AU-12 microscope, Electronic scales Adventurer OHAUS, CO_2 Incubator.

Results and Discussion

The effect of selenium nanoparticles on the viability of cells of A549, SHR, and subline FL lines according to the results of the MTT test

Cell death induced by nanoparticles was assessed by the change in the optical density of the solution relative to the control. The results are shown in **Figure 2**.

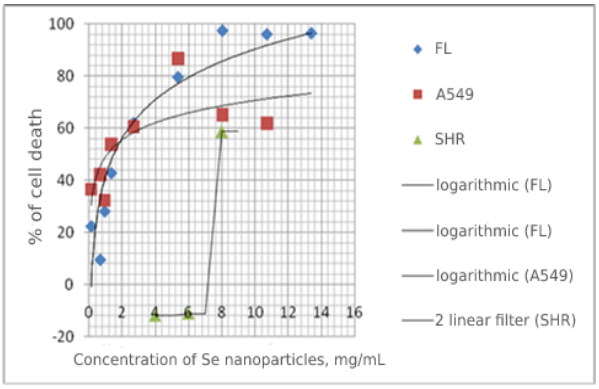

Figure 2. The effect of selenium nanoparticles on cells of various lines

As the results of the MTT test showed, the average inhibitory concentration (IC50) of selenium nanoparticles - a concentration that suppresses the ability of cells to convert tetrazoline salt into formazan by 50%, determined graphically, for cells of the A549 line and the FL subline practically coincided and amounted to 1.3 and 1 mg/ml, respectively. In an experiment on rat cardiomyocytes, the IC50 of selenium nanoparticles was determined at the level of 8 mg/ml.

Thus, the data obtained in the experiment indicate a higher sensitivity of cells of the A549 and FL lines to the action of nanoparticles compared to rat cardiomyocytes. This is probably due to the higher rate of division of transformed cells and amnion cells, as well as the intensity of metabolism.

The effect of nanoparticles on the viability of human lymphocytes according to the results of the MTT test

In an experiment on human lymphocytes that were cultured for 48 hours, it was demonstrated with selenium nanoparticles (IC50 = 100 μg/ml). The average inhibitory concentration was determined graphically. The results of the experiment are shown in **Figure 3**.

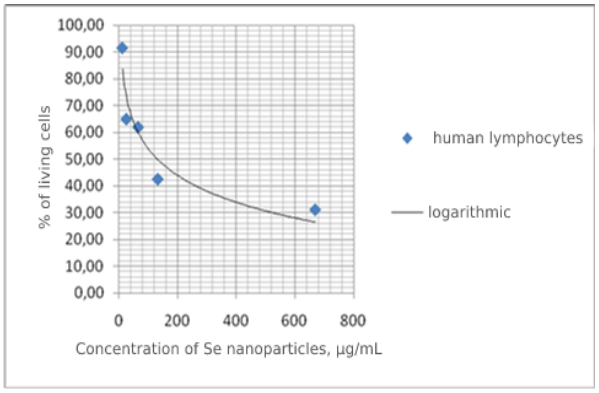

Figure 3. The effect of selenium nanoparticles on human lymphocytes

Comparing the results of the MTT test on human lymphocytes and the previous experiment, it can be concluded that selenium nanoparticles for lymphocytes are an order of magnitude more toxic than for any previously used cell culture.

Study of cytotoxicity of nanoparticles for A549 cells using methylene blue staining

Cells of the A549 line were seeded into 24-well plates, where selenium nanoparticles were introduced. A 0.4% solution of methylene blue was used to assess the viability of the cells. Staining was carried out 8 and 24 hours after the introduction of nanoparticles. The number of cells was counted in 5 arbitrary fields of vision, and the average value was calculated. The results of the experiment are presented in **Table 1**.

Table 1. The effect of selenium nanoparticles in various concentrations on the viability of cells of the A549 line

Experience option	Number of cells					
	8 hours			24 hours		
	Total number of cells	Number of colored cells	% death	Total number of cells	Number of colored cells	% death
selenium nanoparticles, 100 μg/ml	67.2	4.6	6.8	88.4	5.6	6.3
selenium nanoparticles, 300 μg/ml	74	29	37.3	42.2	32.6	77.2

It is shown that with increasing exposure time, the effect of selenium nanoparticles on cells increases. It can be concluded that the method of cell staining in culture with methylene blue is applicable to determine the cytotoxic effect of nanoparticles by assessing the integrity of cell membranes, which corresponds to the data presented in the works of other researchers [40, 41].

Study of cell viability in culture by lactate dehydrogenase activity in culture medium

LDH activity was studied in the A549 cell culture medium with the addition of selenium nanoparticles at concentrations from 100 to 1000 μg/ml, the exposure was 24 hours **(Table 2)**.

Table 2. Results of studying the effect of selenium nanoparticles on LDH activity in A549 cell culture medium

concentration of nanoparticles, μg/ml	LDH activity, mU/ml
100	278
300	302
600	366
800	414
1000	420

As a result, LDH activity at the maximum concentrations of the nanomaterial increased by 1.5 times, which corresponds to the results of other authors [42-44].

Conclusion

Selenium nanoparticles are the most promising objects for study since it has been established that selenium nanoparticles are one of the most promising types of metal nanoparticles [45]. At least 235 types of consumer goods, including toothpaste, dressings, and depilatory products, contain selenium nanoparticles. Probably, selenium nanoparticles will find wide application in the production of textiles, cosmetics, antiseptics, polymers, paint, and varnish materials. Their production and scope of application are increasing, which can lead to a negative impact on human health and the environment.

In the MTT test, the average lethal concentration of selenium nanoparticles for cells of the A549 line and the FL subline practically coincided and amounted to 1.3 and 1 mg/ml, respectively, which is an order of magnitude higher than the average lethal concentration for human lymphocytes. In an experiment on rat cardiomyocytes, the LC50 of selenium nanoparticles was determined at the level of 8 μg/ml.

In an experiment on cells of the A549 line, the suitability of trypan blue staining was evaluated to determine the number of dead (with impaired membrane permeability) cells. The number of dead cells, determined by the number of stained cells, turned out to be somewhat overestimated compared to the results of calculating the difference between living cells in the control and the experiment after flushing rounded and detached cells. It can be concluded that the method of cell staining in culture with methylene blue is applicable to determine the cytotoxic effect of nanoparticles by assessing the integrity of cell membranes.

When studying the effect of nanomaterials on the integrity of the cell membrane, it was found that the activity of LDH at maximum concentrations of nanomaterials increased by 1.5 times.

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Conflict of interest: None

Financial support: None

Ethics statement: The protocol for experiments with laboratory animals complied with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. All patients – lymphocyte donors were involved in the experiment only after signing the agreement for volunteer participation. All raw data are available upon request from the corresponding author.

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