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# Neurotoxicity and gene-expressed profile in brain-injured mice caused by exposure to titanium dioxide nanoparticles

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**Abstract:** Titanium dioxide nanoparticles ($TiO_2$ NPs) are widely used in toothpastes, sunscreens, and products for cosmetic purpose that the human use daily. Although the neurotoxicity induced by $TiO_2$ NPs has been demonstrated, very little is known about the molecular mechanisms underlying the brain cognition and behavioral injury. In this study, mice were exposed to 2.5, 5, and 10 mg/kg body weight (BW) $TiO_2$ NPs by nasal administration for 90 consecutive days, respectively, and their brains' injuries and brain gene-expressed profile were investigated. Our findings showed that $TiO_2$ NPs could be translocated and accumulated in brain, led to oxidative stress, overproliferation of all glial cells, tissue necrosis as well as hippocampal cell apoptosis. Furthermore, microarray data showed significant alterations in the expression of 249 known function genes, including 113 genes upregulation and 136 genes downregulation following exposure to 10 mg/kg BW $TiO_2$ NPs, which were associated with oxidative stress, immune response, apoptosis, memory and learning, brain development, signal transduction, metabolic process, DNA repair, response to stimulus, and cellular process. Especially, significant increases in Col1a1, serine/threonine-protein kinase 1, Ctnnb1, cysteine–serine-rich nuclear protein-1, Ddit4, Cyp2e1, and Krev interaction trapped protein 1 (Krit1) expressions and great decreases in DA receptor D2, Neu1, Fc receptor-like molecules, and Dhcr7 expressions following long-term exposure to $TiO_2$ NPs resulted in neurogenic disease states in mice. Therefore, these genes may be potential biomarkers of brain toxicity caused by $TiO_2$ NPs exposure, and the application of $TiO_2$ NPs should be carried out cautiously. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2013.

**Key Words:** titanium dioxide nanoparticles, brain damage, oxidative stress, gene expression profiling, mice

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## INTRODUCTION

Titanium dioxide nanoparticles ($TiO_2$ NPs) exhibit many specific features such as high stability, anticorrosion, and photocatalytic property.[1] In recent years, $TiO_2$ NPs materials have been focused greatly and widely used in the production of every day products, such as paper, toothpastes, popular sunscreens, cosmetics, and food.[2] However, potential occupational and public exposure to manufactured NPs will increase consumers' concern.

Previous reports have indicated that the impact of nanomaterials on the central nervous system is not negligible, especially for the workers working in nanomaterials manufacturing factories. For instance, $TiO_2$ NPs were suggested to cause oxidative stress and impair dopaminergic function in brain microglia *in vitro*.[3] Furthermore, Wang et al.[4,5] indicated that $TiO_2$ NPs were translocated to the olfactory bulb and caused brain lesions in mice. It was found that $TiO_2$ NPs could be found in brain and damaged the brain of hairless mice.[6] A 14-day or 60-day exposure of $TiO_2$ NPs to female mice could be translocated into the brain, in turn injured the brain, caused oxidative stress, and decrease spatial recognition memory in mice.[7,8] $TiO_2$ NPs was also demonstrated to promote exaggerated neuroinflammation by increasing microglial activation in the pre-inflamed brain of

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mice.[9] The hippocampus is responsible for learning and long-term memory, and the first regions of the brain to have damage.[10] $TiO_2$ NPs were suggested to cause hippocampal apoptosis, activate caspase-3, -9, Bax, and cytochrome c expression, inhibit Bcl-2 expression in hippocampus of mice,[11] and impair the short- and long-term synaptic plasticity in rats' hippocampal dentate gyrus (DG) area.[12] Shimizu et al.[13] showed that maternal exposure of mice to $TiO_2$ NPs altered the expression of genes related to the development and function in the brain of newborn pups. Jackson et al.[14] also reported that maternal inhalation of $TiO_2$ NPs could result in hepatic DNA damage and gene expression in offspring of mice. Although the studies mentioned above were conducted to evaluate the neurotoxic effects of $TiO_2$ NPs, studies on the multiple molecular mechanisms of $TiO_2$ NP-induced neurotoxicity in mice, and global changes in gene expression need to be assessed in the brain-injured mice for long-term exposure. We hypothesize that $TiO_2$ NP-induced brain damages in mice may have special biomarkers of toxicity.

Genome-wide gene expression analysis using DNA microarray, by which expression of thousands of genes can be monitored, has a great advantage to identify the biomarkers of toxicity or specific molecular cascades involved in the complex diseases, and increase the understanding of the process and provide new tools for evaluating brain damages.[15] In this study, we aimed to investigate brain injury by nasal administration with 2.5, 5, and 10 mg/kg body weight (BW) $TiO_2$ NPs for 90 consecutive days and the gene expression profiling of brain-injured mice using microarray analysis. The data on gene expression profiling showed significant changes in genes involved in oxidative stress, apoptosis, memory and learning, immune response, energy metabolism, metabolic process, DNA repair, brain development, signal transduction, response to stimulus, and cellular process in the $TiO_2$ NP-exposed brains. Our findings may provide a reference for future mechanistic studies on the effects of $TiO_2$ NPs in the brain in animals or humans.

