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Molecular mechanism of kidney injury of mice caused by exposure to titanium dioxide nanoparticles

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ABSTRACT

Numerous studies have demonstrated that damage of kidney of mice can be caused by exposure to titanium dioxide nanoparticles (TiO_2 NPs). However, the molecular mechanism of TiO_2 NPs-induced nephric injury remains unclear. In this study, the mechanism of nephric injury in mice induced by an intragastric administration of TiO_2 NPs was investigated. The results showed that TiO_2 NPs were accumulated in the kidney, resulting in nephric inflammation, cell necrosis and dysfunction. Nucleic factor-κB was activated by TiO_2 NPs exposure, promoting the expression levels of tumor necrosis factor-α, macrophage migration inhibitory factor, interleukin-2, interleukin-4, interleukin-6, interleukin-8, interleukin-10, interleukin-18, interleukin-1β, cross-reaction protein, transforming growth factor-β, interferon-γ and CYP1A1, while heat shock protein 70 expression was inhibited. These findings implied that TiO_2 NPs-induced nephric injury of mice might be associated with alteration of inflammatory cytokine expression and reduction of detoxification of TiO_2 NPs.

1. Introduction

In the development of nanotechnology, nanomaterials are recognized to have potential applications due to their larger surface area to volume ratio, which enhances chemical reactivity and easier penetration into cells. Among the various nanomaterials, customarily titanium dioxide nanoparticles (TiO_2 NPs) are regarded as chemical inert, nontoxic and biocompatible material [1–3], they have been widely used in the sunscreen ingredient, pharmaceutical, and paint industries as a colouring material [4–7]. In over ten years, however, TiO_2 NPs toxicology has attracted considerable attention owing to their small sizes, large surface per mass and high reactivity.

A number of investigations have definitely showed that TiO_2 NPs exposure are able to cause injuries in various animal organ types, including lung, liver, spleen, and brain [8–24]. Recently, the toxicity of TiO_2 NPs to kidneys has been reported. Scown et al. had found that TiO_2 NPs were accumulated in the kidney, but had minimal effects on renal functions in rainbow trout [25]. In contradiction, Wang et al. had observed that TiO_2 NPs exposure to mice resulted in higher blood urea nitrogen and creatinine levels and the renal tubule was filled with proteinic liquids [10]. Chen et al. had also observed renal glomerulus dilatation and proteinic liquids filled renal tubule, but no kidney dysfunction was found with TiO_2 NPs-treated mice [26]. Furthermore, TiO_2 NPs were also suggested to induce nephric inflammation and impair nephric functions, which exerted its toxicity through ROS accumulation [27]. However, the molecular mechanism of TiO_2 NPs-induced nephric inflammation remains unclear.

Nephric inflammation and dysfunction are due to altered in kidney regardless of the cause of these diseases. Thus TiO_2 NPs induced nephric inflammation and dysfunction are able to be monitored through inflammatory cytokine expression levels in kidney. To confirm the above hypothesis, mice were continuously exposed to TiO_2 NPs for 90 days by an intragastric administration. The inflammatory cytokine expression in the mouse kidney was determined and the possible mechanism of the TiO_2 NPs induced nephric pathogenesis in mice was discussed.

2. Materials and methods

2.1. Chemicals, preparation and characterization

Nanoparticulated anatase TiO_2 was prepared via controlled hydrolysis of titanium tetrabutoxide. The details of the synthesis

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and characterization TiO_2 NP were previously described by our previous reports [21,28]. The average particle sizes of powder suspended in 0.5% w/v hydroxypropylmethylcellulose K4M (HPMC, K4M) solvent after 12 h and 24 h incubation ranged from 5 to 6 nm. The mean hydrodynamic diameter of TiO_2 NPs in HPMC solvent ranged between 208 and 330 nm (mostly 294 nm), and the zeta potential after 12 h and 24 h incubation was 7.57 mV and 9.28 mV [21].

