Food and Chemical Toxicology 90 (2016) 76–83

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Zinc oxide nanoparticles induce renal toxicity through reactive oxygen species

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ARTICLE INFO

Article history:
Received 21 November 2015
Received in revised form
17 January 2016
Accepted 2 February 2016
Available online 6 February 2016

Keywords:
Apoptosis
Nanoparticles of zinc oxide
Nephrin
Podocytes
ROS

ABSTRACT

Nanoparticles of zinc oxide (ZnO NPs) are applied in many fields nowadays. Consequently, concerns have been raised about its potential harmful effects. The present study focuses on its toxic effect on podocytes and rats. In vitro study, podocytes were treated with different concentrations of ZnO NPs (10, 50 and 100 μg/ml), the viability of cells was decreased as time prolonged according to MTT assay. Meantime, flow cytometry analysis indicated that ZnO NPs induced intracellular accumulation of reactive oxygen species (ROS) and apoptosis. The measurement of superoxide dismutase (SOD) and malondialdehyde (MDA) showed that ZnO NPs decreased SOD level and increased MDA level. Interestingly, pretreatment with N-mercaptopropionyl-glycine, known as a type of ROS scavenger, could inhibit podocyte apoptosis induced by ZnO NPs. Meantime, a loss of nephrin can be detected, which may result in a direct damage to slit diaphragms. In vivo study, adult male Wistar rats were administrated with 3mg/kg/day ZnO NPs for 5 days, body weight and kidney index were significantly reduced. In addition, ZnO NPs decreased the activity of catalase and SOD in kidney cortex in vivo. It could be concluded that ZnO NPs present toxic effect on podocytes and Wistar rats, which was related with oxidative stress.

1. Introduction

The 21st century is regarded by many people as “nanocentury” with the nano-revolution touching almost every aspect of human life such as medical science, drug applications, and defense industries (Long and Ye, 2007). As the nanotechnology develops rapidly, public exposure to nanoparticles is undoubtedly increasing. As a result, the widely application of nanomaterials led to a growing concern on the bioavailability and toxicity of nano-sized materials (Brumfiel, 2003; Igarashi, 2008; Nel et al., 2006; Oberdorster et al., 2005; Nielsen and Lintzeris, 2008).

Although numerous studies on the safety/toxicity of nanoparticles have been published, details have been lacking, however, on the precise mechanisms of injury induced by ZnO NPs. In the present study, the cytotoxic effect of ZnO NPs on podocytes arouses our interest. Nanostructures of ZnO have great potential applications in dyes, paints, textiles, medical diagnosis, sunscreens and cosmetics (Cai, 1995; Mashaghi et al., 2013; Yuranova and Kiwi, 2007). Despite that it is widely used, there are several studies reporting the toxicity of ZnO NPs in bacterial systems, eco-relevant species (Adams et al., 2006; Franklin et al., 2007; Zhu et al., 2008) and vertebrates (Brayner et al., 2006; Heinlaan et al., 2008; Huang et al., 2008). It has been discovered that environmental exposure to ZnO NPs would cause it to stay in the stratum corneum and to accumulate in skin folds and/or hair follicle (Zvyagin et al., 2008). Similarly, the bone, kidney and pancreas were the target organs of 20-nM and 120-nM ZnO (Banyal et al., 2013). On the other hand, exposure to low concentrations of ZnO NPs shows a genotoxic potential mediated by lipid peroxidation and oxidative stress (Sharma et al., 2009). At the same time, several studies reported its damage to a variety of cells such as epidermal cells (Sharma et al., 2009), cancer cells (Akhtar et al., 2012) and liver cells (Sharma et al., 2012). However, there's no related article concerning the toxic effect of ZnO NPs on mouse podocyte cell line.

Podocytes are highly specialized cells, which form multiple interdigitating foot (Pavenstadt et al., 2003). They are

Abbreviations: ZnO NPs, nanoparticles of zinc oxide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SOD, superoxide dismutase; MDA, malondialdehyde; N-MPG, N-(mercaptopropionyl)-glycine; SD, slit diaphragm; GFB, glomerular filtration barrier.

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http://dx.doi.org/10.1016/j.fct.2016.02.002
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interconnected by slit diaphragms (SD) and cover the exterior basement membrane surface of the glomerular capillary(Pavenstadt et al., 2003). Podocytes play critical roles in the maintenance of glomerular filtration barrier (GFB) (Kretzler et al., 1994). In other words, they would take part in the filtration process, which means the harmful substance, for example, ZnO NPs may accumulate around them. Furthermore, kidney is one of targets for ZnO NPs to retain, which may further promote the accumulation of ZnO NPs around podocytes. As a result, it is necessary to evaluate the influence of ZnO NPs on podocytes and to discover its possible mechanism. At the same time, we became interest in the potential impact of ZnO NPs on the necessary protein of podocytes. To date, there have been limited studies relevant to this topic.

