Environ Sci Pollut Res
DOI 10.1007/s11356-014-4002-5

RESEARCH ARTICLE

Subacute toxicity of titanium dioxide (TiO_2) nanoparticles in male rats: emotional behavior and pathophysiological examination

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Received: 19 August 2014 / Accepted: 23 November 2014

Abstract Titanium dioxide nanoparticles (TiO_2 NPs) have a wide range of applications in many fields (paint, industry, medicine, additives in food colorants, and nutritional products). Over the past decade research, TiO_2 NPs have been focused on the potential toxic effects of these useful materials. In the present study, we investigated the effects of subacute exposure to TiO_2 NPs on emotional behavior in adult Wistar rats, the biochemical parameters, and the histology of organs. Animals were injected intraperitoneally (ip) with TiO_2 NPs (20 mg/kg body weight) every 2 days for 20 days. The elevated plus-maze test showed that subacute TiO_2 NPs treatment increased significantly the anxious index (AI) compared to control group. The toxicological parameters were assessed 24 h and 14 days after the last injection of TiO_2 NPs. Subacute exposure to nanoparticles increased the AST/ALT enzyme ratio and LDH activity. However, the blood cell count remained unchanged, except the platelet count increase. Histological examination showed a little inflammation overall. Moreover, our results provide strong evidence that the TiO_2 NPs can induce the liver pathological changes of rats. The intraperitoneal injection of TiO_2 NPs increased the accumulation of titanium in the liver, lung, and the brain. The results suggest that TiO_2 NPs could alter the neurobehavioral performance of adult Wistar rats and promotes alterations in hepatic tissues.

Keywords TiO_2 Nanoparticles · Emotional behavior · Hematological parameters · Biochemical parameters · Liver · Rats

Introduction

Nanosized titanium dioxide (TiO_2 NPs) is used widely in various everyday products as a pigment or additive for paints, paper, ceramics, plastics, foods, and other products (Xie et al. 2011) and can be applied to the medical field for diagnostic or therapeutic tools (Li et al. 2010). Moreover, TiO_2 is used at the nanoscale level in manufacturing (Allen et al. 2005) and to decontaminate water, air, and soil (Esterkin et al. 2005). The TiO_2 nanoparticles in a special top layer of the flagstone are hoped to reduce pollution when activated by sunlight by converting ambient nitrogen dioxide gas into less toxic nitrates. The versatility of TiO_2 is due in part to its useful properties, including being insoluble in aqueous solutions, nonradioactive, not radio-opaque, not costly, easy to prepare in stable suspensions in fine particulate form, and easy to measure by simple chemical techniques (Fabian et al. 2008). A rapid growth in the number of published studies confirms that there is a high level of interest concerning the safety of TiO_2 NPs from the scientific community. Some studies have revealed that TiO_2 NPs are more toxic than TiO_2 fine particles (Zhao et al. 2009; Oberdorster et al. 1994). Recent studies have indicated that TiO_2 NPs are toxic to human organs and cause oxidative stress (Li et al. 2010), so it can be absorbed into the body by inhalation, ingestion, and dermal penetration,

Responsible editor: Philippe Garrigues

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Published online: 10 January 2015

and are distributed to important organ systems, including the lymph, brain, lung, liver, and kidney (Bermudez et al. 2004; Wang et al. 2007). Other studies have shown that it produced pulmonary inflammation, cytotoxicity, and histopathological changes by intratracheally instillation (Warheit et al. 2007), intraperitoneal injection, or oral administration.

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 damaged brain neurocyte and tissue in vitro and in vivo (Hu et al. 2010). Moreover, Wang et al. (2007) and Hu et al. (2010) investigated that with oral gavage, TiO_2 NPs caused a slight brain lesion of mice, such as vacuoles of neurons and fatty degeneration of the hippocampus.

Many exposure and toxicity tests are conducted on rodents rather than human subjects. Two separate studies found that a TiO_2 NPs dose of 5 g/kg body weight did not cause obvious acute toxicity in rats (Wang et al. 2007; Fabian et al. 2008). So, Fabian et al. (2008) confirmed that rats exposed to TiO_2 NPs by a route that allows immediate systemic availability showed an expected tissue distribution but no obvious toxic health effects, no immune response, and no change in organ function. Therefore, they suggested that TiO_2 NPs could be used safely in low doses. Moreover, Adachi et al. (2013) did not find any obvious evidences of nano-TiO_2 particle skin penetration using several morphological methods after the subchronic exposure. However, TiO_2 NPs were shown to be systemically absorbed and distributed into several tissues following administration through single oral dose of 5000 mg/kg, intravenous doses of 5, 160, or 560 mg/kg, intraperitoneal doses of 40–2592 mg/kg, and intra-articular or subcutaneous dose of 5600 mg/kg (Wang et al. 2007; Fabian et al. 2008; Abe et al. 2009; Chen et al. 2009; Patri et al. 2009; Van Ravenzwaay et al. 2009; Wang et al. 2009; Moon et al. 2010). The liver, spleen, lung, and kidney are likely to be the main target organs, while the brain, plasma, or blood cells had no detectable levels of TiO_2. Repeated injections of 5 nm at various doses into the abdominal cavity of mice for 14 days showed translocation into the brain (Ma et al. 2010; Hu et al. 2010). Wang et al. (2008a, b) reported direct uptake of 80 and 155 nm particles by the brain via the olfactory neuronal pathway (i.e., by passing the bloodbrain barrier) following intranasal instillation of 500 μg/mouse every other day for 30 days.

