Toxicity Investigation of Nano-SiO2 on Male Reproductive System in Pubertal Mice

Fanli Sun  
North China University of Science and Technology

Xuying Wang  
North China University of Science and Technology

Pinzheng Zhang  
North China University of Science and Technology

Ziyun Chen  
North China University of Science and Technology

Zhiyi Guo  
North China University of Science and Technology

Xuan Shang (✉ shangx@ncst.edu.cn)  
North China University of Science and Technology  https://orcid.org/0000-0002-1437-3276

Research

Keywords: Puberty, Nanoparticles, Reproductive toxicity, Male fertility, Oxidative stress, Inflammation, Apoptosis

DOI: https://doi.org/10.21203/rs.3.rs-476481/v1

License: ☒ ☑ This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
Abstract

Background

Puberty is a crucial stage to gain reproductive capacity, but it is also a period vulnerable to exogenous materials. While exposure to nanoparticles (NPs) has been linked to toxic responses in reproductive system in previous findings, little is known about the age-dependent effect of NPs, let alone the underlying mechanism. In the present study, we assessed male fertility parameters and explored its mechanism following intraperitoneal exposure to Nano-Silicon dioxide (Nano-SiO$_2$) in mice during puberty.

Methods

40 mice aged 5 weeks were divided into 2 groups after 1 week acclimation and then exposed to 40mg/kg Nano-SiO$_2$ dissolved in saline or vehicle controls by intraperitoneal injection every day over a period of 7-day, respectively. Changes in the structure and function of male reproductive organs were detected after exposure.

Results

Nano-SiO$_2$ exposed through intraperitoneal injection could cause damage to the testicular and epididymal histological architecture and reduce the level of sex hormone (testosterone), leading to a decrease in sperm quality and quantity. Furthermore, Nano-SiO$_2$ could induce oxidative stress and inflammation in male reproductive tissues, indicated by reduced activity of antioxidants (superoxide dismutase, SOD) and increased level of the lipid peroxidation marker (malondialdehyde, MDA), which leads to the activation of cell apoptosis.

Conclusion

Exposure to Nano-SiO$_2$ in pubertal mice could cause toxicity on male reproductive system via inducing oxidative stress and activating TNF-α mediated apoptotic pathway.

Background

During the past forty years, males in multiple countries all over the world have experienced a decline of more than 50% in the quantity and quality of semen which partially responsible for the 10-15% prevalence of infertility[1, 2]. Meanwhile, a spectacular rise in testicular cancer and other male reproductive diseases, including disorders of spermatogenesis, have occurred in all parts of the world [3]. The high incidence of male reproductive disease may be caused by the widespread of environmental pollution and the sensitivity of male reproductive system to exogenous hazards, especially during crucial windows of development.
Puberty is the period of transition between childhood and adulthood, characterised by the development of gonadal maturation and attainment of reproductive capacity [4]. As for male, puberty is thought to occur approximately from postnatal day [PND] 42 to 49 in rodents and the completion of first wave of spermatogenesis is considered as the onset of puberty [5]. In male mice, from day 7 to 35 postpartum (pp), spermatogonia within the seminiferous tubules transform through the differentiated spermatogenic cells to become spermatozoa [6]. The first round of spermatogenesis is followed by additional waves to ensure continuous sperm production throughout the life of the animal. It is worth noting that the starting and running maintenance of spermatogenesis are under the control of hypothalamus-pituitary-gonadal (HPG) axis, one branches of the endocrine system essential for the integrity of male reproductive barrier, which is gradually being established during puberty [5]. Thus, disruptions by various stressors during puberty could have long-term effects on male fertility.

In the last two decades, the vigorous development and broad application of nanotechnology have increased the risk of human exposure to nanoparticles (NPs) and raised concerns about their biological effects. As one of the top five commonly used NPs in the nanotechnology consumer products, silica nanoparticles (SiNPs) have been found in almost all aspects of life, ranging from consumer goods to medicine, due to its unique physicochemical properties such as high levels of electrical conductivity, tissue permeability and resistance to biodegradation in the cellular environment[7-9]. However, the novel characteristics that differs from that of the bulk material has attracted global attention regarding the safety and potential adverse effects of SiNPs [10]. Indeed, studies have shown that NPs could have toxic effects on many organs including brain, liver and lungs, which are the worst affected target organs.

Reproductive toxicity is increasingly becoming considered as an important part of overall toxicology of NPs since reproductive system are believed to be essential for transmitting genetic information to the offspring and sensitive to exogenous hazards. It is reported that exposure generally to SiNPs could affect the development of testis, perturb the production of sex hormones and disturb spermatogenesis and sperm maturation which leading to reduced male fertility [11-13]. There is evidence that those observations could be attributed to oxidative stress toxicity, for scavenging the ROS via antioxidants (N-acetyl-L-cysteine, NAC) can ameliorate the toxicity induced by NPs [14-16]. Those finding have advanced our understanding of how male fertility is affected by NPs, however, the molecular mechanism underlying is still unclear. Moreover, the previous studies mainly focused on the adult stage of administration in nanoparticles-induced male reproductive damage, which fail to see the age-dependent effect of NPs [13, 17]. It is reported that testis at puberty showed a higher sensitivity to exogenous contaminants, exposure to stress during this period might cause diverse outcomes that differs from that are sexually mature [18]. Therefore, it is meaningful to identify the toxic factors on male fertility during puberty and underlying their mechanisms of action to prevent their lasting effects.

