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Mechanisms of nanosized titanium dioxide-induced testicular oxidative stress and apoptosis in male mice

Xiaoyang Zhao^{1†}, Lei Sheng^{1†}, Ling Wang^{2†}, Jie Hong¹, Xiaohong Yu¹, Xuezi Sang¹, Qingqing Sun¹, Yuguan Ze¹ and Fashui Hong^{1,3,4*}

Abstract

Background: Due to the increased application of titanium dioxide nanoparticles (TiO₂ NPs) in the food industry and daily life, their potential toxic effects in humans and animals have been investigated. However, very few studies have focused on testicular oxidative stress and/or apoptosis.

Methods: In order to understand the possible molecular mechanisms of testicular lesions following exposure to TiO₂ NPs, male mice were exposed to 2.5, 5, or 10 mg/kg body weight TiO₂ NPs for 90 consecutive days. Testicular oxidative stress and apoptosis were then evaluated, and the testicular mRNA expression of several genes and their proteins involved in oxidative stress and/or apoptosis was investigated.

Results: TiO₂ NPs entered Sertoli cells and caused severe testicular oxidative damage and/or apoptosis, accompanied by excessive production of reactive oxygen species and peroxidation of lipids, proteins and DNA as well as a significant reduction in antioxidant capacity. Furthermore, exposure to TiO₂ NPs resulted in the up-regulation of caspase-3, Nr1h2, and cytochrome c expression, and caused down-regulation of SOD, CAT, GPx, GST, GR, Cyp1b1, Car3, Bcl-2, Acaa2, and Axud1 expression in mouse testis.

Conclusions: TiO₂ NPs entered Sertoli cells via the blood-testis barrier and were deposited in mouse seminiferous cord and/or Sertoli cells, causing oxidative damage and apoptosis.

Keywords: Titanium dioxide nanoparticles, Testis, Oxidative stress, Sertoli cell apoptosis, Gene expression

Background

The use of nanosized materials in consumer and industrial products has increased due to their distinctive physicochemical properties, including high reactivity, color changes, lower melting temperature, and greater solar radiation absorption. In particular, titanium dioxide nanoparticles (TiO₂ NPs) have been used in crop production, dietary supplements, food additives, food packaging components, medicine, toothpastes, sunscreens, cosmetics, and waste water treatment [1-11]. This widespread use of TiO₂ NPs has inevitably led to harmful

biological responses in humans, and animals [12], particularly in the reproductive system [13].

Komatsu et al. suggested that TiO₂ NPs could migrate to Leydig cells, decrease the viability and proliferation of these cells, and increase the gene expression levels of heme oxygenase-1 and steroidogenic acute regulatory protein of Leydig cells in mouse testis *in vitro* [14]. Hou et al. reported that exposure to TiO₂ NPs led to obvious morphological changes in follicles, a reduction in follicle survival, reduced formation of antral follicles, and inhibition of rat follicle development and oocyte maturation *in vitro* [15]. Suzuki et al. found that TiO₂ NPs entered the cytoplasm of cultured Chinese hamster ovary cells, but not the nucleus [16]. Di Virgilio et al. observed significant agglomeration of TiO₂ NPs on both the plasma membrane and the cytoplasm inducing genotoxic and cytotoxic effects [17]. Exposure to TiO₂ NPs can occur

* Correspondence: hongfsh_cn@sina.com1

†Equal contributors

¹Medical College of Soochow University, Suzhou 215123, China

³Jiangsu Province Key Laboratory of Stem Cell Research, Soochow University, Suzhou 215007, China

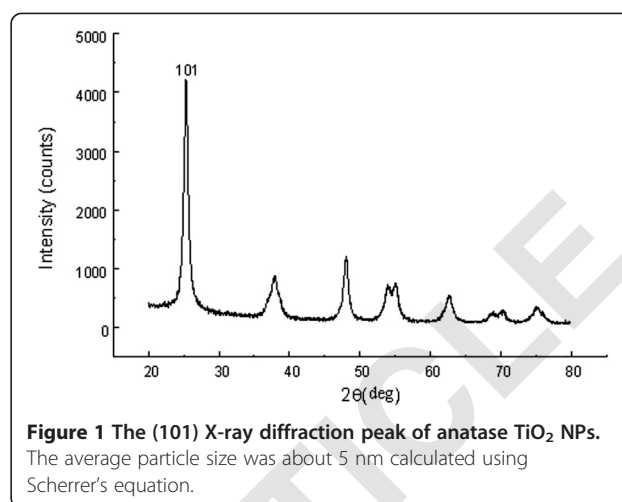
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via inhalation, transdermal absorption, and ingestion, therefore, the ovaries and testes may also be target organs of toxicity in mammals exposed to TiO₂ NPs. The importance of TiO₂ NP exposure has been highlighted in *in vivo* studies. Ema et al. indicated that TiO₂ NPs injected subcutaneously into pregnant mice resulted in male offspring with altered spermatogenesis and histopathological changes in the testes [18]. Takeda et al. also studied pregnant mice injected subcutaneously with TiO₂ NPs, and TiO₂ NPs were observed to migrate to spermatids, Sertoli cells and Leydig cells in the male offspring, resulting in disorganization in seminiferous tubules and reductions in daily sperm production, sperm motility and Sertoli cell number [19]. Male mice exposed to TiO₂ NPs by intraperitoneal injection showed marked reductions in sperm numbers and motility, and elevated abnormal sperm and germ cell apoptosis in the testis [20]. Our recent studies demonstrated that TiO₂ NPs can migrate to the ovary and testis, resulting in injury, a reduction in fertility, lower sperm concentration and sperm motility, higher malformation rates of sperm, and alterations in the gene expression profile in the damaged ovaries and testes in mice [21,22]. Many conditions or events associated with male infertility can induce oxidative stress and germ cell apoptosis. Given that spermatozoa are rich in polyunsaturated fatty acids, they are very susceptible to attack by reactive oxygen species (ROS) [23,24], while protection from lipid peroxidation is mainly due to the activity of glutathione peroxidase (GPx), an enzyme important for maintaining sperm motility and viability [25,26]. The reproductive system of male mammals mainly consists of the testis, epididymis and ductus deferens. The testis produces spermatozoa and the epididymis stores the spermatozoa and completes sperm maturation. We hypothesize that decreased sperm motility and viability caused by exposure to TiO₂ NPs may be associated with testicular oxidative stress and/or apoptosis. To date, no studies have examined whether TiO₂ NPs induce testicular oxidative stress and/or apoptosis by assessing the antioxidant capacity, and alterations in oxidative stress and/or apoptosis-related gene expression in male mice. Thus, the aims of the present study were to test the hypothesis that TiO₂ NPs induce oxidative stress and/or apoptosis in the testis of mice by causing a reduction in antioxidant capacity and alterations in oxidative stress and/or apoptosis-related gene expression. In addition, the mechanisms of TiO₂ NP-induced oxidative stress and/or apoptosis in mouse testis were evaluated.

