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Sub-acute intravenous administration of silver nanoparticles in male mice alters Leydig cell function and testosterone levels

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ABSTRACT

The aim of this study was to determine whether short-term, *in vivo* exposure to silver nanoparticles (AgNPs) could be toxic to male reproduction. Low dose (1 mg/kg/dose) AgNPs were intravenously injected into male *CD1* mice over 12 days. Treatment resulted in no changes in body and testis weights, sperm concentration and motility, fertility indices, or follicle-stimulating hormone and luteinizing hormone serum concentrations; however, serum and intratesticular testosterone concentrations were significantly increased 15 days after initial treatment. Histologic evaluation revealed significant changes in epithelium morphology, germ cell apoptosis, and Leydig cell size. Additionally, gene expression analysis revealed *Cyp11a1* and *Hsd3b1* mRNA significantly upregulated in treated animals. These data suggest that AgNPs do not impair spermatogonial stem cells *in vivo* since treatment did not result in significant decreases in testis weight and sperm concentrations. However, AgNPs appear to affect Leydig cell function, yielding increasing testicular and serum testosterone levels.

1. Introduction

Spermatogonial stem cells (SSCs) provide the foundation for spermatogenesis through their ability to both self-renew and generate daughter cells, which differentiate into spermatozoa. These characteristics make SSCs vital for the maintenance of male fertility and the perpetuation of the species. The complex biological processes underlying spermatogenesis are particularly sensitive to environmental insults [1]. Many chemicals and ultrafine particles have been shown to have a detrimental effect on the testes, either directly, by affecting the germ cells, or indirectly, by acting on the somatic cells [2,3].

Nanoparticles (NPs) have been shown to easily traverse biological membranes such as those across epithelia and the walls of very small capillaries. Thus, following exposure or systemic administration, NPs can easily move throughout the body, potentially affecting any number of different tissues [4–6]. For example, in addition to passing through the blood–brain barrier [7–9], NPs have been shown to penetrate the blood–testis barrier and reach the seminiferous epithelium [5]. NP-related damage to the brain has been shown [9], but few studies have focused on the effect of NPs on the testes.

Among these detrimental substances are silver nanoparticles (AgNPs), which typically range in size from 1 to 100 nm and have become one of the most commonly used nanomaterials in consumer products mostly due to its antibacterial properties. AgNPs may be found as components of bandages, clothing, cosmetics, car wax, toys, and antistatic materials [10,11]. Several previous reports have shown that AgNPs have negative effects on somatic cell lines *in vitro* [12–16]. *In vivo* studies have also shown AgNP-related injuries to the brain, liver, and lung [9,17,18]. AgNPs are additionally a concern for male fertility because they have been found to reach the testes after administration [19–21].

Recently, several studies described the effects of subchronic oral or inhalation toxicity of AgNPs in rodents [19,20]. In each of those studies, the accumulation of silver was observed in the blood and all tested organs, including the liver, spleen, kidneys, thymus, lungs, heart, brain, and testes. To our knowledge, however, no study described the effect of AgNPs on fertility, sex hormone production, or live sperm output and performance.

Recent studies from our lab examined the *in vitro* effects of AgNPs on the mouse spermatogonial stem cell (SSC) line, C18-4, at the molecular level [22–24]. Our data showed that AgNPs interfere with SSC proliferation in a dose-dependent (μg particles per ml culture medium) and particle size-dependent manner

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and that small AgNPs (10–25 nm in diameter) are more likely than larger sized AgNPs (80–130 nm in diameter) to promote apoptosis or the production of reactive oxygen species in these cells. In addition, our data demonstrated that AgNPs are able to disrupt components of the glial cell-derived neurotrophic factor (GDNF) signaling pathway, a pathway essential for self-renewal of SSCs *in vitro* and *in vivo*. Our data revealed that at a concentration of 10 μg/ml, AgNPs specifically interacted with and inhibited the activity of Fyn kinase downstream of GDNF, thereby inhibiting SSC proliferation *in vitro*. These acute *in vitro* effects of AgNPs, if recapitulated *in vivo*, would be deleterious to male fertility.

In vitro studies have established a direct link between AgNPs and SSC toxicity [22,23], and *in vivo* studies have demonstrated the ability of NPs to penetrate the testes and/or negatively affect testis function [5,8,19,20,25–28]. Based on our previous *in vitro* data [23], we hypothesized that AgNPs could directly influence SSC activity *in vivo*, thereby negatively affecting sperm production and fertility. To test this, we administered AgNPs (1 mg/kg/day or 0 mg/kg/day control) intravenously to male mice 5 times over a 12-day period and assessed AgNPs' effect on fertility, sex hormone production, testis histology, and sperm output. Administration of AgNPs resulted in changes in testis histology, increases in serum and intratesticular testosterone, and increases in intratesticular *Cyp11a1* and *Hsd3b1* gene expression. However, AgNPs did not affect sexual behavior, body and testis weights, sperm concentration and motility, or fertility as assessed by pregnancy rate, number of implantations, and gestational survival. Therefore, AgNPs do not appear to be toxic to germline stem cells *in vivo* at the concentration and duration of exposure tested, but our results demonstrate that AgNPs have the potential to significantly alter steroid production and impact testis health.