## MATERIALS AND METHODS

### Chemicals, preparation, and characterization

Nanoparticulate anatase $TiO_2$ was prepared via controlled hydrolysis of titanium tetrabutoxide. Details of the synthesis and characterization of $TiO_2$ NPs were described in our previous reports.[8,16] X-ray diffraction (XRD) measurements showed that $TiO_2$ NPs exhibit 101 peak of anatase. The average particle size of powdered $TiO_2$ NPs that were suspended in 0.5% (w/v) hydroxypropylmethylcellulose (HPMC) K4M solvent after 24 h incubation ranged from 5 to 6 nm, and the surface area of the sample was 174.8 $m^2/g$. The mean hydrodynamic diameter of $TiO_2$ NPs in HPMC solvent ranged from 208 to 330 nm (mainly 294 nm), and the zeta potential after 24 h incubation was 9.28 mV, respectively.[8] The results of XRD, transmission electron microscope (TEM) and dynamic light scattering are available upon request.

### Animals and treatment

It has been demonstrated previously by Wang et al.[18] that sensitivity to $TiO_2$ exposure was higher in CD-1 [Institute for Cancer Research (ICR)] female mice than CD-1 (ICR) male mice. Therefore, CD-1 (ICR) female mice were used in this study. Eighty CD-1 (ICR) female mice (18 $\pm$ 2 g) were purchased from the Animal Center of Soochow University (China). All mice were housed in stainless steel cages in a ventilated animal room. Room temperature of the housing facility was maintained at 24 $\pm$ 2°C with a relative humidity of 60 $\pm$ 10% and a 12-h light/dark cycle in Animal Center of Soochow University (China). Distilled water and sterilized food were available for mice *ad libitum*. Prior to dosing, the mice were acclimated to this environment for 5 days. All procedures used in animal experiments conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals.[18]

A 0.5% HPMC was used as a suspending agent. $TiO_2$ NP powder was dispersed onto the surface of 0.5% (w/v) HPMC, and then the suspending solutions containing $TiO_2$ NPs were treated by ultrasonic for 30 min and mechanically vibrated for 5 min. For the experiment, the mice were randomly divided into four groups ($N = 20$), including a control group (treated with 0.5%, w/v HPMC) and three experimental groups (2.5, 5, and 10 mg/kg BW $TiO_2$ NPs). About the dose selection in this study, we consulted the report of World Health Organization in 1969. According to the report, median lethal dose of $TiO_2$ for rats is larger than 12,000 mg/kg BW after oral administration. In addition, the quantity of $TiO_2$ NPs does not exceed 1% by weight of the food according to the Federal Regulations of US Government. In the present study, we selected 2.5, 5, and 10 mg/kg BW nano-$TiO_2$ exposed to mice by intranasally administration every day. They were equal to about 0.15–0.7 g nano-$TiO_2$ of 60–70 kg BW for humans with such exposure, which were relatively safe doses. The mice were weighed, volume of $TiO_2$ NPs suspensions was calculated for each mouse, and the fresh $TiO_2$ NPs suspensions were administered to the mice by nasal instillation every day for 90 days. Symptoms and/or mortality were observed and carefully recorded each day during the 90-day period.

### Relative weight of brain and preparation of hippocampus

After 90 days, all mice were weighed and then killed after being anesthetized with ether. The brains of all animals were quickly removed and placed on ice. After weighing the body and brain, the relative weight of brain was calculated as the ratio of brain (wet weight, mg) to body weight (g).

### Titanium content analysis of brain

The whole brains were removed from the freezer (−80°C) and thawed. Approximately 0.2 g of the brain was weighed, digested, and analyzed for titanium content. Inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental X7; Thermo Electron Co.) was used to analyze the titanium concentrations in the samples.

### Histopathological examination of brain

For pathologic studies, all histopathologic examinations were performed using standard laboratory procedures.[19] The brains were embedded in paraffin blocks, then sliced (5 μm thickness) and placed onto glass slides. After hematoxylin–eosin staining, the stained sections were evaluated by a

**TABLE I. Changes in Body Weight, Relative Weight of Brain, and Titanium Contents in Brain of Mice After Nasal Administration of $TiO_2$ NPs for 90 Consecutive Days**

| | $TiO_2$ NPs (mg/kg BW) | | | |
|---|---|---|---|---|
| Index | 0 | 2.5 | 5 | 10 |
| Net increase in body weight (g) | 18.89 ± 0.94a | 16.56 ± 0.83a | 14.78 ± 0.74b | 11.27 ± 0.76c |
| Relative weight of brain (mg/g) | 17.98 ± 0.90a | 16.67 ± 0.83a | 14.31 ± 0.72b | 13.04 ± 0.67b |
| Ti content (ng/g tissue) | Not detected | 46.02 ± 2.3a | 83.08 ± 4.15b | 154.66 ± 7.73c |

Different letters indicate significant differences between groups ($p < 0.05$). Values represent mean ± SEM ($N = 20$).

histopathologist unaware of the treatments, using an optical microscope (Nikon U-III Multi-point Sensor System, Japan).