In this study, we investigate the adverse effect of Nano-SiO$_2$ on male reproductive system in mice during puberty, including the histopathological changes in testis and epididymis, and the potential mechanism of toxicity. These findings could provide a scientific basis for evaluation the risk of Nano-SiO$_2$ to
reproductive health and support the application of nanotechnologies by minimizing the adverse effects of nanoparticles in vulnerable populations.

## Results

### Characteristic of Nano-SiO$_2$

Despite the fact that the physicochemical of silica nanoparticles commercially obtained has been preliminary evaluation, it’s necessary to confirm the indicated features [19]. The morphology of 10-20nm Nano-SiO$_2$ in distilled water was visualized by TEM and is presented in Fig. 1A. Nano-SiO$_2$ showed irregularly shaped and tended to form agglomerates. TEM observation was in agreement with the results from Zetasizer measurement (Fig. 1B). The Hydrodynamic size and zeta potential of Nano-SiO$_2$ were 439.6nm and -27.3mV respectively (Fig. 1C), suggesting that Nano-SiO$_2$ in water was prone to form aggregates, which in line with finding from Magdalena Kusaczuk et al [20].

### Reproductive organ coefficients are changed by Nano-SiO$_2$ exposure

It is believed that an organ coefficient is a key biological characteristic for evaluating the function of experimental animals [21]. To determine the general toxicity of Nano-SiO$_2$ in mice, the weight of body was monitored daily during whole experimental period and the results showed that no changes were detected compared to controls. As for the influence of Nano-SiO$_2$ on reproduction, the weights of reproductive organs including testis and epididymis were dissected and measured the day after drug withdrawal. Interestingly, it turned out that the coefficient of testis is significantly decreased ($p<0.05$) while the epididymis index significantly ($p<0.05$) increased post exposure (Fig. 2).

### Adverse effects of Nano-SiO$_2$ on testicular and epididymal pathology

To determine the effect of Nano-SiO$_2$ on reproductive toxicity, we examined the H&E stained cross sections of testis and epididymis under light microscope to assess the pathological changes. The result showed that the cells in seminiferous epithelium, including spermatogenic cells at different stages of division and Sertoli cells, were regularly arranged in control mice (Fig. 3B). In contrast, vacuolization of seminiferous tubules (Fig. 3B) and decreased in the thickness of the seminiferous epithelium were observed in testicular sections (Table S1). Moreover, hemorrhage was detected in the interstitial tissues surrounding Leydig cells of the testicular section (white arrow in Fig. 3B) from exposed mice.

Swellings were present on the cauda epididymis in all Nano-SiO$_2$ treated mice (Fig. 4A), which is consistent with the increased index of epididymis (Fig. 2C). In the initial and caput segment of epididymal tubules in exposed mice, principal cells revealed similar features to that of control group, while the microvilli in the free side of the principal cells were nearly undetectable in the corpus segment (Fig. 4B). The cauda epididymis was the most seriously affected regions in exposed mice compared to controls, the principal cells were shrunken and vacuolated, and some even shedding of epithelium into the lumen (Fig.
4B). What’s more, the basement membrane between epithelium and fibers disappeared in mice post injection, suggesting the reproductive barrier in epididymis might be corrupted.

**Nano-SiO₂ perturbs spermatogenesis and secretion of testosterone**

Spermatogenesis is a complex process occurred in seminiferous tubules which give rise to highly differentiated spermatid. And the outcomes of this process, including daily sperm production (DSP) and sperm quality, are believed to be key indicators of male reproduction. To further determine the effect of Nano-SiO₂ on male fertility, we analyzed the DSP and sperm quality in mice. The results showed that the DSP and sperm count in the exposed group were significantly lower than that in saline group (Fig. 5A). Moreover, different malformed sperm including looped tail, bent tail, curved tail and detached head sperm were observed in exposure mice, which result in the rates of abnormal increased significantly (Fig. 5B, C). The process of spermatogenesis is subjected to the neuroendocrine hypothalamic-pituitary-gonadal (HPG) axis together with local testicular steroids [22]. To reveal the underlying mechanism, we examined the level of the most important androgen, testosterone, in serum. The results showed that the concentration of testosterone markedly decreased in the exposed group (Fig. 6A). What’s more, compared with the control group, the expressions of androgen receptor and genes regulating androgen biosynthesis (luteinizing hormone/ choriogonadotropin receptor, steroidogenic acute regulatory protein) also declined after exposure (Fig. 6B).

**Nano-SiO₂ aggravates oxidative stress and inflammation response**

To get a closer insight into cytotoxicity induced by Nano-SiO₂, the sensitive indices of oxidative stress response were determined by using SOD (superoxide dismutase) and MDA (malondialdehyde) kit. According to the instructions of the protocol, the supernatants were added to the reagents for measuring the activity of SOD or the concentration of MDA. The results showed that the levels of MDA in treated group were markedly increased compared with control and the activity of SOD also changed after exposure (Fig. 7A). Interestingly, the variation trend in epididymis is opposite to those in testis and inconsistent with some reports finding inhibition of SOD activity once oxidative stress occurred (Fig. 7B) [23]. It could be explained that the elevated activity of antioxidant enzymes might be a response towards Nano-SiO₂, while the decreased activities of antioxidant enzymes could be a result of the over-consumption by ROS. The protein concentration was determined by BCA assay.

