

Nanosized titanium dioxide-induced premature ovarian failure is associated with abnormalities in serum parameters in female mice

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Background: Exposure to titanium dioxide nanoparticles (TiO₂ NPs) that are widely used in food, medicine, sunscreen products and cosmetics is reported to cause ovarian damage and lower fertility in animals. However, the potential effects of TiO₂ NPs application on premature ovarian failure (POF) have rarely been evaluated to date.

Methods: In this study, female mice were continuously exposed to TiO₂ NPs at doses of 2.5, 5 or 10 mg/kg via gavage instillation for 30 days, and investigated the serum hormones and autoimmunity markers associated with POF.

Results: Exposure to TiO₂ NPs resulted in POF, reductions in the levels of estradiol, progesterone and inhibin B and increases in luteinizing hormone, follicle-stimulating hormone, follicle-stimulating hormone/luteinizing hormone ratio, anti-Müllerian hormone, thyroid-stimulating hormone, free triiodothyronine, free tetraiodothyronine, anti-nuclear antibody and anti-thyroid peroxidase antibody levels in serum.

Conclusion: Exposure to TiO₂ NPs induced POF triggered by alterations in hormones and autoimmunity markers. Our findings highlight the necessity for significant caution in handling and usage of TiO₂ NPs by female consumers.

Keywords: titanium dioxide nanoparticles, mice, premature ovarian failure, serum hormone levels, autoimmunity levels

Introduction

Titanium dioxide nanoparticles (TiO₂ NPs) are increasingly used as non-toxic, chemically inert and biocompatible pigment products or photocatalysts owing to their high surface area to particle mass ratio and high reactivity and are commercially manufactured for use in industry, comprehensive ecological improvement, food, medical, diagnostic and cosmetic applications in preference to bulk TiO₂.^{1,2} However, the increased use of TiO₂ NPs in various applications is significantly associated with potential human toxicity.

Recent published data support the toxicity of NPs in the reproductive system.^{3,4} For example, Yoshida et al⁵ showed that exposure to black carbon NPs leads to adverse effects on male reproductive function in mice, characterized by increased serum testosterone and partial vacuolation of seminiferous tubules. Bai et al⁶ observed that exposure to water-soluble multiwalled carbon nanotubes leads to oxidative stress in mouse testis. Amorphous nanosilica particles can cross the blood–testis barrier and nuclear membranes of spermatocytes in mouse testis.⁷ In particular, it has been reported that TiO₂ NPs exposure damages follicles, reduces follicular survival and inhibits development and oocyte maturation of rat preantral follicles.⁸ Di Virgilio et al⁹ demonstrated genotoxicity

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and cytotoxicity following TiO₂ or aluminum oxide (Al₂O₃) NPs exposure in Chinese hamster ovary (CHO-K1) cells. Moreover, TiO₂ NPs exposure is associated with reduced sperm number and motility or elevated abnormal sperm number and germ cell apoptosis in mouse testis.¹⁰ Previous studies indicate that TiO₂ NPs cross the blood–testis barrier to reach the testis and accumulate within, causing testicular lesions, sperm malformations and alterations in hormone levels and gene expression profiles in male mice.^{11–14} Importantly, we additionally showed that TiO₂ NPs can translocate to and accumulate in the ovary, leading to reductions in body weight (BW), ovarian indices and fertility, inflammation, follicular atresia and necrosis as well as alterations in gene expression in female mice.^{15–17} Liu et al¹⁸ suggested that nano-zinc oxide (ZnO) exposure affects ovarian development through regulating the number of neuroendocrine cells in the ovary and expression of neuronal factors. This study was conducted to examine the hypothesis that TiO₂ NP-mediated suppression of fertility is also associated with premature ovarian failure (POF) in female mice.

POF is a primary ovarian defect characterized by absent menarche (primary amenorrhea) or premature depletion of ovarian follicles before the age of 40 years. POF is considered an immunological disorder that is potentially associated with several factors, including autoimmune disease,¹⁹ ovarian lymphocytic infiltration,²⁰ presence of anti-ovarian antibodies²¹ and post-immunotherapy reversibility,²² as well as toxics and drugs in women.²³ The defect is biochemically typified by low levels of gonadal hormones (estrogens and inhibins) and high levels of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH])²⁴ and often associated with non-organ-specific autoantibodies, such as anti-nuclear antibodies (ANAs)²⁵ and anti-thyroid peroxidase antibodies (TPO-Abs),²⁶ indicative of altered autoimmunity in these patients. However, the issue of whether TiO₂ NPs exposure triggers alterations in autoimmunity remains unclear at present.

