Nanoparticles Transferred from Pregnant Mice to Their Offspring Can Damage the Genital and Cranial Nerve Systems

Ken Takeda,*,[a] Ken-ichiro Suzuki,[b] Aki Ishihara,[a] Miyoko Kubo-Irie,[a] Rie Fujimoto,[a] Masako Tabata,[a] Shigeru Oshio,[c] Yoshimasa Nihei,[b] Tomomi Ihara,[d] and Masao Sugamata[d]

[a]*Department of Hygiene Chemistry, Faculty of Pharmaceutical Science,* [b]*Department of Pure and Applied Chemistry, Faculty of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278–8510, Japan,* [c]*Department of Hygiene Chemistry, Ohu University School of Pharmaceutical Sciences, 31–1 Misumidou, Tomita-machi, Kooriyama-shi, Fukushima 963–8611, Japan and* [d]*Department of Pathology, Tochigi Institute of Clinical Pathology, 2308–3 Minamiakatsuka, Nogi, Shimotsuga-gun, Tochigi 329–0112, Japan*

(Received September 26, 2008; Accepted October 27, 2008; Published online November 10, 2008)

Nanomaterials are being used increasingly for commercial purposes, yet little is known about the potential health hazards such materials may pose to consumers and workers. Here we show that nano-sized titanium dioxide (TiO_2), which is used widely as a photo-catalyst and in consumer products, administered subcutaneously to pregnant mice is transferred to the offspring and affects the genital and cranial nerve systems of the male offspring. Nanoparticles identified as TiO_2 by energy-dispersive X-ray spectroscopy were found in testis and brain of exposed 6-week-old male mice. In the offspring of TiO_2-injected mice, various functional and pathologic disorders, such as reduced daily sperm production and numerous caspase-3 (a biomarker of apoptosis) positive cells in the olfactory bulb of the brain, were observed. Our findings suggest the need for great caution to handle the nanomaterials for workers and consumers.

Key words —— nanoparticle, titanium dioxide (TiO_2), brain, testis, pregnant mouse, olfactory bulb

INTRODUCTION

Nano-sized particles also known as ultrafine particles, are very tiny particles less than 100 nm in diameter. They are produced daily by activities such as driving, cooking, and generating energy in power plants. Engineered nanomateials are used in sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electonics and will likely be used increasingly in medicine for purposes of diagnosis and drug delivery.[1–4] Nanotoxicology, the evaluation of the safety of engineered nanostructures and nanodevices, is a novel field of toxicology. Materials that are generally thought to be inert may act differently when introduced to the body as nanomaterials.[4–8]

Nanocrystalline titanium dioxide (TiO_2), a noncombustible, odorless powder, is an important material used in commerce. Anatase TiO_2 is currently used in products as diverse as sunscreens and coatings for self-cleaning windows.[9] TiO_2 can generate reactive oxygen species quite efficiently, particularly when exposed to ultraviolet light. The photocatalytic activity of the anatase form of TiO_2 was reported to be higher than that of the rutile form.[10] Gurr and colleagues[11] reported that nano-sized anatase TiO_2 particles induced oxidative DNA damage, lipid peroxidation and micronuclei formations and increased hydrogen peroxide and nitric oxide production in BEAS-2B cells, a human bronchial epithelial cell line, even in the absence of photoactivation. However, the potential toxicity of TiO_2 in the next generation has yet to be examined. In the present study we examined the effects of prenatal exposure to anatase TiO_2 on the genital and cranial nerve systems of male offspring mice.

*To whom correspondence should be addressed: Department of Hygiene Chemistry, Faculty of Pharmaceutical Science, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278–8510, Japan. Tel.: +81-4-7121-3618; Fax: +81-4-7121-3784; E-mail:takedak@rs.noda.tus.ac.jp

MATERIALS AND METHODS

Materials —— TiO_2 particles (anatase form, particle size 25–70 nm, surface area 20–25 m^2/g, a purity 99.9 %) was purchased from Sigma-Aldrich (St Louis, U.S.A.).

Animals —— Pregnant Slc : ICR mice (purchased from Japan SLC Inc., Shizuoka, Japan) (6 mice/group) received subcutaneous injections of 100 μl of 1 mg/ml TiO_2 particles in saline plus 0.05 % Tween 80 at 3, 7, 10, and 14 days postcoitum. Control mice were treated on the same schedule with 0.05 % Tween 80. Male offspring were weighed and killed under anesthesia at 4 days or 6 weeks of age. All experimental animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

Organ Weights —— The weights of the testis, epididymis, and seminal vesicle (including prostate, seminal vesicle, and coagulating gland) bilaterally and brain were measured for each animal, and relative weights (weight of the organ/body weight) were calculated in 6-week-old offspring.