It is reported that oxidative stress is linked to inflammation reciprocally [24]. To further explore the effect of Nano-SiO₂ on reproductive organs at the molecular level, expressions of key inflammatory cytokines were detected. Results demonstrated that the mRNA expression of TNF-α and IL-1β were significantly upregulated after exposure, indicating Nano-SiO₂ treatment produced a robust inflammation response in testis and epididymis (Fig. 8)

**Nano-SiO₂ induces DNA damage in testis and epididymis which leading to cell apoptosis**
It has been found that continuous oxidative stress could cause severe damage to cellular macromolecules such as proteins, lipids and DNA, resulting in cell apoptosis. To understand the concrete mechanism of Nano-SiO$_2$-induced reproductive toxicity, apoptotic cells from testis and epididymal sections were detected by TUNEL staining. As shown in Fig. 9, numerous apoptotic cells were observed in the mice post exposure. Interestingly, the apoptotic sensitive cells in testis were mainly in spermatogenic cells while in epididymis they are both in spermatogenic cells and somatic cells. These data figured out the adverse effect of Nano-SiO$_2$ on male reproductive tissues through activating apoptotic programmed death.

**TNF-α mediated apoptotic signaling pathway is activated by Nano-SiO$_2$**

Given that TNF-α is a biomarker to inflammation induced by NPs and it is considered the master regulator of one of the death-receptor mediated apoptosis pathways, we investigate the expression of proteins involved in TNF-α signaling pathway by western blot. Our results showed that the expression of pro-apoptotic factors including caspase-8 and caspase-3 were upregulated post exposure. Interestingly, the expressions of anti-apoptotic factors, such as bcl-2, also increased after treatment (Fig. 10).

**Discussion**

Environment pollutions and lifestyle modification are believed to be the main cause to the high prevalence of reproductive disorders in recent years [25]. Large-scale production and utilization of NPs have increased the risk of human exposure [26]. Indeed, it has been demonstrated that the accumulation of NPs could cause damage to the structure of reproductive organs in adult model organisms [27]. However, whether there is an age-dependent toxicity of NPs remains to be further studied. Puberty is a crucial stage of maturation of reproductive system while also a period vulnerable to certain environmental factors. Exposure to stressors during or around this period is believed to have enduring consequences on multiple aspects of organism's health [5]. In this study, we aim to evaluate the adverse effect of Nano-SiO$_2$ on male reproductive organisms in pubertal mice and explore their underlying mechanism.

It is believed that an organ coefficient is a key biological characteristic for evaluating the function of experimental animals [21]. In this study, we found that the administration of Nano-SiO$_2$ did not affect the body weight of the mice but caused a significant reduction in the testis coefficient (Fig. 2A-B). The weight of testis is largely dependent on the seminiferous tubules which composed of Sertoli cell and differentiated spermatogenic cells. The decreased testis coefficient suggests that spermatogenesis may be inhabited, resulting in decreased number of sperm [28]. Interestingly, the index of epididymis markedly increased in the mice treated post exposure (Fig. 2C). It's reported that the increased index means hyperemia or edema of organs while the decreased organ index means atrophy or degenerative changes occurred in organs [29]. The inconsistent results indicate that there may be different response towards exposure to Nano-SiO$_2$ in testis and epididymis.
As for testis, exposure to Nano-SiO$_2$ induced vacuolar degeneration of seminiferous tubules, leading to disordered spermatogenesis and decreased quality and quantity of sperm (Fig. 3). It has been verified that SiNPs could damage testicular structure, reduce ATP level and affect expressions of regulatory factors of meiosis in the testis of Wistar rats [11]. In addition, SiNPs have been confirmed to pass through the blood-testis barrier (BTB) and the nuclear membranes of spermatocytes in mice through intravenously administration [30]. Based on the previous studies, we speculate that Nano-SiO$_2$ in mice exposed by intraperitoneal injection could pass through the BTB and be taken up by spermatogenic cells in the luminal compartment of seminiferous tubules directly. Moreover, it's also reported that the inflammation induced by NPs in Leydig cells could reduce testosterone levels, which aggravate the toxicity of NPs on spermatogenic cells through weaken the integrity of the BTB [31]. Therefore, the collapse of seminiferous tubules and the destruction of spermatids in testis could be the direct or indirect effect of NPs.

Epididymis provides a place for sperm maturation which represents a crucial process in male fertility. During this process, sperm acquire both motility and the ability to fertilize. In the present study, lesions of the cauda epididymis were observed after exposure (Fig. 4). The disorganized epithelium and absent of the basement membrane in epididymal tubules implied destruction of blood-epididymis barrier (BEB), which results in stagnation of sperm maturation and post-testicular male infertility (Fig. 5). It is reported that the epididymis in mice is the main target of NPs in the genital area and most of the NPs can reach it through different routes [32]. In male rats, the ratio of the epididymis weight to the body weight increased after gavage with nickel NPs [33]. In another study, the histopathological alterations of epididymis was observed after exposure to Titanium dioxide NPs by oral [34]. Just like in testis, the above abnormality in epididymis could be induced by NPs directly or indirectly. On the organ level, NPs could pass through the barrier and deposit in epididymis, causing irregular cell arrangement and deformed epididymal tubules. On the body level, NPs could change the levels of sex hormones, which is essential for the maintenance of BEB.

As set forth, testosterone is a key factor for the maintenance of male reproductive barriers and fertility. In this study, we found that the concentration of testosterone in serum and expression of genes regulating its biosynthesis in testis were significantly decreased in mice 7 days post Nano-SiO$_2$ (Fig. 6). The change in sex hormone levels after exposure to NPs have been reported in several researches. Intravenous injection of AgNPs in mice could significantly increase the concentration of intratesticular testosterone [35], while the serum testosterone level is markedly reduced after treatment with ZO NPs [36]. Moreover, there are even some studies which show that the testosterone and gonadotropin profiles were not altered by CeO$_2$ NPs or AgNP treatment [37, 38]. The disagreement in various studies might be influenced by different factors, such as particle type, size or the time of exposure. Nevertheless, most of the results showed a decrease in testosterone, which was believed to contribute to severe damage in reproductive organs.

