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Toxicological characteristics of nanoparticulate anatase titanium dioxide in mice

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ABSTRACT

In an effort to examine liver injury, immune response, and other physiological effects in mice caused by intragastric administration of nanoparticulate anatase titanium dioxide (5 nm), we assessed T lymphocytes, B lymphocyte and NK lymphocyte counts, hematological indices, biochemical parameters of liver functions, and histopathological changes in nanoparticulate titanium dioxide -treated mice. Indeed, mice treated with higher dose nanoparticulate titanium dioxide displayed a reduction in body weight, an increase in coefficients of the liver and histopathological changes in the liver. Specifically, in these nanoparticulate titanium dioxide -treated mice, interleukin-2 activity, white blood cells, red blood cells, haemoglobin, mean corpuscular haemoglobin concentration, thrombocytes, reticulocytes, T lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$), NK lymphocytes, B lymphocytes, and the ratio of CD4 to CD8 of mice were decreased, whereas NO level, mean corpuscular volume, mean corpuscular haemoglobin, red (cell) distribution width, platelets, hematocrit, mean platelet volume of mice were increased. Furthermore, liver functions were also disrupted, as evidenced by the enhanced activities of alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, lactate dehydrogenase and cholinesterase, an increase of the total protein, and the reduction of ratio of albumin to globulin, the total bilirubin, triglycerides, and the total cholesterol levels. These results suggested that the liver function damage observed in mice treated with higher dose nanoparticulate titanium dioxide is likely associated with the damage of haemostasis blood system and immune response. However, low dose nanoparticulate anatase TiO_2 has little influences on haemostasis blood system and immune response in mice.

1. Introduction

Titanium dioxide (TiO_2) occurs primarily in the form of the minerals rutile, anatase, brookite, and as the iron-containing mineral ilmenite ($FeTiO_3$). The major source of TiO_2 is ilmenite, while rutile and anatase pigments are mainly manufactured commercially. TiO_2 is a versatile compound that has already been used in nanoparticulate form in a variety of consumer products such as sunscreens that are surface coated with e.g. silica and other cosmetics [1], specialist coatings and paints [2,3], and in industrial photocatalytic processes [4,5]. Therefore, nanoparticulate TiO_2 has probably entered in the environment, even though its current levels are unknown. Generally TiO_2 has been considered biologically inactive in experimental animals [6–9] and humans [10–13]. However, the injury of liver function was recently observed in mice exposed to nanoparticulate TiO_2 [14–17]. Wang et al. reported that high-dose nanoparticulate TiO_2 (25 and 80 nm) with oral gavage increased the ratio of alanine aminotransferase to aspartate aminotransferase, the activity of lactate dehydrogenase and the coefficient of the liver, and caused the hepatocyte necrosis [14]. Liu et al. found that high-dose nanoparticulate anatase TiO_2 (5 nm) with intraperitoneal injection could damage liver function [15] and induced an oxidative attack in the mouse liver [16]. The histopathological changes and hepatocytes apoptosis of the mouse liver were observed; and the liver function damaged and inflammatory cascade by high-doses nanoparticulate anatase TiO_2 (5 nm) with intraperitoneal injection were showed to be closely related to significant alteration of the mRNA and protein expressions of several inflammatory cytokines [17]. However, it remained unclear whether these liver function damages in mice treated with nanoparticulate TiO_2 also have the changes of hematological indices and the reduction of immune response.

Lymphocyte proliferation is the important phase in immune response of the animal body [18,19]. T lymphocyte and B lymphocyte have receptors to identify antigen and mitogen. Could nanoparticulate TiO_2 affect T lymphocytes (such as $CD3^+$, $CD4^+$, and $CD8^+$ cells) and B lymphocyte proliferation in mice when the liver

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function was damaged? In addition, hematological indices are important parameters for the evaluation of the animal and human physiological status. Hematological parameters are closely related to the animal's response to the environment, i.e., any change of hematological indices is indicative of possible effects on the hematological characteristics exerted by the environment where animal live [20]. They can provide substantial diagnostic information once reference values are established under exposure to nanoparticulate TiO_2 conditions. Evaluation of the hemogram involves the determination of the total erythrocyte count (RBC), total white blood cell count (WBC), hematocrit (PCV), haemoglobin concentration (HGB), erythrocyte indices, white blood cell (WBC) differential count and the evaluation of stained peripheral blood films [21]. Thrombocytes (PCT), next to erythrocytes, are known to be one of the most abundant blood cells.