With the establishment of mouse podocyte cell line, which is conditionally immortalized, the study of fully differentiated podocytes in culture has become feasible (Mundel and Reiser, 1997). As a result, the purpose of the study was to assess whether ZnO NPs can induce the apoptosis in podocytes and its impact on a predominate protein, thereby providing basic information for the safe application of ZnO NPs in the future.

2. Materials and methods

2.1. Experimental plan

A brief experimental plan including the selected concentrations and times of the whole study was designed keeping in view the approach for nanomaterials toxicity study as suggested in Vyom Sharma et al. (Sharma et al., 2009), Shichang Liu et al. (Liu et al., 2009) and Jing Xu et al. (Xu et al., 2012)'s report.

2.2. Materials

Rabbit polyclonal anti-nephrin primary antibody was purchased form Abcam, Cambridge, MA, USA. RPMI 1640 culture medium was purchased from GIBCO Invitrogen. The fetal bovine serum (FBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., St Louis, MO, USA. Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was from Bipec Biopharma Corporation, USA. Reactive oxygen species (ROS) testing kit was purchased from Genmed Scientifics Inc, USA. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY, USA). Superoxide dismutase (SOD) assay kit and malondialdehyde (MDA) assay kit were purchased from the Nanking Jiancheng bio-engineering research institute (Nanking, China).

2.3. The characterization of ZnO NPs

The ZnO NPs used in this research was a kind gift from Dr. GG Ren, University of Hertfordshire, England. The characterization of ZnO NPs, such as the size and surface area has been described in our previous work (Zhao et al., 2009).

2.4. Cell culture and preparation of ZnO NPs

Mouse podocytes used in the present study were established by Mundel and Reiser (1997). They were adherent and from a conditionally immortalized cell line. Cells were maintained on plastic culture microplates with RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in the presence of 10 U/ml recombinant mouse interferon-γ (IFN-γ) at 33 °C in a humidified atmosphere of 5% CO_2. In order to induce differentiation, podocytes were cultured at 37 °C without IFN-γ (non-permissive conditions) for two weeks.

The stock solution (10 mg/ml) of ZnO NPs was prepared in Milli-Q water and dispersed by the ultrasonic vibration (Branson Inc., Dabury, CT, USA) for 20 min to prevent from aggregation. Then different concentrations of ZnO NPs were prepared from the suspension.

2.5. Animals

Adult male Wistar rats (200 ± 20 g) were purchased from the Laboratory animal center, Academy of military medical science of people's liberation army. All animal experiments were reviewed and approved by the Animal Research Ethics Committee, School of Medicine, Nankai University.

2.6. Induction of acute toxicity on rats

Adult male Wistar rats were randomly assigned into two groups, i.e. control group (n = 8) and ZnO NPs group (n = 8)). Rats were administrated with 3 mg/kg/day by intraperitoneal injection. At the end of 5 days of administration, rats were killed and kidney cortex were collected and stocked at −70 °C.

2.7. Cell viability assay

The MTT assay was used to assessed the cell viability (Denizot and Lang, 1986). In brief, the cells (1×10^4/ml) were seeded into each well in 96-well culture plates and incubated with various concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for the periods of 3 h, 6 h, 12 h and 24 h. 20 μl MTT was added to each well after the exposure, at a final concentration of 0.5 mg/ml and incubated for another 4 h at 37 °C. The medium was then removed carefully. 150 μl DMSO was added in after that and mixed with the cells thoroughly until formazan crystals were dissolved completely. An ELISA reader (Elx 800, Bio-TEK, USA) was used to measure the mixture at 570 nm. The cell viability was expressed as a percentage of the control. Meanwhile, the concentrations of the ZnO NPs used in assays of SOD and MDA were based on the results of the MTT test.

Cell viability (%) = (the viability of ZnO NPs treated group/the control group) × 100.

2.8. Detection of apoptotic cells by flow cytometry

In order to determine the effect of ZnO NPs on podocyte apoptosis, the apoptosis was assayed by annexin V-FITC and PI staining followed by analysis with flow cytometry (BECKMAN-COULTER, USA). The methodology followed the procedures as described in the annexin V-FITC/PI detection kit. In brief, after treatment with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 12 h, cells were harvested and washed twice with pre-cold phosphate buffer saline (PBS) and resuspended in 1 × binding buffer with a concentration of 1×10^6 cells/ml. The cells were stained with 5 μl annexin V-FITC and 5 μl PI for 15 min at room temperature after that in the dark. Then the cells were analyzed by flow cytometry.

