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Original Article

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. TiO_2 nanoparticles are coated to decrease the potential of harmful effects due to their photoactivity. Rutile-type alumina-coated titanium dioxide nanoparticles (Al_2O_3-TiO_2-NPs) are frequently used in cosmetics to improve their dispersion stability. We herein discuss the effects of Al_2O_3-TiO_2-NPs exposure during pregnancy on mouse spermatogenesis. Pregnant mice were injected five times, once each with 0.1 mL of sequentially diluted concentrations of a Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-NPs that transferred from the mother's body affected spermatogenesis in the offspring.

Key words: Rutile-type, Al_2O_3-TiO_2-NPs, Prenatal exposure, Offspring testes, Abnormal spermatozoa

INTRODUCTION

Titanium dioxide nanoparticles (TiO_2-NPs) are widely used in many commercial products. The forms of TiO_2 that currently used in the industrial setting are roughly divided into the anatase, rutile and blookite types (Warheit *et al*., 2007). Rutile-type TiO_2-NPs are reported to show lower photocatalytic activity and to be 100 times less toxic than anatase-type TiO_2-NPs (Sayes *et al*., 2006). TiO_2-NPs are coated to decrease their potential harmful effects, which occur due to their photoactivity. Rutile-type alumina-coated nanoparticles (Al_2O_3-TiO_2-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 TiO_2-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 TiO_2-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 TiO_2-NPs in the testes of the 12-week-old offspring of prenatally-exposed mice. We found a correlation between the dose of TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2 -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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-NPs in the suspensions was analyzed by dynamic light scattering measurement using a Nano-ZS (Sysmex Co., Hyogo, Japan). To observe the Al_2O_3-TiO_2-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).

Table 1. Characterization of TiO_2 white powder provided by Tayca Co.

TiO^2 (%)	min.90
Main Modifiesrs	Alumina
pH	Neuutral
Cristal Structure	Rutile
Property of surface	Hydrophilic
Particle Size (nm)	35
Specific Surface Area	30-50 (m^2/g)

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-VI-II 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 4ºC. After washing in the same buffer, the samples were post-fixed in 2% OsO_4 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) (JEM-6500F JEOL) 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.