In order to investigate the intrinsic hazard potential of nanoparticulate TiO_2 particles that may have entered into animals and humans from the environment, we studied the toxic effects of nanoparticulate anatase TiO_2 (5 nm) on mice after 30 days of intragastric administration. The immune response in these mice treated with nanoparticulate anatase TiO_2 was measured by T lymphocyte (such as CD3, CD4, and CD8), B lymphocyte and natural killer lymphocyte proliferation, hematological characteristics. We also examined biochemical parameters of liver functions and histopathological changes of these nanoparticulate anatase TiO_2-treated mice.

2. Materials and methods

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 [22]: 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 Ti^{4+} content in the nano-anatase was measured by inductively coupled plasma mass spectroscopy (ICP-MS) and O, C, and H contents in the nano-anatase were assayed by Elementar Analysensysteme Gmbh, showing that Ti, O, C, and H contents in the nano-anatase were 58.114%, 40.683%, 0.232%, and 0.136%, respectively. Bulk TiO_2 (rutile) was purchased from Shanghai Chem. Co. and the average grain size was 10–15 μm.

A 0.5% hydroxypropylmethylcellulose K4 M (HPMC, K4 M) 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 (22 ± 2 g) were purchased from the Animal Center of Soochow University. 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 procedures used in animal experiments were in compliance with the Soochow University ethics committee. Animals were randomly divided into four groups: control group (treated with 0.5% HPMC) and three experimental groups (62.5, 125, 250 mg/kg BW nano-anatase TiO_2). Nanoparticulate anatase TiO_2 (62.5, 125, 250 mg/kg BW) suspensions were given to mice by a intragastric administration every other day for 30 days, respectively. The control group was treated with 0.5% HPMC. The symptom and mortality were observed and recorded carefully everyday for 30 days. After 30 days, all animals were first weighed and then sacrificed after being anesthetized by ether. Blood samples were collected from the eye vein by removing the eyeball quickly. Serum was harvested by centrifuging blood at 2500 rpm for 10 min. The tissues and organs such as liver, spleen and thymus were excised and weighed.

2.3. Coefficients of organs

After weighing the body and tissues, the coefficients of the liver, spleen, thymus to the body weight were calculated as the ratio of tissues (wet weight, mg) to body weight (g).

2.4. Histopathological examination of liver and kidney

For pathological studies, all histopathological examinations were performed using standard laboratory procedures. The tissues 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). The identity and analysis of the pathology slides were blind to the pathologist.

2.5. Biochemical analysis of liver function

Liver function was evaluated with serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), cholinesterase (ChE), total protein (TP), albumin (ALB), globulin (GLB), and total bilirubin (TBIL), triglycerides (TG), total cholesterol (TCHO) using the commercial kits (Bühlmann Laboratories, Switzerland). All biochemical assays were performed using a clinical automatic chemistry analyzer (Type 7170A, Hitachi, Japan).

2.6. Cytometric assay of lymphocyte subsets in peripheral blood

To identify and quantify lymphocyte subsets, cell suspensions were analyzed by flow cytometry. Following red blood cell lysis, cells were stained with anti-mouse monoclonal antibodies against CD3, CD4, CD8, CD19, and NK1.1 (BD Biosciences). Cells were analyzed via four-color flow cytometry on a FACSCaliber (BD Biosciences) in the University of Soochow Immunological Research Center Facility. Lymphocyte subsets, including B cells, CD3 T cells, CD4 T cells, CD8 T cells, double positive thymocytes, double negative thymocytes, and NK cells, were analyzed. The size of each cell population was calculated as the product of the total lymphocyte count recorded by the Hemavet or hemacytometer and the percentage of positive lymphocytes recorded by the flow cytometer. All data were analyzed with BD Biosciences Cellquest analysis software.

