Histomorphometric Alterations Induced in the Testicular Tissues by Variable Sizes of Silver Nanoparticles

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OBJECTIVE: To investigate the histomorphometric alterations induced in testicular tissues by variable sizes of silver nanoparticles (SNPs).

STUDY DESIGN: Male mice (BALB/C) were treated with SNPs (1 mg/kg) using 5 different sizes (10, 20, 40, 60, and 100 nm) for 35 days. Testicular biopsies from all mice under study were examined histomorphologically.

RESULTS: SNPs sized 10 and 20 nm had provoked morphometric changes in the testes of the subjected mice together with the following histological alterations: seminiferous tubules, degeneration, spermatocyte cytoplasmic vacuolation, spermatocyte sloughing, and spermatid giant cell formation. Larger SNPs (40, 60, and 100 nm) induced little or no testicular histomorphometric alterations.

CONCLUSION: The findings of the present work may indicate that subchronic exposure to SNPs could have a deleterious impact on the testicular tissues and spermatogenic process that could affect fertility and reproduction, with smaller SNPs being more toxic than larger ones. (J Reprod Med 2017;62:317–323)

Keywords: morphometric alterations, nanocrystalline materials, nanocrystals, reproduction, seminiferous tubules, silver, silver nanoparticles, spermatid giant cells, spermatids, spermatoblasts, spermatogenesis, testes.

...SNPs could induce toxicological effects on the ... spermatogenic process ...

Silver nanoparticles (SNPs) have unique optical, electrical, and thermal properties and are being used widely in nanomedicine and products for consumption owing to their unique antimicrobial, antifungal, and antiviral properties.[1,2] These fine particles have been widely invested in alginate fibers, wound dressings, medical masks, gels, cosmetics, deodorants, shampoo, sunscreen, footwear, and athletic shirts due to their ability to limit bacterial growth.[3-9] Moreover, SNPs are being used in

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Funded by the Deanship of Scientific Research, King Saud University (research project IRG 14-06).

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Financial Disclosure: The authors have no connection to any companies or products mentioned in this article.

0024-7758/17/6205-06–0317/$18.00/0

biological and chemical sensors and in the imaging of neural tissues.[7] These together have given SNPs potential promise as potent constituents of pharmaceutical, medical, and industrial products.[8]

The small nature of SNPS together with their high surface area to volume ratio enables these particles to enter the tissue components as biological molecules do.[9] In addition, these fine particles have a large functional surface area with dimensions almost the same as those of biological micromolecules, with the possibility of being adsorbed on the surface of these molecules in tissues and body fluids.[6] Nano studies have indicated that SNP toxicity might be related to the charge and functional groups on their surface together with their ability to bind or interact with the tissue and cell components, leading to the production of hydrogen peroxide and reactive oxidative species that could damage plasma membrane and cell organelles, especially the mitochondria and cytoskeleton.[10-15] Some studies have concluded that SNPs could demonstrate DNA damage, cell cycle disturbance, genotoxic and cytotoxic consequences, and reduction in metabolic activity due to their potential to release silver ions.[16-20]

Human exposure to various forms of SNPs is increasing as these particles become more commonly used in products, creating a greater potential for risk to human health. Full attention must be given to determine the safety of these fine particles, with special attention to size, composition, and behavior of these particles. SNPs have been found to induce histological and histochemical alterations in vital organs, including the liver and kidney.[2,7,21-27] Few studies are available on the reproductive toxicity of SNPs associated with the histological alterations induced by these particles on testicular tissues. With this objective, the present study aims to determine the histomorphometric testicular alterations induced by different sizes of SNPs.

Materials and Methods

Animals and Conditions

A total of 42 adult healthy male mice (BALB/C) were used throughout the present study. All mice were randomly divided into 6 groups (a control group and 5 test groups) of 7 animals each, housed at room temperature (24±1°C) with 12 hour light/12 hour dark cycles at the Faculty of Medicine animal facility, University of Jordan. The mice were provided with commercial pellets and tap water *ad libitum*.

