Prenatal exposure to rutile-type alumina-coated titanium dioxide nanoparticles impairs mouse spermatogenesis

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ABSTRACT — The in vivo nanotoxicity of nanoparticles is drawing increased attention as concerns grow over the biosafety of nanotechnology. TiO2 nanoparticles are coated to decrease the potential of harmful effects due to their photocactivity. Rutile-type alumina-coated titanium dioxide nanoparticles (Al2O3-TiO2-NPs) are frequently used in cosmetics to improve their dispersion stability. We herein discuss the effects of Al2O3-TiO2-NPs exposure during pregnancy on mouse spermatogenesis. Pregnant mice were injected five times, once each with 0.1 mL of sequentially diluted concentrations of an Al2O3-TiO2-NPs suspension (1, 10, 100 or 1,000 μg/mL) and received doses of 0.5, 5, 50 and 500 μg, respectively. Prior to injection, the size distribution of the Al2O3-TiO2-NPs was analyzed by dynamic light scattering (DLS) measurement. The average diameter was increased in dose-dependent manner from an average of 153.8 nm to 654.6 nm. The offspring testes were examined at 12 weeks postpartum. The agglomerates in the testicular sections were small (< 200 nm). They were confirmed by the characteristic peaks of the Ti and Al elements on field emission-scanning electron microscope/energy dispersive X-ray spectroscopy (FE-SEM/EDS). Low cellular adhesion and degenerated Sertoli cells were observed in the seminiferous epithelium of all of the Al2O3-TiO2-NP recipient groups by a histological analysis. The detrimental function of the Sertoli cells resulted in the formation of abnormal spermatozoa. The results suggested that Al2O3-TiO2-NPs that transferred from the mother’s body affected spermatogenesis in the offspring.

Key words: Rutile-type, Al2O3-TiO2-NPs, Prenatal exposure, Offspring testes, Abnormal spermatozoa

INTRODUCTION

Titanium dioxide nanoparticles (TiO2-NPs) are widely used in many commercial products. The forms of TiO2 that currently used in the industrial setting are roughly divided into the anatase, rutile and brookite types (Warheit et al., 2007). Rutile-type TiO2-NPs are reported to show lower photocatalytic activity and to be 100 times less toxic than anatase-type TiO2-NPs (Sayes et al., 2006). TiO2-NPs are coated to decrease their potential harmful effects, which occur due to their photocactivity. Rutile-type alumina-coated nanoparticles (Al2O3-TiO2-NPs) are generally used in cosmetics to improve dispersion stability (Liu et al., 2009).

The exposure of pregnant mice to a 400-μg dose of anatase-type TiO2-NPs (25-70 nm) by dorsal subcutaneous injection was shown to cause testicular dysfunction in 6-week-old male offspring. The transfer of anatase-type TiO2-NPs from the mother's body to the offspring testes was confirmed by field emission-scanning electron microscope/energy dispersive X-ray spectroscopy (FE-SEM/EDS) (Takeda et al., 2009). We previously investigated the biodistribution of rutile-type TiO2-NPs in the testes of the 12-week-old offspring of prenatally-exposed mice. We found a correlation between the dose of TiO2-NPs that were injected into the pregnant mice and the number of agglomerates in the offspring testes. However the agglomerates were found to be below 200 nm in size.

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in the testicular sections of all recipient groups, regardless of the dose that was injected during pregnancy (Kubo-Irie et al., 2014).

At present, the effects that exposure to rutile-type Al₂O₃-TiO₂-NPs might have on the testicular function of the next generation are not currently understood. In this study, we sought to evaluate the effects of prenatal exposure to rutile-type Al₂O₃-TiO₂-NPs on spermatogenesis in 12-week-old mice.

**MATERIALS AND METHODS**

**Animals and treatments**

Pregnant ICR mice (at day 2 postcoitum; body weight, approximately 35 g) were purchased from SLC Co. (Shizuoka, Japan). They were maintained in a temperature and light-controlled environment (12 hr light/12 hr dark cycle) with *ad libitum* access to standard rodent food and water. The treatment and care of the mice was approved by the Animal Care and Use Committee of Tokyo University of Science.

