

## Dose-dependent hepatotoxicity effects of Zinc oxide nanoparticles

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Received; 10 May 2015

Accepted; 20 July 2015

### ABSTRACT:

**Objective(s):** Zinc oxide nanoparticles (ZNP) are increasingly used in sunscreens, biosensors, food additives and pigments. In this study the effects of ZNP on liver of rats was investigated.

**Materials and Methods:** Experimental groups received 5, 50 and 300 mg/kg ZNP respectively for 14 days. Control group received only distilled water. ALT, AST and ALP were considered as biomarkers to indicate hepatotoxicity. Lipid peroxidation (MDA), SOD and GPx were detected for assessment of oxidative stress in liver tissue. Histological studies and TUNEL assay were also done.

**Results:** Plasma concentration of zinc (Zn) was significantly increased in 5 mg/kg ZNP-treated rats. Liver concentration of Zn was significantly increased in the 300 mg/kg ZNP-treated animals. Weight of liver was markedly increased in both 5 and 300 mg/kg doses of ZNP. ZNP at the doses of 5 mg/kg induced a significant increase in oxidative stress through the increase in MDA content and a significant decrease in SOD and GPx enzymes activity in the liver tissue. Administration of ZNP at 5 mg/kg induced a significant elevation in plasma AST, ALT and ALP. Histological studies showed that treatment with 5 mg/kg of ZNP caused hepatocytes swelling, which was accompanied by congestion of RBC and accumulation of inflammatory cells. Apoptotic index was also significantly increased in this group. ZNP at the dose of 300 mg/kg had poor hepatotoxicity effect.

**Conclusion:** It is concluded that lower doses of ZNP has more hepatotoxic effects on rats, and recommended to use it with caution if there is a hepatological problem.

**Keywords:** Apoptosis, Nanomaterials, Oxidative stress

### INTRODUCTION

Metal nanoparticles (NPs) and their oxides have a considerable number of present and future applications in the medical and industrial fields. The smaller size and unique properties of the NPs has substantially improved the application of them [1-3]. NPs are considered to be more highly absorbed into the respiratory, skin, and gastrointestinal systems than micron-sized particles because of their unique physicochemical properties, such as their size and surface modifications [4]. Previous studies revealed that administration of NPs to rodents resulted in their accumulation in the various tissues including liver, brain and spleen [5-6].

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Note. This manuscript was submitted on May 10, 2015; approved on July 20, 2015

One of the most popular industrial applications of NPs is sunscreens [7]. The major component of sunscreens is zinc oxide nanoparticles (ZNP), which can effectively absorb ultraviolet light [8]. No published data is available about the daily exposure doses of ZNP in human. However, ZNP increasing use increases the health risk of people exposed to these particles, either occupationally or environmentally. An important factor to be considered in toxicity tests is the diversity of the exposure routes, which includes inhalation, dermal uptake, ingestion and injection [9]. Although most of the exposure to ZNP is via epidermal contact, several studies have reported that ZNP do not permeate the skin [10, 11]. However, small fractions of lip products and sun protection products may accidentally ingest. ZNP may used for its antimicrobial properties in food packaging. ZNP can also be ingested directly when

used in food packaging or drug delivery [12, 13]. Emamifar et al. studied the migration of silver and ZNP in LDPE nano-composite packaging. The zinc (Zn) ions showed an increase in their migration when compared to silver [15]. Workers involved in the synthesis of ZNP can be exposed by unintentional hand-to-mouth transfer of nanomaterials. When discharged accidentally into the environment, ZNP may enter the human body through the food chain [16]. In addition, some of the NPs can be swallowed into and reach the gastrointestinal tract when they are expelled from the mucociliary system of the lungs after inhalation [17]. Oberdorseter et al. demonstrated that following whole-body inhalation of ultrafine carbon particles, the NPs were translocated and accumulated in liver [18]. It has been reported that ZNP administered orally dissolve in the stomach, with Zn ions then absorbed to enter into systemic circulation [19].

