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Neurotoxicological effects and the impairment of spatial recognition memory in mice caused by exposure to TiO_2 nanoparticles

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are now in daily use including popular sunscreens, toothpastes, and cosmetics. However, the effects of TiO_2 NPs on human body, especially on the central nervous system, are still unclear. The aim of this study was to determine whether TiO_2 NPs exposure results in persistent alternations in nervous system function. ICR mice were exposed to TiO_2 NPs through intragastric administration at 0, 5, 10 and 50 mg/kg body weight every day for 60 days. The Y-maze test showed that TiO_2 NPs exposure could significantly impair the behaviors of spatial recognition memory. To fully investigate the neurotoxicological consequence of TiO_2 NPs exposure, brain elements and neurochemicals were also investigated. The contents of Ca, Mg, Na, K, Fe and Zn in brain were significantly altered after TiO_2 NPs exposure. Moreover, TiO_2 NPs significantly inhibited the activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase, Ca^{2+}/Mg^{2+}-ATPase, acetylcholine esterase, and nitric oxide synthase; the function of the central cholinergic system was also noticeably disturbed and the contents of some monoamines neurotransmitters such as norepinephrine, dopamine and its metabolite 3, 4-dihydroxyphenylacetic acid, 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid were significantly decreased, while the contents of acetylcholine, glutamate, and nitric oxide were significantly increased. These first findings indicated that exposure to TiO_2 NPs could possibly impair the spatial recognition memory ability, and this deficit may be possibly attributed to the disturbance of the homeostasis of trace elements, enzymes and neurotransmitter systems in the mouse brain. Therefore, the application of TiO_2 NPs and exposure effects especially on human brain for long-term and low-dose treatment should be cautious.

1. Introduction

Because of its whitening and photocatalytic effects, titanium dioxide (TiO_2) is used widely in the production of every day products such as paper, cosmetics and food. TiO_2 nanoparticles (NPs) are now in daily use including popular sunscreens, toothpastes, and cosmetics [1,2]. Moreover TiO_2 is used at the nanoscale level in manufacturing [3,4] and to decontaminate water, air, and soil by destruction of a pesticide [5–7]. However, the safety and risk of commercialized products has not been examined in detail. If the current trend continues, it will inevitably result in the discharge of synthetic nanomaterials to the environment, potentially threatening human and environmental health.

Recent studies indicate that TiO_2 NPs are toxic to lung, liver, spleen, kidney and gill of animals [8–17]. Many studies have unequivocally showed that exposure to TiO_2 NPs could be translocated into the central nervous system (CNS) via the olfactory pathway and damage brain neurocyte and tissue *in vitro* and *in vivo*. Long et al. found that TiO_2 NPs (25 nm) promoted reactive oxygen species (ROS) production in the mouse microglia, interfered with mitochondrial energy production, and damaged rat dopaminergic neurons in complex brain cultures *in vitro* [18,19]. Wang et al. reported that TiO_2 NP (80 nm) could be translocated to the olfactory bulb through the olfactory nerve system after inhalation or intranasal instillation [11,20], TiO_2 NPs with oral gavage caused a slight brain lesion of mice, such as vacuoles of neurons and fatty degeneration of hippocampus [11], and the intranasal instilled TiO_2 NP caused the obvious scattered Nissl body, large cell somata and an irregular appearance of neurons in the CA1 region of hippocampus, produced higher inflammation responses, in association with the significantly increased tumor necrosis factor alpha (TNF-α) and

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interleukin (IL-1β) levels, significantly increased the lipid peroxidation and protein oxidation in the exposed mice and also induced other specific neurochemicals [21,22]. It was found that TiO_2 NPs (21 nm) could be found in brain and damaged brain of hairless mice [23]. Nanosized TiO_2 (21 nm) was demonstrated to promote an exaggerated neuroinflammatory responses by enhancing microglial activation in the pre-inflamed brain of mice [24]. In our previous study, TiO_2 NPs (5 nm) were found to be accumulated and induced some neurons to turn into filamentous shapes and others into inflammatory cells of mice, and oxidative stress in the mouse brain after injection with various TiO_2 NPs doses, and we speculated TiO_2 NPs might decrease the cognitive function of the mouse brain [25]. All these facts, although based on acute or subchronic experiments, may indicate that the impact of nanomaterials on brain function is not negligible, especially for the workers working in nanomaterials manufacturing. Thus it has become increasingly important to obtain more information on the environmental and biological effects of these nanomaterials in order to forestall any possible deleterious effect caused by their use.

In this article, we investigated the effects of subchronic TiO_2 NPs exposure on brain functions of mice from four aspects: spatial recognition memory, histopathological test, elemental contents and neurochemical changes in brain. Our findings will provide an important theoretical basis for evaluating the toxicity underlying effects of nanomaterials on animals and human.

2. Material and method

2.1. Chemicals and preparation

Nanoparticulate anatase TiO_2 was prepared via controlled hydrolysis of titanium tetrabutoxide. The details of the synthesis are as follows [26]: Colloidal titanium dioxide was prepared via controlled hydrolysis of titanium tetrabutoxide. In a typical experiment, 1 ml of Ti (OC4H9) 4 dissolved in 20 ml of anhydrous isopropanol was added dropwise to 50 ml of double-distilled water adjusted to pH 1.5 with nitric acid under vigorous stirring at room temperature. Then, the temperature was raised to 60 °C and kept 6 h for better crystallization of nanoparticulate TiO_2 particles. The resulting translucent colloidal suspension was evaporated using a rotary evaporator yielding a nanocrystalline powder. The obtained powder was washed three times with isopropanol and dried at 50 °C until complete evaporation of the solvent. The average grain size calculated from broadening of the (101) X-ray diffraction peak of anatase using Scherrer's equation was approximately 5 nm. The value of surface area of the anatase TiO_2 particles was determined to be 174.78 m^2/g by Brunauer–Emmett–Teller (BET) adsorption measurements on a Micromeritics ASAP 2020M + C instrument (Micromeritics Co., USA).

