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Toxicity of zinc oxide nanoparticles on adult male Wistar rats

Roghayeh Abbasalipourkabir [a, *], Hemen Moradi [b], Sadegh Zarei [c], Soheila Asadi [a], Aref Salehzadeh [d], Abolfazl Ghafourikhosroshahi [e], Motahareh Mortazavi [f], Nasrin Ziamajidi [a]

[a] Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[b] Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Clinical Biochemistry, School of Medicine, Yazd University of Medical Sciences, Yazd, Iran
[d] Department of Entomology and Toxicology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[e] Department of Medicinal Chemistry, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran
[f] Department of Anatomical Sciences, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

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ABSTRACT

The purpose of this study was to investigate the effects of zinc oxide nanoparticles (nZnO) on adult male Wistar rats.

Thirty male Wistar rats divided into five groups of six animals each were used for this study. For ten days, Groups one to four continuously received 50, 100, 150 and 200 mg/kg nZnO, respectively. Group five served as the control group. At the end of the study, the rats were sacrificed and histopathological study of the liver and renal tissue, sperm analysis, serum oxidative stress parameters and some liver enzymes were done.

The results of this study showed that nZnO at concentration more than 50 mg/kg lead to significant changes in liver enzymes, oxidative stress, liver and renal tissue and sperm quality and quantity.

In conclusion, the toxicity of nZnO is more significant when the concentration is increased; however, the use of low doses requires further investigation.

1. Introduction

Nowadays, nanotechnology has a vast range of application in diagnosis, drug delivery, food industry, paints, electronics, sports, environmental cleanup, cosmetics, and sunscreens (Al-Suhaibani and El-Morshedi, 2014). Zinc oxide nanoparticles (nZnO) are semiconductor metal oxide nanoparticles that are widely used in biomedical fields as an anticancer drug, a tool for imaging biological systems, and also in cosmetics. Due to its antimicrobial and fungicidal properties, it is used in the food industry and agriculture. Interaction of nanoparticles with biological systems has some unpredictable results, thus understanding their toxicity is essential to prevent their harmful effects on the human body (Sharma et al., 2012; Gerloff et al., 2009; Jin et al., 2009; Rasmussen et al., 2010; John. et al., 2010).

Although there are some reports about oxidative stress of nanoparticles, yet it is not clear how oxidative state could make cells more sensitive to cytotoxic nanoparticles. Oxidative stress would be increased in some pathological situations such as inflammation. Hence, it is important to know how oxidative stress could change the sensitivity of cells to cytotoxic nanoparticles (Heng et al., 2010). Some special features such as high surface area, having 1–100 nm in size, and easy penetration into the cells and proteins, sensing, and detection of biological environments, make inorganic nanoparticles as potential candidate for applications in biomedical fields (Wahaba et al., 2009). As a result of the vast usage of nZnO in different areas, investigating its toxicity is critical, especially in bacterial and mammalian cells (Wang et al., 2010).

In vitro and *in vivo* studies have shown that ZnO nanoparticles have the following toxic effects on the mammalian cell: membrane damage, inflammation, DNA damage, apoptosis and the other effects including the complex cell-cell and cell-matrix interactions and changes in some hormones (Gojova et al., 2007; Jeng and Swanson, 2006; Yang et al., 2009; Osman et al., 2010; Sharma et al., 2011; Fischer and Chan, 2007). ZnO nanoparticles can enter

* Corresponding author. Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, 65176 Hamadan, Iran.
E-mail addresses: abbasalipourkabir@yahoo.com, Abbasalipourkabir@umsha.ac.ir (R. Abbasalipourkabir).

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the body through the gastrointestinal or respiratory system and reach the blood or organs such as the liver (Hussain et al., 2001; Oberdorster et al., 2005). *In vivo* study of ZnO nanoparticles is necessary to understand their long term effects on biological systems (Fischer and Chan, 2007).

The objective of the current study was to investigate the effect of nano ZnO on oxidative stress status in adult male Wistar rats by measuring Maolnedialdehyde (MDA) as an index of lipid peroxidation, total oxidant status (TOS), total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPX), liver enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as a function of the liver, sperm quality and quantity and histopathological changes in the liver and renal tissue.