Daily Sperm Production (DSP) and Morphological Observation of Testis —— Testicular tissue was thawed and weighed after removal of any extracapsular material from the testis. Testes were homogenized in buffer containing 0.05 % Triton X-100 (Nacalai Tesque, Kyoto, Japan) and 0.2 % Eosin Y (Merck, Darmstadt, Germany). The number of sperm nuclei in each suspension was determined by hemocytometer.

Statistical Analysis —— Data were analyzed by Mann-Whitney U test, and differences were considered significant at $p < 0.05$.

Analysis by Field Emission-type Scanning Electron Microscopy (FE-SEM)/Energy-Dispersive X-ray Spectroscopy (EDS) —— The testis or brain tissue was embedded in epoxy resin for FE-SEM/EDS observation. These samples were cut with thickness of approximately 80 nm with an Ultra-Microtome (Leica EM UC6rt, Leica Microsystems Japan, Tokyo, Japan). Each ultra-thin section was placed on a transmission electron microscopy (TEM) grid (Cu 150-B, Okenshoji, Tokyo, Japan) and analyzed by FE-SEM/EDS (Hitachi High-technology, Tokyo, Japan).

Methods of Immunohistochemical Staining of Caspase-3 —— Tissue samples of olfactory from the TiO_2 treated group and the control group were fixed with 10 % buffered formalin and, after routine dehydration, embedded in paraffin. To detect apoptosis in these olfactory under a light microscope, the immunohistochemical staining for caspase-3 (a common enzymatic biomarker of apoptosis) was performed. Paraffin sections 5-μm thick of olfactory samples were stained immunohistochemically by the streptoavidin-biotin method (Histofine SAB-PO kit, Nichirei, Tokyo, Japan). The primary antibody used was anti-human/mouse caspase-3 (active) rabbit IgG (R&D Systems, Inc., Minneapolis, MN, U.S.A.).

RESULTS

TiO_2 powder size was confirmed by FE-SEM (Fig. 1). Male offspring were killed under anesthesia at 4 days or 6 weeks of age. In order to determine the genital toxicity of TiO_2 particles, body

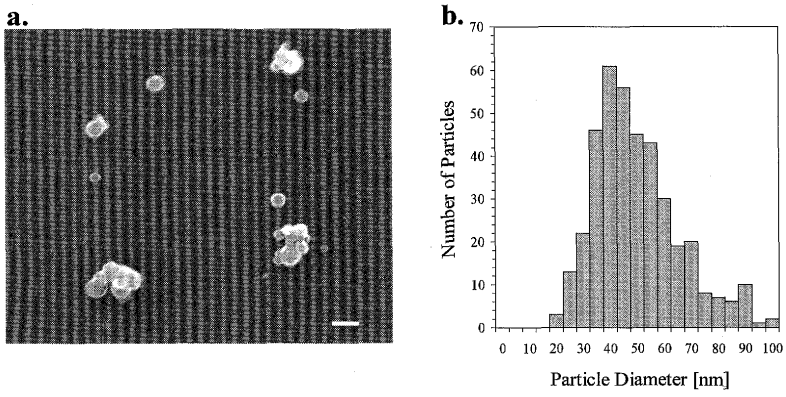

Fig. 1. Distribution of TiO_2 Particle Diameter by FE-SEM

(a) FE-SEM Image of TiO_2 particles (15.0 kV × 80000, Scale bar, 100 nm). (b) Distribution of TiO_2 particle diameters according to FE-SEM analysis. Columns show the diameter of single particles. Diameter of particles was measured on randomly selected area of FE-SEM image.