### Observation of hippocampal ultrastructure

Brains were quickly removed from mice and placed on ice, and then the hippocampi were dissected. Hippocampi were fixed in a fresh solution of 0.1 *M* sodium cacodylate buffer containing 2.5% glutaraldehyde and 2% formaldehyde followed by a 2 h fixation period at 4°C with 1% osmium tetroxide in 50 m*M* sodium cacodylate (pH 7.2–7.4). Staining was performed overnight with 0.5% aqueous uranyl acetate. The specimens were dehydrated in a graded series of ethanol (75, 85, 95, and 100%), and embedded in Epon 812. Ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate, and observed with a HITACHI H600 TEM (HITACHI Co., Japan). Hippocampal apoptosis was determined based on the changes in nuclear morphology (e.g., chromatin condensation and fragmentation).

### Assay of oxidative stress

Superoxide ion ($O_2^{\cdot -}$) in the brain tissues was measured by monitoring the reduction in 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide in the presence of $O_2^{\cdot -}$, as described by Oliveira et al.[20] The detection of $H_2O_2$ in the brain tissues was carried out by the xylenol orange assay.[21]

Lipid peroxidation of brain was determined as the concentration of malondialdehyde (MDA) generated by the thiobarbituric acid reaction as described by Buege and Aust.[22] Protein oxidation of brain was investigated according to the method of Fagan et al.[23] by determining the carbonyl content. DNA of brain was extracted using DNeasy Tissue Mini Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) as described by the manufacturer. Formation of 8-OHdG was determined using the 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Haruoka, Japan). This kit provides a competitive immunoassay for quantitative measurement of the oxidative DNA adduct 8-OHdG. It was carefully performed according to manufacturer's instructions, and using a microplate varishaker-incubator, an automated microplate multireagent washer, and a computerized microplate reader.

### Microarray and data assay

Gene expression profiles in brain tissue isolated from 5 mice in the control and $TiO_2$ NP-treated groups were compared by microarray analysis using Illumina BeadChip purchased from Illumina (San Diego, CA). Total RNA was isolated using the Ambion Illumina RNA Amplification Kit (cat no. 1755) according to the manufacturer's protocol and stored at −80°C. RNA amplification is the standard method for preparing RNA samples for array analysis.[24] Total RNA was then submitted to the Biostar Genechip (Shanghai, China) where RNA quality was analyzed using a BioAnalyzer, and cRNA was generated and labeled using the one-cycle target labeling method. cRNA from each mouse was hybridized for 18 h at 55°C on Illumina Human HT-12 v 3.0 BeadChips, containing 45, 200 probes (Illumina), according to the manufacturer's protocol and subsequently scanned with the Illumina BeadArray Reader 500. This program identifies differentially expressed genes and establishes the biological significance based on the Gene Ontology Consortium database (http://www.geneontology.org/GO.doc.html). Data analyses were performed with GenomeStudio software version 2009 (Illumina), by comparing all values obtained at each time point against the 0 h values. Data were normalized with the quantile normalization algorithm, and genes were considered as detected if the detection *p*-value was lower than 0.05. Statistical significance was calculated using the Illumina Diffscore, a proprietary algorithm that uses the bead standard deviation to build an error model. Only genes with a Diffscore $\leq -13$ and $\geq 13$, corresponding to a *p*-value of 0.05, were considered statistically significant.[25,26]

### Quantitative real-time PCR

The levels of messenger RNA expression of *Xrn1, Apoa1, Apoa2, Apoc3, Cav1, Cyp2e1,* and *Dhcr7* in the mouse brains were determined using real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).[27–29] Synthesized complementary DNA (cDNA) was used for the real-time PCR using primers that were designed using Primer Express Software according to the software guidelines, and PCR primer sequences are available upon request.

### Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). The significant differences were examined by unpaired Student's *t*-test using SPSS 19 software. A *p*-value $<0.05$ was considered as statistically significant.

## RESULTS

### Body weight, relative weight of brain, and titanium accumulation

The net increase in body weight, relative weight of brain, and titanium contents in the mouse brain caused by $TiO_2$ NPs for 90 consecutive days is listed in Table I, respectively. It can be seen that the body weight and relative weight of brain were

