

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

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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 (TiO₂), 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 TiO₂ by energy-dispersive X-ray spectroscopy were found in testis and brain of exposed 6-week-old male mice. In the offspring of TiO₂-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 (TiO₂), 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 nanomaterials are used in sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electronics 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 (TiO₂), a non-

combustible, odorless powder, is an important material used in commerce. Anatase TiO₂ is currently used in products as diverse as sunscreens and coatings for self-cleaning windows.⁹⁾ TiO₂ can generate reactive oxygen species quite efficiently, particularly when exposed to ultraviolet light. The photocatalytic activity of the anatase form of TiO₂ was reported to be higher than that of the rutile form.¹⁰⁾ Gurr and colleagues¹¹⁾ reported that nano-sized anatase TiO₂ 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 TiO₂ in the next generation has yet to be examined. In the present study we examined the effects of prenatal exposure to anatase TiO₂ on the genital and cranial nerve systems of male offspring mice.

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MATERIALS AND METHODS

Materials — TiO₂ particles (anatase form, particle size 25–70 nm, surface area 20–25 m²/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 TiO₂ 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 TiO₂ 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

TiO₂ 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 TiO₂ particles, body

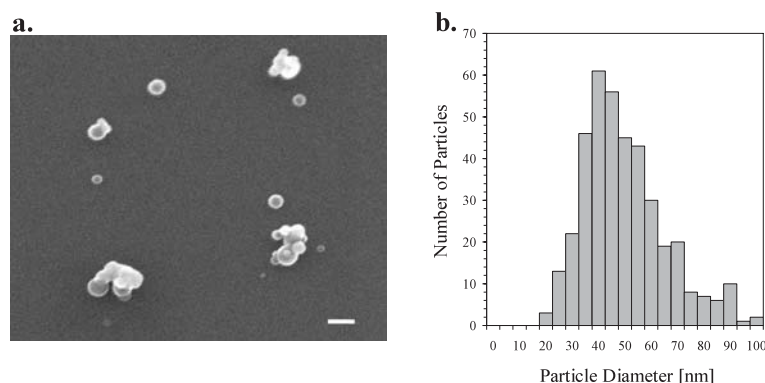


Fig. 1. Distribution of TiO₂ Particle Diameter by FE-SEM

(a) FE-SEM Image of TiO₂ particles (15.0 kV × 80000, Scale bar, 100 nm). (b) Distribution of TiO₂ 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.

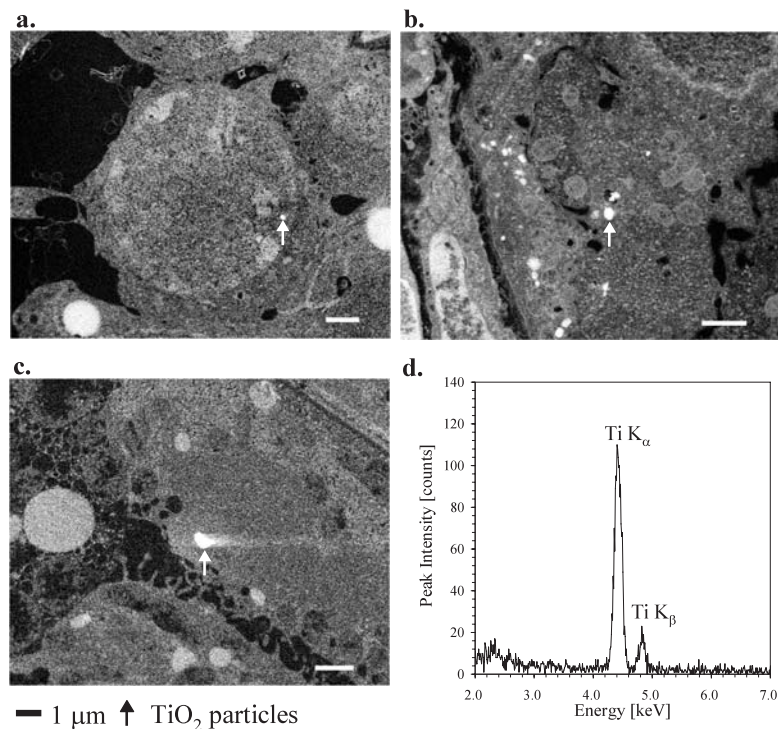


Fig. 2. Detection of TiO₂ Nanoparticles in the Testis of Offspring by EDS

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 TiO₂ by EDS at 7 kV accelerating voltage, 1×10^{-10} A beam current and 100 sec measurement time. Aggregated TiO₂ nanoparticles (100–200 nm) were detected in spermatids (a), Sertoli cells (b) and Leydig cells (c). Scale bars, 1 μ m. TiO₂ particles are indicated by arrows. Particles in the testis were identified respectively as TiO₂ by EDS (d).

