Susceptibility of Young and Adult Rats to the Oral Toxicity of Titanium Dioxide Nanoparticles

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***T**itanium dioxide nanoparticles (TiO_2 NPs) have potential applications as food additives, but concerns persist about their safety. Children are identified as having the highest exposure and may face the greatest health risks. However, the toxicological sensitivity of TiO_2 NPs in different ages is not clear. Here, a comparative toxicity study of TiO_2 NPs in 3-week (youth) and 8-week (adult) old Sprague-Dawley rats is reported following oral exposure at doses of 0, 10, 50, 200 mg kg^{-1} body weight per day for 30 days. The organ mass and histology, blood biochemistry and redox state, intestinal function, and biodistribution of NPs are characterized. The results show that TiO_2 NPs induce different toxic effects on young and adult rats. The liver edema, heart injuries and non-allergic mast cell activation in stomach tissues are found in young rats. On the other hand, only slight injury in the liver and kidney and decreased intestinal permeability and molybdenum contents are found in adult rats. Furthermore, TiO_2 NP exposure can provoke reductive stress (i.e., increased reduced glutathione (GSH)/oxidized glutathione (GSSG) ratios) in plasmas through enhancing the glucose and GSH levels in young rats or reducing the glutathione peroxidase (GSH-Px) acitivity and GSSG levels in adult rats. These results suggest that different ages may require different biomarkers for identifying and monitoring oral toxicity of nanoparticles.*

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1. Introduction

Nanotechnology is an emerging and rapidly growing field that has raised high expectations for a variety of applications in food and food-related industries, including agriculture, food processing, food packaging and dietary supplements.[1] Then a new type of food, called nanofood, is becoming available worldwide. Nanofood is defined that nanotechnology techniques or tools are used during cultivation, production, processing, or packaging of the food.[2] Whereas developments in nanofood aim to improve food quality and enhance nutrient absorption, consumers and researchers worry about the risk of oral uptake of food-related nanoparticles (NPs).[3,4] Some recent studies have shown that indeed there are reasons to suspect that NPs may display toxicological effects on biological systems.[5–7] As nanofood related products are already commercial,[4] it is becoming more and more

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necessary to better understand the potential negative impacts of the food-related NPs on biological systems.

Titanium dioxide (titania, TiO_2) is the most widely used white pigment because of its brightness and very high refractive index. Approximately 5 million tons of pigmentary TiO_2 are used annually worldwide, mainly in the paints, coatings, plastics, papers, inks, foods, pharmaceuticals, cosmetics, and toothpastes.[8] When used as a food colouring, the food-grade TiO_2 (referred to as E171) is considered as an inert and safe material and has been used in many applications for decades. As a common additive in many foods, TiO_2 is used for whitening and brightening foods, especially for confectionary, white sauces and dressings, and certain powdered foods.[9] It has been estimated that in UK the dietary intake of TiO_2 is 5 mg per person per day.[10] However, with the development of nanotechnology, TiO_2 NPs with numerous novel and useful properties are increasingly manufactured and used to replace the conventional TiO_2 materials. Therefore increased exposure can be expected, which has put TiO_2 NPs under toxicological scrutiny.

So far, most studies have addressed the respiratory consequence by air exposure of TiO_2 NPs. Only a few studies have investigated the health effects of TiO_2 NPs via oral exposure.[11] Wang et al. reported that 5 g kg^{-1} body weight (BW) TiO_2 NPs (25 and 80 nm) with a single oral gavage induced the significant lesions of the liver and kidney in mice.[12] Duan et al. found that the liver function damage observed in mice treated with 125 and 250 mg kg^{-1} BW TiO_2 NPs (5 nm) for 30 days is likely associated with the damage of haemostasis blood system and immune response.[13] As to be noted, children are identified as the highest exposures to TiO_2 through foods, because TiO_2 content of sweets is higher than other food products.[8] The above studies suggested that oral exposure of TiO_2 NPs showed some toxicity and children have more chance to exposed. However, no studies have directly compared the adverse effects of TiO_2 NPs exposure on different age groups.

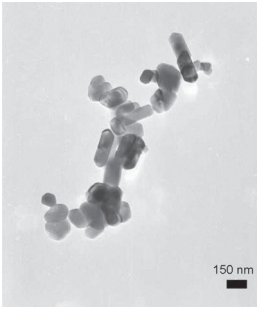
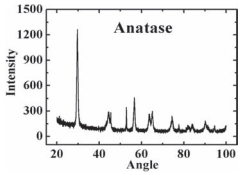
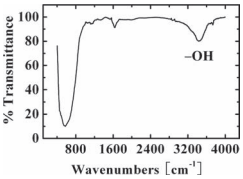
The aim of present study was to know whether young and adult rats show different response after TiO_2 NPs exposure and whether young rats are more susceptible to the adverse effects of TiO_2 NPs. The study was conducted on the healthy Sprague-Dawley male rats of 3-week (youth) and 8-week old (adult) by assessing changes in organ coefficient, histopathology, biochemical parameters, intestinal function, redox state, and biodistribution of titanium after gastrointestinal exposure to TiO_2 NPs for 30 days.

2. Results and Discussion

2.1. Physicochemical Properties of TiO_2 Nanoparticles

It has been demonstrated that the size, surface area, shape, agglomeration, chemical composition, crystal structure, and surface structure of nanomaterials can all strongly affect their biological actions.[14–18] Thus, understanding the physical and chemical properties of nanoparticles (NPs) is necessary to study of biological effects of nanoparticles.[19] As shown in **Table 1**, the TiO_2 NPs were nearly spherical anatase crystals with hydroxyl group on the surface. Its purity was 99.90%. The average size of the TiO_2 NPs was 75 ± 15 nm and the measured Brunauer–Emmett–Teller (BET) specific surface areas of the TiO_2 NPs was 63.95 m^2 g^{-1}.

Table 1. Physicochemical properties of titanium dioxide nanoparticles.

Property	TiO_2 NPs
Shape: TEM image	
Average diameter	[75 ± 15] nm
Specific surface area	63.95 m^2 g^{-1}
Crystal structure	
Surface group	
Purity	99.90%
Hydrodynamic diameter[a)]	
in H_2O	473.6 nm
in artificial gastric juice	1702 nm
in artificial intestinal juice	2081 nm
Zeta potential[a)]	
in H_2O	–33.46 mv
in artificial gastric juice	6.98 mv
in artificial intestinal juice	–2.47 mv

[a)]The particle hydrodynamic diameters and zeta potential was tested in the dose of 1 mg mL^{-1} TiO_2 NPs.

In order to more clearly understand the characteristics of nanoparticles in exposure medium and rat gastrointestinal tract, the hydrodynamic diameter and Zeta potential of TiO_2 NPs in ultrapure water (H_2O), artificial gastric juice (AGJ) and artificial intestinal juice (AIJ) were tested carefully. AGJ (pH = 1.2) and AIJ (pH = 6.8) were prepared according to the methods of Chinese Pharmacopoeia.[20] It is clear that the TiO_2 NPs were converted into larger particles in H_2O, AGJ and AIJ, which is likely due to particle aggregation and the

Table 2. Organ coefficients of rats after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (mean ± SD, $n = 7$).