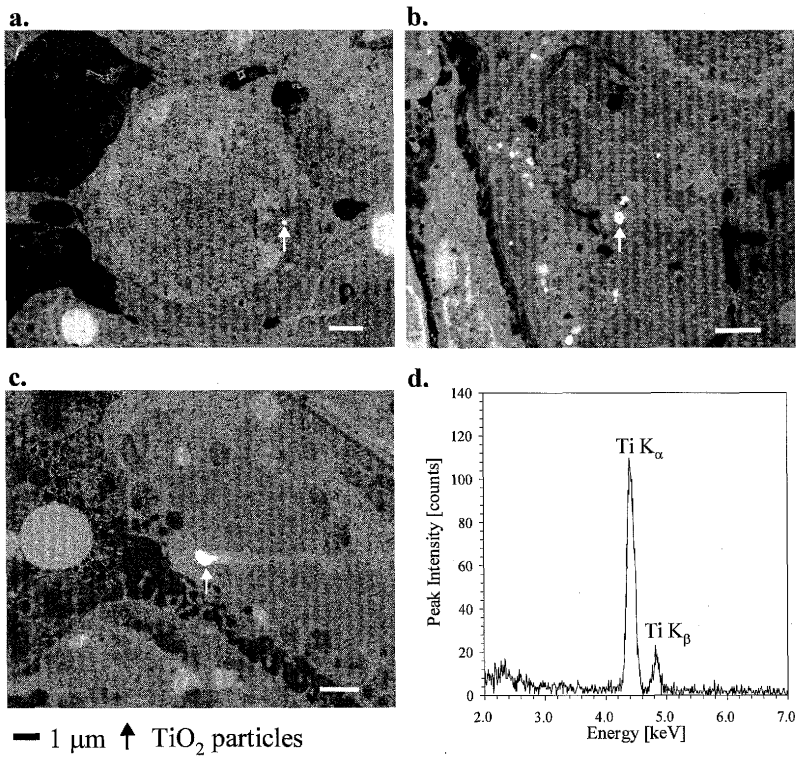

Fig. 2. Detection of TiO_2 Nanoparticles in the Testis of Offspring by EDS

Testes were dissected from 6-week-old mice and fixed. Particles were detected in the cells of testis by TEM and field FE-SEM. The particles were identified as TiO_2 by EDS at 7 kV accelerating voltage, 1×10^{-10} A beam current and 100 sec measurement time. Aggregated TiO_2 nanoparticles (100–200 nm) were detected in spermatids (a), Sertoli cells (b) and Leydig cells (c). Scale bars, 1 μm. TiO_2 particles are indicated by arrows. Particles in the testis were identified respectively as TiO_2 by EDS (d).

and reproduction weights were measured. TiO_2-exposed group had significantly lower body weight (88 % relative to control) and significantly higher weight of epidermis per body weight (117 % relative to control). However, there were no significant changes in the weight of other reproductive organs.

The presence of TiO_2 particles was assessed in testis and brain from 4-day-old and 6-week-old offspring by TEM and FE-SEM. Particles in the testis and brain were identified as TiO_2 by EDS at 7 kV accelerating voltage, 1×10^{-10} A beam current, and 100 sec measurement time.

As shown in Fig. 2, aggregates of TiO_2 nanoparticles (100–200 nm) were detected in Leydig cells, Sertoli cells, and spermatids in the testis at both 4 days and 6 weeks of age. Sperm samples were collected from the cauda epididymis, and sperm motility and morphology were evaluated under phase contrast microscopy. Testes of 6-week-old mice were homogenized, and DSP was examined. Testes were also fixed and stained with standard procedures for examination by light and electron microscopy.

Among 6-week-old mice, the seminiferous tubules of hematoxylin and eosin-stained sections from control mice showed the normal spermatogenic cycle with germ cells and Sertoli cells. Sertoli cells were located regularly in the periphery of the seminiferous tubules and had large nuclei with large nucleoli. Testicular morphology in TiO_2-exposed mice was abnormal compared to that in control mice. In exposed mice, some seminiferous tubules appeared disorganized and disrupted. There were fewer mature sperm in the tubule lumen. The damaged tubules were scattered randomly throughout the testis (Fig. 3). These effects were dependent on the dose of TiO_2 and were significantly higher in the TiO_2 exposed mice than in control mice. DSP per gram of testis, epididymal sperm motility, and the number of Sertoli cells were significantly lower in mice exposed to TiO_2 than in control mice. Sperm morphology did not differ significantly (Fig. 4). These data suggest that prenatal exposure to nano-sized TiO_2 has detrimental effects on mouse spermatogenesis in offspring.