2. Materials and methods

2.1. Materials

Zinc oxide nanopowder was purchased from Iranian Nanomaterials Pioneers Company, NANOSANY (Mashhad, Iran). Its characteristics are presented in Table 1 and transmission electron micrograph (TEM) and crystal characteristics of ZnO nanoparticles are shown in Figs. 1 and 2, respectively.

2.2. Preparation of nanoparticle suspension

A stock suspension was prepared by suspending ZnO nanoparticles in bi-distilled water.

2.3. Experimental design and procedure

Thirty adult male Wistar rats aged 6–8 weeks, weighing 180–200 g were purchased from Pasteur Institute of Iran, IRAN. The animals were housed two rats per plastic cage and allowed to acclimatize under standard conditions (12 h light/dark cycles) for one week. The rats were given free access to distilled water and commercialized food throughout the experiment. The animals were divided into five groups of six animals each. Groups one to four received a dose of 50, 100, 150 and 200 mg zinc oxide nanoparticles (nZnO)/kg body weight and assigned nZnO50, nZnO100, nZnO150 and nZnO200 groups, respectively. The rats were injected intraperitoneally daily for ten days. The control group was injected with bi-distilled water and the effect of shock injection is the same in all groups. At the end of the study, the rats were scarified and blood samples were collected from healthy control and ZnO nanoparticles treated groups by cardiac puncture, using 23 or 26 G needles. The blood samples were allowed to clot at room temperature and centrifuged at 1000 g for 10 min, and serum were separated and analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), glutathione peroxidase (GPX) activities and measurement of Maolnedialdehyde (MDA), total antioxidant capacity (TAC) and total oxidant status (TOS). The rats were also studied for histological examination of liver and renal tissue and sperm analysis. The experimental procedure was approved at the Faculty of Medicine at Hamadan University of Medical Sciences (UMSHA) and the research was conducted according to the guidelines for the care and use of laboratory animals of UMSHA.

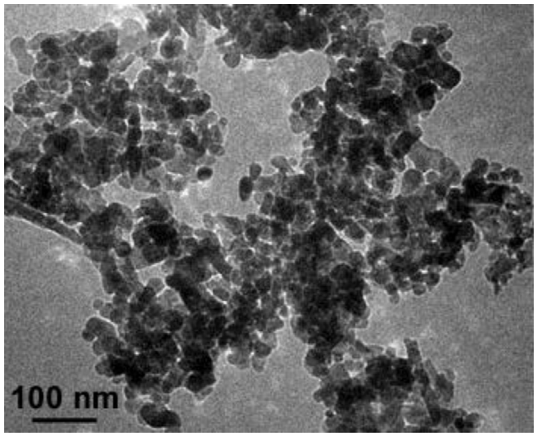

Fig. 1. Transmission Electron Micrograph (TEM) of zinc oxide nanoparticles from Iranian Nanomaterials Pioneers Company, NANOSANY.

2.4. Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide. The method was carried out following the procedure of Marklund and Marklund (1974). In this method, one unit of SOD activity is defined as the amount of enzyme required to inhibit the autoxidation of pyrogallol by 50%. Autoxidation of pyrogallol was quantified at 420 nm for 3 min. Therefore, the rate of decreased optical density between the 1st and 3rd min was expressed as enzyme activity in a UV/Vis Spectrophotometer (Unico S2100, USA) with recorder.

2.5. Measurement of glutathione peroxidase activity

Glutathione peroxidase (GPX) activity was determined based on the potential of the enzyme to reduce H_2O_2. According to the study of Paglia and Valentine (1967), GPX converts H_2O_2 to H_2O and catalyzes GSH to GSSG simultaneously. The optical density of the final mixture was read at 340 nm in UV/Vis Spectrophotometer (Unico S2100, USA) with recorder. Decrease of OD was expressed as enzyme activity, U/L.

