Titanium dioxide nanoparticles oral exposure to pregnant rats and its distribution

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Abstract

Background: Titanium dioxide (TiO2) nanoparticles are among the most manufactured nanomaterials in the industry, and are used in food products, toothpastes, cosmetics and paints. Pregnant women as well as their conceptuses may be exposed to TiO2 nanoparticles; however, the potential effects of these nanoparticles during pregnancy are controversial, and their internal distribution has not been investigated. Therefore, in this study, we investigated the potential effects of oral exposure to TiO2 nanoparticles and their distribution during pregnancy. TiO2 nanoparticles were orally administered to pregnant Sprague-Dawley rats (12 females per group) from gestation days (GDs) 6 to 19 at dosage levels of 0, 100, 300 and 1000 mg/kg/day, and then cesarean sections were conducted on GD 20.

Results: In the maternal and embryo-fetal examinations, there were no marked toxicities in terms of general clinical signs, body weight, food consumption, organ weights, macroscopic findings, cesarean section parameters and fetal morphological examinations. In the distribution analysis, titanium contents were increased in the maternal liver, maternal brain and placenta after exposure to high doses of TiO2 nanoparticles.

Conclusion: Oral exposure to TiO2 during pregnancy increased the titanium concentrations in the maternal liver, maternal brain and placenta, but these levels did not induce marked toxicities in maternal animals or affect embryo-fetal development. These results could be used to evaluate the human risk assessment of TiO2 nanoparticle oral exposure during pregnancy, and additional comprehensive toxicity studies are deemed necessary considering the possibility of complex exposure scenarios and the various sizes of TiO2 nanoparticles.

Keywords: Titanium dioxide nanoparticles, Developmental toxicity, Maternal and fetal distribution, Nanotoxicity

Introduction

Nanotechnology is a rapidly growing field in recent decades and is widely applied in various areas of industry [1]. The use of nanotechnology extends to cosmetics, fabrics and clothing, personal care items, cleaning solutions, sporting equipment and electronics as well as toys for children [2, 3]. Nanomaterials comprise natural, incidental or manufactured material-containing particles with one or more external dimensions in the size range of 1 nm – 100 nm [4]. The size-dependent properties of nanomaterials increase the surface-to-interaction, the possibility of improper interactions with intracellular components and unusual electronic properties, such as electron donation or acceptance [5]. These characteristic properties raise concerns regarding the potential health risk to humans and livestock, as well as the environment [6, 7].

Titanium dioxide (TiO2) nanoparticles are also widely used nanomaterials and are among the top five nanomaterials used in consumer products [8]. TiO2 nanoparticles are used in paints, coatings, plastics, papers, inks, medicines, pharmaceuticals, food products, cosmetics and toothpastes [9–11]. The constant use of TiO2 nanoparticle-containing products increases the possibility of chronic exposure and accumulation in the...
internal organs of humans. In particular, oral and respiratory exposures are considered the most prevalent exposure routes to humans [12]. Oral exposure is an important route for absorption because water, liquid beverages and drug carriers may contain TiO₂ nanoparticles [13]. When TiO₂ nanoparticles (25, 80, and 155 nm; 5 g/kg; single oral dose in mice) enter the circulatory system via oral exposure, they are retained in the internal organs [14].

Several toxicity studies with TiO₂ nanoparticles have been recently conducted; however, there is little toxicological information on TiO₂ nanoparticle exposure during pregnancy. Shimizu et al [15] reported that subcutaneous exposure to TiO₂ nanoparticles (2570 nm; 100 μl suspended at 1 μg/μl) during gestation (gestation days [GDs] 6, 9, 12, and 15) in ICR mice induced changes in gene expression related to brain development, cell death, response to oxidative stress, and mitochondria in the brain during the prenatal period. Takeda et al. [16] reported that subcutaneous exposure of ICR mice to TiO₂ nanoparticles (25 to 70 nm; 16 mg/kg) during gestation (GDs 3, 7, 10 and 14) induced postnatal reproductive toxicities in male offspring, including disrupted seminiferous tubules and tubule lumens with few mature sperm, decreased sperm production and epididymis sperm motility. In addition, TiO₂ nanoparticles were detected in cells of the olfactory bulb and cerebral cortex in these postnatal animals. These previous studies indicated that TiO₂ nanoparticle exposure during pregnancy is able to induce toxic effects. However, the opposite result was also reported: oral exposure of six types of TiO₂ particles, including pigment grade and nanoscale (42, 43, 47, 153, 195 and 213 nm; 100, 300, and 1000 mg/kg; daily, beginning on GDs 6 through 20 in rats), did not induce maternal and embryo-fetal developmental toxicities [17].

The objective of this study was to confirm the maternal and embryo-fetal toxicities of orally exposed TiO₂ nanoparticles during pregnancy. In addition, we also analyzed the internal concentration of titanium in maternal and fetal tissues. The results of this study will contribute to elucidating the potential effects of TiO₂ nanoparticles on humans and support the accurate risk assessment of these nanoparticles at different sizes and under complex exposure scenarios.

Materials and methods

TiO₂ nanoparticles and physicochemical characterization

TiO₂ nanoparticles were obtained from Evonik Industries (Germany) as a fine white powder with a hydrophilic characteristic caused by hydroxyl groups on the surface. The nanoparticles consisted of aggregated primary particles; the mean diameter of the primary particle was approximately 21 nm, and the weight ratio of anatase/rutile was approximately 80/20 according to the manufacturer’s information.