2.7. Hematological parameters determination

Blood samples were collected in tubes containing EDTA as anticoagulant. Red blood cells (RBC), reticulocytes (Ret), white blood cells (WBC), haemoglobin (HGB), platelets (PLT), platelet distribution width (PDW), Red (cell) distribution width (RDW), thrombocytocrit (PCT), hematocrit (HCT), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean platelet volume (MPV) were measured using a hematology autoanalyzer (Cell-DYN 3700).

2.8. Concentration assay of interleukin -2(IL-2)

To determine IL-2 levels of the plasma, enzyme linked immunosorbent assay (ELISA) was performed by using commercial kits that are selective for mouse IL-2 (Biological Marker Laboratory Inc., USA). Manufacturer's instruction was followed. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron, Finland) and IL-2 concentration of samples was calculated from a standard curve.

NO concentration assay in the plasma was performed according to kit protocols (Nanjing Jiancheng Bioengineering Institute). 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 (μmol/L) = $(A_{sample} - A_{blank})/(A_{standard} - A_{blank}) \times 20$(μmol/L).

2.9. Statistical analysis

Results were analyzed by analysis of variance (ANOVA).When analyzing the variance treatment effect ($p \leq 0.05$); the least standard deviation (LSD) test was applied to make a comparison between means at the 0.05 levels of significance.

3. Results

3.1. Growth and the coefficients of organs

During administration, animals were all at growth state. The daily behaviors such as eating, drinking and activity in nanoparticulate TiO_2-treated groups were as normal as the control group. After 30 days, the mice were weighted, various organs were

collected and also weighted. Table 1 shows the net weight increase and the coefficients of the organs to the body weight (milligrams, wet weight of tissues/grams, body weight). No significant differences in the body weight and coefficients of the liver, kidney, spleen, and thymus were found in the 62.5 mg/kg BW nanoparticulate TiO_2-treated group ($p > 0.05$). In contrast, in the 125 and 250 mg/kg BW nanoparticulate TiO_2-treated groups, the net increases of body weight of mice were gradually reduced ($p < 0.05$ or 0.01), while their coefficients of the liver, kidney, spleen and thymus were significantly higher ($p < 0.05$ or $p < 0.01$) than those of the control, suggesting that high-dose nanoparticulate TiO_2 might cause the damage of the organs of mice.

3.2. Liver histopathological observation

The histological photomicrographs of the liver sections are shown in Fig. 1. In the 125 mg/kg BW nanoparticulate TiO_2 group, the liver tissue had no abnormal pathology changes compared with the control, suggesting normal hepatocytes, portal area and integrated hepatic sinusoidal (Fig. 1b). In the 250 mg/kg BW nanoparticulate TiO_2 group, however, the histopathological changes were observed in the liver tissue, i.e., the structure of hepatocytes was blur in the large area, interstitial vessels were congested (Fig. 1c,d).

3.3. Liver function

The serum biochemical parameters were assayed to further evaluate the toxicity of nanoparticulate TiO_2 on the mouse liver. Table 2 lists the changes of biochemical parameters in the mouse liver serum after gavaging with nanoparticulate TiO_2 for consecutive 30 days.