De Louise has reported that ZNP are nontoxic to cultured human dermal fibroblasts [20]. Other reports suggest that ZNP are toxic to dermal fibroblast, neuroblastoma cells and vascular endothelial cells [21-23]. It has been reported that ZNP can induce oxidative stress and apoptosis in HepG2 [24]. However, published toxicity data are still considered inadequate to earn a full understanding of the potential toxicity of ZNP. In present study toxic effects of ZNP on liver was investigated.

## MATERIALS AND METHODS

### *Animals*

In this experimental study, 32 healthy and adult male Wistar rats (8-10 weeks old, 180-200 g) were used. The animals were obtained from Ahvaz Jundishapur University of Medical Sciences, Experimental Research Center, and this study was approved by the ethics committee of Jundishapur University and carried out in an ethically proper way by following the guidelines provided. The animals were kept under standard laboratory conditions (12 h-dark and 12 h- light cycle, relative humidity of  $50 \pm 5\%$  and  $22 \pm 3^\circ\text{C}$ ) for at least 1 week before the experiment and those conditions were preserved until the end of the experiment. Animal cages were kept clean, and commercial food (pellet) and water were provided *ad libitum*.

### *Experimental design*

The rats were randomly divided into 4 groups of 8 animals each. Control group received 0.2 ml normal

saline for 14 days. Experimental groups received 5, 50 and 300 mg/kg ZNP respectively (ZNP-1, 2, 3) for 14 days [25].

After characterization of ZNP, the stock solution (2 mg/ml) was prepared in Milli-Q water and dispersed for 10 min by using a sonicator. The stock solution of ZNP was kept at  $4^\circ\text{C}$  and used within 1 week for the experiments. Just before use, the stock solution diluted in Milli-Q water and prepared by ultrasonication (Solid State/Ultrasonic FS-14; Fisher Scientific) for 15 min to prevent aggregation. To ensure non-aggregation of ZNP before administration, the time interval from preparation to oral gavage was strictly limited in less than 20 min. In addition, 20 min after the preparation, the particle size of ZNP was analyzed by atomic force microscopy (AFM).

Changes in body weight and behavior, and possible appearance of symptoms in the rats were carefully recorded every day after administration of the ZNP. One day after the last administration, after blood sampling, the rats were sacrificed by cervical dislocation under ether anesthesia and liver from each animal were removed quickly and weighed. Small pieces of liver were stored separately in a deep freezer ( $-80^\circ\text{C}$ ) for the MDA, SOD and GPx assay, and *measuring the concentration* of zinc (Zn) in liver. Other pieces were fixed in 10% buffered formalin for histological assessments.

### *Zn content analysis*

Approximately 1 g of the liver tissue was taken and digested in concentrated nitric acid overnight. Next day, 5 ml of concentrated nitric acid and perchloric acid mixture (6:1) was added to each sample and heated at  $80-90^\circ\text{C}$  until the solutions were colourless and clear. The concentrated solutions were diluted to 5 ml with 1% nitric acid. The Zn content was analyzed by an atomic absorption spectrophotometer (ZEE nit 700 P, Analytikjena, Germany). Blood samples were collected via the tail vein to evaluate the plasma Zn concentration. The blood samples were centrifuged at 3000 rpm for 15 minutes at  $4^\circ\text{C}$  to obtain the plasma [26].

### *Biochemical tests*

The blood sample were collected in heparinized centrifuge tube and centrifuged. The plasma enzyme levels including plasma alaine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline

phosphatase (ALP) were determined spectrophotometrically from plasma samples using commercially available kits (Sigma).

#### **Estimation of lipid peroxidation**

Degree of lipid peroxidation in liver tissue homogenate of all the experimental animals was determined in terms of thiobarbituric acid reactive substances (TBARS) formation as previously described [27]. A volume of 500  $\mu$ l of supernatant was mixed with 1.5 ml trichloroacetic acid (10%) and after centrifugation (4,000 $\times$  g for 10 min), 1.5 ml of supernatant was added to 2 ml TBA (0.67%) and heated at 100°C for 30 min. After cooling, the sample was extracted with 2 ml n-butanol and after centrifugation at 4,000 $\times$  g for 15 min, the organic phase was collected. The absorbance was read spectrophotometrically at 535 nm. Values were expressed as nmol per milligram of tissue.