Physicochemical characterization of TiO₂ nanoparticles was confirmed with an additional analytical method. The primary particle size and morphology were analyzed by a transmission electron microscope (JEM-2100F, JEOL, Japan) operating at 200 kV. TiO₂ NPs for transmission electron microscope (TEM) analysis were deposited on carbon-coated nickel mesh grids and were air-dried overnight before analysis. The purity was also analyzed with energy-dispersive X-ray (EDX) analysis on the same TEM images (JEM-2100F TEM equipped with an X-Max® 150 mm² silicon drift detector, Oxford Instruments, UK). The average primary particle size was calculated by measuring at least 100 particles using an image analyzer program (DigitalMicrograph, Gatan Inc., USA). The hydrodynamic diameter and zeta potential of TiO₂ nanoparticles in deionized water (10 mg/ml concentration) were analyzed by the dynamic light scattering (DLS) method (ELS-8000, Otsuka Electronics, Japan).

Animals and experimental design

Nine-week-old specific pathogen free (SPF) female Sprague-Dawley rats were obtained (Orient Bio Inc., Republic of Korea) and permitted a 5-days period of acclimation to the animal room environment. Females were selected for mating on the basis of adequate body weight and freedom from clinical signs of disease or injuries during the acclimation period. Females were mated by placement in the cage of a male that was maintained only for mating without any treatment. Sixty-four mating-proven female rats were selected for this study. The day of sperm and/or vaginal plugs detection was designated as day 0 of gestation. Pregnancy was determined by confirmation of implantation sites on the uterus at the time of final sacrifice.

The animal room environment was automatically controlled according to institutional criteria (target range: temperature of 23 ± 3 °C, relative humidity of 30–70%, approximately 12-h light cycle with 150–300 Lux, and ventilation at 10–20 times/hour). A standard rodent pellet diet irradiated by gamma-ray (PMI Nutrition International, USA) was provided to the animals ad libitum. Titanium was not detected in the rodent pellet diet according to the chemical composition results from the supplier. The animals had ad libitum access to filtered, ultraviolet light-irradiated municipal tap water at all times. Aspen animal bedding material (Bio Lab, Republic of Korea) was sterilized and then provided to the animals in each cage. There were no known contaminants in the food, water and bedding at levels that would be expected to interfere with the results of the study.
TiO$_2$ nanoparticles were suspended in deionized water for administration via the gastrointestinal route. To obtain a homogenized suspension, the dosing formulation was continuously stirred with a magnetic stirrer during the dosing procedure. TiO$_2$ nanoparticles were administered by oral gavage to mated females to evaluate the potential maternal and embryo-fetal development toxicity of TiO$_2$ nanoparticles. This study design refers to the OECD Guideline 414 (Prenatal Developmental Toxicity Study) [18] and was carried out in a good laboratory practice (GLP) facility but was not conducted within the scope of GLP regulations. In addition, we also analyzed the internal distribution of titanium in maternal and fetal tissues after repeated oral exposure during pregnancy. Twelve females per group in the toxicology group (total 48 females) and 4 females per group in the tissue distribution group (total 16 females) were used in this study. TiO$_2$ nanoparticles were administered daily by oral gavage from GDs 6 to 19 at dose levels of 0, 100, 300 and 1000 mg/kg with a dose volume of 10 mL/kg.

All procedures with animals were in compliance with the Animal Protection Act of Korea and the Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research (ILAR). The Korea Institute of Toxicology (KIT) received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) in 1998, which has been renewed regularly. This study was reviewed and assessed by the Institutional Animal Care and Use Committee (IACUC) of KIT.

**In-life maternal examinations**

A mortality observation was conducted twice daily (once at the start of the animal room procedure and once at the end of the animal room procedure). Observation of general clinical signs, including general appearance and behavioral changes, were conducted twice a day during the treatment period (before and after dosing) and once a day during the non-treatment period. During pregnancy, maternal animals were especially monitored for signs of abortion or premature delivery. Body weights and food consumption were measured individually on GDs 0, 6, 9, 12, 15, 17 and 20.

**Cesarean section and fetal morphological examinations**

On GD 20, all toxicology group females were euthanized using CO$_2$ gas to conduct the tissue collection. Maternal tissue collection (approximately 200 mg each), including liver (middle lobe), brain, and blood, was conducted. Fetal tissue collection (approximately 200 mg each), including liver, brain, blood and placenta, was conducted. At least 3 fetuses from a litter were used for fetal tissue collection, and collected samples were pooled by a litter. All collected samples were weighed to quantitatively calculate the tissue distribution and then maintained in frozen condition (approximately –80°C) until titanium content analysis.

For the evaluation of tissue levels of titanium, the samples were digested with a tri-acid mixture. The tri-acid mixture was prepared with concentrated hydrofluoric acid (HF, 49%, J.T. Baker, USA), nitric acid (HNO$_3$, 60%, Matsunoen Chemical LTD, Japan), and hydrogen peroxide (H$_2$O$_2$, 30%, J.T. Baker, USA) mixed in a ratio of 1:4:1, and 12 mL of this mixture was added to each Teflon reaction vessel containing a sample [24–26]. Thereafter, the samples with mixed acid were heated on a graphite digestion system (ODLAB, OD-98-002P, Republic of Korea) for 1 h, and digested residues were made up to
10 mL with 1% (v/v) HNO₃ (pH = 1–2). The final samples were stored at −4 °C before analysis.