The olfactory bulb and the cerebral cortex (frontal and temporal lobes) of 6-week-old mice were examined by TEM and FE-SEM/EDS. Nano-sized TiO_2 particles were detected in cells in brains of 6-week-old mice exposed prenatally to TiO_2

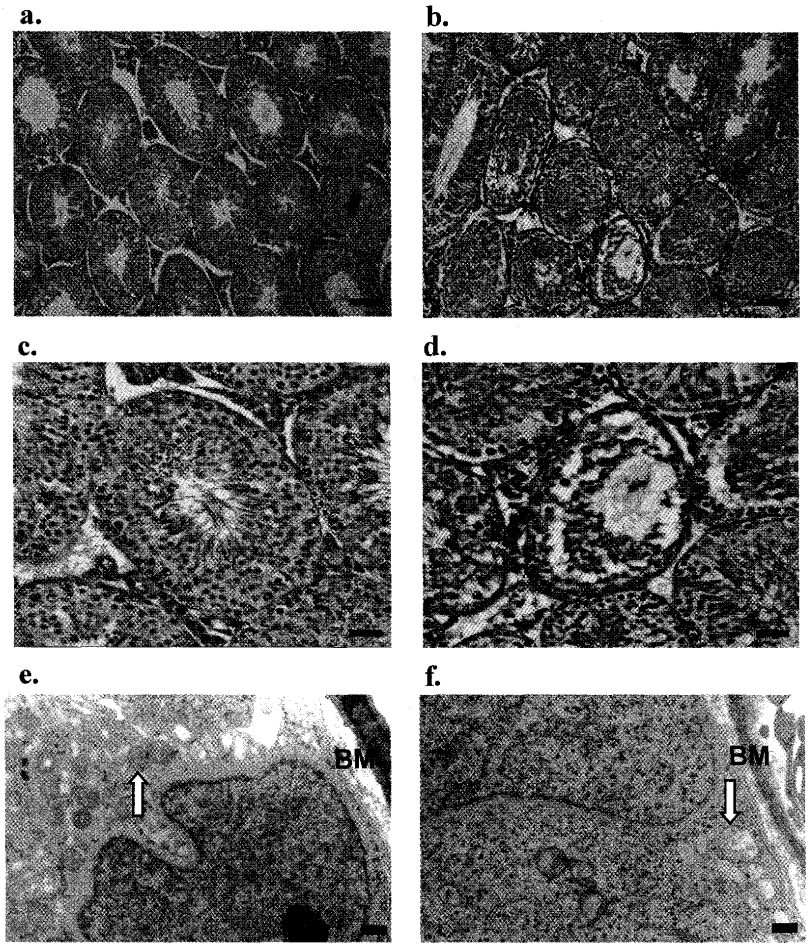

Fig. 3. Morphology of Seminiferous Tubules and Testicular Functions in 6-week-old Mice Exposed Prenatally to TiO_2

Hematoxylin and eosin-stained sections of seminiferous tubules from control mice (a, c) show a normal spermatogenic cycle with germ cells and Sertoli cells. Testicular morphology in TiO_2-exposed mice (b, d) was abnormal compared to that in control mice. Some seminiferous tubules appear disorganized and disrupted. There were fewer mature sperm in the tubule lumen. Damaged tubules were scattered randomly throughout the testis. Scale bars, 100 μm (a, b) and 25 μm (c, d). TEM demonstrating mitochondria (white arrow) of Sertoli cells from control mice (e) and TiO_2-exposed mice (f). Enlargement of mitochondria and disappearance of cristeae were observed (f). Scale bars, 1 μm (e, f). BM; basement membrane.

(Fig. 5, a–e). We believe that the nanoparticles were transferred from the mother to the fetus and moved into the brain because blood-brain barrier was undeveloped.

Numerous cells positive for caspase-3, a common enzymatic marker of apoptosis, were observed under light microscopy in the olfactory bulb of 6-week-old mice exposed prenatally to TiO_2, and the number of caspase-3-positive mitral cells was significantly higher in exposed mice than in control mice (no positive cells, Fig. 6. a, b).

Electron microscopic observations of olfactory bulb revealed that a subset of cells contained cresent-shaped spaces (CSS), which are specific features of apoptosis.[12] Apoptotic granular perithelial (GP) cells, which are scavenger cells that surround vessels in the brain, contained unidentified particulate matter. Occlusion of small vessels and perivascular edema were observed in the prenatally TiO_2-exposed mice.

The abnormalities varied in severity were dependent on the TiO_2 concentration, and were not observed in the control group. These data indicate that prenatal exposure of mice to TiO_2 has a severe negative effect on fetal brain development and carries a risk of various nervous system disorders.

