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Research paper

Toxic effects of TiO_2 NPs in the blood-milk barrier of the maternal dams and growth of offspring

Liyang Yao, Ling Chen, Bolu Chen, Yizhou Tang, Yu Zhao, Shanji Liu, Hengyi Xu *

State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are amongst the most frequently used nanomaterial in everyday consumer products, and their widespread applications have raised concerns of the consequent deleterious effects on human health, particularly to vulnerable populations, such as lactating females remains elusive. Therefore, this study was initiated to investigate the detrimental effects and toxic mechanisms induced by TiO_2 NPs in maternal dams and offspring during the lactation period. Dams were randomly divided into three groups. The water (Control; Group I) and TiO_2 NPs (100 mg/kg; Group II) were orally administered from postnatal day 1–20, respectively. The results indicated that TiO_2 NPs could cause toxicity in the dams, such as pathological damages to mammary gland tissues. The excessive accumulation of TiO_2 NPs could induce oxidative stress in the mammary gland, leading to the dysfunctional blood-milk barrier; besides, TiO_2 NPs could also be transferred to offspring via breastfeeding, causing abnormal development of infant. We further accessed the possible underlying molecular mechanism; for this, we orally administered TiO_2 NPs with vitamin E (100 mg/kg; Group III). The results revealed that toxicity induced by TiO_2 NPs was rescued. Collectively, this study presented the deleterious pathological effects of oral exposure to TiO_2 NPs in the mammary gland tissues and blood-milk barrier via the production of reactive oxygen species (ROS) in dams and developmental concerns in offspring. However, the administration of VE could mitigate the toxic effects induced by the TiO_2 NPs.

1. Background

Nanoparticles (NPs), nanostructures with at least one dimension in size range of 1–100 nm, has gained increasing prominence in recent decades for their extensive application in diverse fields, including medicine, materials science, environmental protection, energy, food and cosmetics, and textile industries (Valentini et al., 2019). Titanium dioxide (TiO_2) NPs are one of the most widely used nanomaterials; it is frequently incorporated as pigment into everyday products, such as paints, toothpaste, sunscreens, ointments, and cosmetics (Kansara et al., 2020). TiO_2 NPs are amongst the most commonly manufactured nanomaterial with an estimated production of approximately 38,000 metric tons each year in the US, and worldwide production of TiO_2 NPs is expected to exceed 2.5 million metric tons per year by 2025 (Coral and Kitchens 2019). The widespread applications of TiO_2 NPs raise concerns about its inevitable exposure to humans and the consequent deleterious effects on human health. Traditionally, TiO_2 NPs were considered to have low toxicity (Wang et al., 2019); however, an increasing number of recent studies have suggested that long-term exposure to TiO_2 NPs can cause potential toxicity to a variety of organisms and impair the physiological characteristics. More recently, Tang et al. (2019) found that TiO_2 NPs could trigger oxidative damage in the liver, gill, and intestinal tissues of Zebrafish. Valentini et al. (2019) indicated that TiO_2 NPs could exert a toxic effect on kidney and liver organs in mice after its exposure Besides, studies have revealed that exposure of TiO_2 NPs can cause damage to the brain of a mouse. Although the toxicity of TiO_2 NPs to human health has been questioned, comprehensive toxicity analysis particularly to vulnerable populations, such as lactating females,

Abbreviations list: TiO_2 NPs, titanium dioxide nanoparticles; VE, vitamin E; ROS, reactive oxygen species; Nrf-2, Nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; HO-1, heme-oxygenase-1; MDA, malondialdehyde; GSH, glutathione; NQO-1, NAD(P)H quinone oxidoreductase; TJ, tight junction; PND, postnatal day; MCH, mean corpuscular hemoglobin; HGB, hemoglobin; PLT, platelets; MCHC, mean corpuscular hemoglobin concentration; RBC, red blood cells; WBC, white blood cells; HCT, hematocrit; MCV, mean corpuscular volume; HE, hematoxylin and eosin; ICP-AES, Inductively Coupled Plasma Atomic Emission Spectrometer; TP, total protein; MLCK, Myosin light chain kinase; OCLN, Occludin; CLDN3, claudin-3; EGF, Epidermal Growth Factor.

* Corresponding author.
E-mail addresses: kidyxu@163.com, HengyiXu@ncu.edu.cn (H. Xu).

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remains highly desirable.

Lactation represents a unique physiologic process by which milk is synthesized and secreted from the mammary glands of the postpartum female to provide nutrition and immune protection to the offspring. Milk can promote the growth and immunity, sensory and cognitive development of infants (Odintsova et al., 2019; Ghozy et al., 2020). It also reduces the risk of infectious diseases and sudden infant death syndrome (Beiker et al., 2020; Pereira et al., 2020). At the same time, the blood-milk barrier, as a basic structure of mammary gland, restricts the intrusion of microbial and exogenous substances constituents into milk (Shin et al., 2019). Thus, the barrier is particularly important for the integrity of milk and the health of newborns. NPs have been known to induce the breakdown of some essential physiological barriers; however, whether NPs can impair the blood-milk barrier remain to be elucidated.

Many papers have demonstrated that nanoparticle can bypass biological barriers via ROS and ROS independent pathway. Such as, Tay, Setyawati et al. (Setyawati et al., 2013; Tay et al., 2017) found nanoparticle could directly bind to the endothelial cells' adherens junction protein VE-cadherin, and follow the VE-cadherin into the cytoplasm during the internalization of VE-cadherin. In 2019 year, Kunovac, Hathaway et al., (2019) reported that TiO_2 NPs would enhance the level of ROS and H_2O_2, the high level of ROS would active the Hif1α/Dnmt1 pathway, in turn to damage the placenta barrier. According to those researches, we still focus on the oxidative stress, a classic pathway signal. Oxidative stress is described as an imbalance between removal and generation of reactive oxygen species (ROS), which may cause damages to all components of the cells and tissues (Tohari et al., 2019). Accumulating pieces of evidence have suggested that excessive generation of ROS and consequent oxidative stress were frequently associated with NPs-induced toxicity in the different organs (Ansar et al., 2018; Guo et al., 2018; Zhou et al., 2019). Moreover, excessive production of ROS can also induce inflammation, apoptosis, and mitochondrial dysfunction, and redox imbalance (Kang et al., 2019, 2020). Nuclear factor erythroid 2-related factor 2 (Nrf-2) has emerged as a highly sensitive regulator of cellular resistance to oxidative stress (Kahroba et al., 2019). Under basal conditions, Nrf2 is sequestered in the cytoplasm by the protein Keap1 (kelch-like ECH-associated protein 1), facilitating ubiquitination and degradation of Nrf2. On exposure to oxidative stress, Keap1 is inactivated through the modification of reactive cysteine residues, promoting the release of stabilized and activated Nrf2 that is translocated in the nucleus and it activates the ARE-dependent gene expression of a series of antioxidative and cytoprotective proteins, including heme-oxygenase-1 (HO-1), malondialdehyde (MDA), glutathione (GSH), and NAD(P)H quinone oxidoreductase (NQO-1) (Nguyen et al., 2009; Dinkova-Kostova and Talalay, 2010). Therefore, the redox-sensitive Keap1/Nrf2/ARE signaling system plays a crucial role in the maintenance of cellular homeostasis, and its activation may indicate the presence of oxidative stress.