Titanium distribution analysis
Inductively coupled plasma mass spectrometry (ICP-MS, ELAN DRC II, Canada) was used to measure titanium concentrations in the collected samples. Instrumental operating conditions were as follows: 1500 W of radiofrequency (RF) power, 0.9 L/min of nebulizer gas flow rate, and 1.5 L/min of auxiliary gas flow rate. Calibration standards of 1, 5, 10, 20, and 40 μg/L for titanium (1000 mg/L, Merck, Germany) were used, and coefficients of determination (R²) for titanium were higher than 0.999. Blank samples, which consisted of solutions without the presence of tissue, were used for the assessment of contamination during the experiments. The digestion method was applied to blank samples in order to measure the likely amounts of titanium contamination. Teflon tubes, 15 mL polypropylene tubes, and chemicals were all potential sources of titanium contamination [26]. For instrumental detection limits (IDLs), 1 mL of the lowest level of calibration standard (1 μg/L) was injected into the ICP-MS seven times (n = 7), and a standard deviation of analytical data was multiplied by a Student’s t value of 3.14. For method detection limits (MDLs) and the limit of quantification (LOQ), 2 mL of 5 μg/L standard was spiked into the seven blank samples, and standard deviations were multiplied by 3.14 and 10, respectively. The final volume in each blank sample was 10 mL. ICP-MS was sensitive enough to quantify all of our samples, and there was no need to improve the IDL of 0.038 μg/L. The MDLs and LOQ for the collected samples were determined to be 0.0001 mg/kg and 0.0002 mg/kg, respectively. The values presented high sensitivity and a satisfactory recovery rate (96.5 ± 2.4%). Maternal samples for non-pregnant subjects were excluded from the concentration analysis, and two samples (one fetal blood at vehicle control and one maternal liver at 100 mg/kg) were excluded from the concentration analysis because they were considered to be contaminated.

Statistical analysis
Statistical analyses for comparisons of the various dose groups with the vehicle control group were conducted using the Pristima System (Version 7.2, Xybion Medical System Co., USA) or SAS/STAT (Version 9.4, SAS Institute Inc., USA). Litter data were statistically evaluated using the litter as a statistical unit. Multiple comparison tests for different dose groups were conducted. Continuous data were examined for variance in homogeneity using Bartlett’s Test. Homogeneous data were analyzed using analysis of variance (ANOVA), and the significance of intergroup differences was analyzed using Dunnett’s test. Heterogeneous data were analyzed using the Kruskal-Wallis test, and the significance of intergroup differences between the control and treated groups was assessed using Dunn’s rank sum test. One-way analysis of covariance (ANCOVA) was used to analyze fetal and placental weight data. The litter size was used as the covariate.

Results
Physicochemical characterization of TiO₂ nanoparticles
The physicochemical characterization of TiO₂ nanoparticles, including analyses of primary shape, primary size, purity, hydrodynamic size and zeta potential, is summarized in Table 1. The majority of the TiO₂ nanoparticles had spherical and anatase crystal shapes with a purity of 100%. The mean primary size of the TiO₂ nanoparticles was 17.8 ± 5.46 nm. The hydrodynamic size of the TiO₂ nanoparticles was 341.5 nm, which indicates that TiO₂ nanoparticles were prone to aggregation and formed a larger size in the vehicle. The zeta potential of the TiO₂ nanoparticles in the vehicle was 35.16 mV.

Mortality and general clinical sign observation
All female rats survived through the end of the study, and no abnormal general clinical signs were observed in any group throughout the study.

Body weights and food consumption
No test item-related changes in body weight and body weight gain were observed during the study period (Table 2). For food consumption, a statistically significant decrease during the study period (92% of control) at 1000 mg/kg was considered test item-related (Table 3). However, this decrease did not have toxicological relevance since it was minimal and there was no correlated decreased body weight or body weight gain during the study period.

Organ weights and gravid uterine weight
There was no test item-related change in absolute and relative organ weights in this study (Table 4). In addition, there was no test item-related change in gravid uterine weight, corrected terminal body weight (body weight on GD 20 minus gravid uterine weight) and net body weight change (corrected terminal body weight minus body weight on GD 6) in this study (Table 5).

Cesarean section and fetal morphological examinations
There was no test item-related change in cesarean section parameters, including corpora lutea, implantation, resorptions (early and late), dead and live fetuses, sex ratio, pre-implantation loss, post-implantation loss, fetal weight, placental weight and placental macroscopic observation (Table 6). In addition, there
was no test item-related change in the fetal external and visceral examinations (Table 7). In the skeletal examination, an increased ossification site of metatarsals in both hindlimbs was only observed at 100 mg/kg, but it was considered incidental since it did not have a dose response and there were no changes in other related parameters (Table 8).

