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Study of Cytogenetic and Cytotoxic Effects of Nanosilver and Silver Sulfate in Germ Cells of Mice in Vivo

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Abstract—The cytogenetic and cytotoxic activities of nanosilver particles and silver sulfate in germ cells in vivo were studied in a model similar to the potential impact on humans for the first time. We investigated nanosilver particles with a diameter of 14 nm coated with gum. The substances were ingested by male CBAB6F1 mice for 14 days with drinking water over a wide range of concentrations: 0.1, 50, and 500 mg/L (0.01, 5, and 50 mg/kg). Silver nanoparticles caused a slight statistically significant increase in the frequency of micronucleated spermatids to 0.57‰. Genomic instability affects the cell kinetics: it resulted in an increase in apoptosis from 3.4% (control) to 6.4% (50 mg/L), and it reduced the level of multinucleated spermatids from 15.6% (control) to 11.1% (50 mg/L). This phenomenon can be seen as a compensatory response aimed at death of genetically damaged cells and the expedited renewal of round spermatids by the disintegration of multinucleated spermatids. The minimally tested concentration of silver sulfate increased apoptotic activity. No other changes in the same mode of action of silver sulfate were observed, indicating a more pronounced effect of silver nanoparticles when compared to its ionic form in the germ cells of mice.

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INTRODUCTION

Nanotechnologies and nanomaterials are becoming more widely used in cosmetics, medicine, the food industry, instrumentation, and other industries. One of the most widely used nanomaterials is nanosilver (NS). Silver has long been used as a disinfectant and antimicrobial agent. At present, increasing attention have been given to nanosilver. It is used for the disinfection of water, in medicine as a basic substance of sterilizing solutions and bandages, and in the food industry and cosmetics as an antiseptic. It is shown that microorganisms are killed by exposure to silver nanoparticles, but at the same time it is found that nanoparticles penetrate easily into the cells of microorgansims and affect their operation.

A lot of research are devoted to the study of toxic and genotoxic effects of nanosilver. However, in general these papers are performed on models in vitro. In experiments in mice in vivo upon intraperitoneal or intravenous introduction, the cytogenetic effect of NS is established in bone-marrow cells [1, 2] and blood reticulocytes [3] and the genotoxic effect is established in the Comet assay in bone marrow cells [2], liver [4], and spleen [5].

The study of the nature of the impact of nanoparticles on germ cells is of great importance, since this may affect human reproduction and the health of future generations. This is particularly important due to the latest data on the gradual decrease in male fertility in different regions of the world. Over the last half of the 20th century, there was a sharp decrease in the number of normal sperm cells (on average, two times) in the ejaculate of men in different regions of the world [6]. The reasons for this are not clear. Cytogenetic studies of testicles in vivo are not widespread due to the insufficient development of methods. The genotoxic effect of NS in the germ cells of mammals was investigated in two studies. C. Ordzonikidze with colleagues [5] identified the genotoxic effect of NS using a test of "abnormality of sperm heads" of mice, J. Gromadzka-Ostrovska et al. [7] used Comet assay on rats. In both studies, the introduction of NS was parenteral. Thus, the mutagenic effect of NS on germ cells of mammals in the natural method of exposure—with drinking water—is still unexplored. The aim of our work was to investigate the effect of NS in germ cells of mice in comparison with the ionic form of silver upon exposure with drinking water.

As the ionic form, silver sulfate (SS) was studied, which, along with other silver salts, is widely used in drugs with antibacterial and wound-healing effects. It is included in dressings, reducing the adhesion of dressings to the wound and reducing pain in patients. In recent years, the production of textile medical