A 0.5%, w/v hydroxypropylmethylcellulose K4M (HPMC, K4M) was used as a suspending agent. Nanoparticulate anatase TiO_2 powder was dispersed onto the surface of 0.5%, w/v HPMC, and then the suspending solutions containing nanoparticulate TiO_2 particles were treated by ultrasonic for 30 min and mechanically vibrated for 5 min.

2.2. Animals and treatment

80 CD-1 (ICR) female mice (24 ± 2 g) were purchased from the Animal Center of Soochow University (Suzhou, China). Animals were housed in stainless steel cages in a ventilated animal room. Room temperature was maintained at 20 ± 2 °C, relative humidity at 60 ± 10%, and a 12-h light/dark cycle. Distilled water and sterilized food for mice were available ad libitum. They were acclimated to this environment for 5 days prior to dosing. All animal procedures were performed in compliance with the regulations and guidelines of the national ethics committee on animal welfare of China. Animals were randomly divided into four groups (each 20 mice): control group (treated with 0.5% w/v HPMC) and three experimental groups (5, 10, and 50 mg/kg BW TiO_2 NPs). TiO_2 NPs (5, 10, and 50 mg/kg BW) suspensions were given to mice by an intragastric administration every day for 60 days, respectively. Animals were weighted before intragastric administration every day. The symptom and mortality were observed and recorded carefully every day for 60 days (death not observed). The animals were regularly handled and weighed before behavioral experiments.

2.3. Behavioral apparatus and method

After 60 days, the acquisition of spatial recognition memory in all animals was detected by Y-mazes. The Y-maze was made of green–blue painted timber and consisted of three arms with an angle of 120° between each two arms. Each arm was 8 cm × 30 cm × 15 cm (width × length × height). The three identical arms were randomly designated: Start arm, in which the mouse started to explore (always open), Novel arm, which was blocked during the 1st trial, but open during the 2nd trial, and Other arm (always open).

The maze was placed in a sound attenuated room with dim illumination. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze, and the observer was always in the same position at least 3 m from the maze.

The Y-maze test consisted of two trials separated by an intertrial interval (ITI) to assess spatial recognition memory. The first trial was 10-min duration and allowed the mouse to explore only two arms (Start arm and Other arm) of the maze, with the third arm (Novel arm) being blocked. After a 1 h ITI, the second trial (retention) was conducted, during which all three arms were accessible and novelty vs. familiarity was analyzed by comparing behavior in all three arms. For the second trial, the mouse was placed back in the maze in the same starting arm, with free access to all three arms for 5 min. By using a ceiling-mounted CCD camera, all trials were recorded on a VCR. Video recordings were later analyzed and the number of entries and time spent in each arm were analyzed. Data were also expressed as percentage of total time and distance spent in arms every 30 s and during the total 5 min [27]. On the second trial, we also assessed which of the arms was entered first as another reflection of recognizing the novel arm—discrimination memory [28]. Because retention in the Y-maze test does not last longer than a few hours, this task can be assessed several times in the same animal [28]. All mice were therefore tested in the Y-maze three times using 1 h ITI.

2.4. Coefficients of organs and preparation of brain

After behavioral detection, all animals were first weighed and then sacrificed after being anesthetized by ether. The livers, spleens, kidneys, lungs, hearts, and brains were excised and weighed accurately. After weighing the body and tissues, the coefficients of liver, spleen, kidneys, lung, heart, and brain to body weight were calculated as the ratio of tissues (wet weight, mg) to body weight (g).

The brains were excised and rinsed in phosphate buffered saline (PBS), and quickly taken and frozen at −80 °C.

2.5. Elemental content analysis

Brains were taken out and thawed. About 0.1–0.3 g of brain were weighed, digested and analyzed for titanium content. Briefly, prior to elemental analysis, the tissues of interest were digested in nitric acid (ultrapure grade) overnight. After adding 0.5 ml H_2O_2, the mixed solutions were heated at about 160 °C using high-pressure reaction container in an oven chamber until the samples were completely digested. Then, the solutions were heated at 120 °C to remove the remaining nitric acid until the solutions were colorless and clear. At last, the remaining solutions were diluted to 3 ml with 2% nitric acid. Inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental X7, Thermo Electron Co., Finland) was used to analyze the titanium, sodium, magnesium, potassium, calcium, zinc, and iron concentration in the samples.

2.6. Histopathological examination of brain

For pathological studies, all histopathological tests were performed using standard laboratory procedures. The brains were embedded in paraffin blocks, then sliced into 5 μm in thickness and placed onto glass slides. After hematoxylin–eosin (HE) staining, the slides were observed and the photos were taken using optical microscope (Nikon U-III Multi-point Sensor System, USA), and the identity and analysis of the pathology slides were blind to the pathologist.

2.7. Assay of enzymatic activities

For enzymatic activity determinations, the brains were homogenized in 10 volumes of 0.15 M NaCl. A quantity of the homogenate was used to determine different enzymatic activities. The activities of acetylcholine esterase (AChE), Ca^{2+}-ATPase, Ca^{2+}/Mg^{2+}-ATPase, and Na^+/K^+-ATPase in the brain were spectrophotometrically measured with commercially-available kits (Nanjing Jiancheng Bioengineering Institute, China). The activity of total nitric oxide synthase (TNOS) in the brain were spectrophotometrically measured with commercially-available kit (Nanjing Jiancheng Bioengineering Institute, China) based on the oxidation of oxyhaemoglobin to methaemoglobin by nitric oxide. Protein concentrations were determined according to the Lowry method [29].