Results

TiO₂ NPs characteristics

XRD measurements showed that TiO₂ NPs exhibited the anatase structure (Figure 1), and the average particle



size calculated from the broadening of the (101) XRD peak of anatase was approximately 5.5 nm using Scherrer's equation. TEM demonstrated that the average size of the powder particles (Figure 2a) and NPs suspended in HPMC solvent after incubation ranged from 5–6 nm, respectively (Figure 2b), which was consistent with the XRD results. The sample surface area was generally smaller than that estimated from the particle size, and it would seem that aggregation of the particles may have caused this decrease. To confirm the dispersion and stability of the TiO₂ NP suspension, the aggregated size and the ζ potential of TiO₂ NPs in HPMC were measured. Following 12, 18, and 24 h incubation, the mean hydrodynamic diameter of TiO₂ NPs in HPMC solvent ranged from 210 to 450 nm (mainly 310 nm), as measured by DLS, which indicated that the majority of TiO₂ NPs were clustered and aggregated in solution. In addition, the ζ potential was 7.57, 8.53, and 9.28 mV, respectively.

Histopathological evaluation

The histological changes in the testis specimens are shown in Figure 3. Unexposed testicular samples showed normal histology. The seminiferous tubules in these samples showed numerous spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, spermatozooids, Sertoli cells and Leydig cells (Figure 3). However, the samples from the mice exposed to TiO₂ NPs exhibited the following severe pathological changes: a significant reduction in the thickness of the germinal layer, a reduction in the number of spermatogonia, rupture of the seminiferous tubules, vacuolation, spermatocyte and Sertoli cell apoptosis or necrosis, and irregular arrangement of spermatocytes and Sertoli cells in the seminiferous tubules (Figure 3). In addition, we also observed a significant number of black agglomerates in the seminiferous tubules exposed to 10 mg/kg of TiO₂ NPs

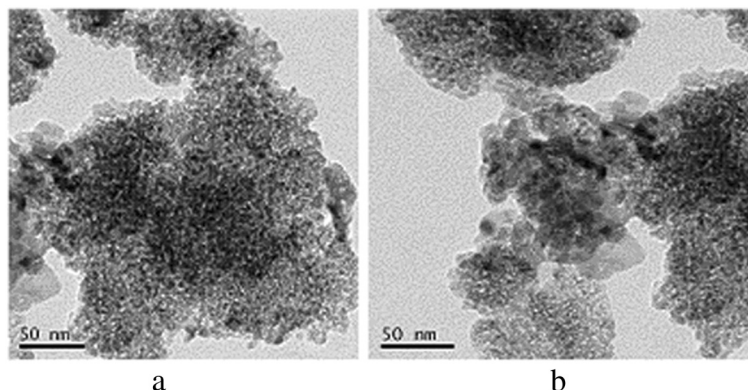


Figure 2 Transmission electron microscope images of anatase TiO₂ NPs. (a) TiO₂ NP powder; (b) TiO₂ NPs suspended in HPMC solvent after incubation for 12 h. TEM images showed that the sizes of the TiO₂ NP powder or TiO₂ NPs suspended in HPMC solvent for 12 h ranged from 5 to 6 nm, respectively.

(Figure 3). Confocal Raman microscopy further showed a characteristic TiO₂ peak in the black agglomerates (148 cm⁻¹), which further confirmed the deposition of TiO₂ in the testis (see Raman intensity insets in Figure 3).

Sperm morphology

As shown in Figure 4, exposure to TiO₂ NPs induced abnormalities in the sperm head and tail. Head abnormalities included amorphous, hookless, banana-shaped and double-headed sperm. TiO₂ NPs also caused the formation of folded, coiled, bent and double-tailed sperm. In addition to these morphological alterations, many sperm tails appeared in the smears of treated mice with detached heads. Sperm also showed an acute bend at the cephalocaudal junction from which fibrils projected out.

Sertoli cell ultrastructure

Changes in the cell ultrastructure in mouse testicular samples are shown in Figure 5. Sertoli cells in the control group contained elliptical nuclei with homogeneous chromatin (Figure 5); however, cell ultrastructure in mouse testicular tissue treated with TiO₂ NPs indicated typical apoptosis, including significant mitochondrial swelling and cristae disappearance, nuclear membrane collapse and chromatin marginalization (Figure 5). In addition, we also observed a significant number of black agglomerates in the cytoplasm and/or nucleus in the TiO₂ NP-exposed testicular tissues (Figure 5). Confocal Raman microscopy showed a characteristic TiO₂ NP peak in the black agglomerate (148 cm⁻¹), which further demonstrated the aggregation of TiO₂ NPs in cells (see the Raman intensity insets in Figure 5). These results suggested that chronic exposure to TiO₂ NPs resulted in Sertoli apoptosis in the testis, which may be due to excessive ROS production and increased peroxidation levels.

Oxidative damage of testis

To confirm the molecular mechanism of TiO₂ NP-induced oxidative stress and/or apoptosis of testicular cells, oxidative indices including O₂⁻, H₂O₂, MDA, PC as well as 8-OHdG levels were examined. As shown in Table 1, O₂⁻, H₂O₂, MDA, and PC levels were significantly elevated and increased with increasing TiO₂ NPs dose ($P < 0.05$). A marked increase in testicular 8-OHdG level was observed in the TiO₂ NP-treated groups ($P < 0.05$ or 0.01), indicating that excessive ROS production led to DNA damage in the testis due to TiO₂ NP-induced toxicity.

Antioxidant defense

The activities of antioxidative enzymes in the testis, including SOD, CAT, APx, GR and GST were examined (Table 2). These enzymes in testes exposed to TiO₂ NPs were significantly decreased with increasing TiO₂ NP dose (Table 2, $P < 0.05$). To further explore the effects of TiO₂ NP stress on antioxidant capacity, the contents of GSH and ascorbic acid in the testis were examined and are shown in Table 2. Marked decreases in GSH and ascorbic acid in the three TiO₂ NP-treated groups were observed ($P < 0.05$). These results suggested that exposure to TiO₂ NPs resulted in decreased ROS removal in mouse testis.

Assay of gene and protein expression

In the present study, GDPH was chosen as the endogenous control gene. The expression level of the GDPH gene was constant, with an expression ratio of almost one in all the samples (data not listed). Therefore, using this gene as a reference, changes in the expression levels of the 13 oxidative stress and/or apoptosis-related genes were evaluated and compared following exposure to TiO₂ NPs for 90 consecutive days (Figure 6). The expression levels of SOD, CAT, GPx, GST, GR, Cyp1b1,

