The effect of silver nanoparticles on the reproductive system of adult male rats: A morphological, histological and DNA integrity study

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Conflict of interest

None declared

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Abstract

Background. Silver nanoparticles (AgNPs) are more often used in various products, and consequently the potential deleterious effects associated with exposure to them are of concern. Several lines of evidence have demonstrated that the toxicity of AgNPs affects different organs and leads to some side effects, including weight loss, inflammation and cell death.

Objectives. The aim of this study was to evaluate the effect of different concentrations of AgNPs on sperm parameters and testicular histology.

Material and methods. In the present study, 28 male adult Wistar rats were categorized into a control group and 3 experimental groups (AgNP-1, AgNP-2 and AgNP-3), intraperitoneally (i.p.) receiving 30, 125 and 300 mg/kg of AgNPs, respectively. Twenty-eight days after injection the epididymes and the testes of each rat were dissected in order to evaluate sperm parameters, sperm chromatin integrity and histomorphometric changes in the testicular tissue.

Results. The results showed a significant decrease in sperm count ($p < 0.0001$), vitality ($p < 0.05$) and morphology changes ($p < 0.001$) in the group receiving 300 mg/kg of AgNPs compared to the control group. A significant decrease was also observed in the number of spermatogonia, Sertoli and Leydig cells in the AgNP-2 and AgNP-3 groups ($p < 0.05$). The evaluation of sperm chromatin did not show any significant differences among the experimental groups ($p > 0.05$).

Conclusions. The data showed some dose-dependent adverse effects of AgNPs on sperm and seminiferous tubules. More experimental investigations are necessary to draw better conclusions regarding the safety of nanoparticles (NPs) on the male reproduction system.

Key words: testis, sperm chromatin, sperm parameters, histomorphometry, silver nanoparticles

Introduction

Silver nanoparticles (AgNPs) are clusters of silver atoms (varying from 1 to 100 nm in diameter) that are used as antibacterial and antimicrobial agents in clinical medicine. Silver nanoparticles are integrated into various food-contact materials, such as plastics used to manufacture food containers, refrigerator surfaces, storage bags, and chopping boards, and are used to preserve foods by inhibiting microorganism growth.^{1,2} Evidence has shown that AgNPs have a potent anti-inflammatory effect.³ Silver nanoparticles are of great importance in the treatment of diseases.⁴

Regardless of their widespread utility, the influence of AgNPs on human health and the mechanisms of their action are not fully understood. Therefore, it is important to survey their potential toxicity in living organisms, especially in mammals, in order to provide a reliable human risk assessment.⁴ Nanoparticles (NPs) like silver can induce reactive oxygen species (ROS) production, which causes oxidative stress and toxicity in various cell types.^{5,6} Silver nanoparticles can pass through cell membranes, the blood-brain barrier and the blood-testis barrier.⁷ The main target organs for AgNP deposition after systemic availability are the spleen, liver and kidneys, while there is less distribution to other organs. Additionally, high levels of silver have sometimes been found in the testes.^{5,6}

The effects of AgNPs on testis and sperm function have been reported. Pothuraju and Kaul reported the effects of these NPs on buffalo sperm parameters, which showed a dose-dependent decrease in sperm viability without a change in sperm motility at a concentration of 50 mg/kg.⁸

However, NPs must be investigated on a case-by-case basis to determine whether an NP will have a positive or negative effect on spermatogenesis, because it is dependent on the chemical composition, size, treatment period, dose level, route, duration of exposure, and recovery time.^{9,10}

Therefore, the main purpose of the present study was to evaluate the effect of different concentrations of AgNPs on sperm parameters and chromatin, and testicular histomorphometry in rats.

Material and methods

Nanoparticle preparation for analysis

We prepared AgNPs as described previously.¹¹ Silver nanoparticles were purchased from US Research Nanomaterials, Inc. (Houston, USA). Based on the specifications provided by the manufacturer, the AgNPs were of 99% purity as a nanopowder, with a nominal diameter of 60–80 nm. The AgNPs were suspended by sonication in deionized water (TKA Fisher Scientific Smart2Pure 30123; Thermo Fisher Scientific, Burladingen, Germany), and then diluted to concentrations of 30, 125 and 300 mg/kg at the time of injection.