2.8. Determination of neurochemicals

The homogenate of brains was centrifuged at 12,000 g for 20 min at 4 °C. The 20 μl supernatant was applied to an HPLC-ECD (Shimadzu, Kyoto, Japan) to determine the concentrations of monoamine neurotransmitter and their metabolites, including dopamine (DA) and its metabolite 3, 4-dihydroxyphenylacetic acid

(DOPAC), 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), and norepinephrine (NE). The 0.1 mol/L sodium acetate/0.01 mol/L citric acid buffer (pH 4.8) containing 0.25 mol/L disodiummedetate, 0.4 mol/L dibutylamine, 1 mol/L sodium octyl sulfate and 82% methanol (v/v) was used as elution solution. The flow rate was 0.8 mL/min. The content of acetylcholine (Ach) was determined by HPLC-ECD. Using Tris–maleate, tetramethyl ammonium chloride and sodium octyl sulfonate as motive phase, the further purification was completed by reverse-phase HPLC [30].

Glutamate (Glu), concentrations were measured using commercially-available kits (Nanjing Jianchen Biological Institute, China). Using the standard Glu stock solutions to produce standard curves, Glu levels in the samples were detected by spectrophotometer at 340 nm, and were expressed as μmol/g prot. Nitric oxide (NO) concentration assay in the brain was performed according to kit protocols (Nanjing Jiancheng Bioengineering Institute, China). The OD value was determined by a spectrophotometer (U-3010, Hitachi, Japan). Results of NO were read with OD value at 550 nm. The result was calculated using the following formula:

$$NO\ (\mu mol/L) = (A_{sample} - A_{blank})/(A_{standard} - A_{blank}) \times 20(\mu mol/L).$$

2.9. Statistical analysis

Statistical analyses were done using SPSS11.7 software (SPSS Co., USA). Data were expressed as means ± standard error (SE). One-way analysis of variance (ANOVA) was carried out to compare the differences of means among multi-group data. Dunnett's test was carried out when each group of experimental data was compared with solvent-control data. Statistical significance for all tests was judged at a probability level of 0.05.

3. Results

3.1. Coefficients of organs to body weight

During the treatment, animals were all at growth state. The daily behaviors such as feeding, drinking and activity in TiO_2 NPs-treated groups were as normal as the control group. Table 1 shows the coefficients of the liver, kidney, spleen, lung, heart, and brain to body weight which were expressed as milligrams (wet weight of tissues)/grams (body weight). No obvious differences were found in the body weight of four groups. The significant differences were not observed in the coefficients of the liver, kidney, spleen, lung, heart, and brain in the 5 mg/kg BW TiO_2 NPs-treated group ($p > 0.05$). However, the coefficients of the liver, kidney, and spleen in the 10, and 50 mg/kg BW NPs-treated groups were significantly higher ($p < 0.05$ or $p < 0.01$) than the control. It can also be seen from Table 1 that with the dose increased, the coefficients of the brain we decreased gradually, and those of 10, and 50 mg/kg BW TiO_2 NPs-treated groups were lower than the control ($p < 0.05$ or $p < 0.01$), while the coefficients of the lung and heart have no obvious changes in the various animal groups ($P > 0.05$). It indicates that higher dose of TiO_2 NPs caused the brain damage of mice, which is confirmed by the further the spatial recognition memory and morphological examination of brain.

Table 1
The increase of net weight and coefficients of organs of mice after intragastric administration with TiO_2 NPs for the consecutive 60 days.

Indexes	TiO_2 NPs (mg/kg BW)			
	0	5	10	50
Net increase of BW (g)	4.70 ± 0.24	5.46 ± 0.27	5.28 ± 0.26	5.20 ± 0.26
Liver/BW (mg/g)	48.05 ± 2.40	51.33 ± 2.57	55.95 ± 2.80*	58.06 ± 2.90**
Kidney/BW (m/g)	15.19 ± 0.76	15.56 ± 0.78	16.05 ± 0.80*	17.46 ± 0.87**
Spleen/BW (mg/g)	5.12 ± 0.26	5.21 ± 0.26	6.12 ± 0.31 *	6.76 ± 0.34**
Lung/BW (mg/g)	8.97 ± 0.45	8.46 ± 0.42	8.13 ± 0.41	7.94 ± 0.40
Heart/BW (mg/g)	6.31 ± 0.32	6.37 ± 0.32	6.48 ± 0.32	6.52 ± 0.33
Brain/BW (mg/g)	16.40 ± 0.80	15.70 ± 0.79	14.91 ± 0.75*	13.67 ± 0.68**

Ranks marked with a star or double stars means that it is significantly different from the control (unexposed mice) at the 5% or 1% confidence level, respectively Values represent means ± SE, $n = 20$.

3.2. Evaluation of spatial recognition memory

Fig. 1 shows effects of TiO_2 NPs on the spatial recognition memory of mice. We can see that the percentage of duration for unexposed mice to visit the novel arm was significantly higher than the start and other arms ($p < 0.01$), the percentage of duration for 5 mg/kg BW TiO_2 NPs mice to explore to visit the novel arm was significantly higher than the start and other arms ($p < 0.05$), respectively. However, the percentage of duration for 10, 50 mg/kg BW TiO_2 NPs mice to explore to visit the novel arm was not statistically significant different from the start and other arms ($p > 0.05$). The results indicated that TiO_2 NPs impaired the spatial recognition memory of mice. The damage of the spatial recognition memory of mice indicates that TiO_2 NPs might be accumulated in the brain, cause brain injury, and alter the contents of electrolyte and neurotransmitter in the mouse brain after intragastric administration of TiO_2 NPs, which are confirmed by the further assays of titanium, morphological examination, electrolyte, and neurotransmitter of the brain.