2.6. Measurement of MDA, TAC and TOS

Total antioxidant capacity (TAC) in serum samples was assessed using ferric reducing antioxidant power assay (FRAP) (Benzie and Strain, 1999). Maolnedialdehyde (MDA) as a lipid peroxidation index was determined using fluorometric thiobarbituric acid method (Botsoglou et al., 1994). The oxidation of ferrous ion to ferric ion accompanied with a number of oxidant species in acidic pH was used for the measurement of total oxidant status (TOS) in serum. The ferric ion was determined by xylenol orange (Erel, 2005).

Table 1
Characteristics of zinc oxide nanoparticles.

Certificate of analysis	Content ZnO%	Cu	Mn	Cd	Pb
	≥99%	≤3 ppm	≤5 ppm	≤9 ppm	≤9 ppm
Particles size nm	Specific surface area m^2/g	Color	Crystal phase	Crystal Morphology	True density g/cm^3
10–30	20–60	Milky white	Single	Nearly spherical	5.606

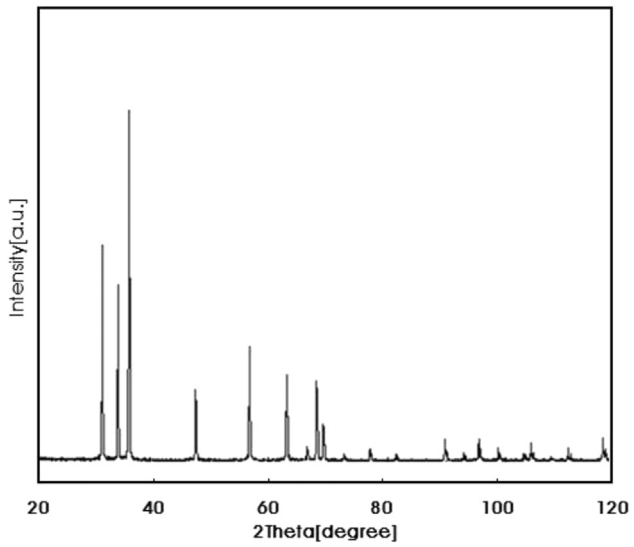

Fig. 2. X-ray diffraction (XRD) of zinc oxide nanoparticles from Iranian Nanomaterials Pioneers Company, NANOSANY.

2.7. Analyzing the enzymes

Animal serum was analyzed for alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using standard diagnostic Pars Azmun Kit (Iran).

2.8. Sperm analysis

At the end of the study after anesthetizing the animals and removing the epididymis, sperm analysis was performed. The sperm count was assessed using a haemocytometer. Also, the qualitative and quantitative morphology and motility of sperms were observed with a light microscope (Ziess, 015447, Germany) and their vitality was studied with Eosin dye. Then, the percentage of live sperms to the total sperms was evaluated with a magnification of 40. The dead sperms were observed in reddish color. The results are expressed as mean ± standard deviation and analyzed using SPSS statistical software and One-Way ANOVA test was analyzed at a significant level of less than 0.05.

2.9. Histopathological study

At the end of the study, the rats were sacrificed and examined for tissue abnormalities. Samples of liver and kidney from all groups were immediately fixed in 10% formalin overnight, embedded in paraffin, cut into 5 μm sections, placed on slides and stained with Hematoxylin-Eosin (H&E). The tissue sections were viewed under a light microscope (Nikon Y-S100, German).

2.10. Statistical analysis

The data were expressed as mean ± standard deviation. For statistical analysis, the experimental values were compared to their corresponding control. One-way analysis of variance (ANOVA) in SPSS software (Version 16.0) was used to illustrate the significant difference between the experimental and control groups. The significant difference was considered to be $p < 0.05$ or less.

3. Results

3.1. Effect of increasing concentration of zinc oxide nanoparticles on oxidant and antioxidant parameters

In this study, blood samples were obtained from the rats at day 10 upon treatment and were used to determine biochemical parameters. The effect of zinc oxide nanoparticles on the oxidant and antioxidant parameters are shown in Table 2. As shown in the table, although there was no significant increase in SOD and GPX activity in all experimental groups compared to the control and between them, an increment was observed in the SOD and GPX activity, when the concentration was increased.