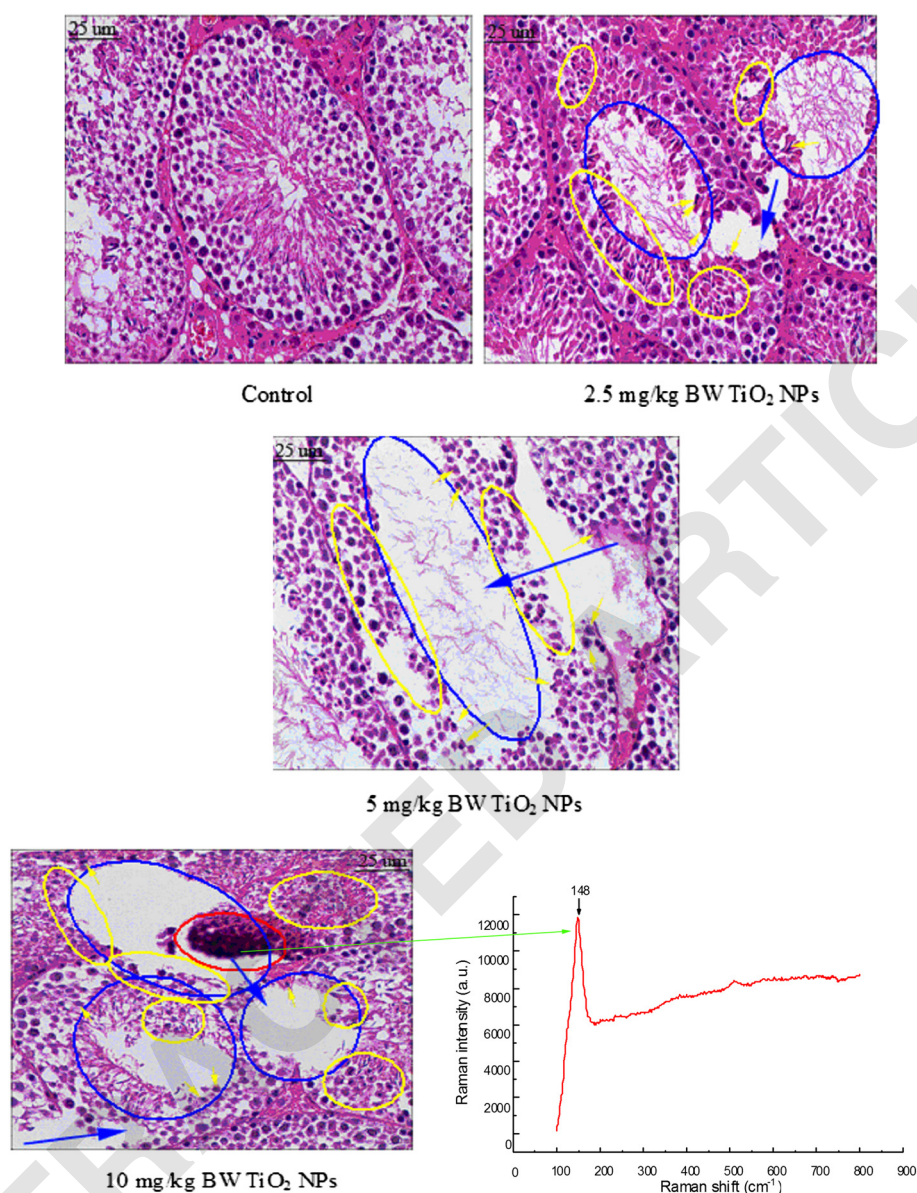


Figure 3 Histopathological observations of mouse testis following intragastric administration of TiO_2 NPs for 90 consecutive days. The blue circle indicates few sperm, breakages or vacuolation, the yellow circle indicates apoptosis and/or necrosis of Sertoli cells, and the blue arrow indicates damaged seminiferous tubule. The red arrows indicate vacuolation, the yellow arrows indicate irregular arrangement of Sertoli cells, and the red circle indicates black agglomerates in the testis. The green arrow spot denotes a representative cell loaded with TiO_2 NPs. The right panel shows the corresponding Raman spectra identifying specific peaks at about 148 cm^{-1} .

Car3, Bcl-2, Acaa2, and Axud1 decreased gradually, whereas the expression levels of caspase-3, Nrbp2, and cytochrome c increased significantly in mouse testis with increasing TiO_2 NP dose ($P < 0.05$ or 0.01), respectively. TiO_2 NP-induced oxidative stress and/or apoptosis-related molecule expression was also examined at the protein level following exposure to various doses of TiO_2 NPs for 90 consecutive days (Figure 7). ELISA showed that TiO_2 NPs caused significant reductions in SOD, CAT, GPx, GST, GR, Cyp1b1, Car3, Bcl-2, Acaa2,

and Axud1 protein expression, and elevated the levels of caspase-3, Nrbp2, and cytochrome c protein expression in mouse testis ($P < 0.05$ or 0.01), respectively.

These results are consistent with the histological findings and Sertoli cell ultrastructure in the testicular sections observed in the treated mice.

Discussion

Studies on the oxidative impairments in the male reproductive system in animals due to nanosized materials are

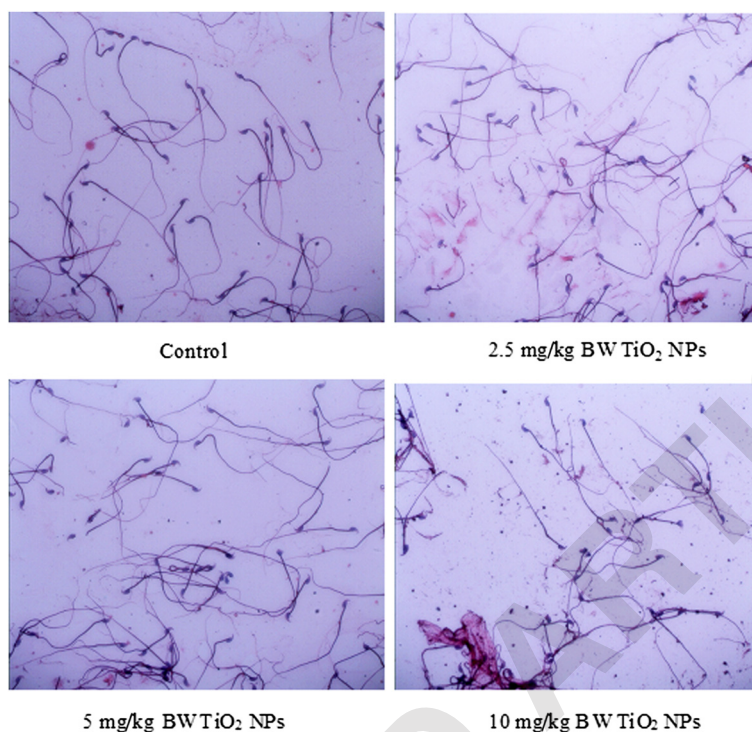


Figure 4 Morphological changes in mouse sperm following intragastric administration of TiO₂ NPs for 90 consecutive days.

limited. In addition, it is not known whether TiO₂ NPs enter Sertoli cells inducing oxidative disturbances and/or apoptosis, and the mechanisms involved have not been defined. This has relevance as elevated oxidative stress in the testicular milieu was demonstrated to have profound implications for testicular physiology and sperm function following exposure to nanosized materials [13,24,27].