Group	Exposure dose	Body weight [BW, g]	Liver/BW [mg g^{-1}]	Kidney/BW [mg g^{-1}]	Spleen/BW [mg g^{-1}]	Testicle/BW [mg g^{-1}]	Lung/BW [mg g^{-1}]	Heart/BW [mg g^{-1}]	Brain/BW [mg g^{-1}]
Youth									
Y0	0 mg kg^{-1} BW	347.6 ± 24.9	32.38 ± 2.83	8.17 ± 0.37	2.35 ± 0.28	8.54 ± 0.44	4.28 ± 0.46	3.92 ± 0.25	4.33 ± 0.41
Y1	10 mg kg^{-1} BW	365.0 ± 31.5	32.87 ± 2.78	8.02 ± 0.44	2.62 ± 0.60	8.35 ± 0.81	4.40 ± 0.29	4.16 ± 0.32	4.10 ± 0.40
Y2	50 mg kg^{-1} BW	344.7 ± 23.6	33.54 ± 3.42	7.97 ± 0.63	2.26 ± 0.37	9.49 ± 0.73	4.18 ± 0.46	3.63 ± 0.17 *	4.30 ± 0.32
Y3	200 mg kg^{-1} BW	354.7 ± 26.6	36.35 ± 2.62	7.95 ± 0.70	2.70 ± 0.67	8.95 ± 1.04	3.97 ± 0.30	3.84 ± 0.19	4.15 ± 0.34
Adult									
A0	0 mg kg^{-1} BW	444.0 ± 16.1	28.98 ± 3.59	6.67 ± 0.35	1.91 ± 0.23	7.38 ± 0.30	3.74 ± 0.32	3.51 ± 0.24	3.54 ± 0.19
A1	10 mg kg^{-1} BW	435.0 ± 34.3	29.23 ± 2.01	6.62 ± 0.40	2.08 ± 0.28	7.57 ± 0.87	3.66 ± 0.23	3.54 ± 0.14	3.57 ± 0.29
A2	50 mg kg^{-1} BW	426.3 ± 35.9	26.93 ± 3.29	6.63 ± 0.75	2.22 ± 0.56	7.50 ± 0.38	3.76 ± 0.65	3.48 ± 0.20	3.54 ± 0.23
A3	200 mg kg^{-1} BW	448.3 ± 23.4	26.76 ± 1.67	6.57 ± 0.47	2.31 ± 0.47	7.27 ± 0.74	3.63 ± 0.33	3.41 ± 0.21	3.39 ± 0.32

Significant difference from group Y0 (* $p < 0.05$).

adsorption of biomolecules (Table 1). Comparatively, the TiO_2 NPs have the smallest average hydrodynamic sizes in H_2O. The data of zeta potentials also showed that the TiO_2 NPs were most stable in H_2O. The stability is due to a large negative zeta potential of TiO_2 NPs in H_2O. Contrastively, the TiO_2 NPs were most unstable and have the biggest average hydrodynamic sizes in AIJ. These results suggested that TiO_2 NPs tended to congregate and form larger particles in gastrointestinal tract.

2.2. Oral Exposure to TiO_2 Nanoparticles Induces Liver, Kidney, and Heart Injury in Young and Adult Rats

During administration, the body weight from each animal was increased (Supporting Information, Figure S1). The daily behaviors such as eating, drinking and activity in TiO_2 NPs-treated groups were as normal as the control group. After 30-day treatment, the rats were sacrificed and weighted, various organs were collected and also weighted. **Table 2** shows the coefficients of the organs to the body weight which are expressed as milligrams (wet weight of tissues)/grams (body weight). No obvious differences were found in the body weight among the four groups in young or adult rats. The significant differences were not observed in the coefficients of the liver, kidney, spleen, testis, lung, heart, and brain of the adult rats. But, for the young rats, the coefficients of heart in the 50 mg kg^{-1} BW TiO_2 NPs-treated group is significantly lower than the control group, suggesting that young rats with TiO_2 NPs treatment should be concerned about the injury of heart.

For further investigation of toxicity, the pathological analysis was conducted. The representative micrographs of the organ sections are shown in **Figure 1**. In young rats, liver edema was evident in the 50 and 200 mg kg^{-1} BW TiO_2 NPs-treated groups. Histology showed hepatic cord disarray, perilobular cell swelling, vacuolization, or hydropic degeneration. But this symptom of liver edema did not appear in the adult rats. Only inflammatory cells infiltration was observed in adult rat liver in the 10 and 50 mg kg^{-1} BW TiO_2 NPs-treated groups. No obvious pathological changes were found in the kidney, spleen, testis, lung and heart of the young and adult rats.

The changes of biochemical parameters in the serum were assayed to further evaluate the toxicity of TiO_2 NPs on rats. As shown in **Table 3**, there were no obvious changes for serum biochemical parameters in adult rats after TiO_2 NPs exposure except the serum total bilirubin (TBIL) and blood urea nitrogen (BUN) levels. The low TBIL levels and high BUN contents showed that TiO_2 NPs in higher doses may cause slight injury in the liver and kidney of adult rats, respectively. On the contrary, in the higher dose of TiO_2 NPs-treated groups (50 and 200 mg kg^{-1} BW) of young rats, the levels of blood glucose (Glu), low density lipoprotein cholesterol (LDL-C), the ratio of alanine aminotransferase (ALT) to aspartate aminotransferase (AST) (ALT/AST) and TBIL were significantly higher than those of the control group, and the activities of AST, alpha-hydroxybutyrate dehydrogenase (HBDH) and creatine kinase (CK) were significantly reduced. The increased TBIL, ALT/AST ratio and decreased activities of AST demonstrated that TiO_2 NPs induced hepatic injury in young rats. The elevated levels of Glu and LDL-C implied that TiO_2 NPs caused metabolism imbalance of glycolipid in the rat liver. It is well known that the high Glu and LDL-C levels are associated with an increased risk in heart disease.[21–23] The decreased activities of HBDH and CK also indicate that the heart function might be injured after exposure to TiO_2 NPs. However, there were no significant changes for BUN and creatinine (Crea) levels in serum after oral administration of TiO_2 NPs in young rats. These results displayed that TiO_2 NPs in higher doses can induce damage in the liver and heart of young rats, but no changes were observed in the kidney of young rats.