DISCUSSION

We show here that anatase TiO_2 nanoparticles administered subcutaneously to pregnant mice are transferred to and affect the genital and cranial nerve systems of the offspring. These findings suggest that anatase TiO_2 can harm the developing fetus in mice. As we observed in TiO_2-exposed mice, we

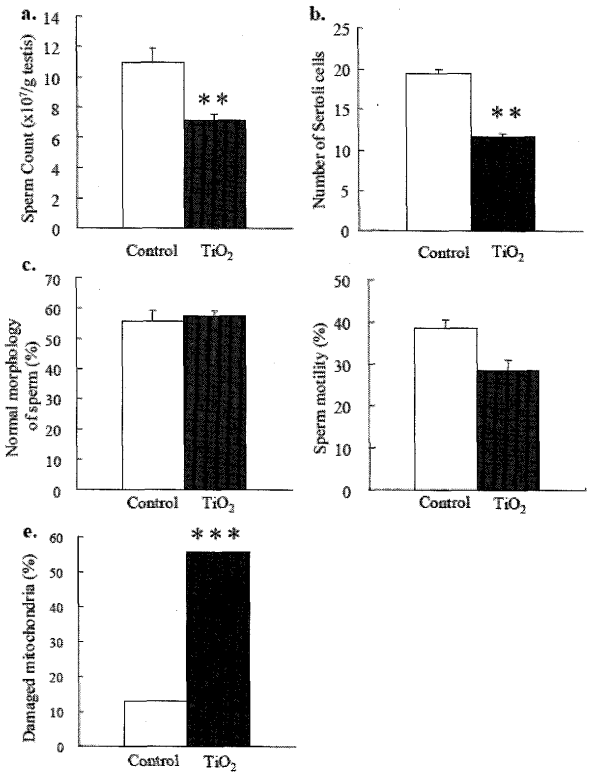

Fig. 4. Effect of Prenatal Exposure to TiO_2 on Seminiferous Tubles and Testicular Functions in 6-week-old Mice

Testis of 6-week-old mice was homogenized on ice, and DSP was determined (a). Sertoli cells in seminiferous tubules were counted (b). Sperm samples were collected from the cauda epididymis, and morphology (c) and sperm motility (d) were determined under phase contrast microscopy. Sertoli cells with damaged mitochondria were counted by TEM (e). Control: $n = 8$, TiO_2: $n = 8$. Presented are the mean ± S.E., where *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

have observed various histologic and functional effects on the male reproductive and central nervous systems in mice exposed prenatally to diesel exhaust (DE)[13–18] and diesel exhaust particles (DEP). The changes in the reproductive and central nervous systems in DE-exposed mice could be reduced by eliminating particles including nano-sized particles with a high-quality filter (unpublished data). Sugamata *et al.*[17] also found that granular perithelial cells, which are scavenger cells, showed signs of apoptosis in the cerebrum and hippocampus of newborn mice exposed prenatally to DE. Furthermore, the cytoplasmic granules of these cells contained nano-sized particles. These observations suggest that exposure of pregnant mice to tiny particles can damage the fetus.

To prevent exposure of the fetus to harmful substances, there is a blood-placenta barrier between the mother and fetus. There is also a blood-brain barrier and blood-testis barrier in the important regions of the brain and genitals, respectively, in adult mice. Our present electron microscopy data indicate that nanoparticles can transfer from pregnant mice into brain and testis of their offspring. These blood barriers are undeveloped or under developed in the fetus, therefore, harmful nanoparticles could easily pass into the brain during the early stages of fetal development.

Nano-sized particles can enter the human body via the lungs and intestines. Whether such particles can penetrate the skin is less clear,[6,7] Kreilgaard[19] suggested that very small TiO_2 particles (*e.g.* 5–20 nm) can penetrate the skin and interact with the immune system. Tinkle *et al.*[20] showed that 0.5- and 1.0-μm particles, in conjunction with motion, penetrate the stratum corneum of human skin and reach the epidermis and, occasionally, the dermis.

There are reports that inhaled or injected nanoparticles enter the systemic circulation[21–23] and migrate to various organs and tissues.[24] If particles enter the body, their distribution is a function of their size and surface characteristics. There may be a critical size beyond which movement of the nanoparticles within the body is restricted. The brain is especially vulnerable to oxygen stress damage, and recent studies have supported our present and previous findings that nanosized particles can be uptaken in brain[25] and enter the central nervous system.[26] Oberdörster *et al.*[27] reported that inhaled nanoparticles could be translocated into brain via the olfactory nerves. Sugamata *et al.*[18] reported previously that specific features of apoptosis were present in Purkinje cells of cerebellum in mice exposed prenatally to DE. In the present study, we observed few apoptotic features in Purkinje cells of TiO_2-exposed mice. DEP and TiO_2 particles may differ in their abilities to induce apoptosis in cerebellum.