and reproduction weights were measured. TiO₂-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 TiO₂ 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 TiO₂ 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 TiO₂ 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 TiO₂-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 TiO₂ and were significantly higher in the TiO₂ 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 TiO₂ than in control mice. Sperm morphology did not differ significantly (Fig. 4). These data suggest that prenatal exposure to nano-sized TiO₂ 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 TiO₂ particles were detected in cells in brains of 6-week-old mice exposed prenatally to TiO₂

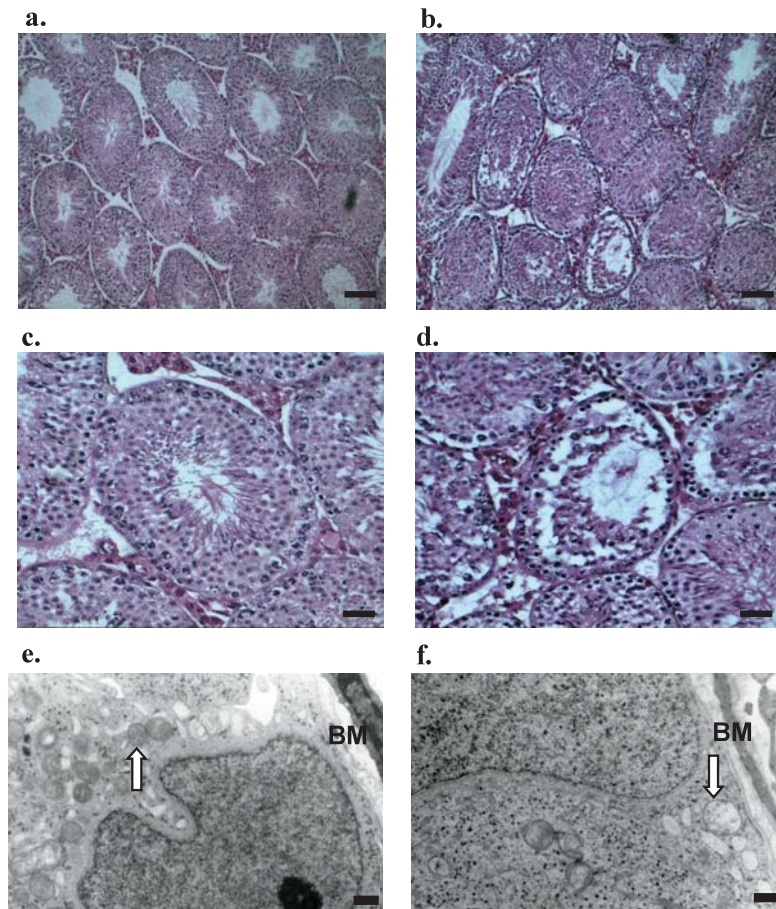


Fig. 3. Morphology of Seminiferous Tubules and Testicular Functions in 6-week-old Mice Exposed Prenatally to TiO_2

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 cristae 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 crescent-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

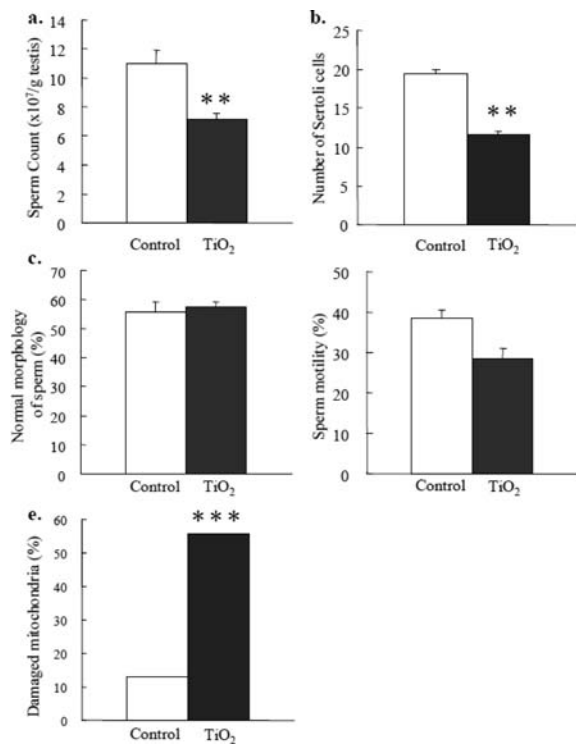


Fig. 4. Effect of Prenatal Exposure to TiO₂ on Seminiferous Tubules and Testicular Functions in 6-week-old Mice

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: $n = 8$, TiO₂: $n = 8$. Presented are the mean \pm 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} Kreilgaard¹⁹ suggested that very small TiO₂ 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- μ m 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 brain²⁵ 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 TiO₂-exposed mice. DEP and TiO₂ particles may differ in their abilities to induce apoptosis in cerebellum.

