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Original Research Article

Resumption of meiotic maturation of oocytes, pre- and post-implantation mortality of embryos under ten-time intravenous treatment of silver nanoparticles in mice

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ABSTRACT

Background: In recent years the use of silver nanoparticles (AgNP) has increased significantly being focused on assessing human health and environmental risks of nanotechnology.
Methods: Research (two series) has been done on white laboratory mice. One dose (20 mg/kg) has been investigated. Frequency of treatment: one time per day for 10 times. Material for the study (ovaries, tubes and uterus) was taken under anesthetic anesthesia on the 10/11th, 21/22nd, 33/34th, 42/43rd days (after the last treatment). Oocyte meiotic maturation, pre- and post-implantation mortality of embryos under ten-time intravenous treatment of AgNP were investigation.
Results: Under the ten-time treatment of AgNPs, the inhibition of reproductive function in female mice occurs: a decrease in the number and quality of ovarian oocytes; after male mice were planted, the females do not get pregnant to 21/22 day. The reproductive function in experimental animals is restored on the 40th day after the last treatment with AgNPs; there is no differences between the values of pre- and post-implantation mortality of embryos on the 33/34th day after male mice were planted; in one of the three experimental animals, 7 live pups were born on the 42/43rd day after the male was planted (in animals of the control group during this period: twice (6 ± 1 ($n = 6$) live pups).
Conclusions: In mice females, under a ten-time treatment of AgNPs, the inhibition of reproductive function takes place; with the termination of the AgNPs treatment, the reproductive function is restored.

Keywords: Embryos, Nanoparticles, Oocytes

INTRODUCTION

Nanomedicine and nanopharmacology develop at a high pace in the search for new drugs their priority being based on silver nanoparticles (AgNPs). AgNPs with their unique optical, electron and antibacterial properties are already widely used in biosensors, photonics, electronics as antimicrobial agents as well as antitumor agents.[1-3] Nowadays, the broad spectrum of AgNPs antimicrobial activity is the main direction of development of AgNPs products, including textiles, food storage containers, antiseptic sprays, catheters and bandages. Despite the promising potential for their use in medicine, the impact of AgNPs on human health (both positive and negative) is still not fully investigated.

In existing research there are data on the toxicity of AgNPs on bacteria, aquatic organisms, and cells.[4] A significant difference in the toxicity of AgNPs (275-fold for mammalian cells and 500-fold for bacteria in in vitro

experiments and the increase in the production of AgNPs), makes it necessary to assess the environmental and human health risks of nanotechnologies. The effects of AgNPs on mammalian cells and tissues require detailed investigation.

An assessment of such effects on the reproductive system is a mandatory part at each stage of the medicine approval process. It is of paramount importance since side effects coming directly from a drug may not only affect humans or animals by but may also be relevant for future generations. This approach is applicable to both, conventional drugs as well as nanoparticles.

Reproductive toxicity studies are conducted either clinically or with the use of animals. From the final stage of maturation of gametes to the blastocyst stage, when implantation becomes inevitable, the reproductive process can be controlled using easily accessible, well-defined primary elements with well-defined functions using international standardized protocols. This period is characterized by significant cytological and molecular rearrangements and, therefore, is particularly sensitive to various impacts.

Therefore, the subtle effects can be detected very accurately. Current research will assess the effect of a dose, method, multiplicity of administration, a size of silver nanoparticles on the reproductive function using female animals, namely on the meiotic maturation of oocytes and the development of embryos. It will provide new data that will facilitate the successful transition of silver nanotechnology to theclinic that requires the development of safe, cost-effective and environmentally friendly AgNPs as well as a deeper understanding of their mechanisms of action in laboratory and during clinical trials.

The aim of the study was to investigate the resumption of meiotic maturation of oocytes and pre- and post-implantation mortality of embryos under ten-time intravenous treatment of silver nanoparticles (20 mg/kg) in mice.