2.2. Animal and treatment

It has been previously demonstrated by Wang et al. that sensitivity to TiO_2 exposure was higher in CD-1 (ICR) female mice than CD-1 (ICR) male mice [1]. Therefore, CD-1 (ICR) female mice were used in this study. 80 CD-1 (ICR) female mice (24 ± 2 g) were purchased from the Animal Center of Soochow University (China). All mice were housed in stainless steel cages in a ventilated animal room. Room temperature of the housing facility was maintained at $24 \pm 2\,^{\circ}C$ with a relative humidity of $60 \pm 10\%$ and a 12-h light/dark cycle. Distilled water and sterilized food were available for mice *ad libitum*. Prior to dosing, the mice were acclimated to this environment for 5 days. All animals were handled in accordance with the guidelines and protocols approved by the Care and Use of Animals Committee of Soochow University (China). All procedures used in animal experiments conformed to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals [29].

The mice were randomly divided into four groups ($N = 20$), including a control group treated with 0.5% w/v HPMC and three experimental groups treated with 2.5, 5, and 10 mg/kg BW TiO_2 NPs, respectively). The mice were weighed, and the TiO_2 NP suspensions were administered to the mice by an intragastric administration every day for 90 days. Any symptom or mortality was observed and recorded carefully everyday during the 90 days. After 90 days, all mice were weighed firstly, and then sacrificed after being anesthetized using ether. Blood samples were collected from the eye vein by removing the eyeball quickly. Serum was collected by centrifuging blood at 2500 rpm for 10 min. Kidneys were collected and weighed.

2.3. Coefficient of kidney

After weighing the body and kidneys, the coefficient of kidney to body weight was calculated as the ratio of kidney (wet weight, mg) to body weight (g).

2.4. Titanium content analysis

Kidneys were removed from the $-80\,^{\circ}C$ and then thawed, and roughly 0.3 g of the kidney was weighed, digested and analyzed for titanium content. Inductively coupled plasma-mass spectrometry (ICP-MS, Thermo Elemental X7, Thermo Electron Company) was used to analyze the titanium concentration in the samples. For the analysis, an Indium concentration of 20 ng/mL was utilized as an internal standard element, and the detection limit of titanium was 0.074 ng/mL. The data were expressed as nanograms per gram fresh tissue.

2.5. Biochemical analysis of kidney functions

Kidney functions were determined by uric acid (UA), blood urea nitrogen (BUN), creatinine (Cr), calcium (Ca) and phosphonium (P). All biochemical assays were performed using a clinical automatic chemistry analyzer (Type 7170A, Hitachi, Japan).

2.6. Histopathological examination of kidney

For pathological studies, all histopathological tests were performed using standard laboratory procedures [30]. The kidneys 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 an optical microscope (Nikon U-III Multi-point Sensor System, USA), and the identity and analysis of the pathology slides were blind to the pathologist.

2.7. Expression assay of inflammatory cytokines

The level of mRNA expression of nucleic factor-κB (NF-κB), NF-κB-inhibiting factor (IκB), tumor necrosis factor-α (TNF-α), macrophage migration inhibitory factor (MIF), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-18 (IL-18), interleukin-1β (IL-1β), cross-reaction protein (CRP), transforming growth factor-β (TGF-β), interferon-γ (INF-γ), cytochrome p450 1A (CYP1A) and heat shock protein 70 (HSP70) in the mouse kidney was determined using real-time quantitative RT polymerase chain reaction (RT-PCR) [31–33], respectively. Synthesized cDNA was used for the real-time PCR by employing primers that were designed using Primer Express Software according to the software guidelines, and PCR primer sequences are available upon request. To determine NF-κB, IκB, TNF-α, MIF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-6, IL-1β, CRP, TGF-β, INF-γ, Bax, Bcl-2, CYP1A1 and HSP-70 levels in the mouse kidney, an enzyme linked immunosorbent assay (ELISA) was performed using commercial kits that are selective for each respective protein (R&D Systems, USA). Manufacturer's instruction was followed. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron, Finland), and the concentrations of NF-κB, IκB, TNF-α, MIF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-6, IL-1β, CRP, TGF-β, INF-γ, CYP1A1 and HSP-70 were calculated from a standard curve for each sample.