Based on the data in Table 2, treatment with nZnO150 and nZnO200 showed a significant increased peroxidation index of lipids, MDA in comparison with the control and other nZnO groups. Although, nZnO100 increased the MDA level significantly compared to the control group, treatment with nZnO150 and nZnO200 led to a significant increase in MDA compared to the control and nZnO50 groups. Treatment with nZnO50 indicated negligible increase in comparison with the control group.

Total oxidant status (TOS) results are shown in Table 2. While, group nZnO200 showed a significant increase in TOS when compared only to the control group, there were no significant changes in TOS levels. However, treatment with nZnO, lead to increased total oxidant status by increasing the concentration. According to the obtained data presented in Table 2, total antioxidant capacity (TAC) decreased insignificantly in all groups when the concentration of zinc oxide nanoparticles was increased. However, group nZno200 showed a significant reduction in total antioxidant capacity compared to the control group.

3.2. Effect of increasing concentration of zinc oxide nanoparticles on two liver enzymes

The effects of the concentration of a variety of zinc oxide nanoparticles on two liver enzymes are presented in Table 3. As shown, although treatment with ZnO nanoparticles increased ALT

Table 2
Effect of treatment with zinc oxide nanoparticles on rats serum oxidant and antioxidant parameters.

Parameters	Groups					
	Control	nZno50	nZno100	nZno150	nZno200	P value
SOD (U/L)	25.29 ± 9.54	29.49 ± 11.85	40.00 ± 7.86	29.93 ± 15.35	30.81 ± 16.37	=0.570
GPX (U/L)	1.76 ± 0.91	1.08 ± 0.49	2.21 ± 0.63	2.20 ± 0.73	1.15 ± 0.50	>0.05
TAC (mmol/ml)	1.24 ± 0.36	1.06 ± 0.20	1.06 ± 0.14	0.95 ± 0.087	0.77 ± 0.10[a*]	>0.05
MDA (μm/l)	0.34 ± 0.21	0.69 ± 0.40	1.21 ± 0.42[a#]	1.46 ± 0.20[a#,bΔ]	1.70 ± 0.17[a#,b#]	=0.001
TOS (mmol/ml)	1.80 ± 1.44	2.28 ± 0.59	2.44 ± 0.39	2.72 ± 0.15	3.20 ± 0.29[a*]	>0.05

All values are expressed as mean ± standard deviation. *p < 0.05; ΔP < 0.01; #p < 0.001. SOD=Superoxide dismutase; GPX = Glutathione peroxidase; TAC = Total antioxidant capacity; MDA = Malondialdehyde; TOS = Total oxidant status.
[a] Comparing with Control.
[b] Comparing with nZno50.

Table 3
Effect of treatment with zinc oxide nanoparticles on liver enzymes of adult male Wistar rats.

Parameters	Groups					
	Control	nZno50	nZno100	nZno150	nZno200	*p* value
AST (U/L)	64.25 ± 2.21	96.00 ± 3.65[a#]	104.00 ± 3.36[a#,b*]	109.25 ± 2.21[a#,b#]	117.50 ± 3.1[a#,b#,cΔ]	<0.001
ALT (U/L)	77.00 ± 2.27	104.50 ± 12.60	104.50 ± 5.25	111.00 ± 4.96[a*]	117.00 ± 5.71[aΔ]	<0.05

All values are expressed as mean ± standard deviation. *p < 0.05; ΔP < 0.01; #p < 0.001. AST = Aspartate aminotransferase; ALT = Alanine aminotransferase.
[a] Comparing with Control.
[b] Comparing with nZno50.
[c] Comparing with nZno100.

activity in all groups, only nZnO150 and nZnO200 led to a significant increase in ALT activity in comparison with the control group. Based on the results, there was a significant increase in AST activity in all treatment groups compared to each other.

3.3. Effect of increasing zinc oxide nanoparticles concentration on sperm quality and quantity

The effect of zinc oxide nanoparticles on sperm quality are presented in Table 4. According to the obtained results the sperm count and vitality of all groups decreased significantly compared to the control group. Furthermore, while sperm motility decreased by increasing the concentration of zinc oxide nanoparticles, the reduction was not significant at the 50 mg concentration. All levels of zinc oxide nanoparticles had a significant impact on sperm morphology.