In the present study, we investigate the effects of intraperitoneal administration of TiO_2 NPs (20 mg/kg body weight) every 2 days for 20 days on the emotional behavior. Additionally, we assess the accumulation of titanium in the organic tissues, the blood cell count, plasma biochemical levels, and the histopathological changes 24 h and 14 days after the last injection of TiO_2 NPs.

Materials and methods

Characterization of NPs

In this research, the TiO_2 NPs were analyzed and provided by the Laboratory of Physics of Materials and Nanomaterials Applied at Environment, College of Sciences in Gabes (Amlouk et al. 2006). The TiO_2 NPs were in the size range, between 20 and 30 nm. The crystalline data were obtained by X-ray diffractometry (XRD; Bruker D8 Advance; 40 KV, 30 mA). The synthesized products were characterized using transmission electron microscopy [(TEM) Tecnai G2-200KV with microanalysis] (Figs. 1 and 2).

TiO_2 NP preparation

TiO_2 NPs were suspended in physiological saline solution (9 ‰ sodium chloride), and the suspension was ultrasonicated for 60 min to disperse completely as well as possible. In order to prevent overheating of the equipment and the suspension, the pulsed mode was used with a second pulse followed by one second off, and the tube containing the solution is placed in a beaker filled with ice, (BANDELIN SONOPLUS, model HD 2070). TiO_2 suspension was vortexed before each injection.

Animals and treatment

Male Wistar rats (SIPHAT, Ben Arous, Tunisia) weighed 100–110 g at the beginning of the experiment. Rats were randomly divided into four groups (n=6), two control and two experimental groups. The animals were housed under controlled conditions of temperature (25 °C) and light (12:12 light/dark). All animals were provided with water and food ad libitum. The experimental protocols were approved by the Faculty Ethics Committee.

Treated animals received ten intraperitoneal injections with moderate dose of TiO_2 NPs (20 mg/kg) every 2 days, and control group received a dose of 9‰ sodium chloride. Half of the rats in each group were killed 1 day after and the remaining half were killed on day 14 (Chen et al. 2009). After weighting the organs, the organ wet weight/BW (mg/g) of the lung, liver, kidneys, thymus, and brain was calculated.

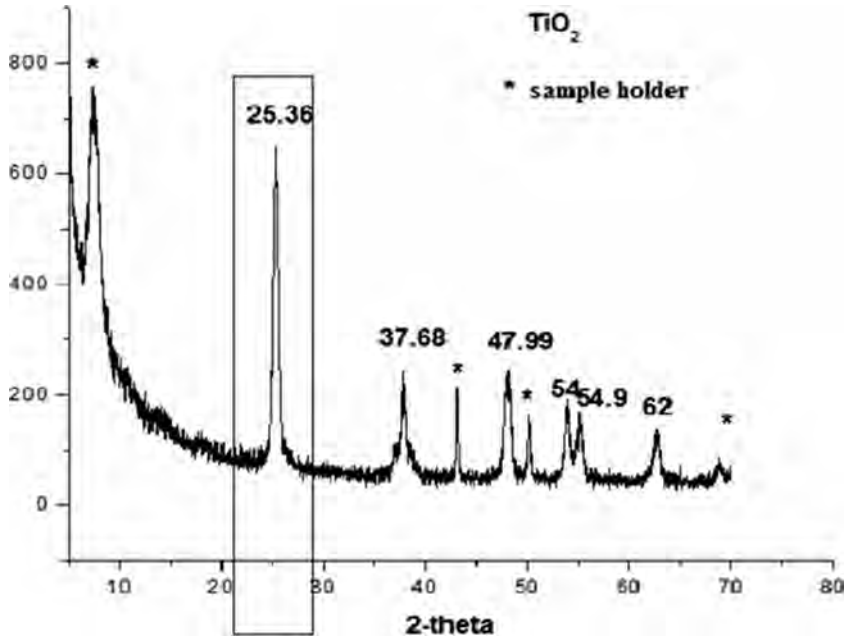

Fig. 1 XRD pattern of nanosized TiO_2 NPs

Behavioral testing

The plus maze was made of clear painted wood. The arm was elevated 60 cm above floor level with two open arms (50× 10 cm) and two closed arms with 50 cm high walls (Maaroufi et al. 2009). Arms of the same type were located opposite from each other. Individual rats were placed on the center of the maze facing an open arm, and the time spent in open and closed arms as well as the path length were recorded during a 5-min period using the videotracking system. The number of entries and the time spent in the open and closed arms and in the center was recorded. In addition, the number of grooming and rears was noted. Then, the anxious index (AI) which is expressed [AI=Closed arm entries/(Closed arm entries+ Open arm entries)*100] was calculated (Pellow et al. 1985).