Oxidative damage can affect many classes of molecules including lipids, proteins, nucleic acids, and sugars. Thus, the cell nucleus, mitochondrial membranes, structural and cytoplasmic proteins, complex carbohydrates, RNA, and DNA may all potentially be affected by oxidative stress [28]. In testicular tissue with high rates of metabolism and cell replication, oxidative stress can be especially damaging, making the antioxidant capacity of the tissue very important. Our data revealed that TiO₂ NPs crossed the blood–testis barrier and were deposited in the seminiferous cord, were translocated to Sertoli cells and/or the nucleus, and caused severe pathological changes such as few sperm, sperm breakage, rupture of the seminiferous tubules, vacuolation, irregular arrangement of Sertoli cells, apoptosis, and significant oxidative impairment in the mouse testis. The pathological changes in the testis were similar to those in our previous report [22]. However, Guo et al. suggested that male mice exposed to rutile TiO₂ NPs (30 nm) or ZnO NPs (30 nm) by intraperitoneal injection for one week showed significantly increased germ cell apoptosis in the testis

without exhibiting significant testicular and/or epididymal lesions [20,29]. Bai et al. suggested that repeated intravenous injections of water-soluble multi-walled carbon nanotubes (20–30 nm) in male mice for 15 days caused oxidative stress in the testis without affecting fertility, and the oxidative stress and testicular damage were repaired by day 60 and 90, respectively [27]. Exposure to black carbon NPs was demonstrated to increase partial vacuolation of the seminiferous tubules [30]. Furthermore, mice intravenously injected with amorphous nanosilica particles (70 nm) for one week, confirmed that the NPs penetrated the blood–testis barrier and the nuclear membranes of spermatocytes, but did not produce any apparent testicular injuries in male mice [31]. We consider that the inconsistent findings from these reports may be due to the different NPs type, size, exposure time, and particularly clearance rates from the circulation and tissues.

Evidence in favor of our central hypothesis was the enhanced levels of MDA, PC and 8-OHdG, as well as ROS levels, and decreased antioxidative enzyme activities including SOD, CAT, GPx, GR and GST and antioxidant contents such as GSH and ascorbic acid in the testes due to TiO₂ NP exposure. The occurrence of oxidative impairments in the testis raises the question as to whether this effect could be interpreted as a primary effect of TiO₂ NPs in the testis as TiO₂ NPs are known prooxidants and their potential to generate free radicals,

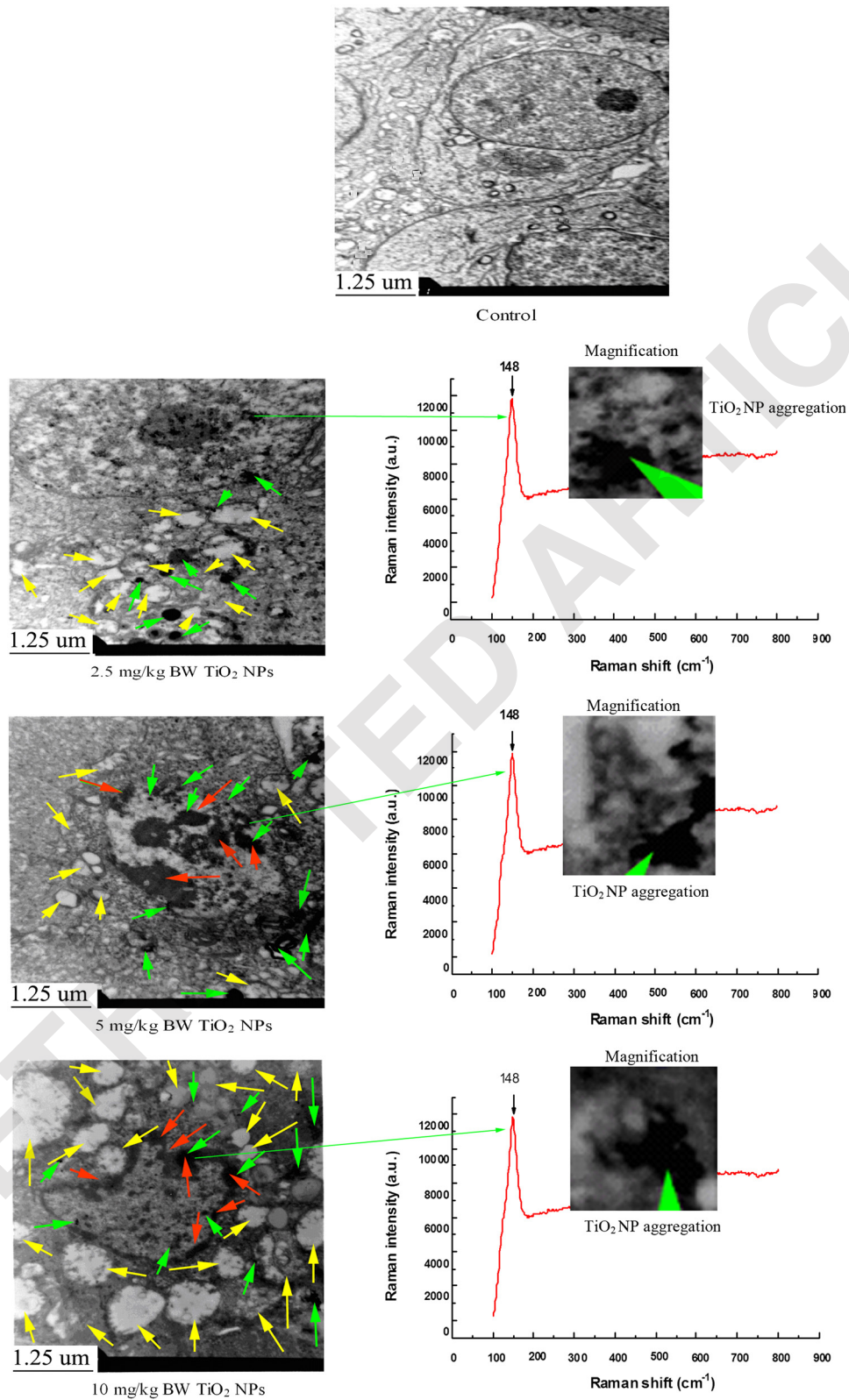


Figure 5 (See legend on next page.)

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Figure 5 Ultrastructure of Sertoli cells in mouse testis caused by intragastric administration of TiO₂ NPs for 90 consecutive days. The yellow arrow indicates mitochondrial swelling, blushing, and/or cristae disappearance, the green arrow indicates TiO₂ NP aggregation in mitochondria, and the red arrow indicates nuclear membrane collapse and chromatin marginalization in Sertoli cells. The green arrow spot denotes a representative cell loaded with TiO₂ NPs. The right panel shows the corresponding Raman spectra identifying specific peaks at about 148 cm⁻¹.

damage DNA, and induce apoptosis/necrosis is well documented *in vitro* [32] and *in vivo* [33]. While, it is difficult to precisely answer this question based on the data obtained in the current study, it can be reasonably postulated that TiO₂ NPs do cause significant oxidative stress in mouse testis.