3.3. Titanium content

The contents of titanium in the mouse brain are shown in Fig. 2. With increasing intragastric administration doses of TiO_2 NPs, the titanium accumulation in brain was statistically significant elevated ($p < 0.01$), while titanium in unexposed mice was not detected.

3.4. Brain histopathological observation

The brain histopathological pictures are illustrated in Fig. 3. In the 5, 10, and 50 mg/kg BW TiO_2 NPs treated groups, the brain tissue had abnormal pathology changes compared with the control, suggesting calcification in neurocyte which is a result of too much calcium depositing and accumulating in brain, proliferation of ependyma and spongiocyte, respectively (Fig. 3b–f). The generation of calcification and calcium deposition are confirmed by the further assays of Ca content of brain.

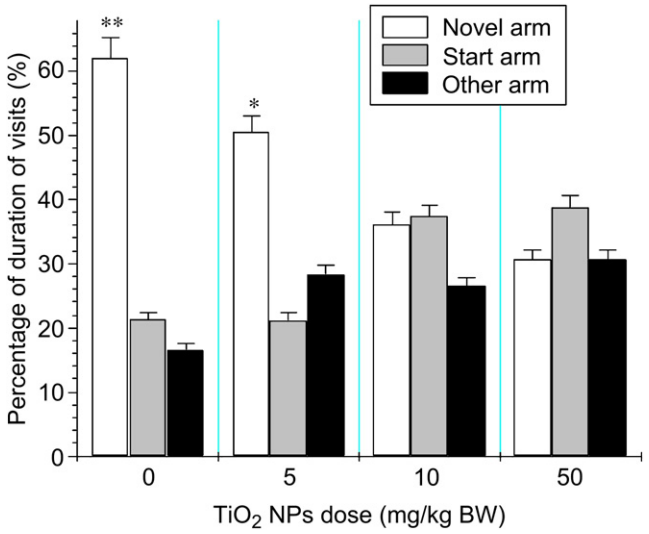

Fig. 1. The percentage of duration for mice visiting the novel, start, and other arms in the Y-maze after intragastric administration with TiO_2 NPs for the consecutive 60 days. Bars marked with a star or double stars mean that novel arm is significantly different from the start and other arms at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 20$.

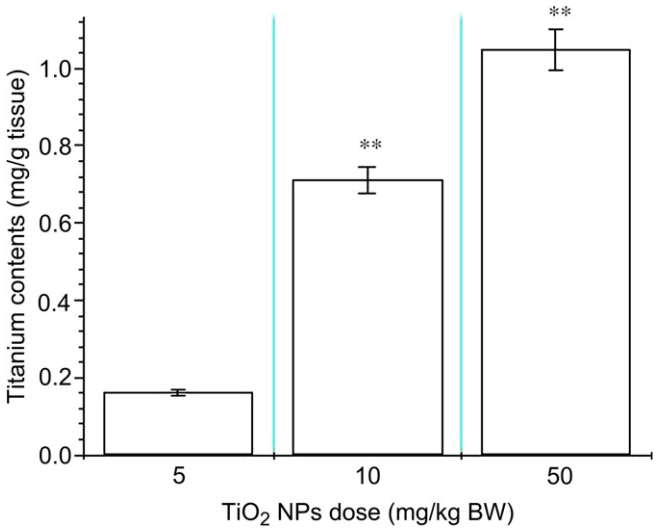

Fig. 2. The contents of titanium in the mouse brain caused by intragastric administration with TiO_2 NPs for consecutive 60 days. Bars marked with a star or double stars means that it is significantly different from the 5 mg/kg BW TiO_2 NPs treated group at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 5$.

3.5. Electrolyte contents

The contents of brain elements such as Ca, Mg, Na, K, Zn and Fe were determined. These elements are important building blocks of cells and play important roles in cell and tissue physiology. The changes of these elements can provide useful information on physiology and pathology of brain. The contents of Ca, Mg, Na, K, Zn and Fe in the mouse brain are listed in Table 2. It can be seen that exposure to TiO_2 NPs has obviously changed the electrolyte contents in the mouse brain. High contents of Ca, and Na were found in brain ($p < 0.05$ or 0.01), while the contents of Mg, K, Zn and Fe were lower in brain from TiO_2 NPs treated groups than those of the control ($p < 0.05$ or 0.01). The alteration of brain electrolyte might be involved in ion pump activities, which are confirmed by the further assays of Na^+/K^+-ATPase, Ca^{2+}-ATPase, Ca^{2+}/Mg^{2+}-ATPase of brain.

3.6. Enzyme activity

Fig. 4 shows that exposure to TiO_2 NPs significantly inhibited the activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase, Ca^{2+}/Mg^{2+}-ATPase ($p < 0.05$ or 0.01), and promoted the activities of AChE, and TNOS in brain ($p < 0.05$ or 0.01). Overall, TiO_2 NPs treatments did significantly affect the activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase, Ca^{2+}/Mg^{2+}-ATPase, AChE, and TNOS of the mouse brain.