3.4. Effect of zinc oxide nanoparticles on the histopathology of liver and kidney

The histopathological features of the liver and kidney are shown in Figs. 3 and 4, respectively. According to Fig. 3 the liver tissue of animals exposed to zinc oxide nanoparticles showed increased Kupffer cells, congestion, inflammation in the liver parenchymal, ballooning, port inflammation and chromatin condensation as a result of apoptosis. Also as shown in Fig. 4, there were pathological changes including the proliferation of glomerular cells, inflammation of interstitial tissue and congestion of glomerulus in kidney of all groups treated with zinc oxide nanoparticles at concentrations above 50 mg/kg body weight.

4. Discussion

Today, the industrial application of ZnO nanoparticles is increasing. As a result of ultraviolet blocking features, ZnO is widely used in sunscreens, cosmetics, and bottle coating (Klaine et al., 2008). Despite all these uses, the possible harmful effects of nanoparticles such as ZnO should be more investigated (Wang et al., 2008).

This study showed that ZnO nanoparticles at concentrations above 50 mg/kg body weight induced significant oxidative stress in adult male Wistar rats. Oxidative stress has been marked a possible mechanism of nanoparticles toxicity (Ahamed et al., 2011). Zinc oxide nanoparticles have been proposed to increase lipid peroxidation and induce oxidative stress, its possible mechanism is involved in the toxicity of nanoparticles (Sharma et al., 2011). In the current study, the potential of ZnO nanoparticles to induce oxidative stress was studied by measuring MDA, TAC, TOS, SOD and GPX in adult male Wistar rats. A significantly increased level of MDA was observed in the serum of the experimental groups treated with ZnO nanoparticles at concentrations above 50 mg/kg body weight compared to the control and nZnO50. The results of this study are comparable to Sharma et al. (2011), who reported that DNA damage in human epidermal cells, is possibly because of lipid peroxidation and oxidative stress. It is suggested that the small size of nanoparticles makes them able to interact with DNA directly (Sharma et al., 2009). According to the study of Hackenberg et al. (2011), DNA damage and inflammation in human nasal mucosa cells *in vitro* due to ZnO nanoparticles even at low concentrations has already been reported.

As shown in Table 2, although in all treatment groups, SOD activity increased and then decreased by increasing the concentration of ZnO nanoparticles, it was not significant, and thus indicates oxidative stress. However, Sharma et al. (2011) reported significantly ($p < 0.05$) reduced SOD activity in cells after 24 h treatment with 0.008, 0.08 and 0.8 μg/ml ZnONPs, compared to unexposed cells (Sharma et al., 2009).

According to the obtained data presented in Table 2, glutathione peroxidase, the other antioxidant enzyme, was reduced in the group treated with higher concentration of ZnO nanoparticles compared to the control group, although it was not significant, showing oxidative stress in the treatment groups. As shown in Table 2, alterations to total oxidant capacity and total oxidant status were significant compared with the control group when animals were treated with 200 mg/kg ZnO nanoparticles, confirming the toxicity of nanoparticles at high concentrations.

Blood biochemical parameters can be useful in the diagnosis of toxicity because they can be easily done in most diagnostic laboratories. Liver disorders can be determined by serological markers such as alanine transaminase, ALT (Wang et al., 2006). The

Table 4
Effect of ZnO nanoparticles on sperm quality and quantity of adult male Wistar rats.

Parameters	Groups					
	Control	nZnO50	nZnO100	nZnO150	nZnO200	*p* value
Count (10^6/ml)	201.6 ± 13.14	136 ± 24.65 [a#]	142.4 ± 13.14 [a#]	143.2 ± 23.73 [a#]	134 ± 8.7 [a#]	<0.001
Motility (%)	70.8 ± 6.7	51.8 ± 5	35.6 ± 14.2 [aΔ]	30.6 ± 5.5 [a#]	32.25 ± 11.34 [a#]	<0.001
Normal Morphology (%)	98 ± 1.4	84.8 ± 6.8 [aΔ]	82.4 ± 1.7 [a#]	81.2 ± 5.9 [a#]	74.75 ± 6.3 [a#/b*]	<0.001
Viability (%)	95 ± 2.7	71.6 ± 6.9 [a#]	74.4 ± 8 [a#]	68.8 ± 2.9 [a#]	71.25 ± 6.7 [a#]	<0.001

All values are expressed as mean ± standard deviation. *p < 0.05; ΔP < 0.01; #p < 0.001.
[a] Comparing with Control.
[b] Comparing with nZno50.