Thirty pregnant mice were divided into four rutile-type Al₂O₃-TiO₂-NP recipient groups and a control group. The nanoparticles were administered via subcutaneous injection. This method causes the nanoparticles to be absorbed by the venules, allowing them to reach the target organs without passing through the liver (Sharpe et al., 1998). In the recipient groups, each mouse was injected once with 0.1 mL of 1, 10, 100 or 1,000 μg/mL Al₂O₃-TiO₂-NPs by dorsal subcutaneous injection without anesthesia on gestational days 5, 8, 11, 14 and 17. It was considered that the administration of a fractionated dose in five injections might reduce the stress on the pregnant mice and that the effects of each injection would be maintained throughout the period of pregnancy. The mice of each group received total doses of 0.5, 5, 50 and 500 μg/mouse, respectively. The mice of the control group received 0.1 mL of saline with 0.05% Tween80, which was injected in the same manner.

**Nanoparticles and characterization**

Rutile-type Al₂O₃-TiO₂-NPs, of 35 nm in primary diameter, were provided by Tayca Co. (Osaka, Japan) (Table 1). The particles were suspended at a concentration of 1 mg/mL in saline (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) containing 0.05% Tween-80 and were sonicated for 30 min just before administration. To detect the size distribution of the particles, ten-fold serial dilutions obtained from the middle layer of the original suspension were prepared. The size distribution of the Al₂O₃-TiO₂-NPs in the suspensions was analyzed by dynamic light scattering measurement using a Nano-ZS (Sysmex Co., Hyogo, Japan). To observe the Al₂O₃-TiO₂-NPs that were dispersed in the suspensions, a drop of the suspension was placed on a formvar-coated copper grid, which was fully drained and allowed to air-dry. The size and shape of the visualized particles were measured using a JEM 1200EX II transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan).

**Light microscopy**

For the histological examinations, the testis was fixed in modified Davidson fixative and embedded in paraffin. Sections (5-μm-thick) were stained with hematoxylin and eosin (HE) and the seminiferous tubules of stages VII-VIII were observed under light microscopy (BX51, Olympus Optical Ltd., Tokyo, Japan).

**Electron microscopy**

The dissected testes were directly fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer overnight at 4°C. After washing in the same buffer, the samples were post-fixed in 2% OsO₄ for 1 hr, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultra-thin (80 nm thick) sections were cut on a Sorvall ultra-microtome MT-2 (Leica Mikrosystem LTD GmbH, Vienna, Austria), and were doubly-stained with uranyl acetate and lead citrate, then observed under TEM. Simultaneously, a scanning electron microscope (SEM) was used to perform an elemental analysis by energy dispersive X-ray spectroscopy (EDX) to identify the presence of Ti and Al elements in the seminiferous epithelium. The ultrastructure of sperm morphology was assessed on longitudinal sections through sperm heads which included the basal plate at the posterior aspect of the nuclei. To evaluate sperm abnormalities in regard to the deformed nuclei and acrosome, about one hundred spermatozoa in each group were counted blindly under TEM images at x 5000 magnification.

| Table 1. Characterization of TiO₂ white powder provided by Tayca Co. |
|-----------------------------|------------------|
| TiO₂ (%) | min.90 |
| Main Modifiers | Alumina |
| pH | Neutral |
| Cristal Structure | Rutile |
| Property of surface | Hydrophilic |
| Particle Size (nm) | 35 |
| Specific Surface Area | 30-50 (m²/g) |
RESULTS

The preparation of the Al₂O₃-TiO₂ nanoparticles

The ten-fold serial dilution of rutile-type Al₂O₃-TiO₂-NPs is shown in Fig. 1a. The suspensions, which contained particles of approximately 35 nm in primary diameter were measured by DLS. In the most diluted concentration (1 μg/mL), the agglomerates were an average of 153.8 nm in diameter. The size of the agglomerates gradually increased in a dose-dependent manner. In the original suspension, the agglomerates were an average of 654.6 nm in size. The agglomerates of Al₂O₃-TiO₂-NPs
were held together by relatively weak forces and individual Al2O3-TiO2-NPs that were observed under TEM were spherical or rod-shaped and were located with a small number of nanoparticles (Fig. 1b).

