

Gestational exposure to titanium dioxide nanoparticles impairs the placentation through dysregulation of vascularization, proliferation and apoptosis in mice

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Background: Titanium dioxide nanoparticles (TiO₂ NPs) have recently found applications in a wide variety of consumer goods. TiO₂ NPs exposure significantly increases fetal deformities and mortality. However, the potential toxicity of TiO₂ NPs on the growth and development of placenta has been rarely studied during mice pregnancy.

Purpose: The objective of this study was to investigate the effects of maternal exposure of TiO₂ NPs on the placentation.

Methods: Mice were administered TiO₂ NPs by gavage at 0, 1 and 10 mg/kg/day from gestational day (GD) 1 to GD 13. Uteri and placentas from these mice were collected and counted the numbers of implanted and resorbed embryo and measured the placental weight on GD 13. Placental morphometry was observed by hematoxylin and eosin staining. The levels of *Hand1*, *Esx1*, *Eomes*, *Hand2*, *Ascl2* and *Fra1* mRNA were assessed by qRT-PCR. Uterine NK (uNK) cells were detected by using DBA lectin. Laminin immunohistochemical staining was to identify fetal vessels. Western blotting and transmission electron micrograph (TEM) were used to assess the apoptosis of placenta.

Results: No treatment-related difference was observed in the numbers of implanted and resorbed embryos and weight of placenta between the groups. However, 1 mg/kg/day TiO₂ NPs treatment significantly reduced the ratio of placenta/body weight on GD 13. The proportion of spongiotrophoblast in the 10 mg/kg/day dose group became higher than that in the control group, yet that of labyrinth was significantly lower in 10 mg/kg/day mice. The expression levels of *Hand1*, *Esx1*, *Eomes*, *Hand2*, *Ascl2* and *Fra1* mRNA markedly decreased in TiO₂ NP treated placentas. Furthermore, TiO₂ NPs treatment impaired the formation of intricate networks of fetal vessels and reduced the number of uNK cells, and inhibited proliferation and induced apoptosis of placenta by nuclear pyknosis, the activation of caspase-3 and upregulation of Bax protein and downregulation of Bcl-2 protein on GD 13.

Conclusion: Gestational exposure to TiO₂ NPs significantly impairs the growth and development of placenta in mice, with a mechanism that seems to be involved in the dysregulation of vascularization, proliferation and apoptosis. Therefore, our results suggested the need for great caution while handling of the nanomaterials by workers and specially pregnant consumers.

Keywords: nanoparticles, titanium dioxide, placenta, proliferation, apoptosis, vascularization

Introduction

Nanoparticles (NPs) are materials with at least one dimension ≤100 nm, and this large surface-to-volume ratio results in unique physicochemical characteristics,

including high reactivity, lower melting temperature, color changes and greater solar radiation absorption, compared to their corresponding bulk materials.¹ Titanium dioxide nanoparticles (TiO₂ NPs) have unusual optical properties and therefore are widely used in various applications, such as sunscreens, food production, dietary supplements, food packaging materials, medicine, toothpastes, cosmetics and waste water treatment.^{2,3} Despite the rapid introduction of nanomaterials to markets and extensive investigations of their toxicological properties, their safety has not yet been well established.

TiO₂ was previously considered as innocuous in particle toxicology, while recent studies on TiO₂ NPs have been reported to cause toxicity in the livers, kidneys, spleens, brains and hearts of animals.^{4–8} For example, TiO₂ NPs exposure induced hydropic degeneration, cloudy swelling and apoptosis of rat hepatocytes, and infiltration of inflammatory cells in portal vein.⁹ Intra-gastric administrations of TiO₂ NPs were accumulated in the kidney, resulting in nephric inflammation, cell necrosis and congestion of mesenchyme blood vessels.⁶ Furthermore, a potentially toxic role of TiO₂ NPs on the reproductive system also raises great concern. TiO₂ NPs exposure was associated with harmful effects on the reproductive system. Some studies showed that TiO₂ NPs could cross the blood–testis barrier to reach the testis and resulted in the decrease of sperm numbers and motility, sperm malformations, alterations in serum sex hormone levels and gene expression in the testes of mice.^{10,11} Komatsu et al demonstrated that TiO₂ NPs were taken up by mouse Leydig TM3 cells *in vitro* and inhibited the proliferation of Leydig cells and increased the expression of heme oxygenase-1 (a sensitive marker for oxidative stress) and steroidogenic acute regulatory protein.¹² TiO₂ NPs were also found to accumulate in the ovary and can impair the equilibrium of sex hormones and alter the gene expression of ovary, and decrease the pregnancy rate and numbers of giving birth.¹³ The treatment of TiO₂ NPs to preantral follicle can lead to morphological changes in them and significantly inhibit follicular development and oocyte maturation in rat.^{14,15} In addition, TiO₂ NPs have a potential risk for embryonic development. Maternal exposure to TiO₂ NPs could suppress the embryonic development of CD-1 mice, resulting in a statistically significant increase in the resorptions, fetal deformities and dead fetuses.^{16,17} Similarly, TiO₂ NP addition to food led to a significant progeny loss in *Drosophila*.¹⁶ Moreover, it was reported that TiO₂ NPs were detected in the placenta by the gamma-ray spectrometer and silver NPs could be transferred to the developing conceptus across placenta.^{18,19} However, little

is known about the effect of TiO₂ NPs on the growth and development of placenta. Therefore, our aim of this work was to explore the effect and mechanism of TiO₂ NPs on the growth and development of placenta *in vivo* during mice pregnancy.

Materials and methods

Materials

TiO₂ NPs (CAS#: 637254) were purchased from Sigma-Aldrich Co., Ltd (St Louis, MO, USA). The average particle size of anatase TiO₂ NPs was <25 nm, while the surface area of sample was 45–55 m²/g. The purity of TiO₂ NPs is 99.7% based on trace metals analysis (≤4,000.0 ppm). TiO₂ NP powder was dispersed on the surface of 0.5% (w/v) tragacanth solution (Xiya Reagent Co. Ltd., Shanghai, People's Republic of China), and the solutions containing TiO₂ particles were treated ultrasonically for 20–30 min and then mechanically vibrated for 3–5 min. The particle sizes of NPs suspended in 0.5% w/v solution (3 mg/mL) following incubation were determined using a Tecnai-10 transmission electron microscope (TEM) (Philips, Amsterdam, the Netherlands). Mean particle size was determined by measuring more than 100 individual particles, which were randomly sampled. The zeta potential of TiO₂ NPs was 9.12 mV.

Animal treatment and tissue collection

Mature mice (Kunming strain) were housed in a consistent environment with a 14 h light: 10 h dark cycle and allowed free access to water and food. The experiment was approved by the Institutional Animal Care and Use Committee of Nanchang University and followed the rules set forth in the Guide for the Care and Use of Laboratory Animals. Adult virgin female mice were mated with fertile males of the same strain to induce pregnancy. The day of finding a vaginal plug was considered as gestational day (GD) 1. Pregnant mice were randomly divided into 3 groups as follows: 1) control group, 2) 1 mg/kg/day TiO₂ NPs group, 3) 10 mg/kg/day TiO₂ NPs group, with 10 mice in each group. The mice were weighed, a volume of TiO₂ NP suspension was calculated for each mouse (0.1 mL/10 g body weight) and the fresh TiO₂ NP suspension was administered by gavage from GD 1 to GD 13. The control group was given an equivalent volume of 0.5% w/v tragacanth solution. Treated mice were weighed and euthanized to collect the liver, spleen, kidney, ovary and placenta tissues at 15:00–16:00 on GD 13 by cervical dislocation. Uterus and placenta from these mice were photographed by using a digital camera (Nikon, Tokyo, Japan) and frozen at –80°C for further analysis.