Vitamin E (VE), a lipid-soluble antioxidant, exhibits excellent antioxidant properties (Miyazawa et al., 2019). Studies have indicated VE as the potential candidate for supplementary antioxidant-based therapeutics owing to its antioxidant properties. Furthermore, the administration of VE eliminated the increased oxidation in multiple sclerosis (Kalita et al., 2020); VE can significantly improve liver histologic changes and function in patients with nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) (Banini and Sanyal, 2019). Thus, VE, as an antioxidant, was used in this study to demonstrate that oxidative stress was the key impaired mechanism induced by TiO_2 NPs toxicity during the lactation period.

In this study, the effects of oral exposure to TiO_2 NPs in postpartum dam rats' blood-milk barrier were evaluated. The permeability of the blood-milk barrier, breast pathology, and changes in milk composition was also assessed; the transcription levels of genes related to oxidative stress, milk composition, and tight junction (TJ) protein were also determined. Collectively, the results provide a theoretical basis for the toxic effects of TiO_2 NPs exposure during lactation.

2. Materials and methods

2.1. Materials and animal treatment

TiO_2 NPs were procured from Aladdin Industrial Corporation (T104943, Lot# D1809001, Shanghai, China). Healthy females and males Sprague Dawley (SD) rats (approximately 200 g), were acquired from the experimental animal center of Nanchang University. The physiochemical properties for the TiO_2 NPs had been reported in our previously papers (Yao et al., 2020; Zhao et al., 2020), and a summary of the physiochemical data of TiO_2 NPs in the Supplementary Materials (Fig. S3). The rats were housed under specific-pathogen-free (SPF) controlled conditions of 25 ± 1 °C temperature, 60% ± 10 humidity with a 12 h light/dark cycle and free access to standard rodent diet and water ad libitum for acclimatization before the start of experiments for one week. All the mice were healthy and had no infection during the experimental period.

The experimental protocols involving rats conformed to the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Review Committee (approval number 0064257, Nanchang University, Jiangxi, China). Female rats were housed for breeding with male rats in the ratio of 3:1 (housed in 5 cages). Once pregnant, female rats were separated and randomly divided into the following three groups: Group I (Control, n = 7) rats were administered with 5 mL/kg of body weight (BW)/day ultrapure water by oral gavage from postnatal day (PND)1 to PND20. Group II (TiO_2 NPs, n = 7) rats were administered with TiO_2 NPs (at the dose of 100 mg/kg of BW/day) by oral gavage. Group III (TiO_2 NPs + VE, n = 7), rats were administrated with TiO_2 NPs (at the dose of 100 mg/kg of BW/day) from PND1 to PND20 along with VE (at the dose of 100 mg/kg). The dose of TiO_2 NPs and VE were as described previously (Al-Rasheed et al., 2018; Mancuso and Barisani, 2019). Accumulating studies indicated that a dose of TiO_2 NPs consumed by adults and children were estimated to be approximately 0.2–0.7 mg/kg of BW/day and 1–2 mg/kg of BW/day in the United States (Yao et al., 2020), respectively. We also considered a safety factor of 100 times of interspecies extrapolation when calculating the exposure dose. Thus, the TiO_2 NPs dose administered was 100 times compared to daily consumption (Chen et al., 2018). The experimental process is shown in Fig. 4A.

3. Toxicity evaluation of maternal rats following exposure to TiO_2 NPs

3.1. Hematological analysis and organ coefficient

On the PND20, the rats were sacrificed using 10% chloral hydrate, and the blood samples and organs of maternal rats were carefully collected. The blood indices, including mean corpuscular hemoglobin (MCH), hemoglobin (HGB), platelets (PLT), mean corpuscular hemoglobin concentration (MCHC), red blood cells (RBC), white blood cells (WBC), hematocrit (HCT) and mean corpuscular volume (MCV) were measured by the Chinese clinical laboratory operator Adicon (Nanchang, Jiangxi, China). Furthermore, the weight data of the organs collected on the PND20 to calculate the organ coefficient using the following Eq. (1):

$$\textbf{Organ coefficient} = \frac{\text{Mass of organ (g)}}{\text{Body weight (g)}} \quad (1)$$

3.2. Histopathological examination

The five pairs of mammary glands were collected for subsequent analyses. The specimens were washed with 1% ice-cold saline and fixed in 10% paraformaldehyde. The specimens were then embedded in paraffin and sectioned (3–5 μm thickness) and stained with hematoxylin and eosin (HE) for histopathological examination. HE-stained sections

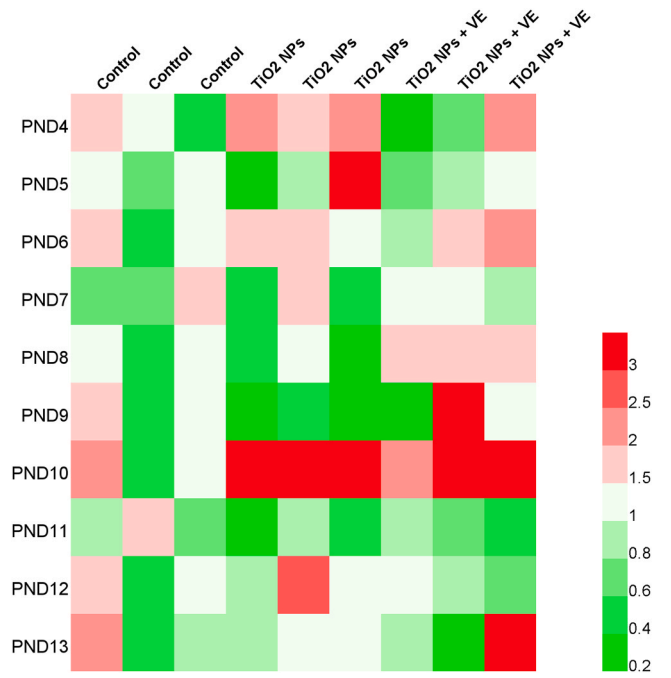

Fig. 1. Alteration in the milk yield following oral administration to different treatments. Values were log10, with green indicating lowest levels, red indicating highest levels, and white indicating median levels.

were observed under a light microscope to determine mammary glands' injury.

3.3. Milk yield

The milk was obtained from dams according to the method described previously (Sampson and Jansen, 1984), the milk yield was measured from PND4 to PND13. On each day, the milk yield was collected twice a day at 8:00 and 14:00, with an interval of 6 h. After 4 h of fasting, pups were weighed before returning to their dams (the total weight was defined as g1). Following a 2 h of suckling, pups were again weighed (the total weight defined as g2). The daily weight gain of pups was calculated by the differences in the weight of the pups at 8:00 (g2-g1) and 14:00 (g4-g3), respectively. The process was repeated for 10 days. The milk yield during the 2 h suckling was calculated according to the following Eq. (2):

$$\text{Milk yield} = \frac{(g2 - g1) + (g4 - g3)}{2} \quad (2)$$

3.4. Food and water intake of the rats after oral exposure to TiO_2 NPs

These data were collected from PND4 to PND13, food, and water consumed by dam rats were recorded during the period of 24 h.

4. Evaluation of oxidative stress markers in mammary glands

4.1. Malondialdehyde (MDA)

For MDA analysis, the mammary glands were harvested after sacrifice and were immediately frozen and stored at −80 °C. The mammary gland tissues were weighed, homogenized in ice-cold PBS with a tissue homogenizer (Bio-Gen PRO200 Homogenizer, Oxford, CT, USA), and the breast homogenates were centrifuged at 3000 g for 15 min at 4 °C. After centrifugation, and the supernatant was collected and subjected to MDA level analysis using the MDA kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

4.2. ROS staining

The ROS level was measured by dihydroethidium (DHE) assay. DHE could be oxidized by intracellular ROS to form ethidium oxide, which intercalates into DNA to generate red fluorescence.