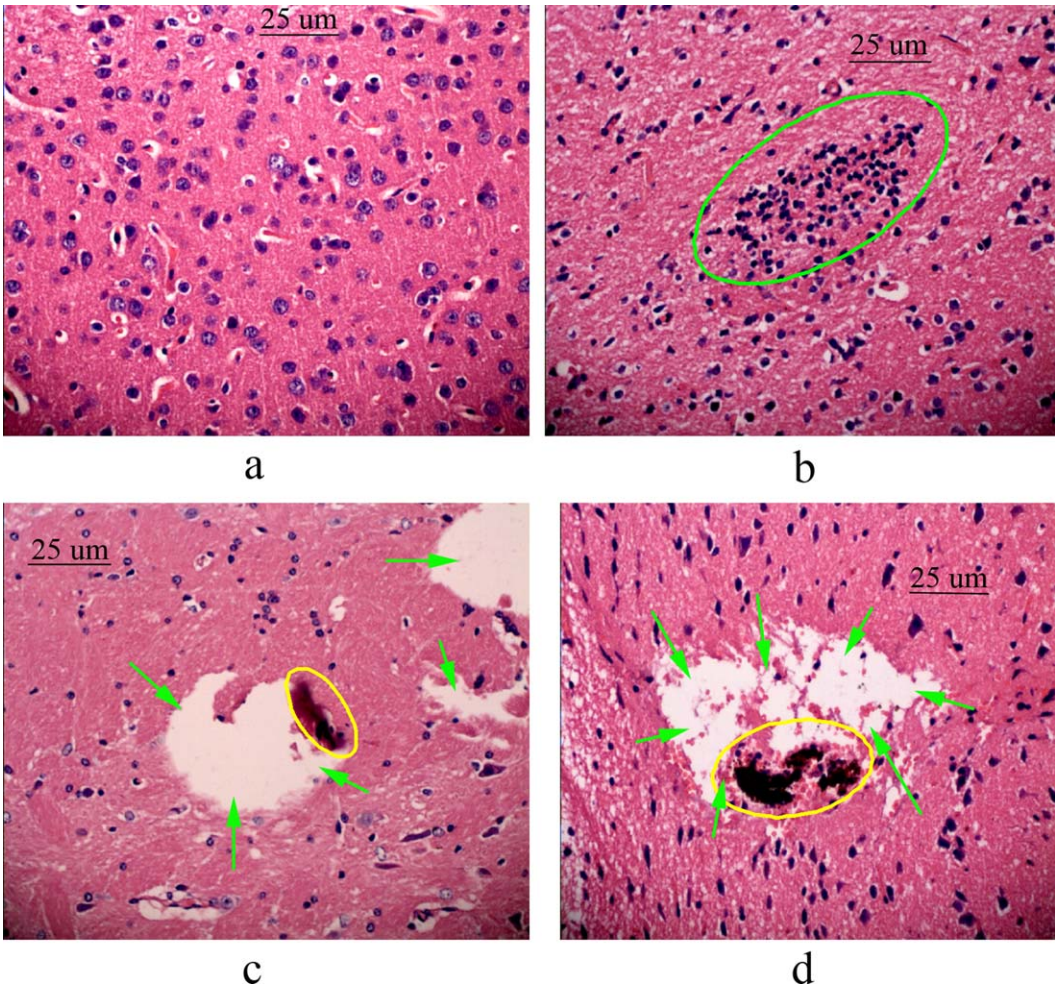


**FIGURE 1**. Histopathological changes in the brain caused by nasal administration of $TiO_2$ NPs for 90 consecutive days. a, Control, (b) 2.5 mg/kg BW $TiO_2$ NPs, (c) 5 mg/kg BW $TiO_2$ NPs, and (d) 10 mg/kg BW $TiO_2$ NPs. Green cycle suggests significant proliferation of all glial cells, blue arrows indicate tissue necrosis, and yellow cycle indicates brown particles or agglomerate in the brain. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly decreased by $TiO_2$ NPs exposure (Table I, $p < 0.05$), while titanium content in brain was significantly detected (Table I), but in the control was not detectable.

### Brain histopathological observations

The histopathological changes in the mouse brain caused by nasal administration with $TiO_2$ NPs for 90 consecutive days are shown in Figure 1. In the control group, we can see the normal architecture of brain [Fig. 1(a)], whereas 2.5 mg/kg BW $TiO_2$ NP-treated group shows overproliferation of all glial cells [Fig. 1(b)] and 5 and 10 mg/kg BW $TiO_2$ NP-treated groups indicate tissue necrosis [Fig. 1(c,d)], respectively. In addition, we also significantly observed brown particles or agglomerates in the 5 and 10 mg/kg BW $TiO_2$ NP-exposed brains [Fig. 1(c,d)], which may be the aggregation of $TiO_2$ NPs in the brain.

### Hippocampal ultrastructure evaluation

The changes in hippocampal cell ultrastructure are presented in Figure 2. It was observed that the hippocampal cell in control mice contained elliptical nucleus with homogeneous chromatin [Fig. 2(a)]. However, with increasing $TiO_2$ NPs dose, the cell ultrastructure in the $TiO_2$ NP-treated mice showed significant irregularity of nuclear membrane, shrinkage of nucleus, chromatin marginalization, and mitochondrial swelling [Fig. 2(b–d)], respectively. These results suggested that long-term exposure to $TiO_2$ NPs caused hippocampal cell apoptosis in mice.

### Oxidative damage of brain

It can be seen from Table II that compared to the unexposed mice, the brains from $TiO_2$ NP-treated mice exhibited greater vulnerability to oxidative stress, with significant increases in the generating rate of $O_2^{\cdot -}$ and $H_2O_2$, and higher levels of MDA, carbonyl and 8-OHdG as degradation products of lipid, protein, and DNA peroxidation ($p < 0.05$).

