Nanosized TiO$_2$-Induced Reproductive System Dysfunction and Its Mechanism in Female Mice

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Abstract

Recent studies have demonstrated nanosized titanium dioxide (nano-TiO$_2$)-induced fertility reduction and ovary injury in animals. To better understand how nano-TiO$_2$ act in mice, female mice were exposed to 2.5, 5, and 10 mg/kg nano-TiO$_2$ by intragastric administration for 90 consecutive days; the ovary injuries, fertility, hormone levels, and inflammation-related or follicular atresia-related cytokine expression were investigated. The results showed that nano-TiO$_2$ was deposited in the ovary, resulting in significant reduction of body weight, relative weight of ovary and fertility, alterations of hematological and serum parameters and sex hormone levels, atretic follicle increases, inflammation, and necrosis. Furthermore, nano-TiO$_2$ exposure resulted in marked increases of insulin-like growth factor-binding protein 2, epidermal growth factor, tumor necrosis factor-α, tissue plasminogen activator, interleukin-1β, interleukin-6, Fas, and FasL expression, and significant decreases of insulin-like growth factor-1, luteinizing hormone receptor, inhibin α, and growth differentiation factor 9 expression in mouse ovary. These findings implied that fertility reduction and ovary injury of mice following exposure to nano-TiO$_2$ may be associated with alteration of inflammation-related or follicular atresia-related cytokine expressions, and humans should take great caution when handling nano-TiO$_2$.

Introduction

The manufacture and application of various synthetic nanoparticles are expanding at a rapid rate, and therefore increased environmental and occupational exposures of these materials seem inevitable. Nanosized titanium dioxide (nano-TiO$_2$) presents various uses in industry, in commerce such as cosmetics, sunscreen, toothpaste, food additives and paints, and even in the treatment of contaminated environments [1–4]. However, toxicological studies suggested that nano-TiO$_2$ had adverse effects on human health and environmental species. The bio-safety of nano-TiO$_2$ is still an argumentative issue.

Toxicological properties of nano-TiO$_2$ have been studied in kidney [5–8], and brain [9–14] of animals. Especially, recent studies demonstrated that exposure to nano-TiO$_2$ resulted in toxicity in reproductive system. For example, nano-TiO$_2$ can reduce sperm density and motility, increase sperm abnormality and germ cell apoptosis of male mice in vivo [15], inhibit follicle development and oocyte maturation of rat in vitro [16], affect development, reproduction, and locomotion behavior of Caenorhabditis elegans [17], induce genotoxicity and cytotoxicity in Chinese hamster ovary cells in vitro [18], and impair zebrafish reproduction [19]. Nano-TiO$_2$ was also administered subcutaneously to pregnant mice and transferred to the offspring and impaired the genital and cranial nerve systems of the male mice offspring [20]. However, the mechanisms of nano-TiO$_2$–induced toxicity in reproductive system have yet to be understood. The present study was designed to investigate histopathological changes of ovary, fertility, and sex hormone levels in female mice following exposure to 2.5, 5, and 10 mg/kg body weight TiO$_2$ NPs 90 consecutive days. Furthermore, the inflammation-related or follicular atresia-related cytokine expressions were also examined by real time RT-PCR and ELISA after female mice exposure to nano-TiO$_2$. Our results showed that nano-TiO$_2$ can induce ovarian dysfunction measured by histological assessment, mating rate, pregnancy rate, number of newborns, and sex hormone levels. Nano-TiO$_2$ also significantly altered the inflammation-related or follicular atresia-related cytokine expressions in the mouse ovary.