Placental morphometry

After counting the numbers of implanted and resorbed embryo and measuring the placental weight on GD 13, tissues were fixed in Bouin's solution for 12 h, dehydrated in gradient ethanol, embedded in paraffin blocks, sliced to 6 μm thickness and placed on the glass slides coated with poly-L-lysine. Then sections stained with hematoxylin and eosin (H&E) were examined and photographed using a Nikon DS-Fi1 microscope (Nikon) at a 10 \times objective magnification for morphological evaluation. The areas of whole placenta, placental spongiotrophoblast and placental labyrinth were measured using sections, with the maximum areas for the layer of whole placenta, using ImageJ software (v. 1.48, National Institutes of Health). The average of the areas was calculated using 5 serial sections from 10 individuals. All placentas were analyzed without treatment knowledge to avoid bias.

Immunohistochemistry

Tissue sections were deparaffinized in xylene, and hydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in PBS for 10 min. Nonspecific binding was blocked in 5% bovine serum albumin (BSA) in PBS for 60 min. Then, the sections were incubated in rabbit anti-laminin (1:1,200, Sigma-Aldrich), rabbit anti-PCNA (1:1,000; Cell Signaling, Danvers, MA, USA) or biotinylated DBA lectin (1:300; Sigma-Aldrich) overnight at 4°C. After washing in PBS 3 times for 5 min each time, the sections were incubated with a secondary antibody for 60 min at 37°C followed by fresh diaminobenzidine (ZSGB-BIO, Beijing, People's Republic of China) solution, together with counter-staining with Harris' hematoxylin. Experimental results were observed by light microscopy, and analyses of immunohistochemical staining were taken using an NIS-Elements analysis system (Nikon). Each slide was measured in a randomly selected 10 fields under the same magnification ($\times 400$) and light intensity. Mean values of optical density for positive cells were calculated using 5 placenta sections from 10 individuals in each group.

Placental genes expression

Relative expression of placental genes was detected by real-time polymerase chain reaction (PCR) using $2^{-\Delta\Delta\text{CT}}$ method. Total RNA was extracted from placental tissues with RNAiso Plus solution (Takara, Tokyo, Japan) and cDNA was generated using PrimeScriptTM II cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. Gene expression of each sample was examined in a 20 μL reaction volume containing 10 μL of 2 \times Brilliant SYBR Green Mix (Takara), 2 μL of template cDNA, 0.5 μM primers and

300 nM reference dyes using the ABI thermal cycler 7500 (Applied Biosystems, Waltham, MA, USA). Relative expression of each transcript was normalized to 18S. The primers for placenta-specific genes were described previously.²⁰

Observation of placental ultrastructure by TEM

Placenta tissue blocks of about 1 mm^3 in size were placed in 2.5% glutaraldehyde phosphate buffer overnight and fixed in 1% osmium acid for 1 h. Staining was performed for 30 min with 2% uranium acetate solution. The specimens were dehydrated in a graded series of ethanol (50%, 70%, 90% and 100%). Tissues were embedded in Epon 812, sectioned (120 nm thickness), stained with 4% uranyl acetate and lead citrate, and observed with a Tecnai-10 TEM (Philips). Placental apoptosis was analyzed based on the changes in nuclear morphology.

Western blot analysis

Tissues were homogenized and lysed in the ice-cold radioimmunoprecipitation assay buffer supplemented with phenylmethylsulfonyl fluoride solution and a phosphatase inhibitor cocktail (Applygen Technologies, Beijing, People's Republic of China). Protein content was detected by using the BCA protein assay kit (Applygen Technologies). A total of 20 μg protein extracts were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis, and then transferred onto the nitrocellulose blotting membrane (Corning Life Sciences, New York, NY, USA). Nonspecific binding was blocked with 5% BSA for 1 h at room temperature and blotting membranes were incubated with the following primary antibodies at 4°C overnight: rabbit anti-Bcl-2, Bax, caspase-3 (Casp-3) and mouse anti- β -actin (Cell Signaling). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at 1:8,000 dilutions and visualized via enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). The relative band intensity was acquired by using the Quantity One software. The results were corrected for background, normalized to β -actin expression.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software (IBM Corporation, Armonk, NY, USA). Values are presented as means \pm standard error of mean. The results were analyzed by using Student's *t*-test between two groups or using 1-way analysis of variance (ANOVA) followed by least-significant difference's post-hoc test. Statistical significance for all tests was judged at a probability level of 0.05 ($P < 0.05$).

Results

TiO₂ NPs characteristics

X-ray-diffraction (XRD) measurements showed that TiO₂ NPs exhibited the anatase structure, and the average particle size calculated from the XRD peak of anatase was <25 nm using Scherrer's equation (Figure S1). In addition, TEM analyses indicated that the average size of TiO₂ powder particles suspended in tragacanth solution was about 10 nm (Figure 1), which was almost consistent with the XRD results.

Effect of TiO₂ NPs on the organ weights

Absolute and relative organ weights of pregnant mice exposed to TiO₂ NPs are shown in Table 1. At the dose levels tested, TiO₂ NPs had no significant effect on the absolute and relative weights of liver, spleen, kidney and ovary on GD 13 (all $P>0.05$, Table 1).

Effect of TiO₂ NPs on embryo implantation and placental weight

Although TiO₂ NPs treatment slightly increased the numbers of resorbed embryos, statistical analysis indicated that there was no significant difference between TiO₂ NP-treated group and control group (Figure 2A and B). In addition, no detectable changes were noted in the numbers of viable embryos (Figure 2B) and absolute weight of placenta (Figure 3A and B). However, 1 mg/kg/day TiO₂ NPs significantly reduced the ratio of placental weight to body weight compared to control group on GD 13 ($P<0.05$, Figure 3C).

Effect of TiO₂ NPs on placental structure

We observed placental histopathologic changes by H&E staining. On GD 13, treatment with 1 mg/kg/day TiO₂

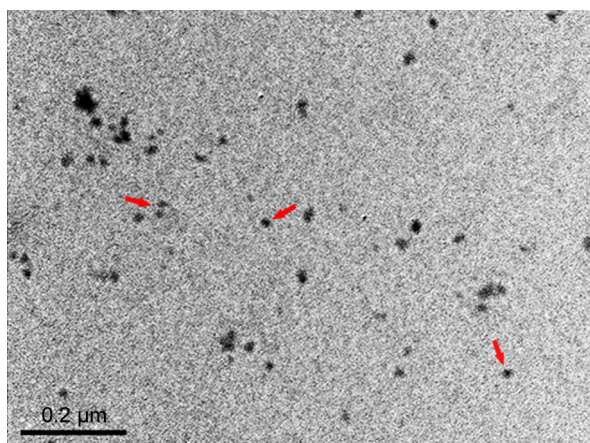


Figure 1 TEM image of TiO₂ NPs.