### Changes in gene expression profile

Treatment with 10 mg/kg BW $TiO_2$ NPs resulted in the most severe brain damage and these tissues were used to

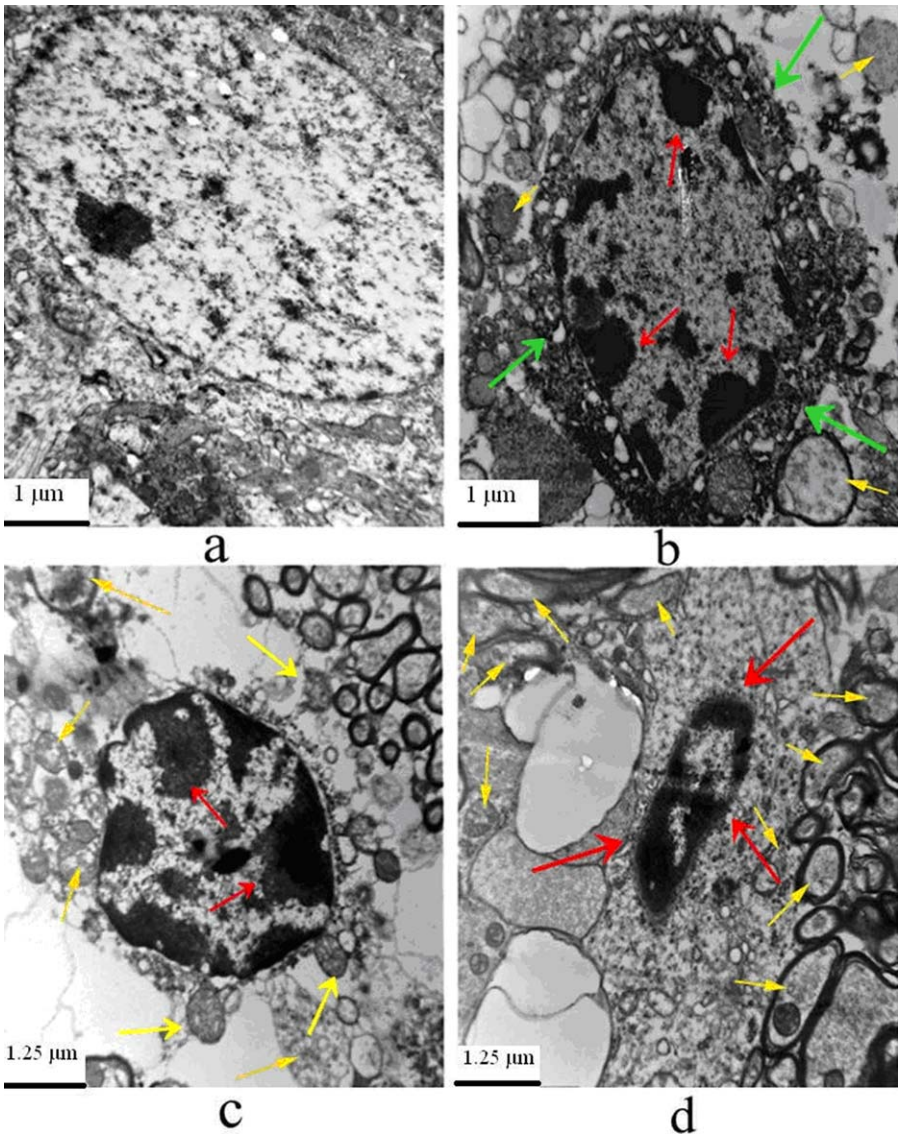


**FIGURE 2.** Ultrastructure of hippocampal cell of mice caused by nasal administration of $TiO_2$ NPs for 90 consecutive days. a, Control, (b) 2.5 mg/kg BW $TiO_2$ NPs, (c) 5 mg/kg BW $TiO_2$ NPs, and (d) 10 mg/kg BW $TiO_2$ NPs. Green arrows indicate irregularity of nuclear membrane, significant shrinkage of nucleus. Red arrows suggest chromatin marginalization. Yellow arrows exhibit mitochondria swelling. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

determine gene expression profiles to further explore the mechanisms of brain damage induced by $TiO_2$ NPs. The brain known gene expression profiles was analyzed with Illumina Bead Chip, and the results are listed in Supporting Information Table IS. Of the known functional genes altered, 113 genes were upregulated and 136 genes were downregulated. The 249 genes was classified using the ontology-driven clustering algorithm included with the PANTHER

**TABLE II. Oxidative Damage in Brain of Mice Caused by Nasal Administration of $TiO_2$ NPs for 90 Consecutive Days**

| | $TiO_2$ NPs (mg/kg BW) | | | |
|---|---|---|---|---|
| Oxidative Stress | 0 | 2.5 | 5 | 10 |
| $O_2^{\cdot -}$ (nmol/mg prot. min) | 64.91 ± 3.25a | 75.27 ± 3.76b | 99.18 ± 4.96c | 122.35 ± 6.12d |
| $H_2O_2$ (nmol/mg prot. min) | 78.05 ± 3.90a | 89.17 ± 4.46b | 108.25 ± 5.41c | 137.70 ± 6.89bd |
| MDA (μmol/ mg prot) | 0.63 ± 0.03a | 0.99 ± 0.05b | 1.78 ± 0.09c | 2.42 ± 0.12d |
| Carbonyl (μmol/mg prot) | 1.04 ± 0.05a | 1.72 ± 0.09b | 2.96 ± 0.15c | 4.93 ± 0.25d |
| 8-OHdG (mg/g tissue) | 0.36 ± 0.02a | 0.46 ± 0.02b | 0.75 ± 0.04c | 0.98 ± 0.05d |

Different letters indicate significant differences between groups ($p < 0.05$). Values represent mean ± SEM ($N = 5$).