5. The effects of dams' to TiO_2 NPs exposure on the nutrient quality of milk

Milk samples were collected from dams at PND19 in different groups. The dams were anesthetized using 10% chloral hydrate, and oxytocin was injected intraperitoneally. Then, the content of protein and lactose in the milk was determined.

5.1. The total protein in milk

The total protein was quantitatively measured using the Coomassie Brilliant Blue method (Bradford, 1976). Using a series of dilutions of bovine serum albumin (BSA) protein (0, 0.2, 0.4, 0.6, 0.8, 1 mL) solution, the calibration curve was prepared, and the protein content present in the milk was calculated by measurements of absorption at 595 nm.

5.2. The lactose content in milk

The lactose content in milk was measured according to established SN/T 0871–2000 method. The preparation of reagents: precipitant included 4.5% Barium hydroxide (GB 630–78, Shanghai, China) and 5% zinc sulfate (CNS:7446–20–0, Aladdin, AR, 99.5%); the chromogenic reagents were a mixture of 1% phenol (CNS:108–95–2, Aladdin, AR), 5% sodium hydroxide (CNS:1310–73–2, Aladdin, AR), 1% picric acid and 1% sodium bisulfate (Mackln, CAS: 7681–38–1) in a volume of 1:2:2:1; lactose solution (purity≥ 99). Briefly, harvested milk was centrifuged with 2 mL 4.5% Barium hydroxide and 0.5 mL 5% zinc sulfate at 2000 r/min in 2 min. Then the supernatant was collected, and 0.5 mL of supernatant was added to 2.5 mL chromogenic reagent (consisted of 1% phenol (CNS:108–95–2, Aladdin, AR), 5% sodium hydroxide (CNS:1310–73–2, Aladdin, AR), 1% picric acid and 1% sodium bisulfate (Mackln, CAS: 7681–38–1) in a volume of 1:2:2:1), the solution was heated in boiling water bath for 6 min. Finally, the absorbance was measured at 520 nm, and a standard curve was created with lactose samples of known concentrations.

6. Inductively coupled plasma atomic emission spectrometer (ICP-AES) analysis for Ti content

The mammary glands of maternal rats and milk, including colostrum (PND2) and mature milk (PND19), were collected for the analysis of the Ti content. Briefly, each sample was digested at 280 °C with the digestion solution (HNO_3: 10 mL; $HClO_4$: 2 mL) in a microwave oven until the solution dried out. The digested sample was diluted with 25 mL of ultrapure water. Using Indium (20 ng/mL) as an internal standard, the content of Ti was determined by the inductively coupled plasma-mass spectrometry (ICP-AES).

7. The effects of dams' TiO_2 NPs exposure on offspring via breastfeeding

To evaluate the effects of TiO_2 NPs on offspring, the bodyweight of pups, length of tail, and body were measured during the period of breastfeeding. Moreover, in this study, the hematological analysis and organ coefficient were also determined at different time points during lactation.

8. Quantitative real-time PCR (qRT-PCR)

For qRT-PCR, the mammary glands were harvested after sacrifice

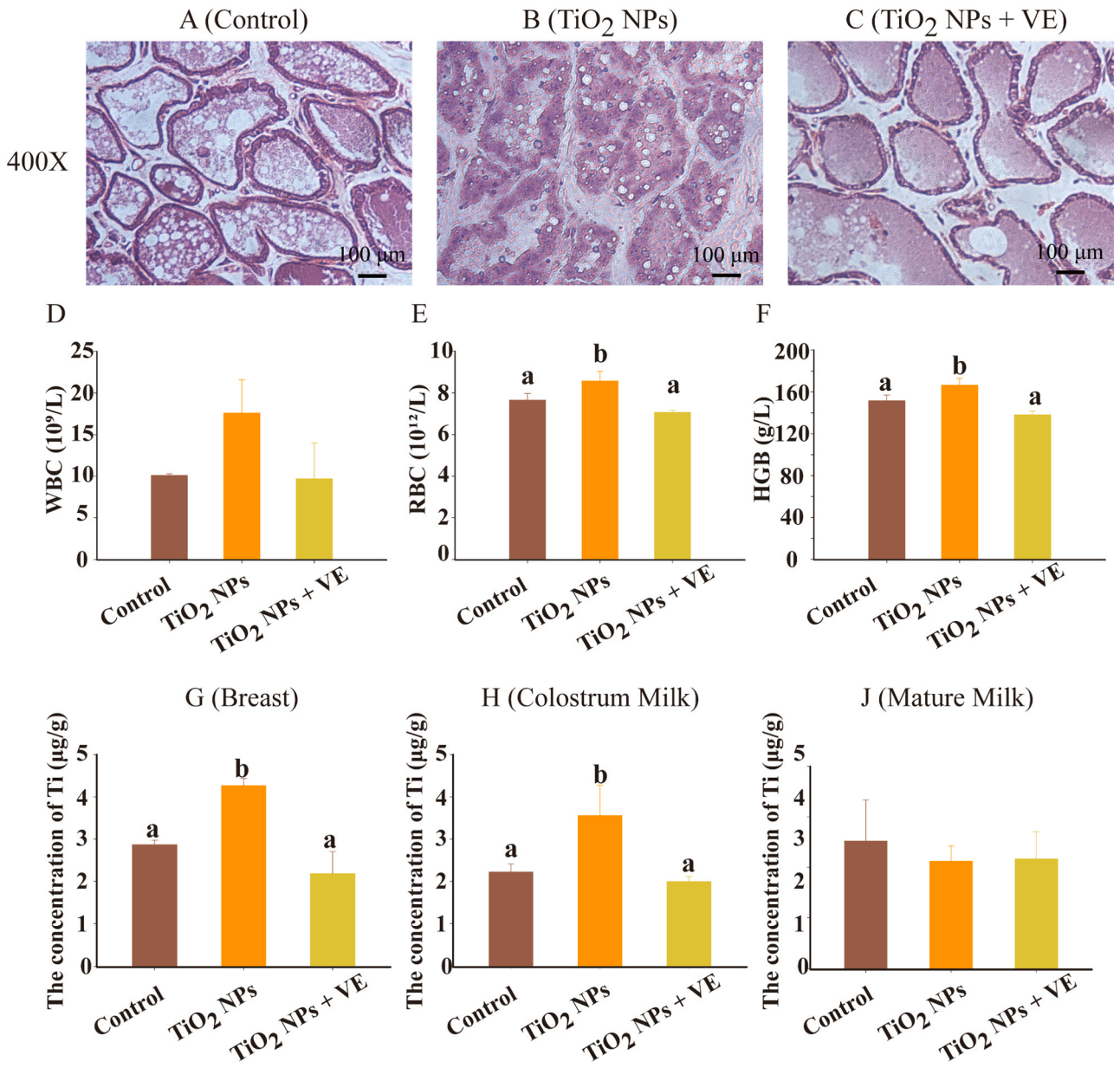

Fig. 2. Effects of TiO_2 NPs on pathological injury in dams. Mammary gland tissues from each experimental Group were obtained, sectioned, and stained with HE (original magnification 400 ×). Mammary gland tissues of (A) Control (B) TiO_2 NPs (C) TiO_2 NPs + VE. Blood biochemical characteristics of dams as evaluated on PND19. (D) WBC, (E) RBC and (F) HGB. The distribution of TiO_2 NPs following oral exposure during lactation, the breast (G) and mature milk (J) were collected on the PND19, the colostrum (H) was collected on the PND2. b compared to a, $P < 0.05$; ab compared to a and b, $P > 0.05$; c compared a and b, $P < 0.05$. Data were presented as mean ± SD.