TiO₂ NP-induced oxidative damage in the testis responded to modulation of antioxidant capacity. Decreased antioxidant enzymes, ascorbic acid and GSH markedly aggravated TiO₂ NPs induced oxidative damage in the testis as shown by elevated MDA and ROS levels in the cytosol and mitochondria. The major antioxidant enzymes in mammals are SOD, CAT, and GPX, the latter necessitating a number of other enzymes, e.g. GR and GST, required for the recycling or elimination of GSH. All of these antioxidant enzymes are expressed in the testis [34-36]. SOD converts O₂⁻ to H₂O₂ and O₂, and CAT and GPX reduce H₂O₂ to H₂O and O₂. GR reduces glutathione disulfide (GSSG) to the sulfhydryl form, GSH, which is an important cellular antioxidant. Elevated GR activity can increase the GSH/GSSG ratio, which is required for ascorbic acid regeneration [37]. Furthermore, GST, previously known as ligandin, is from a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyze the conjugation of GSH to xenobiotic substrates for the purpose of detoxification [38]. In the present study, our data suggest that exposure to TiO₂ NPs significantly decreased the activities of SOD, CAT, GPX, GR, and GST, and their gene and protein levels, resulting in a higher level of ROS production, increased ROS toxicity, testicular tissue and cell damage in male mice following exposure to TiO₂ NPs. A number of non-enzyme factors also function as antioxidants in the testis. Of these, ascorbic acid and GSH have proven efficacy in reducing testicular oxidative stress by

removing ROS either directly or indirectly by being involved in the ascorbic acid–GSH cycle under different conditions [39-43]. Ascorbic acid is considered to be a major antioxidant in the testis, and neutralizes ROS and prevents sperm agglutination. It is an electron donor in redox systems, prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by the hydrogen peroxide radical [44]. Oral administration of 200 mg of ascorbic acid together with vitamin E and GSH for a period of 2 months significantly reduced hydroxyguanine (8-OH-dG) levels in spermatozoa and increased the sperm count [45]. In the present study, however, the depletion of ascorbic acid and GSH in the testis caused by TiO₂ NPs was associated with increases in ROS, MDA, PC, and 8-OH-dG, suggesting that the testis was using antioxidant defenses to prevent oxidative stress, which caused severe oxidative damage in the mouse testis. The oxidative damage and reductions in antioxidants caused by TiO₂ NPs were consistent with those in mouse liver [46], kidney [47,48], lung [49,50], brain [51], and ovary [21].

The blood-testis barrier is formed by the tight junctions of Sertoli cells [52]. Therefore, TiO₂ NP deposition in the testis is closely related to seminiferous tubule injury or apoptosis of Sertoli cells following exposure to TiO₂ NPs. To understand the mechanisms of oxidative stress and/or apoptosis, the expression of oxidative stress and/or apoptosis-related genes and their proteins in the testis was detected and is listed in Figures 6 and 7. Of the genes involved in apoptosis and/or oxidative stress, caspase-3, Nrpb2, and cytochrome c were markedly up-regulated, while Cyp1b1, Car3, Bcl-2, Acaa2, Axud1, SOD, CAT, GPX, GR, and GST were significantly down-regulated in the mouse testis. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic

Table 1 Oxidative stress in testis of male mice caused by intragastric administration with TiO₂ NPs for 90 consecutive days

Oxidative stress	TiO ₂ NPs (mg/kg BW)			
	0	2.5	5	10
O ₂ ⁻ (nmol/mg prot·min)	45 ± 2.42	57 ± 2.96*	78 ± 4.28**	103 ± 5.29***
H ₂ O ₂ (nmol/mg prot·min)	63 ± 3.49	73 ± 3.82*	95 ± 4.86**	125 ± 6.36***
MDA (nmol/ mg prot)	570 ± 29.62	1030 ± 51.72*	1880 ± 95.08**	2580 ± 130.55***
PC (nmol/mg prot)	960 ± 48.75	1170 ± 58.93*	2220 ± 112.56**	4070 ± 205.57***
8-OHdG (ng/g tissue)	310 ± 15.86	3390 ± 169.78*	6380 ± 320.81**	8660 ± 435.63***

*p < 0.05, **p < 0.01, and ***p < 0.001. Values represent mean ± SD (N = 5).

Table 2 Alterations of antioxidative capacity in mouse testis after intragastric administration with TiO₂ NPs for 90 consecutive days

Enzyme activity	TiO ₂ NPs (mg/kg BW)			
	0	2.5	5	10
SOD (unit/mg protein·min)	51 ± 2.71	35 ± 1.79*	23 ± 1.39**	13 ± 0.72***
CAT (unit/mg protein·min)	73 ± 3.82	53 ± 2.79*	35 ± 1.86**	25 ± 1.39***
GSH-Px (unit/mg protein·min)	57 ± 2.96	43 ± 2.29*	29 ± 1.56**	18 ± 1.15***
GST (unit/mg protein·min)	50 ± 2.65	37 ± 2.06*	22 ± 1.2**	15 ± 0.8***
GR (unit/mg protein·min)	59 ± 3.23	50 ± 2.65*	36 ± 1.85**	29 ± 1.56***
GSH (ng/g tissue)	2360 ± 118.56	1910 ± 95.53*	1090 ± 55.56**	510 ± 25.68***
Ascorbic acid (ng/g tissue)	2050 ± 103.28	1480 ± 75.22*	850 ± 43.55**	420 ± 22.67***

*p < 0.05, **p < 0.01, and ***p < 0.001. Values represent mean ± SD (n = 5).

(mitochondrial) pathways [53-56]. It has been reported that TiO₂ NPs induced apoptosis in mouse spleen by activating caspase-3 [57]. The present study showed that caspase-3 expression in mouse testis was increased, in concordance with the above-mentioned report on the effects of TiO₂ NPs in mouse spleen. Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli. Cytosolic cytochrome c is necessary for initiation of the apoptotic program, suggesting a possible connection between Bcl-2 and cytochrome c, which is normally located in the mitochondrial intermembrane space. Cells undergoing apoptosis were found to have elevated cytochrome c in the cytosol and a corresponding decrease in the mitochondria. Overexpression of Bcl-2 prevented the efflux of cytochrome c from the mitochondria and the initiation of apoptosis [58,59]. In the present study, Bcl-2 expression was significantly decreased, whereas cytochrome c was markedly increased in the TiO₂ NP-exposed mouse testis, suggesting that Bcl-2 may play an important role in TiO₂ NP-induced Sertoli apoptosis in the mouse testis. These results were similar to those of TiO₂ NPs-induced apoptosis in mouse spleen [60]. Nrbp2 belongs to the protein kinase superfamily, and was found to be expressed in a subset of tumor cells in human medulloblastoma and protected nestin-positive neural progenitors against apoptosis during differentiation [61]. Nrbp2 upregulation in the mouse testis may prevent Sertoli cell apoptosis and may be a response to TiO₂ NP-induced toxicity. Cao et al. indicated that Acaa2 abolished Bcl-2/adenovirus E1B 19 kD-interacting protein 3 (BNIP3)-mediated apoptosis and mitochondrial damage [62]. *Axud1* is a member of the gene family encoding nuclear proteins which contain cysteine- and serine-rich domains, and is often considered to be related to reduced apoptosis [63]. Therefore, Sertoli apoptosis in testicular tissue caused by TiO₂ NPs may be due to a marked reduction in Acaa2 and Axud1 expression. Of the genes and their proteins involved in oxidative stress, SOD, CAT, APX, GR, and GST were highly suppressed. SOD catalyses the dismutation of superoxide anion by