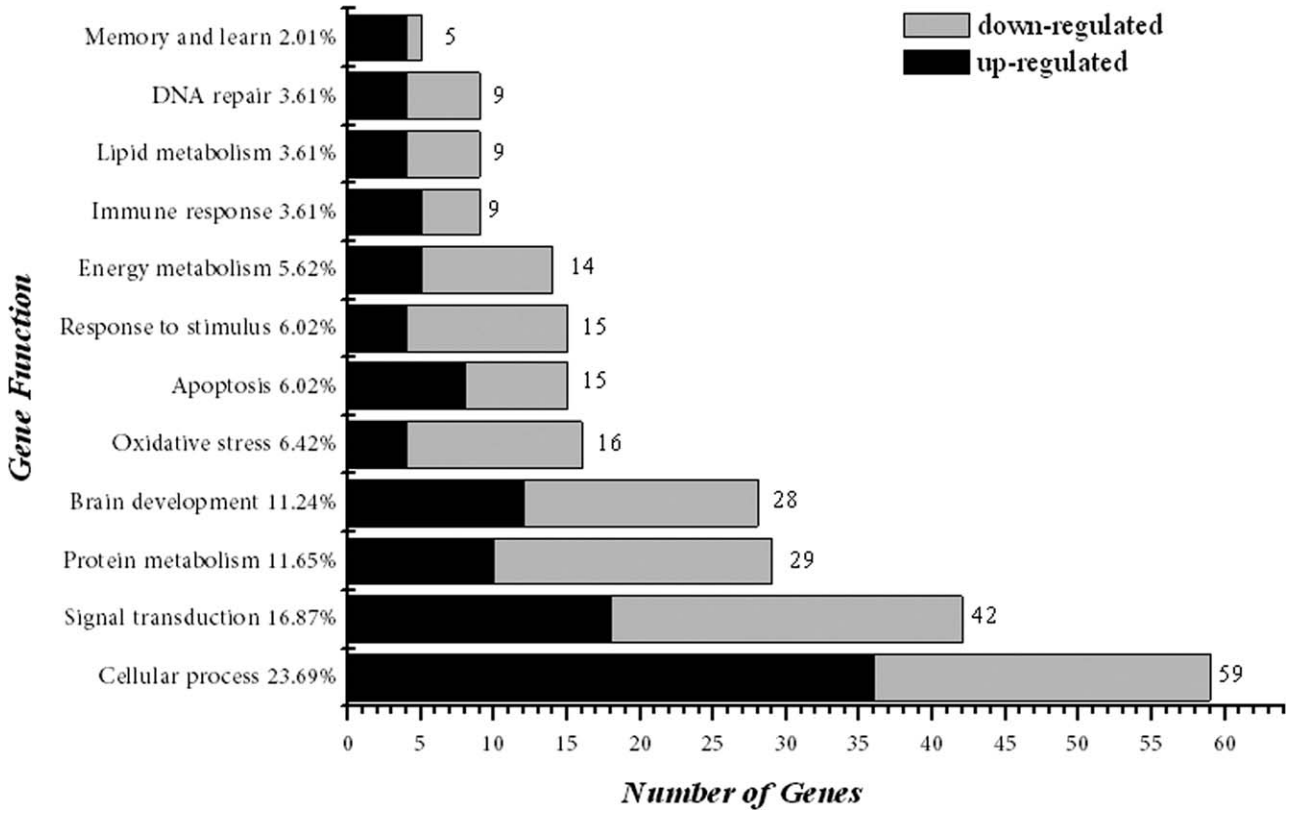


**FIGURE 3**. Gene expression changes in the mouse brain after intragastric administration of 10 mg/kg BW $TiO_2$ NPs for 90 consecutive days. Selection of overrepresented biological categories that include differentially expressed genes. Rows represent gene categories and the percentage of the genes included in each category to the total number of differentially expressed genes, and the number of genes is indicated on the right side of each bar.

Gene Expression Analysis Software (www.pantherdb.org/), which were associated with oxidative stress, apoptosis, memory and learning, immune response, energy metabolism, protein metabolism, lipid metabolism, DNA repair, apoptosis, brain development, signal transduction, response to stimulus, and cellular process, respectively (Fig. 3), and the function of 175 genes was unknown.

### Quantitative reverse-transcriptase PCR

To verify the accuracy of the microarray analysis, seven genes that demonstrated significantly different expression patterns were further evaluated by qRT-PCR due to their association with oxidative stress, immune response, lipid metabolism, brain development, and signal transduction. These five genes including Cyp2e1, Apoa1, Apoa2, Xrn1, and Apoc3 were upregulated, whereas two genes including Dhcr7, and Cav1 were downregulated (Table III). The qRT-PCR analysis of all seven genes displayed expression patterns comparable with the microarray data (i.e., either up- or downregulation; Supporting Information Table IS).