Nanoparticle characterization

The size distribution of AgNPs was measured by dynamic light scattering (DLS) (Zetasizer Nano ZS apparatus ZEN 3600; Malvern Panalytical, Malvern, UK) at 25°C and was started 2 min after the cuvette was placed in the DLS apparatus to reach thermal equilibrium. Measurements were conducted 24 h after the suspensions were prepared.^{11,12} A Philips-EM 208 transmission electron microscopy (TEM) and a scanning electron microscope (SEM) (Philips, Amsterdam, the Netherlands) were also employed to monitor the size and morphological changes in AgNPs.

Animals

Twenty-eight adult male Wistar rats weighing 200–250 g were kept under standard conditions (12-hour light/dark cycle at 22–24°C, with free access to water and food). All of the procedures were carried out with minimal stress and discomfort in accordance with national guidelines and protocols approved by the Institutional Animal Ethics Committee of Hamadan University of Medical Sciences, Iran.

Experimental design

The animals were weighed and randomly divided into 4 groups ($n = 7$ per group): a control group and 3 experimental groups (AgNP-1, AgNP-2 and AgNP-3), receiving a single intraperitoneal (i.p.) dose of AgNPs at 30, 125 and 300 mg/kg concentrations, respectively. The animals in the control group were injected with distilled water. The animals were sacrificed 4 weeks after the injection. In rats, the duration of the spermatogenesis cycle is approx. 48–56 days. A half of this cycle and 2 cycles of the seminiferous epithelium takes 28 days.^{10,13}

Sperm collection and count

After 28 days, the left cauda epididymis of each animal was dissected and placed in a Petri dish containing Ham's F10 medium (Sigma-Aldrich, St. Louis, USA). The dishes were placed in the incubator for 15 min. Approximately 10 μ L of the diluted sperm suspension was transferred to each counting chamber of a hemocytometer and allowed to stand for 5 min. The cells which settled during this time were counted by a light microscope (Zeiss, Munich, Germany) at $\times 200$ magnification.¹³

Sperm morphology

The sperm morphology was also determined as described previously.¹⁴ Morphological abnormalities were classified as headless sperm, a flattened head, a reduced hook or banana head, a pin or nail head, a bent neck, a bent tail, a kinked tail, and multiple abnormalities. The percentage

of normal morphology of 100 spermatozoa per rat was assessed by light microscopy.

Sperm motility

Aliquots of the sperm suspension prepared for analysis were placed on a slide. The spermatozoa were classified as motile or immotile. The percentage of motility was evaluated for each of the animals used.¹⁵

Sperm viability

The sperm viability was also determined using eosin stain as described before.¹⁴ Eosin penetrated non-viable, dead spermatozoa with disrupted membranes, which appeared stained in red.

Histological evaluation

The left testes were removed and fixed in a 10% buffered formalin solution for 2 weeks, and were then embedded in paraffin. The paraffin blocks were cut into 5-micrometer slices. The sections (3 per animal) were stained with hematoxylin and eosin (H&E).¹⁰ Twenty-five seminiferous tubules from each testis were randomly evaluated on circular cross-sections, and the spermatogonia, spermatocyte, spermatid, and Leydig and Sertoli cells were counted.

Parameters of seminiferous tubules

For each set of 25 seminiferous tubules from each testis, the parameters, including area, circumference and diameter, were determined under a light microscope equipped with Motic Moticam 2000 2.0M Pixel camera (Motic, Kowloon, Hong Kong).¹⁰

Evaluation of sperm nuclear chromatin

For the evaluation of sperm nuclear chromatin, 3 different techniques were applied.