### Table 1 Physicochemical characterization of titanium dioxide nanoparticles

| Physicochemical          | TiO$_2$ nanoparticles |
|--------------------------|-----------------------|
| Primary Shape (TEM Image)|                       |
| Primary Size             | 17.8 ± 5.46 nm        |
| Purity                   | 100%                  |
| Hydrodynamic Size        | 341.5 nm              |
| Zeta Potential           | 35.16 mV              |

### Table 2 Body weights and body weight gain of TiO$_2$ nanoparticles exposed pregnant females during the pregnancy

| Pregnant Females (N) | 0       | 100     | 300     | 1000    |
|----------------------|---------|---------|---------|---------|
| Body Weight (g)      |         |         |         |         |
| Gestation day 0      | 235.4 ± 8.88* | 235.4 ± 8.93 | 235.6 ± 9.19 | 235.5 ± 9.70 |
| Gestation day 6      | 275.5 ± 12.08 | 277.4 ± 11.41 | 274.5 ± 10.31 | 276.8 ± 13.64 |
| Gestation day 9      | 288.1 ± 13.17 | 288.4 ± 12.59 | 286.6 ± 9.74 | 286.7 ± 14.86 |
| Gestation day 12     | 307.5 ± 14.00 | 307.3 ± 13.20 | 307.6 ± 10.38 | 306.3 ± 18.14 |
| Gestation day 15     | 323.5 ± 15.19 | 325.8 ± 15.92 | 326.7 ± 12.20 | 321.0 ± 19.40 |
| Gestation day 17     | 342.8 ± 17.30 | 346.9 ± 16.35 | 349.2 ± 11.13 | 341.4 ± 18.53 |
| Gestation day 20     | 389.3 ± 26.47 | 399.1 ± 22.46 | 400.1 ± 14.68 | 388.7 ± 21.15 |
| Body Weight Gain (g) |         |         |         |         |
| Gestation day 6–20 (treatment period) | 113.8 ± 16.81 | 121.7 ± 15.08 | 125.6 ± 9.16 | 111.9 ± 10.30 |

*Values are presented as mean ± S.D

### TiO$_2$ nanoparticle distribution in tissues

The titanium contents were analyzed in maternal tissues (liver, brain and blood) and fetal tissues (liver, brain, blood and placenta) after the oral exposure of TiO$_2$ nanoparticles during pregnancy (Fig. 1). Titanium concentrations in maternal liver, maternal brain and placenta at 1000 mg/kg were elevated compared to the
concentration in control animals. In addition, at 300 mg/kg, titanium concentrations in the maternal brain and placenta were also slightly elevated. Moreover, there was no titanium concentration change in the maternal blood, fetal liver, fetal brain or fetal blood.

**Discussion and conclusion**

Oral exposure to TiO$_2$ nanoparticles is one of the most prevalent exposure scenarios because humans are frequently exposed to TiO$_2$ nanoparticles contained in food products, liquid beverages and drugs [27, 28]. In this study, we evaluated the potential effects of oral exposure to TiO$_2$ nanoparticles during pregnancy and their distribution in maternal organs as well as fetuses. TiO$_2$ nanoparticles were administered by oral gavage to pregnant Sprague-Dawley rats at doses of 0, 100, 300 and 1000 mg/kg. In-life and terminal experimental endpoints, including general clinical signs, body weight

Table 3  Food consumption of TiO$_2$ nanoparticles exposed pregnant females during the pregnancy

| TiO$_2$ nanoparticles (mg/kg) | 0  | 100 | 300 | 1000 |
|-----------------------------|----|-----|-----|------|
| Pregnant Females (N)        | 12 | 12  | 12  | 12   |
| Food Consumption (g)        |    |     |     |      |
| Gestation day 0–6           | 24.3 ± 2.70$^{a}$ | 23.3 ± 1.95 | 24.0 ± 2.00 | 22.9 ± 1.55 |
| Gestation day 6–9           | 26.0 ± 2.60  | 25.4 ± 1.97  | 25.7 ± 1.44  | 23.7 ± 1.77$^{a}$ |
| Gestation day 9–12          | 26.6 ± 2.84  | 24.8 ± 1.50  | 26.0 ± 1.35  | 23.5 ± 2.70 |
| Gestation day 12–15         | 26.0 ± 2.19  | 25.6 ± 2.22  | 26.2 ± 1.70  | 24.1 ± 2.38 |
| Gestation day 15–17         | 27.1 ± 1.92  | 26.9 ± 2.37  | 28.0 ± 2.12  | 25.6 ± 2.52 |
| Gestation day 17–20         | 27.7 ± 2.43  | 27.1 ± 2.02  | 28.5 ± 2.05  | 26.2 ± 2.10 |
| Gestation day 6–20 (treatment period) | 26.7 ± 2.15 | 25.9 ± 1.73 | 26.8 ± 1.39 | 24.5 ± 2.04$^{**}$ |