Nanoparticles

Naked spherical SNPs (10, 20, 40, 60, and 100 nm; Sigma-Aldrich, USA, with mass concentration of 0.02 mg/mL in aqueous citrate buffer) were used in the study. The specified size of these SNPs was confirmed by Transmission Electron Microscope techniques.

Experimental Protocol

Following a period of stabilization (7 days), all members of all groups were exposed to intraperitoneal injection with a daily single dose of SNPs (0, 10, 20, 40, 60, and 100 nm) for 35 days.

All animals were handled and all experiments were conducted in accordance with the protocols approved by the University of Jordan Local Animal Care ethical committee, while the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

Organ Weight Monitoring

The mice were killed by cervical dislocation and the testes of each dissected mouse were removed carefully, weighed in grams (absolute organ weight) on the dissection day. The relative testes weight for each mouse was calculated according to the following equation of Aniagu et al[28]:

Relative organ weight = (Absolute testes weight [g] / Body weight of mouse [g]) × 100.

Organ Index

From the obtained data the amount of change on the relative ratio of the testes (T_x) of mice subjected to different sizes of SNPs for 35 days was calculated according to the following equation[29]:

$$T_x = \frac{\text{Average weight of the experimental testes/ Average weight of the experimental animals}}{\text{Weight of the control testes/ Average weight of the control animals}}.$$

Histological Processing

Biopsies from the left testis from each mouse of all groups were cut out rapidly, fixed in 10% neutral buffered formalin, and then dehydrated with ascending grades of ethanol (70%, 80%, 90%, 95%, and 100%). Dehydration was then followed by

clearing the tissue samples in 2 changes of chloroform before being impregnated with 2 changes of melted paraffin wax, embedded, and blocked out. Testicular sections (4–5 μm) from all experimental mice of all groups were prepared for hematoxylin and eosin (H&E) stain according to Bancroft and Stevens.[30] The histological processing and histological examination of all testicular samples were carried out in the Toxicological Unit, Zoology Department, King Saud University.

TUNEL Staining

Terminal dUTP nick end labeling (TUNEL) assay was performed by using an in situ TUNEL Apoptosis Detection Kit (GenScript, USA). Known positive control sections were used while negative control sections were incubated in the label solution only without terminal transferase.

Statistical Analysis

The amount of change on the relative ratio of the organ weight to body weight of mice subjected to different sizes of SNPs for 35 days±standard deviation (SD) for each group after treatment with different sizes of SNPs was calculated and expressed from the obtained data. The significant differences between SNP-treated groups and the control group were tested by Student's *t* test, where p values <0.05 were considered statistically significant.

Results

Macroscopic Alterations

No mortalities or signs of toxicity were detected in any of the experimental groups of the present study. Also, no macroscopic anomalies were seen in the appearance and behavior of mice subjected to the different sizes of SNPs. Mice of all treated groups, except 20 nm, showed a slight decrease in the relative ratios of total testis weight to body weight and testis index (Table I) (Figure 1). The percentage absolute testes weights were not significantly (p value >0.05) affected in all treated mice (Figure 2).

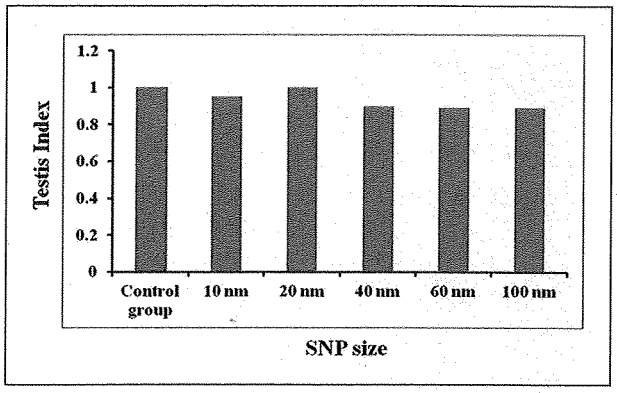

Figure 1 Testes index for control and SNP-treated groups.