2.8. Statistical analysis

Statistical analyses were conducted using SPSS 11.7 software. Data were expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was carried out to compare the differences of means among multi-group data. Dunnett's test was performed when each dataset was compared with the solvent-control data. Statistical significance for all tests was judged at a probability level of 0.05 ($p < 0.05$).

3. Results

3.1. Coefficient of kidney and titanium accumulation

Significant increases of the coefficients of kidney ($p < 0.05$ or $p < 0.01$) were caused by TiO_2 NPs exposure for consecutive 90 days (Fig. 1). Furthermore, with increasing TiO_2 NPs dose, the obvious accumulation of titanium in the kidney occurred ($p < 0.01$) (Fig. 2). These results show that the accumulation of titanium in the kidney was associated with the coefficients of kidney of mice. The increase of kidney indices caused by TiO_2 NPs exposure may be related to the nephric dysfunction and tissue injury, which are confirmed by the further assays of biochemical parameters and histopathological observation of kidney of mice.

3.2. Biochemical parameters in serum of kidney

The changes of biochemical parameters in the blood serum of mice kidney caused by TiO_2 NPs exposure are presented in Table 1.

Table 1
The changes of biochemical parameters in the blood serum of mice after intragastric administration with TiO_2 NPs for 90 days.

Indexes	TiO_2 NPs (mg/kg, BW)			
	0	2.5	5	10
UA (μmol/L)	222.56 ± 11.13	160.21 ± 8.01*	110.88 ± 5.54**	96.76 ± 4.84**
Cr (μmol/L)	8.81 ± 0.44	9.75 ± 0.49*	11.68 ± 0.58**	13.19 ± 0.66**
BUN (mmol/L)	9.28 ± 0.46	8.11 ± 0.41*	7.05 ± 0.35**	6.32 ± 0.32**
Ca (mmol/L)	2.43 ± 0.12	2.48 ± 0.12	2.51 ± 0.13	2.71 ± 0.14
P (mmol/L)	3.28 ± 0.16	3.33 ± 0.16	3.46 ± 0.17	3.52 ± 0.18

Ranks marked with an asterisk or double asterisks means it is statistically significant different from the control (unexposed mice) at the 5% or 1% confidence level, respectively. Values represent means ± SD, $N = 10$.

With TiO_2 NPs dose increased, the contents of Ca and P of nephric function parameters were not significant compared with the control group ($p > 0.05$). However, the Cr was increased, and the UA, and BUN were decreased gradually ($p < 0.05$ or $p < 0.01$), respectively; demonstrating that long-term exposure to low dose TiO_2 NPs impaired nephric functions in mice.

3.3. Histopathological evaluation of kidney

Fig. 3 presents the histopathological changes of kidneys in mice treated by TiO_2 NPs exposure. In the 2.5 mg/kg BW TiO_2 NPs treated group, the nephric tissue is significantly showed to inflammatory cell infiltration and congestion of mesenchyme blood vessel (Fig. 3b). In the 5 mg/kg BW TiO_2 NPs treated group, inflammatory cell infiltration, congestion of mesenchyme blood vessel and spotty necrosis of renal tubular epithelial cells were observed (Fig. 3c). Furthermore, a large area of necrosis of renal tubular epithelial cells was detected in the 10 mg/kg BW TiO_2 NPs treated group (Fig. 3d). The findings indicate that the kidney injury was related to a dose-dependent manner of TiO_2 NPs exposure.