METHODS

Research (two series) has been done on white laboratory mice: 36 females (10 weeks, 20-22 g) and 3 males. All experiments was certified by biomedical ethics committee of Bogomoletz institute of physiology and compliance with all requirements for work with laboratory animals (International European Convention for the Protection of Vertebrate Animals, Strasbourg, 1986). After the experiments, anesthetized animals were put down by Nembutal and dislocation of the cervical vertebrae.

The objective status of the animals was assessed before the start and during the experiment, (appearance, general motor activity, need for food and water, 2 times a week, determined body weight).

Animals were divided into two groups:

1 - control (n=12), physiological solution (0.3 ml), 2 - experimental (n = 24), AgNPs (20 mg/kg, 0.3 ml). The substance was administered intravenously (the tail vein) during 10 days for each groups of mice.

Characterization of nanoparticles AgNPs- 30 nm (concentration: 8 mg/ml for metal, shape: spherical, color: brown, reagents used for synthesis: silver nitrate (AgNO3), (BioXtra,> 99% (titration, Sigma-Aldrich); carbonate potassium (K2CO3) (99,995% trace metals basis, Sigma-Aldrich); Tannin (ACS reagent, Sigma-Aldrich) synthesized at the Ovcharenko Institute of biocolloidal chemistry NAS of Ukraine according to the original protocol (by chemical condensation).

Method of treatment

Intravenous. One dose (20 mg/kg) has been investigated. Frequency of treatment: one time per day for 10 times (for all animals in each group). Control animals injected with physiological solution 10 times.

First series

There has been investigated the effect of AgNPs on the resumption of meiotic maturation of oocytes (dissolution of the germinal vesicle) and the oocytes with atypical morphology (unevenly granulated cytoplasm and signs of fragmentation of the latter) were counted. Groups of animals: 1 - control (n = 6), 2 - AgNPs (20 mg/kg, n = 12 (3×4 animals).

Material for the study (ovaries) was taken under anesthetic anesthesia on the 10/11th, 21/22th, 33/34th, 42/43th days (after the last injection).

Second series

There has been investigated the effect of AgNPs on pre- and post-implantation embryo mortality. The introduction of substances has been performed daily for 10 days. Groups of animals: 1- control (n = 6), 2 - AgNPs (20 mg/kg, n = 12 (3×4 animals). One day after the last injection, males were planted to female in a ratio of 1:4. Duplication and further manipulation with the embryos were carried out according to the method. The experimental material was collected (ovaries, tubes and uterus) under anesthetic anesthesia on investigated terms: 10/11th, 21/22nd, 33/34th days after a male was put together with a female.

The study was completed (on the 42/43th day after male maturity), with birth in a group of animals under the ten-time administration of AgNPs in one female of three 7

live pups (in animals of the control group during this period: twice (6±1 (n = 6) live pups).

Oocytes cultivation

The oocytes have been isolated mechanically from the ovaries of mice in a non-enzymatic way (without cumulus cells and in cumulus-oocyte-cell complexes) and units/one ovary have been counted. Then the oocytes from one group were collected and distributed into separate chambers, 10-20 oocytes each. All control and experimental oocytes were cultured under the same conditions (a sterile box, cameras with 0.4 ml culture medium DME and 15 mM HEPES, Ca2+ concentration of 1.71 mM, temperature 37° C, duration 2 hours). Morphological study of oocytes was performed under a microscope MBS-10 after 2 hours of cultivation (% of total): the oocytes which restored the meiotic maturation (resumption meiosis) and were at metaphase I stage (germinal vesicle break-down) and oocytes with atypical morphology (unevenly granulated cytoplasm and fragmentation characteristics of the latter) have been counted.

Fetal mortality in mice

Female control and experimental groups were crossed with intact males. Counted: A - number of live embryos; B - number of seats of resorption (number of dead embryos); B - number of corpora lutea of pregnancy. Indicators of pre-and post-implantation death was calculated using the formula: ((B-A + B) / B) • 100% and (B / (A + B)) • 100%.

