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Nephrotoxic effects of low-dose zinc oxide nanoparticles in rats

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ABSTRACT

Background: The use of zinc oxide nanoparticles (ZNPs) in various products such as biosensors, sunscreens and nourishment added substances is increasing.

Objectives: In the present work the impacts of ZNP on kidney of rats was explored.

Materials and Methods: In this experimental study male Wistar rats were used. Trial groups received 5, 50 and 300 mg/kg ZNP (ZNP-1 to ZNP-3) for 2 weeks. Control group received only normal saline. blood urea nitrogen (BUN), creatinine (Cr) and uric acid were considered as biomarkers to indicate nephrotoxicity. To evaluate the oxidative stress in the kidney, malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) enzymes activity were measured. Histopathological and apoptotic cell death was also evaluated in renal tissues.

Results: Blood density of zinc (Zn) was significantly increased in the low dose-treated rats. Concentration of Zn in the renal tissue was significantly increased in the high dose treated rats. Weight of kidney was significantly elevated in both ZNP-1 and ZNP-2 groups. ZNP resulted in a significant increment in MDA content in the low measurements and a critical reduction in the activity of the SOD and GPx enzymes. Exposure of ZNP at low dose induced a significant elevation in blood concentration of BUN, Cr and uric acid. Treatment with low dose of ZNP caused a significant increase in histological changes and apoptotic index. ZNP at the high dose induced poor nephrotoxicity.

Conclusion: In conclusion, ZNP has dose-dependent nephrotoxic effects on rats and lower doses have more toxic action.

Implication for health policy/practice/research/medical education:

In this study we demonstrated that zinc oxide nanoparticles (ZNPs) dose-dependently induced nephrotoxicity by elevation blood biochemical markers and generation of stress oxidative. ZNPs dose-dependently induced structural changes and increased apoptosis in renal tissue. Lower doses of this nanoparticle have more nephrotoxicity effects. The results of this study highlighted the requirement for alert during the utilization of ZNPs to prevent health impacts.

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1. Background

Compared with micron-sized particles, nanoparticles (NPs) due to its diverse physicochemical properties (size and surface modifications) are absorbed by the respiratory system, skin, and gastrointestinal tract (1). It has been accounted for that NPs accumulate in liver, kidneys, brain and spleen of rodents (2-5). Metal NPs are broadly utilized as a part of different businesses. Their small size and unique physico-chemical properties

increase their application (6-8). NPs are mainly used in cosmetic and sunscreen products (9). Zinc oxide nanoparticle (ZNP) is the primary segment of sunscreens (10). There is no detailed information in the literature about the everyday exposure amount of ZNP in human. However, the expanding utilization of ZNP can increase the health risk of people exposed to this substance. Although ZNP mainly is used as cosmetics, it cannot absorb through the skin (11-13). However, small pieces

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of lipstick and sunscreen may be accidentally ingested. This NP may also be swallowed directly through food bundling or medication delivery (14,15). Emamifar et al demonstrated that zinc (Zn) ions considerably migrate in low-density polyethylene (LDPE) nanocomposite packaging (16).

When releasing into the surroundings, it might enter the body via the food way (17). ZNP dissolves in the stomach and converts to the Zn ions and enters into the bloodstream (18). De Louise indicated that ZNP is nontoxic to dermal fibroblasts of human in vitro (19). Other studies show that ZNP has toxic effects on dermal fibroblast, neuroblastoma cells and endothelial cells (20-22). Sharma et al have demonstrated that apoptosis and oxidative stress occur on ZNP in human liver cells (23). However, insufficient information is available in the literature to fully understand the toxic potential of ZNP particularly on kidney.

2. Objective

The present study aimed to examine the toxic and apoptotic effects of ZNP on kidney of rats.

3. Materials and Methods

3.1. Animals

Thirty-two, healthy male Wistar rats (160-180 g, 8-12 weeks old) were used. The rats were obtained from Experimental Research Center of Ahvaz Jundishapur University of Medical Sciences. The rats were exposed to 12 hours of darkness and 12 hours of light, with a relative humidity of $50 \pm 5\%$ and $22 \pm 3^\circ\text{C}$ condition. The rats have free access to commercial food (pellet) and water.