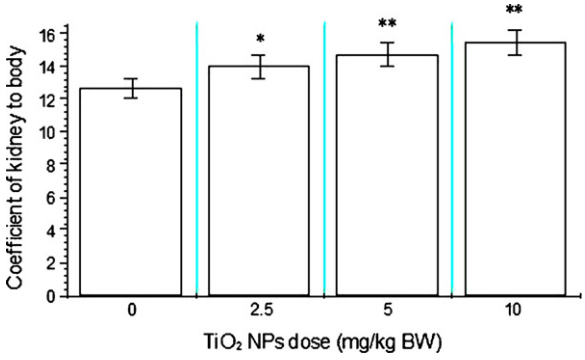

Fig. 1. The coefficients of kidney of mice by an intragastric administration with TiO_2 NPs for consecutive 90 days. Bars marked with an asterisk or double asterisks means it is significantly different from the control (unexposed mice) at the 5% or 1% confidence level, respectively. Values represent means ± SD, $N = 20$.

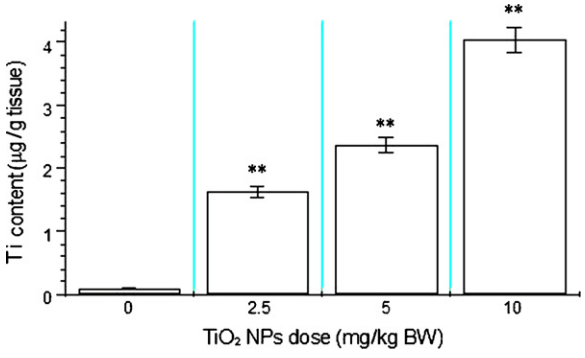

Fig. 2. The contents of titanium in the mouse kidney by an intragastric administration with TiO_2 NPs for 90 days. Bars marked with an asterisk or double asterisks means it is statistically significant different from the control (unexposed mice) at the 5% or 1% confidence level, respectively. Values represent means ± SD, $N = 5$.

3.4. Cytokine expression

To further confirm the role of molecular mechanism in the TiO_2 NPs-induced kidney injury, the changes of the inflammation-related genes or detoxification-related genes and their proteins expression in mice caused by TiO_2 NP exposure were detect using real time RT-PCR and ELISA (Tables 2 and 3). The mRNA expression levels of NF-κB, TNF-α, MIF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-1β, CRP, TGF-β, INF-γ, and CYP1A1 were increased significantly in the TiO_2 NP treated groups ($p < 0.05$ or 0.01). Interestingly, IκB and HSP-70 expression levels were decreased significantly compared with control group ($p < 0.05$ or 0.01).

4. Discussion

In this study, effects of TiO_2 NPs on the mouse kidney were studied. After intragastric administrations with 2.5, 5, and 10 mg/kg BW of TiO_2 NPs for 90 consecutive days, significant increases of the kidney indices (Fig. 1) and titanium accumulation in mouse kidneys (Fig. 2) were observed, coupled with increase of Cr level, decrease of BUN, UA excretion (Table 1), induced inflammatory response and necrosis of kidneys (Fig. 3). Previous study indicated that abnormal pathological changes of the mouse kidney and the nephric dysfunction were not able to be triggered by intraperitoneal injection with 5 mg/kg BW TiO_2 NPs for 14 days, but with 50, 100 and 150 mg/kg BW TiO_2 NPs exposure, impairment of kidney functions and severe inflammatory response of kidney were observed [27]. Wang et al. also observed that the 2-week exposure to the 5 g/kg BW TiO_2 NPs by a gavage caused nephric dysfunction and tissue damage of mice [10]. In this study, molecular evidences were provided to prove TiO_2 NPs induced nephric dysfunction and inflammation of mice by alteration of gene expression levels of the cytokines involved in inflammatory response or detoxification. NF-κB is known as a critical intracellular mediator of the inflammatory cascade, and it binds to inhibitory proteins (IκBs) which prevent NF-κB from migrating to the nucleus from cytoplasm. When an appropriate inducer existed, IκBs are phosphorylated and degraded, allowing nuclear uptaking of NF-κB and initiating gene transcriptions, including MIF, the proinflammatory cytokines of TNF-α, IL-1β, IL-6, IL-8, IL-18, CRP, and anti-inflammatory cytokines of IL-2, IL-4, and IL-10 [34]. TGF-β is proved to be involved with a dual-role as an anti-inflammatory and a profibrotic cytokine. IFN-γ and TNF are essential for primary defense against infection [35,36], and mice that lack these two cytokines or their cognate receptors succumb to infection rapidly [37]. In response to TiO_2 NPs stimulation, our results suggested that TiO_2 NPs exposure for 90 consecutive days could significantly up-regulate mRNA expression levels of several relative inflammatory cytokines genes, including NF-κB, TNF-α, MIF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-1β, CRP, TGF-β, and