Taking into account the present findings, TiO_2 NPs induced liver and heart injuries in young rats, but slight injury in the liver and kidney of adult rat. Corresponded to the previous studies,[12,13] the liver is the most sensitive target organ for TiO_2 NPs toxicity by the oral routes. Furthermore, the

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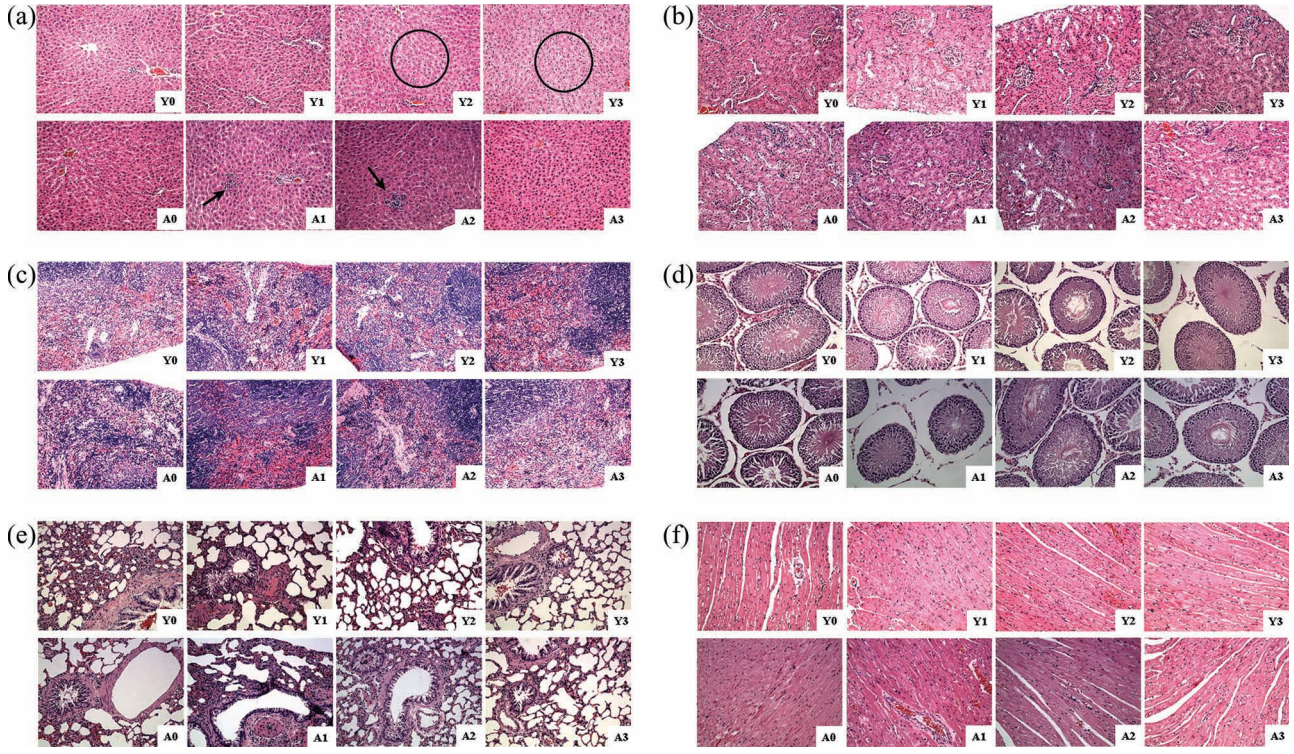

Figure 1. The representative histological photomicrographs of the liver (a), kidney (b), spleen (c), testis (d), lung (e), and heart (f) in young and adult rats after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (objective: 20×). Three rats in each group were used for histological examination ($n = 3$). Circles indicate the liver edema in young rats. Arrows indicate inflammatory cells infiltration in adult rat liver. No pathological changes were found in kidney, spleen, testis, lung and heart tissues. Y0-Y3: young rats, A0-A3: adult rats, Y0 & A0: control group (0 mg kg^{-1} BW), Y1 & A1: low-dose exposure group (10 mg kg^{-1} BW), Y2 & A2: middle-dose exposure group (50 mg kg^{-1} BW), Y3 & A3: high-dose exposure group (200 mg kg^{-1} BW).

toxic effects were more severe in young rather than adult animals. Besides liver, an increased risk of cardiac injury in youth should be carefully considered after TiO_2 NPs exposure.

2.3. Oral Exposure to TiO_2 Nanoparticles Induces Gastrointestinal Function Change in Young and Adult Rats

All materials given orally are in close contact with the gastrointestinal tract. In this study, the effects of TiO_2 NPs on gastrointestinal tract were evaluated. Histological studies of the young and adult rats pointed out that TiO_2 NPs expouse did not lead to observed pathological changes in stomach and small intestine (Supporting Information, Figure S2). However, the decreased levels of D-lactate and the lowered activity of diamine oxidase (DAO) in plasma presented a low level of permeability of intestinal barrier in the TiO_2 NPs-treated groups, especially in the adult rats (**Figure 2**a,b). Under normal conditions, the intestinal barrier allows nutrients to pass into the body while protecting against infectious agents, allergens, and other harmful substances.[24] The reduced intestinal permeability indicated that intestinal barrier function was upregulated after TiO_2 NPs challenge.

On the other hand, decreased intestinal permeability can be the cause of poor absorption and lead to malnutrition, even with a normal amount of food intake. Mahler et al. reported that acute oral exposure of chicken to polystyrene nanoparticles can disrupt iron transport and chronic exposure can cause remodelling of the intestinal villi, which increased the surface area available for iron absorption.[25] It is suggested that oral exposure to nanoparticles can affect nutrient absorption. Thus, the influence of TiO_2 NPs on elemental contents such as titanium (Ti), molybdenum (Mo), cobalt (Co), strontium (Sr), manganese (Mn), rubidium (Rb), iron (Fe), copper (Cu), zinc (Zn), calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and phosphorus (P) in the rat blood was evaluted. The results exhibited no obvious differences in elemental content except a significant decrease in the content of Mo in adult rats (Figure 2c). Whether the low Mo absorption was related to the decreased intestinal permeability and if other biological events could happen due to the upregulated barrier function need further investigation.

Besides intestinal permeability, the mast cell activation was studied to evaluate the adverse effects of TiO_2 NPs on the gastrointestinal function. As one of the most important members of immune system in gastrointestinal tract, the mast cells are regarded as key effector and immunoregulatory cells in IgE-associated allergic disorders and certain innate and adaptive immune response.[26] It plays a crucial role in the maintaining of gastrointestinal function. Recently, some studies have suggested that nanoparticles can potentiate allergic responses. Classical examples of allergies indicate

Table 3. Biochemistry assay of serums in the rats after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (mean ± SD, $n = 7$).