and stored in RNA stabilization Reagent at −80 °C. The total RNA was extracted using the total RNA miniprep kit (Axygen Scientific, Union City, CA, USA) according to the manufacturer's protocol. The purity and concentration of the RNA samples were determined with NanoDrop® ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, United States). cDNA was synthesized using the Takara PrimeScript™ RT reagent kit (Cat#RR047A, Lot#AK2802). The primers (Table S4) were synthesized from Qingke (Huna, China). qRT-PCR was performed to determine gene expression of target genes using the TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) on the 7900HT fast real-time System (Applied Biosystems, Foster City, CA, USA). The qRT-PCR was performed by initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 1 min, and annealing at 72 °C for 38 s. The $2^{-\Delta\Delta CT}$ method was used to determine relative gene expression, which was normalized to the amount of the reference gene *GAPDH*. All experiments were performed at least in triplicate for each gene.

9. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Differences between groups were assessed using the analysis of variance (ANOVA) followed by the least-significant difference (LSD) test. All statistical analyses were performed using SPSS Statistics software version 22.0 (SPSS, Inc., Chicago, IL, USA). $p < 0.05$* and $p < 0.01$** were considered statistically significant.

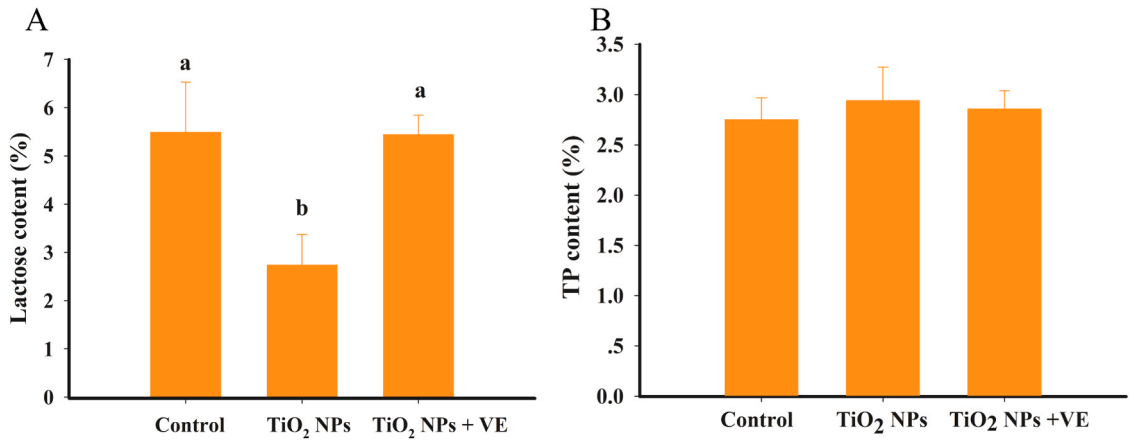

Fig. 3. The effect on the milk composition following oral exposure to TiO_2 NPs (from PND1 to PND20). (A) The lactose content was measured on the PND19, (B) The TP content was measured on the PND19 using the Coomassie Brilliant Blue method. b compared to a, $P < 0.05$; ab compared to a and b, $P > 0.05$; c compared a and b, $P < 0.05$. Data were presented as mean ± SD.

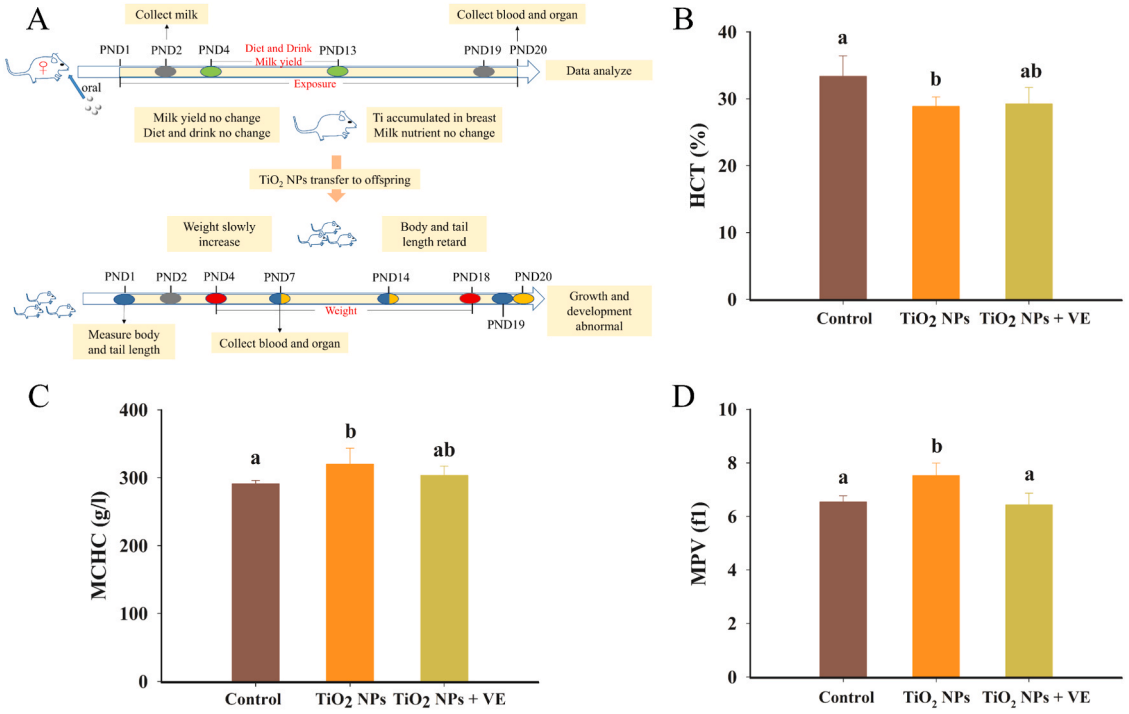

Fig. 4. The flow chart of experiment (A) and the change of blood biochemical characteristics of pubertal rats was evaluated on PND19. (B) HCT, (C) MCHC and (D) MPV. b compared to a, $P < 0.05$; ab compared to a and b, $P > 0.05$; c compared a and b, $P < 0.05$. Data were presented as mean ± SD.

10. Result

10.1. Acute and subacute Toxicity of the TiO_2 NPs in the lactating dams

After oral administration with TiO_2 NPs for 20 consecutive days (from PND1 to PND20), the effect of water and diet did not show significant difference among the three groups (Tables S2 and S3); similarly, the milk yield and organ coefficient of dams indicated no significant differences (Fig. 1, Table S1 and Fig. S1). The blood biochemistry data of dams revealed that RBC and HGB exhibited increasing trends in Group II compared to the Group I and Group III (Fig. 2E, F and Table S5).