Table 2
Effect of TiO_2 NPs on the amplification of regulating the inflammation, immune and oxidative stress gene mRNA of the mouse kidney by real-time PCR analysis after intragastric administration with TiO_2 NPs for consecutive 90 days.

Ratio of gene/actin	TiO_2 NPs (mg/kg, BW)			
	0	2.5	5	10
NF-κB/actin	0.30 ± 0.015	0.36 ± 0.018*	0.57 ± 0.029**	0.88 ± 0.044**
IκB/actin	0.71 ± 0.036	0.58 ± 0.029*	0.42 ± 0.021**	0.32 ± 0.016**
TNF-α/actin	0.08 ± 0.004	0.11 ± 0.006*	0.18 ± 0.009**	0.31 ± 0.016**
MIF/actin	0.21 ± 0.011	0.32 ± 0.016*	0.49 ± 0.025*	0.76 ± 0.038**
IL-2/actin	0.06 ± 0.003	0.09 ± 0.005*	0.15 ± 0.008**	0.23 ± 0.012**
IL-4/actin	0.07 ± 0.004	0.09 ± 0.005*	0.16 ± 0.008**	0.27 ± 0.014**
IL-6/actin	0.09 ± 0.005	0.13 ± 0.007*	0.18 ± 0.009**	0.31 ± 0.016**
IL-8/actin	0.15 ± 0.008	0.19 ± 0.010*	0.31 ± 0.016**	0.51 ± 0.026**
IL-10/actin	0.12 ± 0.006	0.17 ± 0.009*	0.24 ± 0.012**	0.38 ± 0.019**
IL-18/actin	0.28 ± 0.014	0.31 ± 0.016	0.46 ± 0.023**	0.61 ± 0.031**
IL-1β/actin	0.21 ± 0.011	0.33 ± 0.017**	0.42 ± 0.021**	0.58 ± 0.029**
CRP/actin	0.42 ± 0.021	0.53 ± 0.027*	0.65 ± 0.033**	0.88 ± 0.044**
TGF-β/actin	0.26 ± 0.013	0.38 ± 0.019*	0.53 ± 0.027**	0.70 ± 0.035**
INF-γ/actin	0.20 ± 0.010	0.26 ± 0.013*	0.41 ± 0.021**	0.58 ± 0.029**
CYP1A1/actin	0.28 ± 0.014	0.36 ± 0.018*	0.66 ± 0.033**	1.01 ± 0.051**
HSP-70/actin	0.41 ± 0.021	0.32 ± 0.016*	0.25 ± 0.013**	0.11 ± 0.006**

Ranks marked with an asterisk or double asterisks means it is statistically significant different from the control (unexposed mice) at the 5% or l% confidence level, respectively. Values represent means ± SD, $N = 5$.

INF-γ, and decrease IκB expression. The obvious alterations of these cytokines' expression indicated the involvement of inflammatory responses in TiO_2 NPs-induced kidney toxicity. Studies had showed that TiO_2 NPs promoted the expression of inflammatory cytokines in the lung, liver, spleen and brain of rat and mice [9,12,38–41]. Increases of NF-κB expression in the mouse liver were also detected due to the significant increases of NF-κB-inducible kinase and IκB kinase expression and decrease of IκB expression after treated with TiO_2 NPs for 60 days [17].