The injection of TiO2 nanoparticles into pregnant mice

The injection protocol is shown in Fig. 1c. The mice delivered their pups on gestational day 19. In the 0, 0.5, 5, 50 and 500 μg recipient groups, the numbers of dams and the male to female sex ratios of the pups for each group were as follows: 4 (dams) and 46 (25 male: 21 female) pups; 5 and 66 (35:31); 6 and 68 (31:37); 5 and 57 (26:31); and 5 and 70 (41:29), respectively. Each group was weaned on postnatal 21. The reproductive organs developed fully and no structural malformations were detected in the offspring at 12 weeks. The general toxicity in each of the Al2O3-TiO2 recipient groups was indistinguishable from that in the control group at 12 weeks postpartum. The treatment and care of the mice was approved by the Animal Care and Use Committee of Tokyo, University of Science.

The characterization and localization of Al2O3-TiO2-NPs in the testicular sections

Some Al2O3-TiO2-NP agglomerates were comprised of a small number of nanoparticles that were held together by relatively weak forces. The agglomerates were detected in sections of the seminiferous epithelium in the 500 μg recipient group and were found to be smaller than 200 nm in diameter by SEM (Fig. 1d). To confirm whether or not the particles contained the Ti and Al elements, FE-SEM/EDS was performed. Ti and Al were indicated by the X-ray spectrum peaks of 4.51 keV and 1.62 keV, respectively (Fig. 1e).

The effects of prenatal exposure on the seminiferous tubules

The seminiferous tubules of hematoxylin and eosin-stained sections in the control group (stages VII-VIII) showed that the organization of spermatogenic cells was oriented from the basal to the adluminal compartments. Mature spermatozoa were located in the adluminal compartment (Fig. 2a). In contrast, the spermatogenic cells in the in the 500 μg Al2O3-TiO2-NP recipient group were disorganized, the seminiferous epithelia displayed low adhesion and loose cellular spaces were found (Fig. 2b). In the control group, TEM revealed that the spermatogenic cells, spermatogonia, spermatocytes, spermatids and spermatozoa associated with Sertoli cells, were oriented from the basal to the adluminal compartments (Fig. 2c). However, in the 0.5 μg Al2O3-TiO2-NP recipient group, the spermatogenic cells displayed a disrupted spermatogenic orientation. Spermatozoa were enveloped with the vacuolated cytoplasm of Sertoli cells in the basal compartment. The Sertoli cells were deformed and had loose contact with their neighboring cells (Fig. 2d). The irregularly shaped Sertoli cell nucleus was positioned at the upper part of spermatocytes in the 500 μg Al2O3-TiO2-NP recipient group. Two electron-dense degenerated materials were observed in the cytoplasm of the Sertoli cells (Fig. 2e). The Sertoli cell nuclei were not attached at the basal membrane, close to where the mature sperm heads were positioned.

Many phagosomes and small vacuoles and damaged mitochondria were found in the cytoplasm of the Sertoli cells (Fig. 2f).

The ultrastructural analysis of the abnormal spermatozoa in the testis at 12 weeks

Abnormal spermatozoa were observed in the 500 μg Al2O3-TiO2-NP recipient group (Fig. 3). The lateral side of a spermatozoon with a tapered head, outlined with a tapered acrosome was observed to be bent backwards. The flagella axoneme was arranged normally with a layer of mitochondria. A cytoplasmic droplet remained at the nucleus neck (Fig. 3a). The lateral side of a spermatozoon with a triangular head had a creator at the posterior side of the nucleus. The acrosome covered the triangular nuclear head (Fig. 3b). The lateral side of a spermatozoon with a round head had an acrosome with a disorganized filaments layer at the ectoplasmic specialization between the acrosome and the Sertoli cells. The filaments layer is an important structure for the sperm heads detached from Sertoli cells (spermiation). The flagella axoneme was arranged normally with a layer of mitochondria (Fig. 3c). A spermatozoon with a narrow head was outlined by an acrosome and a layer of filaments at ectoplasmic specialization that were arranged normally in the sagittal view (Fig. 3d). A spermatozoon with a collapsed head was covered by a deformed acrosome (Fig. 3e). A spermatozoon with a small head showed fully-condensed nuclei with an acrosome, but the flagellum was not organized and a mitochondria were gathered at the nucleus neck (Fig. 3f). The incidence of abnormal sperm showed a tendency to increase as the concentrations of Al2O3-TiO2-NPs increased in the Al2O3-TiO2-NP recipient group (Fig. 4).