2. Methods

2.1. Silver nanoparticles

Citrate-coated AgNPs of approximately 10 nm size were purchased from nanoComposix, Inc. (San Diego, CA, US) at a supplied stock concentration of 1.00 mg/ml. The AgNP size distribution after 1:10 dilution of stock nanomaterial in phosphate buffered saline (PBS) was measured by dynamic light scattering (DLS; Brookhaven Instruments, Holtsville, NY), and the mean diameter was found to be 14 nm (Fig. 1A). Additionally, the size and morphology was assessed through transmission electron microscopy (TEM; Fig. 1B).

2.2. Transmission electron microscopy of AgNPs

One drop of stock nanoparticle solution diluted 1:10 in PBS was placed on a formvar plastic and carbon-coated copper grid that was placed atop of a small piece of Parafilm. Excess sample was removed with filter paper and the grid was submerged in a solution of 2% ammonium molybdate for 2 min. Excess fluid was again removed using filter paper, and the grid placed into a grid box, which was then covered with drierite desiccant crystals for 10 min. The grid was then examined and photographed with a Hitachi H600 Transmission Electron Microscope at 20,000× or higher magnification.

2.3. Mice

Male and female *CD1* mice (4- to 5-weeks old) were obtained from Charles River Laboratories (Wilmington, MA, US) and maintained in the animal facilities of the College of Veterinary Medicine at the University of Illinois Urbana-Champaign and the Institute for Biosciences and Technology, Texas A&M Health Science Center. At both facilities, mice were housed at 25 °C with a 12 h light, 12 h dark photoperiod and given tap water and a standard rodent chow diet. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois Urbana-Champaign and the Institute for Biosciences and Technology, Texas A&M Health Science Center, and all experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Prior to administration of the first dose, mice were acclimated for 1 week. In sum, 72 males were randomly divided into 6 sets of 12 mice each, and each set of 12 mice were randomly divided into two separate cohorts: vehicle (control group) and 1 mg/kg AgNP (per dose) treated. Each set was used for the different time points and analyses as discussed below. The concentration of 1 mg/kg was chosen to achieve serum concentrations roughly equivalent to the lowest *in vitro* dose found with effects in our previous studies, 10 μg/ml [23].

Nanoparticle suspensions were freshly prepared for each animal exposure by diluting stock nanoparticle solution 1:10 in phosphate buffered saline (PBS), pipetting up and down, and then immediately injecting into the tail veins of treated mice. Particles were diluted in PBS to obtain a physiological solution suitable for intravenous injection and no agglomeration was observed visually or through DLS analysis during the short (approximately 0.5–2 min) duration between dilution and injection (or DLS analysis). Control mice were given an equivalent amount of PBS containing a 1:10 dilution of 2 mM citrate buffer *via* the tail vein. Mice were given 5 injections (1 injection every 3 days on days 0, 3, 6, 9, and 12) (Fig. 2).

Three sets of control and treated mice were utilized for isolation at 15 and 120 days post-initial treatment: two sets for the isolation of testes for ICP-MS analysis at 15 and 120 days and the other

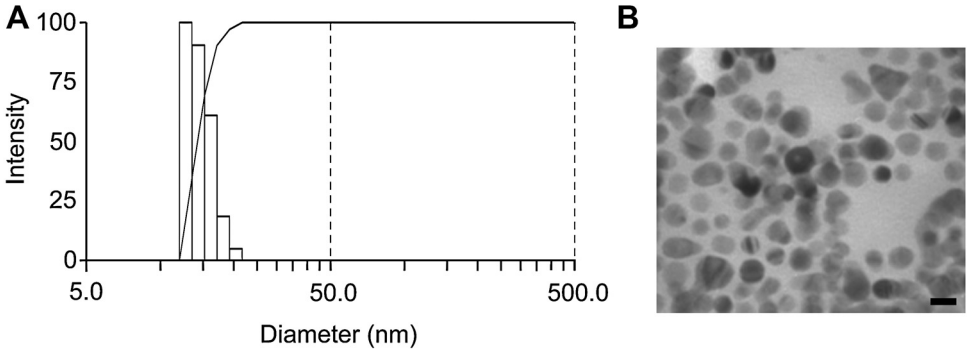

Fig. 1. Size characterization of the silver nanoparticle suspension. Size distribution (Panel A) as determined by dynamic light scattering and transmission electron micrograph (Panel B) of the silver nanoparticles once diluted in PBS. Scale bar equals 10 nm.