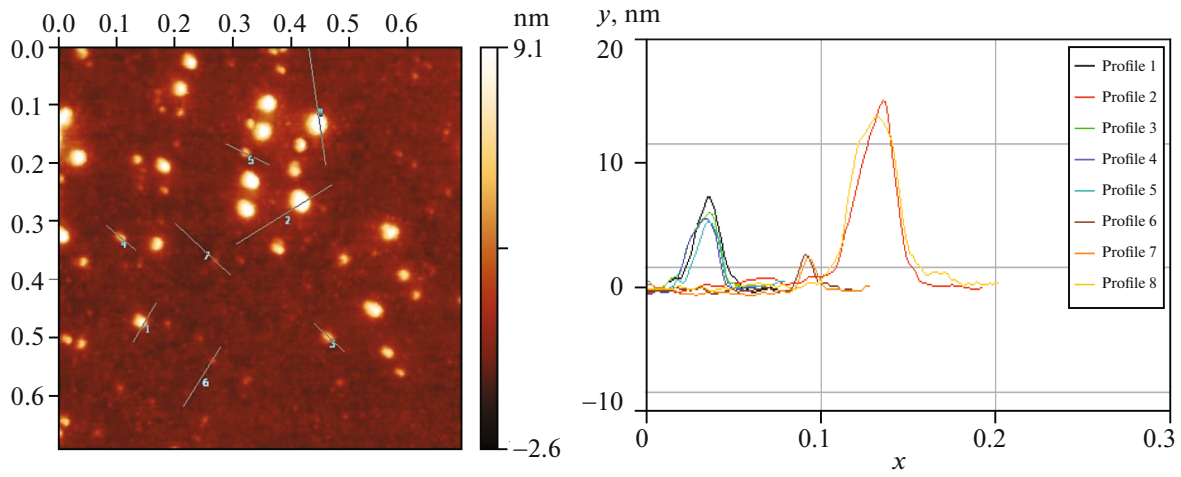

Fig. 1. (Color online) Silver nanoparticles of the KND-S-K type. On the left is a photo of Ag 14 silver nanoparticles on an atomic force microscope synthesized by Multi Mode SPM technology, Digital Inst., Inc., in tapping-mode regime using a Nanoworld NCH-50 silicon probe with a force constant of 42 N/m and resonant frequency of 320 kHz (Institute of Atomic and Molecular Sciences, Taipei, Taiwan); on the right is a cross section (profile) of nanoparticles.

products has began using SS: surgical gowns, sheets, and mattresses for patients with burns and wounds. SS is used to disinfect water in large containers in shipping. There are tablets for the disinfection of water under field conditions on the basis of SS. This material is considered low-toxic (LD_{50} for rats = 5000 mg/kg [8]), but its mutagenic properties by in vivo experiments are studied negligibly. In this regard, data on the mutagenicity and cytotoxicity of SS in germ cells of mice are of independent interest.

To assess the mutagenic effect in the study, we used the multiorgan karyological test [9]. Together with micronuclei, other karyological indicators were considered, which greatly expanded the information content of the method.

MATERIALS AND METHODS

The mutagenic activity of commercial drugs of spherical silver particles is studied stabilized with arabic gum 1 : 7 by weight. Particle size is 14 ± 0.2 nm. CAS RN HSD771500. Nanosilver is manufactured by Centoza Factoring NP SPE LLC (Russia) according to TOR 9197-009-77342998-11 (Fig. 1).

As the ionic form, SS (Ag_2SO_4, CAS RN 10294-26-5) was used, which dissociates into ions in water (solubility in water is 0.79 g/100 g of H_2O at 20°C). SS is made in Russia according to TOR 6-09-370374, reagent grade.

For the preparation of suspensions/solutions of SS and gum, bottled drinking water of 1 quality category was used.

The experiment was performed on male CBAB6F1 mice weighing 25–35 g of the propagation of the Research Center of Biomedical Technologies, Russian Academy of Medical Sciences (Stolbovaya branch). Studies are carried out in accordance with the rules adopted by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (Strasbourg, 1986) and the requirements of the "Rules of Performance of Works Using Experimental Animals" (Appendix to the Order of the Ministry of Health of the USSR from 08.12.1977, no. 755). Animals were kept on a standard diet with free access to water and food.

Eight groups containing seven mice per group were formed. Substances were introduced to the organisms of animals with drinking water as suspensions–solutions at concentrations of 0.1, 50, and 500 mg/L for 14 days. Upon taking into account water consumption, mice received substances in doses of 0.01, 5, and 50 mg/kg per day. As a negative control, two groups of mice were used: intact animals receiving bottled drinking water and mice receiving a solution of arabic gum coated with nanosilver particles. Its concentration corresponded to the concentration in the nanosilver slurry of 500 mg/L.