Here, female mice were continuously exposed to TiO₂ NPs at doses of 2.5, 5 or 10 mg/kg via gavage instillation for 30 days. Changes in serum parameters were assessed with the aim of establishing whether TiO₂ NPs exposure is linked to POF and the underlying mechanisms in mice.

Methods

Chemicals

Nanoscale TiO₂ (anatase, TiO₂ content > 99.5%) was kindly provided by Professor Yang Ping (Chemical College of Soochow University, Suzhou, China). TiO₂ NPs were characterized according to the procedures in the previous studies.^{27,28}

The average particle size of TiO₂ NPs suspended in 0.5% (w/v) hydroxypropyl methylcellulose (HPMC) solvent after 24 h incubation ranged from 5 to 6 nm based on X-ray diffraction patterns (Mercury CCD Corporation, Japan) and Tecnai G220 transmission electron microscopy (TEM) (FEI Corporation, USA) data, and hydrodynamic diameters of TiO₂ NPs in suspension ranged from 208 to 330 nm (mainly ~294 nm) as determined by dynamic light scattering (Brookhaven Instruments Corporation, USA). The surface area of TiO₂ NPs was determined to be 174.8 m²/g based on Brunauer–Emmett–Teller adsorption measurements on a Micromeritics ASAP 2020M+ C instrument (Micromeritics Corporation, USA) and ζ potential was determined to be 9.28 mV using the Zeta PALS+BI-90 Plus analyzer (Brookhaven Instruments Corp.).^{27,28}

Ethics approval

All animal experiments were conducted during the light phase and approved by the Animal Experimental Committee of Soochow University (ethical approval number: 2111270). Procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Animals and treatment

Female specific pathogen-free (SPF) mice aged 4 weeks were used for this study. In total, 200 SPF female and 40 SPF male mice (18 ± 2 g) were purchased from the Animal Center of Soochow University (China). All mice were housed in stainless steel cages in a ventilated animal room. The room temperature of the housing facility was maintained at 24°C ± 2°C and a relative humidity of 60% ± 10% under a 12-h light/dark cycle. Distilled water and sterilized food for mice were available ad libitum. After acclimatization to laboratory conditions for 1 week, mice were fasted for 3 h prior to TiO₂ NPs administration.

TiO₂ NP powder was dispersed onto the surface of 0.5% w/v HPMC, and the suspension containing TiO₂ NPs was treated ultrasonically for 30 min and mechanically vibrated for 5 min. Mice were randomly divided into four groups (n = 50 each), including a control group treated with 0.5% w/v HPMC and three experimental groups administered 2.5, 5 or 10 mg/kg of TiO₂ NPs. Next, mice were weighed, and the volume of fresh TiO₂ NPs suspensions required for each animal was calculated. Fresh TiO₂ NPs suspensions were administered to mice via a gavage needle every day for 30 days. For appropriate dose selection, we referred to a report of the World Health Organization in 1969, according to which LD₅₀ of

TiO₂ for rats is >12 g/kg BW after oral administration. We additionally referred to previous studies,^{15,16} which showed that the National Institute for Occupational Safety and Health (NIOSH) recommends exposure limits of 2–3 mg/m³ for fine TiO₂ and 0.3–1 mg/m³ for ultrafine (including nanoscale engineered) TiO₂.²⁹ The doses of TiO₂ NPs selected for the study (2.5, 5 and 10 mg/kg) were equivalent to ~0.15–0.7 g exposure for humans with 60–70 kg BW, representative of relatively safe doses.

Mating of animals

To evaluate the effects of TiO₂ NPs on fertility, we treated three groups of female mice (10 per mating group). In total, 10 male and 10 control or treated female mice from each group were placed in a common cage for mating after 30 days of TiO₂ NPs administration. Mating success was confirmed based on the formation of a white suppository at the mouth of the vagina tracked via vaginal smearing under an optical microscope (U-III Multi-point Sensor System; Nikon Corporation, Tokyo, Japan). Mating success was assessed by counting the number of pregnant mice.

BW and ovary weight

After 30 days, all mice were weighed. Blood samples were collected from the eye vein following rapid removal of the eyeball. After collecting blood, all mice were sacrificed via cervical dislocation, and ovaries of all animals were quickly removed, weighed, placed on ice and dissected and frozen at –80°C (with the exception of 40 ovaries that were used for histopathological examination). Serum was collected by centrifuging blood at 1,200× *g* for 10 min.