Notes: A representative TEM image of TiO₂ NPs suspended in tragacanth solution is shown. Red arrows show TiO₂ NPs.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; TEM, transmission electron microscope.

Table 1 Absolute and relative organ weights of mice on GD 13

Parameters	TiO ₂ NPs (mg/kg/day)		
	0	I	10
Liver weight (g)	2.45±0.29	2.37±0.11	2.43±0.36
Liver organ coefficient (%)	5.77±0.56	5.48±0.22	5.44±0.23
Spleen weight (g)	0.39±0.08	0.30±0.04	0.32±0.05
Spleen organ coefficient (%)	0.91±0.18	0.70±0.13	0.72±0.04
Kidney weight (g)	0.20±0.006	0.21±0.008	0.21±0.013
Kidney organ coefficient (%)	0.48±0.009	0.48±0.006	0.46±0.008
Ovary weight (mg)	8.90±0.000	9.20±0.001	10.00±0.001
Ovary organ coefficient (‰)	0.21±0.001	0.21±0.002	0.22±0.001

Note: Values are presented as means ± SE (n=10).

Abbreviations: GD, gestational day; TiO₂ NPs, titanium dioxide nanoparticles; SE, standard error.

NPs had no obvious effect on the area of spongiotrophoblast and labyrinth relative to control group (all $P>0.05$, Figure 4A–C). Nevertheless, 10 mg/kg/day TiO₂ NP treatment significantly increased the area of placental spongiotrophoblast and reduced the area of labyrinth (all $P<0.05$, Figure 4B and C).

Effect of TiO₂ NPs on the expression of placental marker genes

To investigate underlying mechanisms of the dysregulation of spongiotrophoblast and labyrinth development in mice treated with TiO₂ NPs, we examined the expression levels of placental marker genes. Compared with the control, quantitative PCR results showed that all doses of TiO₂ NPs significantly reduced levels of *Hand1*, *Esx1*, *Eomes*, *Hand2*, *Ascl2* and *Fra1* mRNA (all $P<0.001$, Figure 5A–F).

Effect of TiO₂ NPs on the fetal vessels and numbers of uNK cells of decidua

Laminin immunohistochemical staining (a fetal vessels marker) showed that there were numerous intricate fetal vessels in normal control placental labyrinth (Figure 6A and D). However, 1 and 10 mg/kg/day TiO₂ NP treatment resulted in the collapse of fetal blood vessels (Figure 6B and C and E and F). DBA lectin staining (a novel uNK-specific marker) indicated that administration of 10 mg/kg/day TiO₂ NPs significantly decreased the numbers of uNK cells in the placental decidua compared with control group ($P<0.001$, Figure 7G), whereas 1 mg/kg/day dose did not significantly affect the numbers of uNK cells.

TiO₂ NPs treatment inhibited the proliferation and induced the apoptosis of placenta

Compared to control group, TiO₂ NP administration significantly reduced the numbers of PCNA-positive cells in the

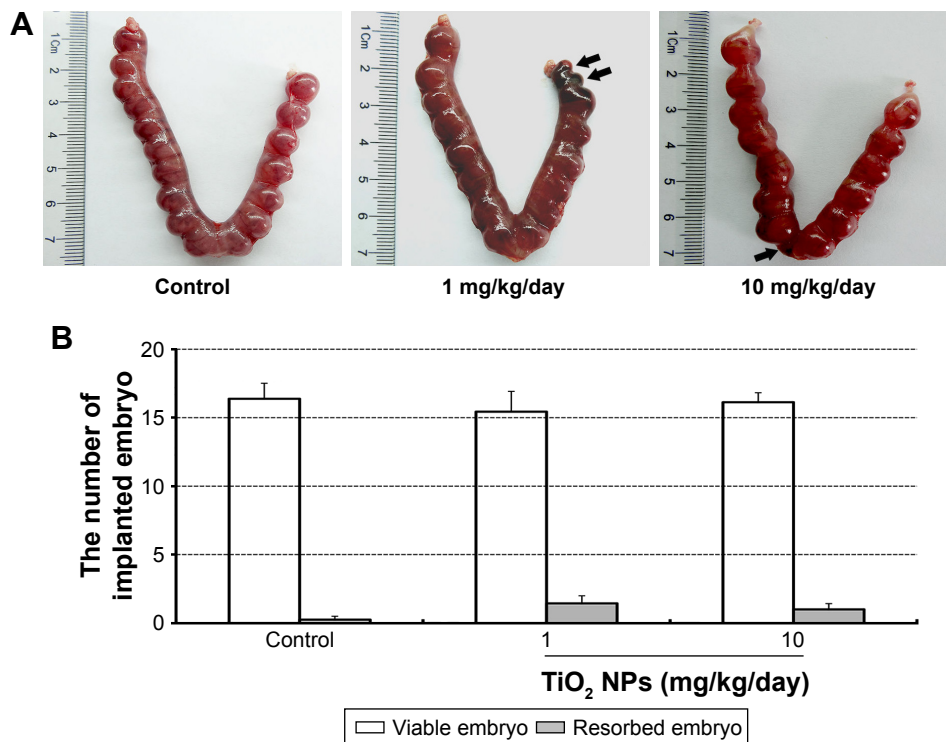


Figure 2 Effect of TiO₂ NP exposure on the numbers of implanted embryo.

Notes: (A) Representative images of implanted embryo in uteri on GD 13; black arrows show resorbed sites. (B) The numbers of implanted embryo in GD 13 mice; white bar represents viable embryos and gray bar represents resorbed embryos. Results are shown as mean \pm SEM of 10 animals.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; GD, gestational day; SEM, standard error of mean.

placental labyrinth ($P < 0.05$ or $P < 0.001$, Figure 8A and B). Furthermore, TEM analysis showed that TiO₂ NPs aggregated in the nucleus of placental cells (Figure S2), and nuclear pyknosis, mitochondrial swelling, expansion of sliding

endoplasmic reticulum and abscission of rough endoplasmic reticulum ribosome were observed in the TiO₂ NP-treated placental tissues (Figure 9B and C). Western blot analysis showed that intact Casp-3 was cleaved to active fragments