After the experiment was carried out, cervical dislocation was performed on the 15th day, testicles were isolated, and smears were made for microscopic study. Slides of testicle cells were prepared ex tempore according to the original method based on the preparing of suspension of germ cells, fixation with ethanol-acetic acid (3 : 1), the nucleus staining with 2.5% aceto-orcein, and finishing painting of cytoplasm with a 1% light green solution [9, 10]. Before microscopic analysis, drugs were encrypted. This method allows one to count the undamaged isolated of testicular cells of different stages of maturation. However, the cells of late stages of spermatogenesis with cytoplasm (round spermatids) that formed after the second meiotic divi-

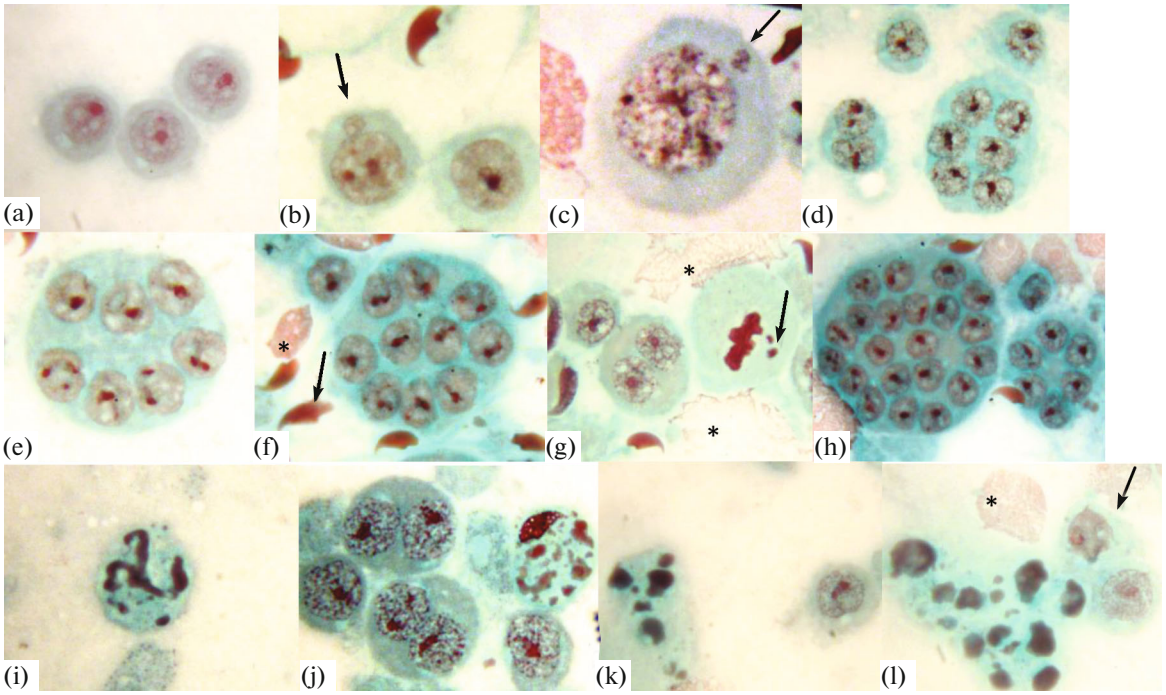

Fig. 2. (Color online) Microphoto of germ cells of mice. Aceto-orcein staining, light green. Magnification of ×1000. Olympus BX-41 microscope. (a) Three mononuclear spermatids without pathology; (b) two mononuclear spermatids, one of them with a micronucleus (arrow); (c) mononuclear spermatid with micronucleus (arrow); (d) two mononuclear spermatids, one binuclear and one hexanuclear; (e) spermatid with seven nuclei; (f) mononuclear spermatid, spermatid with 10 nuclei and sperm heads (arrow); (g) mononuclear, binuclear spermatids, dividing cell in the metaphase stage with two lagging chromosomes or fragments of chromosomes, which in daughter cells can form a micronucleus (arrow); (h) multinucleated spermatids with 21 nuclei, spermatid with 6 nuclei and mononuclear spermatid; (i) cell in the apoptosis, the nucleus is destroyed; (j) two mononuclears spermatids, two binuclear spermatids, and the cell in apoptosis with the nucleus disintegrates into fragments; (k) mononuclear spermatid and the cell in apoptosis with destroyed nucleus; and (l) mononuclear spermatid, spermatid at the stage of becoming a sperm cell (arrow) and four closely located cells in apoptosis. Asterisks denote the nuclei of Leydig cells.