Aniline blue staining

Aniline blue (AB) selectively stains lysine-rich histones and has been used for distinguishing sperm chromatin condensation anomalies. For this purpose, air-dried smears were prepared from fresh sperm samples of each rat and fixed in 3% buffered glutaraldehyde in a 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min. For light microscopy evaluation, 100 spermatozoa were counted in each slide and pale-blue spermatozoa were considered normal, while dark-blue-stained ones were treated as abnormal spermatozoa.¹³

Toluidine blue staining

Toluidine blue (TB) is a metachromatic dye which measures the rate of sperm nuclear chromatin condensation via binding to phosphate groups of DNA strands. For this staining, air-dried sperm smears were fixed in fresh 96% ethanol:acetone (1:1) at 4°C for 30 min, and then hydrolyzed in 0.1 N hydrochloric acid (HCl) at 4°C for 5 min. Next, the slides were rinsed thrice in distilled water for 2 min, and finally stained with 0.05% TB for 10 min. The component of staining buffer was 50% citrate phosphate (pH 3.5).¹⁶ For light microscopic assessment, using $\times 100$ magnification, the chromatin quality of the spermatozoa was determined according to metachromatic staining of sperm heads in light blue (TB-) and purple (TB+).

Acridine orange staining

Acridine orange (AO) is a fluorescence probe for measuring the susceptibility of sperm nuclear DNA to in situ acid-induced denaturation. Sperm DNA integrity was determined by AO staining. For this purpose, the smears were first air-dried and then fixed overnight in Carnoy's solution (methanol:glacial acetic acid, 3:1). Each sample was stained for 10 min in freshly prepared AO (0.19 mg/mL) in citrate phosphate buffer (pH 2.5) for 10 min. The smears were evaluated on the same day with an Olympus fluorescence microscope (Zeiss) with a 460-nanometer filter. The duration of illumination was limited to 40 s per field. The percentage of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample was calculated.¹⁶

Statistical analysis

Statistical analysis was performed using SPSS v. 16 (SPSS Inc., Chicago, USA) and the variables were analyzed by one-way analysis of variance (ANOVA), the Kruskal-Wallis test and the Mann-Whitney U test. The Tukey method was used for the following multiple comparison tests. All data was expressed as mean $\pm$ standard deviation (SD). The statistical level of significance was set at $p < 0.05$.

Results

Characterization of silver nanoparticles

As previously mentioned, the DLS analysis demonstrated a broad hydrodynamic diameter peak with an average size of 250 nm (nominally < 100 nm in diameter, but actually ~ 250 nm) (Fig. 1).¹¹ The diameters of NPs observed under TEM were in agreement with the DLS results (Fig. 2).¹⁷ According to the SEM micrographs, the AgNPs were almost spherical (Fig. 3), but had a tendency to agglomerate or aggregate in the solution.

Fig. 1. Dynamic light scattering results of the characterization of nanosilver used in the study

Fig. 2. Transmission electron microscopy (TEM) image of silver nanoparticles (AgNPs); scale bars indicate 10 nm

Fig. 3. Scanning electron microscopy (SEM) image of silver nanoparticles (AgNPs); bars indicate 200 nm

Body weight

There were no significant differences ($p > 0.05$) in mean body weight between the treated groups and the control group (data not shown).

Sperm parameters

Table 1 shows the effect of AgNPs on sperm parameters. A significant reduction in sperm vitality ($p < 0.05$), normal sperm morphology ($p < 0.001$) and sperm count ($p < 0.0001$) was observed in the AgNP-3 group, while there were no significant differences in sperm motility in the AgNP-injected groups compared to the control rats.

Testicular histomorphometry

The data showed a significant reduction in the number of spermatogonia, Sertoli and Leydig cells in the AgNP-2 and AgNP-3 groups ($p < 0.05$), whereas there were no significant differences in the number of spermatocytes and spermatid cells among the 4 study groups (Fig. 4).

Parameters of seminiferous tubules

We observed a significant reduction in the diameter, circumference and mean area of seminiferous tubules in the AgNP-2 group compared to the control group ($p < 0.001$) (Table 2).

Fig. 4. The effect of silver nanoparticles (AgNPs) on testicular histomorphometry (mean $\pm$ SD)

* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$: (compared to controls); results presented as mean $\pm$ standard deviation (SD).

Assessment of sperm DNA and chromatin

Sperm DNA and chromatin assay is shown in Fig. 5. As can be seen in Fig. 6, there were no significant differences between the experimental and control groups regarding AB, TB and AO staining ($p > 0.05$).