According to previous studies, about 30-80% of male infertility could be attributed to oxidative stress-mediated injure [39]. In the present study, the direct measurement of the activity of antioxidant enzyme
(SOD) and the level of MDA provided strong evidence for the involvement of oxidative stress caused by Nano-SiO$_2$ (Fig. 7). Oxidative stress is an important mechanism of the cytotoxic actions of NPs via generating excessive ROS [40]. The imbalance of the oxidative-redox status could cause a wide variety of DNA damage including chromosomal fragmentation, DNA strand breakages and the induction of gene mutation, which finally initiate the process of apoptosis. In our study, we have detected apoptotic cells in testis and epididymis sections post exposure using TUNEL staining (Fig. 9). Indeed, there have been some reports about the NPs mediating apoptosis in somatic cells. A study in human umbilical vein endothelial cells (HUVECs) demonstrated that SiNPs could trigger vascular endothelial cell injury via ROS-mediated MAPK/Bcl-2 apoptosis pathway [41]. In another study, JNK-mediated apoptosis pathway was triggered in primary astrocytes by ZnO NPs-induced oxidative stress [42]. Moreover, the results that antioxidative quercetin was able to ameliorate changes in the male reproductive parameters induced by TiO$_2$ NP suggests that oxidative stress could be a driving force for male fertility [43]. Thus, oxidative stress induced apoptosis has been considered as one of the most important causes of male infertility.

It is reported that oxidative stress is linked to inflammation reciprocally in the NPs-induced toxicity [24]. Inflammation response is a defensive response of the body to inflammatory factors and local lesions. Our results showed that exposure to Nano-SiO$_2$ could induce inflammation response in testis and epididymis via stimulating the release of pro-inflammatory mediators, including TNF-α and IL-1β (Fig. 8). It has been reported that inflammation response induced by NPs could cause toxicity and promote cell death through receptor-induced apoptosis [44]. TNF-α, a major production of inflammation response, regulates a diverse range of cellular responses, including cell survival and apoptosis. Indeed, TNF-α/TNFFR-mediated apoptosis has been verified to play a key role in fibrosis of pneumoconiosis, a class of interstitial lung diseases caused by inhalation of dust containing SiO$_2$. It's known that TNF-α could induce apoptosis by activating caspase-8 and -3, but also inhibit apoptosis via overexpression of anti-apoptotic genes such as Bcl-2. In our study, we found that both pro-apoptotic and anti-apoptotic pathway mediated by TNF-α were activated in the testis and epididymis of mice post exposure (Fig. 10). However, the changes we observed in the mice model suggested that the pro-apoptotic pathway may occupy the leading position in the toxicity caused by Nano-SiO$_2$. In conclusion, those findings suggest that the male reproductive system in mice during puberty is sensitive to Nano-SiO$_2$ exposure. Despite the variation tendency of organ index in testis and epididymis was inconsistent, the abnormality in those organs were both caused by NPs-induced oxidative stress and activation of the apoptosis.

**Conclusion**

In summary, the present study reveals that intraperitoneally administration of Nano-SiO$_2$ to pubertal mice could induce oxidative stress and inflammation by disturbing oxidative-redox status and levels of cytokines in testis and epididymis, leading to disordered spermatogenesis and sperm dysmaturity. The oxidative stress and inflammation have been considered as the driving force for augmented male reproductive function caused by NPs, followed by DNA damage and apoptosis. A further study found that TNF-α mediated pro-apoptotic pathway was activated while the anti-apoptotic pathway was inhibited in
testis and epididymis post exposure. To the best of our known, this is the first report about the
cytotoxicity of Nano-SiO$_2$ on male reproductive system during puberty. Those findings can broaden our
understanding of safe applications of Nano-SiO$_2$ in medicine or other fields, especially for the pubertal
male.

**Methods**

**Animal treatments**

Experimental animals were purchased from HFK Bioscience CO. LTD (Beijing, China). 40 adolescent male
C57BL/6 mice aged 4 weeks with mean weight of 22 ± 2g were randomly divided into 2 groups (n=5).
Every five mice were raised in a box and housed at controlled environment (temperature: 23°C; light: 12-h
light/dark cycle) with free access to sterilized water and food. After one week acclimation, mice were
treated with vehicle (the control group) or Nano-SiO$_2$ at 40mg/kg dosage per mouse through
intraperitoneal injection. At 7 days after injection, the mice were sacrificed and the organs were collected
(Fig. S1). All animals experimental protocols were approved by the Committee on the Ethics of Animal
Experiments of the North China University of Science and Technology, China (Ethical review number:
LAEC-NCST-2020159).

**Particle characteristics**

Nano-SiO$_2$ (99.5% trace metals basis, 5-20nm particle size in TEM) was purchased from Sigma-Aldrich
Chemical Co. (St. Louis, MO, USA). The shape of Nano-SiO$_2$ was measured by transmission electron
microscope (TEM) (JEM-2800, Japan) and the zeta potential in distilled water was detected using a
Zetasizer, which reflecting the stability of nanoparticles (NPs) (Zetasizer Nano zs90, UK). Before using,
Nano-SiO$_2$ was vortexed for 1 min and then sonicated for 5 min (120W output; Thermo Scientific, USA).

**Relative reproductive organ weight quantification**

Body weight was monitored throughout the entire experimental period. After executed, testis and
epididymis were collected and weighted. The relative weight of testis (gonadosomatic) and epididymis
(epididymal somatic indices) were calculated as the percentage of testicular or epididymal weight in
relation to the total body weight.