sion are the most interesting. Spermatids are not divided, but DNA damage taking place in the previous stages of spermatogenesis may appear in them in the form of micronuclei (Fig. 2). In other words, spermatids are an indicator of DNA damage or spindle apparatus proteins formed in the previous stages of maturation of germ cells. These cells are morphologically well visualized at the noted staining method, and micronuclei can be determined in them. From each animal, 1000 round spermatids were analyzed, and among them the frequency of cells with micronuclei was analyzed: as indicator of cytogenetic action. In addition to that, the sum of spermatids with two or more nuclei (multinucleated spermatids) and cells in apoptosis were taken into account.

The micronucleus is a chromatin body with a round or oval shape with a smooth continuous edge no larger than 1/3 of the nucleus, was clearly separate from the nucleus in the cytoplasm, not refracting light, with the same intensity of staining and chromatin image as in the main nucleus and located in the same plane with nucleus. Also, in the suspension smears, spermatids with two or more nuclei and apoptotic figures were taken into account, which were cells with a round or oval shape containing many (in the form of clusters) fragments of destroyed nucleus or nuclei in the cytoplasm were taken into account. It was not possible to determine the type of cells in apoptosis (Fig. 2).

A microscopic analysis of encoded slides was carried out using a Leica DM5000B microscope with oil immersion at ×1000.

Statistical treatment of the results was performed using Excel and STATISTICA programs for Windows. The average values of all indicators in the groups and 95% confidence intervals (CI) were determined; a comparison of the indicators in experimental and control groups was carried out using χ^2 criterion. Differences were considered statistically significant at $P < 0.05$. A correlation analysis was performed using Spearman's criterion.

RESULTS OF STUDY

The results of an experimental study of the cytogenetic and cytotoxic effect of silver in nano- and ionic form and in the cells of testicles of mice, studied using karyological analysis, are presented in the table.

Karyological indicators of analysis of the testicle cells of mice treated with silver in nano- and ionic forms

Studied groups of mice	Indicators, per 1000 cells. $X_{av.}$; (CI-95%)		
	Spermatids with micronuclei	Spermatids with two or more nuclei	Cells in apoptosis
Control, without exposure	0	156 (128–182)	34.1 (16.5–51.7)
Control, gum	0.29(0–0.74)	156 (140–173)	40.6* (7.5–73.6)
Nanosilver 0.1 mg/L	0.57*(0–1.30)	142* (91.5–193.4)	47.4*** (14.5–80.3)
Nanosilver 50 mg/L	0.57*(0–1.30)	111*** (63.2–159)	64.6*** (19.9–109)
Nanosilver 500 mg/L	0	138** (103–172)	23,6** (5.8–41.4)
Silver sulfate 0.1 mg/L	0.29(0–0.74)	145 (97.4–192)	56.9*** (2.4–111)
Silver sulfate 50 mg/L	0.14(0–0.49)	143 (116–170)	38.7 (6.6–70.8)
Silver sulfate 500 mg/L	0	151 (131–172)	36.6 (18.0–55.1)

Significant differences from the control group at $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$ upon the comparison of the data on χ^2.

In the control group of intact mice, spermatids with micronuclei are not observed. The cytogenetic and cytotoxic effect of gum is not revealed. The average value of the frequency of spermatids with micronuclei was 0.29‰ at the top level of a confidence interval of 0.74‰.

After exposure of NS at concentrations of 0.1 and 50 mg/L, a low but statistically significant cytogenetic effect is revealed. The upper limit of the confidence interval is 1.3%, which is less than the data of historical control for polychromatic erythrocytes of the bone marrow and cells of other organs (2%), including those presented in monograph [9].

It is interesting to note the phenomenon of reducing multinucleated cells after using of all studied concentrations of NS compared with the control, most pronounced at a concentration of nanoparticles of 50 mg/L. The fraction of multinucleated spermatids decreased in relation to the control at about 30%.