#### **Superoxide dismutase (SOD) activity assay**

The assay for SOD activity was made according to the method of Suttle [28] using Ransod kit (Randox Labs, Crumlin, UK). This method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from xanthine oxidase reaction), which is assayed in a spectrophotometer at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as 1 unit of SOD, and specific activity is expressed as units per milligram of tissue.

#### **Glutathione peroxidase (GPx) activity assay**

GPx activity was determined using the Ransel kit (Randox Labs., Crumlin, UK) according to Paglia and Valentine [29]. GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized Glutathione (GSSG) was immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance was monitored with a spectrophotometer at 340 nm. One GPx unit is defined as 1  $\mu$  mol of NADPH consumed per minute, and specific activity is reported as units per milligram of tissue.

#### **Histology changes**

The formalin fixed samples were embedded in paraffin, sectioned (5  $\mu$ m) and stained with haematoxylin and eosin (H&E). Six microscopy stained

slides per animal were examined for signs of histological features, such as inflammatory-cell infiltration, hepatocyte vacuolization (fatty deposits) and congestion of red blood cells (RBC).

#### **TUNEL assay**

For detection of apoptosis at a single cell level, based on labeling of DNA strand breaks, the In Situ Cell Death Detection Kit, POD (Roche, Germany) was used. The paraffin sections were dewaxed and rehydrated by standard methods. Proteases were added and incubated with 5% of appropriate normal serum for 30 min at 37°C. The slides were washed in phosphate buffered saline. The sections were permeabilized (2 min, on ice) and incubated with TUNEL reaction mixture (60 min, 37°C). Anti-fluorescein-AP was added and incubated (30 min, 37°C). Subsequently, the sections were washed in PBS and incubated for 20 min with substrate. They were analyzed by light microscopy. A cell was considered TUNEL-positive when the nuclear staining was intense, dark brown and homogenous. Apoptotic index was calculated by dividing the number of TUNEL-positive hepatocytes in a randomly focused field by the total number of hepatocytes in that field, and the result was multiplied by 100. The apoptotic indexes of 10 randomly field for each slide were evaluated and the mean apoptotic index of each case was calculated [30]. Four slides/ animal were used in this study.

#### **Statistical analysis**

The data were analyzed using one-way ANOVA followed by post hoc LSD test and were presented as the mean  $\pm$  SD.  $p < 0.05$  was considered significant.

## **RESULTS**

#### **Nanoparticle characterizations**

AFM revealed the size and morphology of the synthesized particles. The complexes appear spherical with a mean size that is inferior to 100 nm as can be seen in Fig. 1.

#### **Organ weight**

No animal was found dead or in a moribund state during the experiment. There were also no treatment related clinical signs such as lethargy, ataxia, etc. However, body weight in ZNP-1 group was significantly lower than control group ( $p < 0.05$ ). Liver weights were significantly increased in ZNP-1 group. In ZNP-2 group, liver weight was slightly increased and body weight was similar to control animals. ZNP-3 group showed a significant increase in liver weight ( $p < 0.05$ ). Body weight was not significantly changed in this group. These results are shown in Table 1.

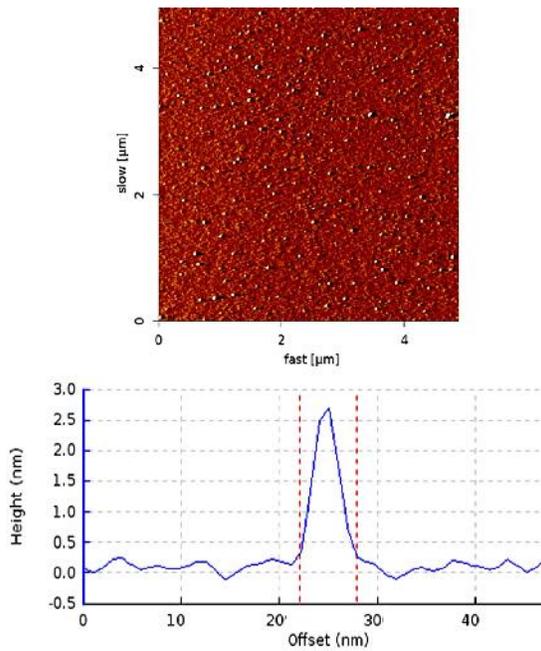


Fig. 1. AFM image of NPs showed distinct spherical particles in size range between 20 and 30 nm