$^{a}$Values are presented as mean ± S.D

**Table 4** Absolute and relative organ weights of TiO$_2$ nanoparticles exposed pregnant females

| TiO$_2$ nanoparticles (mg/kg) | 0  | 100 | 300 | 1000 |
|-------------------------------|----|-----|-----|------|
| Pregnant Females (N)          | 12 | 12  | 12  | 12   |
| Adrenal glands (g)            | 0.08 ± 0.010$^{a}$ | 0.07 ± 0.010 | 0.07 ± 0.010 | 0.07 ± 0.009 |
| Organ to terminal body weight ratio (%) | 0.02 ± 0.003 | 0.02 ± 0.003 | 0.02 ± 0.003 | 0.02 ± 0.002 |
| Brain (g)                     | 1.86 ± 0.090  | 1.87 ± 0.089  | 1.83 ± 0.064  | 1.90 ± 0.045 |
| Organ to terminal body weight ratio (%) | 0.49 ± 0.047 | 0.49 ± 0.037 | 0.47 ± 0.021 | 0.50 ± 0.024 |
| Heart (g)                     | 1.09 ± 0.106  | 1.10 ± 0.087  | 1.12 ± 0.066  | 1.10 ± 0.103 |
| Organ to terminal body weight ratio (%) | 0.29 ± 0.028 | 0.28 ± 0.026 | 0.29 ± 0.018 | 0.29 ± 0.026 |
| Kidneys (g)                   | 2.05 ± 0.211  | 2.03 ± 0.160  | 2.06 ± 0.189  | 2.06 ± 0.192 |
| Organ to terminal body weight ratio (%) | 0.54 ± 0.049 | 0.52 ± 0.034 | 0.53 ± 0.047 | 0.55 ± 0.050 |
| Liver (g)                     | 14.19 ± 1.000 | 14.48 ± 1.059 | 15.16 ± 1.186 | 14.70 ± 0.823 |
| Organ to terminal body weight ratio (%) | 3.75 ± 0.229 | 3.75 ± 0.185 | 3.89 ± 0.237 | 3.88 ± 0.138 |
| Pituitary gland (g)           | 0.01 ± 0.002  | 0.01 ± 0.003  | 0.01 ± 0.002  | 0.01 ± 0.002 |
| Organ to terminal body weight ratio (%) | 0.004 ± 0.0006 | 0.004 ± 0.0006 | 0.004 ± 0.0005 | 0.004 ± 0.0005 |
| Spleen (g)                    | 0.70 ± 0.116  | 0.69 ± 0.073  | 0.73 ± 0.115  | 0.71 ± 0.077 |
| Organ to terminal body weight ratio (%) | 0.19 ± 0.028 | 0.18 ± 0.018 | 0.19 ± 0.028 | 0.19 ± 0.019 |
| Lung (g)                      | 1.35 ± 0.105  | 1.33 ± 0.112  | 1.35 ± 0.122  | 1.28 ± 0.107 |
| Organ to terminal body weight ratio (%) | 0.36 ± 0.032 | 0.35 ± 0.034 | 0.35 ± 0.030 | 0.34 ± 0.021 |
| Right ovary (g)               | 0.06 ± 0.012  | 0.07 ± 0.011  | 0.06 ± 0.011  | 0.06 ± 0.011 |
| Organ to terminal body weight ratio (%) | 0.02 ± 0.003 | 0.02 ± 0.003 | 0.02 ± 0.003 | 0.02 ± 0.003 |
| Left ovary (g)                | 0.06 ± 0.009  | 0.06 ± 0.011  | 0.06 ± 0.014  | 0.06 ± 0.012 |
| Organ to terminal body weight ratio (%) | 0.02 ± 0.002 | 0.01 ± 0.003 | 0.02 ± 0.004 | 0.02 ± 0.003 |
| Thymus (g)                    | 0.40 ± 0.084  | 0.37 ± 0.076  | 0.43 ± 0.066  | 0.37 ± 0.080 |
| Organ to terminal body weight ratio (%) | 0.11 ± 0.023 | 0.10 ± 0.019 | 0.11 ± 0.0175 | 0.10 ± 0.023 |

$^{a}$Values are presented as mean ± S.D
changes, food consumption, macroscopic findings, organ weights, cesarean section parameters and fetal morphology, including external, visceral, and skeletal aspects, were examined. There were no TiO₂ nanoparticle-related toxicological findings related to maternal and embryo-fetal development toxicity parameters during the study. In addition, increased titanium concentrations in the maternal liver, maternal brain and placenta were observed after high dose oral exposure during pregnancy.

The molecular mechanism of TiO₂ nanoparticle-induced toxicity is regarded as the induction of inflammation and generation of reactive oxygen species (ROS). The accumulation of TiO₂ nanoparticles induces chronic inflammation, which leads to the formation of ROS and cell proliferation [29]. Previous studies have demonstrated that TiO₂ nanoparticle exposure induces the expression of inflammatory cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-6 and IL-18 [30–32]. In addition, the role of free radicals in DNA damage [27, 33], ROS-induced activation of p53-mediated DNA damage [28] and cell-derived oxidants involved in the induction of mutagenesis [34] after TiO₂ nanoparticle exposure were investigated. Although the exact pathophysiological mechanism is not clear, these multifactorial events related to the induction of inflammation leading to the production of ROS would be the major cause in TiO₂ nanoparticle-induced toxicity.

Experimental animal studies were also conducted to evaluate the potential effects of TiO₂ nanoparticle exposure. An acute oral toxicity study in mice (25, 80 and 155 nm, 5000 mg/kg) reported no obvious acute toxicity, but hepatic and renal injury was observed after high dose oral exposure during pregnancy.

| Table 5 | Gravid uterine weight, corrected terminal weight and net body weight change of TiO₂ nanoparticles exposed pregnant females during the pregnancy |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| TiO₂ nanoparticles (mg/kg) | 0 | 100 | 300 | 1000 |
| Pregnant Females (N) | 12 | 12 | 12 | 12 |
| Gravid uterine weight (g) | 76.91 ± 21.44‌ ‌ | 89.31 ± 15.33‌ ‌ | 81.92 ± 6.91 | 78.37 ± 13.18 |
| Corrected terminal body weight (g) | 312.37 ± 16.87 | 309.78 ± 16.54 | 318.14 ± 12.20 | 310.37 ± 20.59 |
| Net body weight change (g) | 36.88 ± 11.43 | 32.40 ± 8.89 | 43.69 ± 8.34 | 33.54 ± 10.82 |