Serum parameter analysis

Plasma concentrations of anti-Müllerian hormone (AMH), inhibin B, estradiol (E2), progesterone (P), LH, FSH, prolactin (PRL), thyroid-stimulating hormone (TSH), free triiodothyronine (fT3) and free tetraiodothyronine (fT4) were detected with the aid of commercial kits (Bühlmann Laboratories AG, Switzerland). The levels of ANAs and anti-TPO-Abs were measured using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Inc., Minneapolis, MN, USA). All biochemical assays were performed using an automated clinical chemistry analyzer (Type 7170A; Hitachi Ltd., Tokyo, Japan).

Histopathological evaluation of ovary

All histopathological examinations were performed using standard laboratory procedures. Five sets of ovarian tissues from mice were embedded in paraffin blocks, sliced

into 5 µm sections and placed on separate glass slides (five slices from each kidney). After hematoxylin–eosin staining, sections were evaluated by a histopathologist blinded to the treatments under an optical microscope (U-III Multi-point Sensor System; Nikon Corporation).

Statistical analysis

Data are presented as mean ± SD. Statistical analyses were performed using SPSS 19.0 software (IBM Corporation, Armonk, NY, USA) and compared via one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test. Differences were considered statistically significant at *p*-values < 0.05.

Results

Body and ovarian weights

Figure 1 depicts the effects of TiO₂ NPs exposure on net increase in body and ovarian weights. TiO₂ NPs treatment induced a significant reduction in the net increase in BW (–7.81% to –35.87%) and ovarian weight (–16.32% to –61.44%), compared with the control group (*p* < 0.05). The decrease in ovarian weight caused by TiO₂ NPs is suggestive of ovarian atrophy and may be related to tissue injury or POF, as confirmed by histopathological observation of mouse ovaries.

Reproduction

In response to increasing TiO₂ NP doses, the mating and pregnancy rates of mice were significantly decreased by 14%–31% and 17%–40%, respectively, as shown in Figure 2 (*p* < 0.05). The decreased pregnancy rate may be associated with POF triggered by TiO₂ NPs exposure.

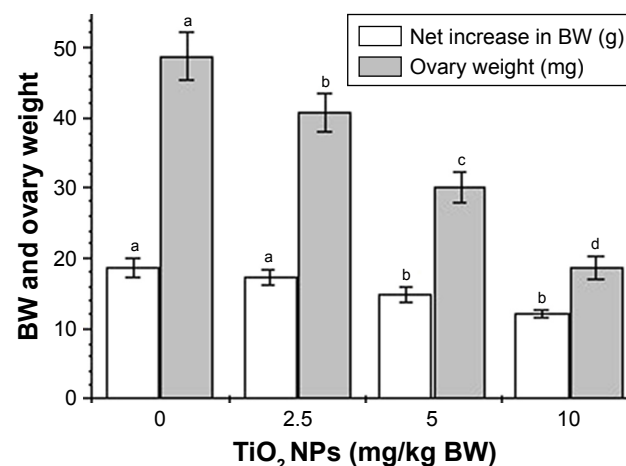


Figure 1 Effects of TiO₂ NPs on net increase in BW and ovarian weight after gavage administration for 30 days.

Notes: Different letters within the same parameter indicate significant differences between groups (*p* < 0.05). Values represent mean ± SD (*n* = 10).

Abbreviations: BW, body weight; TiO₂ NPs, titanium dioxide nanoparticles.

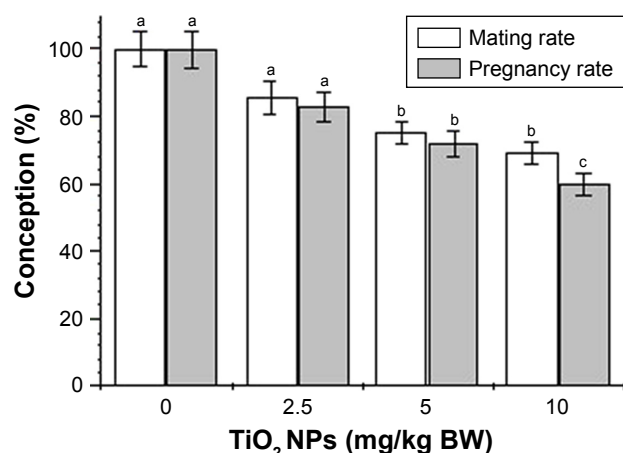


Figure 2 Effect of TiO₂ NPs on conception in female mice after gavage administration for 30 days.