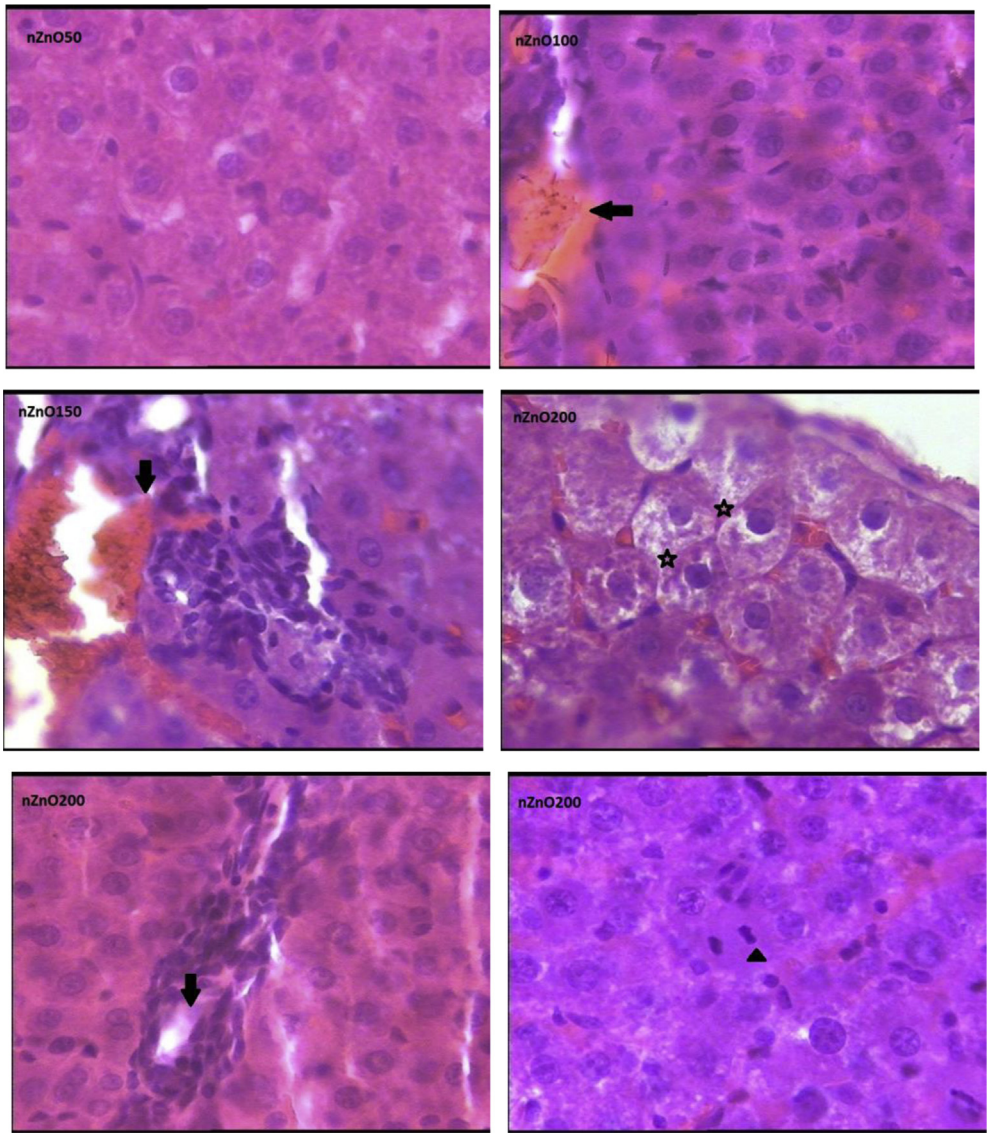

Fig. 3. Histopathological findings of rat's liver after treatment with ZnO nanoparticles for ten days (H&E, x40). (nZnO50) Kupffer cells proliferation, (nZnO100) Kupffer cells proliferation and congestion (arrow), (nZnO150) Liver parenchymal inflammation and congestion (arrow), (nZnO200 Ballooning (stars), (nZnO200) Port inflammation (arrow), (nZnO200) Apoptosis (arrow head).