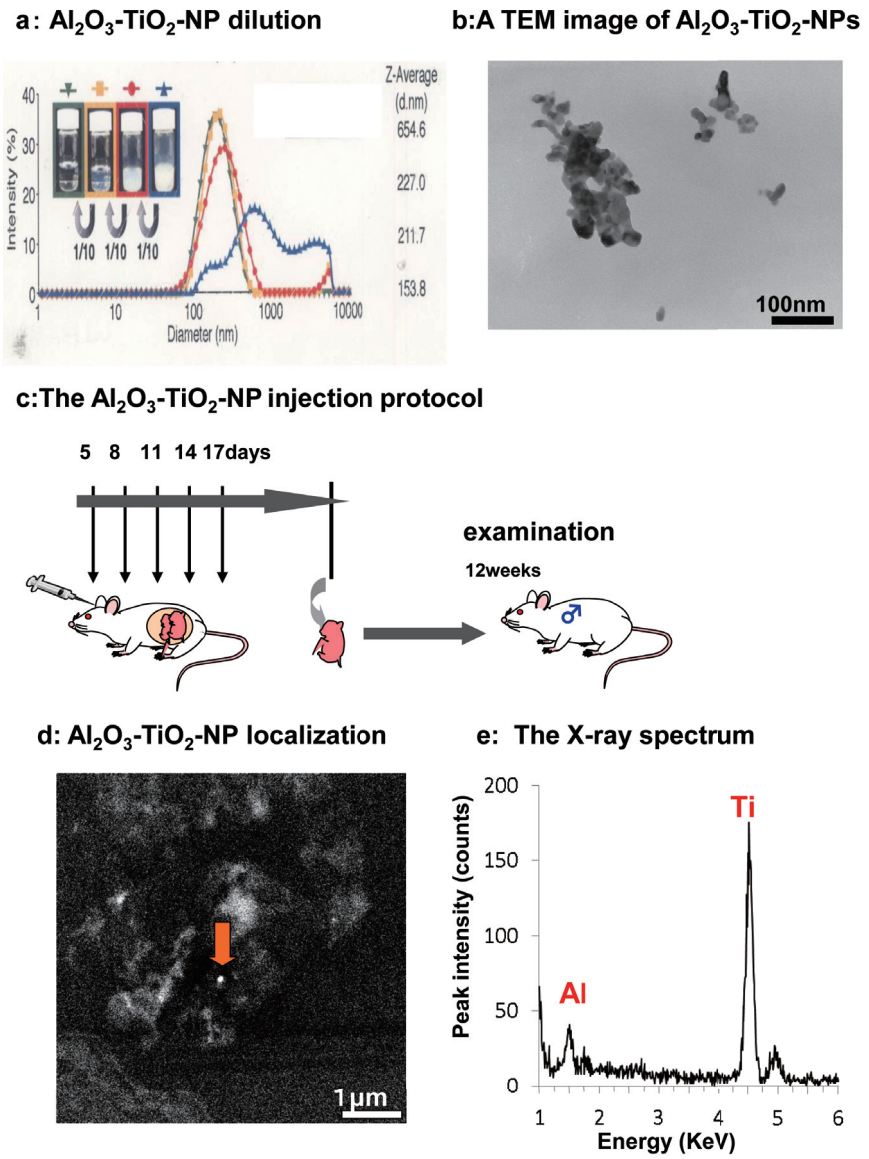

Fig. 1. Experimental design. a: The ten-fold serial dilution of rutile-type Al_2O_3-TiO_2-NPs. b: The characterization of 1 μg/mL Al_2O_3-TiO_2-NP suspension. Agglomerates of various sizes were found to consist of particles of 35 nm in primary diameter. c: The Al_2O_3-TiO_2-NP injection protocol. Al_2O_3-TiO_2-NPs were subcutaneously injected into pregnant ICR mice. A single 0.1 mL dose was administered on gestational days 5, 8, 11, 14 and 17. d: The localization of Al_2O_3-TiO_2-NPs in a testicular section observed under FE-SEM/EDS. e: The X-ray spectrum. The peaks of Ti and Al at 4.51 keV and 1.62 keV, respectively in the same observed particles (arrow).

RESULTS

The preparation of the Al_2O_3-TiO_2 nanoparticles

The ten-fold serial dilution of rutile-type Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-NPs

were held together by relatively weak forces and individual Al_2O_3-TiO_2-NPs that were observed under TEM were spherical or rod-shaped and were located with a small number nanoparticles (Fig. 1b).

The injection of TiO_2 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 Al_2O_3-TiO_2 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 Al_2O_3-TiO_2-NPs in the testicular sections

Some Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-NPs 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 Al_2O_3-TiO_2-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 Al_2O_3-TiO_2-NPs increased in the Al_2O_3-TiO_2-NP recipient group (Fig. 4).

DISCUSSION

This is the first study to investigate the effects of prenatal exposure to the less-toxic rutile-type Al_2O_3-TiO_2-