3.7. Neurotransmitters

We can see from Table 3 that neurotransmitter contents of ACh, Glu, and NO in the mouse brain from the TiO_2 NPs treated groups were higher than those of the control ($p < 0.05$ or 0.01). The contents of monoamine neurotransmitter and related metabolites in the mouse brain caused by exposure to TiO_2 NPs are shown in Fig. 5. Compared with the controls, the levels of NE, DA, DOPAC, 5-HT, and 5-HIAA in the brain of mice decreased significantly by exposure to TiO_2 NPs ($p < 0.05$ or 0.01). It suggested that TiO_2 NPs could alter neurotransmitter contents in the mouse brain.

4. Discussion

In this study, 5, 10, and 50 mg/kg BW TiO_2 NPs (5 nm) suspensions were given to the ICR mice by an intragastric administration every day for 60 days, respectively. In the 10, and 50 mg/kg BW TiO_2 NPs-treated groups, the higher coefficients of the liver, kidney, and spleen and the lower coefficients of the brain were observed ($p < 0.05$ or $p < 0.01$). We did not find obvious differences for the coefficients of the lung and heart of three treated groups. Our previous study also suggested that 50, 100, and 150 mg/kg BW TiO_2 NPs (5 nm) were injected into abdominal cavity of mice every day for 14 days, and the higher coefficients of the liver, kidney, and spleen and the lower coefficients of the brain were observed [12,25]. Wang et al. reported that when a fixed large dose of 5 g/kg BW of TiO_2 suspensions (25 and 80 nm) was administrated by a single oral gavage, the coefficient of liver after 2 weeks was significantly increased, while the coefficients of spleen and kidney changed a little [11]. The discrepancy between our study and others is most likely attributed to differences in the sizes or the types of nanoparticles or the treatment methods. Nevertheless, these studies did demonstrate that TiO_2 NPs in higher dose had toxicity to the liver, kidney, spleen and brain of mice.

The results represented the first assessment of the effect of TiO_2 NPs on spatial recognition memory of animals. According to Dellu et al. [28], the two-trial Y-maze task is a specific and sensitive test of spatial recognition memory in rodents. Our data supported this view by showing that there were always significant arm effects on percentage measures of total duration of visits and number of visits during the retention test. For exposure to TiO_2 NPs mice, the time spent in the unfamiliar novel arm, and or the frequency mice entered, were not statistically significant different from the familiar start and other arms after 1 h ITI, however, for unexposed mice, the time spent in the unfamiliar novel arm, and or the frequency mice entered, were higher than those familiar start and other arms (Fig. 1). This suggested that mice are highly sensitive to their spatial and contextual environment. Moreover, our data are consistent with previous findings in CD1 mice which demonstrated a very high level of novelty exploration [28]. The first choice for novel arm seems to be testing discrimination memory. In the retention test, mice have to make a choice between the novel arm (unfamiliar) and the other arm (familiar) when they are released from the start arm in the Y-maze. Exposure to TiO_2 NPs mice showed a lower score in discrimination memory than unexposed mice. Our findings demonstrate that TiO_2 NPs may impair spatial recognition memory of mice in the Y-maze.

This impairment of spatial recognition memory of mice caused by exposure to TiO_2 NPs can be possibly ascribed to the accumulation of TiO_2 NPs in brain (Fig. 2) and brain injury involving in calcification and Ca deposition in neurocyte, proliferation of ependyma and spongiocyte (Fig. 3b–f), which may indirectly or directly disturb the homeostasis of trace elements, neurotransmitters and enzymes in brain. There are two classifications of calcification on the brain, such as metastatic calcification and dystrophic calcification. The former is when calcification is caused by too much calcium or phosphorus in the blood. The latter deals with calcification as a result of inflammation or damage to the site [31]. Due to TiO_2 NPs invasion to the brain (Fig. 2), the calcification of mouse brain caused by exposure to TiO_2 NPs is ascribed to dystrophic calcification. The proliferation of spongiocyte caused by exposure to TiO_2 NPs indicated that TiO_2 NPs caused pathological changes of neuron in the mouse brain, and the proliferation of ependyma ascribed to damages of the transport of ions, small molecules, and water between the cerebrospinal fluid and neuropil in the mouse brain caused by exposure to TiO_2 NPs [32]. Therefore, the behavioral development may be affected.

In this study, after the mice were exposed to TiO_2 NPs, the Ca levels in brain significantly increased (Table 2), which was consistent with histopathological observation of the brain tissue (Fig. 3b–f). Moreover, the Ca^{2+}-ATPase, a Ca^{2+} binding protein and