Discussion

The main purpose of the present study was to evaluate the effect of different concentrations of AgNPs on sperm parameters and chromatin, and testicular histomorphometry. Our in vivo data revealed that AgNPs could reduce sperm parameters, such as normal sperm morphology, sperm vitality and sperm count. Silver nanoparticles caused a decrease in Sertoli and Leydig cell number. To better understand the role of AgNPs, we observed the

Table 1. The effect of AgNPs on sperm parameters

Parameters	Control	30 mg/kg	125 mg/kg	300 mg/kg
Motility [%]	62.8 ±3.03	44.4 ±8.41	53.8 ±4.18	42 ±5.8
Vitality [%]	96.7 ±0.56	94.5 ±0.71	97 ±0.72	93.1 ±1.37
Normal morphology [%]	96.7 ±0.56	92.5 ±0.75	93.5 ±0.57	89.5 ±1.87**
Sperm count [×10 ⁶ /mL]	53.1 ±1.30	51.6 ±1.48	49.0 ±1.6	36.0 ±1.7***

AgNPs – silver nanoparticles; * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ (compared to controls); results presented as mean ± standard deviation (SD).

Table 2. The effect of AgNPs on seminiferous tubule parameters in testes

Feature	Control	30 mg/kg	125 mg/kg	300 mg/kg
Area [×10 ⁻⁸ m ²]	11.6 ±0.19	11.27 ±0.17	10.44 ±0.18*	11.62 ±0.19
Circumference [×10 ⁻³ m]	1.20 ±0.01	1.18 ±0.00	1.18 ±0.04*	1.20 ±0.00
Mean diameter [×10 ⁻³ m]	0.36 ±0.00	0.36 ±0.00	0.34 ±0.00*	0.36 ±0.00

AgNPs – silver nanoparticles; * $p < 0.001$ (compared to controls); results presented as mean ± standard deviation (SD).

Fig. 5. Sperm chromatin integrity assay

A – acridine orange (AO), green-stained spermatozoa were considered to be AO-negative (non-denatured DNA); B – AO, orange-red-stained spermatozoa were considered abnormal (denatured DNA); C – toluidine blue (TB), unstained or pale blue-stained spermatozoa were considered normal; D – TB, dark blue-stained spermatozoa were considered abnormal; E – anilin blue (AB), unstained or pale blue-stained spermatozoa were considered normal; F – AB, dark blue-stained spermatozoa were considered abnormal.

Fig. 6. The effect of AgNPs on sperm nuclear chromatin

AgNPs – silver nanoparticles; AB – anilin blue; TB – toluidine blue; AO – acridine orange; results presented as mean ± standard deviation (SD).

sperm chromatin packaging by AB, TB and AO staining. Silver nanoparticles did not destroy sperm DNA or chromatin integrity in any of the 3 concentration levels (30, 125 and 300 mg/kg).

In the past decade, with the development of nanotechnology, there has been a remarkable increase in application

of NPs and nanomaterials in everyday products in industrial and medical sciences.¹⁸ Despite the beneficial effects of NPs on improving the quality of life, there is in turn an increase in the risk of human as well as animal exposure to these nanomaterials through various routes. The toxic role of NPs, especially AgNPs, has been under investigation by researchers. In vitro studies have shown that NPs induce necrosis, apoptosis and mitochondrial dysfunction in mouse spermatogonia stem cells.¹⁹ Similarly, in vivo studies have confirmed the in vitro observations and have suggested that the deleterious effects of NPs are related to their chemical composition, size and dosage.²⁰

The i.p. route is one of the most neglected routes for testing AgNPs toxicity. The i.p. route delivers substances into circulation faster than the oral route. In this study, rats were treated with AgNPs at concentrations of 30, 125 and 300 mg/kg. These dosage levels were selected based on a previous 28-day oral toxicity study by Kim et al.²¹ The pharmacokinetics of substances administered i.p. is more similar to this seen after oral administration.²²

Consistent with previous reports, our results showed a significant reduction in sperm function in a dose-dependent manner. A high dose of AgNPs could affect conventional sperm characteristics, such as normal morphology and viability. Sleiman et al. indicated that AgNPs decreased sperm parameters in the pre-pubertal period of rats.²³ Sperm count is one of the most sensitive tests for spermatogenesis, since it gives the cumulative result of all stages of sperm production and is highly correlated with fertility.