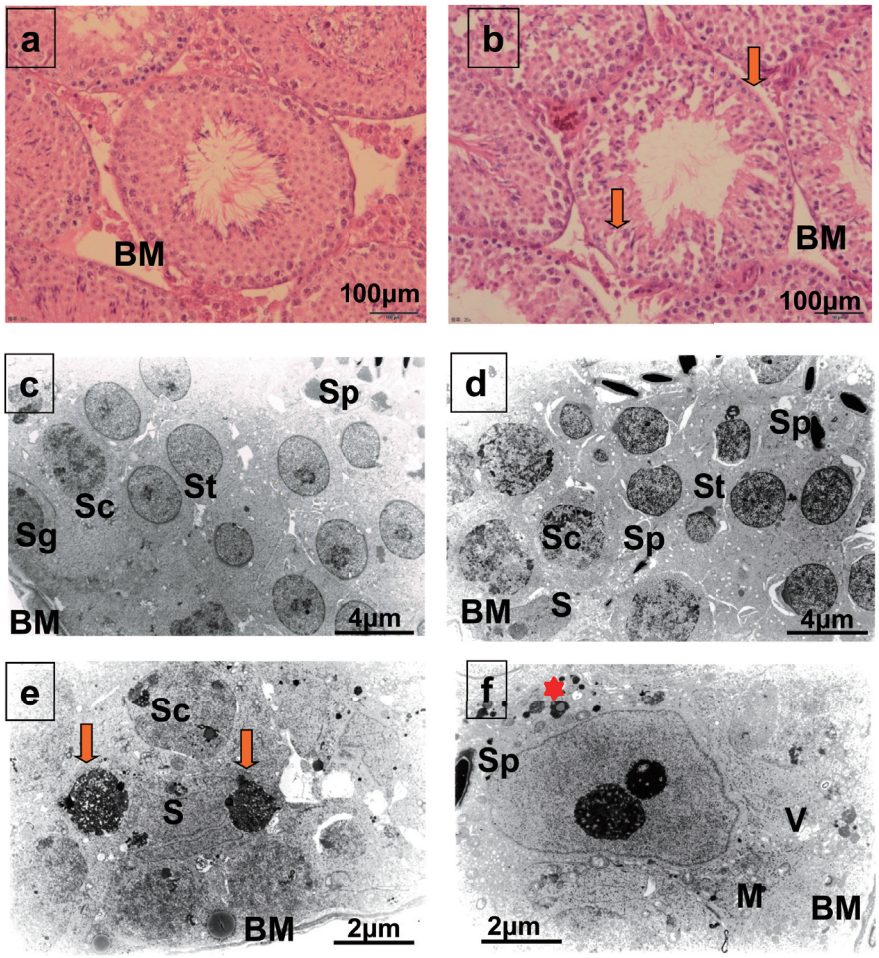

Fig. 2. The effects of prenatal exposure on the seminiferous tubules at 12 weeks. An HE-stained cross section of seminiferous tubules. a: The control group. Mature spermatozoa were located in the adluminal compartment. b: The 500 μg Al_2O_3-TiO_2-NPs recipient group. The seminiferous epithelia displayed low adhesion and loose cellular spaces between the spermatogenic cells. TEM images. c: Part of the seminiferous epithelium in the control group. The spermatogenic cells were normally arranged from the basal to the adluminal compartments. d: Part of the seminiferous epithelium in the 0.5 μg recipient group. A spermatozoa enveloped with the vacuolated cytoplasm of Sertoli cells was found in the basal compartment. e: Part of the seminiferous epithelium in the 500 μg recipient group. Note the irregularly-shaped Sertoli cell nucleus and degenerated materials in the cytoplasm. f: A Sertoli cell in the 500 μg recipient group. Phagosomes (star), small vacuoles (V) and damaged mitochondria (M) found in the cytoplasm. BM, basal membrane; S, Sertoli cell nucleus; Sc, spermatocyte; Sg, spermatoginia; Sp, spermatozoon; St, spermatid.

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_2O_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_2O_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,

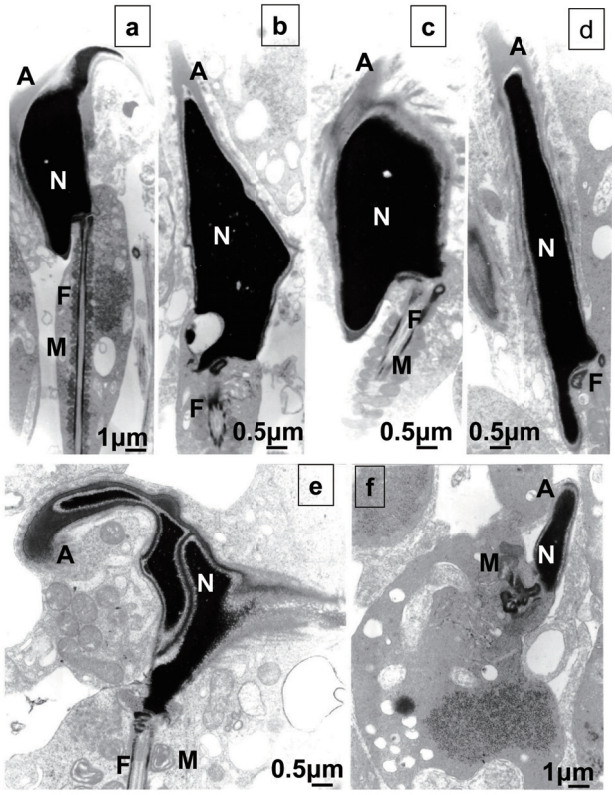

Fig. 3. An ultrastructural analysis of the abnormal spermatozoa in the testis at 12 weeks in the 500-μg recipient group. The different morphological features of the abnormal spermatozoa (a, tapered head; b, triangular head; c, round head; d, narrow head; e, collapsed head; f, small head). The mature sperm nuclei detached from Sertoli cells (spermiation) were located in the basal compartment in various locations. A, acrosome; F, flagellum; M, mitochondria; N, sperm nuclei.

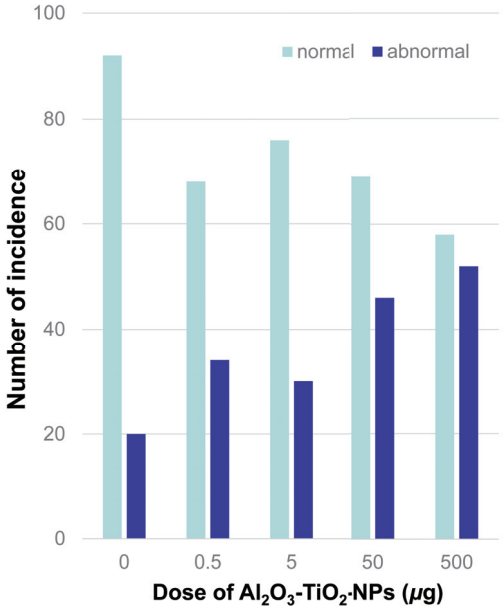

Fig. 4. Dose-dependency in the incidence of abnormal sperm. The incidence of abnormal sperm tended to increase in a dose-dependent fashion at Al_2O_3-TiO_2-NP concentrations of between 0 and 500 μg in the Al_2O_3-TiO_2-NP recipient group.

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 TiO_2-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 Al_2O_3-TiO_2 -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 Al_2O_3-TiO_2 –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 Al_2O_3-TiO_2 -NPs may be hazardous to the next generation. Further studies will be necessary to clarify the mechanism underlying the effects of Al_2O_3-TiO_2 –NPs *in vivo*.

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

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