Notes: Different letters within the same parameter indicate significant differences between groups ($p < 0.05$). Values represent mean \pm SD ($n = 10$).

Abbreviations: BW, body weight; TiO₂ NPs, titanium dioxide nanoparticles.

Histopathological evaluation

The histological changes in mouse ovary are presented in Figure 3. Normal development of primary follicles from control ovary was observed (Figure 3). However, samples

from the 2.5 mg/kg TiO₂ NP-exposed group showed not only atrophic secondary follicles but also a large number of primary atretic follicles and disposed disorder or apoptosis of granule cells. Similarly, the 5 mg/kg TiO₂ NP-exposed group exhibited a large increase in primary atretic follicle number and disposed disorder or apoptosis of granule cells, and the 10 mg/kg TiO₂ NP-exposed group showed severe inflammatory cell infiltration along with increased primary atretic follicle content and disposed disorder or apoptosis of granule cells in the ovary (Figure 3). Our collective results support the theory that TiO₂ NPs exposure induces POF in female mice.

Serum parameters

As shown in Table 1, serum FSH, LH, FSH/LH ratio, AMH, PRL, TSH, fT3 and fT4 levels were significantly increased, whereas inhibin B, E2 and P levels were markedly reduced with increasing TiO₂ NP doses ($p < 0.05$). Notably, the levels of serum ANAs and TPO-Abs involved in the autoimmune response were gradually increased with exposure to increasing concentrations of TiO₂ NPs in female mice (Table 1, $p < 0.05$).

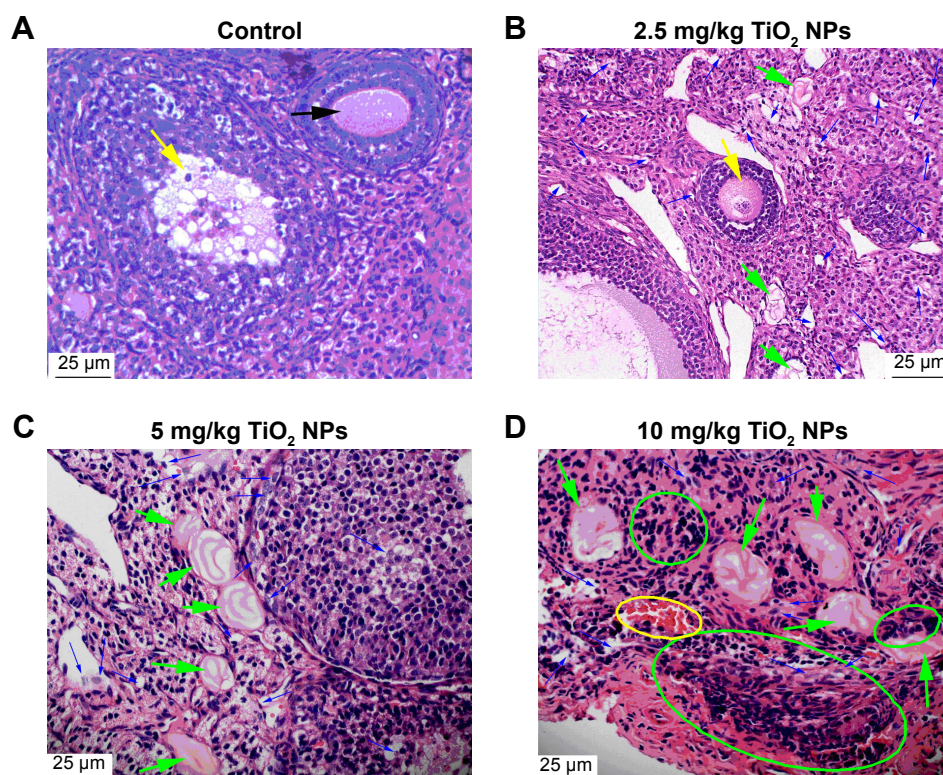


Figure 3 Histopathological examination of ovary of mice following gavage administration of TiO₂ NPs for 30 days.

Notes: (A) Control groups (unexposed mice) present normal development of primary follicles (black arrow) and secondary follicles (yellow arrow); (B) the 2.5 mg/kg TiO₂ NP-exposed group shows atrophic secondary follicle (yellow arrow), primary follicle atresia (green arrow) and apoptosis of granule cells (blue arrow); (C) the 5 mg/kg TiO₂ NP-exposed group shows large primary follicle atresia (green arrow) and granule cell apoptosis (blue arrow), and (D) the 10 mg/kg TiO₂ NP-exposed group shows severe inflammatory cell infiltration (green circle), congestion (yellow circle), significant primary follicle atresia (green arrow) and disposed disorder or apoptosis of granule cells (blue arrow).