Materials and Methods

Preparation and Characterization of Nano-TiO$_2$

Nanoparticulate anatase TiO$_2$ of the synthesis and characterization of nano-TiO$_2$ was described in our previous reports [13,21]. In a typical experiment, 1 mL of Ti (OC$_4$H$_9$)$_4$ was...
dissolved in 20 ml of anhydrous isopropanol, and was added dropwise to 50 mL of double-distilled water that was adjusted to pH 1.5 with nitric acid under vigorous stirring at room temperature. The temperature of the solution was then raised to 60°C, and maintained for 6 hours to promote better crystallization of nanoparticulate TiO₂ particles. Using a rotary evaporator, the resulting translucent colloidal suspension was evaporated yielding a nanocrystalline powder. The obtained powder was washed three times with isopropanol, and then dried at 50°C until the evaporation of the solvent was complete. A 0.5% w/v hydroxypropylmethylcellulose (HPMC) K4M was used as a suspending agent. TiO₂ powder was dispersed onto the surface of 0.5% w/v

Figure 1. Changes of body weight and relative weight of ovary of mice caused by intragastric administration of nano-TiO₂ for 90 consecutive days. Different letters indicate significant differences between groups (p<0.05). Values represent means ± SE (N=10).
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Different letters indicate significant differences between groups (p < 0.05). Values represent means ± SE (N = 5).

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Table 2. Hematological and biochemical parameters in female mice by intragastric administration of nano-TiO2 for 90 consecutive days.

| Index                  | Nano-TiO2 (mg/kg BW) | 0       | 2.5     | 5       | 10      |
|------------------------|----------------------|---------|---------|---------|---------|
| WBC (10^9/L)           | 7.68 ± 0.39a         | 5.78 ± 0.29b | 4.08 ± 0.20c | 2.76 ± 0.14d |
| LYMPH (10^9/L)         | 6.05 ± 0.30a         | 3.79 ± 0.19b | 2.29 ± 0.11c | 1.68 ± 0.08d |
| NEUT (10^9/L)          | 1.62 ± 0.08a         | 1.18 ± 0.06b | 0.81 ± 0.04c | 0.52 ± 0.03d |
| RBC (10^12/L)          | 9.45 ± 0.47a         | 8.57 ± 0.43b | 7.59 ± 0.38c | 6.89 ± 0.34d |
| HGB (g/L)              | 146.12 ± 7.30a       | 131.55 ± 6.40b | 127.11 ± 6.40b | 116.55 ± 5.9c |
| ALT (U/L)              | 19.29 ± 0.96a        | 22.53 ± 1.13b | 26.38 ± 1.32c | 35.82 ± 1.79d |
| AST (U/L)              | 77.27 ± 3.86a        | 86.25 ± 4.31b | 97.56 ± 4.88c | 115.39 ± 5.77d |
| ALP (U/L)              | 92.81 ± 4.64a        | 108.66 ± 5.43b | 118.61 ± 5.93c | 136.86 ± 6.84d |
| LDH (U/L)              | 659.66 ± 32.98a      | 715.38 ± 35.77b | 796.71 ± 39.84c | 896.79 ± 44.84d |
| UA (μmol/L)            | 228.76 ± 11.44a      | 157.39 ± 7.87b | 105.26 ± 5.26c | 89.87 ± 0.49d |
| Cr (μmol/L)            | 8.45 ± 0.422a        | 9.89 ± 0.49b   | 12.23 ± 0.61c  | 14.68 ± 0.73d  |
| BUN (mmol/L)           | 9.56 ± 0.48a         | 8.02 ± 0.40b   | 6.88 ± 0.34c   | 6.05 ± 0.30d   |

Different letters indicate significant differences between groups (p < 0.05). Values represent means ± SE (N = 5).