3.2. Experimental design

The rats were casual divided into 4 groups. Control group was given 0.2 mL normal saline for 2 weeks. Trial groups received 5, 50 and 300 mg/kg ZNP respectively (ZNP-1, 2 and 3 groups) for 2 weeks (24). The stock solution of (ZNP) sigma was set up in Milli-Q water and scattered for 15 minutes by utilizing a sonicator. The stock solution was maintained at 4°C and used within 7 days for the examination. Exactly before administration, the stored solution was diluted and ultrasonicated (Ultrasonic FS-14; Fisher) for 20 minutes to prevent assembly. The time interval from ultrasonication to oral gavage was less than 20 minutes to guarantee non-conglomeration of the NPs. The particle size and prepared morphology of the NPs was analyzed by using an atomic force microscopy (AFM).

Twenty-four hours after the last administration, blood tests were gathered, the animals were euthanized and their kidneys were expelled and weighed. Left kidneys

were maintained in -80°C for assessments of MDA levels, SOD and GPx activities, and Zn contents. Right kidneys for histological evaluation were expelled in 10% formalin.

3.3. Zn content examination

As previously mentioned, 0.5 g of kidney tissue is stored in nitric acid overnight. Condensed nitric acid and perchloric acid blend (6:1) was included and warmed at $80-90^\circ\text{C}$ until the point when the arrangements were dismal. A nuclear ingestion spectrophotometer (ZEEnit 700 P, USA) was utilized to analyze the Zn content. The blood tests were gathered and centrifuged for 15 min at 3000 rpm to assess the plasma Zn substance (25).

3.4. Biochemical experiments

The blood samples (from tail vein) were gathered in heparinized tube and centrifuged. The plasma concentrations of blood urea nitrogen (BUN), creatinine (Cr) and uric acid were determined spectrophotometrically by using accessible kits (Sigma).

3.5. Assessment of lipid peroxidation

Level of lipid peroxidation in renal tissue was measured as already described (26). Briefly, the kidney tissues were homogenate and 500 μL of supernatant from each sample was added to 1.5 mL trichloroacetic acid (10%) and centrifuged for 15 minutes at $5000\times g$. Then, 1.5 mL of the supernatant was mixed with 2 mL TBA (0.67%) and boiled for 0.5 hours. After cooling, 2 mL n-butanol was added to each sample and centrifuged at $5000\times g$ for 20 minutes. The absorbance was read at 535 nm by a spectrophotometer.

3.6. Superoxide dismutase enzyme activity

The SOD enzyme action was measured by utilizing Ransod unit (Randox Labs, UK). In this method, a water-soluble formazan dye upon reduction with superoxide anion is produced. The price of the diminishment with a superoxide anion is directly identified with the xanthine oxidase (XO) action and is prevented by SOD. The inhibition activity of SOD can be determined by a spectrophotometer at 505 nm (26).

3.7. Glutathione peroxidase enzyme activity

GPx action was assessed utilizing the Ransel unit (Randox Labs., UK) as already described by Paglia et al (27). In this strategy, glutathione peroxidase (GPx) diminishes the cumene hydroperoxide test while oxidizing GSH to GSSG. The made GSSG is diminished to GSH with utilization of NADPH by glutathione reductase. The abatement of NADPH is corresponding to GPx activity and easily measured by a spectrophotometer at 340 nm.

3.8. Histology changes

In this examination, 6 microscopy of hematoxylin and Eosin recolored slides per animal were analyzed for assessment of histological components, for example, nuclear pyknosis, collection of inflammatory cells, brush border loss and gathering of red platelets (RBC). The normal rate of every criteria were dictated by isolating the quantity of tubules with a histologic criteria, for example, brush border loss, nuclear pyknosis and cytoplasm swelling and in a randomly microscopic field by the aggregate number of tubules in a similar field and the outcome duplicated by 100.

Permeation of inflammatory cells and blockage of RBCs were evaluated into 4 classes: normal (0), weak (1), moderate (2) or intense (3) and the averages were considered (28).

3.9. TUNEL assay

TUNEL staining was performed using an Intracellular Cell Detection Kit, POD (Invitrogen, Germany). Paraffin slides were incubated in proteinase K for 0.5 hours at 24°C. The sections were then washed with PBS (buffer phosphate solution) and incubated for 1 hour at 37°C with a TUNEL reaction mixture. After washing by PBS, the sections were incubated with anti-fluorescein-AP for 30 minutes at 37°C. Then, the slides were washed in deionized water and incubated with DAB substrate for 5 minutes. Intense, dark brown and homogenous stained nucleus was considered as TUNEL-positive cells. Percentage of TUNEL-positive cells (apoptotic index) was calculated in 10 randomly field for each slide (24). Three slides/animal were used for this method.