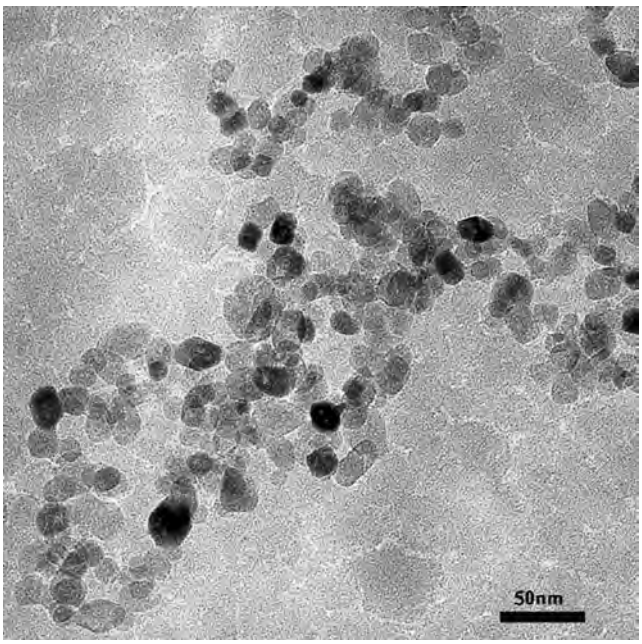

Fig. 2 TEM characterization of titanium dioxide nanoparticles

Blood biochemical and hematology analysis

Animals were first weighed and blood samples were collected via the ocular vein. Then, they were sacrificed. Serum was collected by centrifugation (4000*g* for 15 min). Liver function was evaluated based on the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), uric acid, creatinine, and glucose. These biochemical parameters were determined by an automated biochemical analyzer. Hematological parameters examined in this study included white blood cells (WBC) count, red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Ht), platelet (PLt), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH).

Ti content analysis in tissues

Fractions of sample tissues (liver, kidney, lung, and brain) were weighed and dried in oven set at 60 °C, to constant weight, and then their dry weight is determined with a precision balance. The samples were placed in test tubes and the

volume is added 3 ml/100 mg dry weight of nitric acid. The tubes are then heated to 120 °C in a thermoblock (WTW TB_2-type). After cooling tubes, minerals were diluted with 10 ml of deionized water (Takashima et al. 1978). Titanium content was analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The detection limit of titanium was 0.002 mg/L.

Histopathological examination

Immediately after sacrifice, the liver, lung, kidney, and brain were excised and weighted. Fractions of tissues were harvested and washed with ice-cold saline, and then they were fixed in a 10 % neutral buffered formalin solution, embedded in paraffin, and used for histopathological examination. Sections were cut of about 5 μm thick, deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E).

Data presentation and statistical analysis

Results are expressed as the mean±standard error to the mean (S.E.M) and were subjected to the unpaired Student's *t* test. A *p* value less than 0.05 was considered significant (*$p<0.05$).

Results

The average size distribution of TiO_2 NPs

X-ray diffraction measurements show that TiO_2 NPs exhibit the anatase structure (Fig. 1), and the average grain size calculated from the broadening of the XRD peak of anatase was roughly 30 nm using Scherer's equation:

$$D = 0.9\lambda \Big/ \beta\cos\theta,$$

Table 1 The behavior of control and TiO_2-NP-treated rats in the plus maze

Variables	C	TiO_2
Time in the open arms (s)	22.34±6.68	14.72±7.07
Time in the close arms (s)	219.20±21.70	246.37±15.02
Time in the center (s)	58.45±15.66	38.90±10.38
Grooming	3.91±0.49	4.66±0.51
Number of rears	12.16±1.06	10.41±1.24

The data represent the mean±S.E.M, $n=12$

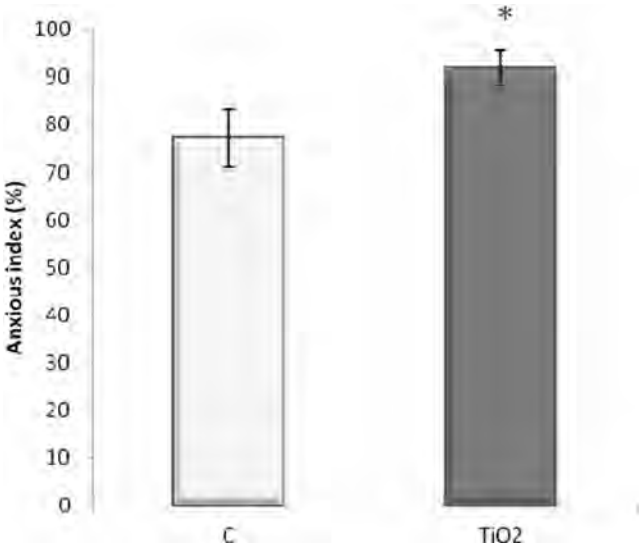

Fig. 3 Anxious index (AI) of control and (ip) injected rats with TiO_2 NPs. Values represent mean±S.E.M, $n=12$. *Significantly different from the control group ($p<0.05$, Student's *t* test)

where λ is the wavelength of the X-ray radiation, β is the full width at half maximum (FWHM) in radians of the XRD peak, and θ is the angle of diffraction.

The MET showed that TiO_2 NPs had a uniformly scattered situation with a nanocrystalline structure (Fig. 2).