Regardless of the particle size, TiO_2 has only minimal effects in adult rodents.[28] However, numerous *in vitro* studies revealed that TiO_2 nanoparticles cause oxidative stress-mediated toxicity in diverse cell types including skin fibroblasts,[29] alveolar macrophages.[30] Long *et al.*[31] showed that mouse microglia engulfed the TiO_2 particles and, for 2 hr, released bursts of reactive oxygen molecules that interfered with mitochondrial energy production. This did not damage the microglia, however, prolonged exposure to such compounds can damage neurons. Greater surface area per mass renders nano-size particles more active biologically than larger particles of the same chemical makeup.

Numerous studies regarding the effects of ul-

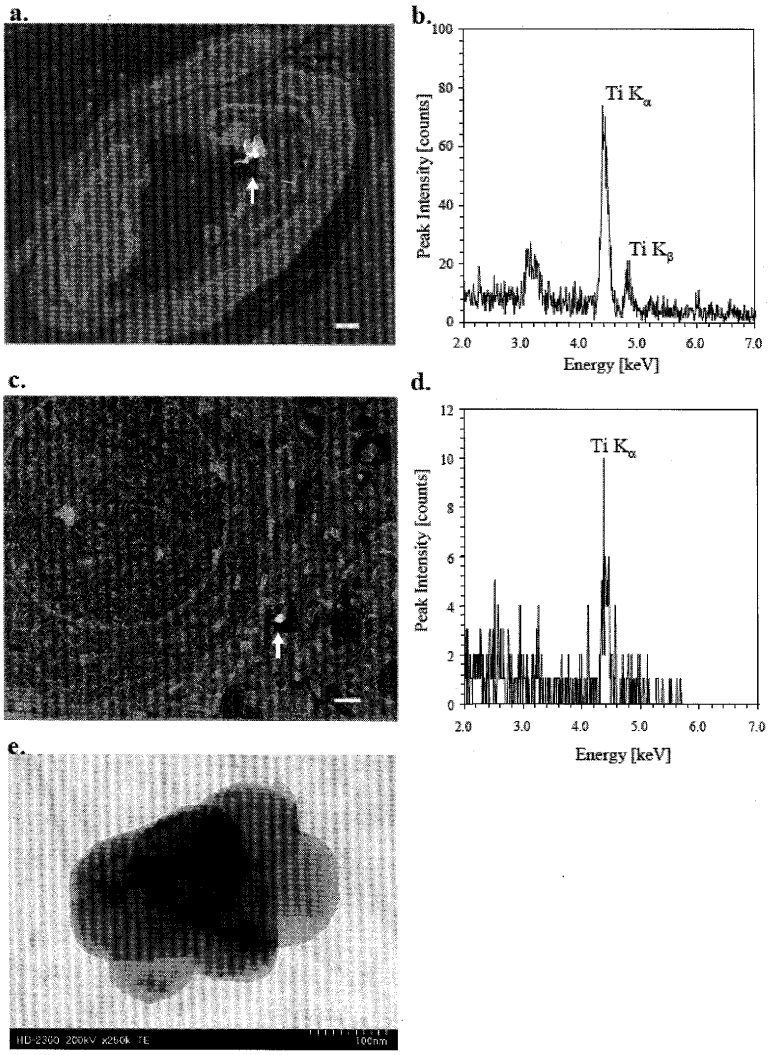

Fig. 5. Detection of TiO_2 Nanoparticles in the Olfactory Bulb and Cerebral Cortex of Brain of Offspring of TiO_2-exposed Mice by EDS

Olfactory bulb and cerebral cortex were dissected from 6-week-old mice and fixed. Particles were detected by TEM and FE-SEM. Photographs demonstrating aggregated TiO_2 nanoparticles (100–200 nm) in endotherial cells of olfactory bulb (a), and nerve cell fibers in cerebral cortex (c). Scale bars, 1 μm. TiO_2 particles are indicated by arrows. Particles in the brain were identified respectively as TiO_2 by EDS at 15 kV (b) and 7 kV (d) accelerating voltage, 1×10^{-10} A beam current and 100 sec measurement time. Electron micrograph demonstrating magnified aggregated TiO_2 particles in nerve cells in cerebral cortex (e).