**Daily sperm production**

After weighted, the tunica albuginea were removed to release the seminiferous tubules into a
homogenizer tube. Then the tissues were homogenized in 1ml of PBS for 2 min and left to stand for
60min at room temperature. Equal volumes of sample were taken to count sperm heads in a
haemocytometer chamber. DSP was calculated as: DSP=N/4.48. Where N means the total number of
spermatids per sample and 4.48 is a fixed coefficient standing for the number of days for a spermatid to
develop from stage 14 to stage 16 in mice, which are resistant to homogenization [17].
Histopathological examination

After blood collection, the mice were killed and tissues were rapidly removed and weighted. Unilateral testis and epididymis were fixed in Bouin's fixing solution, dehydrated in a graded series of ethanol and xylene solutions, embedded in paraffin and then sectioned into 5μm sections and mounted onto the glass slides. After stained with hematoxylin and eosin (H&E, BASO), the slides were observed and pictured using optical microscope (Olympus X71, Japan).

Evaluation of sperm parameters

One side of cauda epididymis was dissected from mice after execution, cut into pieces and then put into a 1.5 ml Eppendorf tube containing 200 μl TYH medium at 37°C for 30 min to allow sperm get out. After incubation, the sperm suspension was divided into two aliquots. One part was diluted with TYH at a ratio of 1:2 and then added to the hemocytometer for counting analysis. The others were washed with PBS and placed on a clean glass slide. After fixation with 4% paraformaldehyde, the sperm were stained with Coomassie Brilliant Blue G250 and assessed under high power (×40) of a light microscope to evaluate the sperm morphology. At least 100 spermatozoa were counted [45].

RNA extraction and quantitative Real-time PCR

Testis and epididymis samples were sliced and homogenized in 1 ml Trizol Reagent (Ambition) and then 2ug of total RNA were reverse-transcribed to cDNA using Advantaged RT for PCR Kit (Takara) according to the manufacturers' instructions. cDNAs were subsequently subjected to real-time PCR analysis using specific primers synthesized by Sangon Biotech (Beijing, China) listed in Table S2 with a 2X M5 HiPer Realtime PCR Super mix (Mei5bio). A melting curve analysis was performed to exclude nonspecific product amplification. The expression levels of the target genes were normalized to Actb content, and the relative fold change were calculated based on the 2^{-ΔΔCt} comparative method [46].

Oxidative stress measurement

Total proteins in testis and epididymis tissues were extracted in PBS containing protease inhibitor cocktail (P8340, Sigma) with an ultrasonic cell crusher on ice. The supernatants were collected and then added to the reagents for measuring the levels of MDA and SOD according to the instructions of the oxidation-antioxidation assay kits (A003-1, A001-3, Jiancheng).

TUNEL assay

Apoptotic cells on testis or epididymal paraffin sections were detected using One Step TUNEL Assay Kit (Beyotime) according to the manufacturer's protocol. Images were taken with a Nikon fluorescent microscope.

Western blotting
Proteins from mouse testis and epididymis were extracted in RIPA containing protease inhibitor cocktail (Beyotime). After short sonication, samples were incubated on ice for 30 min and then centrifuged at 12,000rpm for 10 min. Then, the protein concentration was determined by using bovine serum albumin (BSA) as a standard with BCA protein assay kit (Sangon Biotech). Equal amounts of proteins were separated on 10% SDS-PAGE gels and transferred onto PVDF membrane. Membranes were blocked with 5% nonfat milk for 1h, followed by incubation with primary antibodies, including anti-TNF-α (GTX110520, GENETEX), anti-NF-κB (ARG51013, Arigo), anti-Caspase-8 (AF6442, Affinity), anti-Caspase-3 (AF6311, Affinity), anti-Bcl-2 (AF6139, Affinity), anti-Actin (AF7018, Affinity), overnight at 4℃. Second antibody labeled with HRP (Beyotime) and the ultrasensitive ECL chemiluminescence detection kit (ZD310A, ZOMABIO) were used to visualize specific protein bands. ACTB was used as protein control.

Testosterone levels

Blood was collected through avulsion of the eyeball, incubation at room temperature for 30 min and then centrifuged at 5000 rpm for 30 min. The supernatant was collected as serum and stored at -80℃ until analysis. The levels of testosterone in serum were diluted with phosphate buffered saline (PBS) at the ratio of 1:4 and assayed in triplicate using specific enzyme-linked immunosorbent assay (ELISA) commercial kits according to the manufacturers’ protocol (Cayman). The concentration of each sample was determined using the equation obtained from the standard curve plot. All samples were examined twice when fell out the standard curve. The variation coefficients of inter-assay were 2.8-7.7% [17].

Statistical analysis

All results are presented as means ± standard error (SE) (n≥3) and examined for their statistical significance of differences by Student’s t-test. All comparisons were considered significantly different when p-value < 0.05.

Abbreviations

NPs: nanoparticles; SiNPs: silica nanoparticles; Nano-SiO₂: Nano-Silicon dioxide; DSP: daily sperm production; SOD: superoxide dismutase; MDA: malondialdehyde; NAC: N-acetyl-L-cysteine; TEM: transmission electron microscope; H&E: hematoxylin-eosin staining; HPG: hypothalamic-pituitary-gonadal; PBS: phosphate buffered saline; ELISA: enzyme-linked immunosorbent assay; SE: standard error; BTB: blood-testis barrier; BEB: blood-epididymis barrier; MAPK: Mitogen activated protein kinases (MAPKs); JNK: c-Jun N-terminal Kinase; BSA: bovine serum albumin

Declarations

Acknowledgments

We would like to thank Fang Yang and Hong Xu for the technical assistance in western blot technique, Heliang Liu and Xiaohui Hao for the generous contribution of antibodies, and many of our colleagues for
Authors’ contributions

XS conceived and designed the study; XS, FS, XW, ZC and PZ performed experiments and interpreted data; XS and ZG drafted the manuscript. All authors read and approved the final version.