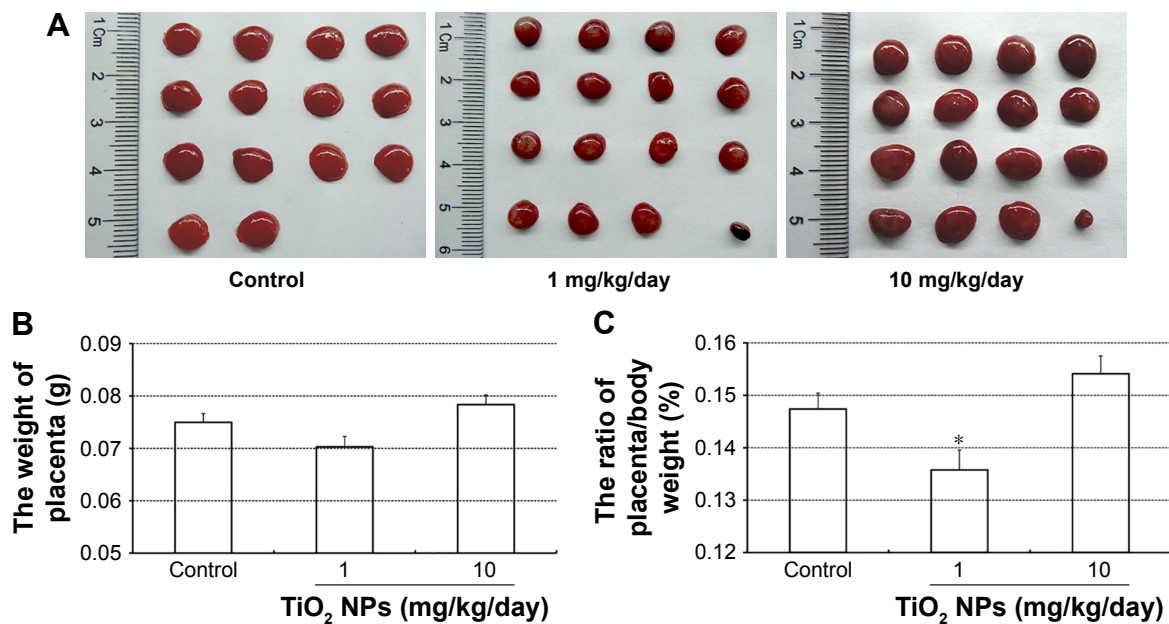


Figure 3 Effect of TiO₂ NP exposure on the weight of placenta.

Notes: (A) Representative images of placentas collected on GD 13; (B) the weight of placenta; (C) the ratio of placenta/body weight on GD 13. The data are presented as means \pm SEM of 10 animals. * $P < 0.05$ compared with control.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; GD, gestational day; SEM, standard error of mean.

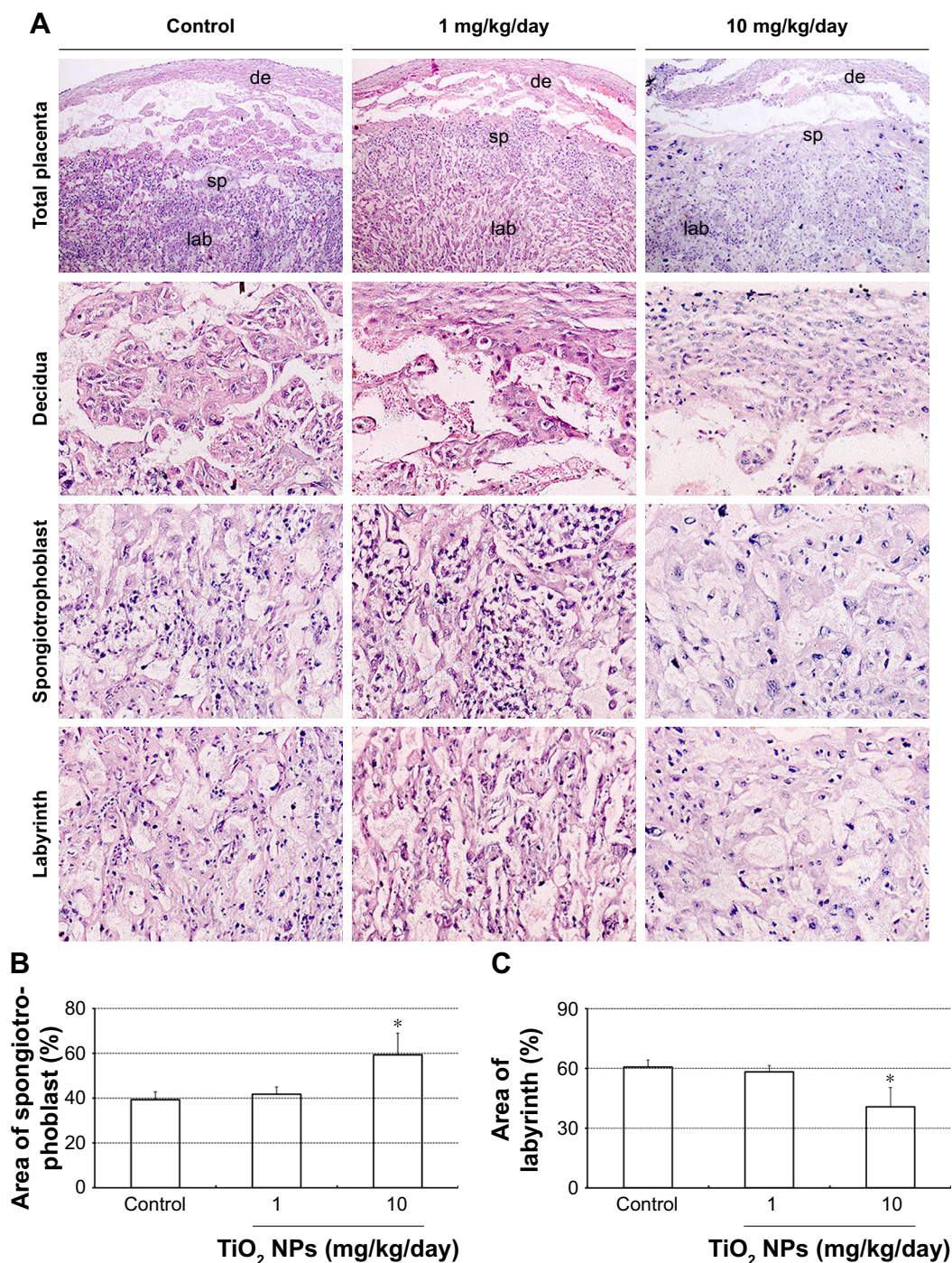


Figure 4 Effect of TiO₂ NP exposure on placental histopathology.

Notes: (A) Representative images of placental section observed by H&E staining. Scale bar, 50 μ m. (B) The ratio of spongiotrophoblast/total area of placenta (%). (C) The ratio of labyrinth/total area of placenta (%). Data are represented as means \pm SEM of 10 animals. * $P < 0.05$ compared with control.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; H&E, hematoxylin and eosin; SEM, standard error of mean; de, decidua; sp, spongiotrophoblast; lab, labyrinth.

after TiO₂ NP treatment on GD 13 (Figure 10A) and expression levels of active fragments significantly increased in a dose-dependent manner ($P < 0.001$, Figure 10D). In addition, TiO₂ NP treatment also significantly increased the expression level of Bax protein ($P < 0.05$ or $P < 0.001$, Figure 10C and D). However, the expression level of Bcl-2 protein was

significantly downregulated on GD 13 ($P < 0.01$ or $P < 0.001$, Figure 10B and D).