DISCUSSION

This is the first study to investigate the effects of prenatal exposure to the less-toxic rutile-type Al2O3-TiO2-
NPs on spermatogenesis in offspring. While the nanoparticles that are currently used in cosmetic preparations and sunscreens are not believed to pose any risk to human skin or health (Nohynek et al., 2010), their presence in such a wide range of products makes it extremely important to investigate their potential effects on human health. The in vivo translocation and biodistribution of rutile-type Al$_2$O$_3$-TiO$_2$-NPs from the mother’s body to the offspring’s testes are key factors in evaluating their toxicity. It has been demonstrated that nanomaterials could easily cross the placental barrier in ex vivo human placental perfusion (Wick et al., 2010) in pregnant mice (Takeda et al., 2009; Yamashita et al., 2011; Kubo-Irie et al., 2014). The electron dense particles detected in the seminiferous epithelium were confirmed to be Al$_2$O$_3$-TiO$_2$-NPs by the X-ray spectrum peaks of the observed particles (Ti, 4.51 keV; Al, 1.62 keV). This analysis directly shows that the nanoparticles were localized in the seminiferous epithelium.
even in the offspring of mice in the low-dose groups.

The fetal stage has been demonstrated to be the period in which proper development is most easily affected (Takeda et al., 2009). The prenatal exposure of mice to carbon black (14 nm) (200 μg) was shown to affect reproductive function, damage the seminiferous epithelium and reduce the DSP numbers in male offspring (Yoshida et al., 2010). It is possible that the damage of the seminiferous epithelium by TiO2-NPs is caused by a characteristic of their nano-size, which can induce reactive oxygen species or oxidative stress in mice (Trouiller et al., 2009) as it has also been reported that nanotubes that accumulated in the mice testes, generated oxidative stress and decreased the thickness of the seminiferous epithelium in the testes at 15 days after injection (Bai et al., 2010). This was demonstrated to increase testicular oxidative stress, which leads to an increase in germ cell apoptosis and subsequent hypospermatogenesis (Turner and Lysiak, 2008). Regarding herbicide and heavy metal exposure during gestation and lactation, cadmium and simazine were found to interfere with the function of male reproductive system in offspring (Ji et al., 2011; Park and Bae, 2012).

The number of damaged mitochondria in Sertoli cells that were involved in abnormal sperm formation was reported to be increased in the offspring of pregnant mice that were exposed to diesel exhaust (Kubo-Irie et al., 2011). Damaged mitochondria were also found in the Sertoli cells in mice testes after the inhalation of lead, cadmium or a lead-cadmium mixture (Bizarro et al., 2003). The seminiferous epithelium in rat testes was reported to be damaged after the repeated oral administration of Ag-NPs for 90 days (Thakur et al., 2014). Ultrafine particles localized in the mitochondria of incubating cells of a murine macrophage cell line induced oxidative stress and mitochondrial damage (Lui et al., 2003). These reports suggested that ultrafine particles in the Sertoli cells were detrimental to spermatogenesis. Similar sperm abnormalities to those induced by the inhalation of diesel exhaust were observed after Al2O3-TiO2-NPs exposure by TEM in our
study. The acrosome is an important cellular organ, can act as a trigger. The damage of the acrosome structure causes failure at the time of fertilization. The deformed nucleus and flagellum appeared impede sperm movement. An increase in the number of abnormal spermatozoa is related to infertility (Kubo-Irie et al., 2005). With regard to the phagocytic function of the Sertoli cells, rat Sertoli cells in primary cultures have been shown to phagocytize apoptotic cells (Pineau et al., 1991), and mouse Sertoli cells have been shown to be responsible for the phagocytic elimination of apoptotic spermatogenic cells in vivo (Maeda et al., 2002; Nakagawa et al., 2005).

In conclusion, FE-SEM/EDS and TEM observation was useful for showing the position of the nanoparticles that localized in the seminiferous epithelium. The results demonstrate that the biodistribution of Al2O3-NPs in the offspring testes could affect spermatogenesis and that they resulted in abnormal spermatozoa. The incident of abnormal sperm were a tendency to increase in line with the concentrations which suggested that the high dose of Al2O3-TiO2-NPs may be hazardous to the next generation. Further studies will be necessary to clarify the mechanism underlying the effects of Al2O3-TiO2-NPs in vivo.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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