Regardless of the particle size, TiO₂ has only minimal effects in adult rodents.²⁸ However, numerous *in vitro* studies revealed that TiO₂ 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 TiO₂ 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-

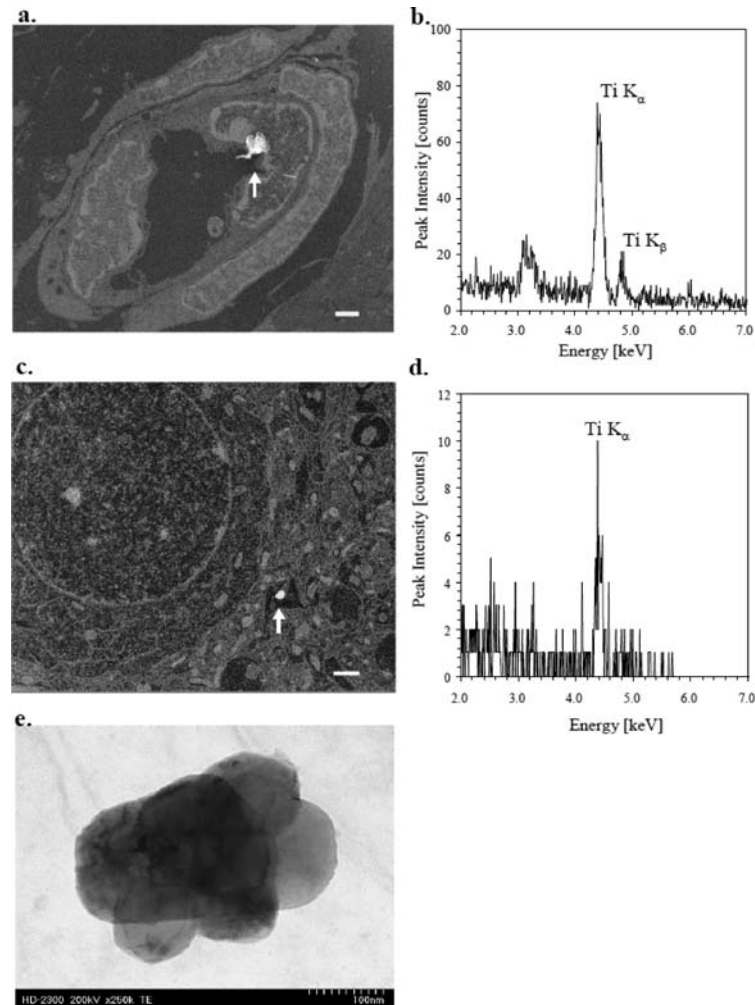


Fig. 5. Detection of TiO₂ Nanoparticles in the Olfactory Bulb and Cerebral Cortex of Brain of Offspring of TiO₂-exposed Mice by EDS

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 TiO₂ nanoparticles (100–200 nm) in endothelial cells of olfactory bulb (a), and nerve cell fibers in cerebral cortex (c). Scale bars, 1 μm. TiO₂ particles are indicated by arrows. Particles in the brain were identified respectively as TiO₂ 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 TiO₂ particles in nerve cells in cerebral cortex (e).

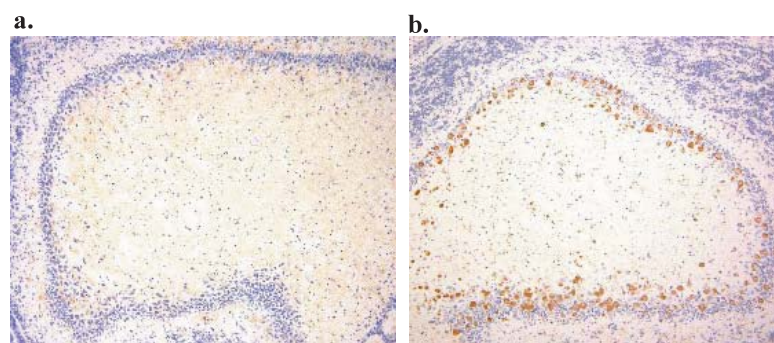


Fig. 6. Immunohistochemical Staining of Caspase-3 in Olfactory Bulb of 6-week-old Mice

(a) Control mice, (b) mice exposed prenatally to TiO₂. Numerous caspase-3 positive mitral cells are visible and the number of positive cells in TiO₂-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 TiO₂ 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.

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