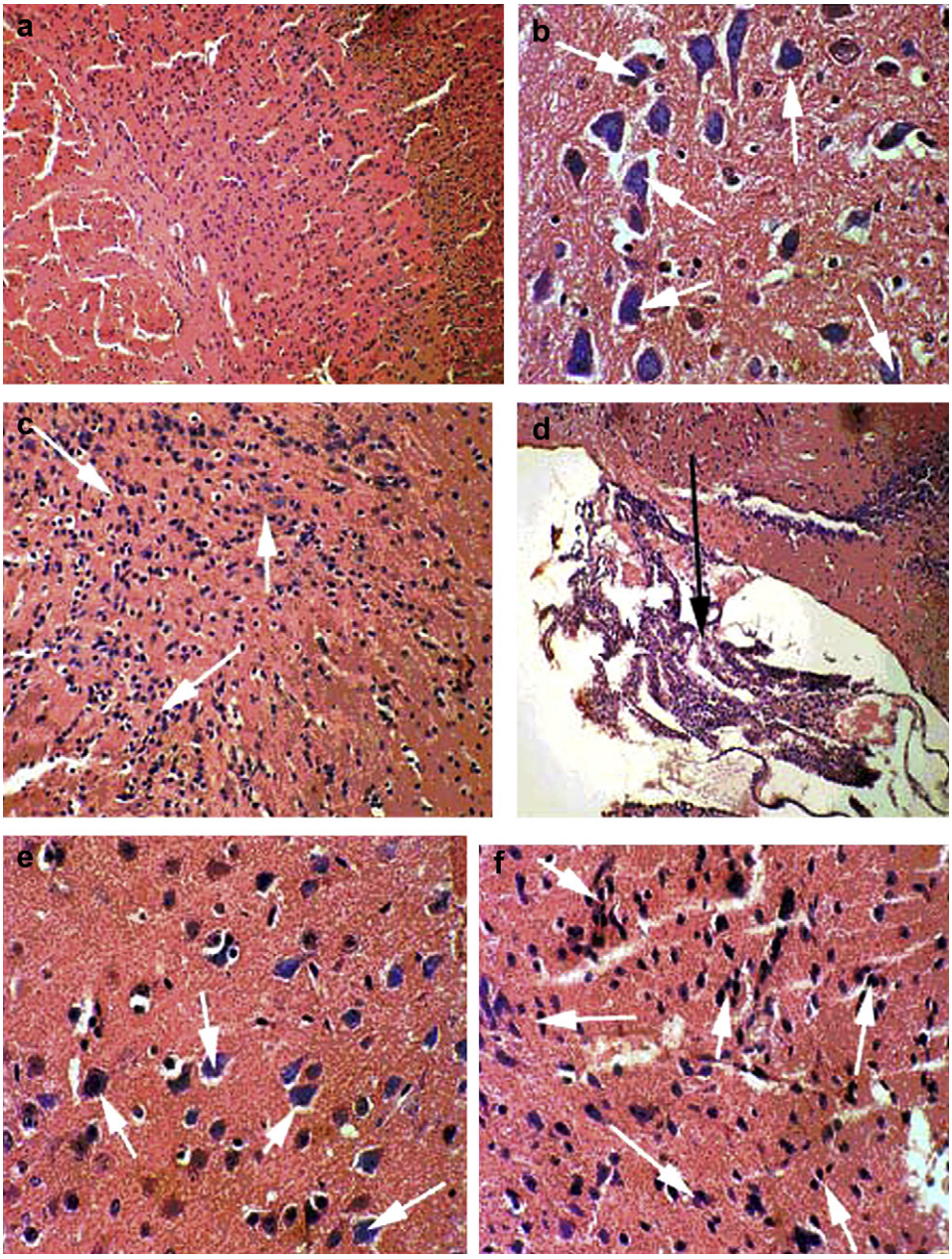

Fig. 3. Histopathology of the brain tissue in ICR mice caused by intragastric administration with TiO_2 NPs for consecutive 60 days. (a) Control group (100×) shows normal brain architecture; (b) 5 mg/kg BW TiO_2 NPs group (400×): arrow indicates calcification deposition in neurocyte; suggesting a result of too much calcium depositing and accumulating in the brain; (c) 10 mg/kg BW TiO_2 NPs group (200×): arrow indicates spongiocyte proliferation, suggesting pathological changes of neuron; (d) 10 mg/kg BW TiO_2 NPs group (100×): arrow indicates ependyma proliferation; (e) 50 mg/kg BW TiO_2 NPs group (400×) : arrow indicates calcification deposition in neurocyte, suggesting a result of too much calcium depositing and accumulating in the brain; (f) 50 mg/kg BW TiO_2 NPs group (400×) : arrow indicates spongiocyte proliferation, suggesting pathological changes of neuron. The section was stained with HE and examined by light microscopy.

being crucial for maintenance of neuronal Ca^{2+} homeostasis [33], was also significantly inhibited when exposed to TiO_2 NPs (Fig. 4). Therefore, the imbalance of the Ca level and Ca^{2+} binding protein, caused by TiO_2 NPs accumulated in brain, can disturb the ion homeostasis and cause a series of physiological disorders in the central nervous system (CNS).

Mg that is bonded to Ca^{2+}/Mg^{2+}-ATPase in a competitive manner with Ca, inhibits Ca influx, activates Ca^{2+}/Mg^{2+}-ATPase pump, and increases the Ca efflux. Our data suggested that TiO_2 NPs caused Mg reduction and Ca increase in the mouse brain (Table 2). The present work also demonstrated an obvious reduction of Ca^{2+}/Mg^{2+}-ATPase (Fig. 4). We thought that TiO_2 NPs might impair Ca^{2+}/Mg^{2+}-ATPase activity by down regulating the interaction between Ca and Mg ions. The net result is calcium homeostasis imbalance, which may cause cellular injury.

In CNS, the increase of the neuronal excitability would consume more cell energy. It has been shown that approximately one half or more of total ATP generated in brain at resting state is consumed by Na^+/K^+-ATPase to maintain proper transmembrane ionic gradients [34]. Establishing and maintaining high K^+ and low Na^+ in the cytoplasm are required for normal resting membrane potentials and various cellular activities. The ionic homeostasis maintained by the Na^+/K^+-ATPase is also critical for cell growth, differentiation, and cell survival [35]. Our results also suggested that K^+ contents

Table 2
The electrolyte contents in the mouse brain after intragastric administration with TiO_2 NPs for the consecutive 60 days.