Silver nanoparticles could damage sperm membranes and/or penetrate the cells, subsequently increasing free radicals, including ROS, that cause membrane lipid peroxidation and, in consequence, loss of motility and viability, and can injure the sperm membrane and flagellum structure, eventually leading to sperm motility and morphology perturbation.^{6,19,24}

Importantly, based on our data, AgNPs caused a significant reduction in testicular morphological characteristics, such as the diameter, area and circumference of seminiferous tubules, at a concentration of 125 mg/kg, compared to the control group. In contrast to our result, Gromadzka-Ostrowska et al. showed a significant increase in the testicular morphology of animals treated with 200 nm of AgNPs at a dose of 5 mg/kg 28 days after intravenous injection.¹⁰ Furthermore, Miresmaeili et al. showed that there were no significant changes in seminiferous tubule diameter in animals treated with oral AgNP administration at doses of 25, 50, 100, and 200 mg/kg after 48 days (the time period of spermatogenesis in rats).²⁵ Many in vivo studies have shown that chemicals, hypoxia or metals such as chromium, cadmium or lead can decrease the diameter of seminiferous tubule epithelial cells.¹⁰ Accordingly, it seems that AgNPs have a dual effect on testicular morphological characteristics: at higher concentrations they reduce these features, and at lower doses increase them. Intraperitoneal injection of AgNPs might influence the endocrine system, and, consequently, may manipulate endogenous testosterone and estradiol levels; hence, these endocrine alterations might protect seminiferous tubules at high concentrations.²³

Moreover, a histological evaluation of the testis tissue indicated a significant decrease in the mean number of spermatogonia, Leydig and Sertoli cells at doses of 125 and 300 mg/kg compared to controls. In support of our finding, Miresmaeili et al. showed a significant decrease in the number of primary spermatocyte and spermatid cells at doses of 50, 100 and 200 mg/kg.²⁵ It has been shown that AgNPs markedly decreased spermatogonial stem cell proliferation by some intracellular pathways.²⁶

Silver nanoparticles, like other NPs, can damage the DNA structure of Leydig cells, leading to apoptosis. During spermatogenesis, histones are replaced by protamines in the nuclear DNA. Anilin blue can determine the protamines deficiency in sperm chromatin. The presence of disulfide bonds in the chromatin of mature sperm cells can prevent the denaturation of DNA, and TB can measure

the rate of sperm nuclear chromatin condensation and DNA fragmentation via binding to DNA phosphate. Purple sperm heads showed DNA damage. Acridine orange is a fluorescence probe for the detection of denatured or single-stranded DNA in spermatozoa, and red fluorescence in sperm heads showed DNA damage or altered chromatin structure in sperm cells.¹⁶ Our study also showed no significant changes in chromatin integrity.

Our results were in agreement with the findings of various studies which reported no significant correlation between sperm chromatin condensation and sperm parameters, including motility, vitality, normal morphology, and sperm count.^{27,28} In contrast, other studies reported significant correlations between sperm DNA fragmentation and poor sperm morphology.^{29,30}

Conclusions

Collectively, these findings indicate the effect of AgNPs on the male reproductive system. Normal sperm morphology and viability was disrupted by AgNPs. Silver nanoparticles at a high concentration (300 mg/kg) also reduced the number of testicular tubules, and spermatogonia, Sertoli and Leydig cells. Spermatozoa DNA was not affected by AgNPs, which needs more investigation on an intracellular level. The in vitro results indicated that the harmful effect of AgNPs is related to their chemical composition, size, dosage, means of administration, and duration of exposure.³¹ Further investigation is necessary to clarify the mechanisms of NP-induced male reproductive dysfunction.

Given these findings, we can conclude that AgNPs pose a potential risk to male fertility, depending on their dose, and taking into consideration the protective role of AgNPs in relation to some parameters, a study over a long period of time should be carried out in our future work.

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