Group	Exposure dose	Glu [mmol L^{-1}]	TCHO [mmol L^{-1}]	TG [mmol L^{-1}]	HDL-C [mmol L^{-1}]	LDL-C [mmol L^{-1}]	TP [g L^{-1}]	ALB [g L^{-1}]	GLB [g L^{-1}]	ALB/GLB
Youth										
Y0	0 mg kg^{-1} BW	5.04 ± 0.98	1.59 ± 0.22	0.82 ± 0.29	0.60 ± 0.07	0.23 ± 0.03	70.86 ± 2.85	36.51 ± 1.29	34.34 ± 1.74	1.06 ± 0.04
Y1	10 mg kg^{-1} BW	5.20 ± 1.18	1.76 ± 0.27	1.04 ± 0.37	0.61 ± 0.07	0.29 ± 0.04	70.43 ± 1.40	35.60 ± 1.14	34.83 ± 0.39	1.02 ± 0.03
Y2	50 mg kg^{-1} BW	6.61 ± 0.73 **	1.91 ± 0.30	0.59 ± 0.13	0.60 ± 0.07	0.43 ± 0.09 **	71.57 ± 1.62	36.54 ± 0.98	35.03 ± 1.38	1.04 ± 0.05
Y3	200 mg kg^{-1} BW	6.44 ± 0.36 **	1.69 ± 0.36	0.63 ± 0.13	0.58 ± 0.08	0.36 ± 0.05 **	69.86 ± 2.79	35.99 ± 1.27	33.87 ± 1.75	1.06 ± 0.04
Adult										
A0	0 mg kg^{-1} BW	5.73 ± 1.57	1.65 ± 0.35	0.84 ± 0.30	0.62 ± 0.10	0.26 ± 0.06	70.43 ± 2.70	35.66 ± 1.35	34.77 ± 1.71	1.03 ± 0.04
A1	10 mg kg^{-1} BW	5.13 ± 1.09	1.72 ± 0.25	0.72 ± 0.20	0.67 ± 0.06	0.27 ± 0.06	70.71 ± 3.64	35.34 ± 1.67	35.37 ± 2.23	1.00 ± 0.04
A2	50 mg kg^{-1} BW	4.88 ± 0.88	1.83 ± 0.37	0.52 ± 0.11	0.64 ± 0.07	0.29 ± 0.05	72.17 ± 2.23	35.67 ± 1.03	36.50 ± 2.35	0.98 ± 0.08
A3	200 mg kg^{-1} BW	5.06 ± 0.36	1.61 ± 0.28	0.82 ± 0.37	0.59 ± 0.07	0.24 ± 0.05	68.86 ± 2.54	34.54 ± 1.58	34.31 ± 1.60	1.01 ± 0.06

Group	Exposure dose	ALT [U L^{-1}]	AST [U L^{-1}]	ALT/AST	TBIL [μmol L^{-1}]	LDH [U L^{-1}]	HBDH [U L^{-1}]	CK [U L^{-1}]	BUN [mmol L^{-1}]	Crea [μmol L^{-1}]
Youth										
Y0	0 mg kg^{-1} BW	47.57 ± 4.83	209.14 ± 31.91	0.23 ± 0.04	1.27 ± 0.21	1852.86 ± 467.30	890.43 ± 344.28	2792.00 ± 294.97	6.93 ± 1.08	52.57 ± 5.32
Y1	10 mg kg^{-1} BW	44.43 ± 7.68	179.71 ± 36.59	0.25 ± 0.06	1.23 ± 0.19	1731.29 ± 306.55	770.71 ± 206.29	2083.29 ± 622.24	7.13 ± 1.26	52.29 ± 3.73
Y2	50 mg kg^{-1} BW	54.43 ± 7.09	152.14 ± 28.20 **	0.37 ± 0.07 **	1.47 ± 0.18	1313.86 ± 346.95	510.86 ± 168.34 **	2154.43 ± 914.55	6.49 ± 1.10	52.86 ± 3.07
Y3	200 mg kg^{-1} BW	48.86 ± 6.39	157.57 ± 31.83 **	0.32 ± 0.06 *	2.11 ± 0.30 **	1499.43 ± 383.68	617.28 ± 222.54 *	1658.00 ± 685.07 *	6.44 ± 1.11	49.71 ± 2.75
Adult										
A0	0 mg kg^{-1} BW	52.43 ± 6.16	170.43 ± 38.31	0.32 ± 0.07	1.81 ± 0.13	1548.86 ± 393.39	692.86 ± 328.89	2045.86 ± 520.32	5.60 ± 0.91	53.43 ± 3.10
A1	10 mg kg^{-1} BW	47.43 ± 5.65	178.57 ± 54.36	0.28 ± 0.05	2.09 ± 0.41	1753.71 ± 545.80	835.71 ± 439.23	2176.86 ± 777.33	6.43 ± 0.94	53.00 ± 8.74
A2	50 mg kg^{-1} BW	46.00 ± 5.66	167.83 ± 31.24	0.29 ± 0.08	1.90 ± 0.29	1560.33 ± 336.45	690.83 ± 232.51	1789.17 ± 459.89	8.42 ± 1.96 ++	54.33 ± 5.96
A3	200 mg kg^{-1} BW	51.57 ± 9.05	177.00 ± 19.21	0.29 ± 0.03	1.37 ± 0.50 +	1647.29 ± 44.32	709.29 ± 27.40	2041.86 ± 337.52	7.26 ± 0.61 +	50.29 ± 4.57

Significant difference from group Y0 (* $p < 0.05$, ** $p < 0.01$); Significant difference from group A0 (+ $p < 0.05$, ++ $p < 0.01$).

that people with asthma and rhinitis are more susceptible to the short term acute effects of particle exposure.[27,28] Results from animal models demonstrated that lung exposure to TiO_2 NPs can modulate asthmatic responses by aggravating pulmonary inflammation and airway hyper-responsiveness,[29] and transdermal exposure to TiO_2 NPs can exacerbate atopic dermatitis symptoms by elevating proinflammatory molecules in the skin and increasing serum levels of IgE and histamine.[30] Moreover, Chen et al reported that a mixture of anatase and rutile TiO_2 NPs can directly stimulate histamine release from RBL-2H3 mast cells without allergen sensitization.[31] These finding sparked us to further study whether oral-exposed TiO_2 NPs can directly lead to allergic reactions and histamine release from mast cells in healthy animals.

It was seen that TiO_2 NPs exposure induced an increase in the number of mast cells in stomach tissues, but no significant changes were found in the small intestine (**Figure 3**a–d). Especially in young rats, the number of mast cells in stomach tissues showed a dramatic enhancement in the 200 mg kg^{-1} BW TiO_2 NPs-treated group compared with the control. However, the levels of histamine and IgE in serum were not elevated after TiO_2 NPs exposure (Figure 3e,f). The results indicated that TiO_2 NPs exposure induced IgE-independent activation of mast cells, but did not trigger histamine release and allergic inflammation in healthy rats. The age of rats might affect the response of gastrointestinal tract to ingested TiO_2 NPs, which presented mast cell activation in young rats, but intestinal permeability decrease in adult rats.

2.4. Oral Exposure to TiO_2 Nanoparticles Induces the Redox State Change in Young and Adult Rats

The redox status, which represents the reduction potential or reducing capacity in biological systems, plays a critical role in