All concentrations of NS caused a statistically significant alteration in the fraction of cells in apoptosis; the direction of these alterations is characterized by an unimodal curve, as well as for the level of cytogenetic damages, and is inversely related to the fraction of multinucleated nuclear spermatids.

SS had no effect on the frequency of spermatids with micronuclei and multinucleated nuclear spermatids; at the lowest investigated concentration it caused an increase of apoptosis.

Correlation analysis using the rank Spearman correlation coefficient showed a significant relationship of apoptosis indicators and frequency of spermatids with micronuclei both in the action of NS ($R = 0.84$, $N = 4$, $P < 0.05$) and SS ($R = 0.93$, $N = 4$, $P < 0.05$).

DISCUSSION

In this study we tried to simulate a real situation with the possibility nanosilver intake with drinking water upon using NS as antiseptic. In connection with this, NS was studied in the range of fairly low doses, starting from 0.1 mg/L. In practice, as antimicrobial drugs for application to mucous membranes, Protargolum or Collargolum drugs are applied: the colloidal dispersion of silver particles of submicron size which contain silver in significantly higher concentrations. The concentration of silver in a 5% solution of Protargolum is approximately 400 mg/L, and in a 2% solution of Collargolum it is 1400 mg/L. Upon admission into an organism with drinking water and the use of low concentrations, the mutagenic effect of NS in the germ cells of mammals is investigated for the first time. In the control group of mice, no micronuclei were found. The data on the control level of micronuclei in spermatids of mice are previously presented in two of our other studies. In one of them we did not observe spermatids with micronuclei [10]; in the other, the frequency of spermatids with micronuclei was 0.33‰ (CI 0.06–1.34) [11].

NS at doses of 0.01 and 5 mg/kg caused a very small but statistically significant increase in the frequency of round spermatids with micronuclei when compared to the control group (Table 1, Figs. 2a–2c). These results find indirect confirmation in [7], which describes an increasing level of DNA damages in the germ cells of rats when NS particles are administered intravenously with a diameter of 20 nm in doses of 5 and 10 mg/kg are administered intravenously. In the experiment on mice, a significant effect of NS in germ cells is revealed in the test of "anomaly of sperm heads" with the use of NS in a concentration of 170 mg/L [5]. Lower doses or concentrations of the substance are not studied. Summarizing these data, one can assume that NS induces a very weak, but significant, mutagenic effect in mammalian germ cells in the range from 0.01 to about 20 mg/kg. At a dose of 50 mg/kg (this study), cells with micronuclei were not revealed. This is a known effect for mutagens for experiments in vivo, high doses of which lead to the death of geneti-

cally damaged cells and another mechanism of organism protection comes into action [12].

Very low frequencies of spermatids with micronuclei (0.57%, the upper level of CI 1.30%) after exposure of NS raise the question of its real effect on germ cells. Two facts evidence the mutagenic effect of NS. First, there is a statistically significant difference from the control, and, secondly, the absence of an effect in the other five groups upon the action of gum and SS.

Using the karyological test allowed us to quantitatively determine the frequency of multinucleated spermatids in the testicles of intact mice after exposure of NS and SS (Figs. 2d–2h). Such a possibility has appeared due to the application of the suspension method of slide preparation, in which spermatids "swim out" from the testicular tubules and lay separate. This allows one to determine the number of nuclei in each of them. Most spermatids are mononuclear. Binuclear spermatids are less common; however, we note spermatids with 4, 8, 16, or even 20 nuclei. Interestingly, multinucleated spermatids are present in control mice, and their fraction is approximately 15.6% (CI is 12.8–18.2%). In our other two experiments, the frequency of multinucleated spermatids of control animals was 13.1% (CI is 12.2–13.9) [11] and 16.5% (CI is 12.5–20.5%) [10], i.e., very close to the results of this experiment. When exposed to gum and CC, multinucleated spermatids were presented with a frequency corresponding to the control level. A statistically significant reduction in the frequency of multinucleated spermatids is noted in all dose groups of NS. The importance of multinucleated spermatid and, their way of formation and changing nuclearity under the influence of some factors is not clear yet. Studying these cells for intact mice using a transmission electron microscope showed that they are formed by the fusion of spermatids by expanding tight intercellular bridges joining epithelial spermatogenic cells [13, 14]. The presence of multinucleated spermatids is determined for all rabbits of the control group at the age of 15 weeks and older [13]. In the control group of rats, multinucleated giant spermatids were not detected on histological slides; they appeared only for the experimental rats after the day of ligation of the spermatic cord [15]. In our three studies, multinucleated spermatids were present on suspension slides in the testicles of control mice and, under the influence of NS, their frequency was statistically significantly reduced. We assume that multinucleated cells are a reserve for the rapid replenishment of mononuclear spermatids when exposed to toxicants. This is especially important under the inhibition conditions of proliferation in the presence of NS [16].