## DISCUSSION

The results of this study indicate that $TiO_2$ NPs were translocated and deposited in brain, in turn decreased body weight, relative weight of brain (Table I), led to overproliferation of all glial cells, tissue necrosis (Fig. 1), and hippocampal cell apoptosis (Fig. 2), respectively. The $TiO_2$ NP-induced brain injuries were also coupled with reactive oxygen species (ROS) overproduction and peroxidation of lipid, protein, and DNA in brain (Table II). Numerous studies have demonstrated that $TiO_2$ NPs could cross the blood–brain barrier, accumulate in brain, and damage brain.[4–811] Therefore, we speculated that the brain injury following long-term exposure to $TiO_2$ NPs may be involved alterations in gene expression in brain. To identify the molecular mechanisms of multiple genes working together following exposure to $TiO_2$

**TABLE III. RT-PCR Validation of Selected Genes From Microarray Data**

| Function | Gene | ΔΔCt | Fold | Microarray |
|---|---|---|---|---|
| Oxidative stress | Cyp2e1 | 6.668674 | 2.1569803 | 6.620695 |
| | Dhcr7 | 3.996619 | 0.292235632 | 0.4750227 |
| Immune response | Cav1 | 3.890061 | 0.280051294 | 0.3158298 |
| Lipid metabolism | Apoa1 | 5.888985 | 2.305283209 | 2.322022 |
| | Apoa2 | 6.186024 | 3.008274049 | 5.897682 |
| Brain development | Xrn1 | 8.0084 | 0.406132931 | 2.584194 |
| Signal transduction | Apoc3 | 6.431832 | 0.771829451 | 2.18969 |

ΔCt = gene Ct − actin Ct, ΔΔCt = ΔCt of treated mice − ΔCt of control mice, and fold = POWER(2, −ΔΔCt).

NPs, microarray assays of brain RNA were performed to establish a global gene expression profile. These assays suggested that the expressions of 424 genes were obviously altered, and 249 of these genes were involved in oxidative stress, apoptosis, memory and learning, metabolic process, immune response, DNA repair, signal transduction, brain development, response to stimulus, and cellular process (Supporting Information Table IS), respectively. The main results are discussed below.

As showed in the results, $TiO_2$ exposure resulted in oxidative stress and overproliferation of all glial cells, tissue necrosis, and hippocampal cell apoptosis in brain (Figs. 1 and 2). Oxidative stress of brain caused by $TiO_2$ NPs had been demonstrated to be related to reduction of antioxidant capacity.[7] In our previous study, $TiO_2$ NPs was also shown to mediate apoptosis in the hippocampus in mice through the induction of ROS and activation of caspase-3, -9, Bax, and cytochrome c expression and inhibition of Bcl-2 expression in hippocampus of mice.[11] In this study, 16 genes (6.42% of 249 genes) genes involved in oxidative stress were significantly altered (Supporting Information Table IS, Fig. 3). Of the genes altered, 4 genes were upregulated, and 12 genes were downregulated. For instance, Cyp2e1 was increased with a Diffscore of 32.4 in the $TiO_2$ NP-exposed group (Supporting Information Table IS). Cytochrome P450 2E1 (Cyp2e1) is a member of cytochrome P450 enzymes and metabolize various endogenous substrates, such as steroids and fatty acids, and xenobiotics, including drugs, toxins, and carcinogens.[30] Studies *in vivo* and *in vitro* have shown that increased expression of Cyp2e1 could metabolize ethanol to acetaldehyde and 1-hydroxyethyl radicals, which could react with other cellular macromolecules and cause toxicity in rat liver.[31,32] According to the Cyp2e1-mediated oxidative stress pathway, we speculate that exogenous Cyp2e1 inducers such as acetone, ethanol, and benzene as well as $TiO_2$ NPs could regulate overexpression of Cyp2e1, which further promoted the use of $H^+$ from reduced nicotinamide adenine dinucleotide phosphate and $H_2O$ and produced nicotinamide adenine dinucleotide phosphate and $O_2^{\cdot-}$, resulting in peroxidation and aggravate pathologic changes in $TiO_2$ NP-exposed mice. 7-Dehydrocholesterol reductase (Dhcr7) plays a role in drug-induced malformations and inhibits cholesterol biosynthesis, which severely impair brain development.[33] In this study, Dhcr7 was decreased with a Diffscore of $-13.71$ (Supporting Information Table IS), suggesting the weaken ability of brain removing free radicals and aggravating lipid peroxidation situation (Table II). Krev interaction trapped protein 1 (Krit1) is a gene responsible for limiting the accumulation of intracellular oxidants and prevents oxidative stress-mediated cellular dysfunction and DNA damage by enhancing the neurons capacity to scavenge intracellular ROS through an antioxidant.[34] Therefore, increased Krit1 expression with a Diffscore of 21.66 (Supporting Information Table IS) impaired brain cell redox homeostasis by $TiO_2$ NPs exposure (Table II). The pathophysiological changes or apoptosis of brain may be also related to changes in cytokines and chemokines expression. Our results show that 15 genes (6.02% of 249 genes) involved in apoptosis were significantly altered (Supporting Information Table I, Fig. 3). Among the genes altered, eight genes were upregulated, and seven genes were downregulated (Supporting Information Table IS). For example, cysteine–serine-rich nuclear protein-1 (Csrnp1) and Ddit4 increased with Diffscores of 40.4 and 26.92 in the $TiO_2$ NP-exposed group (Supporting Information Table IS), respectively. Csrnp1 has been identified an immediate early gene and strongly give response to interleukin 2 in the brain immune response.[35] Ddit4 is strongly induced in a hypoxia-inducible factor-1 dependent manner, and is a downstream target in the PI3-kinase-Akt pathway, and is believed to exert strong antiapoptotic effects and promote cancer growth.[36] Cui et al.[37] also identified that the overexpression of Ddit4 induced the accumulation of ROS, in turn led to DNA oxidation, and finally caused hepatocyte apoptosis in mice under $TiO_2$ NP-induced toxicity. Sialidase 1 (lysosomal sialidase), also known as Neu1, is a mammalian lysosomal neuraminidase that leads to sialidosis, which has been shown to enhance recovery from spinal cord contusion injury when injected in rats.[38] In the study, Neu1 was downregulated with a Diffscore of $-41.98$ (Supporting Information Table IS), supporting the overt evidence of neuronal loss or neuropathology in the $TiO_2$ NP-treated mice. Fc receptor-like molecules (Fcrls) are a class of proteins that resemble Fc receptors and are preferentially expressed by B lymphocytes which are immune signals.[39] Fcrls reduction with a Diffscore of $-64.70$ (Supporting Information Table IS) suggested that long-term exposure of $TiO_2$ NPs weakened brain immune ability and resulted in apoptosis or necrosis of brain.