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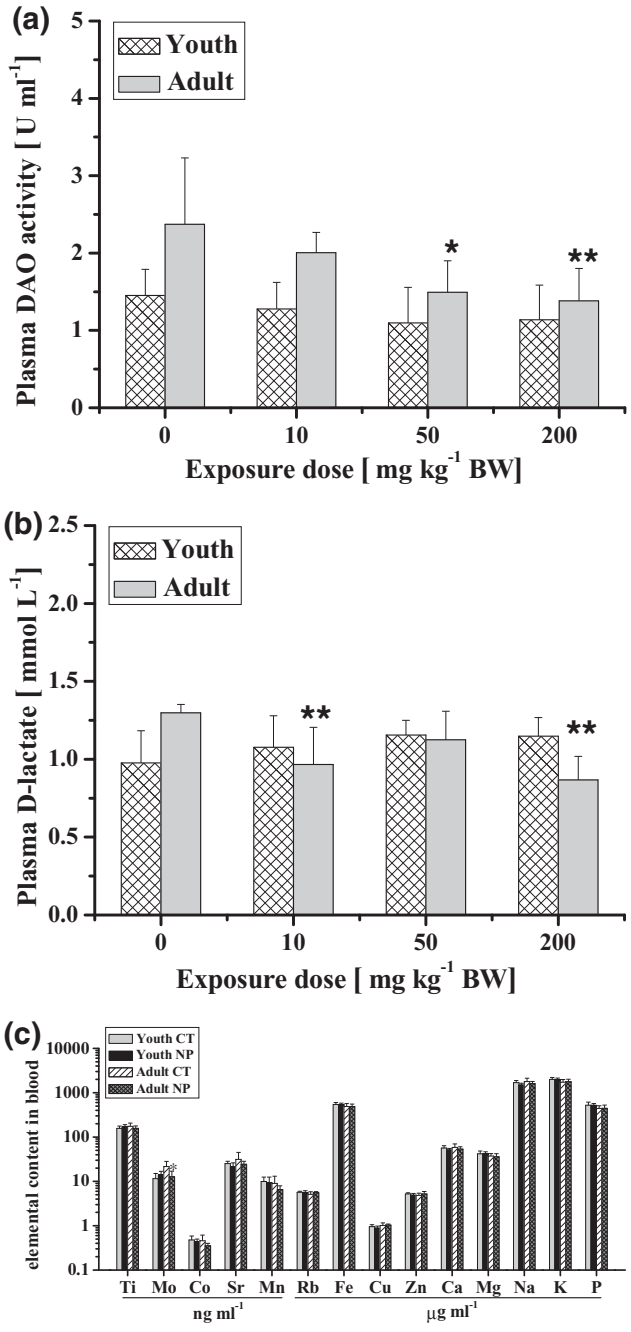

Figure 2. Change of intestinal permeability in young and adult rats after TiO_2 nanoparticle exposure. The activity of diamine oxidase (DAO) (a) and level of D-lactate (b) in rat plasma after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (mean ± SD, $n = 6$). The elemental contents in the rat blood (c) after gastrointestinal exposure to 200 mg kg^{-1} BW TiO_2 nanoparticles for 30 days (mean ± SD, $n = 6$). Significant difference between exposure and control groups in young or adult rats (* $p<0.05$, ** $p<0.01$). CT: control group (0 mg kg^{-1} BW), NP: TiO_2 nanoparticle exposure group (200 mg kg^{-1} BW).

regulation of homeostasis of organism. Disorder of the redox status is related with the onset and/or propagation of damage from oxidative or reductive stress in biological systems. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) is well recognized as a reliable index to evaluate the status of redox.[32] In present study, TiO_2 NPs exposure significantly increased plasma GSH/GSSG ratios in both young and adult rats (**Figure 4**a), which indicated that the animals were under reductive stress.

However, the reductive stress in the plasmas of young and adult rats was provoked through different pathway. As shown in Figure 4b,c, the elevated plasma GSH/GSSG ratios attributed to the higher levels of GSH in young rats, but attributed to the reduced GSSG levels in adult rats. Furthermore, the decreased activities of glutathione peroxidase (GSH-Px) in TiO_2 NPs-exposed group were responsible for the low GSSG levels and high GSH/GSSG ratios in adult rats (Figure 4d). On the contrary, the activities of GSH-Px in TiO_2 NPs-exposed group of young rats were obviously increased with the high GSH contents and GSH/GSSG ratios. As shown in Figure 4e, the elevated levels of glucose were found in TiO_2 NPs-exposed group of young rats. The higher GSH/GSSG ratios in young rats were associated with higher glucose levels. It is demonstrated that the glucose levels have a great impact on cellular redox status.[33] The glucose can accelerate the glutathione reductase (GSSG-R) reaction by supplying NADPH, leading to higher GSH concentration and a higher GSH/GSSG ratio (Figure 4f).[34] It is suggested that the glucose levels were critical in regulating redox state in young rats under TiO_2 NPs exposure. The plasma GSH/GSSG ratios are increased during an elevation of the glucose concentration.

Similar to oxidative stress, reductive stress has also been shown to have a deleterious effect on organism. Some studies indicated that the reductive stress linked to heart disease and the excessive levels of GSH could harm the heart.[35,36] In this work, the cardiac injury, including the significant change of the coefficients of heart and serum levels of Glu, LDL-C, HBDH and CK, was corresponded to high plasma GSH content and GSH/GSSG ratio in young rats. However, previous studies mostly focus on the nanoparticles-induced oxidative stress (i.e. decreased GSH/GSSG ratios) and toxicity, which always found after nanoparticles enter the body and direct contact with tissues and cells.[6] This finding, for the first time, links reductive stress to the toxicity of nanoparticles. The low contents of titanium in the blood (**Figure 5**d) indicated that the TiO_2 NPs can not direct contact with the blood, which might be one of the causes inducing reductive stress but not oxidative stress. To generalize the influence of TiO_2 NPs on redox state and explain the mechanism of reductive stress induced by TiO_2 NPs, further investigations are still required.

2.5. Biodistribution of TiO_2 Nanoparticles in Young and Adult Rats after Oral Exposure

The TEM images clearly showed that some particles with a diameter of approximately 60–200 nm were adhered to the intestinal villi and absorbed in the stomach and small intestine tissue (Figure 5a,b). Although TiO_2 NPs exposure resulted in hydropic swelling and vacuolar degeneration of the liver cells in young rats and vacuolar degeneration of the liver cells in adult rats, no particles were found in the liver of young and adult rats(Figure 5c). The contents