In the 62.5 mg/kg BW treatment, there were no significant changes for all the parameters compared with the control group ($p > 0.05$). In the higher dose of nanoparticulate TiO_2-treated (125, and 250 mg/kg BW) groups, however, the activities of ALT, ALP, AST, LDH, ChE, and TP were significantly higher than those of the control group ($p < 0.05$ or $p < 0.01$), and the ratio of ALB to GLB (A/G) and TBIL were significantly reduced ($p < 0.05$ or $p < 0.01$). The increased activities of ALT, ALP, AST, LDH, ChE, TP, and the decreased ratio of ALB to GLB, and TBIL levels demonstrated that nanoparticulate TiO_2-induced hepatic injury. Furthermore, as the increased enzymatic activities and the reduced ratio of ALB to GLB, and TBIL levels are dose-dependent, the nanoparticulate TiO_2-induced hepatic injury should be dose-dependent as well. TG and TCHO from the 125 and 250 mg/Kg BW-treated groups were higher than those of the control group ($p < 0.05$ or 0.01). These results indicate that nanoparticulate TiO_2 in higher doses caused metabolism imbalance of lipids in the mouse liver.

Table 1
The increase of net weight and coefficients of organ of mice by intragastric administration with nanoparticulate anatase TiO_2 for consecutive 30 days.

Indexes	Nanoparticulate anatase TiO_2 (mg/kg BW)			
	0	62.5	125	250
Net increase of BW (g)	7.12 ± 0.36	6.91 ± 0.35	$6.06 \pm 0.30^{*}$	$5.57 \pm 0.28^{**}$
Liver/BW (mg/g)	40.56 ± 2.03	44.92 ± 2.25	$46.77 \pm 2.34^{*}$	$51.52 \pm 2.58^{**}$
Kidney/BW (mg/g)	13.56 ± 0.68	14.52 ± 0.73	$15.23 \pm 0.77^{*}$	$17.38 \pm 0.87^{**}$
Spleen/BW (mg/g)	3.69 ± 0.18	4.05 ± 0.20	$4.45 \pm 0.22^{*}$	$4.97 \pm 0.25^{**}$
Thymus/BW (mg/g)	2.22 ± 0.11	2.79 ± 0.14	$3.08 \pm 0.15^{*}$	$3.26 \pm 0.16^{**}$

Ranks marked with a star or double stars means it is significantly different from the control (no nanoparticulate) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 20$.

3.4. Hematological parameters

Results of hematological study (Table 3) indicate that WBC, RBC, HGB, MCHC, PCT, and Ret of the nanoparticulate TiO_2-treated mice were gradually reduced, while MCV, MHC, RDW, PLT, HCT, MPV of these mice were gradually elevated with increasing doses of nanoparticulate TiO_2. Except for PDW, all the parameters mentioned-above from the higher dose nanoparticulate TiO_2 groups showed significant differences from those of control ($p < 0.05$ or 0.01).

3.5. Parameters of immunologically competent cells

Table 4 exhibits the parameters of immunologically competent cells in mice caused by intragastric administration with different doses of nanoparticulate TiO_2 for consecutive 30 days. According to Table 4, the immunologically competent cells of CD3, CD4, and CD8 caused by 250 mg/kg BW nanoparticulate TiO_2, and B cell and NK cell caused by 62.5, 125, 250 mg/kg BW were significantly lower than those of control ($p < 0.05$ or 0.01). In addition, the ratio of CD4 to CD8 of mice from the 250 mg/kg BW group was significantly decreased compared with that of control ($p < 0.05$).

3.6. IL-2 and NO levels of serum

The nanoparticulate TiO_2-induced IL-2 protein and NO levels of serum were examined after intragastric administration with various doses of nanoparticulate TiO_2 suspensions for consecutive 30 days (Table 5). Nanoparticulate TiO_2 caused significant reduction of IL-2 protein and increase of NO of serum levels in a dose-dependent manner ($p < 0.05$ or 0.01).

The results described-above showed that the reduction of immunologically competent cells was observed in the treated mice. The reduction of immunology is able to induce a decrease of the expression level of IL-2 and an increase of NO in nanoparticulate TiO_2-treated mice.