In this study, significant increase of the CYP1A expression and reduction of HSP70 expression were observed (Tables 2 and 3). CYP1A and HSP70 were selected since they represent different processes that the cells follow to detoxify and/or defend against environmental toxicants [42]. Differences of gene expression of CYP1A and HSP70 were then used to explain the toxic characteristic signatures of TiO_2 NPs. It is well known that CYP1A induction is activated by the aryl hydrocarbon receptor (AHR) pathway, and its protein plays an essential function in the biotransformation and detoxification of endogenous and exogenous compounds. It is a widely accepted environmental biomarker, useful for monitoring the biological effects of several xenobiotic groups, including heavy metals [42]. De Jongh et al. showed that administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to male C57BL/6J mice had caused the increases of both CYP 1A1 and CYP 1A2 hepatic protein levels [43]. In this study the high level expression of this gene and its protein products indicated that TiO_2 NPs may cause kidney intoxication in mice. Likewise, higher level expression of HSP70 is often associated with a cellular response to a harmful stress or to adverse life conditions. The reduction of the HSP70 expression in the kidney by exposure to TiO_2 NPs indicated a slow biotransformation or

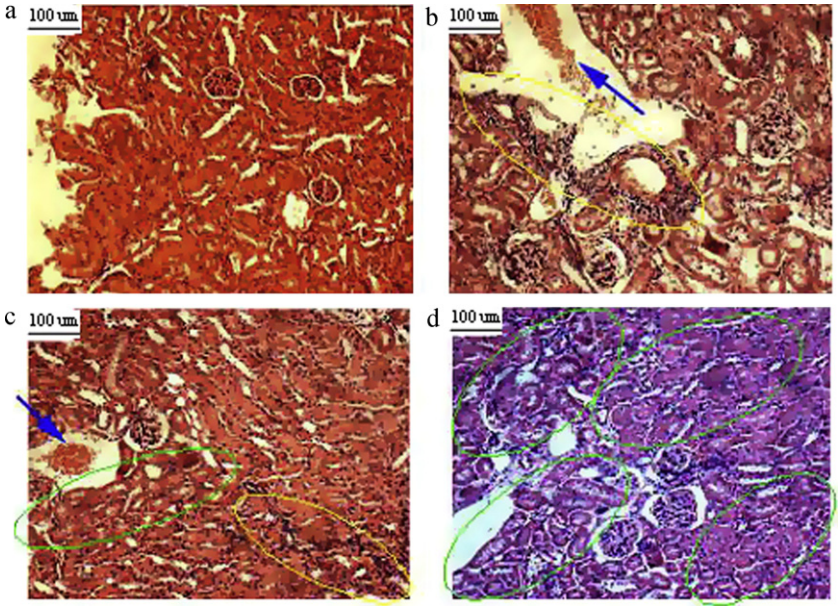

Fig. 3. Histopathological observation of kidney caused by an intragastric administration with TiO_2 NPs for consecutive 90 days. (a) Control group (unexposed mice) presents integrated glomerulars and normal kidney tubulars; (b) 2.5 mg/kg TiO_2 NPs group presents inflammatory cell infiltration (yellow cycle) and congestion of mesenchyme blood vessel (blue arrow); (c) 5 mg/kg TiO_2 NPs group indicates inflammatory cell infiltration (yellow cycle), congestion of mesenchyme blood vessel (blue arrow) and spotty necrosis of renal tubular epithelial cell (green cycle); (d) 10 mg/kg TiO_2 NPs group indicates severe necrosis of renal tubular epithelial cell (green cycles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.) The scale bar presented at the upside of each photomicrograph indicated 100 μm.