Funding

The work was supported by the grants from the Tangshan Science and Technology Bureau (no. 20130212b), the grants from the Science and Technology Research Project of Hebei Province Universities (no. JQN2020002), the grants from Department of Health of Hebei Province (no. 20210049), and the Undergraduate Innovation and Entrepreneurship Project of North China University of Science and Technology (no. X2020085).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

All animal experiments were reviewed and approved by the Laboratory Animal Ethics Committee of North China University of Science and Technology.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to disclose.

Author details

1School of Public Health, North China University of Science and Technology, Tangshan, Hebei 063210, People’s Republic of China.

References

1 Carlsen E, Giwercman A, Keiding N, Skakkebaek NE: Evidence for decreasing quality of semen during past 50 years. Bmj 1992;305:609-613.

2 Levine H, Jorgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, Pinotti R, Swan SH: Temporal trends in sperm count: A systematic review and meta-regression analysis. Human reproduction
update 2017;23:646-659.

3 Skakkebaek NE, Rajpert-De Meyts E, Buck Louis GM, Toppari J, Andersson AM, Eisenberg ML, Jensen TK, Jorgensen N, Swan SH, Sapra KJ, Ziebe S, Priskorn L, Juul A: Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. Physiological reviews 2016;96:55-97.

4 Abreu AP, Kaiser UB: Pubertal development and regulation. The lancet Diabetes & endocrinology 2016;4:254-264.

5 Kane L, Ismail N: Puberty as a vulnerable period to the effects of immune challenges: Focus on sex differences. Behavioural brain research 2017;320:374-382.

6 Zindy F, den Besten W, Chen B, Rehg JE, Latres E, Barbacid M, Pollard JW, Sherr CJ, Cohen PE, Roussel MF: Control of spermatogenesis in mice by the cyclin d-dependent kinase inhibitors p18(ink4c) and p19(ink4d). Molecular and cellular biology 2001;21:3244-3255.

7 Hansen SF, Michelson ES, Kamper A, Borling P, Stuer-Lauridsen F, Baun A: Categorization framework to aid exposure assessment of nanomaterials in consumer products. Ecotoxicology 2008;17:438-447.

8 Salata O: Applications of nanoparticles in biology and medicine. Journal of nanobiotechnology 2004;2:3.

9 Esmaeilou M, Moharamnejad M, Hsankhani R, Tehrani AA, Maadi H: Toxicity of zno nanoparticles in healthy adult mice. Environmental toxicology and pharmacology 2013;35:67-71.

10 Croissant JG, Fatieiev Y, Khashab NM: Degradability and clearance of silicon, organosilica, silsesquioxane, silica mixed oxide, and mesoporous silica nanoparticles. Advanced materials 2017;29

11 Zhang L, Wei J, Duan J, Guo C, Zhang J, Ren L, Liu J, Li Y, Sun Z, Zhou X: Silica nanoparticles exacerbates reproductive toxicity development in high-fat diet-treated wistar rats. Journal of hazardous materials 2020;384:121361.

12 Xu Y, Wang N, Yu Y, Li Y, Li YB, Yu YB, Zhou XQ, Sun ZW: Exposure to silica nanoparticles causes reversible damage of the spermatogenic process in mice. PloS one 2014;9:e101572.

13 Zhang J, Ren L, Zou Y, Zhang L, Wei J, Li Y, Wang J, Sun Z, Zhou X: Silica nanoparticles induce start inhibition of meiosis and cell cycle arrest via down-regulating meiotic relevant factors. Toxicology research 2016;5:1453-1464.

14 Zhang X, Luan J, Chen W, Fan J, Nan Y, Wang Y, Liang Y, Meng G, Ju D: Mesoporous silica nanoparticles induced hepatotoxicity via nlrp3 inflammasome activation and caspase-1-dependent pyroptosis. Nanoscale 2018;10:9141-9152.
15 Lu Y, Xu S, Chen H, He M, Deng Y, Cao Z, Pi H, Chen C, Li M, Ma Q, Gao P, Ji Y, Zhang L, Yu Z, Zhou Z: Cdse/zns quantum dots induce hepatocyte pyroptosis and liver inflammation via nlrp3 inflammasome activation. Biomaterials 2016;90:27-39.

16 Hussein MMA, Gad E, Ahmed MM, Arisha AH, Mahdy HF, Swelum AA, Tukur HA, Saadeldin IM: Amelioration of titanium dioxide nanoparticle reprotoxicity by the antioxidants morin and rutin. Environmental science and pollution research international 2019;26:29074-29084.

17 Skovmand A, Jensen ACO, Maurice C, Marchetti F, Lauvas AJ, Koponen IK, Jensen KA, Goericke-Pesch S, Vogel U, Hougaard KS: Effects of maternal inhalation of carbon black nanoparticles on reproductive and fertility parameters in a four-generation study of male mice. Particle and fibre toxicology 2019;16:13.

18 Li X, Yao Z, Yang D, Jiang X, Sun J, Tian L, Hu J, Wu B, Bai W: Cyanidin-3-o-glucoside restores spermatogenic dysfunction in cadmium-exposed pubertal mice via histone ubiquitination and mitigating oxidative damage. Journal of hazardous materials 2020;387:121706.