2.9. Measurement of ROS

The generation of ROS was measured using intracellular oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA) at 37 °C for 20 min in the dark. The cells were pre-loaded in a 6-well plate for 24 h for stabilization. Then cells were washed and incubated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h. After exposure, cells were washed with PBS. Then they were resuspended at a concentration of 1×10^6 cells/ml.

The cells were detected and analyzed by flow cytometry. In addition, the H_2O_2 level of rats' kindey tissue was measured using the kit (Beyotime Biotechnology, Haimen, China) according to the manufacture's protocol.

2.10. SOD assay

The generation of total SOD (T-SOD) for the cells and kidney cortex was measured by assay kit (Beyotime Biotechnology, Haimen, China). Briefly, the podocytes were incubated in a 6-well plate for 24 h for stabilization. Then cells were treated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h. After exposure, the level of SOD was evaluated using a UV–visible spectrophotometer (V-530UV/UISNIR Spectrophotometer, Jasco, Japan) at 550 nm followed the manufacturer's instructions. The percentage of SOD activity was obtained as follows: % T-SOD activity = (the activity of treated group/the control group) × 100.

2.11. MDA assay

The MDA level in podocytes was measured by MDA assay kit. At first, the podocytes were incubated in a 6-well plate for 24 h for stabilization. Then cells were treated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h. After exposure, the level of MDA was evaluated in accordance with the manufacturer's instructions. The MDA level in control group was defined as 100%, while the MDA level in the rest groups was expressed as a percentage of the MDA level of the control group.

2.12. Catalase assay

The levels of catalase activity of kidney cortex in rats were measured using the kit (Beyotime Biotechnology, Haimen, China) according to the manufacture's protocol.

2.13. Western blotting

Protein preparation: The podocytes were cultured in 6-well culture plates with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h and lysed in lysis buffer containing a proteinase inhibitor cocktail (1:100 dilutions). Lysates were centrifugated at 12,000 rpm for 10 min at 4 °C. The supernatant was collected and total proteins were quantified by bicinchonic acid assay according to manufacturer's instructions. Then the protein samples were mixed with loading buffer and boiled at 95–100 °C for 5 min.

Semiquantitative immunoblotting was carried out as followed: Proteins were separated on polyacrylamide gels by SDS-PAGE and transferred to polyvinylidene difluoride (nitrocellulose) membrane in Tris–glycine buffer at 300 mA for 1 h. Membranes were blocked with 5% fat-free milk blocking buffer for 2 h at room temperature and then incubated overnight at 4 °C with the respective primary rabbit polyclonal anti-nephrin antibodies (working dilution 1:5000) and rabbit polyclonal anti-β-actin IgG (working dilution 1:2000). After that, membranes were washed three times with Tris-buffered saline/Tween 20 (TBST) buffer and exposed to horseradish peroxidase-conjugated secondary antibodies (working dilution 1:2500) for 1 h at the room temperature. Blots were washed another three times with TBST and detected with chemiluminescent HRP substrate (Immobilon Western). Figures showed representative results from experiments repeated at least three times.

2.14. The effect of N-(2-mercaptopropionyl)-glycine

To assess the effect of ROS on cell apoptosis, cells were pretreated with N-MPG (300 μmol/L, Liu, Xu et al.) known as a kind of ROS scavenger (Mitsos et al., 1986). Then the cells were exposed to 100 μg/ml ZnO NPs, the cell apoptosis was measured by flow cytometry.

2.15. Statistical analysis

The results were expressed as mean ± SEM. The statistical significance was assessed by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison post test using the SPSS (11.5) software. The significant difference was taken as $P < 0.05$.

3. Results

3.1. Effects of ZnO NPs on cell viability

As shown in Fig. 1, when the concentration and time period increased, the cell viability was decreased with a concentration and time-dependent manner. The 100 μg/ml ZnO NPs caused a dramatically decrease in the cell viability after 3 h, 6 h, 12 h and 24 h incubation ($P < 0.05$). With time increasing, the significant cytotoxicity of 100 μg/ml ZnO NPs was observed after 3 h ($P < 0.05$). ANOVA analysis and Dunnett's test revealed that ZnO NPs decreased the cell viability both in a concentration-dependent and time-dependent manner.

3.2. The podocyte apoptosis induced by ZnO NPs

As shown in Fig. 2(a~d), podocytes were incubated with different concentrations of the ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 12 h, and the apoptotic rate of podocytes was increased from 4.85% in control group to 40.46% ($P < 0.05$) after the cell exposure to the ZnO NPs for 12 h (Fig. 2f). It can be inferred from the results that ZnO NPs induced the podocyte apoptosis in a concentration-dependent manner. But pre-treated with ROS scavenger N-MPG decreased the apoptotic rate of podocytes (Fig. 2e).