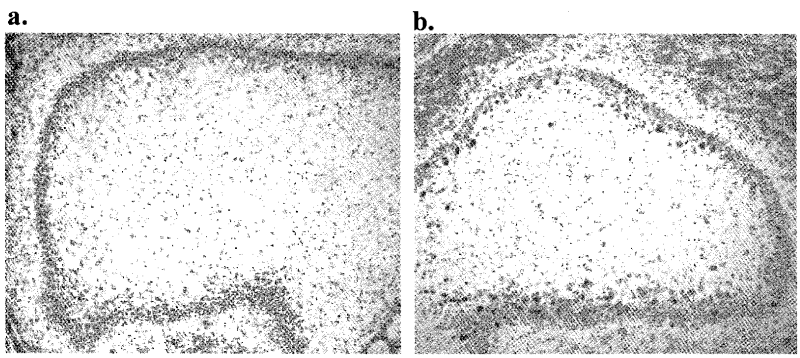

Fig. 6. Immunohistochemical Staining of Caspase-3 in Olfactory Bulb of 6-week-old Mice

(a) Control mice, (b) mice exposed prenatally to TiO_2. Numerous caspase-3 positive mitral cells are visible and the number of positive cells in TiO_2-exposed mice is significantly higher compared with that in control mice.

trafine particle pollutants on respiratory and circulatory systems have been reported. However, little is known about the effect on the genital and central nervous systems. Our present and former findings suggest that widespread use of TiO_2 and other nanoparticles including ultrafine particulates in air might affect unborn children, especially development of their reproductive and nervous systems. Therefore, research into the risk of exposure to nanoparticles, into removal of nanoparticles from the environment, and into methods to protect against toxicity of such particles is important.

Acknowledgements We thank Dr. I. Sugawara of Research Institute of Tuberculosis for help with preparation of testis samples for histology. We thank the graduate and undergraduate students in the Takeda and Nihei laboratories. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in Aid for the Private University Science Research Upgrade Promotion Business Academic Frontier Project and a Grant-in Aid for Health and Labour Sciences Research Grants, Research on Risk of Chemical Substances, from the Ministry of Health, Labour and Welfare.

REFERENCES

1) Mazzola, L. (2003) Commercializing nanotechnology. *Nature Biotechnol.*, **21**, 1137–1143.
2) Paull, R., Wolfe, J., Hebert, P. and Sinkula, M. (2003) Investing in nanotechnology. *Nature Biotechnol.*, **21**, 1144–1147.
3) Salata, O. V. (2004) Applications of nanoparticles in biology and medicine. *J. Nanobiotechnology*, **2**, 3.
4) Nel, A., Xia, T., Mädler, L. and Li, N. (2006) Toxic potential of materials at the nanolevel. *Science*, **311**, 622–627.
5) Oberdörster, G., Oberdörster, E. and Oberdörster, J. (2005) Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect.*, **113**, 823–839.
6) Donaldson, K., Stone, V., Tran, C. L., Kreyling, W. and Borm, P. J. A. (2004) Nanotoxicology. *Occup. Environ. Med.*, **61**, 727–728.
7) Hoet, P. H. M., Brüske-Hohlfeld, I. and Salata, O. V. (2004) Nanoparticles—known and unknown health risks. *J. Nanobiotechnology*, **2**, 12.
8) Amurao, C. V. (2006) Nanotechnology—It's a small (and scary) world after all. *Occupational Health Tracker*, **9**, 3–6.
9) Wolf, R., Matz, H., Orion, E. and Lipozencic, J. (2003) Sunscreens—the ultimate cosmetic. *Acta Dermatovenerol. Croat.*, **11**, 158–162.
10) Sayes, C. M., Wahi, R., Kurian, P. A., Liu, Y., West, J. L., Ausman, K. D., Warheit, D. B. and Colvin, V. L. (2006) Correlating nanoscale titania structure with toxicity: A cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. *Toxicol. Sci.*, **92**, 174–185.
11) Guur, J. R., Wang, A. S., Chen C. H. and Jan, K. Y. (2005) Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epitherial cells. *Toxicology*, **213**, 66–73.
12) Ihara, T., Yamamoto, T., Sugamata, M., Okumura, H. and Ueno, Y. (1998) The process of ultrastructural changes from nuclei to apoptotic body. *Virchows Arch.*, **433**, 443–447.
13) Takeda, K., Tsukue, N. and Yoshida, S. (2004) Endocrine-disrupting activity of chemicals in diesel exhaust and diesel exhaust particles. *Environ. Sci.*, **11**, 33–45.
14) Fujimoto, A., Tsukue, N., Watanabe, M., Sugawara, I., Yanagisawa, R., Takano, H., Yoshida, S. and Takeda, K. (2005) Diesel exhaust affects immunological action in the placenta of mice. *Environ. Toxicol.*, **20**, 431–440.
15) Yoshida, S., Ono, N., Tsukue, N., Oshio, S., Umeda, T., Takano, H. and Takeda, K. (2006) In utero exposure to diesel exhaust increased accessory reproductive gland weight and serum testosterone concentration in male mice. *Environ. Sci.*, **13**, 139–147.
16) Ono, N., Oshio, S., Niwata, Y., Yoshida, S., Tsukue, N., Sugawara, I., Takano, H. and Takeda, K. (2007) Prenatal exposure to diesel exhaust impairs mouse spermatogenesis. *Inhal. Toxicol.*, **19**, 275–281.
17) Sugamata, M., Ihara, T., Takano, H., Oshio, S. and Takeda, K. (2006) Maternal diesel exhaust exposure damages newborn murine. *J. Health Sci.*, **52**, 82–84.
18) Sugamata, M., Ihara, T., Sugamata, M. and Takeda, K. (2006) Maternal exposure to diesel exhaust leads to pathological similarity to autism in newborns. *J. Health Sci.*, **52**, 486–488.
19) Kreilgaard, M. (2002) Influence of microemulsions on cutaneous drug delivery. *Adv. Drug Deliv. Rev.*, **54**, 77–98.
20) Tinkle, S. S., Antonini, J. M., Rich, B. A., Roberts, J. R., Salmen, R., DePree, K. and Adkins, E. J. (2003) Skin as a route of exposure and sensitization in chronic beryllium disease. *Environ. Health Perspect.*, **111**, 1202–1208.
21) Takenaka, S., Karg, E., Roth, C., Schulz, H., Ziese-