Effects on emotional behavior

The results from the elevated plus maze showed that the TiO_2-NP-injected group entered less frequently and spent less time in the open arms than control group (Table 1). Moreover, subacute TiO_2 NPs treatment increased significantly the anxious index (AI) (91.78±3.68 vs 77.13±6.12, $p<0.05$) (Fig. 3).

Effects on body and relative organ weight

All animals were weighed at the beginning and the end of treatment. TiO_2 NPs treatment did not affect the body weight of the injected rats (Fig. 4). Table 2 showed the coefficient of the liver, kidney, lung, and brain to body weight expressed as milligrams (wet weight of tissues) per gram (body weight) and also the thymus index (%). No obvious significant differences were observed in the coefficients of the liver (46.26±4.03 for S1 and 38.11±1.05 for S2 vs 40.39±0.71, $p>0.05$), kidney (9.13±0.86 for S1 and 7.94±0.1 for S2 vs 8.06±0.23, $p>0.05$), and brain (7.92±0.15 for S1 and 7.88±0.16 for S2 vs 8.02±0.1, $p>0.05$), but the coefficient of the lung was significantly higher in TiO_2-NP-treated rats and sacrificed after 14 days (S2) than the control group (8.04±0.33 vs 6.08

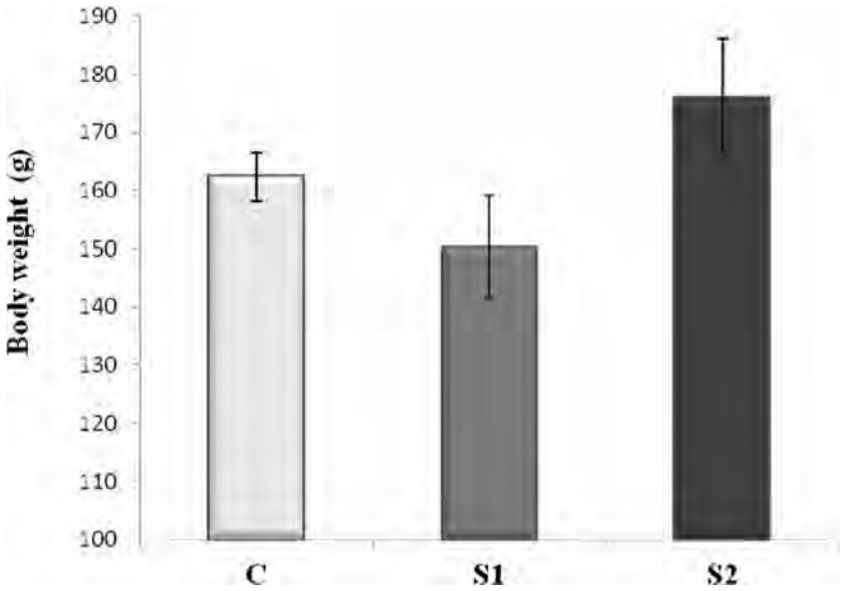

Fig. 4 Effect of subacute treatment of TiO_2 NPs on the body weight of male control rats (*C*) and treated rats sacrificed 1 day (*S1*) or 14 days (*S2*) after the last injection. Each value represents the mean of six determinations±S.E.M

±0.20, $p<0.05$). Regarding the index, the treatment decreased significantly the thymus index (1.33±0.24 for S1 vs 1.94± 0.25; $p>0.05$); this change seems to be reversible after 14 days.

Effects of exposure to TiO_2 NPs on hematological and biochemical parameters of rats

Table 3 showed the changes of hematological and biochemical parameters induced by TiO_2 NPs injection. The concentration of hemoglobin, the hematocrit, and the percentage of red blood cells and white blood cells remained unchanged, but the platelet count increased significantly for treated rats and sacrificed after 1 day after the last injection (S1) than the control group (1092.2±85.41 vs 928.6±52.27, $p<0.05$). TiO_2 NPs treatment increased significantly the aspartate aminotransferase/alanine aminotransferase ratio (AST/ALT ratio) (4.92±0.43 for S1 and 5.59±0.93 for S2 vs 2.98±0.22, $p>0.05$), and it also increased the LDH and glucose levels for treated rats sacrificed 14 days after the last injection (S2) (1138.15± 126.94 vs 808.69±47.77, $p<0.05$ and 117.74±3.93 vs 153.69±8.89, $p<0.05$, respectively). In contrast, the serum levels of uric acid and creatinine remained unchanged compared to the control group.

Titanium concentration in different organs

The contents of titanium in the rat organs are shown in Table 4. With intraperitoneal injection, the titanium may cause significant accumulation in the rat liver and lung ($p<0.05$) at different times (1 day and 14 days after the last injection). The titanium accumulation in the brain increased significantly for treated rats and sacrificed after 1 day after the last injection (S1) than the control group (2.05±0.12 vs 0.70±0.07, $p<0.05$).