Microscopic Alterations

Control Mice. Examination of the control mice testicular histological sections revealed normal, intact seminiferous tubules at various stages of spermatogenesis together with normal intertubular tissues (Figure 3A–C).

Mice Exposed to 10 nm SNPs. In comparison with the control group the testes of mice treated with 10 nm SNPs for 35 days showed occasional seminiferous tubule ghost degeneration with spermatocyte cytoplasmic vacuolation (Figure 4A). Moreover, mild intertubular edema was also detected (Figure 4B).

Mice Exposed to 20 nm SNPs. The testes of mice treated with 20 nm SNPs for 35 days had spermatocyte sloughing and occasional appearance of

Table I *Change in Relative Ratio of Total Testes Weight to Body Weight of Mice Subjected to Different Sizes of SNPs for 35 Days*

Dose	Average total testis weight (g)	Average body weight (g)	Relative testis weight	Testis index (T_x)
Control group	0.25±0.02	30.83±1.18	0.83±0.28	1.0
10 nm	0.24±0.02	31.03±2.21	0.79±0.10	0.95
20 nm	0.23±0.03	27.77±3.96	0.83±0.18	1.0
40 nm	0.22±0.05	29.57±3.31	0.75±0.15	0.90
60 nm	0.23±0.03	31.27±2.77	0.74±0.11	0.89
100 nm	0.22±0.01	29.51±1.69	0.74±0.14	0.89

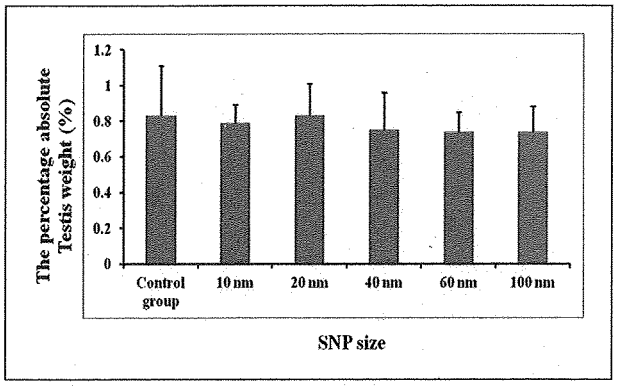

Figure 2 The percentage absolute total testes weight of control and SNP-treated mice.

spermatid giant cells in the lumen of some seminiferous tubules. Spermatid detachment and accumulation of desquamated spermatocytes together with spermatids and cellular debris were also observed in the lumen of the seminiferous tubules (Figure 5A–B).

Mice Exposed to 40 nm SNPs. Mice treated with 40 nm SNPs for 35 days showed almost normal testicular tissues with occasional seminiferous tubule epithelial degeneration.

Mice Exposed to 60 or 100 nm SNPs. The testicular tissues of mice exposed to 60 or 100 nm SNPs for 35 days showed almost normal testicular structures.

Apoptosis Detection

In comparison with the control sections, sloughing spermatocytes in the testicular tissues of mice subjected to 10 or 20 nm SNPs for 35 days failed to stain by TUNEL method (Figure 6).

Discussion

Silver nanoparticles are the most widely invested NPs in consumer products and medicine due to their unique properties.[31] These fine particles have been used in different sectors of the medical field, including prevention, diagnosis, and treatment. Nanosilver particles of 9–50 nm in size are being utilized in many products as antimicrobial agents, medical device coating, and as biosensors for disease diagnosis. The number of SNP-containing products has grown from 300 in 2006 to 1,300 by the beginning of 2014.[32] Routine exposure to SNP clinical applications includes wound dressings and nanosilver-coated medical devices such as venous catheters, drainage catheters, contact lenses, dental instruments, and biosensors. In addition, these particles are invested in nanosilver bone cement, topical cream, meshes to bridge large wounds, and in numerous assays as biological tags for quantitative detection.