Discussion

Recently, information on the toxicity of TiO₂ NPs in the liver and kidneys and in male and female reproductive

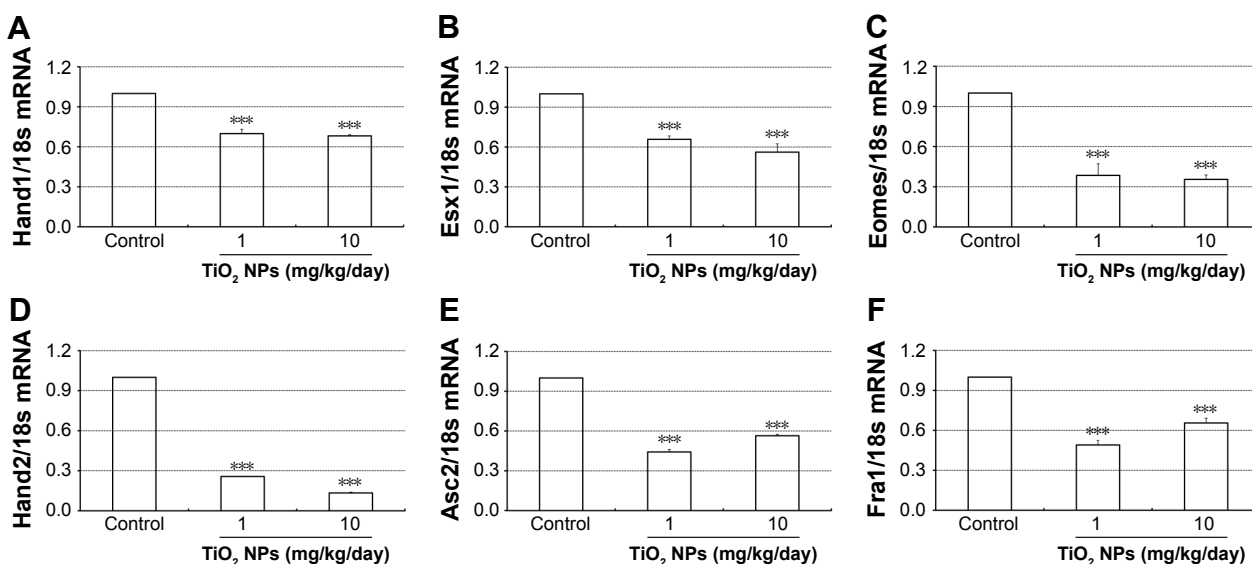


Figure 5 Effect of TiO₂ NP treatment on placental gene expression.

Notes: (A–F) Relative expression levels of *Hand1* (A), *Esx1* (B), *Eomes* (C), *Hand2* (D), *Ascl2* (E) and *Fra1* mRNA (F) in mice placentas treated by control, 1 and 10 mg/kg/day TiO₂ NPs on GD 13. mRNA levels were quantified using reverse transcription-quantitative polymerase chain reaction and normalized to 18S rRNA. Data are presented as means ± SEM of 6 animals. ****P*<0.001 compared to control.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; GD, gestational day; SEM, standard error of mean.

system injury^{5,6,9,15} is increasing. However, the evidence for placental developmental toxicity in mammals was limited. In this study, our results demonstrated that 1 mg/kg/day TiO₂ NP treatment significantly reduced the ratio of placenta/body weight in mice on GD 13. Histopathologically, compared with control group, the area of placental spongiotrophoblast significantly increased and labyrinth was markedly reduced in the 10 mg/kg/day dose group. The expression levels of

Hand1, *Esx1*, *Eomes*, *Hand2*, *Ascl2* and *Fra1* mRNA were significantly inhibited in TiO₂ NP-treated placentas. TiO₂ NP administration dramatically disrupted labyrinth vascularization of placentas and reduced the numbers of uNK cells. Moreover, we found that TiO₂ NP treatment significantly inhibited the proliferation and induced apoptosis of placenta by the activation of Casp-3, upregulation of Bax and down-regulation of Bcl-2 protein.

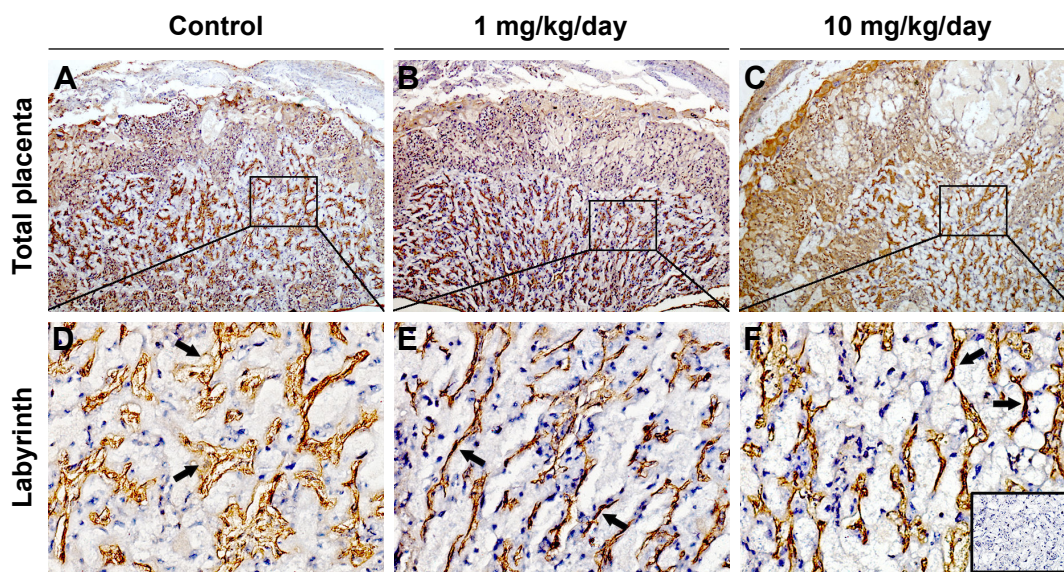


Figure 6 Effect of TiO₂ NP treatment on the labyrinth vascularization of placenta.

Notes: (A–C) Laminin immunohistochemical staining of mice placentas treated by control (A), 1 (B) and 10 mg/kg/day (C) TiO₂ NPs. Boxed areas in A–C were imaged with four-times higher magnification (D–F, respectively). Inset of F shows immunostaining of a negative control-stained section (primary antibody was replaced by normal rabbit serum). Black arrows show fetal vessels.

Abbreviation: TiO₂ NPs, titanium dioxide nanoparticles.