Histopathological examination

Histopathological examination of the tissues indicated that intraperitoneal injection of TiO_2 NPs (20 mg/kg body weight)

Table 2 Effects of subacute treatment of titanium dioxide nanoparticles (TiO_2) on the relative weight of different organs in male rats

	Liver (mg/g)	Kidney (mg/g)	Lung (mg/g)	Brain (mg/g)	Thymus index (%)
C	40.39±0.71	8.06±0.23	6.08±0.20	8.02±0.10	1.94±0.25
S1	46.26±4.03	9.13±0.86	7.92±0.63	7.92±0.15	1.33±0.24*
S2	38.11±1.05	7.94±0.10	8.04±0.33*	7.88±0.16	1.82±0.19

The data represent group averages (mean±S.E.M, $n=6$). Male control rats (C) and treated rats sacrificed 1 day (S1) or 14 days (S2) after the last injection
*Significantly different from the control group ($p<0.05$, Student's t test)

Table 3 Effects of exposure to TiO_2 NPs on hematological and biochemical parameters of control rats and TiO_2-NP-treated rats sacrificed 1 day (S1) or 14 days (S2) after the last injection

	Control	S1	S2
WBC (10^3/mm^3)	13.42±1.70	12.62±1.66	12.78±1.05
RBC (10^3/mm^3)	7.42±0.13	7.64±0.12	7.04±0.18
Hb (g/dl)	12.90±0.33	13.25±0.16	12.63±0.39
Ht (%)	41.66±1.00	45.76±0.74	41.60±0.99
PLt (10^3/mm^3)	928.60±52.27	1092.20±85.41*	1039.60±68.34
MCV	56.20±0.62	60.50±0.86	56.38±0.87
MCH	17.68±0.46	17.32±0.27	17.55±0.29
AST/ALT	2.98±0.22	4.92±0.43*	5.59±0.93*
LDH (U/I)	808.69±47.77	1017.54±109.48	1138.15±126.94*
Uric acid (mg/dl)	1.48±0.13	1.57±0.14	1.68±0.08
Creatinine (mg/dl)	0.50±0.02	0.51±0.08	0.40±0.04
Glucose (mg/dl)	153.69±8.89	134.76±5.08	117.74±3.93*

The data represent group averages (mean±S.E.M, n=6)

WBC white blood cells, *RBC* red blood cells, *Hb* hemoglobin, *Ht* hematocrit, *PLt* platelet, *MCV* mean corpuscular volume, *MCH* mean corpuscular hemoglobin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *LDH* lactate dehydrogenase

*Significantly different from the control group (C) (p<0.05, Student's t test)

every 2 days for 20 days did not show significant changes in morphology and pathological lesions in organs (Figs. 5, 6, 7, and 8).

Discussion

In order to evaluate the toxicity of nanoparticles, several exposure routes were chosen for experimental animal studies. These include oral, inhalation, intratracheal, subcutaneous injection, intravenous, and intraperitoneal administration. In this study, adult rats were injected intraperitoneally with TiO_2 NPs (20 mg/kg body weight) every 2 days for 20 days. Animal behavior, blood biochemistry, hematology, titanium biodistribution, and histopathology were investigated. Our results showed that TiO_2 NPs induced serious effects on the emotional behavior. In the plus maze test, TiO_2 NPs treatment increased the anxious index of rats. These results could be interpreted in terms of reduced activity and exploratory drive instead of anxiety modifications. Cui et al. (2014) suggested that the depressive-like behaviors observed in adult rats following exposure to TiO_2 NPs could be related to the oxidative damage in the central nervous system (CNS). The CNS is a potentially susceptible target for TiO_2 NPs. Kim et al. (2013) indicated that central administration of TiO_2-NPs induced behavioral deterioration in freely moving intact animals. The Morris water maze test and the passive avoidance test showed that exposure to TiO_2 NPs significantly impaired learning and memory in offspring (Mohammadipour et al. 2014). Hu et al. (2010) showed that TiO_2 NPs exposure decreased the contents of some monoamines neurotransmitters such as norepinephrine, dopamine, and its metabolite. These abnormalities in the monoaminergic systems may be associated with psychiatric diseases such as schizophrenia, depression anxiety, and attention-deficit hyperactivity disorder (Ressler and Nemeroff 2000; Tamminga 2006). Our investigation showed that subacute exposure to TiO_2 NPs increased the accumulation of titanium in the brain of rats 24 h after the last injection. The nanoparticles from the blood circulation could influence endothelial cell membrane integrity and/or disrupt the blood–brain barrier and may induce vesicular transport to gain access into the CNS. However, the histopathology analysis of the brain showed normal architecture in treated rats.

Blood cell count analysis is normally used to detect the hematological toxicity of different chemicals. In this study, the results indicated that, 14 days after intraperitoneal administration of TiO_2 NPs, no significant hematological toxicity could be observed. However, 24 h after treatment, we noted a significant increase of platelet count. Platelets are characterized by expert functions in assisting and modulating inflammatory reactions and immune responses (Hundelshausen and Weber 2007). Although no significant changes were found in the body weight and the relative weight for liver, kidney and brain in TiO_2-NPs-treated rats. However, TiO_2 NP administration increased the relative weight of lung tissues. Both increase and decrease of the organ coefficients may be caused by TiO_2 NPs excretion or accumulation in the organs,

Table 4 Titanium concentration (μg/g) in different organs of control and treated male rats