A quantitative accounting of apoptosis by morphological criteria (Figs. 2i–2l) also gave interesting results. Upon ×100, cells in the apoptosis on the drug look like bright "blots." Magnification of ×1000 allows one to consider these structures and take them into account when calculating 1000 spermatids. In the control group of mice, the frequency of the cells in apoptosis was 3.4% (CI is 1.65–5.17%). Gum significantly increased the frequency of such cells up to 4%, and NS at a dose of 5 mg/kg increased them up to 6.5%. A higher dose of NS (50 mg/kg) caused a statistically significant decrease in the fraction of cells in the apoptosis up to 2.4% when compared with the control. From this, we can conclude that NS in a high dose blocks this type of cell death. Activation of the apoptosis upon the action of NS is noted in many studies in vitro and in vivo. On the culture of embryonic stem cells and mice embryonic fibroblasts, a significant activation of apoptosis is revealed when using Annexin V and MTT assay [17]. The induction of apoptosis and formation of reactive oxygen on the action of NS was determined in cultured human HepG2 liver cells [18]. The induction of necrosis and apoptosis is identified in L-929 fibroblasts; the smallest nanoparticles (10 nm) were the most toxic [19]. In the experiments in vivo, it is demonstrated that NS can cross the blood–brain barrier and induce apoptosis in the cells of the rat brain [20]. These data are consistent with the activation of apoptosis in germ cells of mice detected by us upon the action of NS in rather low concentrations. The lowest concentration of SS also caused an increase of apoptosis in the testes. In gum-treated group, the frequency of cells in apoptosis corresponded to the control level (table).

An important issue is the mechanism of action of NS on the genetic structures in the germ cells. Disinfecting properties of silver were used long before microorganisms were discovered. It is generally accepted that its antibacterial action is caused by oxidation, the release of ions, and their reaction with thiol groups of peptidoglycans of the cell wall and proteins of the cell membrane of bacteria and bacterial cell lysis. Moreover, silver ions bind to the DNA of bacteria, interfering with replication and protein synthesis [21]. Apparently, this mechanism also takes place upon the introduction of NS to the organism of mammals. However, the worry of researches regarding NS is related with the possibility of its direct entry into the cell and/or nucleus, as well as the generation of reactive oxygen species (ROS), which can significantly enhance toxicity. Thus, now three "candidates" are identified for the role of genotoxic agent: silver nanoparticles themselves, generated silver ions, or ROS. Each of them is capable of damaging DNA; thus is found in many experiments on the cell cultures in vitro. However, the damage of the genetic structures of sex cells in vivo is dependent on their distribution in the organism, the possibility of entering the testis, their overcoming the blood–testis barrier, and their introduction into cytoplasm and nucleus of spermatids. In one of the few in vivo experiments, the genotoxic effect of NS and increasing ROS level in the cells of the bone marrow of mice are shown after a single