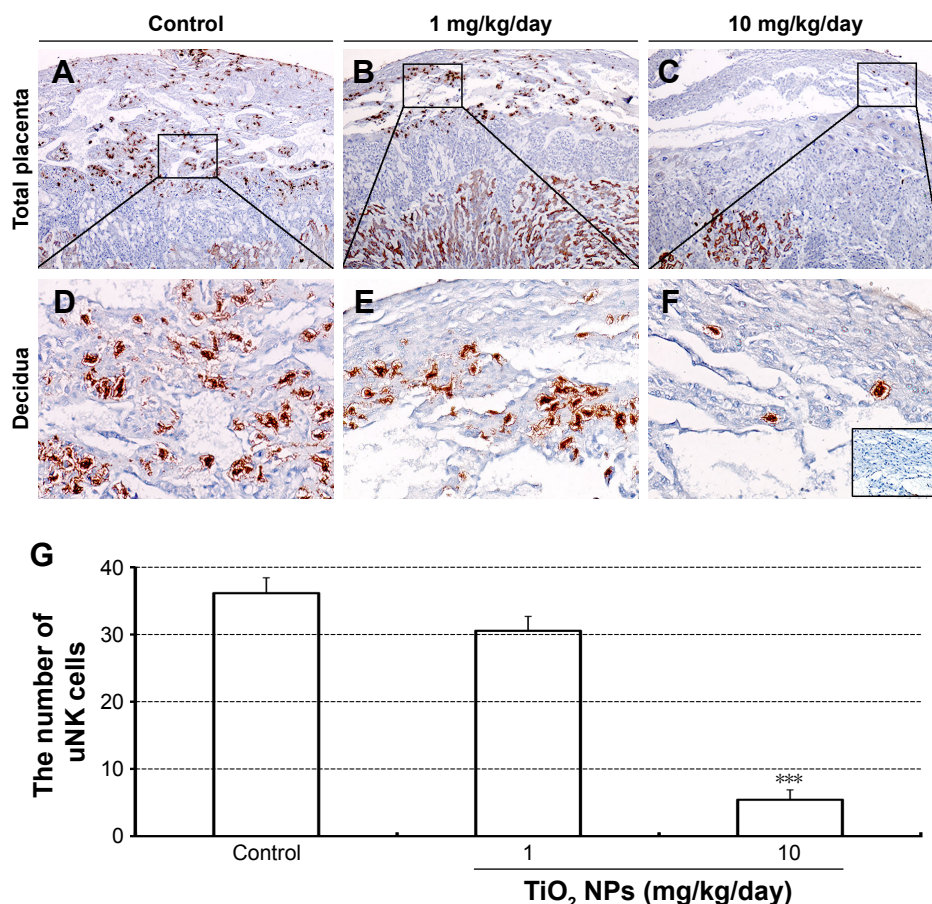


Figure 7 Effect of TiO₂ NP exposure on the numbers of uNK cells in placental decidua.

Notes: (A–C) DBA lectin staining of whole placental sections collected from animals, which were administered by control (A), 1 (B) and 10 mg/kg/day (C) TiO₂ NPs. Boxed areas in A–C were imaged with four-times higher magnification (D–F, respectively). Inset of F shows immunostaining of a negative control section (stained with the addition of 0.1 M N-acetyl-D-galactosamine to the DBA lectin incubation). (G) The numbers of uNK cells in placental decidua of mice on GD 13. Data are presented as the means ± SEM of 6 animals. ****P*<0.001 compared with control.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; GD, gestational day; SEM, standard error of mean.

Under our experimental conditions, statistical analysis showed that treatment with different doses of TiO₂ NPs did not affect absolute and relative organ weights of pregnant mice, including liver, spleen, kidney, ovary and body weight. This is almost consistent with Wang et al's result,²¹ in which treatment with 5 g/kg TiO₂ NPs in adult mice did not induce significant abnormal pathology changes in the spleen, lung, testis, ovary and heart tissues. However, female mice treated with 25 and 80 nm TiO₂ NPs for 2 weeks showed high coefficients of liver.²² Upregulation of blood urea nitrogen level and pathology change of kidneys were also observed in the experimental groups. In addition, Hong et al's study showed that chronic exposure to TiO₂ NPs for 9 months led to significant decreases in body weight and increases in kidney organ coefficient compared with those of control group.²³ Nevertheless, Ag NP exposure on gestational days 6–19 in rats did not affect maternal body weight, organ weight (brain, liver, spleen, kidney, heart and ovary), and fetal and

placental weights.²⁴ These results suggested that effects of TiO₂ NPs on the organ weight are probably exposure time dependent, dose dependent, and nanoparticle size and nano-material dependent.

In addition, we also found that the numbers of implanted and resorbed embryos and the weight of placenta were not significantly affected by different doses of TiO₂ NPs; only 1 mg/kg/day TiO₂ NPs significantly reduced placental organ coefficient on GD 13. Consistent with our findings, Warheit et al found that the numbers of implantation sites, early and late resorptions were all comparable to control group values for every dose level tested, which were 0, 100, 300 or 1,000 mg/kg/day TiO₂ NPs in Sprague-Dawley rats from GD 6 through GD 20.²⁵ The authors concluded that TiO₂ NP exposure produced no evidence of maternal or developmental toxicity at any dose level. However, Zhao et al's study demonstrated that female mice who were exposed to 2.5, 5 and 10 mg/kg TiO₂ NPs by intragastric administration for 90

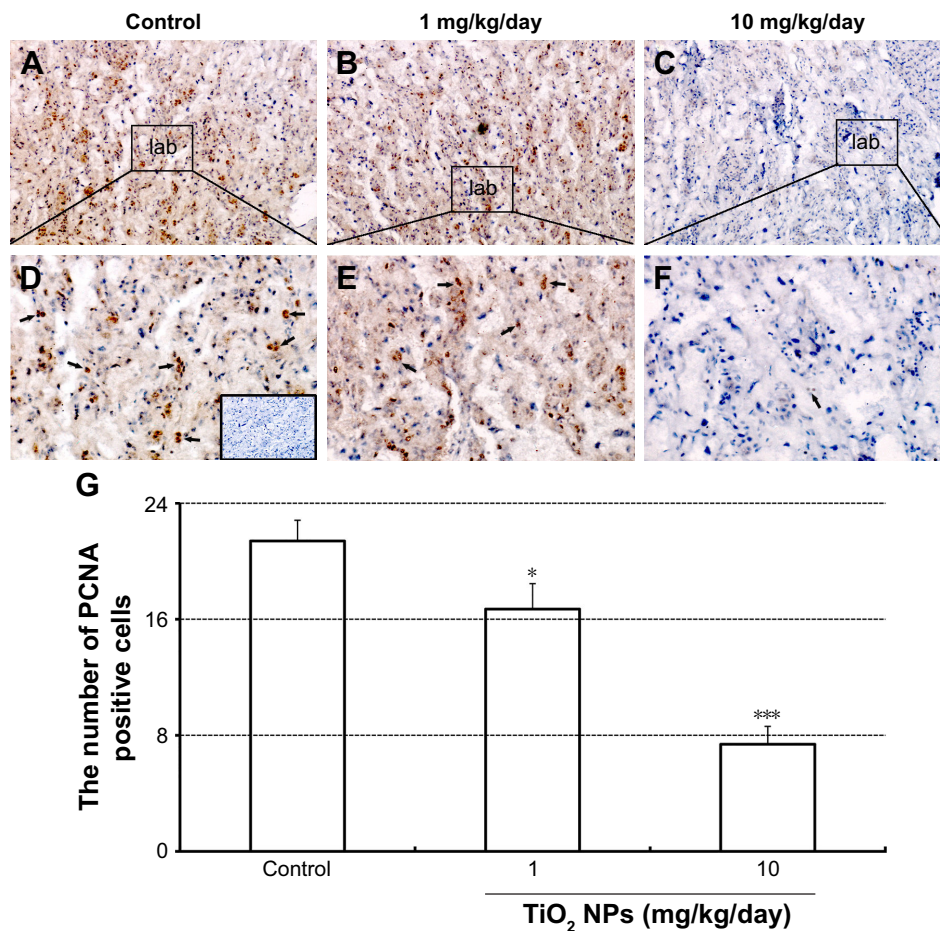


Figure 8 Effect of TiO₂ NP exposure on the proliferation of placenta.