*Values are presented as mean ± S.D

| Table 6 | Caesarean section results of TiO₂ nanoparticles exposed pregnant females during the pregnancy |
|---------|----------------------------------------------------------------------------------------------------------------------------------|
| TiO₂ nanoparticles (mg/kg) | 0 | 100 | 300 | 1000 |
| Pregnant Females (N) | 12 | 12 | 12 | 12 |
| Corpora lutea (N) | 14.1 ± 1.83 | 14.6 ± 1.98 | 13.9 ± 1.31 | 14.1 ± 1.56 |
| Implantation (N) | 12.8 ± 3.81 | 14.4 ± 2.19 | 13.6 ± 1.38 | 13.2 ± 2.55 |
| Early resorptions (N) | 0.4 ± 0.67 | 0.2 ± 0.58 | 0.4 ± 0.67 | 0.4 ± 0.67 |
| Late resorptions (N) | 0.0 ± 0.00 | 0.2 ± 0.39 | 0.0 ± 0.00 | 0.0 ± 0.00 |
| Dead fetuses (N) | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 | 0.0 ± 0.00 |
| Fetal death (resorptions + dead fetuses) | 0.4 ± 0.67 | 0.3 ± 0.65 | 0.4 ± 0.67 | 0.4 ± 0.67 |
| Live fetuses (N) | 12.4 ± 3.55 | 14.1 ± 2.54 | 13.2 ± 1.34 | 12.8 ± 2.45 |
| Sex ratio (%, male) | 48.1 ± 18.87 | 63.0 ± 14.98 | 45.2 ± 7.01 | 53.7 ± 13.75 |
| Pre-implantation loss (%) | 10.0 ± 22.13 | 1.4 ± 3.19 | 2.4 ± 3.57 | 7.0 ± 12.07 |
| Post-implantation loss (%) | 2.7 ± 4.31 | 2.7 ± 5.98 | 3.0 ± 4.65 | 3.0 ± 5.18 |
| Fetal weight (g) | 4.24 ± 0.25 | 4.38 ± 0.30 | 4.23 ± 0.25 | 4.21 ± 0.25 |
| Covariate adjusted means | 4.22 | 4.40 | 4.23 | 4.20 |
| Placental weight (g) | 0.50 ± 0.06 | 0.49 ± 0.05 | 0.54 ± 0.14 | 0.49 ± 0.05 |
| Covariate adjusted means | 0.49 | 0.49 | 0.54 | 0.49 |
| Placental macroscopic observation | NAD | NAD | NAD | NAD |

NAD No Abnormalities Detected

*Values are presented as mean ± S.D

**(No. of corpora lutea - No. of implantation) / No. of corpora lutea) × 100

*[(No. of implantation – No. of live fetuses) / No. of implantation] × 100
histopathological examination [14]. A repeated oral toxici

ity study in rats (< 50 nm; 0.16, 0.4 and 1 g/kg for 14
days) revealed disturbances in metabolism and the gut
microflora environment caused by slight injury to the
liver and heart, as shown by urianalysis with nuclear
magnetic resonance (NMR) [35]. The results from
other experimental animal studies indicated that the
absorption of TiO2 nanoparticles is able to enter the
systemic circulation and induce organ injuries and
inflammation [12].

Reproductive and developmental toxicity potentials
of TiO2 nanoparticles were also reported in previous
studies with zebrafish, mice and rats. In zebrafish studies,
TiO2 nanoparticles (20 nm; 5 mg/mL, 21 nm; 0.01, 10
and 1000 μg/mL and 240–280 nm in water; 0.1 μg/mL)
induced deformities in the cardiovascular system,
pread effects but enhanced the metabolism of pentachlorophenol (PCP) and caused oxidative damage and developmental toxicity when co-exposed with PCP [39]. In mouse studies, subcutaneous TiO2 nanoparticle exposure (2570 nm; 100 μ suspended at 1 μg/μl; GDs 6, 9, 12, 15 and 25, 70 nm; 16 mg/kg; GDs 3, 7, 10 and 14) during pregnancy induced genital and cranial nerve system damage in the offspring and altered gene expression in the brain during the prenatal period [15, 16]. Intravenous TiO2 nanoparticle (35
mm; 0.8 mg/animal; GDs 16 and 17) exposure during
pregnancy induced smaller uteri and fetuses, and TiO2
nanoparticles were found in the placenta, fetal liver and
fetal brain [40]. In contrast, Warheit et al. [17] reported
that oral exposure to different sized TiO2 particles (42,
43, 47, 153, 195 and 213 nm; 100, 300, and 1000 mg/kg;
daily beginning on GDs 6 through 20 in rats) did not in-
duce toxicities during pregnancy. Our study results con-
firm that oral exposure to TiO2 nanoparticles during
pregnancy did not induce toxic effects in maternal ani-
mals or embryo-fetal development endpoints. This find-
ing is consistent with the study reported by Warheit et
al. [17], although the analyzed primary particle size of
the TiO2 nanoparticles was different from that in the
previous study.