19 Breznan D, Das DD, O'Brien JS, MacKinnon-Roy C, Nimesh S, Vuong NQ, Bernatchez S, DeSilva N, Hill M, Kumarathasan P, Vincent R: Differential cytotoxic and inflammatory potency of amorphous silicon dioxide nanoparticles of similar size in multiple cell lines. Nanotoxicology 2017;11:223-235.

20 Kusaczuk M, Kretowski R, Naumowicz M, Stypulkowska A, Cechowska-Pasko M: Silica nanoparticle-induced oxidative stress and mitochondrial damage is followed by activation of intrinsic apoptosis pathway in glioblastoma cells. International journal of nanomedicine 2018;13:2279-2294.

21 Zhang R, Zhang L, Jiang D, Zheng K, Cui Y, Li M, Wu B, Cheng S: Mouse organ coefficient and abnormal sperm rate analysis with exposure to tap water and source water in nanjing reach of yangtze river. Ecotoxicology 2014;23:641-646.

22 Huleihel M, Lunenfeld E: Regulation of spermatogenesis by paracrine/autocrine testicular factors. Asian journal of andrology 2004;6:259-268.

23 Guo C, Xia Y, Niu P, Jiang L, Duan J, Yu Y, Zhou X, Li Y, Sun Z: Silica nanoparticles induce oxidative stress, inflammation, and endothelial dysfunction in vitro via activation of the mapk/nrf2 pathway and nuclear factor-kappab signaling. International journal of nanomedicine 2015;10:1463-1477.

24 Khanna P, Ong C, Bay BH, Baeg GH: Nanotoxicity: An interplay of oxidative stress, inflammation and cell death. Nanomaterials 2015;5:1163-1180.

25 Barazani Y, Katz BF, Nagler HM, Stember DS: Lifestyle, environment, and male reproductive health. The Urologic clinics of North America 2014;41:55-66.

26 Murugadoss S, Lison D, Godderis L, Van Den Brule S, Mast J, Brassinne F, Sebaihi N, Hoet PH: Toxicology of silica nanoparticles: An update. Archives of toxicology 2017;91:2967-3010.
27 Wang R, Song B, Wu J, Zhang Y, Chen A, Shao L: Potential adverse effects of nanoparticles on the reproductive system. International journal of nanomedicine 2018;13:8487-8506.

28 Takahashi O, Oishi S: Testicular toxicity of dietary 2,2-bis(4-hydroxyphenyl)propane (bisphenol a) in f344 rats. Archives of toxicology 2001;75:42-51.

29 Zhou Q, Yue Z, Li Q, Zhou R, Liu L: Exposure to pbse nanoparticles and male reproductive damage in a rat model. Environmental science & technology 2019;53:13408-13416.

30 Morishita Y, Yoshioka Y, Satoh H, Nojiri N, Nagano K, Abe Y, Kamada H, Tsunoda S, Nabeshi H, Yoshikawa T, Tsutsumi Y: Distribution and histologic effects of intravenously administered amorphous nanosilica particles in the testes of mice. Biochemical and biophysical research communications 2012;420:297-301.

31 Lan Z, Yang WX: Nanoparticles and spermatogenesis: How do nanoparticles affect spermatogenesis and penetrate the blood-testis barrier. Nanomedicine 2012;7:579-596.

32 Zhao H, Gu W, Ye L, Yang H: Biodistribution of pamam dendrimer conjugated magnetic nanoparticles in mice. Journal of materials science Materials in medicine 2014;25:769-776.

33 Kong L, Tang M, Zhang T, Wang D, Hu K, Lu W, Wei C, Liang G, Pu Y: Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats. International journal of molecular sciences 2014;15:21253-21269.

34 Morgan AM AE-HM, Noshy PA.: Reproductive toxicity investigation of titanium dioxide nanoparticles in male albino rats. Journal of Pharmacy and Pharmaceutical Sciences 2015;4(10):34–49.

35 Garcia TX, Costa GM, Franca LR, Hofmann MC: Sub-acute intravenous administration of silver nanoparticles in male mice alters leydig cell function and testosterone levels. Reproductive toxicology 2014;45:59-70.

36 Lafuente D, Garcia T, Blanco J, Sanchez DJ, Sirvent JJ, Domingo JL, Gomez M: Effects of oral exposure to silver nanoparticles on the sperm of rats. Reproductive toxicology 2016;60:133-139.

37 Adebayo OA, Akinloye O, Adaramoye OA: Cerium oxide nanoparticle elicits oxidative stress, endocrine imbalance and lowers sperm characteristics in testes of balb/c mice. Andrologia 2018;50

38 Mathias FT, Romano RM, Kizys MM, Kasamatsu T, Giannocco G, Chiamolera MI, Dias-da-Silva MR, Romano MA: Daily exposure to silver nanoparticles during prepubertal development decreases adult sperm and reproductive parameters. Nanotoxicology 2015;9:64-70.

39 Tremellen K: Oxidative stress and male infertility—a clinical perspective. Human reproduction update 2008;14:243-258.
40 Ahmed MM, Hussein MMA: Neurotoxic effects of silver nanoparticles and the protective role of rutin. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 2017;90:731-739.

41 Guo C, Yang M, Jing L, Wang J, Yu Y, Li Y, Duan J, Zhou X, Li Y, Sun Z: Amorphous silica nanoparticles trigger vascular endothelial cell injury through apoptosis and autophagy via reactive oxygen species-mediated mapk/bcl-2 and pi3k/akt/mtor signaling. International journal of nanomedicine 2016;11:5257-5276.

42 Wang J, Deng X, Zhang F, Chen D, Ding W: Zno nanoparticle-induced oxidative stress triggers apoptosis by activating jnk signaling pathway in cultured primary astrocytes. Nanoscale research letters 2014;9:117.