Electrolyte (μg/g tissue)	TiO_2NPs (mg/kg BW)			
	0	5	10	50
Ca	105.88 ± 5.29	130.49 ± 6.52*	170.79 ± 8.54*	229.68 ± 11.48**
Mg	689.59 ± 34.48	669.71 ± 33.49	658.66 ± 32.93*	597.22 ± 29.86**
Na	4153.7 ± 207.69	4293.61 ± 214.68*	458.39 ± 22.92*	5068.50 ± 253.43**
K	15964.28 ± 798.21	15879.62 ± 793.98	14088.98 ± 704.45*	12179.21 ± 608.96***
Zn	102.39 ± 5.12	84.61 ± 4.23*	68.18 ± 3.41**	59.26 ± 2.96**
Fe	131.53 ± 6.58	111.48 ± 5.57	86.29 ± 4.31**	76.66 ± 3.83**

Ranks marked with a star or double stars means that it is significantly different from the control (unexposed mice) at the 5% or 1% confidence level, respectively Values represent means ± SE, $n = 5$.

(Table 2) and Na^+/K^+-ATPase activity (Fig. 4) significantly decreased, and Na^+ content increased in brain from TiO_2 NPs-treated mice (Table 1). Based on the idea that cellular energy expenditure does increase linearly with the frequency of action potentials [36], and repetitive firing will result in large increases of Na^+ influx and K^+ efflux. So it was that Na^+/K^+-ATPase could not export intracellular redundant Na^+ and/or import extracellular K^+ timely, which would result in the accumulation of intracellular Na^+ and loss of intracellular K^+, and consequently, reducing the Na^+ and K^+ electrochemical gradient, and disturbed the ionic homeostasis and the physiological functions of neurons, thereby impaired spatial recognition memory of mice caused by exposure to TiO_2 NPs. Zhao et al. suggested that nano-ZnO solution could lead to an enhancement in the current amplitudes of I_{Na} and I_K by increasing the opening number of sodium channels, delaying rectifier potassium channels, and enhancing the excitability of neurons, which lead to Na^+ influx and the accumulation of intracellular Na^+, as well as K^+ efflux plus the loss of cytoplasmic K^+ in rat hippocampal CA3 pyramidal neurons [37].

In brain, zinc plays an important role in neurotransmission by modulating the activity of Glu and gamma aminobutyric acid receptors [38]. A reduction in brain zinc content can impair the spatial memory in adult rats [39]. Further, the reduced dietary zinc intake has been found to induce behavioral changes, including impaired short-term memory [40–42]. Collectively, these facts implicate zinc in learning and memory processing. In the present work, exposure to TiO_2 NPs obviously decreased the Zn contents in the mouse brain (Table 2). This decrease may possibly affect the function of zinc-containing glutaminergic neurons. Therefore, the damage of zinc homeostasis likely results in the impairment of learning abilities.

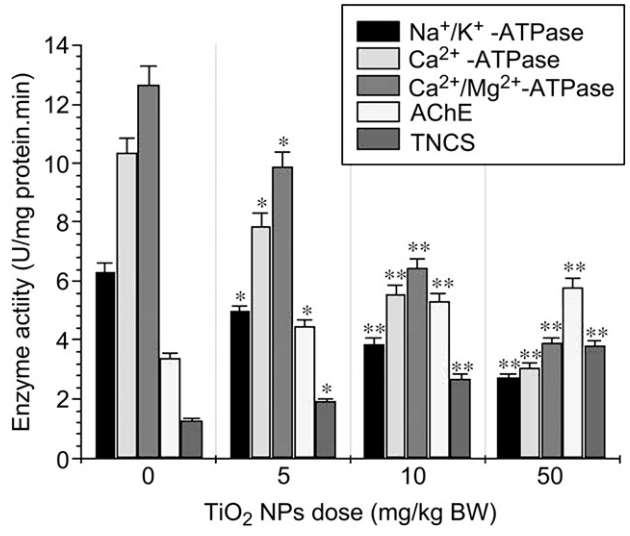

Fig. 4. Effects of TiO_2 NPs on the enzyme activities in the mouse brain after intragastric administration with TiO_2 NPs for the consecutive 60 days. Bars marked with a star or double stars means that it is significantly different from the control (unexposed mice) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 5$.

It is well known that the cholinergic system in CNS plays an important role in cognitional function, and that brain cholinergic hypofunction causes dementia with symptoms such as memory loss and disorientation in cerebrovascular or Alzheimer's disease [43]. It has also been reported that hypoxia induces a reduction of memory and judgment that is associated with a decrease in acetylcholine synthesis [44]. Earlier study has shown that TiO_2 NPs could alter the AChE activity in the mouse brain [25]. Hence, the Ach level, partly modulated by AChE, would also be influenced. In this study, we observe that TiO_2 NPs significantly increased AChE activity (Fig. 4) and Ach content (Table 3) in the mouse brain. It has been demonstrated that the duration of Glu application can enhance the formation of long-term potentiation in CNS [45]. The excitatory neurotransmitter-Glu in brain was significantly increased after TiO_2 NPs entering the mouse brain (Table 3). The binding of Glu and NMDA receptors can disrupt calcium homeostasis (Ca^{2+} influx) and activate calcium-dependent protease, i.e. nitric oxide synthase (NOS) (Fig. 4), causing the over production of NO in the mouse brain exposed to TiO_2 NPs (Table 3). NO plays dual function as a free radical and signal molecule of neurotransmitter in organisms [46–48]. Because of the existence of ROS in the mouse brain after exposure to TiO_2 NPs [25], NO could be oxidized to peroxinitrite (ONOO−) radical to cause the damaged neurons, as found in the pathological observations (Fig. 3). Therefore, the damage of spatial recognition memory caused by exposure to TiO_2 NPs can be explained only by the cholinergic system.

Although involvement of the central cholinergic system is well established in learning process, the role of other neurotransmitter systems cannot be ignored. Several neurotransmitter systems have been investigated to assess hypothesized mechanisms underlying the decline in recent memory abilities in normal aging and in Alzheimer's disease [49,50]. 5-HT and DA are two major monoamine neurotransmitters with a multitude of functions in brain.