To evaluate pathological effect of TiO_2 NPs on the mammary glands, the five pair mammary gland were harvested and stained with HE. Apparent histopathological changes were observed in Group II (Fig. 2A–C), after exposure of TiO_2 NPs, the alveolar wall was thickened, and the diameter of alveolar was significantly reduced compared to the Group I. The postpartum exposure to TiO_2 NPs severely impaired cell membrane structures.

10.2. Transmission of TiO_2 NPs from exposed dams to offspring through milk

To investigate whether the TiO_2 NPs could be transferred to the offspring through the breastfeeding, the Ti content in the mammary glands and milk were determined (Fig. 2G–J). In Group II, the Ti content was significantly higher in the mammary glands compared to Group I (Fig. 2G). Similarly, in the colostrum, Group II also presented a higher tendency of Ti content compared to Group I (Fig. 2H). However, interestingly, in the mature milk, the Ti content of Group II was presented at normal levels compared to Group I (Fig. 2J). It was noted that the

content of Ti in intestine and stomach of infants were significantly increased compared to the Group I (Fig. S2). Taken together, these findings indicated that oral exposure to TiO_2 NPs in lactating dams promoted its accumulation in the mammary glands and facilitated its transfer to offspring through the milk during feeding.

10.3. Effect of dams' exposure to TiO_2 NPs oral on the composition of milk

As TiO_2 NPs are accumulated in the mammary glands of dams and milk, causing damages to the mammary glands; therefore, the composition of the milk was also assessed to evaluate the effect of TiO_2 NPs exposure. In this study, the total protein (TP) was no significantly altered after different treatments (Fig. 3B); however, the lactose content was significantly lower in Group II (Fig. 3A) compared to Group I. The lactose content of milk returned to normal after VE treatment compared to the Group II.

10.4. Effects of dams' exposure to TiO_2 NPs on growth and development of offspring

Next, we investigated the adverse effects on offspring following dam exposure to TiO_2 NPs during lactation. The relative indicators of growth and development were measured, such as Hematological Analysis, Body weight, Body length, Tail length and Organ Coefficient. The level of HCT, MCHC, and MPV was significantly altered (Fig. 4 and Table S6). As compared to group I, the levels of MCHC and MPV were significantly upregulated in the group II, and the levels of HCT were significantly downregulated. Besides, the body length and tail length were gradually decreasing following TiO_2 NPs exposure at PND1, PND7, PND14, and PND20 (Fig. 5A, B, C, D); however, the effects returned to normal after treatment with VE. The results of the organ coefficient were significantly abnormal (Fig. 6). On PND7 (Fig. 6A), the organ coefficients of the heart and liver were significantly downregulated compared with Group I, and after treatment with VE, the organ coefficient presented trend towards recovery. The organ coefficient of liver and spleen were significantly upregulated in Group II compared to the Group I after 14d exposure (Fig. 6B), the organ coefficient of Group III was significantly ameliorated. On the PND20 (Fig. 6C), the organ coefficient of lung and brain of the Group II presented a significant upregulation compared to the Group I. At the same time, the newborn body weights were also recorded (Fig. 6D), the body weights after exposure to TiO_2 NPs were found to be lower compared to the Group I from PND4 to PND18.

10.5. Expression of oxidative stress related genes and protein markers

In order to elucidate the possible molecular mechanisms of blood-milk barrier destruction, the expression of genes related to oxidative stress and marker genes was determined. The expression levels of MDA and ROS, as markers of the cause of oxidative stress, were determined. The level of ROS and MDA presented a significant upregulation in Group II; however, after treatment with VE, these markers showed a significant downregulation (Fig. 7B, C). The results implied that the TiO_2 NPs could induce oxidative stress in the maternal mammary gland. To further elucidate the underlying mechanism, the expression levels of genes related to oxidative stress were determined using qRT-PCR (Fig. 7A). The genes involved in the Nrf-2/HO-1 antioxidative pathway were analyzed, the level of *Nrf-2* was significantly upregulated in the Group II compared to Group I. Furthermore, the gene expression of *HO-1* presented a significant decrease after treatment with the TiO_2 NPs, and the expression was recovered in the Group III. The expression levels of genes related to Tight junction (*MLCK* and *OCLN*) in the Group II were also

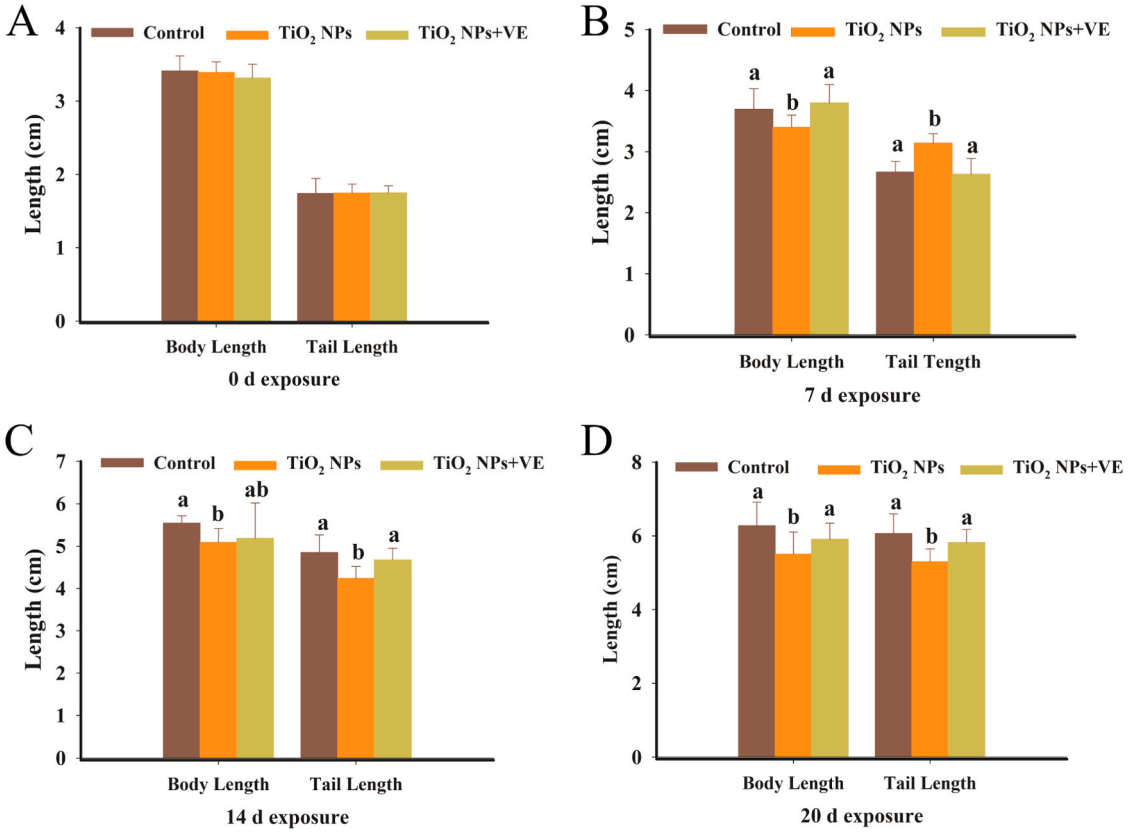

Fig. 5. The change in body length and tail length following different exposures during the lactation period. The body length and tail length were measured on exposure day 0 (A), 7(B), 14(C), and 20 (D). b compared to a, $P < 0.05$; ab compared to a and b, $P > 0.05$; c compared a and b, $P < 0.05$. Data were presented as mean ± SD.