Table 3
Effect of TiO NPs on the inflammatory cytokine protein levels of the mouse kidhey by ELISA analysis after intragastric administration with TiO NPs for consecutive 90 days.

Protein expression	TiO_2 NPs (mg/kg, BW)			
	0	2.5	5	10
NF-κB (ng/g tissue)	34.62 ± 1.73	38.13 ± 1.91	52.95 ± 2.65**	89.96 ± 4.50**
IκB (g tissue)	18.71 ± 0.94	14.26 ± 0.71*	10.77 ± 0.54**	7.86 ± 0.39**
TNF-α (ng/g tissue)	72.83 ± 3.64	81.66 ± 4.08*	171.26 ± 8.56**	327.79 ± 16.39**
MIF (ng/g tissue)	269 ± 13	582 ± 29**	2749 ± 137**	3129 ± 156**
IL-2 (ng/g tissue)	66.45 ± 3.32	73.28 ± 3.66*	87.39 ± 4.37**	94.46 ± 4.72**
IL-4 (ng/g tissue)	44.99 ± 2.25	49.39 ± 2.47	57.78 ± 2.89*	71.19 ± 3.56**
IL-6 (ng/g tissue)	6.95 ± 0.35	8.23 ± 0.41*	9.67 ± 0.48**	13.99 ± 0.70**
IL-8 (ng/g tissue)	32.93 ± 1.65	38.99 ± 1.95*	42.98 ± 2.15**	53.37 ± 2.67**
IL-10 (ng/g tissue)	5.96 ± 0.30	7.12 ± 0.36*	8.35 ± 0.42**	10.66 ± 0.53**
IL-18 (ng/g tissue)	6.17 ± 0.31	8.29 ± 0.41*	12.25 ± 0.61**	19.99 ± 1.00**
IL-1β (ng/g tissue)	88.94 ± 4.45	105.77 ± 5.29*	168.81 ± 8.44**	196.42 ± 9.82**
CRP (μg/g tissue)	38.68 ± 1.93	53.91 ± 2.70*	74.85 ± 3.74**	95.93 ± 4.80**
TGF-β (ng/g tissue)	21.69 ± 1.08	34.71 ± 1.74**	51.99 ± 2.60**	72.48 ± 3.62**
TGF-γ (ng/g tissue)	19.50 ± 0.98	28.57 ± 1.43**	47.92 ± 2.40**	63.81 ± 3.19**
CYP1A1 (ng/g tissue)	12.02 ± 0.60	21.17 ± 1.06**	36.59 ± 1.83**	52.88 ± 2.64**
HSP-70 (ng/g tissue)	11.95 ± 0.60	7.14 ± 0.36*	5.56 ± 0.28**	3.78 ± 0.19**

Ranks marked with an asterisk or double asterisks means it is statistically significant different from the control (unexposed mice) at the 5% or l% confidence level, respectively. Values represent means ± SD, $N = 5$.

detoxification and decreased response to the adverse effects experienced in the kidney [44–47]. About the dose selection in this study, we consulted the report of World Health Organization in 1969. According to the report, LD50 of TiO_2 for rats is larger than 12,000 mg/kg BW after oral administration. In the present study, we selected 5, 10, and 50 mg/kg BW TiO_2 NPs exposed to mice every day. They were equal to about 0.15–0.7 g TiO_2 NPs of 60–70 kg body weight for humans with such exposure, which were relatively safe doses. However, we think, attention should be aroused on the application of TiO_2 NPs and their potential long-term exposure effects especially on human beings.

In conclusion, the present study shows that mice treated with 2.5, 5 and 10 mg/kg BW TiO_2 NPs for 90 consecutive days resulted in significant increases of NF-κB, TNF-α, MIF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, IL-1β, CRP, TGF-β, INF-γ, CYP1A expression and significant decrease of HSP70 expression, leading to the increase of kidney indices, inflammatory responses and cell necrosis in mouse kidney.

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