The results of the present work have shown that subchronic exposure to SNPs causes a slight decrease on the relative ratio of total testis weight to body weight and testis index, while the percentage of absolute testis weight was not significantly affected in any of the treated mice. One study reported that chronic exposure to 56 nm SNPs for 90 days induced testis enlargement, in the left testis, particularly.[23] Together, these findings may support previous reports indicating that SNPs could cross the blood-testes barrier and accumulate in the testes.[31,33]

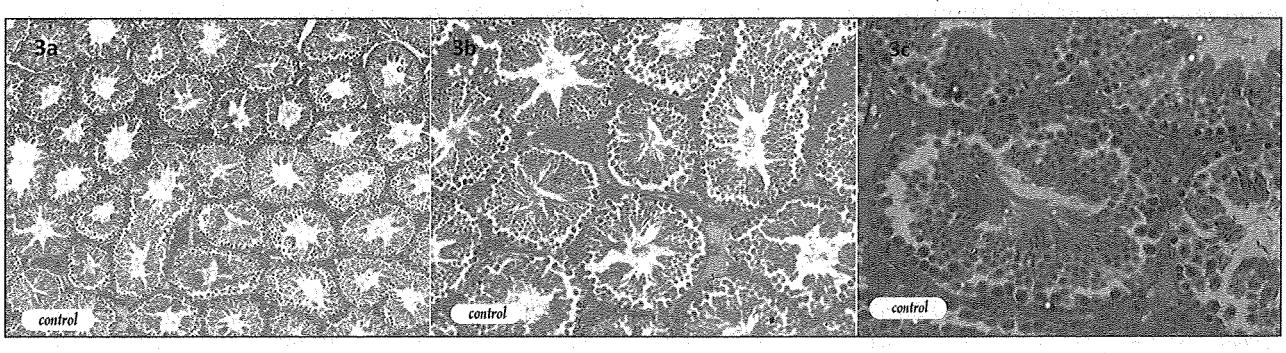

Figure 3 Light photographs of sections in the testes of control mice demonstrating (A) normal testicular histological structure (H&E stain, ×150), (B) normal seminiferous tubules together with normal interstitial tissues (H&E stain, ×300), and (C) normal pattern of spermatogenesis (H&E stain, ×750).

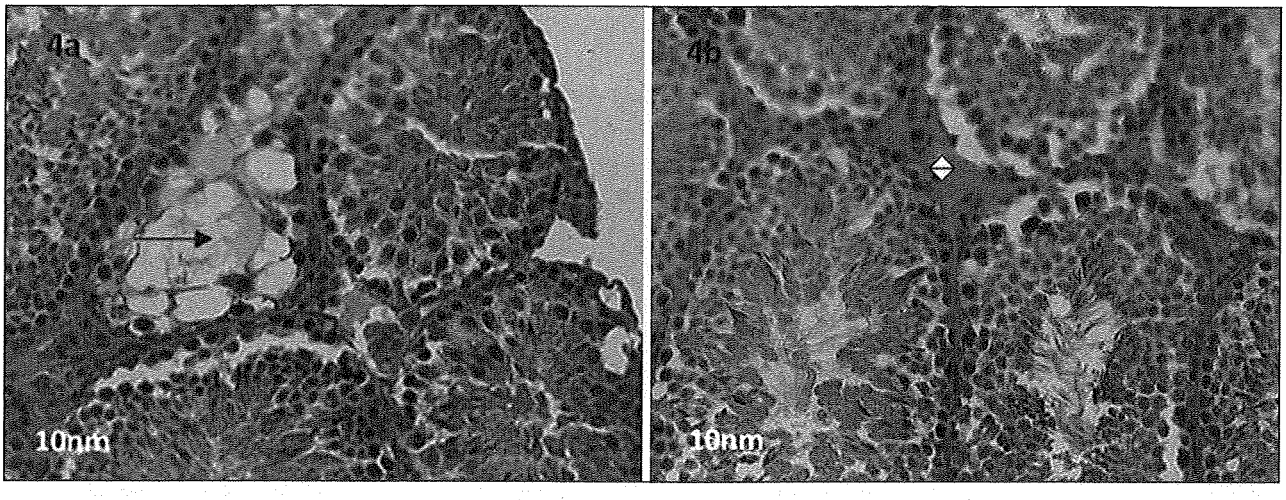

Figure 4 Light photographs of sections in the testis of a mouse that received 10 nm SNPs for 35 days demonstrating (A) degenerative spermatogenic cells with cytoplasmic vacuolation (arrow) and (B) mild intertubular edema (double triangles). (A–B) H&E stain, ×450.