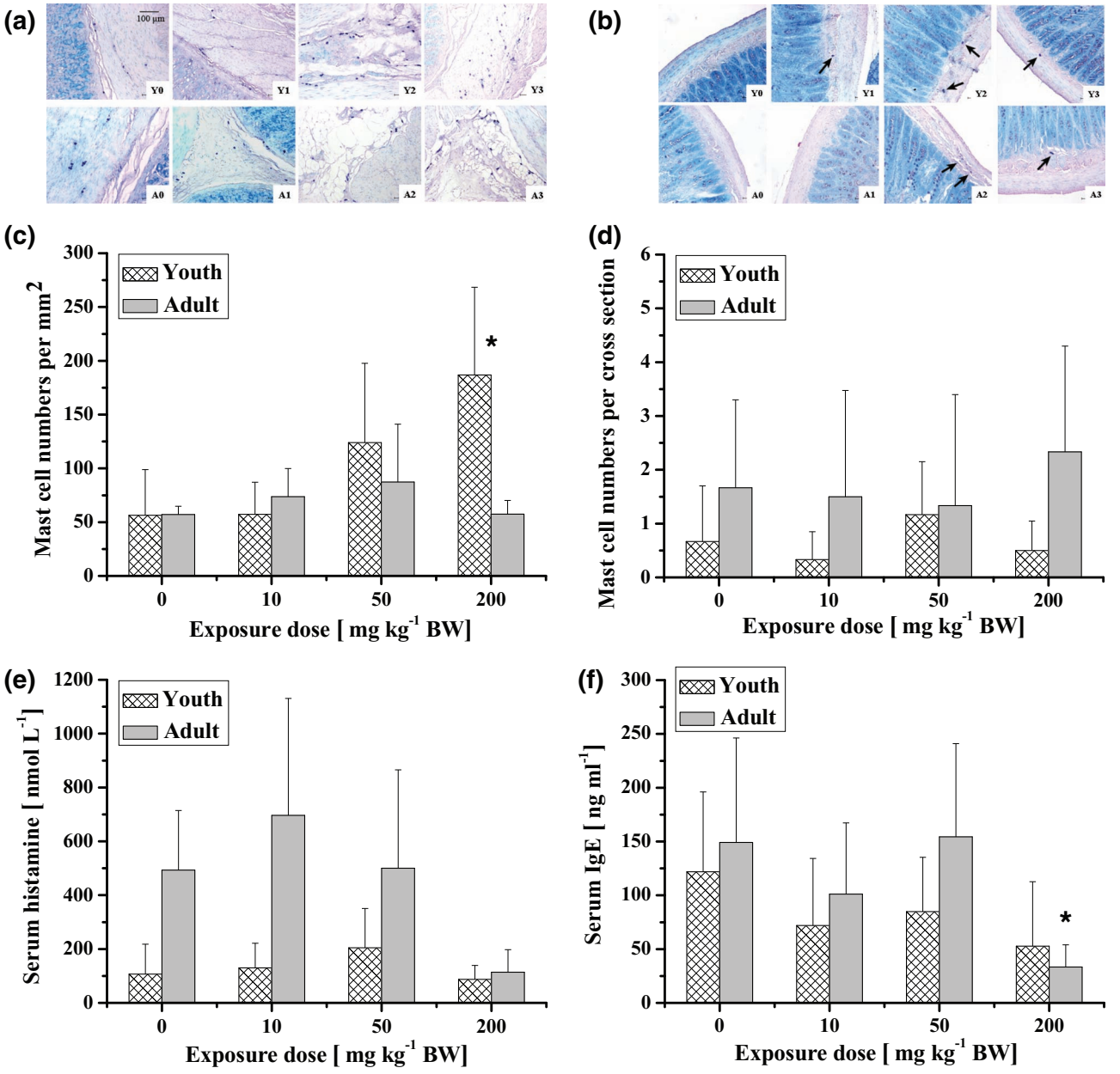

Figure 3. The activation of mast cells in gastrointestinal tract of young and adult rats after TiO_2 nanoparticle exposure. Photomicrographs of mast cell in rat stomach (a) and small intestine (b) after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (objective: 20×). Three rats in each group were used for histological examination (n = 3). The number of mast cell in rat stomach (c) and small intestine (d) after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (mean ± SD, n = 3). The level of histamine (e) and IgE (f) in rat serum after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (mean ± SD, n = 6). Significant difference between exposure and control groups in young or adult rats (* $p<0.05$, ** $p<0.01$). Y0-Y3: young rats, A0-A3: adult rats, Y0 & A0: control group (0 mg kg^{-1} BW), Y1 & A1: low-dose exposure group (10 mg kg^{-1} BW), Y2 & A2: middle-dose exposure group (50 mg kg^{-1} BW), Y3 & A3: high-dose exposure group (200 mg kg^{-1} BW).

of titanium in the blood, liver, kidney and spleen of young and adult rats in the TiO_2 NPs-treated groups were not significantly different to the control group after oral exposure to 200 mg kg^{-1} BW TiO_2 NPs for 30 days (Figure 5d). These results displayed that the rate and extent of absorption of TiO_2 NPs from the gastrointestinal tract is very low, and these nanoparticles located in the mucosa of stomach and small intestine did not translocate into the systemic circulation.

It is generally agreed that absorption of nanoparticles by the oral route increases with decreasing particle diameter.[37] Based on the high hydrodynamic sizes of TiO_2 NPs in artificial gastric juice (AGJ) and artificial intestinal juice (AIJ), the TiO_2 NPs quickly aggregated into larger particles (1702~2081 nm) in gastrointestinal tract, which may contribute to the low absorption and translocation rates.[38] Furthermore, the low level of permeability of intestinal barrier could lead to low efficiency of the translocation of TiO_2 NPs.