Statistical analysis

For the statistical analysis of the results the software package Origin 8Pro (Origin Lab Corp., North., MA, USA) and spreadsheets «Microsoft®Excel2003» have been used. Student's t test was performed for continuous variables. P<0.05 was considered statistically significant. The statistical analysis of the research results conducted by using analysis of variance ANOVA followed the comparison of mean values between groups by Newman-Coles test using the statistic program-6.

RESULTS

Resumption of meiotic maturation of oocytes

It has been established that a ten-time treatment of AgNPs reduces the number of oocytes isolated from one ovary on the 10/11th day and the 21/22th day to, respectively, 1.57±1.0 units/one ovary (p <0.05, n = 6) and 4.3±1.2 units/one ovary (p<0.05, n = 6) compared with control values of 12.0±1.0 units/one ovary (n = 6); restoration of oocyte count in the ovary that occurs on the 33/34th day and on the 42/43th day is 9.2±1.62 and 10.6±0.8 units/one ovary - to control values (Table 1).

Thus, the ten-time treatment of AgNPs (20 mg/kg) leads to reduction in the number of oocytes that restore meiosis in vitro (p <0.05, n = 3) at all investigated terms (10/11th, 21/22nd, 33/34th, 42/43rd days) in comparison with the following values in control animals.

Table 1: Resumption of meiotic maturation of oocytes under a ten-time treatment of AgNPs (20 mg/kg).

Group of animals	Oocytes that restored meiosis %
Control	84.83 ± 2.27
10/11 day	-
21/22 day	28.47 ± 4.23*
33/34 day	54.21 ± 6.37*
42/43 day	63.15 ± 3.41*

*p <0,05 - the probability of differences in the average data experimental groups (n = 3) with respect to such values in the control group of animals (n = 6).

Ten-time treatment effect of AgNPs on pre- and post-implantational embryonic mortality in mice

Data of pre- and post-implantational embryonic mortality under ten-time treatment of AgNPs are presented in Table 2.

Table 2: Pre and post-implantational embryonic mortality in mice under the ten-time treatment of AgNPs (20 mg / kg).

Days/group of animals	Embryonic mortality, %	
	Pre-implantation	**Post-implantation**
Control	2.76±0.74	1.38±0.37
10/11	-	-
21/22	-	-
33/34	3.41±2.72	1.87±1.43

Animals of the experimental group were not pregnant on the 10/11th and the 21/22nd days after male mice were planted to female but were pregnant on 33/34 day (two out of three animals in the experimental group).

The study was completed (42/43 days after males were planted to female), with the birth in a group of animals under the ten-time treatment of AgNPs in one female of three 7 live pups (in animals of the control group during this period: twice (6±1 (n = 6) live pups).

Thus, under the ten-time treatment of AgNPs (20 mg/kg), there occurs inhibition of reproductive function in female mice: a decrease in the number and quality of ovarian oocytes was established; after males were planted to females did not get pregnant up to 21/22 day.

The reproductive function in experimental animals is restored on the 40th day after the last treatment of AgNPs; there are no differences between the values of pre- and post-implantation mortality of embryos on the 33/34th day after a male mice were planted; in one of the three experimental animals, 7 live pups were born on the

42/43rd day after a male was planted (in animals of the control group during this period: twice (6±1 (n = 6) live pups).

DISCUSSION

Intravenous (IV) treatment (injection) of silver can still provide valuable information on the processes taking place in vivo AgNPs which have to overcome the main barriers (like skin, lungs, gastrointestinal tract) and in terms of putting it into circulation for clinical purposes (for example: dressings materials, intravascular medical devices for diagnosis, to drug delivery). Since this study was not designed to simulate scenarios of human exposure to avoid the limited systemic exposure due to the existence of cellular barriers in the skin, gastrointestinal tract and lungs, in order to assess the potential systemic toxicity, we used intravenous (IV) treatment of AgNPs: nanoparticles 30 nm (shape: spherical, color: brown).