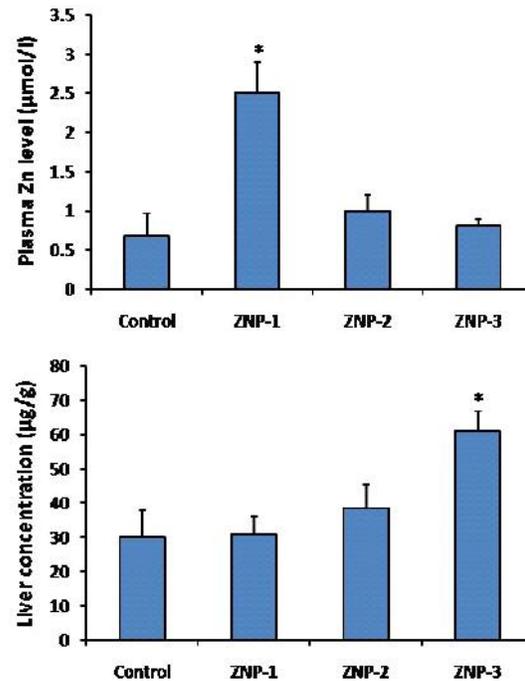


Fig. 2. Plasma and liver concentrations of Zn in control and experimental groups. Values are expressed as mean  $\pm$  SD for 8 rats. \* $p < 0.05$

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

Group	body weight (g)	Liver weight (mg)
Control	241 $\pm$ 18.29	10.1 $\pm$ 1.2
ZNP-1	218 $\pm$ 21.3*	16.2 $\pm$ 3.3**
ZNP-2	238 $\pm$ 14.3	11.6 $\pm$ 0.8
ZNP-3	240 $\pm$ 16.7	15.2 $\pm$ 2.4**

Values expressed as mean  $\pm$  SD for 8 rats. \* $p < 0.05$ , \*\* $p < 0.01$ , \* indicate comparison to control group

**Absorption of ZNP**

In ZNP-1 group, concentration of Zn in blood was significantly increased in compare to control group. Zn contents of liver tissue was similar to control group ( $p > 0.05$ ). In ZNP-2 group, concentration of Zn in blood was similar to control group. Zn contents of liver tissue was slightly increased in compare to control group ( $p > 0.05$ ). In ZNP-3 group, concentration of Zn in blood was slightly higher than control group ( $p > 0.05$ ). Zn contents of liver tissue was significantly increased in compare to control or ZNP-1 groups ( $p < 0.01$ ). These results are shown in Fig. 2.

**Biochemical tests**

ZNP-1 group showed a significant increase in plasma levels of ALT, AST and ALP compared to control group ( $p < 0.001$ ). Plasma levels of all biochemical tests were significantly increased in ZNP-2 group ( $p < 0.01$ ). In ZNP-3 group, biochemical tests were not significantly changed. These findings are depicted in Fig. 3.

**MDA level, SOD and GPx activities**

ZNP-1 showed a significant increase in the hepatic level of MDA when compared the control animals. GPx and SOD activities were significantly decreased in ZNP-1 group compared to the control ( $p < 0.001$ ). These effects were less in ZNP-2 group. There were not significant changes in MDA, SOD and GPx in ZNP-3 group in compare to the control group. These results are reported in Fig. 4.