Abbreviation: TiO₂ NPs, titanium dioxide nanoparticles.

Table 1 Changes in serum parameters involved in autoimmune response in female mice caused by gavage administration of TiO₂ NPs for 30 days

Serum parameters	TiO ₂ NPs (mg/kg BW)			
	Control	2.5	5	10
FSH (IU/L)	2.28 ± 0.31 ^a	3.98 ± 0.45 ^b	4.05 ± 0.41 ^b	6.79 ± 0.78 ^c
LH (IU/L)	2.35 ± 0.26 ^a	3.31 ± 0.36 ^b	3.39 ± 0.39 ^b	4.90 ± 0.58 ^c
Ratio of FSH/LH	0.97 ± 0.14 ^a	1.10 ± 0.17 ^a	1.41 ± 0.18 ^b	1.49 ± 0.21 ^b
AMH (pmol/L)	10.79 ± 1.25 ^a	11.21 ± 1.35 ^a	14.86 ± 1.61 ^b	19.09 ± 2.08 ^c
Inhibin B (pg/mL)	36.59 ± 3.27 ^a	28.82 ± 3.12 ^b	27.66 ± 2.49 ^b	15.03 ± 1.15 ^c
E2 (pmol/L)	152.16 ± 12.31 ^a	145.29 ± 16.74 ^a	120.51 ± 14.31 ^b	111.36 ± 9.91 ^b
P (nmol/L)	30.29 ± 3.02 ^a	25.25 ± 2.76 ^b	23.67 ± 2.43 ^b	11.72 ± 1.29 ^c
PRL (μg/L)	0.87 ± 0.11 ^a	1.02 ± 0.16 ^a	1.75 ± 0.22 ^b	1.89 ± 0.25 ^b
TSH (pg/mL)	53.4 ± 5.67 ^a	40.9 ± 4.25 ^b	38.1 ± 3.92 ^b	34.8 ± 3.61 ^b
ft3 (pmol/L)	18.71 ± 1.94 ^a	24.75 ± 2.88 ^b	27.96 ± 2.93 ^b	30.77 ± 3.74 ^b
ft4 (μg/L)	97.45 ± 9.87 ^a	131.23 ± 15.13 ^b	199.10 ± 20.62 ^c	210.62 ± 23.51 ^c
ANA (ng/L)	25.25 ± 2.71 ^a	29.44 ± 3.31 ^a	36.87 ± 3.82 ^b	38.56 ± 4.09 ^c
TPO-Ab (mU/L)	149.05 ± 15.48 ^a	160.53 ± 19.15 ^a	213.08 ± 22.63 ^b	226.09 ± 24.12 ^c

Notes: Different letters within the same parameter indicate significant differences between groups ($p < 0.05$). Values represent mean ± SD ($n = 5$).

Abbreviations: AMH, anti-Müllerian hormone; ANA, anti-nuclear antibody; BW, body weight; FSH, follicle-stimulating hormone; ft3, free triiodothyronine; ft4, free tetraiodothyronine; LH, luteinizing hormone; PRL, prolactin; TiO₂ NPs, titanium dioxide nanoparticles; TPO-Ab, thyroid peroxidase antibody; TSH, thyroid-stimulating hormone.

Discussion

To determine the potential effects of TiO₂ NPs exposure on POF of female mice, we focused on histopathological changes in ovary, fertility, and hormone and anti-ovarian antibody levels in serum in the present study. Exposure to 2.5, 5 and 10 mg/kg BW TiO₂ NPs for 30 days led to significant reductions in body and ovarian weights (Figure 1) and mating and pregnancy rates (Figure 2), severe inflammation, and follicle atresia as well as apoptosis of granule cells in the ovary (Figure 3), consistent with our previous findings.^{15–17} These characteristics contribute to POF following TiO₂ NP-induced toxicity, which may be attributable to TiO₂ NP accumulation in ovary¹⁵ or biokinetics in vivo after oral application.^{30–32} Furthermore, TiO₂ NPs induced a decrease in inhibin B, E2 and P levels and an increase in FSH, LH, FSH/LH ratio, AMH, PRL, TSH, ft3, ft4, ANA and TPO-Ab levels in mouse sera (Table 1), as discussed subsequently.