The findings of the present study showed that 10 and 20 nm SNPs could induce testicular alterations in the form of spermatocyte sloughing and moderate seminiferous tubule degeneration. These findings are in line with a recent ultrastructural study by Thakur et al[34] in which 20 nm SNPs induced disappearance of the normal testicular histological feature with structural damage including depletion and necrosis of spermatocytes and Leydig cells. In addition, the results of the present work are consistent with the report from the Norwegian Institute of Public Health, which indicated damaged testicular cells due to SNP exposure.[31] Moreover, particle-size-dependent cellular toxicity was reported by Kim et al,[20] who found that 10 nm SNPs were more toxic than were the larger sizes (50 and 100 nm).

Spermatocytes and spermatid sloughing, as demonstrated by the present study, might indicate that SNPs could induce cytotoxicity. The fail-

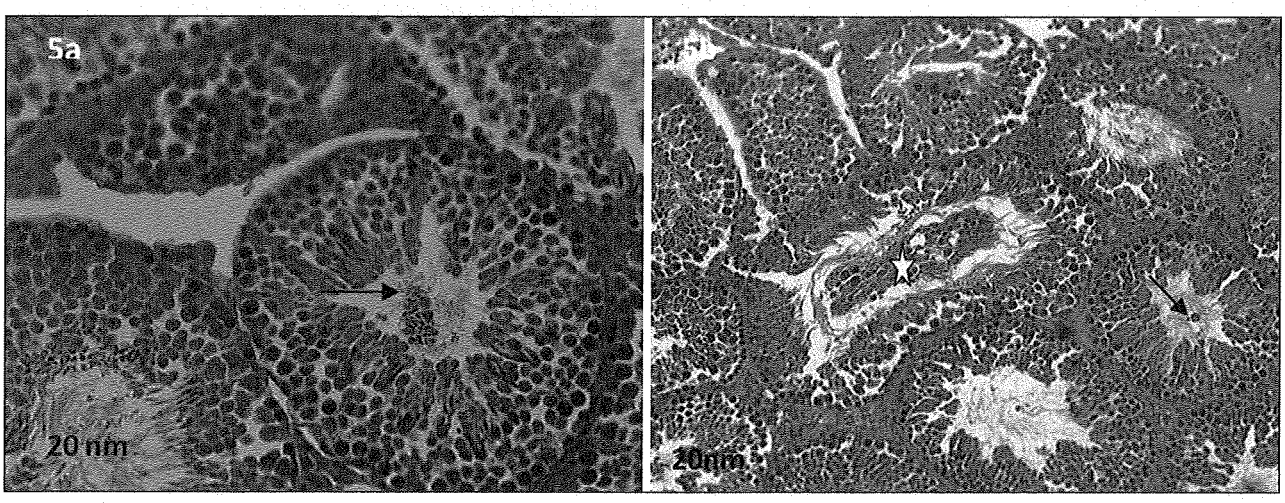

Figure 5 Light photographs of sections in the testis of a mouse that received 20 nm SNPs for 35 days demonstrating (A) spermatocytes sloughing (arrow) (H&E stain, ×450) and (B) accumulation of desquamated spermatocytes (star) and spermatid giant cell (arrow) (H&E stain, ×300).