3.10. Ethical issues

The research followed the tenets of the Declaration of Helsinki. This examination was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (# IR.AJUMS.REC.1395.404). Prior to the trial, the protocols such as animal care, nutrition and prescribing method, anesthesia and euthanasia procedures were confirmed to be in accordance with the guidelines of the moral committee of this university.

3.11. Statistical analysis

A one-way analysis of variance (ANOVA) and then post hoc LSD or Tukey tests were used to data analysis. Data were expressed as the mean \pm standard deviation (SD). A P value less than 0.05 was considered statistically significant.

Results

4.1. Characterization of ZNP

The size and morphology of the ZNP were uncovered

by AFM. As can be observed in the Figure 1, the NPs indicated circular morphology and a molecule measure appropriation practically homogeneous, with a mean size that is substandard compared to 100 nm.

4.2. Organ weight

In the ZNP-1 (low dose treatment) group, body weight was significantly decreased in comparison to the control group ($P < 0.05$), while renal weights were significantly increased in this group. Kidney and body weights were not significantly changed in ZNP-2 group. A significant increase in renal weight was observed in the high dose-treated animals ($P < 0.05$), while body weight in these animals did not change significantly (Table 1).

4.3. Absorption of ZNP

As shown in Figure 2, Zn concentration in blood was significantly incremented in the low dose-treated rats (ZNP-1 group). Zn contents of renal tissue were not significantly changed in this group. Zn concentration in blood in the ZNP-2 group did not change significantly, while Zn content of renal tissue was slightly higher in this group. Zn concentration in blood was slightly increased in high dose-treated rats (ZNP-3 group), while contents of Zn in renal tissue were significantly elevated compared to the low dose-treated rats or control group ($P < 0.01$).

4.4. Biochemical tests

A significant increment in blood amounts of BUN, Cr

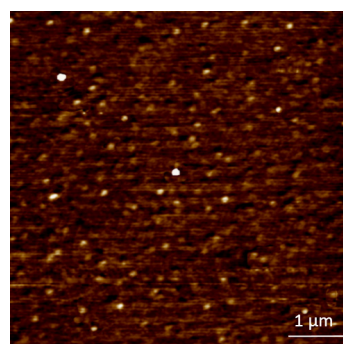


Figure 1. AFM image of ZNP nanoparticles showed spherical morphology and a particle size distribution almost homogeneous with dimensions of less than 100 nm.

Table 1. Kidney and body weight for control and experimental groups

Group	Body weight (g)	Kidney weight (mg)
Control	256.3 \pm 19.3	0.82 \pm 0.12
ZNP-1	213.6 \pm 13.8*	1.32 \pm 0.21*
ZNP-2	242.4 \pm 17.5	0.78 \pm 0.10
ZNP-3	249 \pm 19.7	1.21 \pm 0.15*

Values expressed as mean \pm SD for 8 rats.

* $P < 0.05$. * indicates comparison to the control group.

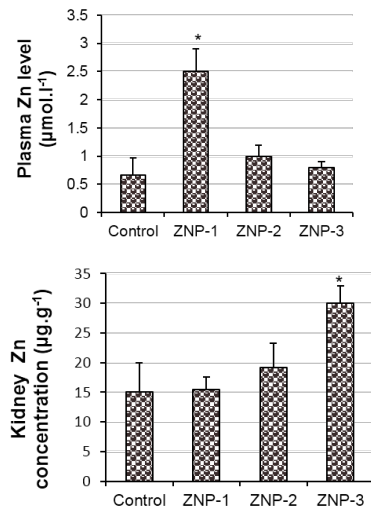


Figure 2. Plasma and kidney concentrations of Zn in control and experimental groups. Values are expressed as mean \pm SD for 8 rats. * $P < 0.01$, ** $P < 0.001$. * indicates comparison to the control group.

and uric acid in the low dose-treated rats was observed ($P < 0.001$). In the ZNP-2 group, blood levels of biomarkers were significantly increased ($P < 0.01$). The biochemical tests were not significantly changed in the high dose-treated animals (Figure 3).