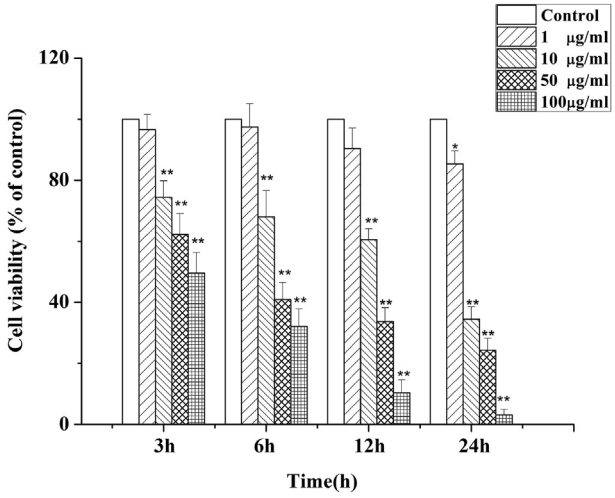

Fig. 1. Effects of ZnO NPs on podocyte viability determined by MTT assay. Cells were treated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 3 h, 6 h, 12 h and 24 h. Results presented the means of three separate experiments, and error bars represent the standard error of the mean. **P < 0.01 compared with the control group.

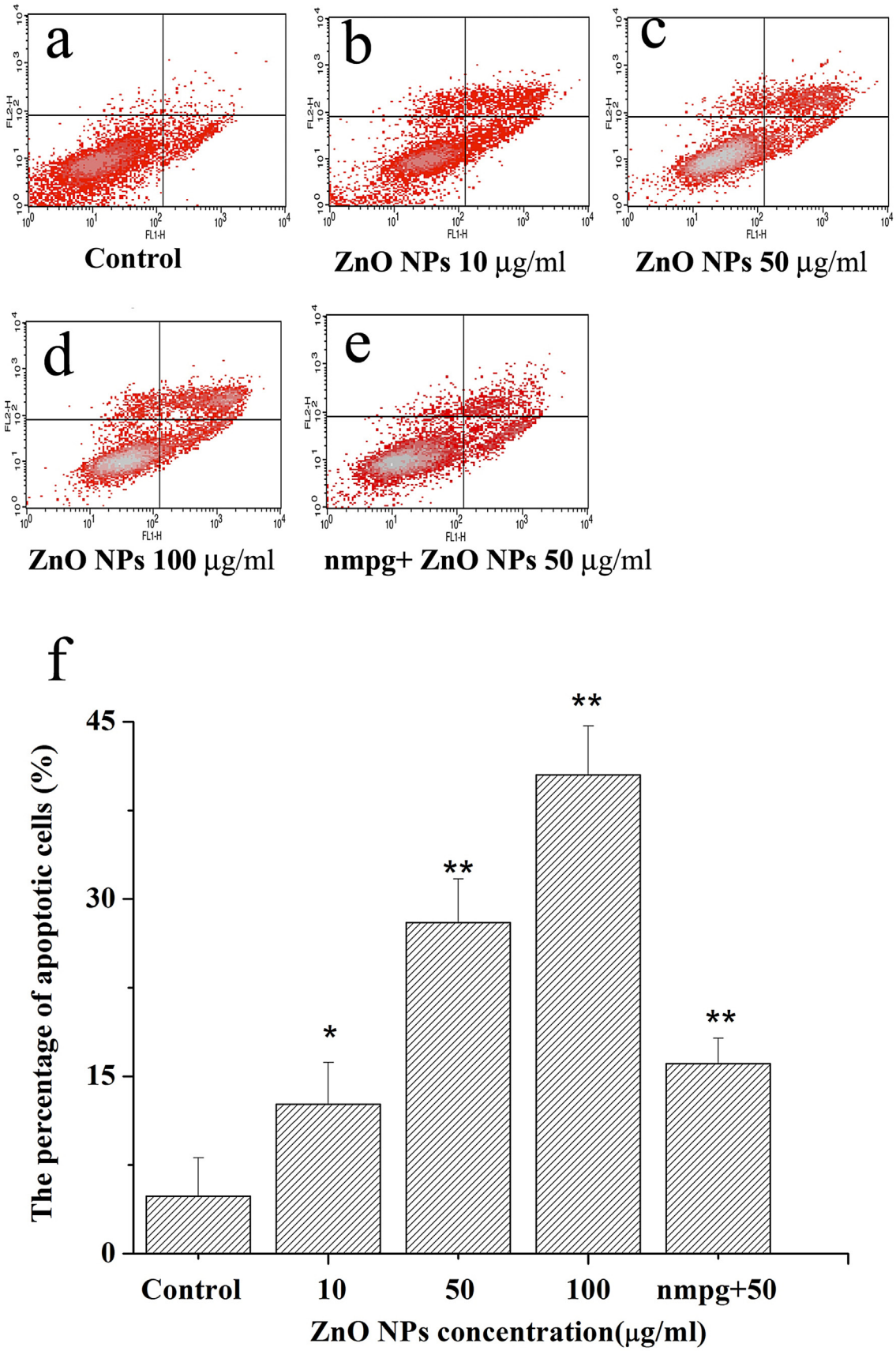

Fig. 2. Effects of ZnO NPs on apoptosis in podocytes by flow cytometry. The flow cytometry assay was carried out in order to detect the apoptotic rate of cells using cells incubated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 12 h. Then the flow cytometry assay was carried out in order to detect the apoptotic rate of cells. (a) Control, (b) 10 μg/ml, (c) 50 μg/ml, (d) 100 μg/ml ZnO NPs, (e) 50 μg/ml + N-MPG treated cells' apoptotic rate and (f) the corresponding linear diagram of flow cytometry was shown. Results presented the means of three separate experiments, and error bars represent the standard error of the mean. **P < 0.01 compared with the control group.

3.3. Measurement of ROS generation

DCFH-DA can passively enter the cell and react with the ROS to produce a fluorescent compound dichlorofluorescein (DCF). The generation of the ROS was demonstrated by the increased fluorescence intensity of oxidized DCF. As shown in Fig. 3, the ratio of DCF-positive cells was distinctly increased in the ZnO NPs treated group (10 μg/ml, 50 μg/ml and 100 μg/ml) compared to that of control group.

3.4. SOD assay

The cellular level of T-SOD was dramatically decreased when the cells were cultured in media containing different concentrations of ZnO NPs for 6 h as shown in Fig. 4(a). In contrast to the control group, the level of SOD in the cells under the treatment of different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) decreased to 74.85%, 64.55% and 54.13% respectively.

3.5. MDA assay