nis, A., Heinzmann, U., Schramel, P. and Heyder, J. (2001) Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. *Environ. Health Perspect.*, **109**, 547–551.

22) Nemmar, A., Hoet, P. H., Vanquickenborne, B., Dinsdale, D., Thomeer, M., Hoylaerts, M. F., Vanbilloen, H., Mortelmans, L. and Nemery, B. (2002) Passage of inhaled particles into the blood circulation in humans. Circulation. *Circulation*, **105**, 411–414.

23) Meiring, J. J., Borm, P. J., Bagate, K., Semmler, M., Seitz, J., Takenaka, S. and Kreyling, W. G. (2005) The influence of hydrogen peroxide and histamine on lung permeability and translocation of iridium nanoparticles in the isolated perfused rat lung. *Particle and Fibre Toxicology*, **2**, 3.

24) Samet, J. M., DeMarini, D. W. and Malling, H. V. (2004) Do airborne particles induce heritable mutations? *Science*, **304**, 971–972.

25) Lockman, P. R., Oyewumi, M. O., Koziara, J. M., Roder, K. E., Mumper, R. J. and Allen, D. D. (2003) Brain uptake of thiamine-coated nanoparticles. *J. Control. Release*, **93**, 271–282.

26) Kreyling, W. G., Semmler, M., Erbe, F., Mayer, P., Takenaka, S., Schulz, H., Oberdörster, G. and Ziesenis, A. (2002) Translocation of ultrafine insoluble iridium particles from lung epithelium to extrapulmonary organs is size dependent but very low. *J. Toxicol. Environ. Health A*, **65**, 1513–1530.

27) Oberdörster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Kreyling, W. and Cox, C. (2004) Translocation of inhaled ultrafine particles to the brain. *Inhal. Toxicol.*, **16**, 437–445.

28) Warheit, D. B., Brock, W. J., Lee, K. P., Webb, T. R. and Reed, K. L. (2005) Comparative pulmonary toxicity inhalation and instillation studies with different TiO_2 particle formulations: impact of surface treatments on particle toxicity. *Toxicol. Sci.*, **88**, 514–524.

29) Wamer, W. G., Yin, J. J. and Wei, R. R. (1997) Oxidative damage to nucleic acids photosensitized by titanium dioxide. *Free Radic. Biol. Med.*, **23**, 851–858.

30) Renwick, L. C., Donaldson, K. and Clouter, A. (2001) Impairment of alveolar macrophage phagocytosis by ultrafine particles. *Toxicol. Appl. Pharmacol.*, **172**, 119–127.

31) Long, T. C., Saleh, N., Tilton, R. D., Lowry, G. V. and Veronesi, B. (2006) Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. *Environ. Sci. Technol.*, **40**, 43–46.