Since a dose of 20 mg/kg body weight in mice is equivalent to the human dose of 0.81 mg/kg body weight, it corresponds to about 100 (97, 29) mg for a person of 60 kg, according to the principles of conversion of doses animals to humans.[5]

We have chosen a dose of 20 mg/kg body weight, since it exceeds (2-10 times) the doses used in previous studies IV and does not cause significant side effects in animals.[6-8]

There are data about intravenous (IV) treatment of AgNPs.[9,10] So, silver was found in the main organs after AgNPs administration done in different ways.[11,12] There is also evidence of distribution of silver in tissues after oral administration in rats.[12] The highest concentrations of silver regardless of their coverage and regarding all sizes of particles were found in spleen and liver, and lungs, kidneys and brain 24 hours after IV. The largest share of silver that reaches the blood is filtered by the liver and excreted through the gall.[6,7,13]

The scheme of silver treatment has been described depending on the size in the liver and it has been suggested that absorption of silver by the Kupffer cells hardly occurs after administration of 100 nm AgNPs, while the introduction of 10 nm and 40 nm AgNPs led to the absorption of silver by both endothelial cells and hepatocytes, in addition to the Kupffer cells. Only occasionally, silver was identified in gallbladder of 10 nm AgNP-treated mice (within the epithelial cells of the gallbladder and endothelial cells of blood vessels). In lungs silver was found in the alveolar walls, capillaries, and interstitial tissue.[13]

The embryo development under conditions of nanoparticle treatment is in the focus of attention of scientists. It has been established that in vitro silver nanoparticles 50 microM inhibited pre-implantation development of mice embryos.[14] There are also data that silver nanoparticles do not have any effect on mice embryos.[15] The differences in body weight, food consumption, weight body index pregnancy, fetal death, fetal weight and placental sex ratio, morphological changes between groups have been evaluated. It has been established that the repeated oral administration of AgNPs during pregnancy causes oxidative stress in the liver tissue at ≥100 mg/kg/day, but does not cause changes in the development of embryo and fetus under conditions of administration of doses up to 1000 mg/kg/day.[16] The rate of blastocysts after micro-injection of nanoparticles in 2-cell stage mouse embryos (nanoparticles of gold, 67.3% silver nanoparticles: 61.5% false, 66.2%, processing control: 79.4%) did not differ in experimental and control groups. Using polymerase chain reaction (PCR) in a real-time analysis of six major ontogenetic genes (CVC, BCL2L2, TP53, OCT4, NANOG, Dnmt3a) no effect of nanoparticles in gene expression of blastocyst has been registered. Unlike silver nanoparticles, the influence of comparable concentrations of Ag+ ions resulted in the immediate arrest of development of the embryo.[15]

Previously, we have obtained data that rates of embryonic mortality had not changed the conditions a ten-time treatment AgNPs (2 mg/kg and 4 mg/kg).[17] In this work, we have shown for the first time that under the conditions of the ten-time treatment of AgNPs (20 mg / kg):

1) Up to 21/22 days, the animals are not pregnant.

2) There are no differences between the pre- and post-implantation mortality rates of embryos in animals on the33/34th day after the male fertilization.

In existing research there are data about the impact of nanoparticles on oocytes.[18-20] There have been published data about the effect nanoparticles of gold, silver, and gold-silver alloy, which were covered with bovine serum albumin (BSA) on in vitro cumulus-oocytes-cell-complexes of pigs.[20] Meiotic maturation of oocytes was assessed after 46 hours of cultivation in vitro in the presence of different types of nanoparticles and silver nitrate in the environment all the time. Maturation in this case is defined as the percentage of oocytes that reflects the metaphase plate and the formed polar body (the second meiotic division) in 350 oocytes per group. The concentration of nanoparticles was 10 mg/ml, and all particles were conjugated to bovine serum albumin (BSA).[19]

Previously, we were shown that the ten-time AgNPs treatment (2 mg/kg and 4 mg/kg) results in inhibition of oocytes meiotic maturation in mice; a single- and five-time AgNPs treatment (2 mg/kg and 4 mg/kg) increases the number of apoptotic cells, while the ten-time AgNPs treatment results in an increase of the apoptotic and necrotic follicular cells surrounding oocytes.[17] In this work, we showed for the first time that under the

conditions of ten-time treatment of AgNPs (20 mg/kg) there is a decrease in the number and quality of oocytes in the ovary up to the 33/34th day after the male fertilization.