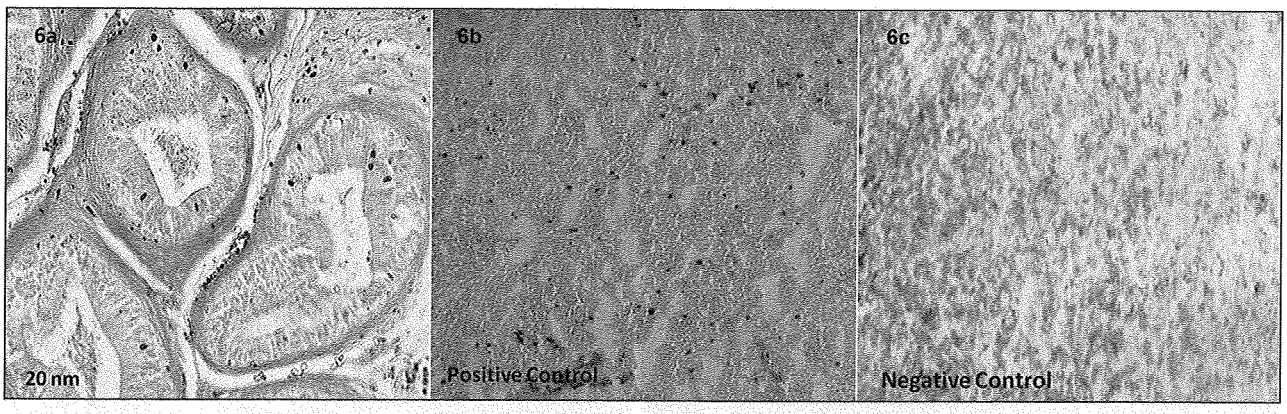

Figure 6 Light photographs of (A) TUNEL-stained testis section of a mouse that received 20 nm SNPs for 35 days, demonstrating no apoptotic nuclei of the detaching germ cells (TUNEL assay, ×450), (B) positive control section for apoptosis after incubation in TUNEL stain working solution demonstrating apoptotic nuclei stained dark brown (TUNEL assay, ×200), and (C) control section for apoptosis after incubation in the label solution only without terminal transferase to work as negative control (no positive stain is seen, ×200).

ure to detach spermatocytes for the purpose of staining with TUNEL stain might indicate that SNPs cause no DNA fragmentation and the detached germ cells are not apoptotic ones but instead might be the result of the disruption of the physical interaction of these cells due to SNP toxicity. On the other hand, the induced seminiferous degeneration might indicate SNPs' interference in the dynamic process of spermatogenesis. Several studies report cytotoxicity and oxidative stress with exposure to SNPs. Subchronic exposure to these particles shows accumulation of these particles in the testicular tissues and induced germ cell death by SNPs' oxidative stress.[17,33] Some studies reveal that SNPs could cause a decrease in the epididymal sperm count and DNA damage in spermatocytes together with change in seminiferous tubule morphometry.[35]

The morphometric abnormalities and histological alterations in the testicular tissues as seen in the present work reveal that smaller SNPs (10 and 20 nm) were more toxic and induced more testicular damage than did larger ones (40, 60, and 100 nm). This finding is in agreement with other studies that considered the size of SNPs a key factor of the toxicity of these nanoparticles.[36] Smaller-size SNPs might have more impact on the germ cell cytoskeleton and spermatogenesis than do the larger ones, with potential risk to male fertility and reproduction, where smaller NPs have much greater surface area to volume and have longer circulating residue than do the larger ones. In addition, some studies indicate that SNPs' potential toxicity could be related to dissolution rate, reactive surface area, and bioavailability, where smaller particles release more silver ions due to their greater surface area to mass ratio.[15] Oxidative dissolution of smaller particles may produce more hydrogen peroxide, depletes more dissolved oxygen, generates more intracellular reactive oxygen species, and leads to more cellular damage than the dissolution of the larger ones does.

The findings of the present study may indicate that subchronic exposure to SNPs could induce toxicological effects on the testicular tissues and spermatogenic process, with potential risks and possible consequences on fertility and reproduction. The results of the current work also indicate that different sizes of SNPs have different impacts on the testicular tissues, where smaller SNPs are more toxic to the testes than are the larger ones.

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