4. Discussion

Our results showed that the intragastric administration of nanoparticulate anatase TiO_2 of 125 or 250 mg/kg (BW) everyday ("every other day" was described in M&M) for 30 days can significantly decrease the body weight and increase the coefficients of the liver, kidney, spleen and thymus of mice. We observed blur in the large area and congestion in the liver tissue. The damages of liver function was also caused by 250 mg/kg BW nanoparticulate anatase TiO_2, as evidenced by the increased activities of ALT, ALP, AST, LDH, ChE, TP, and the decreased ratio of ALB to GLB, TBIL levels. Wang et al. [14] reported that two weeks after a fixed large dose of 5 g/kg BW of nanoparticulate TiO_2 suspensions (25 and 80 nm) was administrated by a single oral gavage, the coefficient of the liver was significantly increased, while the coefficients of the spleen and kidney changed only a little, and the hydropic degeneration around the central vein was prominent and the spotty necrosis of hepatocyte in the liver tissue to 80 nm and fine TiO_2 particles, but no significant histopathological change in the liver tissues to the 25 nm TiO_2 particles. Liu et al. [15,16] also observed the higher coefficients of the liver, kidney, and spleen of mice, the damage of liver function, inflammatory cascade and histopathological changes of the liver caused by intraperitoneal injection of higher doses (50, 100, and 150 mg/kg BW) of nanoparticulate TiO_2 (5 nm) for 14 days.

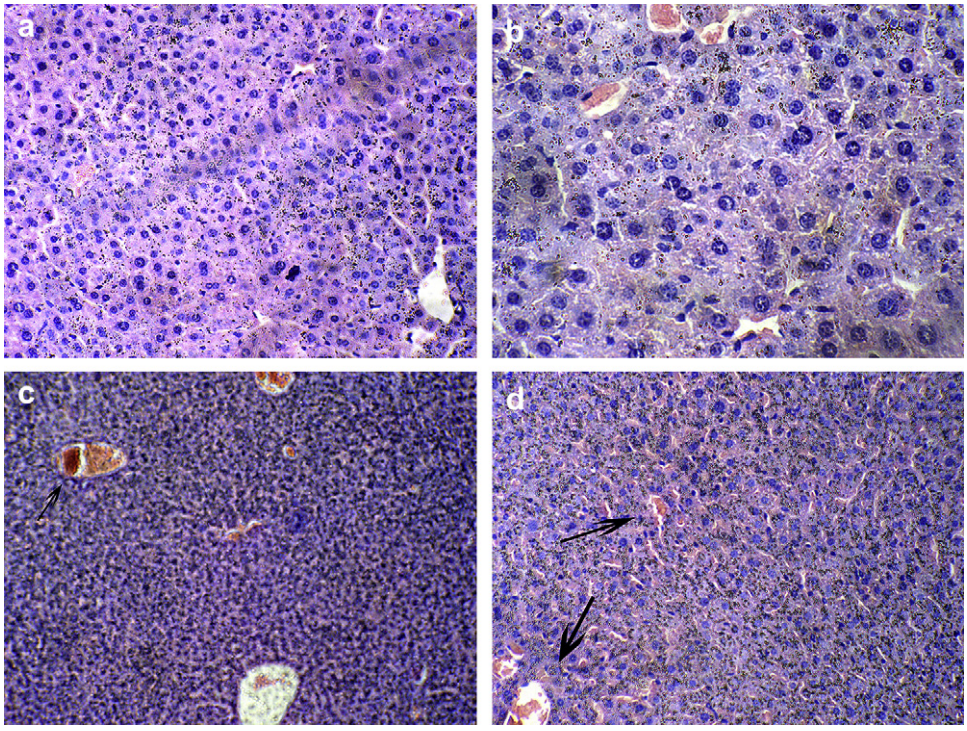

Fig. 1. Histopathology of the liver tissue (100× or 400×) in female mice caused by intragastric administration with nanoparticulate anatase TiO_2 for consecutive 30 days. (a) Control group (100×) indicates that the hepatocytes are integrated and well arranged; (b) 125 mg/kg BW nanoparticulate TiO_2 group (400×) indicates that the portal area and hepatocytes are normal, hepatic sinusoidal is integrated; (c) 250 mg/kg BW nanoparticulate TiO_2 group (100×) indicates that the structure of hepatocytes are blur in the large area, interstitial vessels are congested (arrow); (d) 250 mg/kg BW nanoparticulate TiO_2 group (200×) indicates that the structure of hepatocytes are blur in the large area, interstitial vessels are congested (arrows).