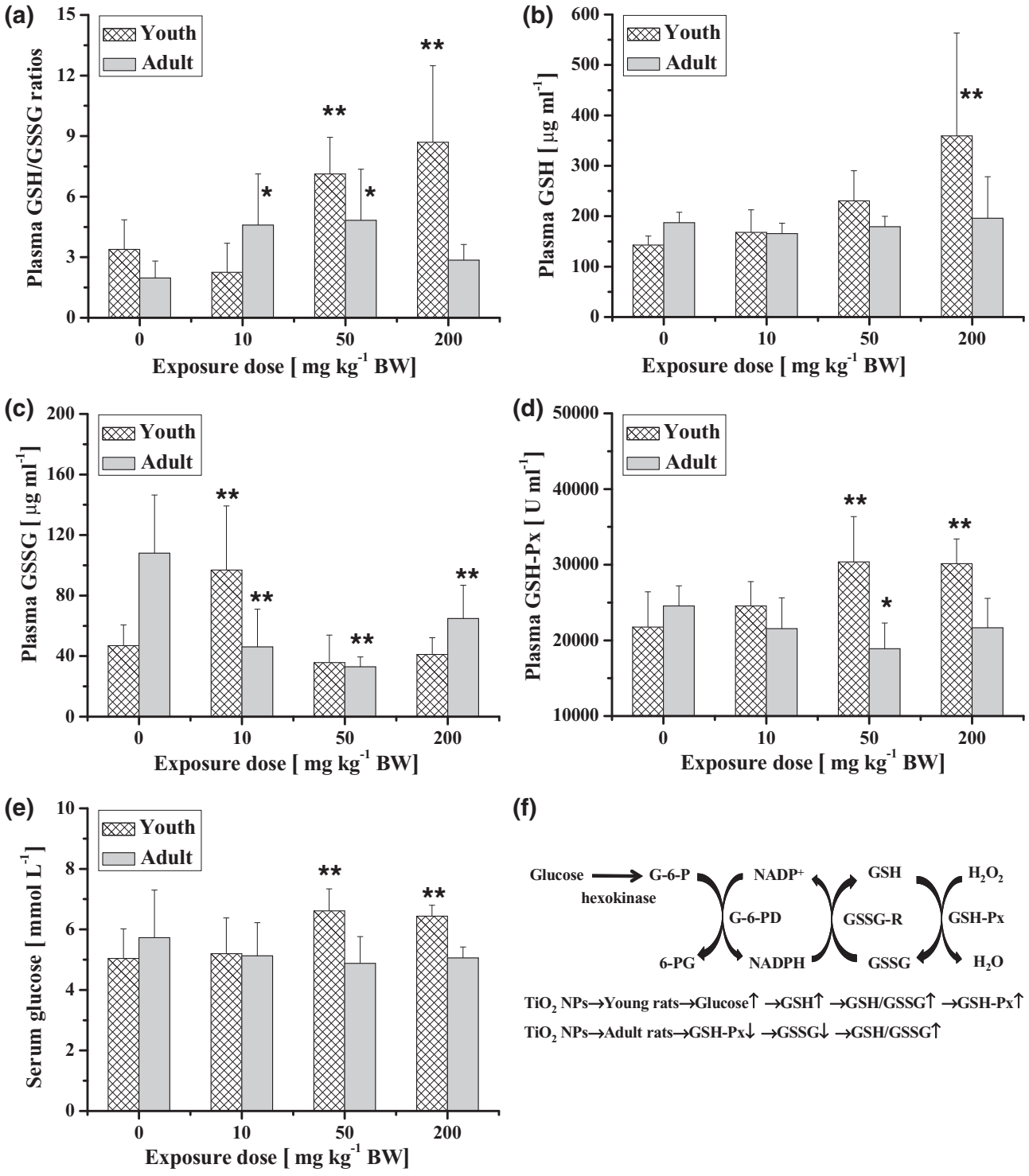

Figure 4. Change in the redox state in rat blood after TiO_2 nanoparticle exposure. The reduced glutathione/oxidized glutathione (GSH/GSSG) ratios (a), GSH (b) and GSSG (c) contens, activity of glutathione peroxidase (GSH-Px) (d) and glucose contens (e) in the rat blood after gastrointestinal exposure to TiO_2 nanoparticles for 30 days (mean ± SD, $n = 7$). The relationships among glucose, GSH, GSSG, and GSH-Px (f) in young and adult rats after TiO_2 nanoparticle exposure. Significant difference from control group (0 mg kg^{-1} BW) in young or adult rats (* $p < 0.05$, ** $p < 0.01$). G-6-PD: glucose-6-phosphate dehydrogenase, G-6-P: glucose-6-phosphate, 6-PG: 6-phosphogluconate, GSSG-R: glutathione reductase.

3. Conclusion

The young rats seem to be more susceptible to nanoparticle exposure than the adult rats. For the young rats, the liver and heart injuries and non-allergic mast cell activation in stomach tissues were induced by the high dose of TiO_2 NPs. On the other hand, only slight injury in the liver and kidney and decreased intestinal permeability and blood Mo contents

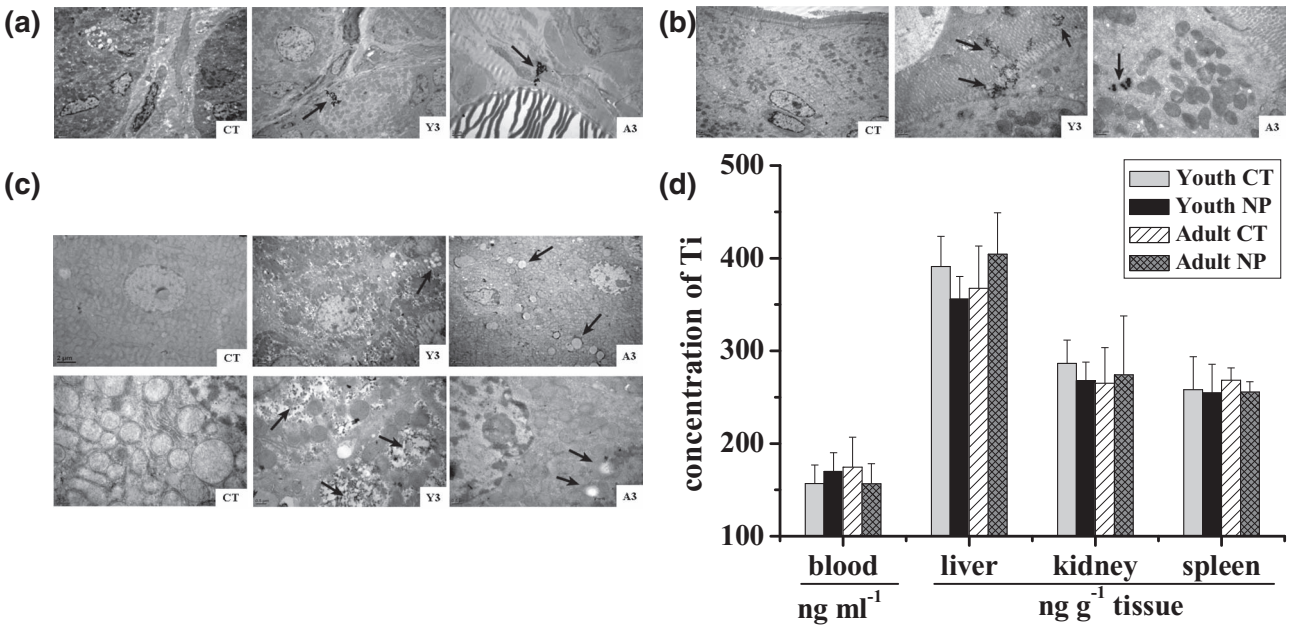

Figure 5. The TEM images of the mucosa of the stomach (a) and small intestine (b) and the liver (c) in rats after gastrointestinal exposure to 200 mg kg^{-1} BW TiO_2 nanoparticles for 30 days. The titanium (Ti) elemental contents (d) in the rat blood, liver, kidney and spleen after gastrointestinal exposure to 200 mg kg^{-1} BW TiO_2 nanoparticles for 30 days (mean ± SD, $n = 6$). CT: control group (0 mg kg^{-1} BW), NP: TiO_2 nanoparticle exposure group (200 mg kg^{-1} BW). Y3: high-dose exposure group (200 mg kg^{-1} BW) in young rats. A3: high-dose exposure group (200 mg kg^{-1} BW) in adult rats.

were found in adult rats. Therefore, our study provides sound evidence that it is necessary to consider age difference when we set up the recommended daily intake of TiO_2 NPs in food.

Moreover, present study shows that TiO_2 NPs exposure can provoke reductive stress (i.e. increased GSH/GSSG ratios) in the plasmas of young and adult rats through different mechanism, which differs from previous studies. The NPs-induced high glucose level is related to a high GSH conten and GSH/GSSG ratio in blood, which might link to heart injury in the young rats. It is clear that children will eat sugar more frequently than adult, and the TiO_2 content of sweets is higher than other food products.[7] Considering the complex relationships among TiO_2 NPs, glucose and heart injury, it is necessary to control the dietary TiO_2 NPs intake for children. Further study is needed to understand the synergetic effect of TiO_2 NPs and glucose on child and adolescent health.

4. Experimental Section

Nanoparticle Characterization: The titanium dioxide nanoparticles (TiO_2 NPs) were purchased from Shanghai Aladdin Reagent Co. Ltd, China. The size and shape of the particles was characterized by transmission electron microscopy (TEM, JEOL JEM-200CX). The purity of the particles was analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, IRIS Advantag, TJA, USA). The crystal structure of the particles was identified by X-ray powder diffractometry (XRD, PANalytical's X'Pert PRO, X'Celerator). The surface functional group of the particles was determined using a fourier transform infrared spectrometer (FTIR, Nexus 470, Thermo Nicolet, USA). The specific surface area (SSA) of the particles was measured according to Brunauer–Emmett–Teller (BET) method (Quantachrome, Autosorb 1, Boynton, FL, USA).