4.5. MDA level, SOD and GPx activities

A significant increment in the kidney amount of MDA was observed in the low dose-treated rats. GPx and SOD activities were significantly reduced in this group ($P < 0.001$). The GPx and SOD activities and MDA contents were significantly changed in the ZNP-2 group ($P < 0.05$). No significant change in MDA contents, SOD and GPx activities was observed in high dose-treated rats (Figure 4).

4.6. Histological assessments

All kidney sections revealed a normal appearance in control group. Kidney structure was damaged in ZNP-1 group. Histological criteria such as brush border loss, pyknosis in proximal cells, and accumulation of RBCs and leukocytes were significantly increased in low dose-treated animals ($P < 0.001$). The histological criteria were significantly reduced in ZNP-2 group in comparison to the low dose-treated rats. In the high dose-treated rats, the histological criteria were not significantly changed in comparison to the control (Table 2 and Figure 5).

4.7. TUNEL staining

In the control group, spontaneous apoptosis was observed in some normal tubules. In the low dose-treated rats, apoptosis was observed in many proximal tubules and percentage of apoptosis was significantly

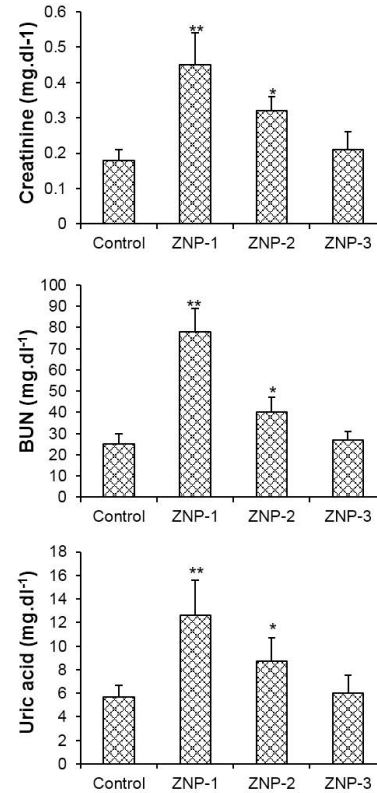


Figure 3. Biochemical tests of control and experimental groups. Values are expressed as mean \pm SD for 8 rats. * $P < 0.01$, ** $P < 0.001$. * indicates comparison to the control group.

increased ($P < 0.001$). The apoptotic index In the ZNP-2 group was also significantly higher than the control group ($P < 0.01$). In the high dose-treated rats, no significant alterations in percentage of apoptosis were observed (Figures 6 and 7).

5. Discussion

Our results have demonstrated that ZNP dose-dependently induces nephrotoxicity by generation oxidative stress, histological changes and apoptosis. Low dose of ZNP induced more nephrotoxicity. The exact mechanism of this finding is unclear. At high concentrations, NPs tend to generate aggregations with a diameter of more than 100 nm. These large NPs can be easily removed by macrophages (29).

Previous studies revealed that heavy doses of various NPs such as iron, silver or titanium dioxide aggregate in larger particles after inhalation or intra-tracheal instillation. These large aggregates are removed by the macrophages (29,30). Therefore, low ZNP concentrations with smaller than 100 nm may release more than high concentrations to bloodstream and different tissues (and its toxicity). As indicated in the results, plasma Zn levels at lower doses in ZNP-treated animals increased significantly.

Our findings are in agreement with those of Pasupuleti

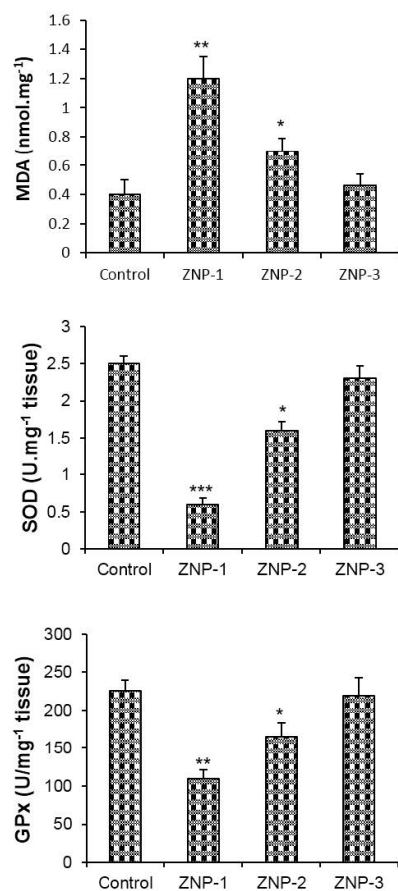


Figure 4. MDA level, SOD and GPx activities of control and experimental groups. Values are expressed as mean ± SD for 8 rats. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. *indicates comparison to the control group.