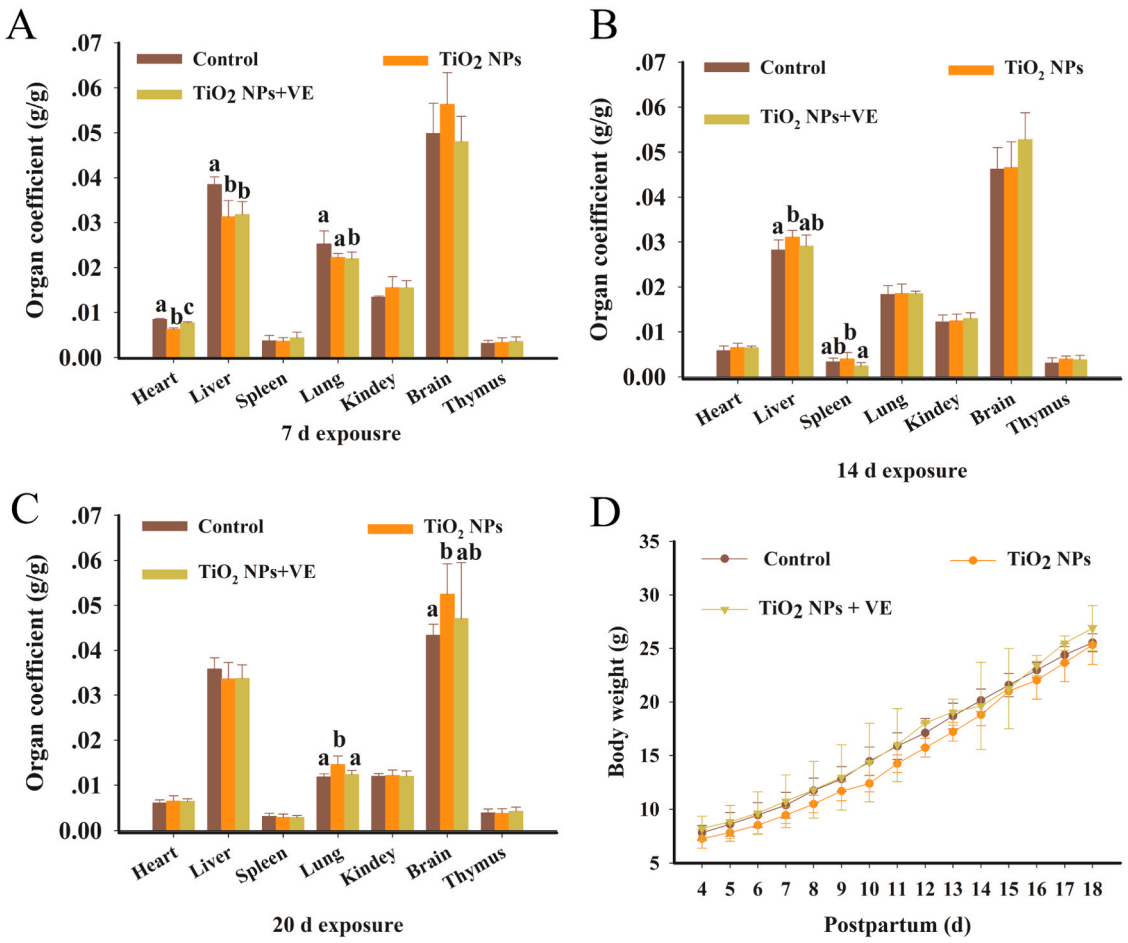

Fig. 6. The toxic effects on the growth and development of offspring following different treatments. (A) The organ coefficient was assessed on the PND7; (B) The organ coefficient was assessed following 14 days exposure; (C) The organ coefficient was measured following 20 days exposure; (D) The change in weight data were collected from PND4 to PND18. b compared to a, $P < 0.05$; ab compared to a and b, $P > 0.05$; c compared a and b, $P < 0.05$. Data were presented as mean ± SD.

found to be significantly increased compared to the Group I. However, the expression of *CLDN3* was significantly downregulated after TiO_2 NPs exposure in the Group II. The transcript levels of *EGF* and *α-Lactalbumin* showed no alteration, indicating that the nutritive value of milk did not change.

11. Discussion

TiO_2 NPs are produced abundantly and used extensively as colorant for food, medications, toothpaste, paints, and cosmetics because of their unique properties, including high stability, whiteness, and brightness, antibacterial and photocatalytic properties. An increasing number of studies have suggested that oral exposure is one of the most prevalent routes of exposure as TiO_2 NPs are added to a variety of food products, liquid beverages, and drugs (Talamini et al., 2019), which significantly increases the risk of exposure to vulnerable populations, such as lactating females. Thus, the present study aimed to assess the adverse effect of exposure to TiO_2 NPs on the blood-milk barrier in the maternal dam mice and the development of offspring during the lactation period.

Change in weight, the consumption of diet and drinking water, are considered as basic parameter to assess the levels of toxicity. During the period of exposure, the effect of diet and drinking water on the dams were normal from PND4 to PND13 (Tables S2 and S3), which were consistent with the reports of Kim et al. (2008) and Gromadzka-Ostrowska et al. (2012). The body weight of dams was not recorded because of the weight body weight fluctuations. Additionally, the milk yield (Fig. 1 and Table S1) also did not change considerably among the three groups (Cai et al., 2019), suggesting that TiO_2 NPs did not alter the milk yield. However, on hematological analysis, the levels of RBC and HGB showed an increasing trend between Group I and Group II (Fig. 2E and F). According to the report, we can infer that pathological injury improved the level of RBC and HGB (De Jong et al., 2019). And, WBC levels showed an increased trend on the Group II, although it had no difference among these group, which indicated inflammation may be presented (Fig. 2D). Mammary glands are the essential organs that, in the female mammals, produce milk for the sustenance of the offspring. After exposure to TiO_2 NPs, the alveolar wall thickened, the alveolar diameters were also significantly reduced, and the cell membrane structures were also severely damaged (Guo et al., 2019; Zhang et al., 2015). Taken together, the findings suggested that TiO_2 NPs could damage the structure of the mammary gland during lactation.

Several studies have demonstrated that nanoparticle could transfer from dams to offspring. Yang et al. (2018) found that quantum dots (QDs) could be transferred to breast milk via blood circulation, then could be transmitted to offspring via breastfeeding, leading to the abnormal development of offspring. Morishita et al. (2016) found that both intravenously and orally administered Ag nanoparticles were accumulated in the breast milk and then transferred to the pups, although the retention of Ag in the infants, the development of offspring showed no significant changes. In this study, we showed that in group II, the Ti concentration was significantly higher than Group I, suggesting that orally administered TiO_2 NPs could accumulate in the mammary gland via blood circulation (Zhang et al., 2014; Zhang et al., 2015). In the colostrum (PND2), the Ti concentration in Group II was significantly