This discrepancy in reproductive and developmental
results among previous studies is considered to be
caused by differences in exposure routes, animal species,
physicochemical properties of the nanoparticles, etc. In
fact, a gastrointestinal absorption study of silver nano-
particles reported that nanoparticles were aggregated
and changed their physical properties in the stomach,
and the degree of these changes was especially influ-
enced by the particle size of the nanoparticles [41]. This
study indicates that oral exposure to nanoparticles is
able to alleviate the toxicity by inducing the loss of char-
acteristic properties of nanoparticles before they enter
the systemic circulation when compared to directly sys-
temically exposed routes, such as intravenous and inhal-
ation routes. Quantitative biokinetics of TiO2
nanoparticle studies with oral and intravenous exposure
also proved that internal exposure was much higher with
intravenous than with oral exposure [42, 43]. These dif-
fences in internal exposure might result in different in-
teractions and binding to blood proteins and biomolecules with TiO2 nanoparticles, which will subse-
sequentially affect uptake in organs and tissues [44]. More-
over, species differences in metabolism and placentation
are considered to be important factors for birth defects
occurrence [45]. In fact, the birth defect levels for se-
lected developmental toxicants differ among animal spe-
cies and humans [46].
The distribution analysis of maternal and fetal tissues in this study showed that TiO$_2$ nanoparticles were retained in the maternal liver, maternal brain and placenta at 1000 mg/kg after repeated oral exposure during pregnancy. At 300 mg/kg, the TiO$_2$ nanoparticle level was also slightly elevated in the maternal brain and placenta. However, it was indiscernible in the maternal blood, fetal liver, fetal brain and fetal blood of all the TiO$_2$ nanoparticle-treated groups. Other studies have also reported that TiO$_2$ nanoparticles can be absorbed into the systemic circulation and then distributed to internal organs. Single oral exposure of different sized TiO$_2$ particles (25, 80, and 155 nm; 5 g/kg) in mice yielded retention of the particles in the liver, spleen, kidneys, brain and lung, but there was no detection of the particles in blood. The distribution level in each tissue varied depending on the TiO$_2$ particle size [14]. Another single oral exposure study of TiO$_2$ nanoparticles (70 nm; 30–80 μg/kg) in rats reported that the TiO$_2$ particles that crossed the intestinal membrane accounted for less than

Table 8  Fetal skeletal examination results of TiO$_2$ nanoparticles exposed pregnant females during the pregnancy

| TiO$_2$ nanoparticles (mg/kg) | 0    | 100  | 300  | 1000 |
|------------------------------|------|------|------|------|
| Fetal Skeletal Examination   |      |      |      |      |
| No. of litters examined      | 12   | 12   | 12   | 12   |
| No. of fetuses examined      | 77   | 88   | 83   | 79   |
| Malformation                 |      |      |      |      |
| No. of affected litters (%)  | 0 (0)| 0 (0)| 0 (0)| 0 (0)|
| No. of affected fetuses (%)  | 0 (0)| 0 (0)| 0 (0)| 0 (0)|
| Variation                    |      |      |      |      |
| Ribs, Full thoracic supernumerary rib |
| No. of affected litters (%)  | 0 (0)| 2 (17)| 0 (0)| 0 (0)|
| No. of affected fetuses (%)  | 0 (0)| 2 (2)  | 0 (0)| 0 (0)|
| Ribs, Short thoracic supernumerary rib |
| No. of affected litters (%)  | 2 (17)| 4 (33)| 3 (25)| 2 (17)|
| No. of affected fetuses (%)  | 5 (6) | 9 (10) | 4 (5) | 2 (2) |
| Skull, Large fontanelle      |      |      |      |      |
| No. of affected litters (%)  | 3 (25)| 2 (17)| 0 (0)| 0 (0)|
| No. of affected fetuses (%)  | 4 (5) | 2 (2)  | 0 (0)| 0 (0)|
| Thoracic centrum, Asymmetric ossification |
| No. of affected litters (%)  | 0 (0)| 0 (0) | 1 (8) | 0 (0)|
| No. of affected fetuses (%)  | 0 (0)| 0 (0) | 1 (1) | 0 (0)|
| Thoracic centrum, Bipartite ossification |
| No. of affected litters (%)  | 0 (0)| 0 (0) | 1 (8) | 2 (17)|
| No. of affected fetuses (%)  | 0 (0)| 0 (0) | 2 (2) | 3 (4) |
| Thoracic centrum, Dumbbell ossification |
| No. of affected litters (%)  | 2 (17)| 1 (8) | 0 (8) | 2 (17)|
| No. of affected fetuses (%)  | 3 (3) | 1 (1)  | 0 (0)| 2 (3) |
| No. of ossification centers  |      |      |      |      |
| Sternebra                     | 5.9 ± 0.18 $^a$ | 5.9 ± 0.20 | 6.0 ± 0.08 | 5.8 ± 0.30 |
| Metacarpals in both forelimbs | 7.8 ± 0.38 | 8.0 ± 0.14 | 8.0 ± 0.12 | 7.9 ± 0.12 |
| 1st phalanges in both forelimbs | 1.8 ± 1.39 | 2.7 ± 1.20 | 2.1 ± 1.12 | 2.6 ± 1.12 |
| Metatarsals in both hindlimbs  | 8.0 ± 0.09 | 8.3 ± 0.38 $^o$ | 8.0 ± 0.09 | 8.0 ± 0.03 |
| 1st phalanges in both hindlimbs | 0.2 ± 0.58 | 0.1 ± 0.20 | 0.0 ± 0.06 | 0.0 ± 0.00 |
| Cervical vertebra             | 1.3 ± 1.29 | 1.3 ± 1.00 | 1.4 ± 1.23 | 0.9 ± 0.86 |
| Sacral and caudal vertebra    | 8.5 ± 0.45 | 8.7 ± 0.49 | 8.5 ± 0.50 | 8.5 ± 0.34 |