**Histological Analysis**

Under light microscope, liver lobular structures in control group was clear and regular, and single layer of

hepatocytes arranged around the central vein in a radial pattern (Fig. 5-A). In ZNP-1 treated rats normal liver lobular structures were damaged. The hepatocytes showed swelling and vacuolization. Severe sinusoidal congestion, hemorrhage and inflammatory cell infiltration were also observed (Fig. 5-B). These histological changes were considerably decreased in ZNP-2 group (Fig. 5-C). In ZNP-3 group, no considerable changes in histology of liver were observed.

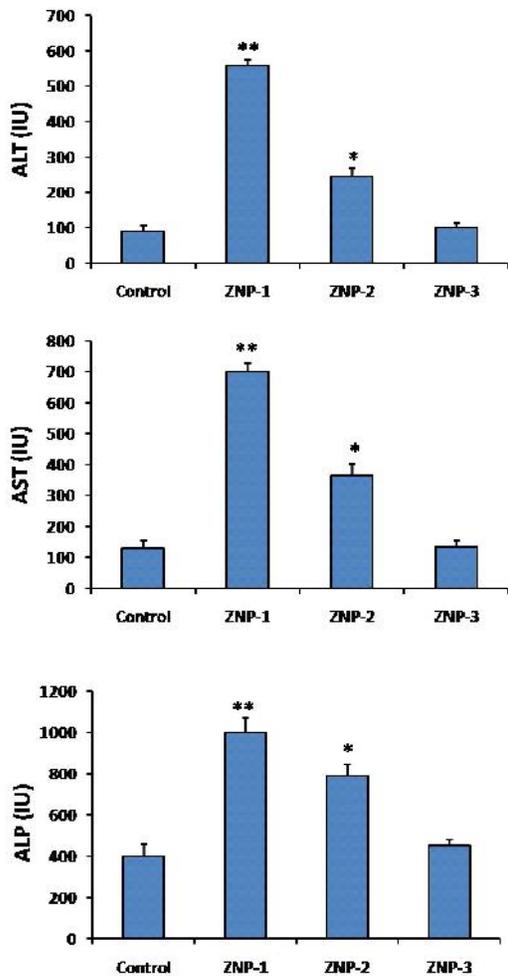


Fig. 3. Biochemical tests of control and experimental groups. Values expressed as mean ± SD for 8 rats. \*p < 0.01, \*\*p < 0.001