et al, in which the lower doses of ZNP had more toxicity effects in comparison to the higher doses (31). On the other hand, in the study of Talebi et al, ZNP at the higher doses had more toxic effects on the mouse germ cells in compare to the lower doses (24). A study revealed that ZNP had no cytotoxic effects on astrocytes and hepatocytes of normal rat (32). In contrast, Guan et al reported that ZNP has cytotoxic effects on L02 (human liver cells) and HEK293 (derived from human embryonic kidney cells) cell lines (33). Khorsandi et al, have also demonstrated hepatotoxicity of ZNP in rats (34). Interestingly, oxidative stress and apoptosis can be induced by ZNP in vitro (35).

Table 2. Histology assessments in control and experimental groups

Histology criteria	Control	ZNP-1	ZNP-2	ZNP-3
Pyknosis (%)	0.11 ± 0.09	4.9 ± 0.83**	1.8 ± 0.62*	0.21 ± 0.05
Brush border loss (%)	0.23 ± 0.08	15.2 ± 1.3**	4.6 ± 0.81**	0.34 ± 0.04
Congestion of RBCs	0.06 ± 0.01	3.1 ± 0.33**	1.9 ± 0.43*	0.09 ± 0.01
Infiltration of leukocytrs	0.00 ± 0.00	2.3 ± 0.35	1.2 ± 0.12**	0.00 ± 0.00

Values are expressed as mean ± SD for 8 rats. **P* < 0.01, ***P* < 0.001. *indicates comparison to the control group.

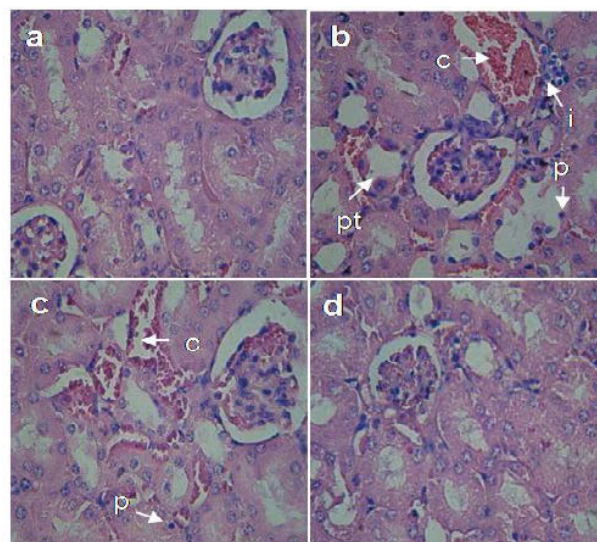


Figure 5. Light microscopy of cross sections of H&E stained kidneys from control and experimental groups. (A) Control groups; (B) ZNP-1 group; (C) ZNP-2 group. (D) ZNP-3 group. i: infiltration of leukocytes, p: pyknosis, pt: proximal tubule, c: Congestion of RBC. Magnification: ×400.

Toxic effects of NPs depend on various factors such as existing disease, genetics, exposure duration, sizes and agglomeration state (36,37). Inhaling carbon nanotubes cause acute inflammatory responses in the mice lungs, but this does not happen in the rats. (38,39). These studies suggest NPs have species-dependent toxicity. As shown in the result, ZNP has been able to significantly increase the measures of biomarkers (BUN, Cr, and uric acid). The plasma measures of each of these markers are susceptible to any type of kidney disorder. When kidney damages, these biomarkers (which are inside the proximal cells of nephrons) release into the bloodstream. Hence, elevation concentration of them indicates proximal cells destruction (28). As revealed in histological assessments, proximal tubules were damaged in low dose treatment of ZNP.