Previous study suggested that the reduction in learning and memory of mice caused by $TiO_2$ NPs was closely related to marked increases in acetylcholine, glutamate, and NO and significant decreases in norepinephrine, 5-hydroxy tryptophan, and its metabolite 5-hydroxy indole acetic acid, and dopamine (DA) as well as its metabolite 3,4-dihydroxyphenylacetic acid.[8] Whether these alterations were related to alteration in gene expression in the brain was unclear.[8] In the present study, there were four genes associated with memory and learning processes in brain (Supporting Information Table IS, Fig. 3). Of the genes altered, Col1a1, serine/threonine-protein kinase 1 (Sgk1), and Ctnnb1 were upregulated with the Diffscores of 24.30, 21.80, and14.80, respectively, and only DA receptor D2 (Drd2) was downregulated with a Diffscore of $-18.46$ (Supporting Information Table IS). Collagen, type I, alpha 1, also known as Col1a1, encodes the major component of type I collagen[40] and increased DNA methylation.[41] Increased Col1a1 expression suggested that $TiO_2$ NPs exposure may link with the inhibition of brain collagen synthesis, damage the normal structure of brain tissue, lessen sensitive to stimulus, and contribute to the deficiency of memory and learning of mice. Sgk1 is a member of kinase subfamily and encodes a serine/threonine protein kinase that response to cellular hydration or swelling,[42] activating certain potassium, sodium, and chloride channels.[43] Significant induction of Sgk1 expression demonstrated the insertion of channels into the

plasma membrane through an alteration of trafficking, enhanced the slow delayed potassium rectifier current in the neurons under $TiO_2$ NP-induced toxicity, which is accorded with an electrolyte analysis of potassium loss as our previous study.[8] MacDonald et al.[44] has implicated it as an integral component in the Wnt signaling pathway. Increased Ctnnb1 expression revealed that upregulation of β-catenin may activate the kinase cascades of Wnt signaling pathway, which cooperated with other genes such as Ndp and Sostdc1 in the signal transduction category (Fig. 3), and ultimately affected episodic memory of mice by $TiO_2$ NPs exposure. Drd2 encodes the D2 subtype of the DA receptor, and the regulation of D2R surface expression by the calcium sensor neuronal calcium sensor-1 in the DG controls exploration, synaptic plasticity and memory formation in mice.[45] In this study, decreased Drd2 expression shows that $TiO_2$ NPs exposure inhibited neuroendocrine function, reward and reinforcement, and cognition.[46] Therefore, the decrease in learning and memory ability induced by $TiO_2$ NPs may be associated with an increase in Col1a1, Sgk1, and Ctnnb1 expressions and reduction in Drd2 expression in brain.

## CONCLUSION

$TiO_2$ NPs could be translocated to the brain and deposited in the brain, which in turn led to ROS overproduction, high levels of lipid, protein, and DNA peroxidation, significant proliferation of all glial cells, necrosis of brain tissues, and hippocampal cell apoptosis of mice. The neurotoxicities may be closely associated with significant alterations of 249 genes expression involved in oxidative stress, apoptosis, memory and learning, brain development, lipid metabolism, DNA repair, signal transduction, immune response, and response to stimulus in the brain-injured mice. In particular, significant increases in Col1a1, Sgk1, Ctnnb1, Csrnp1, Ddit4, Cyp2e1, and Krit1 expression levels and great decreases in Drd2, Neu1, Fcrls, and Dhcr7 expression levels following $TiO_2$ NPs exposure resulted in neurogenic disease states in mice. Therefore, these genes may be potential biomarkers of brain toxicity caused by $TiO_2$ NPs exposure.

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