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Table 1. Hematological and biochemical parameters in female mice by intragastric administration of nano-TiO2 for 90 consecutive days.
and 10 mg/kg body weight of nano-TiO2 of fresh solutions). The female mice were randomly divided into four groups (each group N = 100), including a control group (treated with 0.5% w/v HPMC) and three experimental groups (2.5, 5, and 10 mg/kg body weight) after oral administration. In addition, the quantity of TiO2 nanoparticles does not exceed 1% by weight of the food according to the Federal Regulations of US Government. In the present study, we selected 2.5, 5, and 10 mg/kg BW TiO2 NPs exposed to mice by intragastric administration every day. They were equal to about 0.15–0.7 g TiO2 NPs of 60–70 kg body weight for humans with such exposure, which were relatively safe doses. For the mice, the female mice were weighed, and the fresh nano-TiO2 suspensions within 30 min were administered to the mice by intragastric administration every day for 90 min. Any symptom or mortality was observed and recorded carefully every day during the 90 days.

Hematological Parameters Determination

Titanium Content Analysis

To evaluate the effect of nano-TiO2 on the fertility and growth of newborns, we treated three groups of female mice (10 in each mating group) for 90 days. After last day of nano-TiO2 administration, 10 male and 10 control or treated female mice from each group were put in a common cage for mating. The number of newborns from each pregnant mouse were counted and weighed. The ovaries of all animals were quickly removed and placed on ice and then dissected and frozen at −80°C except for 40 ovaries for histopathological examination, respectively.

Mating of Animals

Table 2. Effects of nano-TiO2 on conception of female mice, number of newborns, and weight of neonates after intragastric administration of nano-TiO2 for 90 consecutive days.

| Index                          | Nano-TiO2 (mg/kg BW) |
|-------------------------------|----------------------|
|                               | 0  | 2.5 | 5  | 10 |
| Mating rate (%)               | 100±5a | 85±4.25b | 75±3.75c | 65±3.25d |
| Pregnancy rate (%)            | 100±5a | 81±4.05b | 72±3.6c  | 58±2.90d |
| Number of newborns            | 14±0.7a | 10±0.5b  | 8±0.4c   | 6±0.3d   |
| Weight of neonates female mice in 1st after birth (g) | 1.54±0.077a | 1.52±0.076a | 1.45±0.073b | 1.40±0.07b |
| Weight of neonates male mice in 1st after birth (g) | 1.57±0.079a | 1.54±0.077a | 1.47±0.074b | 1.41±0.07b |
| Survival rate of young mice in 28th day after birth (%) | 98±4.90a | 89±4.45b  | 81±4.05c | 72±3.60d |

Different letters indicate significant differences between groups (p<0.05). Values represent means ± SE (N = 5).

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Table 3. Effects of nano-TiO2 on sex hormone levels in sera of female mice.

| Hormone level | Nano-TiO2 NPs (mg/kg BW) |
|---------------|--------------------------|
|               | 0  | 2.5 | 5  | 10 |
| E2 (pmol/L)   | 83.66±4.18a | 91.09±4.554b | 101.98±5.10c | 111.88±5.59d |
| P4 (nmol/L)   | 34.32±1.75a | 30.11±1.50b  | 26.49±1.32c | 23.42±1.17d |
| LH (IU/L)     | 0.12±0.006a | 0.06±0.003b  | 0.03±0.002c | 0.02±0.001d |
| FSH (IU/L)    | 0.48±0.024a | 0.42±0.021b  | 0.37±0.018c | 0.28±0.014d |
| PRL (µg/L)    | 0.60±0.030a | 0.64±0.032a  | 0.67±0.033a | 0.73±0.036a |
| T (ng/dL)     | 71.13±1.56a | 61.55±1.08b  | 55.01±2.75c | 49.02±2.45d |
| SHBG (nmol/L) | 0.43±0.021a | 0.42±0.021a  | 0.42±0.021a | 0.42±0.021a |

Different letters indicate significant differences between groups (p<0.05). Values represent means ± SE (N = 5).

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Relative Weight of Ovary

After weighing the body and ovary, the relative weight of ovary was calculated as the ratio of ovary (wet weight, mg) to body weight (g).

Hematological Parameters Determination

Blood samples were collected in tubes containing EDTA as anticoagulant. Red blood cells (RBC), lymphocytes (LYMPH), reticulocytes (Ret), white blood cells (WBC), haemoglobin (HGB) were measured using a hematology autoanalyzer (Cell-DYN 3700).