abnormal increase of serum ALT concentrations, may suggest liver hepatotoxicity (Lynch et al., 2005). The results of this study showed that apart from the nZnO50, and nZnO100 groups, the activity of ALT increased significantly in all groups compared to the control and nZnO50 groups. Although treatment with ZnO nanoparticles at 50 mg/kg body weight had no toxic effect on the liver, its concentration at 100 mg/kg body weight and above produced a dose-dependent toxicity in the liver. Therefore, a high dose of ZnO nanoparticles might decrease its efficiency by increasing its toxicity. Sharma et al. (2011) reported significantly ($p < 0.05$) higher levels of ALT compared to the control mice followed by treatment with 300 mg/kg Zinc oxide nanoparticles. It was suggested that to find liver damage, ALT activity is usually determined with AST (Wang et al., 2006). The current study also reported the impact of ZnO nanoparticles on AST activity in the treatment groups. The statistically significant increased activity of AST in all groups in comparison with the control group may be pointed to the liver damage. Similar results were reported by Wang et al. (2008) that there were insignificant differences in plasma biochemical parameters between the group that was treated with micro-scale zinc powder and the group treated with nano-scale zinc powder. However, this result may be due to the high doses of zinc powder that cause toxic effects in organs such as liver.

The results of the current study showed the impact of zinc oxide nanoparticles on sperm quality in a dose-dependent manner in rats. This observation is similar to that of Talebi et al. (2013) which showed that dose of 50 and 300 mg/kg zinc oxide nanoparticles significantly reduced sperm count, motility and increased abnormality in mice. It was suggested that the toxicity of ZnO particles may increase from Zn ions, diffusion of particles into and their contact with organs (Manzo et al., 2013). Therefore, the risk of gonadotoxicity of zinc oxide nanoparticles should also be considered.

In this study, the histopathological findings confirmed the results obtained for the effect of zinc oxide nanoparticles on serums oxidant and antioxidant status in the rats. Similar result was also reported in the study of Saman et al. (2013) that the administration of different concentrations of ZnO nanoparticles (100, 200 and