However, the previous reports didn't find the reduction of body weight of mice caused by nanoparticulate TiO_2 [14–16]. The discrepancy between our study and others is most likely attributed to differences in the treatment methods and treatment times. Nevertheless, all studies did demonstrate that nanoparticulate TiO_2 in higher dose had serious toxicity to the mouse liver. The present study indicates that the liver injury of mice is triggered by nanoparticulate anatase TiO_2 reduction of immune response that resulted in disruption of the liver tissue, liver function, and inflammatory cascade.

The liver toxicity and its molecular pathogenesis caused by nanoparticulate TiO_2 have been reported [14–17], but its hematological and cellular pathogenesis is not known. The knowledge of the hematological characteristics is an important tool that can be used as an effective and sensitive index to monitor physiological and pathological changes in animals and humans. In order to evaluate the physiological and pathological status of mice caused by intragastric administration with nanoparticulate TiO_2 for consecutive 30 days, we measured the hematological parameters in mice. The treatment with higher dose nanoparticulate TiO_2 caused a significant increase ($p < 0.05$ or 0.01) in MCV, MHC, RDW, PLT, HCT, MPV, and a significant decrease ($p < 0.05$ or 0.01) in WBC, RBC, HGB, MCHC, PCT, and Ret blood levels, thereby indicating haemostasis blood system damages in mice. For example, the reduction of RBC, and enhancement of MCV, MHC, RDW of mice caused by

Table 2
The changes of biochemical parameters in the blood serum of mice by intragastric administration with nanoparticulate anatase TiO_2 for consecutive 30 days.

Indexes	Nanoparticulate anatase TiO_2 (mg/kg BW)			
	0	62.5	125	250
ALT (U/L)	29 ± 1.45	36 ± l.80	44 ± 2.22*	59 ± 2.95**
AST (U/L)	168 ± 40	149 ± 7.45	201 ± 10.05*	245 ± 12.25**
ALP (U/L)	90 ± 4.50	94 ± 4.70	114 ± 5.70*	133 ± 6.65**
LDH (U/L)	1035 ± 51.75	1182 ± 59.10	1230 ± 61.50*	1795 ± 89.75**
ChE (U/L)	1179 ± 58.95	1223 ± 61.15	1447 ± 72.35*	1681 ± 84.05*
TP (g/L)	51.0 ± 2.55	56.90 ± 2.85	61.32 ± 3.07*	65.91 ± 330*
A/G	1.40	1.47	1.22*	1.13*
TBIL (mmol/L)	1.4 ± 0.07	1.1 ± 0.06	0.8 ± 0.04*	0.5 ± 0.03**
TChol (mmol/L)	2.33 ± 0.12	2.59 ± 0.13	2.95 ± 0.15*	3.13 ± 0.16**
TG (mmol/L)	1.55 ± 0.08	1.99 ± 0.10	2.3 ± 0.12*	2.48 ± 0.12**

Ranks marked with a star or double stars means it is significantly different from the control (no nanoparticulate anatase) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 10$.

Table 3
Hematological parameters in mice by intragastric administration with nanoparticulate anatase TiO_2 for consecutive 30 days.