The MDA level was distinctly increased when cells were treated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h as shown in Fig. 4b. The MDA level increased to 105.52%, 123.33% and 140.10% compared with that of control group.

3.6. Western blotting detection of nephrin

There was a significant loss of the protein when treated with the concentration of 100 μg/ml as shown in Fig. 5. However, when treated with the concentration of 10 μg/ml, there had not been obvious difference compared with that of control group. In a word, the loss of protein can be detected when podocytes were incubated with ZnO NPs.

3.7. Acute toxicity on rats

As shown in Fig. 6, the boy weight of ZnO NPs group (3 mg/kg/day) was markedly decreased compared with that of control group ($P < 0.05$) (Fig. 6a). In addition, the index of kidney was also significantly decreased in ZnOt NPs group, which preliminary indicated that there was toxicity of ZnO NPs on kidney (Fig. 6b).

3.8. The level of SOD and catalase in kidney cortex

As shown in Fig. 7, ZnO NPs significantly attenuated the SOD activity to 4.44 ± 0.18 unit/mg protein compared to control group (5.91 ± 0.39 unit/mg protein) ($P < 0.05$) (Fig. 7a). At the same time, ZnO NPs also reduced the catalase activity to 55.33 ± 6.86% ($P < 0.05$) (Fig. 7b). Our results in vivo were consistent with the results on podocytes experiments.

4. Discussion

In present study, the cytotoxicity of the ZnO NPs on podocytes and rats' kidney were assessed. In podocytes experiment, the detection of the cell apoptosis by flow cytometry demonstrated that ZnO NPs might induce the apoptosis of podocytes by enhancement of intracellular ROS generation, while ROS scavenger N-MPG could decrease the cellular apoptosis caused by ZnO NPs [Fig. 2(a~e)]. Also, the viability of podocytes incubated with different concentrations of nanoparticles was investigated. It was found that ZnO NPs decreased the viability of podocytes in a concentration and time-dependent manner [Fig. 2f]. ANOVA analysis