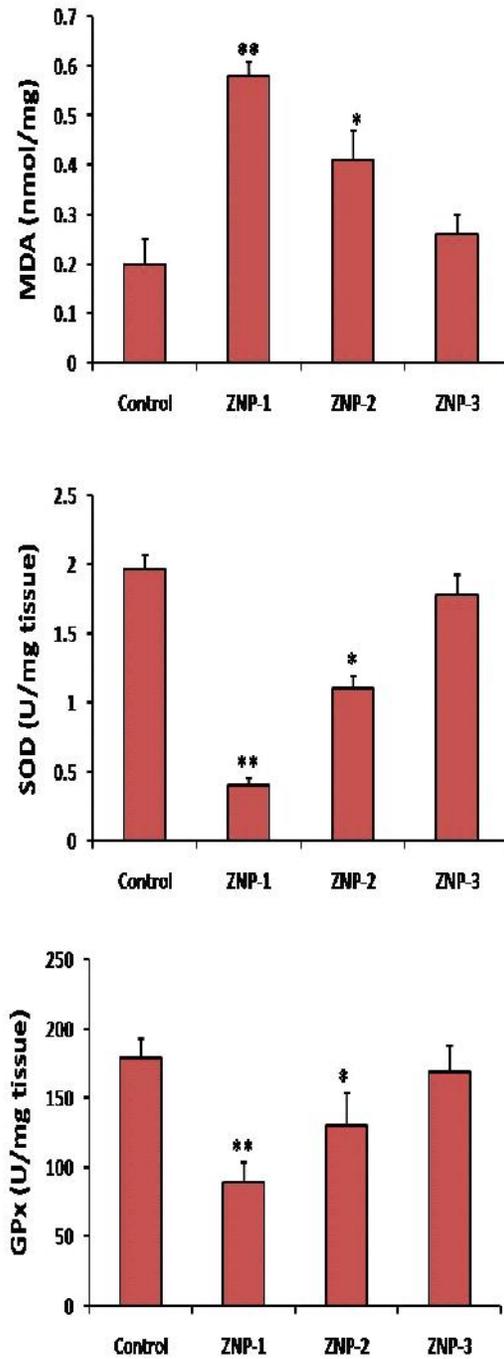


Fig. 4. MDA level, SOD and GPx activities of control and experimental groups. Values are expressed as mean ± SD for 8 rats. \*p < 0.01, \*\*p < 0.001

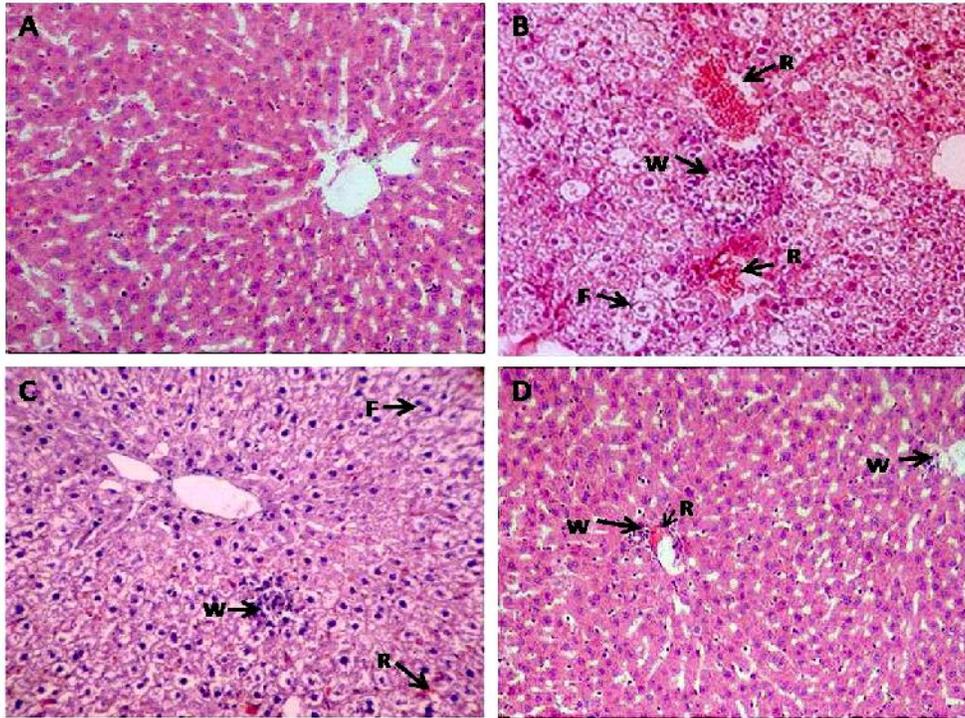


Fig. 5. Light microscopy of cross sections of H&E stained liver from control and experimental groups. A, Control groups; B, ZNP-1 group; C, ZNP-2 group. D, ZNP-1 group. WBC: White blood cells, F: Fat deposit, C: Congestion of RBC. Magnification:  $\times 250$