Serum Parameters Determination

Biochemical parameters were evaluated by serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), uric acid (UA), blood urea nitrogen (BUN), creatinine (Cr). All biochemical assays were performed using a clinical automatic chemistry analyzer (Type 7170A, Hitachi, Japan).
Figure 3. Histopathological observation of ovary of mice caused by intragastric administration of nano-TiO$_2$ for 90 consecutive days. (a) control groups (unexposed mice) present normal development of primary follicle and secondary follicle; (b) 2.5 mg/kg nano-TiO$_2$-exposed group: green cycle suggest inflammatory cell infiltration, yellow arrows indicate atretic follicle, red arrows present apoptosis or tissue necrosis; (c) 5 mg/kg nano-TiO$_2$-exposed group: green cycle suggest severe inflammatory cell infiltration, yellow cycles present nano-TiO$_2$ deposition, yellow arrows indicate atretic follicle, red arrows present apoptosis or tissue necrosis; (d) 10 mg/kg nano-TiO$_2$-exposed group: green cycle suggest severe inflammatory cell infiltration, yellow arrows indicate atretic follicle, red arrows present tissue necrosis, yellow cycle may show aggregation of nano-TiO$_2$ in ovary. Arrow A spot is a representative cell that not engulfed the nano-TiO$_2$, while arrow B spot denotes a representative cell that loaded with nano-TiO$_2$. The right panels show the corresponding Raman spectra identifying the specific peaks at about 148 cm$^{-1}$. doi:10.1371/journal.pone.0059378.g003
progesterone (P4), luteinizing hormone (LH), follicle stimulating hormone binding globulin (SHBG) using commercial kits (Type 7170A; Hitachi Co., Japan).

**Table 4.** Effect of nano-TiO2 on the levels of cytokine gene mRNA expression in mouse ovary.

| Ratio of gene/actin | Nano-TiO2 (mg/kg BW) |
|---------------------|----------------------|
|                     | 0        | 2.5       | 5         | 10        |
| IGF-1/actin         | 1.53±0.068a | 0.92±0.046b | 0.58±0.029c | 0.32±0.016d |
| IGFBP-2/actin       | 0.41±0.021a | 0.69±0.035b | 0.99±0.050c | 1.37±0.069d |
| EGF/actin           | 0.71±0.036a | 1.03±0.052b | 1.38±0.069c | 1.75±0.088d |
| TNF-α/actin         | 0.27±0.014a | 0.43±0.022b | 0.69±0.035c | 0.97±0.049d |
| tPA/actin           | 0.07±0.004a | 0.28±0.014b | 0.42±0.021c | 0.56±0.028d |
| LHR/actin           | 0.46±0.023a | 0.25±0.013b | 0.12±0.006c | 0.05±0.003d |
| INHa/actin          | 0.95±0.048a | 0.61±0.031b | 0.38±0.019c | 0.12±0.006d |
| IL-1β/actin         | 0.22±0.011a | 0.39±0.020b | 0.68±0.034c | 1.05±0.053d |
| IL-6/actin          | 0.09±0.005a | 0.25±0.013b | 0.48±0.024c | 0.76±0.038d |
| Fas/actin           | 0.55±0.028a | 0.76±0.039b | 1.06±0.053c | 1.67±0.084d |
| Fasl/actin          | 0.33±0.017a | 0.54±0.027b | 0.86±0.043c | 1.13±0.057d |
| GDF-9/actin         | 1.07±0.054a | 0.72±0.036b | 0.46±0.023c | 0.29±0.015d |

Different letters indicate significant differences between groups (p<0.05). Values represent means ± SE (N = 5). doi:10.1371/journal.pone.0059378.t004

**Sex Hormone Assays**

Sex hormones were evaluated with serum levels of estradiol (E2), progesterone (P4), luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), testosterone (T), and sex hormone binding globulin (SHBG) using commercial kits (Bühlmann Laboratories, Switzerland). All biochemical assays were performed using a clinical automatic chemistry analyzer (Type 7170A; Hitachi Co., Japan).