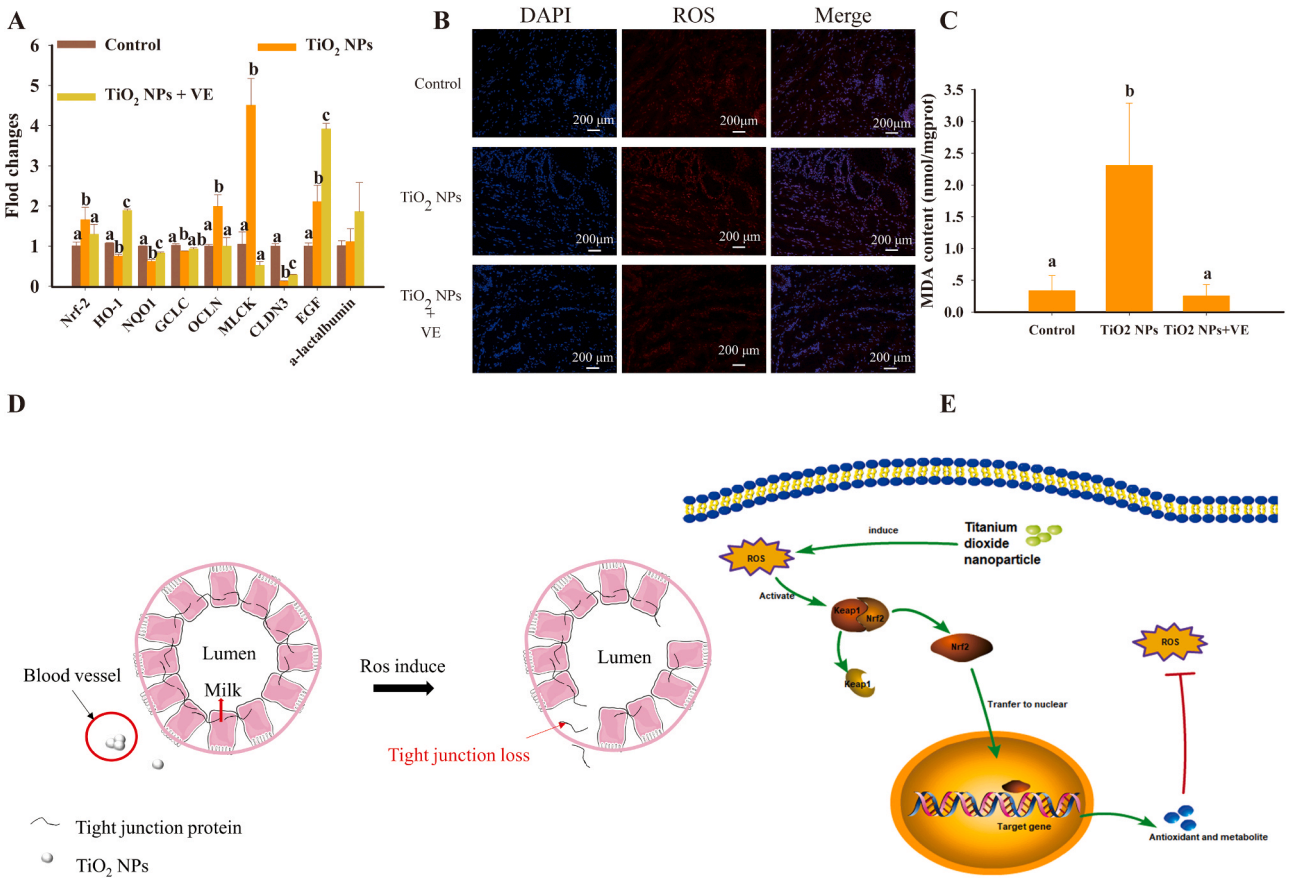

Fig. 7. The oxidative stress caused dysfunctional blood-milk barrier. (A) The expression of genes related to oxidative stress, Tight junction, and milk production was determined. (B) The levels ROS generation in mammary gland were also determined following different treatments. The nuclei of normal cells presented as blue (DPAI), the ROS presented as red. (C) The MDA content in mammary glands was determined on PND20. b compared to a, $P < 0.05$. Data were presented as mean ± SEM. The possible mechanism underlying toxicity (D) TiO_2 NPs accumulated in the mammary gland via blood circulation, leading to a dysfunctional blood-milk barrier. (E) ROS activated the Nrf-2/Keap1 signaling pathway. Data were presented as mean ± SEM. b compared to a, $P < 0.05$; c compared a and b, $P < 0.05$. Data were presented as mean ± SD.

upregulated (Fig. 2H). Interestingly, in mature milk (PND19), the level of Ti content did not increase significantly in Group II (Fig. 2J). It is well-recognized that the lactation cycle of a rat is about 21 days, and there is still an adequate amount of milk that could be collected on the PND19 (Nagasawa, 1979). In this study, the decreasing concentration of Ti was noted in the milk, which may be not attributed to the deficiency of milk yield at the end of the lactation period. According to the report by Morishita (Morishita et al., 2016) et al., TiO_2 NPs can be more easily transferred to breast milk in the early lactation period than in the late lactation period. At the same time, we found the TiO_2 NPs accumulated in the intestine and stomach of infants (Fig. S2). Collectively, the data suggested that TiO_2 NPs could be transferred to offspring via breastfeeding.

Breast milk remains the preferred mode of feeding infants during the lactation period, and the components of milk play significant roles in the growth and development of pups (Bautista et al., 2019). Epithelial growth factor (EGF) has crucial roles in promoting the growth of the gut and various epidermal/epithelial tissues, which also prevent bacterial infection (D'Alessandro et al., 2010). Lactose is the most common whey protein, which provides protective functions to the infants (Suri et al., 2019). The total protein as an essential constituent of the milk did not show any significant changes and the gene expression of *EGF* was de novo expression. Suggesting that oral exposure to TiO_2 NPs did not change the nutrient quality of breast milk. It was noted that the lactose content of TiO_2 NPs was decreased compared to the control group at the PND19 (Fig. 3A). While, the level of *α-Lactalbumin* also did not present any significant alteration in its expression in this study, which plays a crucial role in the synthesis of lactose (Kessler et al., 2019). The results were consistent with previous reports (Cai et al., 2019), we could infer that the synthesis of lactose was normal. The process of synthesis of lactose was normal, and the lactose content of milk reduced. Why? Stelwagen (Stelwagen et al., 1995) et al. have explained that when the blood-milk barrier was dysfunction, the level of lactose in the milk would decrease, and the element of Na, K, and CL in the breast milk can increase. The reduce of lactose transferred to the blood from milk, and Na of blood may be transferred to the milk via the damaged blood-milk barrier. Consistently, Fig. 3A and Table S4 represented that the content of lactose and Na in milk decreased and increased, respectively, in this study. These findings suggested that the blood-milk barrier dysfunction may be occurred after the oral administration of TiO_2 NPs. Next, we would confirm the hypothesis that the blood-milk barrier may be damaged by TiO_2 NPs.

Blood-milk barrier, as a basic structure of mammary gland (Guo et al., 2019), comprised of the tight junction of epithelial cells (Lee et al., 2019a, 2019b). *OCLN* represents a vital tight junction protein, and its destruction directly indicates the injury of the tight junction between epithelial cells (Bendriem et al., 2019). In this context, Brun et al. (2014) found that nanoparticle could injury the tight junction with the high expression of the *OCLN* gene. Furthermore, Kazakova et al., (2020) suggested that *MLCK* plays a vital role in maintaining the function of the endothelium barrier. Besides, the increased levels of *MLCK* may cause actin contraction and open the paracellular pathways. The findings of the present study also revealed a damaged tight junction and dysfunctional blood-milk barrier due to dams' exposure to TiO_2 NPs during the

lactation period (Liu et al., 2018). At the same time, Martínez et al. (2012) found that some CLDNs family protein can strengthen the function of a barrier, including the *CLDN-3*. The downregulation of *CLDN-3* gene expression in our study also suggested that the barrier was impaired. Collectively, these findings (the lactose content and Na content in the breast milk and gene expression of genes related to the tight junction) indicated that the blood-milk barrier was damaged after oral exposure to TiO_2 NPs during the lactation period. Overall, TiO_2 NPs would damage the mammary gland and blood-milk barrier, while the process of milk synthesize and secretion were not influenced.