The method of preparing artificial gastric juice (AGJ) and artificial intestinal juice (AIJ) was according to the 2010 edition of Chinese Pharmacopoeia.[20] The artificial gastric juice (AGJ, pH = 1.2) was prepared using 10 g L^{-1} pepsin (3800 units mg^{-1}) and 45 mmol L^{-1} HCl. The artificial intestinal juice (AIJ, pH = 6.8) was made with 10 g L^{-1} trypsin (2500 units mg^{-1}) and 6.8 g L^{-1} KH_2PO_4. The pH was adjusted to 6.8 using 0.1 mol L^{-1} NaOH. After the TiO_2 NPs were dispersed in ultrapure water (H_2O), AGJ or AIJ to obtain a final concentration of 1 mg mL^{-1}, the suspensions were supersonicated for 15 min to break up aggregates. The particle hydrodynamic diameters and Zeta potentials were tested using the ZetaSizer Nano ZS90 (Malvern Instruments Ltd, Malvern, UK).

Animal and Experimental Design: The healthy Sprague-Dawley male rats of 3-week old and 8-week old represent young and adult animals respectively, were supplied and bred by the Department of Laboratory Animal Science, Peking University Health Science Center. The rats were fed a commercial pellet diet and deionized water *ad libitum*, and kept in plastic cages in a 20 ± 2 °C and 50–70% relative humidity room with a 12:12 h light-dark cycle. After one week accommodation, 28 young rats and 28 adult rats were randomly divided into eight groups (each 7 rats): Y0 (young control group), Y1 (young low-dose exposure group), Y2 (young middle-dose exposure group), Y3 (young high-dose exposure group), A0 (adult control group), A1 (adult low-dose exposure group), A2 (adult middle-dose exposure group) and A3 (adult high-dose exposure group). The animal experiments were carried out in accordance with the Guiding Principles in the Use of Animals in Toxicology adopted by Society of Toxicology.

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The TiO_2 NPs were dispersed in ultrapure water and ultrasonic vibrated for 15 min. In order to obtain homogenized suspension, the particle dispersion solution was stirred on vortex agitator before every use. The intragastric doses were selected based on the intake of dietary TiO_2 particles in the UK, which has been estimated to be about 5 mg person^{-1} day^{-1},[9] equivalent to approximately 0.1 mg kg^{-1} body weight (BW) per day. In this study, the 100 times dose of the potential human exposure (10 mg kg^{-1} BW) was used as the low-dose TiO_2 NPs exposure in rats. The young and adult rats were intragastrically administrated with 0, 10, 50 and 200 mg kg^{-1} BW TiO_2 NPs, respectively, once a day for 30 consecutive days. The symptom and mortality were observed and recorded carefully every day for 30 days (death not observed). The body weight of rats was tested every 4–6 days. During the experiments, no significant changes in the body weight of the exposed young or adult rats were found (supplemental data, Figure S1). After 30 days, all animals were weighed and sacrificed. The blood samples were collected from the femoral artery in the groin area. Serum was harvested by centrifuging blood at 3000 rpm (1500 g) for 10 min. The tissues and organs such as liver, kidney, spleen, testis, lung, heart, brain, stomach and small intestine were excised and weighed.

Coefficients of Organs: After weighing the body and tissues, the coefficients of the liver, kidney, spleen, testis, lung, heart, brain to the body weight were calculated as the ratio of tissues (wet weight, mg) to body weight (g).

Blood Biochemical Assay: Liver function was evaluated with serum levels of total protein (TP), albumin (ALB), globulin (GLB), ALB/GLB ratio, alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALT/AST ratio, and total bilirubin (TBIL). Nephrotoxicity was determined by blood urea nitrogen (BUN) and creatinine (Crea). The glycolipid metabolism was judged by the levels of fasting blood glucose (Glu), total cholesterol (TCHO), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C). The enzymes of creatine kinase (CK), lactate dehydrogenase (LDH) and alpha-hydroxybutyrate dehydrogenase (HBDH) were assayed for evaluating cardiac damage. All biochemical assays were performed using a clinical automatic chemistry analyzer (Type 7170A, Hitachi, Japan).

Histological Examination: 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 (Olympus BX50, Moticam 2306, Japan). The identity and analysis of the pathology slides were blind to the pathologist.

The mast cells (MC) in the stomach and small intestine tissues were identified histochemically by using a toluidine blue staining method. Intact or partially degranulated mast cells were counted using Nikon Eclipse E400 microscope and at least five randomly selected visual fields were examined in each section (eyepiece 10×, objective 20×). The mast cell density was calculated and recorded as mast cell numbers per square millimeter of tissue.

Biomarker Assay: The concentrations of histamine and IgE in serum were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (rat histamine, SPI-Bio, France; rat IgE, GenWay Biotech, USA).

Plasma was harvested via centrifugation of heparin-anticoagulated whole blood at 3000 rpm (1500 g) for 10 min. The levels of D-lactate and the activity of diamine oxidase (DAO) in plasma were detected to evaluate the permeability and intergrity of intestinal barrier. The D-lactate levels was measured using the colorimetric method according to the manufacturer's protocol (D-lactate Assay Kit, BioVision, USA). The activity of diamine oxidase (DAO) was determined by the reaction of cadaverine dihydrochloride (Sigma, USA) as the same method described in reference lecture.[39]

For redox state assay, the levels of reduced (GSH) and oxidized (GSSG) glutathione were measured using *o*-phthalaldehyde (OPT, Sigma, USA) as fluorescent reagent according to the method of Hissin and Hilf.[40] The GSH/GSSG ratio were calculated from the GSH and GSSG concentrations. The activity of glutathione peroxidase (GSH-Px) were tested using commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Elemental Content Analysis: The heparin-anticoagulant blood and liver, kidney, spleen tissues were taken out and thawed. About 0.1–0.3 g of each tissue were weighed and 1 mL of whole blood were taken accurately. All tissues and blood samples were digested and analyzed for elemental content. Briefly, prior to elemental analysis, the tissues and blood samples 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, Elan DRC II, PerkinElmer, USA) and inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP6000, Thermo Electron Corp., USA) was used to analyze the titanium (Ti), molybdenum (Mo), cobalt (Co), strontium (Sr), manganese (Mn), rubidium (Rb), iron (Fe), copper (Cu), zinc (Zn), calcium (Ca), magnesium (Mg), sodium (Na), potassium (K) and phosphorus (P) concentration in the samples.

TEM Observation: The liver and the mucosa of the stomach and small intestine were cut up into small pieces (1 mm^3) and immediately fixed in 2.5% glutaraldehyde (pH 7.4) overnight. Then the samples were treated according to the general protocols for TEM study. The ultra-thin sections (70–100 nm) were stained with lead citrate and uranyl acetate. The specimens were examined using JEOL JEM-1400 electron microscopy.

Statistical Analysis: Data were expressed as means ± SD and analyzed with SPSS 12.0. Independent-samples T test was used to assess the significant difference between two experimental groups. One-way variance (ANOVA) with LSD or Games-Howell tests was applied to evaluate the statistical significance of differences between the experimental groups and the controls. A p value less than 0.05 was considered to be statistically significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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