**Histopathological Examination of Ovary**

For pathologic studies, all histopathologic examinations were performed using standard laboratory procedures. The ovaries were embedded in paraffin blocks, then sliced (5 μm thickness) and placed onto glass slides. After hematoxylin–eosin (HE) staining, the stained sections were evaluated by a histopathologist unaware of the treatments, using an optical microscope (Nikon U-III Multi-point Sensor System, Japan).

**Confocal Raman Microscopy in Ovarian Sections**

Raman analysis was performed using backscattering geometry in a confocal configuration at room temperature in a HR-800 Raman microscope system equipped with a 632.817 nm HeNe laser (JY Co., France). Laser power and resolution were approximately 20 mW and 0.3 cm⁻¹, respectively, while the integration time was adjusted to 1 s. Ovaries were embedded in paraffin blocks, then sliced into 5 μm in thickness and placed onto glass slides. The slides were dewaxed, hydrated, and then scanned under the confocal Raman microscope.

**Expression Assay of Cytokines**

The levels of mRNA expressions of insulin-like growth factor-1 (IGF-1), insulin-like growth factor-binding protein 2 (IGFBP-2), epidermal growth factor (EGF), tumor necrosis factor (TNF-α), tissue plasminogen activator (tPA), luteinizing hormone receptor (LHR), inhibin-α (INHa), interleukin-1β (IL-1β), IL-6, Fas, and GDF-9 in mouse ovary tissue were determined using real-time quantitative RT polymerase chain reaction (RT-PCR) [22–24]. Synthesized cDNA was used for the real-time PCR by employing primers that were designed using Primer Express Software according to the software guidelines, and PCR primer sequences are available upon request. To determine levels of protein expressions of IGF-1, IGFBP-2, EGF, TNF-α, tPA, LHR, INHa, IL-1β, IL-6, Fas, Fasl, and GDF-9 in the mouse ovary tissue, ELISA was performed using commercial kits that are selective for each respective protein (R&D Systems, USA). Manufacturer’s instruction was followed. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron, Finland), and the concentrations of IGF-1, IGFBP-2, EGF, TNF-α, tPA, LHR, INHa, IL-1β, IL-6, Fas, Fasl, and GDF-9 were calculated from a standard curve for each sample.

**Table 5.** Effects of nano-TiO2 on the levels of cytokine protein expression in mouse ovary.

| Protein expression (ng/g tissue) | Nano-TiO2 (mg/kg BW) |
|---------------------------------|----------------------|
|                                 | 0        | 2.5       | 5         | 10        |
| IGF-1                           | 117.62±5.88a | 92.29±4.61b | 74.19±3.71c | 61.24±3.06d |
| IGFBP-2                         | 34.38±1.72a | 40.41±2.02b | 47.30±2.36c | 61.61±3.08d |
| EGF                             | 41.22±2.06a | 65.38±3.27b | 82.85±4.14c | 102.42±5.12d |
| TNF-α                           | 20.00±1.00a | 31.03±1.75b | 50.14±2.51c | 71.49±3.57d |
| tPA                             | 11.32±0.56a | 18.50±0.93b | 26.44±1.32c | 34.22±1.71d |
| LHR                             | 39.53±1.98a | 29.73±1.49b | 21.76±1.09c | 15.21±0.76d |
| INHa                            | 82.90±4.14a | 66.77±3.34b | 52.18±2.61c | 38.85±1.94d |
| IL-1β                           | 22.98±1.15a | 30.52±1.53b | 38.20±1.91c | 47.38±2.37d |
| IL-6                            | 10.99±0.55a | 19.19±0.96b | 31.59±1.58c | 42.04±2.10d |
| Fas                             | 43.37±2.17a | 64.47±3.22b | 89.99±4.50c | 125.98±6.30d |
| Fasl                            | 31.45±1.57a | 42.24±2.11b | 54.05±2.70c | 67.11±3.35d |
| GDF-9                           | 85.37±4.27a | 66.70±3.33b | 47.85±2.39c | 31.19±1.56d |