	Liver	Kidney	Lung	Brain
C	0.39±0.19	2.22±0.82	1.79±0.32	0.70±0.07
S1	40.97±11.17 *	2.90±0.64	23.16±4.28 *	2.05±0.12 *
S2	115.55±11.40 *	3.09±1.45	12.20±3.20 *	1.15±0.52

Titanium concentration (μg/g) in the liver, kidneys, lungs, and brain in control rats (C) and treated rats sacrificed 1 day (S1) or 14 days (S2) after the last injection. Each value represents the mean of six determinations± S.E.M

*p<0.05 compared to control (Student's t test)

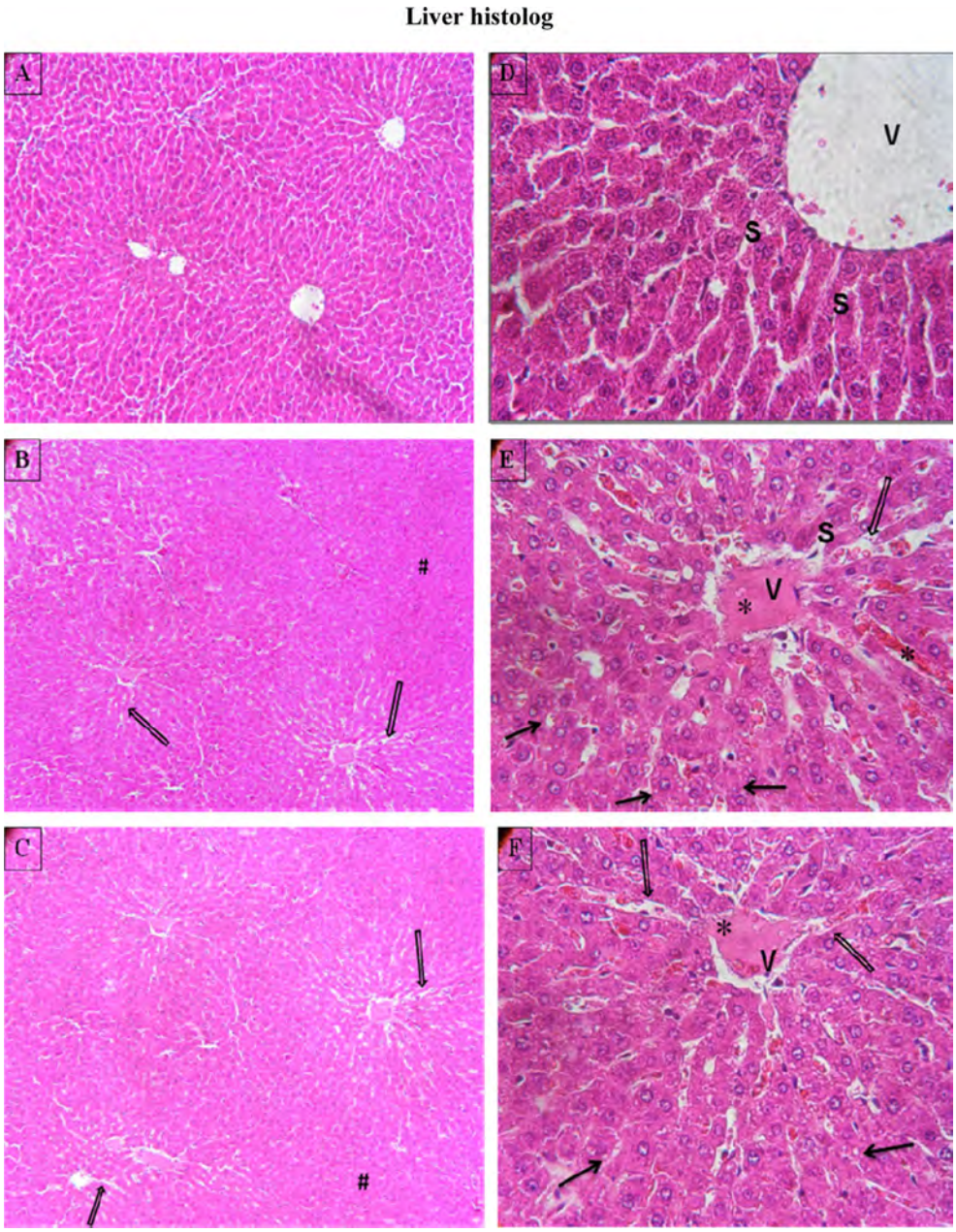

Fig. 5 Histopathology of the liver tissue (H&E stain) in male rats caused by intraperitoneal administration with TiO_2 NPs. The rats were sacrificed 1 day (**b**, **e**) or 14 days (**c**, **f**) after the last injection and control (**a**, **d**). **a**, **b**, **c** ×100 and **d**, **e**, **f** ×400. ⟹ Sinusoïdal dilatation, → vacuoles, * congestion, # cellular space, v vein, s sinusoid

which could cause certain histopathological changes (Xu et al. 2013). In this study, symptoms of subacute toxicity such as the increase of the AST/ALT enzyme ratio and LDH activity were observed in 14 days after the last injection of TiO_2 NPs. ALT and AST exist in the liver, heart, and other organs. When the organs injured, the activities of ALT and AST in serum would increase. It is well known that LDH is an important isoenzyme in glycolysis and glyconeogenesis and widely exists in the heart, liver, lung, and many other tissues. When the tissues are subjected to injury, LDH would leak into the serum of blood from organs or cells, which resulted in the increase of LDH activity and its isoenzyme in the corresponding organs (Ma et al. 2009). The results of this study indicated that intraperitoneal injection of TiO_2 NPs can induce histopathological changes of liver, including congestion, prominent vasodilatation, and vacuolization, thus leading to the damage of liver function. Our study showed that liver injury could be related to the obvious titanium accumulation in this organ. In addition, the accumulation of titanium in the liver of rats sacrificed 14 days after the last injection