$^a$Values are presented as mean ± S.D

$^o$Significant difference at p < 0.01 level compared with the control group
0.6% of the applied dose; however, the TiO\textsubscript{2} particles were still distributed in the liver, lungs, kidneys, brain, spleen, uterus and skeleton after 7 days of exposure [43]. A single intravenous administration study of TiO\textsubscript{2} nanoparticles (20–30 nm; 5 mg/kg) in rats reported that the nanoparticles were mainly retained in the liver as well as the spleen and kidney but were not detected in the blood, brain and lymph nodes [47]. A single intraperitoneal administration of TiO\textsubscript{2} nanoparticles (100 nm; 324–2592 mg/kg) in mice yield retention in the spleen as well as liver, kidney and lung, but nanoparticles were not detected in the heart. The distribution level was changed depending on the sample collection time after administration [48]. These TiO\textsubscript{2} nanoparticle distribution studies concluded that the liver and kidney were the most commonly observed internal organs into which the particles were distributed after they were absorbed into the systemic circulation regardless of the administration route and particle size. However, it was also concluded that the TiO\textsubscript{2} nanoparticle distribution can change depending on the administration route, particle size and tissue sampling time.

One intriguing distribution result in this study was the relatively high level of TiO\textsubscript{2} nanoparticles detected in the brain. Distribution studies of TiO\textsubscript{2} nanoparticles in the brain have not been comprehensively conducted, but several studies have suggested that TiO\textsubscript{2} nanoparticles can be deposited into the brain. Wang et al. [14] reported that acute orally exposed TiO\textsubscript{2} particles (25, 80 and 155 nm; 5 g/kg) in mice were deposited in the brain and induced fatty degeneration in the hippocampus. Li et al. [49] also reported that intratracheal instillation of TiO\textsubscript{2} particles (3 nm; 13.2 mg/kg, once a week for 4 weeks) in mice induced brain injury through oxidative stress. Taken together, these previous studies indicate that TiO\textsubscript{2} nanoparticles are able to penetrate the blood–brain barrier, and these results were consistent with our study results.

No marked toxicities were observed in maternal animals and embryo-fetal development in this study design, but this finding does not indicate that TiO\textsubscript{2} nanoparticles are completely safe during pregnancy. Generally, two species (commonly rats and rabbits) are required in this type of study to evaluate toxicity during pregnancy [50]. In fact, rats and rabbits might be able to exhibit different teratogenic results during pregnancy [45]. Moreover, it is noteworthy that TiO\textsubscript{2} nanoparticles (75 nm; 10, 50 and 200 mg/kg; daily oral for 30 days) are able to induce liver edema (revealed by histopathological examination) and reductive stress (shown by biochemical assays) [51]. This result indicates that the toxicity of TiO\textsubscript{2} nanoparticles can be detected by more sensitive and/or different parameters. In this regard, additional reproductive endpoints, including fertility, parturition, postnatal development and hormone analysis, were not investigated, and these parameters will support the accurate safety assessment of TiO\textsubscript{2} nanoparticles. Considering the possibility of complex TiO\textsubscript{2} nanoparticles exposure scenarios and their various particle sizes, it is necessary to conduct additional studies to evaluate the potential adverse effects of TiO\textsubscript{2} nanoparticles.

In conclusion, we systemically investigated the maternal and embryo-fetal effects of orally exposed TiO\textsubscript{2} nanoparticles during pregnancy in rats. In addition, we analyzed the titanium distribution during pregnancy using maternal and fetal tissues. As a result, there were
no TiO\textsubscript{2} nanoparticle-related toxicity findings in maternal animals or with respect to embryo-fetal development in this study design, and the titanium content was increased in the maternal liver, maternal brain and placenta with high-dose exposure to TiO\textsubscript{2} nanoparticles. The results of this study can be used to evaluate the human risk assessment of TiO\textsubscript{2} nanoparticles during pregnancy, and additional toxicity studies are considered necessary to elucidate the effects of TiO\textsubscript{2} nanoparticles under various exposure scenarios and at different particle sizes.

Abbreviations

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care International; ANCOVA: Analysis of covariance; ANOVA: Analysis of variance; BET: Brunauer–Emmett–Teller; GD: Gestation day; GLP: Good laboratory practice; H\textsubscript{2}O\textsubscript{2}: Hydrogen peroxide; HF: Hydrofluoric acid; HNO\textsubscript{3}: Nitric acid; IACUC: Institutional Animal Care and Use Committee; ICP–MS: Inductively coupled plasma mass spectrometry; IDL: Instrumental detection limits; ILAR: Institute for Laboratory Animal Research; LOQ: Limit of quantification; MLD: Method detection limits; PCP: Pentachlorophenol; ROS: Reactive oxygen species; SPF: Specific pathogen free; TiO\textsubscript{2}: Titanium dioxide

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Authors’ contributions

JL, KP, SYN and WJY conceived and designed the present study. JL, JSJ and SYK conducted the animal experiments, and MKP, SDC and UJK conducted the titanium distribution analysis. JL and JSJ analyze the physicochemical properties of particles. JL and WJY interpreted the data and wrote the manuscript. All authors reviewed and approved the final manuscript.

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Availability of data and materials

The relevant datasets supporting the conclusions of this article are included within the article, and all datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal protocols were prepared in accordance with the Animal Protection Act of Korea and Guide for the Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research (ILAR). This animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of NT.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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