Notes: (A–B) PCNA immunohistochemical staining of whole placental sections collected from animals, which were administered by control (A), 1 (B) and 10 mg/kg/day (C) TiO₂ NPs. Boxed areas in A–C were imaged with two-times higher magnification (D–F, respectively). Inset of D shows immunostaining of a negative control section (primary antibody was replaced by normal rabbit serum). (G) The numbers of PCNA-positive cells in placental labyrinth of mice on GD 13. Data are presented as means ± SEM of 6 animals. **P*<0.05, ****P*<0.001 compared with control.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; lab, labyrinth; GD, gestational day; SEM, standard error of mean.

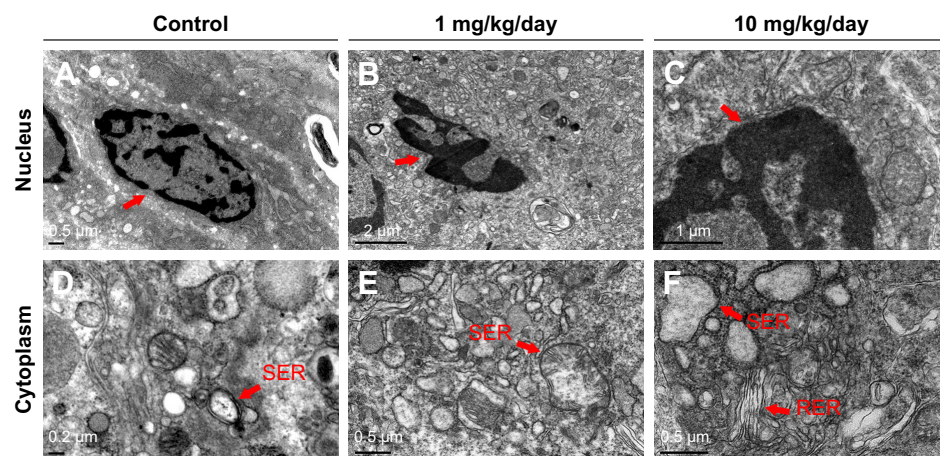


Figure 9 Effect of TiO₂ NP exposure on the placental ultrastructure by TEM evaluation.

Notes: (A–F) Representative TEM images of placental sections collected from animals, which were administered by control (A, D), 1 (B, E) and 10 mg/kg/day (C, F) TiO₂ NPs. (A–C) Representative TEM images of cell nucleus parts. Red arrows show nucleus. (D–F) Representative TEM images of cell cytoplasm parts.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; TEM, transmission electron microscope; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum.

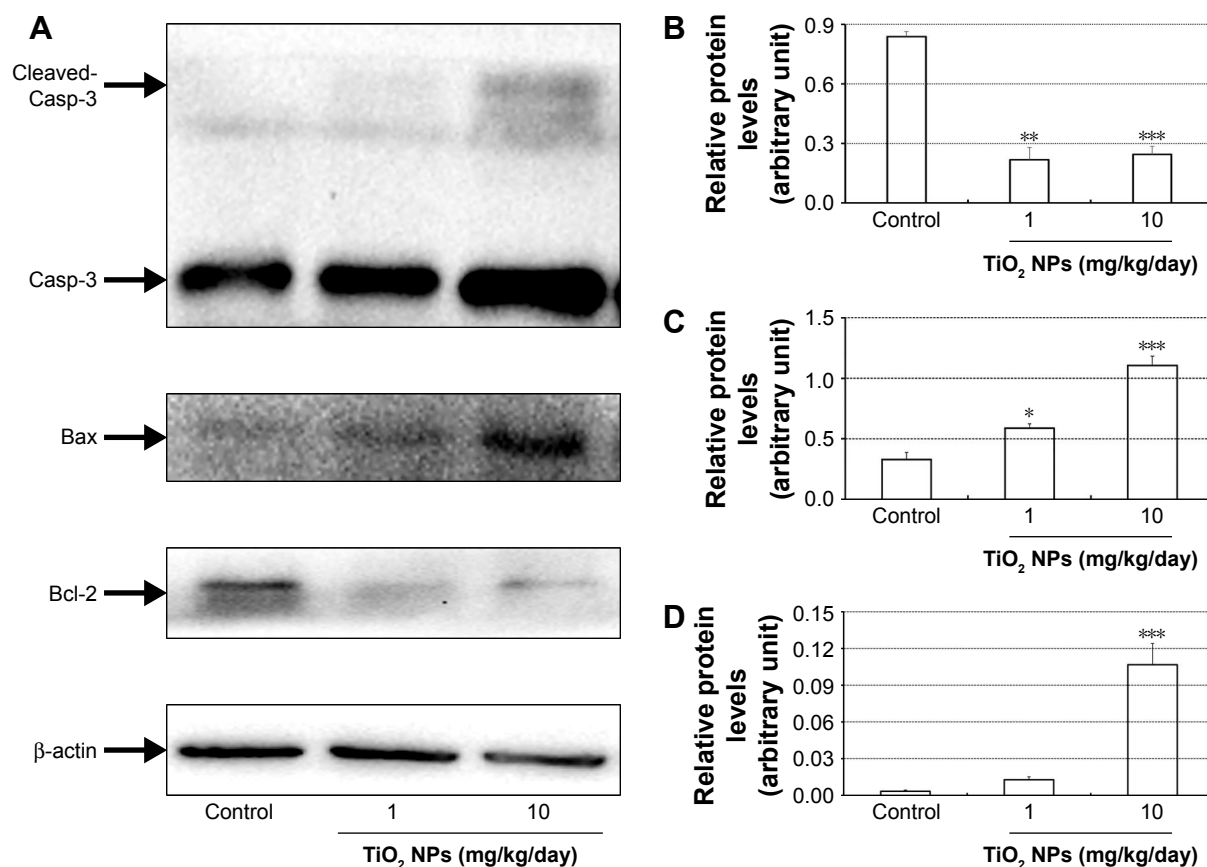


Figure 10 Effect of TiO₂ NP exposure on the apoptosis of placenta.

Notes: (A) Representative immunoblotting images of Casp-3, Bax and Bcl-2 proteins in the TiO₂ NP-treated placentas. (B–D) Densitometric values from Western blot analyses of Bcl-2 (B), Bax (C) and cleaved-Casp-3 (D) proteins. The data are normalized to β -actin expression (but cleaved-Casp-3 is normalized to Casp-3) and shown as mean \pm SEM of 6 animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

Abbreviations: TiO₂ NPs, titanium dioxide nanoparticles; Casp-3, caspase-3; SEM, standard error of mean.

consecutive days, resulted in significant reduction of relative weight and function of ovary, and pregnancy rate and number of newborns.¹⁵ Reduced pregnancy rate, and fertility may be a result of ovarian injury because the fertility of treated females was tested by caging with males of proven fertility after 90 days.^{13,15}

In this study, the significant reduction of developmental related genes *Hand1*, *Esx1*, *Eomes*, *Ascl2* and *Fra1* expression was observed in TiO₂ NP-treated placental tissues. After implantation, *Hand1* expression is restricted to placental trophoblast cells and essential for the differentiation of trophoblast.²⁶ *Esx1* is required for mouse labyrinthine development and mesoderm formation.²⁷ In addition, *Eomes* and *Fra1* also play a crucial role in the labyrinthine development.^{28,29} Therefore, downregulation of *Hand1*, *Esx1*, *Eomes* and *Fra1* expression would severely impair the development of labyrinth and result in the downregulation of labyrinth area in the 10 mg/kg/day dose group. *Ascl2* is essential to spongiotrophoblast maintenance and the development of extraembryonic trophoblast lineage in mice.