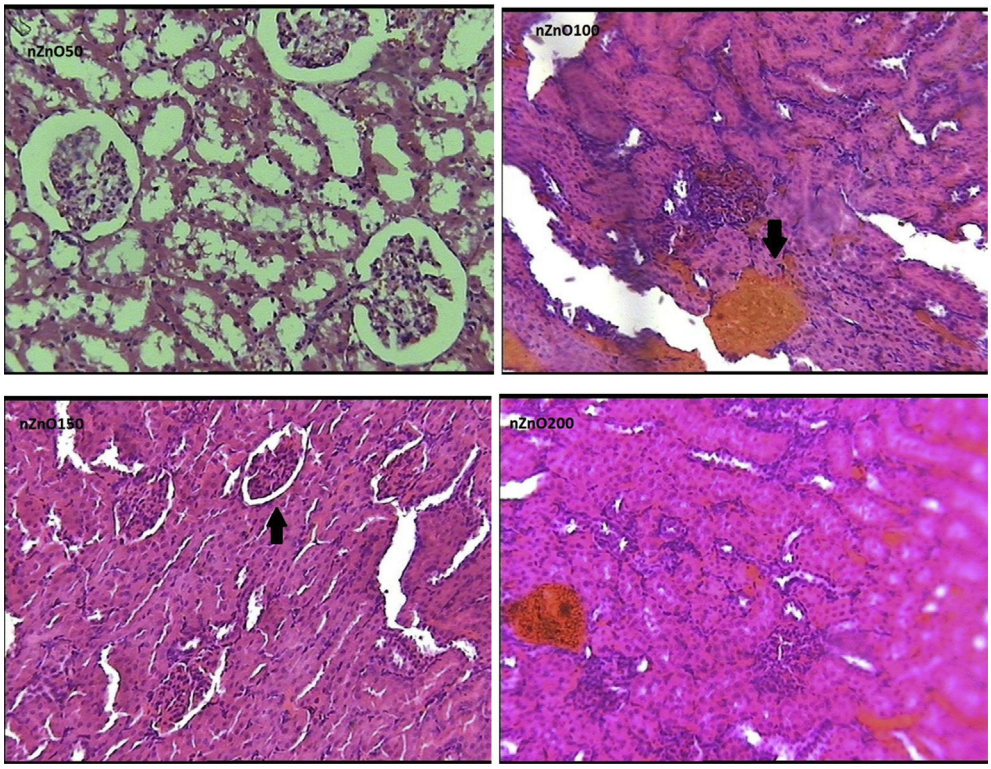

Fig. 4. Histopathological findings of rat's kidney after treatment with ZnO nanoparticles for ten days (H&E, x40). (nZnO50) no abnormality, (nZnO100) Glomerulus congestion (arrow), (nZnO150) Glomerulus proliferation (arrow), (nZnO200) Interstitial tissue inflammation.

400 mg/kg body weight) resulted in apoptosis and congested blood vessel in liver tissue of male adult Wistar rats. It was suggested that the toxic effect of ZnO nanoparticles maybe through their accumulation in the liver and induction of intracellular reactive oxygen species (ROS) production. However, increased levels of ROS results in reduced mitochondrial membrane potential (MMP) as well as increased apoptotic protein, Bax (Esmaeillou et al., 2013). In another study by AL-Taee and Hamdani (2013), it was observed that the pathological damage in the liver may be due to increased cellular oxidative stress including disturbed superoxide dismutase (SOD), glutathione peroxidase activities and increased peroxidation of lipids. These results are concurrent with a study that reported liver damage caused by high-dose of zinc salt such as zinc acetate intraperitoneal administration (Wang et al., 2010). In a previous study it has been reported that the mechanism of dose- and time-dependent effect of ZnO nanoparticles in the liver may be raised from oxidative stress generation, lipid peroxidation, and cell membrane and DNA damage (Najafzadeh et al., 2013).

The histopathology results of the present study also revealed toxicological impact through the kidney. With regard to the excretion of xenobiotic products through the kidney, pathological renal damage is expected (Esmaeillou et al., 2013). However, some biochemical parameters such as BUN and creatinine can be determined to understand the ZnO nanoparticles-induced nephrotoxicity. As a result, it seems the toxicity of zinc oxide nanoparticles is more significant when the concentration is increased. However, the use of ZnO nanoparticles even at low doses requires further investigation.

5. Conclusion

Although, the use of zinc oxide in the form of nanoparticles is very common, but data related to harmful effect in human is not sufficient and requires more researches. Some reports on its toxicological properties have encouraged us to investigate the toxicity of Zinc oxide nanoparticles, as well as the enhanced liver enzymes and oxidative stress index in rat as an animal model. The toxicity of zinc oxide nano particles on adult male Wistar rats treated by peritoneal injection of various concentrations of ZnO nanoparticles was assessed by measuring some liver enzymes, oxidative stress factors, histopathological examination of liver and kidney and sperm analysis. The results showed significant increase in liver enzymes starting from ZnO nanoparticles concentration of 100 mg/kg animal body weight. Also, it was found that ZnO nanoparticles at concentrations above 50 mg/kg resulted in significantly enhanced SOD and non-significant decreased GPX, suggesting oxidative stress in treatment groups compared to the control group. Although the current findings indicate that the significant toxicity effects of ZnO nanoparticles appear at concentrations above 50 mg/kg body weight of animals, it is not yet clear whether the use of a low dose of ZnO nanoparticles (50 mg/kg and below) in industrial materials such as cosmetic products and sunscreen is justified. However, further research is needed to study the impact of ZnO nanoparticles on human health and safety.

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Transparency document

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