43 Khorsandi L, Orazizadeh M, Moradi-Gharibvand N, Hemadi M, Mansouri E: Beneficial effects of quercetin on titanium dioxide nanoparticles induced spermatogenesis defects in mice. Environmental science and pollution research international 2017;24:5595-5606.

44 Wallach D, Kang TB, Kovalenko A: Concepts of tissue injury and cell death in inflammation: A historical perspective. Nat Rev Immunol 2014;14:51-59.

45 Shang X, Shen C, Liu J, Tang L, Zhang H, Wang Y, Wu W, Chi J, Zhuang H, Fei J, Wang Z: Serine protease prss55 is crucial for male mouse fertility via affecting sperm migration and sperm-egg binding. Cellular and molecular life sciences : CMLS 2018;75:4371-4384.

46 Schmittgen TD, Livak KJ: Analyzing real-time pcr data by the comparative c(t) method. Nature protocols 2008;3:1101-1108.

Figures
Figure 1

Characterization of Nano-SiO2. (A) The transmission electron microscope image of Nano-SiO2 showed a tendency to form aggregates. (B) The hydrodynamic sizer and (C) zeta potential of Nano-SiO2 in distilled water.
Nano-SiO2 affects male reproductive organ coefficients in mice. (A) There is no significant difference in the daily body weights of mice between different groups during the whole experimental period. (B, C) The testis coefficient is marked decreased while the epididymis index increased remarkably after administrating with Nano-SiO2 for 7 days.
Figure 3

Nano-SiO2 causes damage to the structure of testis and decreases daily sperm production. (A) The tendency to become less in the testis can be detected in morphology observations post treatment. (B) The H&E staining of testicular paraffin sections show a significant degeneration in the seminiferous tubules. (C) The daily sperm production (×106) in the testis decreases sharply, mean ± SE. Black arrows represent spermatogonium, arrowheads represent spermatocyte, white arrows represent hemorrhage in interstitial tissue, and asterisks represent vacuoles. Images are captured from three independent experiments and the pictures showed in the figure are representative.
Figure 4

Nano-SiO2 causes damage to the structure of epididymal tissue. (A) The pathological lesions in the epididymal tubule can be observed from perspective of morphology after exposure. (B) The H&E staining of epididymal paraffin sections show that the microvilli in free side of the principal cells is absent from the corpus segment and swelling can be detected in epididymal tubules from the cauda segment. (Black arrows represent the absent of microvilli in the free side of principal cells; Hollow arrowheads represent the basal lamina of the epithelium; Red arrowheads represent the epithelium were shrunken; Asterisk represent cells shedding from epithelium). Images are captured from three independent experiments and the pictures showed in the figure are representative.
Nano-SiO2 decreases sperm quality and quantity. (A) Sperm from the cauda epididymis is freed in TYH buffer and then counted under the microscope field. The sperm count in mice post exposure significantly decreased compared with the control (*p < 0.05). (B) The Coomassie Brilliant Blue staining of sperm from cauda epididymis indicates Nano-SiO2 can induce malformation of sperm. (Arrowheads and arrows represent malformed sperm including looped tail, bent tail, curved tail and detached head sperm.) (C) The percent of abnormal sperm in exposure group is marked increased. At least 200 sperm from each mouse were examined. Data are showed as mean ± SE, n=3 of each group.
Nano-SiO2 perturbs the secretion of testosterone and decreases the mRNA expression of genes relating to the production of testosterone in mice. (A) Plasma testosterone level in the serum is detected by ELISA kit and the result shows a decrease in the mice post exposure. (B, C, D) Relative mRNA expression of androgen receptor (Ar), luteinizing hormone/ choriogonadotropin receptor (Lhcgr) and steroidogenic acute regulatory protein (Star) detected by qRT-PCR are significantly decreased in treated mice. The housekeeping gene Actb is used for expression normalization. Data was shown as mean ± SE of at least three mice per group. *p < 0.05 compared with controls.
Nano-SiO2 induces oxidative stress in testis and epididymis. (A) The activity of SOD detected with Superoxide Dismutase (SOD) assay kit in the testis is markedly decreased while there is a remarkable increase in the epididymal tissue after exposure. (B) The concentration of MDA detected with Malondialdehyde (MDA) assay kit in testicular and epididymal tissue is significantly increased after exposure. Data was presented as mean ± SE of at least three mice per group. *p < 0.05 compared with control.

Inflammation response is induced by Nano-SiO2 in the testis and epididymis. Real-time PCR assessment of mRNA levels for inflammatory cytokines (TNF-α, IL-1β) shows a remarkable increase in the expression...
of those genes. The housekeeping gene Actb is used for expression normalization. Data is presented as mean ± SE of at least three mice per group. *p < 0.05 versus control.

Figure 9

Nano-SiO2 induces DNA-damage in testis and epididymis which leading to cell apoptosis. TUNEL staining is used to detect the apoptotic cells in the paraffin sections of testis and epididymis, the results show that there is a significant increase of apoptotic cells in mice treated with Nano-SiO2. Images are captured from two independent experiments and the pictures showed in the figure are representative.
Figure 10

TNF-α mediated pro-apoptotic pathway is activated in the testis and epididymis post exposure. Mice exposed to Nano-SiO2 exhibit an increase in protein levels of TNF-α mediated pro-apoptotic genes (TNF-R II, NF-κB, cleaved-Caspase8, cleaved-Caspase3) and anti-apoptotic genes (Bcl-2) in testicular (A, B) and epididymal tissues (C, D) as determined with Western blot. *p < 0.05, n = 3 each group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarydata.docx