Indexes	Nanoparticulate anatase TiO_2 (mg/kg BW)			
	0	62.5	125	250
WBC (10^9/L)	4.92 ± 0.25	3.33 ± 0.17*	3.08 ± 0.15*	2.25 ± 0.11**
EBC (10^{12}/L)	9.26 ± 0.46	8.21 ± 0.41	7.53 ± .38*	5.15 ± 0.26**
HGB (g/L)	168 ± 8	148 ± 7	125 ± 6*	105 ± 5**
MCV (fL)	53.15 ± 2.66	55.59 ± 2.78	59.28 ± 2.96*	66.47 ± 3.32**
MCH (pg)	17.6 ± 0.9	18.2 ± 0.9	18.5 ± 0.9	19.0 ± 1.0*
MCHC (g/L)	358 ± 18	346 ± 17	329 ± 16*	306 ± 15**
RDW (%)	15.78 ± 0.79	15.14 ± 0.76	16.46 ± 0.82	19.72 ± 0.99*
PLT (10^9/L)	423 ± 21	482 ± 24	605 ± 30**	701 ± 35**
PCT (%)	39.05 ± 1.95	29.11 ± 1.45	17.08 ± 0.85*	11.01 ± 0.55**
Ret (%)	6.56 ± 0.33	4.79 ± 0.24*	2.06 ± 0.10**	1.18 ± 0.06**
HCT (%)	40.11 ± 2.00	42.05 ± 2.10	45.06 ± 2.25	55 ± 2.75*
PDW (%)	16.33 ± 0.82	18.75 ± 0.94	17.31 ± 0.87	16.77 ± 0.84
MPV (fL)	6.2 ± 0.3	6.6 ± 0.3	7.3 ± 0.4*	8.l ± 0.4**

Ranks marked with a star or double stars means it is significantly different from the control (no nanoparticulate anatase) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 10$.

Table 4
Flow cytometric parameters in mice by intragastric administrate with nanoparticulate aititase TiQ: for consecutive 30 days.

Nanoparticulate (mg/kg BW)	CD3(%)	CD4(%)	CD8(%)	CD4/CD8	B Cell(%)	NK cell(%)
0	54.02 ± 2.70	75.30 ± 3.77	59.40 ± 2.97	1.27 ± 0.06	18.00 ± 0.90	80.72 ± 4.04
625	72.80 ± 3.64	74.16 ± 3.21	61.05 ± 3.05	1.21 ± 0.06	12.20 ± 0.61*	70.80 ± 3.54*
125	56.80 ± 2.84	72.36 ± 3.62	59.25 ± 2.96	1.22 ± 0.06	8.60 ± 0.43**	65.50 ± 3.28**
250	31.62 ± 1.58**	40.54 ± 2.03**	36.5 ± 1.83**	1.11 ± 0.06*	5.30 ± 0.27**	56.10 ± 2.81**

Ranks marked with a star or double stars means it is significantly different from the control (no nanoparticulate anatase) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 10$.

nanoparticulate TiO_2 suggested giant corpuscle anemia, and the increase of PLT and MPV showed a possible effect of nanoparticulate TiO_2 on blood coagulation, causing a severe damage of platelets but improving the metabolic function of the bone marrow. Nanoparticles can affect endothelial function, leading to sequestration of red cells and platelets, a response that could in theory impair circulation and promote thrombosis [23]. Takenaka et al. suggested that nanoparticulate CdO and nanoparticulate Ag particles can enter blood from the rat lung [24,25]. Liu et al. reported that intracheal instillation of nanoparticulate ZnO induced significantly pulmonary inflammation and marked body weight loss accompanied by anemia [26]. The significant decrease of RBC count and HGB concentration caused by higher dose nanoparticulate anatase TiO_2 could cause a marked decrease in O_2 content in the blood, then might decrease metabolism and immune response of mice.

$CD4^+$ cells can induce proliferation and differentiation of other cells, and produce growth factors of T cell or IL-2. And $CD8^+$ cells have cytolysis activity or cytotoxicity, and they can regulate $CD4^+$ cells. Therefore, the level of T lymphocytes can determine the immunological state or central link of immune levels of organisms [27]. Our data suggested that the intragastric administration of higher dose nanoparticulate anatase TiO_2 decreased significantly the proliferation of T lymphocytes (including CD3, CD4, and CD8), B lymphocyte and natural killer (NK) lymphocyte, and the ratio of CD4 to CD8 of mice, and showed a disturbance of cellular immune function and an inhibition of the immune response of mice. The decrease of CD4/CD8 ratio implies that $CD4^+$ cells proliferated less efficiently than $CD8^+$ cells of mice caused by nanoparticulate anatase TiO_2, and immune responses associated with $CD8^+$ cells might be stronger than those associated with $CD4^+$ cells in mice.