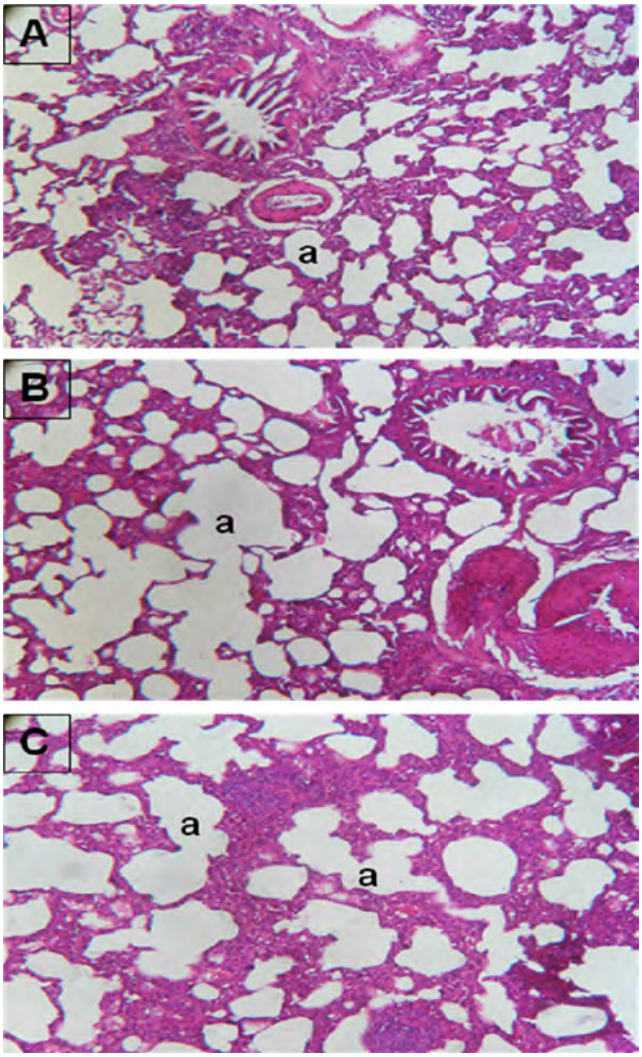

Fig. 6 Histology of the lungs of control rats (**a**) and treated rats sacrificed 1 day (**b**) or 14 days (**c**) after the last injection, ×100, alveolus (*a*)

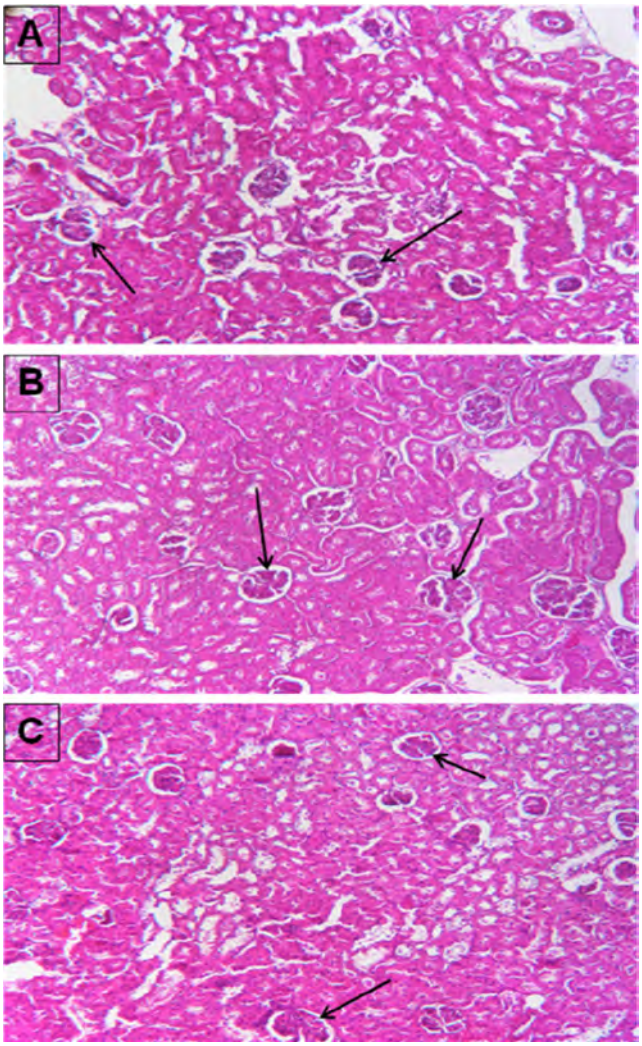

Fig. 7 Histology of kidneys of control rats (**a**) and treated rats sacrificed 1 day (**b**) or 14 days (**c**) after the last injection, ×100. The *arrows* represent the glomerulus