In *Ascl2* mutant placentas, spongiotrophoblast cells and their precursors are absent and chorionic ectoderm is reduced.³⁰ In di-(2-ethylhexyl) phthalate-treated placenta, the area of spongiotrophoblast and expression levels of *Ascl2* mRNA dramatically decreased in mice on GD 13.²⁰ However, the area of placental spongiotrophoblast significantly increased in the 10 mg/kg/day TiO₂ NP group compared to control group. This suggested that the development of spongiotrophoblast with exposure to TiO₂ NPs was not closely related to the downregulation of *Ascl2*. The confirmation of the underlying mechanism requires further investigation.

Vascularization of placental labyrinth is essential for the growth and development of placenta and establishment of successful pregnancy.^{31,32} Our results evidenced that TiO₂ NP treatment resulted in placental vascular collapse and it impaired the formation of an intricate network of fetal vessels. Similar to our results, Yamashita et al found that spiral artery canals failed to form and blood flow was significantly reduced in the fetal vascular sinuses of 70 nm TiO₂ NP-treated mice.¹⁹ Stapleton et al's study showed that TiO₂ NP exposure

significantly impaired the microvascular reactivity of uterine and coronary circulations of female offspring.³² Furthermore, Stapleton et al reported that thrombosis was observed in the pulmonary vascular system of TiO₂ NP-treated mice, which could be induced by the blocking of blood vessels with TiO₂ particles.³³ In addition, uNK cells are short-lived, terminally differentiated and the most abundant lymphocyte in the uterus, and play a vital role in vascularization, spiral arteriole modification and placentation.^{34–36} Our results showed that 10 mg/kg/day TiO₂ NP treatment significantly decreased the numbers of uNK cells in the placental decidua compared with control group. This suggests that reduction of branched fetal vessels may partly result from the downregulation of uNK cells in the TiO₂ NP-treated group. Previous research indicated that *Esx1*, *Hand2* and *Fra1* were closely related with vascularization.^{27,29,37} Therefore, our study showed that downregulation of *Esx1*, *Hand2* and *Fra1* mRNA in TiO₂ NP-treated placenta possibly also contributed to the decrease of fetal vessels within the labyrinth.

We also observed whether defective growth and development of placentas exposure TiO₂ NPs were associated with the proliferation and apoptosis changes of placenta. PCNA staining indicated that TiO₂ NP treatment significantly decreased the proliferation of placental trophoblast. TEM analysis showed that nuclear pyknosis, expansion of sliding endoplasmic reticulum and abscission of rough endoplasmic reticulum ribosomes were observed in the TiO₂ NP-treated placental tissues. Genes involved in apoptosis cleaved-Casp-3 and Bax proteins were markedly upregulated, while Bcl-2 was significantly downregulated in the mice placentas. In concordance with our report, Li et al found that TiO₂ NPs induced apoptosis in mouse spleen by activating Casp-3.³⁸ Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of harmful stimuli.³⁹ In our present study, Bcl-2 expression was significantly reduced, suggesting that Bcl-2 may play an important role in TiO₂ NP-induced trophoblast apoptosis in the mice placentas. It has been shown that exposure to TiO₂ NPs resulted in the upregulation of Casp-3, Nrbp2 and cytochrome c expression, and caused downregulation of SOD, CAT, GST and Bcl-2 expression in the Sertoli cells of mouse testis.²² Consequently, Sertoli cell apoptosis in testicular tissue caused by TiO₂ NPs may be involved in oxidative stress. However, underlying mechanisms of TiO₂ NP-induced placental apoptosis in mice requires further investigation.

Conclusion

Our results provide evidence that gestational exposure to TiO₂ NPs significantly impairs the growth and development

of placenta during mice pregnancy with a mechanism that seems to be involved in vascularization, proliferation and apoptosis pathways. The present study provides new insight into the mechanism of TiO₂ NP-induced fetal mortality and deformities. Therefore, we believe that the unnecessary supplementation of commercial products with TiO₂ NPs should be restrained during pregnancy, until we are sure that it has no detrimental effects on reproductive health.

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Author contributions

LZ, XX, YZ and HK conceived and designed the study. LZ, XX and DY, YD and JO carried out all the experiments. BY, DL and DZ performed Western blot analysis. LZ, YZ, and HK drafted the paper. All the authors contributed toward statistical analysis and revising the paper, read and approved the final manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

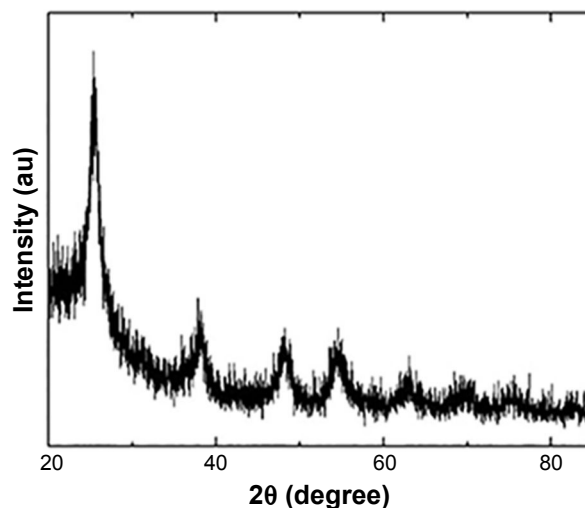


Figure S1 The x-ray diffraction peak of anatase TiO_2 NPs.

Note: X-ray-diffraction (XRD) measurements showed that TiO_2 NPs exhibited the anatase structure, and the average particles size calculated from the XRD peak of anatase was <25 nm using Scherrer's equation (sigma-aldrich Co., Ltd).

Abbreviation: TiO_2 NPs, titanium dioxide nanoparticles.

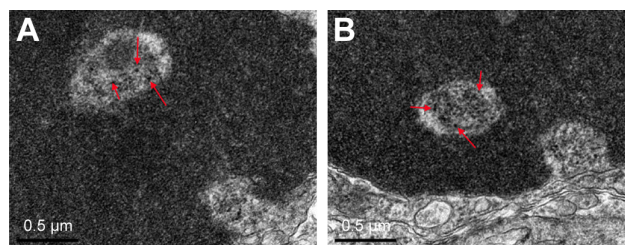


Figure S2 TEM evaluation of placental cell ultrastructure in pregnant mice caused by an intragastric administration with 10 mg/kg/day TiO_2 NPs for 13 consecutive days. (A, B) Arrows indicate that TiO_2 NPs aggregated in the nucleus of placental cells.

Abbreviations: TiO_2 NPs, titanium dioxide nanoparticles; TEM, transmission electron microscope.

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