successive oxidation-reduction of the transition metal at the enzyme's active site [64]. CAT converts hydrogen peroxide to hydrogen and water. GPX catalyzes the breakdown of H₂O₂ and organic hydroperoxides. GPX can directly reduce phospholipid hydroperoxides inside biological membranes to lipid and water, and is essential for survival and important in repairing damage due to lipid peroxidation [65]. GPX also protects against mitochondrial apoptosis and decreases in mitochondrial ATP generation in the presence of oxidative stress, and is strongly expressed in the mitochondria of testis and spermatozoa [66,67]. GR plays a key role in oxidative stress by converting GSSG to GSH. Increased GR activity can elevate the GSH/GSSG ratio, which is required for ascorbic acid regeneration [37]. GST is able to conjugate GSH to the toxic reactive compounds, 4-hydroxynonenal and cholesterol α -oxide, which are generated during the oxidation of membranes. The GPx activity of some GST proteins also suggests that they may be important in the detoxification of organic hydroperoxides [68]. Thus, decreased expression of SOD, CAT, GPX, GR, and GST in the mouse testis aggravated TiO₂ NP-induced oxidative stress. Car3 has been demonstrated to rapidly glutathionylate *in vivo* and *in vitro* when cells are exposed to oxidative stress, and it is one of the most carbonylated proteins in rodent liver [69]. Overexpression of Car3 decreased steady-state levels of intracellular ROS, increased the proliferation rate, and protected cells against H₂O₂-induced apoptosis [69]. Cyp1b1 is a member of the cytochrome P450 family of proteins and can remove cellular oxygenation products which induce oxidative stress, while a lack of Cyp1b1 increases oxidative stress *in vivo* and *in vitro* [70]. Therefore, the generation of oxidative stress in the TiO₂ NP-exposed mouse testis may also be closely associated with significant reductions in Car3 and Cyp1b1 expression and removal of ROS.

In summary, TiO₂ NPs entered Sertoli cells via the blood-testis barrier and were deposited in mouse testicular tissue and/or Sertoli cells, causing testicular oxidative damage and cell apoptosis. Furthermore, testicular

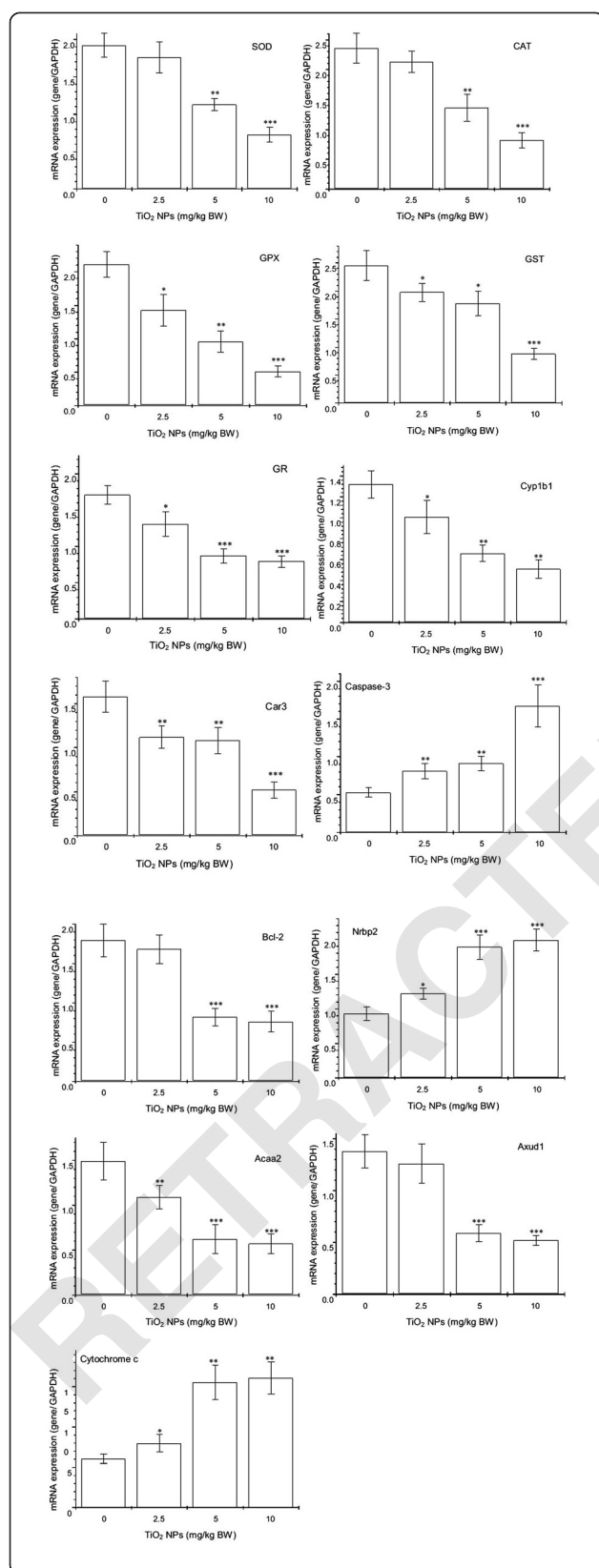


Figure 6 Alterations in the mRNA expression of oxidative stress and/or apoptosis-related genes in mouse testis caused by intragastric administration of TiO₂ NPs for 90 consecutive days. *p < 0.05, **p < 0.01, and ***p < 0.001. Values represent mean ± SD (n = 5).

damage following exposure to TiO₂ NPs may be related to significant overproduction of ROS, peroxidation of lipids, proteins and DNA, and a significant reduction in antioxidant capacity. Sertoli cell apoptosis caused by TiO₂ NPs was associated with up-regulation of caspase-3, Nr1p2, and cytochrome c expression, and down-regulation of SOD, CAT, GPx, GST, GR, Cyp1b1, Car3, bcl-2, Acaa2, and Axud1 expression in mouse testis. Therefore, the application of TiO₂ NPs should be performed with caution to reduce potential risks to humans.

Materials and methods

Chemicals

Anatase TiO₂ nanoparticles were prepared via controlled hydrolysis of titanium tetrabutoxide. The details of TiO₂ NP synthesis were previously described [71,72]. Hydroxypropylmethylcellulose (HPMC) 0.5% w/v was used as a suspending agent. TiO₂ powder was dispersed onto the surface of a 0.5% w/v HPMC solution, and then the suspension containing TiO₂ particles was treated ultrasonically for 15–20 min and mechanically vibrated for 2 or 3 min.