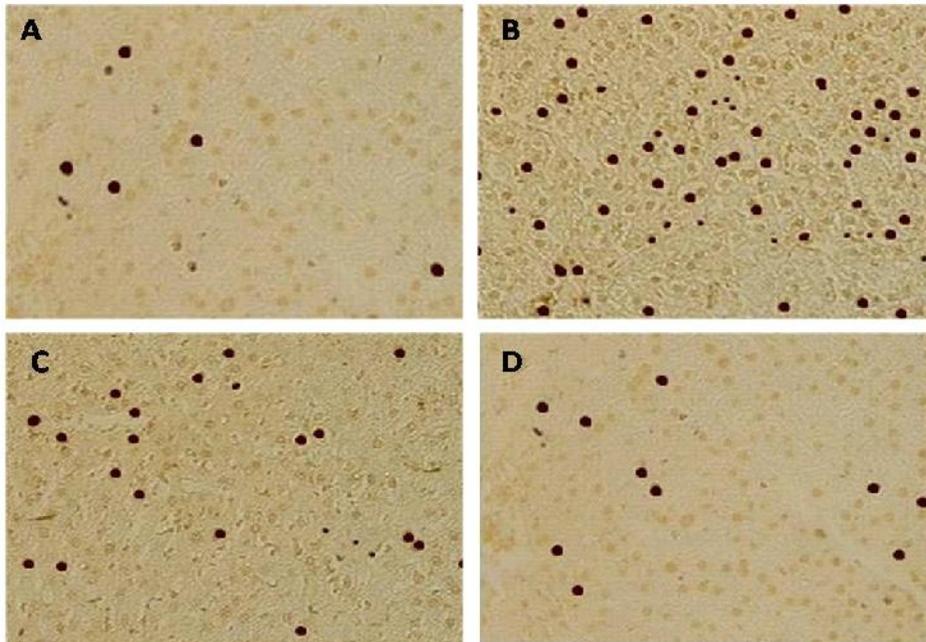


Fig. 6. Light microscopy of cross sections of TUNEL stained liver from control and experimental groups. A, Control groups; B, ZNP-1 group; C, ZNP-2 group. D, ZNP-1 group. Magnification:  $\times 250$

**TUNEL assay**

Spontaneous apoptosis was mainly observed in some hepatocytes of normal liver from the control group (Fig. 6). In ZNP-1 group, apoptosis was observed in all lobules and apoptotic index significantly increased in compare to the control group ( $p < 0.001$ ). In the ZNP-2 group, apoptotic index was also significantly higher than control group ( $p > 0.001$ ). In ZNP-3 group, no significant changes in apoptotic index were observed in compare to control group. The results of TUNEL assay are shown in Figs 6 and 7.

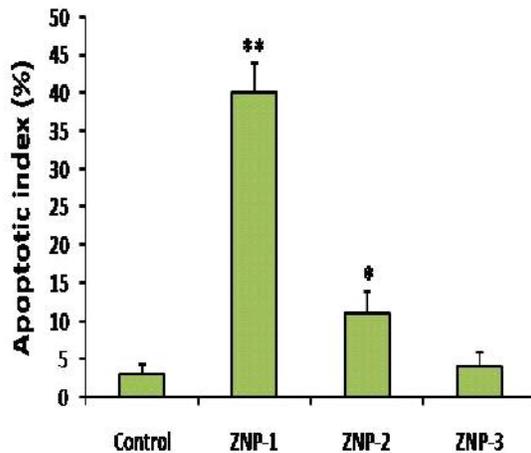


Fig. 7. Apoptotic index of control and experimental groups. Values are expressed as mean  $\pm$  SD for 8 rats. \* $p < 0.01$ , \*\* $p < 0.001$

**DISCUSSION**

Our results demonstrated that ZNP had concentration-dependent toxic effects on liver inducing stress oxidative, apoptosis induction and histological changes. We found that lower doses had more toxicity.

Despite intensive research efforts, reports of cellular responses to nanomaterials are often inconsistent and even contradictory. Pasupuleti et al. found that the incidences of toxicity were higher in lower doses of nano-size zinc oxide compared to higher dose [31]. However, Talebi et al. have demonstrated that ZNP at the doses of 50 and 300 mg/kg body weight have toxic effects on mouse spermatogenesis while no significant changes in spermatogenesis was reported at the dose of 5 mg/kg [25]. Akhtar et al. showed that ZNP has no

impact on normal rat astrocytes and hepatocytes [32]. On the other hand, Guan et al. have reported that ZNP has toxic effects on human hepatocyte (L02) and human embryonic kidney (HEK293) cells [33]. Interestingly, Wang et al. have reported that ZNP induces oxidative stress and apoptosis in cultured primary astrocytes [34].

Adverse effects of NPs on human health depend on individual factors such as genetics and existing disease, as well as exposure, and NPs chemistry, size, shape, agglomeration state, and electromagnetic properties [35].