Table 3
Effects of TiO_2 NPs on neurotransmitters in the mouse brain after intragastric administration with TiO_2 NPs for the consecutive 45 days.

Indexes	TiO_2 NPs (mg/kg BW)			
	0	5	10	50
ACh (μg/ml)	37.69 ± 1 88	47.48 ± 2.37*	58.96 ± 2.95**	64.93 ± 3.25**
GLu (μmol/g protein)	667 ± 33	873 ± 44*	1077 ± 54**	1368 ± 68**
NO (μmol/g protein)	10.91 + 0.55	13.17 ± 0.66*	16.32 ± 0.82**	19.77 ± 0.99***

Ranks marked with a star or double stars means that it is significantly different from the control (unexposed mice) at the 5% or 1% confidence level, respectively Values represent means ± SE, $n = 5$.

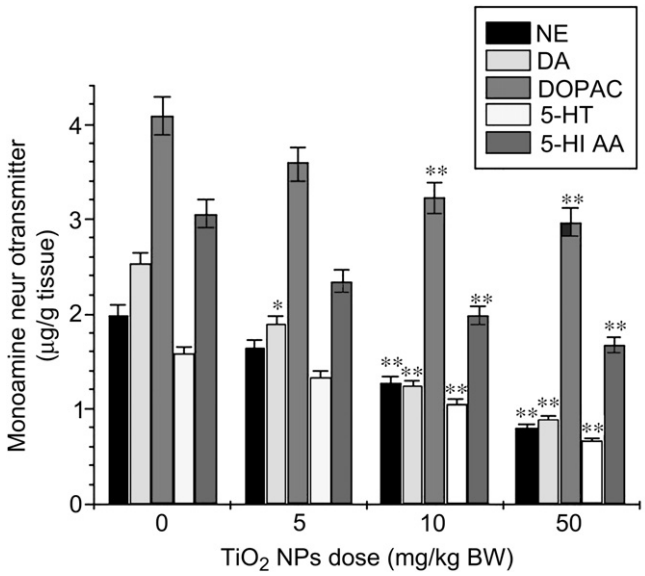

Fig. 5. Effects of TiO_2 NPs on the levels of monoamine neurotransmitters in the mouse brain after intragastric administration with TiO_2 NPs for the consecutive 60 days. Bars marked with a star or double stars means that it is significantly different from the control (unexposed mice) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 5$.

Previous studies have shown that metabolism of monoamines neurotransmitters changed significantly when aging, and moreover, the contents of NE, DA and 5-HT were reduced in cerebrum cortex of aged mice. Also dopaminergic lesioning of the prefrontal cortex leads to cognitive dysfunctions, dopamine depletion of the mesoseptal dopaminergic projections leads to a decrease in working memory [51]. It has been demonstrated that manganese oxide nanoparticles (40 nm) induced DA depletion in a cultured neuronal PC-12 cells and this process might be related with increased ROS [52]. Wang et al. found the slight change of HIAA and NE level in the hippocampus of mice exposed to the 21 nm- and 280 nm-Fe_2O_3 particles [53]. In this study, we observed that TiO_2 NPs significantly decreased the levels of NE, 5-HT and its metabolites 5-HIAA, DA and its metabolites DOPAC in the mouse brain (Fig. 5). From Table 2, we observed a significant decrease of the Fe content in the TiO_2 NPs treated mice. The role of intraneuronal iron in metabolism is varied and involves the following: synthesis and packaging of neurotransmitters; uptake and degradation of the neurotransmitters into other iron-containing proteins that may directly or indirectly alter brain function through peroxide reduction, amino acid metabolism and fat desaturation, thus altering membrane functioning [54]. For example, iron is a cofactor for many iron-containing enzymes that are essential for the production of monoamine neurotransmitter [55]. Thus, iron deficiency would be expected to lead to decreased activities of these enzymes, so as to affect the contents of biogenic amines.

All of these results indicated that exposure to TiO_2 NPs may impair the learning and memory abilities and the exact mechanisms of this impairment need to be further studied.

TiO_2 is an inert and poorly soluble matter. A potential exposure route for general population is the oral ingestion because TiO_2 is used as a food additive in toothpaste, capsule, cachou, and so on. The quantity of TiO_2 does not exceed 1% by weight of the food according to the Federal Regulations of US Government.

In 1969, WHO (1969) reported that the LD50 of TiO_2 for rats is larger than 12,000 mg/kg body weight after oral administration. In the article, the oral doses of 5, 10, and 50 TiO_2 NPs mg/kg BW every day are equal to about 0.3–0.35, 0.6–0.7, and 3–3.5 g TiO_2 NPs of 60–70 kg body weight for humans such exposure/intake, respectively, which are safe for humans. However, the application of TiO_2 NPs and exposure effects especially on human brain for long-term and low-dose treatment should be cautious.

5. Conclusions

Our studies observed the decline of neurobehavioral performance and morphological signs of brain damage of mice caused by exposure to TiO_2 NPs. Furthermore, we also observed that both the trace elemental contents and the neurotransmitter levels in brain changed after an intragastric administration with TiO_2 NPs for the consecutive 60 days. These were the first results to confirm the of damage of spatial recognition memory behavior caused by exposure to TiO_2 NPs, which are of important significance for environmental or occupational safety following various exposures to TiO_2 NPs. Therefore, our study aroused the attention of TiO_2 NPs application and exposure effects especially on human brain for long-term and low-dose treatment.

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Appendix

Figures with essential colour discrimination. Figs. 1, 2 and 3 in this article may be difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.07.011.

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