The particle sizes of both the powder and nanoparticles suspended in 0.5% w/v HPMC solution (5 mg/L) following incubation were determined using a TecnaiG220 transmission electron microscope (TEM) (FEI Co., USA) operating at 100 kV, respectively. In brief, particles were deposited in suspension onto carbon film TEM grids, and allowed to air-dry. The mean particle size was determined by measuring > 100 randomly sampled individual particles. X-ray-diffraction (XRD) patterns of TiO₂ NPs were obtained at room temperature with a charge-coupled device (CCD) diffractometer (Mercury 3 Versatile CCD Detector; Rigaku Corporation, Tokyo, Japan) using Ni-filtered Cu K α radiation. The average aggregate or agglomerate size of the TiO₂ NPs after incubation in 0.5% w/v HPMC solution (5 mg/L) for 0, 12 and 24 h was measured by dynamic light scattering (DLS) using a Zeta PALS + BI-90 Plus (Brookhaven Instruments Corp., USA) at a wavelength of 659 nm. The scattering angle was fixed at 90°.

Ethics statement

All experiments were conducted during the light phase, and were approved by the Animal Experimental Committee of Soochow University (Grant 2111270) in accordance with the National Institutes of Health

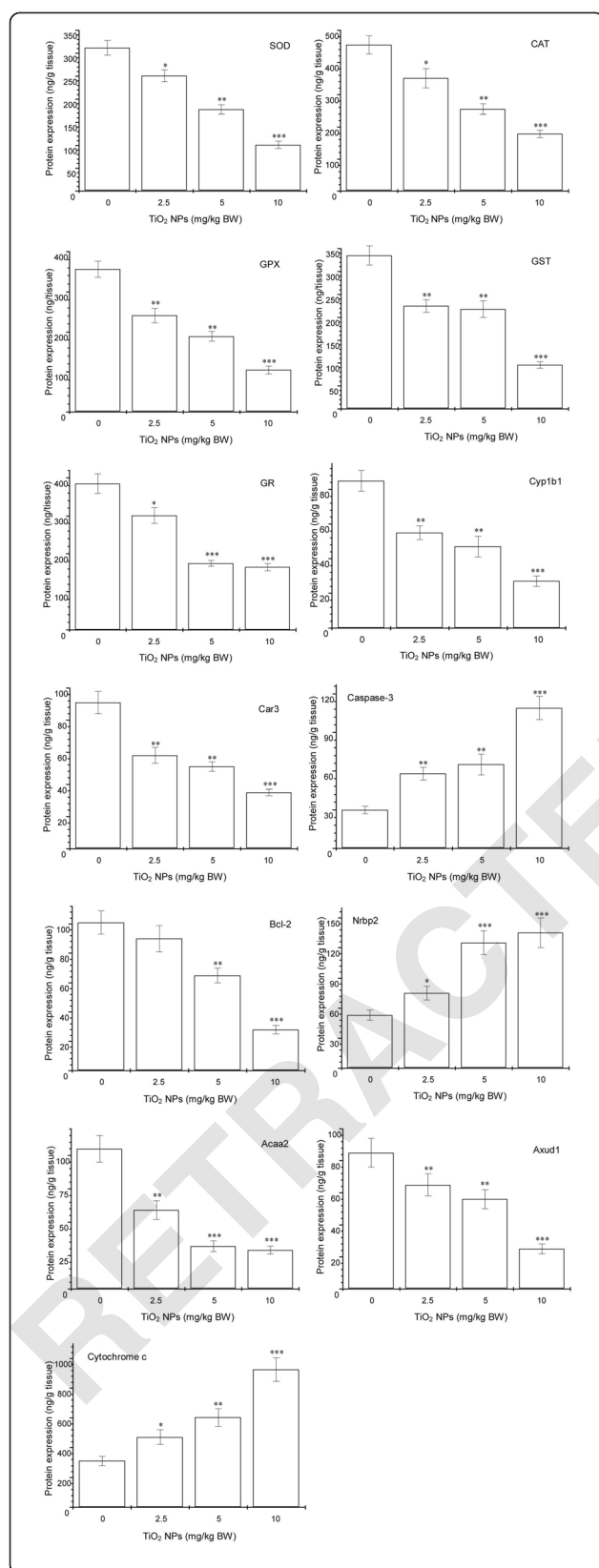


Figure 7 Alterations in the expression of oxidative stress and/or apoptosis-related proteins in mouse testis caused by intragastric administration of TiO_2 NPs for 90 consecutive days. *p < 0.05, **p < 0.01, and ***p < 0.001. Values represent mean \pm SD (n = 5).

Guidelines for the Care and Use of Laboratory Animals (NIH Guidelines).

Animals and treatment

One hundred sixty CD-1 (ICR) male mice (24 ± 2 g) were purchased from the Animal Center of Soochow University (China). The mice were housed in stainless steel cages in a ventilated animal room. The room temperature in the housing facility was maintained at $24 \pm 2^\circ\text{C}$, with a relative humidity of $60 \pm 10\%$ and a 12-h light/dark cycle. Distilled water and sterilized food were available *ad libitum*. Before treatment, the mice were acclimated to this environment for five days. All the animals were handled in accordance with the guidelines and protocols approved by the Care and Use of Animals Committee of Soochow University (China).

TiO_2 NP powder was dispersed onto the surface of 0.5% w/v HPMC and the suspension containing TiO_2 NPs was treated ultrasonically for 30 min and mechanically vibrated for 5 min. The mice were randomly divided into four groups (n = 40 in each group), including a control group treated with 0.5% w/v HPMC and three experimental groups treated with 2.5, 5, or 10 mg/kg TiO_2 NPs. The mice were weighed, a volume of TiO_2 NP suspension was calculated for each mouse, and the fresh TiO_2 NP suspension was administered by gavage using a gavage needle each day for 90 days. Symptoms, growth status, eating, drinking, activity and mortality were observed and carefully recorded daily during the 90-day period. After 90 days, the mice were weighed, anesthetized using ether, and then sacrificed. The testes were quickly removed, placed on ice, dissected, and then frozen at -80°C (except those for histopathological examination).

Histopathological examination of testis

All histopathological tests were performed using standard laboratory procedures [73]. Five sets of testicular tissues from 5 mice were embedded in paraffin blocks, sliced into 5 μm thick sections and then placed onto glass slides (five slices from each testis). Following hematoxylin–eosin staining, the slides were observed and photographed using an optical microscope (Nikon U-III Multi-point Sensor System, USA). The pathologist was blind to the identity and analysis of the pathology slides.

Observation of testis ultrastructure

Testes (n = 5 in each group) were fixed in a fresh solution of 0.1 M sodium cacodylate buffer containing 2.5%

glutaraldehyde and 2% formaldehyde followed by a 2 h fixation period at 4°C with 1% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2-7.4). Staining was performed overnight with 0.5% aqueous uranyl acetate. The specimens were dehydrated in a graded series of ethanol (75, 85, 95, and 100%), and embedded in Epon 812. Ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate, and observed with a HITACHI H600 TEM (HITACHI Co., Japan). Testicular apoptosis was determined based on the changes in nuclear morphology (e.g., chromatin condensation and fragmentation).