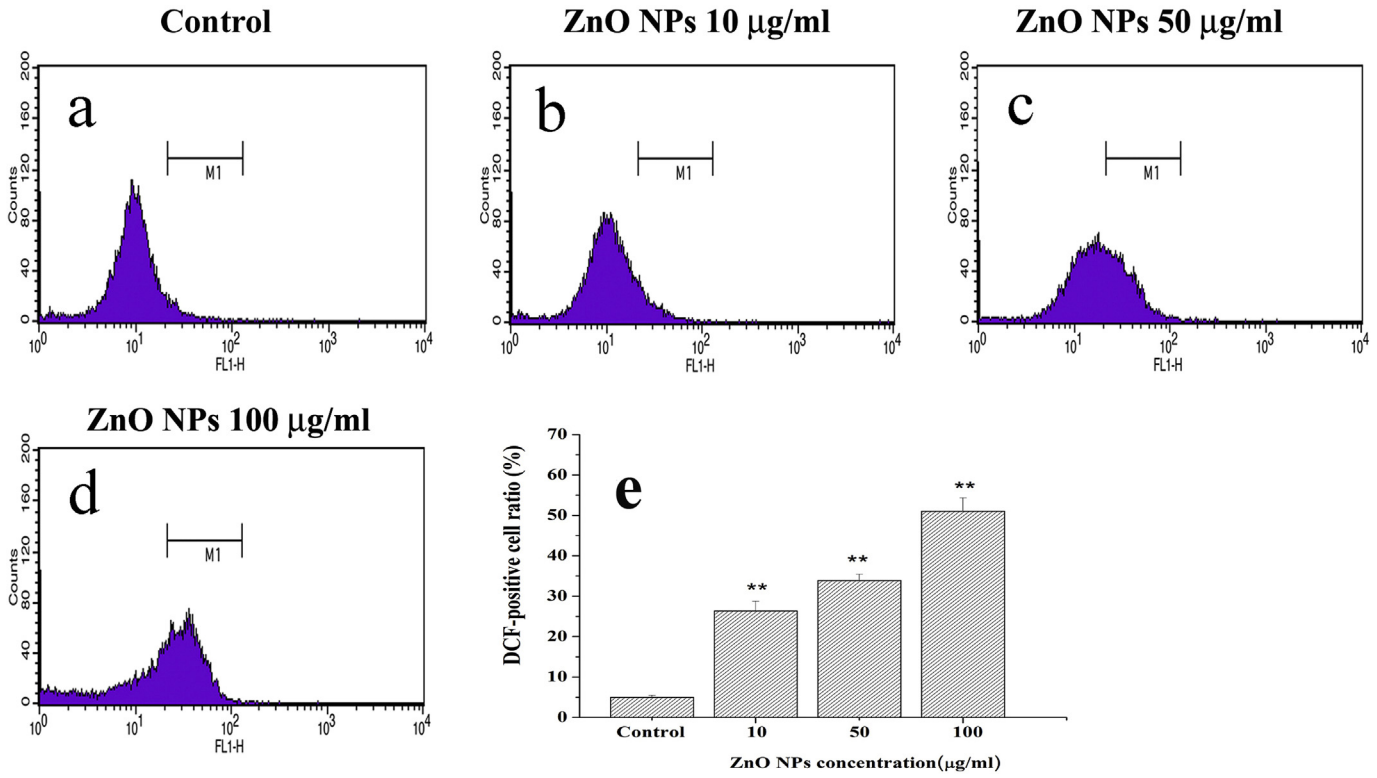

Fig. 3. Effects of ZnO NPs on ROS generation. Cells were treated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h. Then the flow cytometry assay was carried out for the detection of ROS generation. (a) Control, (b) 10 μg/ml, (c) 50 μg/ml, (d) 100 μg/ml and (e) The corresponding linear diagram of flow cytometry was shown. Data were presented as mean ± S.E.M. of three independent experiments. **$P < 0.01$ compared with the control group.

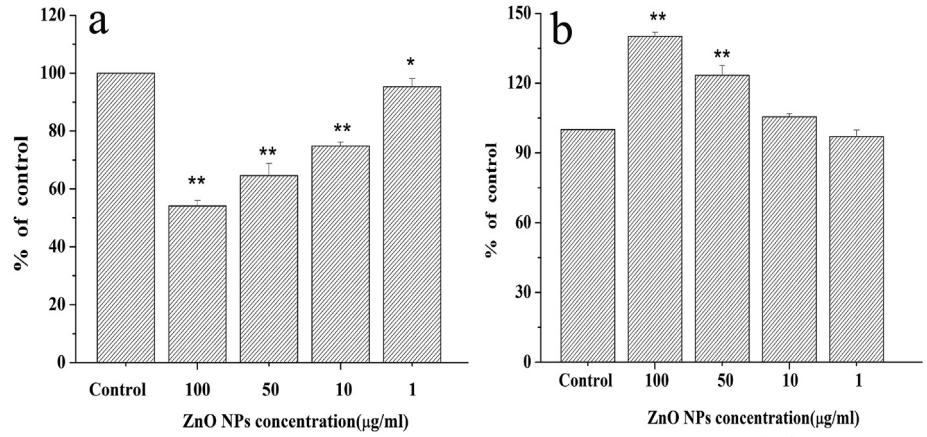

Fig. 4. Effects of ZnO NPs on the decrease of SOD and the elevation of MDA level in podocytes. Cells treated with different concentrations of ZnO NPs (10 μg/ml, 50 μg/ml and 100 μg/ml) for 6 h. (a) The T-SOD level and (b) the MDA level was shown. Data were presented as mean ± S.E.M. of three independent experiments. *P < 0.05 compared with the control group, **P < 0.01 compared with the control group.