Gonadotrophin-releasing hormone (GnRH), FSH and LH regulate ovarian function through a sensitive feedback system. Elevated serum FSH in the early follicular phase, a consequence of reduced ovarian function, is predictive of impaired pregnancy outcomes after infertility treatment,³³ and a combination of low FSH and high LH levels can be utilized to predict pregnancy outcomes.³⁴ The FSH:LH ratio has been successfully applied as a predictor of pregnancy outcomes in infertile women.²³ Elevated serum FSH, LH and FSH:LH ratio observed in the present study are clearly suggestive of impaired ovarian function due to TiO₂ NPs exposure in mice.

AMH is a dimeric glycoprotein generated by granulosa cells from pre-antral and antral follicles that mainly function to inhibit follicular development from the primordial to primary follicular stage. During the menstrual cycle, serum AMH levels are relatively stable.³⁵ Additionally, AMH is reported to be a better predictor of pregnancy outcome than other hormonal parameters.^{36,37} Inhibin B, produced by granulosa cells in antral follicles, has been proposed as an effective marker of follicular growth. Low serum inhibin B levels are associated with elevated FSH levels, leading to reduction in oocyte quality and fertility.³⁵ Therefore, increased AMH and decreased inhibin B levels in serum induced by TiO₂ NPs exposure may be closely linked to POF generation in mice.

Estrogen plays important roles in oocyte maturation, embryo quality and fertilization.^{38,39} The most important estrogen is E2 in serum,¹⁰ which, in combination with FSH and age, is predictive of pregnancy outcomes after in vitro fertilization treatment.⁴⁰ Progesterone is the most important hormone for endometrial development, implantation and maintenance of pregnancy,⁴¹ and its activity during the luteal phase can increase pregnancy as well as live birth rates after infertility treatment.^{42,43} In the present study, lower serum E2 and P levels were associated with reduced mating and pregnancy rates in TiO₂ NP-exposed female mice.

Thyroid hormones influence ovarian function directly and indirectly through elevation of PRL levels and alterations in GnRH secretion. Ovarian insufficiencies often occur in women with thyroid dysfunction.⁴⁴ Increased serum PRL levels are related to menstrual disorders because

of their restraining effect on pulsatile GnRH secretion as well as inhibition of FSH and LH release.⁴⁵ Importantly, hyperprolactinemia is suggested to be associated with ovulatory dysfunction,⁴⁶ and both hypothyroidism and hyperprolactinemia are linked to infertility. However, the specific association between pregnancy outcome and serum TSH levels is a subject of controversy at present. For example, Cramer and co-workers suggested that infertile women with increased serum TSH levels have lower pregnancy rates than those with normal TSH levels.⁴⁷ Arojoki et al reported low serum TSH levels in women with infrequent menstruation and female infertility and high serum TSH levels in women with unexplained infertility and ovulatory dysfunction.⁴⁸ In our study, PRL, TSH, ft3 and ft4 levels were significantly increased in TiO₂ NP-exposed female mice, suggesting that these factors are correlated with ovulatory dysfunction and low fertility due to POF.

Earlier reports indicate that 10.5% infertile women are positive for antithyroid antibodies (ATAs),⁴⁸ and TPO-Abs are associated with the TSH levels.⁴⁹ For instance, TPO-Ab-positive women have significantly elevated TSH levels, whereas a number of women with normal TSH levels are positive for TPO-Abs.^{50,51} The presence of ATAs in euthyroid women may be related to fertility problems, including increased abortion rate and incidence of infertility.²⁴ In addition, the incidence of ANAs in patients with POF was demonstrated to be higher than that in age-matched hypogonadotropic patients with secondary amenorrhea, refuting a potential role of hypoeestrogenism in the formation of ANAs.²⁴ Data from this study collectively suggest that exposure to TiO₂ NPs induces significant elevation of serum ANA and TPO-Ab levels in female mice, which may also be associated with altered autoimmunity, leading to POF.

Conclusion

Exposure of female mice to TiO₂ NPs results in POF characterized by a reduction in reproductive ability along with decreased levels of inhibin B, E2 and P and increased FSH, LH, FSH/LH ratio, AMH, PRL, TSH, ft3, ft4, ANA and TPO-Ab levels in serum. Our results support the crucial need to improve public awareness of the hazards of oral exposure to TiO₂ NPs for female consumers.

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Disclosure

The authors report no conflicts of interest in this work.

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