was higher than that of rats decapitated 24 h following last injection. However, we noted a marked increase of titanium accumulation in the lung of both TiO_2-NP-treated groups compared to control group. The predominant deposition of TiO_2 NPs in these organs could be dependent on physical/mechanical processes leading to a highly agglomerated state. Histological examination showed no obvious pathological effects in the lung of TiO_2-NP-treated rats. Previous studies have demonstrated that accumulation of TiO_2 NPs can be observed in the liver, lung, kidneys, and spleen after intraperitoneal, intravenous, or dermal administration (Chen et al. 2009; Wu et al. 2009). After inhalation exposure in rats, TiO_2 NPs have been found to accumulate in the lung, leading to phagocytosis (Bermudez et al. 2004; Grassian et al. 2007). Fabian et al. (2008) reported the accumulation of TiO_2 NPs in the liver, spleen, lung, and kidneys after intravenous administration of 5 mg/kg TiO_2 NPs in rat. In our study, we noted any significant risk for kidney injury following TiO_2 NPs treatment. Plasmatic uric acid and creatinine levels remained unchanged. Moreover, TiO_2 NPs produced no significant histopathological modifications in the kidney compared to controls. This result could be related to the rapid elimination of titanium from the kidney tissue.

In conclusion, TiO_2 NPs could alter the neurobehavioral performance of adult Wistar rats and promote alterations in hepatic tissues. The results of this in vivo study suggest that elution of metal ions is an important factor in the toxicity of TiO_2 NPs, but there are many points which are unclear and further studies are necessary.

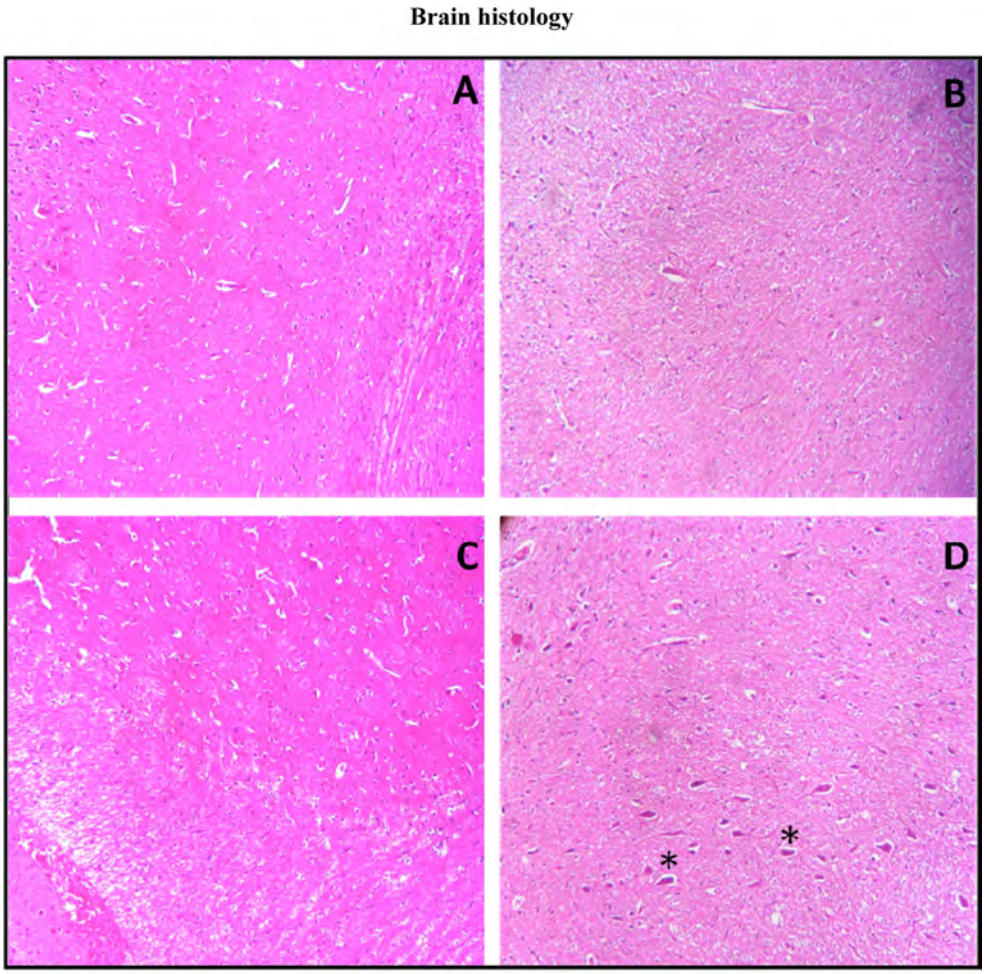

Fig. 8 Histological analyses in the brain tissues in rats treated with TiO_2 NPs. Control rats (**a**, **c**) and treated rats sacrificed 1 day (**b**) or 14 days (**d**) after the last injection, magnification ×400. The histological analyses in the brain tissues did not show significant changes in morphology of neurons (*asterisks*)

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