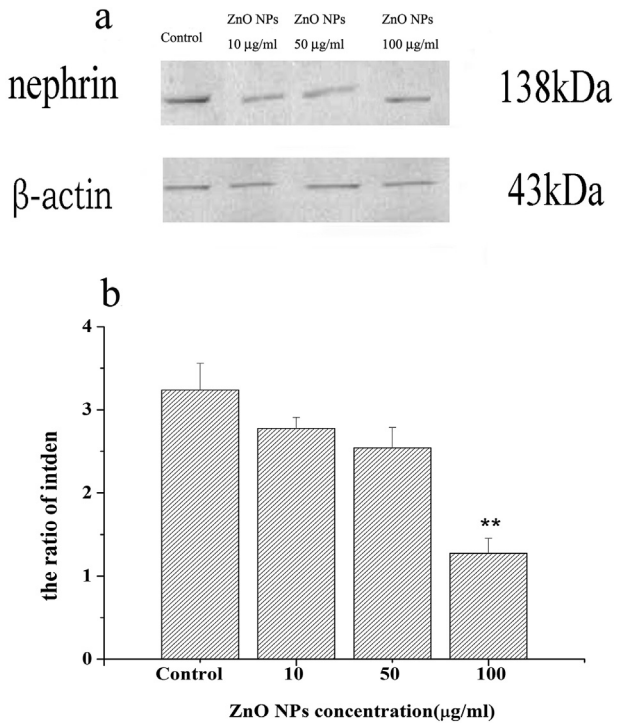

Fig. 5. Immunoblotting analysis of nephrin in podocytes. The expression of nephrin and β-actin were detected by Immunoblotting in podocytes. Cells treated with different concentrations of ZnO NPs (10 mg/ml, 50 mg /ml and 100 mg/ml) for 6 h. (a) Representative picture of protein expression of nephrin and β-actin; (b) Quantitative analysis of protein expression of nephrin. Data were presented as mean ± S.E.M. of three independent experiments. **P.

demonstrated that there was a statistical significance between different groups. As a result, it could be concluded that ZnO NPs induced the apoptosis and decreased the cell viability of podocytes. In acute toxicity study, administration of ZnO NPs for 5 days resulted in the decreased body weight and kidney index of rats (Fig. 6), it preliminary suggested that ZnO NPs presented kidney toxicity.

The production of free radicals has been discovered in a diverse range of nanomaterials caused damage, which can be one of the primary mechanisms of toxicity in nanoparticles (Nel et al., 2006). It may result in the oxidative stress, inflammation and consequent damage to proteins (Donaldson and Stone, 2003). Thus, in our in vitro study, we investigated the level of ROS, the damage to the respiratory chain to trigger the apoptotic process, and SOD, one of the antioxidant marker enzymes against ROS. It can be observed that the contents of ROS were increased significantly (Fig. 3). Meantime, depletion in the SOD level was found on 6 h exposure to different concentration-treated cells [Fig. 4(a)]. These indicated a condition of oxidative stress due to imbalance in the ROS formation and antioxidant defence system of cells. Oxidation of lipid stimulation can also produce the end-products of lipid peroxidation, such as MDA (Termini, 2000). As a result, the formation of MDA is determined as another symbol of oxidative stress. In order to evaluate the consequence of oxidative stress induced by ZnO NPs from another aspect, the formation of MDA was measured. An increase in the formation of MDA was observed compared to that of control group [Fig. 4(b)], indicating lipid peroxidation on exposure to ZnO NPs. On the other hand, in our in vivo study, we detected both of the catalase activity and SOD activity (Fig. 7). Our data showed that ZnO NPs significantly reduced the activity of catalase and SOD, which indicated that administration of ZnO NPs for 5 days could obviously disturb the balance of antioxidant system in vivo, these results were consistent with the results of the in vitro study.