Confocal raman microscopy of testicular sections

Raman analysis was performed using backscattering geometry in a confocal configuration at room temperature using a HR-800 Raman microscope system equipped

with a 632.817 nm HeNe laser (JY Co., Fort-de-France, Martinique). It was reported that when the size of the TiO₂ NPs reached 6 nm, the Raman spectral peak was 148.7 cm⁻¹ [74]. Laser power and resolution were approximately 20 mW and 0.3 cm⁻¹, respectively, while the integration time was adjusted to 1 s. The testicular specimens (*n* = 5 in each group) were embedded in paraffin blocks, sliced into 5-µm thick sections, and placed onto glass slides. The slides were dewaxed, hydrated, and then scanned using the confocal Raman microscope.

Oxidative stress assay

Reactive oxygen species (ROS) (O₂⁻ and H₂O₂) production and levels of malondialdehyde (MDA), protein carbonyl (PC), and 8-hydroxy deoxyguanosine (8-OHdG) in the testicular tissues (*n* = 5 in each group) were assayed using commercial enzyme-linked immunosorbent assay

Table 3 Real time PCR primer pairs

Gene name	Description	Primer sequence	Primer size (bp)
Refer- GAPDH	mactin-F	5'- TGTGTCCGTCGTGGATCTGA -3'	
	mactin-R	5'- TTGCTGTTGAAGTCGCAGGAG -3'	150
SOD	mSOD F	5'-CTGGACAAACCTGAGCCCTAA-3'	
	mSOD R	5'-TCCCCAGCAGCGGAATAA-3'	242
CAT	mCATF	5'-AGCGACCAGATGAAGCAGTG-3'	
	mCAT R	5'-GGGTGACCTCAAAGTATCCAAA-3'	241
GSH-Px	mGSH-Px F	5'-GGGACTACACCGAGATGAACG-3'	
	mGSH-Px R	5'-TCCGCAGGAAGGTAAAGAGC-3'	231
GST	mGST F	5'-CCGCTCTTTGGGGCTTTAT-3'	
	mGST R	5'-GGTTCTGGGACAGCAGGGT-3'	191
GR	mGR F	5'- TTCTGTTTCATGGCGTGAGTACC -3'	
	mGR R	5'- CCCTTGGCACCTATTCAGTT -3'	150
Cyp1b1	mCyp1b1-F	5'-TTAGTATGCTTTTCGCTGTGACA -3'	
	mCyp1b1-R	5'-GGGGAGTTATTCCTGGGTTATTA-3'	81
Car3	mcar3 F	5'-GCTCTGCTAAGACCATCC-3'	
	mcar3 R	5'-ATTGGCGAAGTCGGTAGG-3'	160 bp
Caspase-3	mcaspase-3 F	5'-CTGACTGGAAGCCGAAACTC-3'	
	mcaspase-3 R	5'-GACTGGATGAACCACGACCC-3'	203
Bcl-2	mbcl-2 F	5'-TGTGGTCCATCTGACCCCTCC-3'	
	mbcl-2 R	5'-ACATCTCCCTGTTGACGCTCT-3'	224
Nr1p2	mnrbp2 F	5'-TGCAGTGCAACCTGGAAA-3'	
	mnrbp2 R	5'-AGGAAACCAGGGTGGCTC-3'	188
Acaa2	macaa2 F	5'-TGTGTCAGAAATGTGCGCTTC-3'	
	macaa2 R	5'-CAAGGCGTATCTGTACAGTC-3'	123
Axud1	mAxud1-F	5'-CGCCCTTCATTAGCTGATGTT -3'	
	mAxud1-R	5'-CAGAGCCTGCGTTTCTTGG-3'	117
Cytochrome c	mcytochrome c F	5'-CATCCCTTGACATCGTGCTT-3'	
	mcytochrome c R	5'-GGGTAGTCTGAGTAGCGTCGTG-3'	250

PCR primers used in the gene expression analysis.

kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's instructions.

Antioxidant capacity

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S epoxide transferase (GST), and glutathione reductase (GR), and the contents of reduced glutathione (GSH) and ascorbic acid in the testicular tissues ($n = 5$ in each group) were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's instructions.

Assay of gene and protein expression

Total RNA was extracted from individual testes using from the homogenates was isolated using Tripure Isolation Reagent (Roche, USA) according to the manufacturer's instructions. Probes and cycling condition were optimized in accordance with MIQE guidelines for PCR [75]. Synthesized cDNA was used for the real-time PCR by employing primers designed using Primer Express Software according to the software guidelines. PCR primers used in the gene expression analysis are listed in Table 3. Gene expression levels were calculated as a ratio to the expression of the reference gene, GAPDH and data were analyzed using the $\Delta\Delta C_t$ method. The probes for SOD, CAT, GPx, GST, GR, Cyp1b1, carbonic anhydrase III (Car3), caspase-3, Bcl-2, nuclear receptor binding protein 2 (Nrpb2), acetyl-coenzyme A acyltransferase 2 (Acaa2), Axin upregulated 1 (Axud1), and cytochrome c were designed by the manufacturer and purchased from Shinegene Company (Shanghai, China). The RT-qPCR data were processed with the sequence detection software version 1.3.1 following the method of Schefe et al. [76].

To determine protein levels of SOD, CAT, GPx, GST, GR, Cyp1b1, Car3, caspase-3, Bcl-2, Nrpb2, Acaa2, Axud1, and cytochrome c in the testes, total protein from the frozen testicular tissues ($N = 5$ in each group) from experimental and control mice was extracted using Cell Lysis Kits (GENMED SCIENTIFICS INC.USA) and quantified using BCA protein assay kits (GENMED SCIENTIFICS INC.USA). ELISA was performed using commercial kits that were selective for each respective protein (R&D Systems, USA), following the manufacturer's instructions. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron, Finland), and the concentrations of SOD, CAT, GPx, GST, GR, Cyp1b1, Car3, caspase-3, Bcl-2, Nrpb2, Acaa2, Axud1, and cytochrome c were calculated from a standard curve for each sample.

Statistical analysis

All results are expressed as means \pm SD. One-way analysis of variance (ANOVA) was carried out to compare

the differences of means among the multi-group data using SPSS 19 software (SPSS, Inc., Chicago, IL, USA). Dunnett's test was performed when each dataset was compared with the solvent control data. Statistical significance for all tests was judged probability level of 0.05 ($P < 0.05$).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: FH, XZ, LS, and LW. Performed the experiments: FH, XZ, LS, LW, JH, and XY. Analyzed the data: FH, XZ, LS, LW, JH, XY, YZ, XS, and QS. Contributed reagents/materials/analysis tools: YZ, XS, and QS. Wrote the paper: FH, XZ, LS, and LW. All authors read and approved the final manuscript.

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Author details

¹Medical College of Soochow University, Suzhou 215123, China. ²Library of Soochow University, Suzhou 215123, China. ³Jiangsu Province Key Laboratory of Stem Cell Research, Soochow University, Suzhou 215007, China.

⁴Cultivation base of State Key Laboratory of Stem Cell and Biomaterials built together by Ministry of Science and Technology and Jiangsu Province, Suzhou 215007, China.

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