Different letters indicate significant differences between groups (p<0.05). Values represent means ± SE (N = 5). doi:10.1371/journal.pone.0059378.t005
Statistical Analysis

All results are expressed as means ± standard error (SE). The significant differences were examined by unpaired Student’s t-test using SPSS 19 software (USA). A p-value <0.05 was considered as statistically significant.

Results

Body Weight, Relative Weight of Ovary and Titanium Accumulation

The body weight, relative weight of ovary, titanium accumulation in the mouse ovary caused by exposure to nano-TiO₂ for 90 consecutive days are exhibited in Figs. 1, 2, respectively. It can be seen that with increased nano-TiO₂ doses, the body weight (Fig. 1 a) and relative weight of ovary (Fig. 1 b) were significantly decreased (P<0.05 or P<0.01), while titanium contents in the ovary were significantly increased (Fig. 2, P<0.01).

Hematological and Biochemical Parameters

Results of hematological detection indicate that WBC, LYMPH, NEUT, RBC, and HGB in the nano-TiO₂-treated female mice were significantly reduced with increased exposure doses (P<0.05 or p<0.01, Table 1). It can be also seen from Table 1 that nano-TiO₂ exposure significantly increased the activities of ALT, AST, ALP, LDH, and the levels of Cr, and reduced UA and BUN in sera (P<0.05 or p<0.01, Table 1), respectively.

Reproduction

With increased nano-TiO₂ exposed doses, decreased mating rate, pregnancy rate, number of newborns and weight of neonates of mice were significantly observed in Table 2 (P<0.05). In addition, nano-TiO₂ led to reduction of survival of young mice at 28th day after birth (P<0.05).

Histopathological Evaluation

Figure 3 presents histological changes in ovary. Normal development of primary follicle and secondary follicle from the control ovary was observed (Fig. 3 a, b). In the nano-TiO₂-treated groups, however, a large of atretic follicles, severe inflammatory cell infiltration, and necrosis were observed (Fig. 3 d-e), respectively. In addition, we also observed significant black agglomerates in the ovary samples exposed to 10 mg/kg of nano-TiO₂ (Fig. 3e). Confocal Raman microscopy further showed a characteristic nano-TiO₂ peak in the black agglomerate (148 cm⁻¹), which further confirmed the deposition of nano-TiO₂ in the ovary (see spectrum B in the Raman inset in Fig. 3f). The results also suggest that exposure to nano-TiO₂ dose-dependently deposited in the ovary, thus severely resulted in the ovarian injuries.

Cytokines Expression

To confirm molecular mechanisms of nano-TiO₂ on the ovary injury, the expression of the inflammation-related genes or follicular atresia-related genes and their proteins in the ovary were examined (Tables 4, 5). It can be observed that exposure to nano-TiO₂ resulted in significant increases of IGFBP-2, EGF, TNF-α, IPA, IL-1β, IL-6, Fas, and FasL expression, while obviously decreased IGF-1, LHR, INH-α, and GDF-9 expression in the ovary compared with the control (P<0.05 or 0.01), which

Sex Hormone Levels

As shown in Table 3, serum E2 levels were gradually increased (P<0.05), contrary, P4, LH, FSH, and T were significantly decreased in female mice (P<0.05) with increased nano-TiO₂ exposed doses. However, no significant differences between serum PRL and SHBG levels in the nano-TiO₂ exposed female mice and those of control were observed (P>0.05).
are consistent with the trends of fertility reduction and ovary injury.