Our study showed that the obvious decrease of $CD4^+$ cell proliferation caused by nanoparticulate anatase TiO_2 significantly decreased IL-2 activity of the mouse serum. IL-2 is mainly originated from $CD4^+$ cells. And the inhibition of IL-2 activity can decrease the proliferation of T lymphocytes and the activation of other immunologically competent cells, which can cause the reduction of cellular immune function of mice. To our knowledge, there are no published values for nanomaterial particles on lymphocyte proliferation in animals and humans. The previous research of molecular pathogenesis on liver demonstrated that nanoparticulate anatase TiO_2 could significantly stimulate the mRNA expressions and increase protein levels of several inflammatory cytokines, including NF-κB, MIF, TNF-α, IL-6, IL-1β, CRP, IL-4, and IL-10 [17].

Table 5
The Changes of IL-2 and NO in the blood serum of mice by intragastric administration with nanoparticulate anatase TiO_2 for consecutive 30 days.

Index	Nanoparticulate anatase TiO_2 (mg/kg BW)			
	0	62.5	12.5	2.50
IL-2 (pg/ml)	1.25 ± 6.25	1.08 ± 5.40*	8.3 ± 4.15**	5.7 ± 2.85**
NO (μmol/L)	3.34 ± 0.17	5.58 ± 0.28**	7.21 ± 0.36**	9.66 ± 0.48**

Ranks marked with a star or double stars means it is significantly different from the control (no nanoparticulate anatase) at the 5% or 1% confidence level, respectively. Values represent means ± SE, $n = 10$.

Our data also suggested that NO levels of the mouse serum from nanoparticulate anatase TiO_2-treated mice were higher than that of control ($p < 0.01$), showing that nanoparticulate anatase TiO_2 could induce considerable production of NO in mice. Our recently article reported that nanoparticulate anatase TiO_2 promoted significantly NO release in the mouse brain [28]. NO is closely related to cellular immune function by mediating NK cells to kill YAC-1 lymphocyte tumor [29]. Therefore, we thought that nanoparticulate anatase TiO_2 could promote NO generation, thus inhibited immune activity of NK cells, and caused the reduction of natural killer capacity of NK cells that is first line of defense for antitumor or antivirus immune in mice. The significant reduction of T lymphocytes, B lymphocyte, NK lymphocyte, IL-2 activity, and the NO increase indicated that the damage of liver function may be involved in nanoparticulate anatase TiO_2-induced the reduction of immune response of mice.

5. Conclusion

Our study showed that mice treated with 125 and 250 mg/kg BW nanoparticulate anatase TiO_2 for consecutive 30 days display decreased body weight, increased coefficients of the liver, kidney, spleen and thymus and seriously damaged liver function. We also observed that these nanoparticulate anatase TiO_2-treated mice decrease WBC, RBC, HGB, MCHC, PCT, Ret, and T lymphocytes, the ratio of CD4 to CD8, B lymphocytes, NK lymphocytes, and IL-2 activity, but increase MCV, MHC, RDW, PLT, HCT, MPV, NO. But 62.5 mg/kg BW dose nanoparticulate anatase TiO_2 has little influences on haemostasis blood system and immune response in mice. It is very likely that liver function damage in mice caused by higher nanoparticulate anatase TiO_2 is closely associated with the damage of haemostasis blood system and immune response. However, for humans such exposure/intake (several 10 g per person per day) is impossible to generation in the environment and daily living.

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Appendix

Figures with essential color discrimination. Fig. 1 of this article may be difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.10.003.

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