intraperitoneal injection [2]. In our experiment, our colleagues have identified an increase in the activity of glutathione reductase in erythrocytes upon the introduction of NS and SS and reducing antiradical activity of blood serum upon the introduction of NS [22]. According to the authors, the biological effects of NS in animals are both due to the impact of solubilized Ag+ ions and the reaction of cells to the nanoparticles themselves. These data indicate the generalized joint action of NS, silver ions, and ROS. The possibility of NS entering the testicles is evidenced by paper [23]. The authors used transmission electron microscopy to reveal the presence of silver nanoparticles in the spermatids and sperm of rabbits after the intravenous administration of the substance at a dose of 0.6 mg/kg. In [24], the possibility of accumulation of NS in the testicles is noted after inhalation. Consequently, NS may enter testicles, overcome blood–testis barrier, and be introduced into germ cells. The possibility of NS penetrating into human sperm cells and reducing their survival is shown using transmission electron microscopy in an experiment in vitro [25]. It is believed that particles with a diameter of 8–10 nm may enter the nucleus through the nuclear pores and, at a diameter of 15–60 nm, they may remain in the cytoplasm of interphase cells and damage genetic structures during cell division, when the nuclear membrane breaks down into fragments. The absorption of NS particles by clathrin-mediated endocytosis and macropinocytosis, as well as their presence in the nucleus and mitochondria, is shown in the experiments in vitro on the culture of human lung fibroblasts IMR-90 [16]. Apparently, this way of exposure is also characteristic for germ cells of mice. Nanoparticles can damage proteins of the spindle apparatus and chromatin and disrupt DNA synthesis and chromosome segregation, i.e., cause a clastogenic and/or aneugenny effect that may appear in the form of micronuclei in daughter cells (spermatids). In our study, the frequency of spermatids itself with micronuclei increased upon the action of silver nanoparticles. The average diameter of nanoparticles is 14 nm, but some of them did not exceed 10 nm (Fig. 1) and could enter the nucleus through nuclear pores. It can be assumed that the cytogenetic effect of NS on germ cells can depend on both the direct damaging effect of silver nanoparticles and the silver ions, entering not only through the bloodstream, but also released directly in the cell or nucleus. This mechanism was called a Trojan horse. This effect is shown in [26] in vitro on human bronchial epithelial cells. Interestingly, in this study the possibility of the direct genotoxic action of NS particles is rejected in the test of DNA comets, since the effect was determined after 24 h instead of after 4 hours of exposure. The authors suggest that the genotoxic effect in this case is determined by the interaction of nanoparticles with the enzymes of DNA repair. The damaging effect of ROS, which react with many macromolecules, including DNA, also cannot be ignored. The above mentioned ability of silver nanoparticles to be introduced into mitochondria can lead to the disruption of redox processes and induce the formation of ROS and free radicals in the cell [2]. In [23], an increased level of ROS is noted in the sperm of rabbits after the administration of NS in comparison with the control. Thus, both silver nanoparticles themselves and solubilized Ag^+ ions and/or ROS can be "agents of influence," and such an action can be both generalized and local. In living objects, everything is more complicated than it might seem at first glance. However, from the viewpoint of genetic safety, the fact is important that silver in the form of nanoparticles is more toxic than its ionic forms or particles submicron in size. In this study, the effect of the ionic form of silver is noted only in the minimum concentration and only by the activation of apoptosis; however, it can be assumed that the mechanism of the action of NS in testicles is largely related to the receipt of nanoparticles, which can act directly or generate ions and ROS, and they are the greatest danger. This should be considered both in the regulation of the substance and in risk assessment.

In general, data on the negative action of NS 14 nm are obtained in rather low concentrations of 0.1 and 50 mg/L. Increasing the frequency of spermatids with micronuclei is noted. Together with with cytogenetic effect apoptosis is activated, aimed at removing genetically damaged cells, and the fraction of multinucleated spermatids is reduced. We assume that multinucleated spermatids are a "reserve" for the rapid reduction of the cell population by their decay into high-grade mononuclear ones. And then the decrease in the frequency of multinucleated spermatids we noted can be regarded as a compensatory process, aimed at replacing the genetically defective cells. These effects should be taken into account upon replacing traditionally used silver for nanosilver, especially if their size is comparable to the cell structures. The experiment shows that they are not safe even for germ cells protected by the testes barrier.

CONCLUSIONS

For the first time, the mutagenic effect of NS and SS on the experimental model of germ cells in vivo is studied close to conditions of their effect on the human body. It is established that NS 14 nm, given to mice with drinking water at concentrations of 0.1 and 50 mg/L for 15 days, induces a small statistically significant increase in the frequency of spermatids with micronuclei, which indicates the cytogenetic effect. The activation of apoptosis and decrease in the fraction of multinucleated spermatids under the action of NS can be viewed as a compensatory effect aimed at regeneration of the cell population. Silver sulfate in the minimally tested concentration increased apoptotic activity. No other changes in the same mode of action of silver sulfate was observed, indicating a more pro-

nounced effect of silver nanoparticles when compared to its ionic form on the germ cells of mice.

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