Discussion

To confirm effects of reproductive system of female mice caused by 90 consecutive days exposure to low dose of nano-TiO₂, the present study was designed to investigate the changes of ovarian morphology, fertility, hormone levels and expression of relevant genes and their proteins in mouse ovary. Our findings indicated that the oral nano-TiO₂ with 2.5, 5, and 10 mg/kg BW doses for 90 consecutive days led to atretic follicle increases, severe inflammatory response and necrosis in the ovary (Fig. 3). Increased atretic follicles were closely associated with premature ovarian failure following nano-TiO₂-induced toxicity. Furthermore, the present study also suggested that exposure to nano-TiO₂ reduced mating rate, pregnancy rate, number of newborns and growth of neonates (Table 2) and altered sex hormone levels, including a significant increase of E2 concentration and great decreases of P₄, LH, FSH, and T concentrations in the sera (Table 3). Decreased mating capacity of female mice following exposure to nano-TiO₂ may be associated with imbalance of sex hormone levels. Folicular atresia is not only the break-down of the ovarian follicles, but also is hormonally controlled apoptosis. Therefore, FSH and LH reduction by exposure to nano-TiO₂ resulted in the follicular atresia in mouse ovary. Theoretically, increased effect on E2 levels may be due to the activation of cytochrome P450 aromatase, which converts T into E2 [25]. Elevated E2 and decreased T caused by nano-TiO₂ may be related to activate cytochrome P450 aromatase, promoting transformation from T to E2, but it needs to study in future. Taken together, increased atretic follicles and decreased fertility were due to reduction of FSH, P₄, LH, and T levels in the nano-TiO₂ treated female mice. In addition, T has wide ranging roles in ovarian function, including granulosa cells, theca cells, oocytes, and interstitial cells, because T enhances IGF-I and IGF-I receptor mRNAs in primates [26]. Activins, inhibins, GDF-9, and TGF-β of growth and differentiation factors can influence follicular development [27]. Therefore, the ovarian injuries and changes of sex hormone levels in female mice may be due to nano-TiO₂ alter the expression of relevant genes and their proteins in the ovary. To identify the mechanisms of multiple cytokines working together caused by nano-TiO₂, mRNA and protein expression of IGFBP-2, EGF, TNF-α, tPA, LHR, INH-α, IL-1β, IL-6, Fas, Fasl and GDF-9 from ovary were examined. The assays indicated that the levels of these cytokines were significantly altered (Tables 4, 5). The main results are discussed below.

In mammals, most of ovarian follicles undergo atresia during development, and only a few differentiate to mature finally. Apoptosis occurs in ovarian follicular granulosa cell of majority of animals during follicular atresia, which is the cause of atretic initiation and progression [28]. Therefore apoptosis is suggested to be regulated by various factors and atretogenic factors [29]. Intrafollicular IGF-1 plays a critical role in the enhanced response (estradiol production) of the future dominant follicle to the small rise in FSH that initiates the follicular wave. The binding of IGF to its receptors is strongly modulated by a family of six high-affinity IGF-binding proteins (IGFBPs). IGFBP-2 inhibits IGF effect on gonadotropin-induced follicular growth and differentiation. EGF is involved in regulation of ovarian cell proliferation and differentiation. So, EGF, IGF-1 and gonadotropins are determined to be survival factors, but IGFBPs are atretogenic factors [30]. Luo and Zhu demonstrated that FSH concentration, and IGF-1 expression were decreased, contrary IGFBP-2 and EGF expression were increased in process of induced follicular atresia of female rat [31]. Our data indicated that the levels of IGFBP-2 and EGF expression were significantly elevated, whereas IGF-1 expression was greatly inhibited in the nano-TiO₂-treated ovary (Tables 4, 5), suggesting that follicular atresia caused by nano-TiO₂ (Fig. 3) may be involved in increased IGFBP-2 and EGF, and decreased IGF-1 in the ovary.