It is also another proof to support the hypothesis that the AgNPs toxicity is primarily linked to Ag+ bioavailability. It is important to stress that our data suggest that oocytes in mammals have the potential for the deployment of compensatory mechanisms of action under conditions of Ag+ administration at doses lower than those causing obvious toxicity. Probably, the differential toxicity of nanosilver may be connected with different coatings that are often applied to the AgNPs surface to achieve a stabilizing effect.[21] In addition, all the characteristics of NPs need to be considered: size, shape, crystalline structure, aggregation, chemical composition, surface properties (surface charge, surface area), solubility, and porosity. Further studies are needed for the elucidation of mechanisms of the AgNPs influence on germ and somatic cells administering AgNPs of different sizes and coatings.

Despite the fact that the toxicity appears to be caused by oxidation and inflammation, it is still not entirely clear what is responsible for the toxic effects of silver: whether this is the form of nanoparticles, or whether this is silver ions alone during oxidation of the metal.[4,22,23] There is evidence that AgNPs influence the induction of oxidative damage, change the regulation of enzymes, which are responsible for removing free radicals, change regulation of genes expression, which are involved in apoptosis and disregulation of cellular structures involved in storage, detoxification and metabolism of metals in various organs.[24,25] Silver ions were equally toxic as both metal particles containing 80% silver and pure AgNP indicating that at least their toxic potential was similar.[19]

Consequently, under conditions of ten-time administration of AgNPs (20 mg kg), the inhibition of reproductive function in female females is observed: a decrease in the number and quality of ovarian oocytes is established. The reproductive function in experimental animals is restored 37 days after the last administration of AgNPs; there are no differences between the values of pre- and post-implantation mortality of embryos on the 33/34th day after male planting; in one of the three experimental group animals, 7 live pups were born on the 42/43rd day after the male fertilization (in animals of the control group during this period: twice (6±1 (n = 6) live pups).

Present results confirm that the in vitro maturation of oocytes and evaluation of pre- and post-implantation embryos are sensitive systems for research related to nano-toxicology in which subtle effects can be visualized. Using such test systems in the future will contribute to deepening the understanding of the possible effects of nanoparticles on female reproduction. Also, future research could lead to the development of clear specifications of nanoparticles (from the dose size) under the appropriate biological conditions.

Despite the fact that existing literature data give grounds to confirm that, despite the promising potential for the use of AgNPs in medicine as well as the conducted toxicological studies AgNPs, it is still difficult to make univocal conclusion about the extent of their impact on the human body. At present, little is known about the specific effects of the AgNPs impact on the environment and it is practically impossible to reliably estimate the environmental risks associated with their production and use.

These data confirm that the oocytes and embryos make a sensitive system for studies involving nanotoxicology, which can be visualized by thin-effects. Using this test system in the future it will contribute to a deeper understanding of the possible effects of nanoparticles on female reproduction.

Studies that will assess the effect of different doses, the way the multiplicity of the treatment of various sizes AgNPs on reproduction using female animals gain topicality. Such studies will provide new data that will contribute to a fuller understanding of AgNPs mechanisms of action in the laboratory as well a successful transition of silver nanotechnology into the clinic.

CONCLUSION

Taken together, it can be argued that in female mice, under the ten-time treatment of AgNPs (20 mg/kg), the inhibition of reproductive function takes place; with the termination of AgNPs treatment, the reproductive function is restored.

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