Our results also demonstrated that ZnO NPs caused statistically significant protein damage on podocytes at different concentrations after the exposure period of 6 h (Fig. 5). We detected a protein that is essential to the normal function of podocytes, nephrin. It was found that when treated with the concentration of 100 μg/ml, there was a dramatically protein loss in podocytes. Previous study demonstrated that the deletion of nephrin in mice (Rantanen et al., 2002; Hamano et al., 2002), resulted in protein leak. So the loss of nephrin may result in the failure of the filtration process and lead to the unsuccessful urine formation process. As there is a well-documented link between nanoparticles and oxidative stress, one of the possible modes that can be suggested for ZnO NPs induced protein damage may be lipid peroxidation and oxidative stress (Xia et al., 2006). At the same time, Vyom Sharma et al. reported that ZnO NPs had a DNA damaging potential to human epidermal cells (Sharma et al., 2009). This suggested that ZnO NPs may interfere in the protein synthesis process, which requires the completeness of the DNA. It may further explain the damage caused by ZnO NPs.

5. Conclusion

In conclusion, the present study indicated that ZnO NPs induced

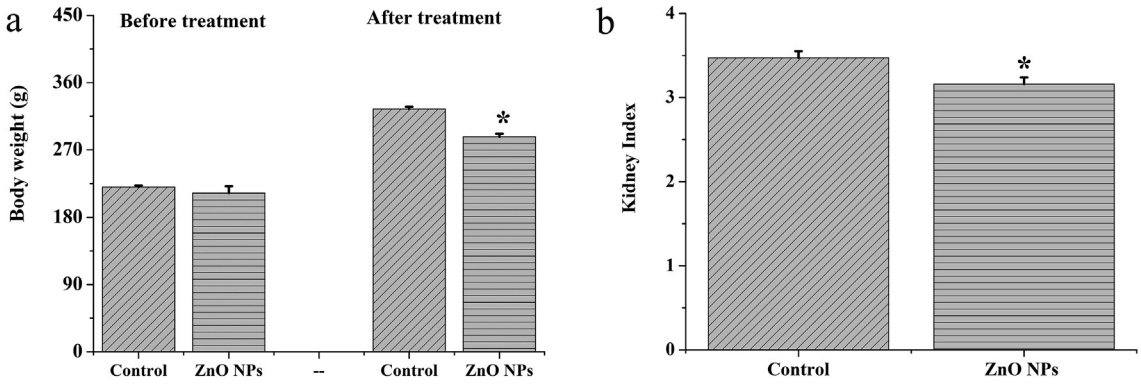

Fig. 6. Analysis of body weight and kidney index of rats. Adult male Wistar rats were treated with ZnO NPs 3 mg/kg/day for 5 days, and the body weight (a) and kidney index (b) were detected. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, comparison with Control group.

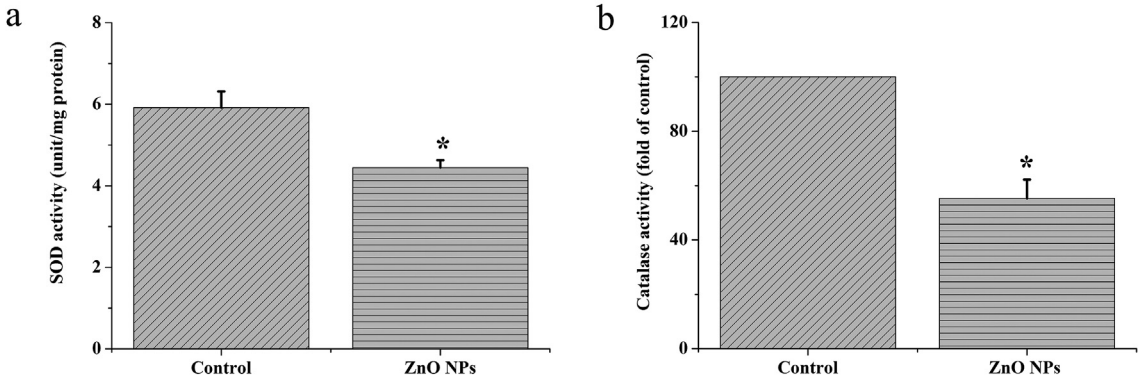

Fig. 7. The level of SOD and catalase in kidney cortex. The level of SOD and catalase in kidney cortex were analyzed. (a) The level of SOD; (b) The level of catalase. Data are expressed as mean ± S.E.M. $^{*}P < 0.05$, comparison with Control group.

kidney toxicity via oxidative stress, which accompanied with the elevated level of ROS, MDA and decreased level of SOD in vitro and in vivo. At the same time, we discovered that ZnO NPs was harm to nephrin, one essential protein of podocytes, which may lead to a direct damage to the SD. In conclusion, ZnO NPs can harm kidney via oxidative stress. These may give us more useful information on the impact of ZnO NPs on human health.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by grant from the National Natural Science Foundation of China (31271074, 31400986, 81571804) and the National Basic Research Program of China (2011CB944003).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.02.002.

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