Renal weights were significantly increased in both low and high doses treated rats. The increase in the kidney weight may be due to congestion of RBCs, infiltration of leukocytes and accumulation of ZNP. In the high dose-treated rats, Zn concentration of kidney was significantly more than control while histopathological alterations were not observed in the kidney of this animals. Thus,

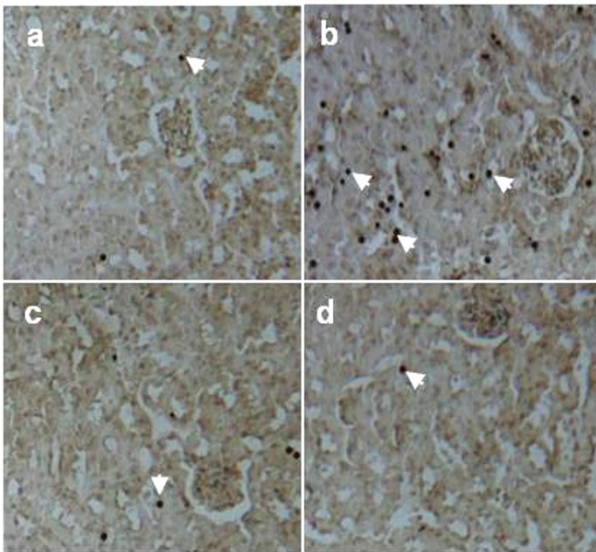


Figure 6. Light microscopy of cross sections of TUNEL stained kidneys from control and experimental groups. A, Control groups; B, ZNP-1 group; C, ZNP-2 group. D, ZNP-1 group. Arrows indicate TUNEL-positive cells (Magnification; $\times 250$).

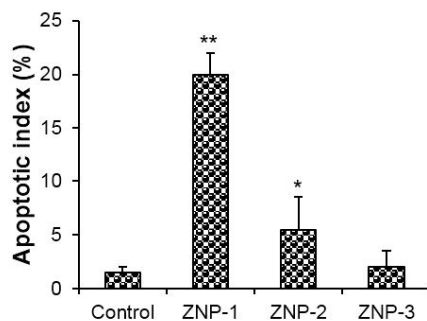


Figure 7. Apoptotic index of control and experimental groups. Values are expressed as mean \pm SD for 8 rats. * $P < 0.01$, ** $P < 0.001$. *indicates comparison to the control group.

the increase in the renal weight of high dose-treated rats may be associated with Zn aggregation in this tissue. Proximal tube defects, RBC congestion and leukocyte leakage indicate the destructive effects of ZNP in low doses on renal tissue. In addition, the accumulations of RBCs and inflammatory cells may be a reason for increasing the kidney weight.

In this research, the activity of the SOD and GPx enzymes has been reduced and the increase in MDA levels in the kidney tissues is indicative of oxidative stress and induces lipid peroxidation with a low dose of ZNP. Sharma et al showed that ZNP can damage DNA and cell toxicity in HepG2 cells through induction of oxidative stress (25). Xiao et al showed that administration of 3 mg/kg of ZNP for 5 days induced elevation of MDA and reduction in SOD enzymes activity in rat livers (42). In addition to necrosis cell death, apoptosis is also seen in the kidney of the animal after treatment with ZNP.

Necrosis and apoptosis are dependent process (40), specifically, when apoptosis includes a large number of cells (41). Wilhelm et al reported that after treatment with ZNP, cell death of apoptosis and necrosis occur together in macrophages (42). Park et al revealed that ZNP had the highest cytotoxic effect on human cancer cells (A549) among six different types of NPs, which was identified by DNA analysis and apoptosis studies (43). Apoptotic effects of ZNP in human dermal fibroblasts have reported by Meyer et al (20). Roy et al have also showed apoptotic effect of ZNP in macrophages (44). Toxic effects of ZNP on kidney have also reported in some previous studies (43). Xiao et al showed that ZNP had toxic effects on podocytes (45). Yan et al reported that 1000 mg/kg ZNP causes mitochondria and cell membrane impairment in kidney of rats (46).

6. Conclusions

In the present study, we found that lower doses of ZNP exhibited nephrotoxicity and induced apoptosis in proximal cells of rats. The exact mechanism of ZNP-induced nephrotoxicity is not obtained from this study. Future studies are needed to clarify the signaling pathways of apoptosis in ZNP-treated animals.

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Authors' contribution

All the authors contributed to laboratory work and involved in the preparation of the manuscript. LK contributed to statistical analysis and preparation of the abstract. ZJ was involved in the preparation of the abstract and background. AHM participated in the preparation of statistical analysis, results, and discussion and also conclusion parts of the paper. All the authors have approved the latest version of manuscript.

Conflicts of interest

The authors declared no conflicts of interest.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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