To assess the biological effects of TiO_2 NPs transferred into offspring via the impaired blood-milk barrier, the bodyweight of offspring breastfed by dams that received TiO_2 NPs was recorded and results revealed that the bodyweight of these offspring was always lesser than Group I. Hematological parameters determined at the PND20, revealed that hematological markers, including HCT, MCHC, and MPV of the Group II were significantly different from that of group I. Yan et al. (2012) suggested that the decreased HCT level may be related to the symptoms of anemia; the increase of MCHC could be attributed to RBCs hemolysis (Suckow et al., 2012); together Sun et al. (2014) found the level of MPV may be decreased or increased during the inflammation. Considering these studies, we inferred that the development of infants was impaired. The data on body and tail length were also collected at different times; which indicated that during lactation, the body and tail length in filial offspring of the Group II was significantly retarded compared to the Group I, the result was consistent with Fu et al. (2015) Organ index was the most sensitive marker of an effect of toxicity, as significant differences in organ weight of experimental animals may occur when any morphological changes were presence. To explore side effects in the newborn, the organ index was collected at the PND7, PND14, and PND20. It is well known that increased spleen coefficient is associated histologically with serious congestion in red pulp, the enhancement of extramedullary hematopoiesis and reduction of lymphoid follicles in white pulp (Piao et al., 2013), which was association with our results. According our data, the liver coefficient was increased, indicates that oral the TiO_2 NPs cause dropsy, congestion and inflammation of the infant liver, which may have led to the changes seen in the liver coefficient (Magaye et al., 2014). And the lung coefficient is considered as sign of pulmonary edema (Yu et al., 2015). The abnormal coefficient indicated that the lung may be damaged. The heart coefficient of the TiO_2 NPs group was significantly lowered, indicated that an adverse effect of TiO_2 NPs exposure on heart development (Huang et al., 2017). Brain coefficient plays an important role in evaluation of the brain and nerve injury (Ma et al., 2010). Given that the brain coefficients have increased after TiO_2 NPs treatments, there may have some adverse effects such as congestion, edema, or hypertrophy in the brain of infant after breastfeeding from the maternal rats injected TiO_2 NPs (Cao et al., 2013). Those results demonstrated that the development of infant organs was impaired throughout the lactation period. Thus, growth and development of offspring were significantly impaired because of the transmission of TiO_2 NPs through breastfeeding, but not due to the yield and composition of milk.

Next, we investigated the possible mechanism underlying the blood-milk barrier dysfunction. Several studies have reported that the generation of oxidative stress by TiO_2 NPs was predominant reasons behind tissue damages (Hu et al., 2019; Zhou et al., 2019). MDA, as an indicator of oxidative stress (Li et al., 2019), was used to reflect the activation of oxidative stress in this study. ROS dissociates the Nrf-2 from its inhibitor Keap1 (Park et al., 2019), the dissociated Nrf-2, then translocated to the nuclei and activates target genes, including *HO-1*, *NQO-1,* and *GCLC* (Shin et al., 2019). In this study, the MDA content was significantly upregulated in group II (Fig. 7C), and the fluorescence intensity of ROS was also increased significantly (Fig. 7B), which suggested that the oxidative stress might have occurred in the mammary gland (Wang et al., 2018; Fadda et al., 2019). To further verify that TiO_2 NPs induce oxidative stress in the mammary gland, the expression level of genes related to oxidative stress was determined. The expression level of *Nrf-2* was significantly increased when dams were exposed to TiO_2 NPs, indicating that the *Nrf-2* antioxidant signal pathway was activated to protect the cells from oxidative stress (Li et al., 2016). *NQO-1* participates in detoxification (Shin et al., 2019), and its downregulation is always accompanied by the overproduction of ROS (Kandeil et al., 2019). *HO-1*, as a cytoprotective enzyme, can regulate antioxidative responses (Yokoji-Takeuchi et al., 2020). *GCLC,* as a rate-limiting enzyme in the process of *GSH* synthesis (Zhang et al., 2019), represents a crucial organ antioxidant. In this study, the lower expression of *HO-1* and *GCLC* genes indicated the process of antioxidation was impaired. Collectively, these findings indicated that the predominant mechanism underlying the impaired blood-milk barrier system was the production of oxidant stress.

Overall, the study suggested that TiO_2 NPs could induce oxidant stress, leading to an impaired blood-milk barrier. To further verify that the oxidant stress was the main mechanism of the induced-toxicity, oral administration of TiO_2 NPs was supplemented with VE. VE exhibits excellent antioxidant and anti-inflammatory properties. Moradi et al. (2019) found that VE can mitigate the toxicity of oxidative stress, which was induced by TiO_2 NPs. We found that after treatment with VE, the adverse effects were significantly ameliorated, such as the toxicity to dams was alleviated, and the development of infants was improved. However, the toxicity of TiO_2 NPs cannot be completely alleviated, suggesting that TiO_2 NPs activated the other mechanism of toxicity besides oxidant stress. Thus, further studies are warranted to explore other putative mechanisms.

12. Conclusion

Overall, the study presented the deleterious pathological effects of oral exposure to TiO_2 NPs in dams and developmental concerns in the offspring. The results indicated that exposure to TiO_2 NPs could cause severe pathological damages to the mammary glands. The excessive accumulation of TiO_2 NPs in mammary glands did not cause any change in the nutrition of breast milk but could be transferred to offspring through an impaired blood-milk barrier. Additionally, the accumulation of TiO_2 NPs in newborns can negatively influence their growth and development. Notably, the study also deduced that the oxidative stress was the predominant toxic mechanism associated with impaired blood-milk barrier system following exposure of TiO_2 NPs (Fig. 7D and E). To further verify this conjecture, VE was used in supplementation with TiO_2 NPs, and results revealed that the toxicity induced by TiO_2 NPs was rescued, indicating that the use of VE could ameliorate the deleterious effects of TiO_2 NPs exposure during the lactation period. Although further investigations are warranted, this study provided the theoretical foundation for the toxic effects of TiO_2 NPs exposure in dams during lactation.

CRediT authorship contribution statement

Liyang Yao: Conceptualization, Validation, Formal analysis, Writing - original draft. **Ling Chen:** Investigation, Writing - review & editing. **Bolu Chen:** Investigation, Writing - review & editing. **Yizhou Tang:** Validation, Writing - review & editing. **Yu Zhao:** Formal analysis, Writing - review & editing. **Shanji Liu:** Software, Investigation. **Hengyi Xu:** Methodology, Supervision, Funding acquisition, Writing - review & editing.

Associated content

Supporting Information: The milk yield following oral administration to different treatments (Table S1). The effect of diet and drink on maternal dams after oral exposure (Table S2, 3). The content of Cl, Na and K on the mature milk (Table S4). Blood biochemical characteristics of dams and pubertal rats (Table S5, 6). Genes and Primers selected for

RT-qPCR (Table S7). The effect on organ coefficient of maternal rats after different treatment (Figure. S1).

Data availability

The data used to support the findings of this study are included within the article.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111762.

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