Granulosa cells produce estrogen which can synergistically promote FSH-induced self-production of LHR and aromatase activity in cells. In contrast to estrogen, androgen is capable of inducing follicular atresia. Inhibin has also been demonstrated to be an atretic factor. To form bioactive dimers linked by disulfate bonds, one α subunit combines with one of the two β subunits will form two types of inhibin, and combination of two types of β subunits will form three types of actin. Inhibin and actin are involved in coordination between gonadotropins or other factors in regulation of follicular selection, development and atresia [32]. tPA is responsible for the cumulus cell expansion, dispersion and oocyte maturation. Yan et al indicated that tPA expression was significantly increased, but LHR and inhibin subunits were not expressed in the follicle undergoing atresia in rats [33]. Our findings also showed that nano-TiO₂ greatly promoted tPA expression, but inhibited LHR and INH-α expression in the ovary (Tables 4, 5), which may lead to follicular atresia in female mice (Fig. 3).

It had been suggested that Fas can be not only found in murine oocytes obtained from atretic follicles [34], but also in granulosa cells of follicles undergoing atresia in the ovary [35]. FasL was also demonstrated to express in the granulosa cells and antral atretic follicles from rat [35], and in granulosa cells of atretic follicles from mouse ovary [36]. Fas and FasL expression in the ovary raise the possibility of Fas-FasL interaction as a mediator of apoptosis during follicular atresia [37]. In the present study, the significant increases of Fas and Fasl expression in the ovary are observed following nano-TiO₂ induced toxicity (Tables 4, 5), which may conduct to atretic follicle formation of female mice (Fig. 3).

As we know, TNF-α induces apoptosis in several cellular models, and is produced locally in the rat, ovine ovarian granulosa cells and oocytes, and may act as a paracrine regulatory factor [38]. IL-1β, IL-6 and androgens produced locally in the ovary are also demonstrated to induce follicular atresia [39]. GDF-9 is a growth factor secreted by oocytes in growing ovarian follicles, which is essential for normal follicular development [27]. GDF-9-deficient female mice are infertile because of an early block in folliculogenesesis at the type 3b primary follicle stage [40]. Increased levels of TNF-α, IL-1β and IL-6 expressions are also demonstrated to be closely associated with inflammation generation in human and animals. The previous studies suggested that expressions of TNF-α, IL-1β and IL-6 were significantly elevated in the nano-TiO₂ exposed lung [41–44], liver [45–49], kidney [5,8,50], and spleen [5,19,51–53] of animals. The present findings showed that nano-TiO₂ exposure markedly promoted expression of TNF-α, IL-1β and IL-6, but significantly inhibited GDF-9 expression in mouse ovary (Tables 4, 5), which resulted in inflammation and follicular atresia in mouse ovary (Fig. 3). A scheme that links the nano-TiO₂ and the changes of IGFBP-2, IGF-1, EGF, tPA, LHR, INH-α, Fas, Fasl, GDF-9, TNF-α, IL-1β, and IL-6 is depicted in Figure 4.

Conclusion

In the present study, we demonstrate that the exposure to nano-TiO₂ could result in the fertility reduction, ovarian inflammation and follicular atresia in a dose-dependent manner, which were
closely related to reduction of immunity, biochemical dysfunction, imbalance of sex hormones, and changes of IGFBP-2, IGF-1, EGF, tPA, LHR, INH-b, Fas, Fasl, TNF-α, IL-1β, IL-6, and GDF-9 expressions in the ovary. Therefore, our findings suggested the need for great caution to handle the nanomaterials for workers and consumers.

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Author Contributions

Conceived and designed the experiments: FH XZ YZ GG XS BL. Performed the experiments: FH XZ YZ GG XS. Analyzed the data: FH XZ YZ GG XS BL. Contributed reagents/materials/analysis tools: BL SG LS QS JC ZC RH LW. Wrote the paper: FH XZ.

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