Song et al. suggested that the morphologies, ion release rate of NPs as well as the species-specific vulnerabilities of cells should all be considered when explaining and extrapolating toxicity test results among particles and among species (36).

Administration of carbon nanotube (20-50 nm) within the trachea causes acute inflammatory effects in the lungs in mice but not in rats [37, 38]. This suggests that toxic effects of NPs are dependent on the species used. In this study, ZNP significantly elevated the levels of ALT, AST and ALP. The levels of these enzymes are indicative of the functional efficiency of the liver, and are very sensitive to any disease process of the liver [39].

Due to damaged liver cells, such enzymes (which are inside the hepatocytes) are released into the blood. Therefore, a high amount of these enzymes indicates destruction of liver cells. In the present study, the decreased SOD and GPx activity and elevated MDA levels in liver indicated that the presence of oxidative stress and lipid peroxidation response were generated by ZNP exposure. It has been reported that oxidative stress mediated DNA damage and cytotoxicity induced by ZNP in HepG2 cells [24]. The role of oxidative stress in the mechanism of NPs induced hepatotoxicity has also been reported by Sha et al. [40].

Mechanism of the generation of the oxidative stress after NPs treatment is not clear, but Singh et al. [41] indicated that it is related to the large particle surface area. As shown in results, liver weights were significantly increased in ZNP-1 and ZNP-3 groups. It may have been the result of RBC congestion and WBC infiltration as well as accumulation of ZNP in liver tissue. Zn content of liver in ZNP-3 was significantly higher than control while histological changes were

not observed in this group. Thus, the increase in liver weight of ZNP-3 group relates to accumulation of ZNP in this organ.

The destruction of lobular structure, vacuolization of hepatocytes (fat deposits), congestion of RBC and infiltration of leukocytes indicate necrotic effects of ZNP at lower doses on liver tissue. On the other hand, these accumulations of fat, RBCs and leukocytes can increase the weight of liver. The appearance of inflammatory cells in hepatic tissue suggests that the ZNP at low dose can interact with proteins and enzymes in the interstitial tissue of the liver, interfering with the antioxidant defense mechanism and leading to generation of reactive oxygen species, which in turn may induce an inflammatory response [42].

Swelling of hepatocytes together with dilatation of the central vein and blood sinusoids indicate that these NPs may affect permeability of the cell membrane in hepatocytes and the endothelial lining of blood vessels. Hepatocytes fatty deposits might be due to lipid peroxidation that leads to rough endoplasmic damage and detachment of the cytoplasmic lipoprotein which indicate abnormal fat metabolism [43].

The abnormal retention of lipids in hepatocytes might indicate toxic injury to the liver in the form of hepatocytes lipolysis by ZNP.

In addition to necrosis, apoptosis were also observed in liver tissue of ZNP treated animals. Park et al. found that, out of 6 different NPs, ZNP were the most cytotoxic to A549 cells, as assessed by DNA fragmentation and apoptosis experiments [44]. Both apoptosis and necrosis has been reported to occur in cells treated with ZNP. ZNP can induce necrosis and apoptosis in macrophages [45]. Meyer et al. have reported that ZNP can induce apoptosis in human dermal fibroblasts [21]. Sharma et al. have also demonstrated that ZNP even at low concentrations possess a genotoxic potential in human epidermal cells [46].

In addition, severe oxidative stress is associated with lysosomal membrane permeabilization and subsequent necrosis, which is controlled by complicated signaling pathways [47, 48]. It has also been reported that apoptosis and necrosis are not completely independent process [49], In particular, when a large number of cells undergoing apoptosis [50]. As mentioned above ZNP at low dose showed more toxicity. The mechanism of this finding is not clear. It has been demonstrated that

NPs at high concentrations tend to cluster, forming aggregates often larger than 100 nm. Larger NPs (>100 nm) can be readily phagocytized by macrophages [51]. Results of studies involving inhalation or intra-tracheal instillation of high concentrations of NPs (silver, iron, India ink, or titanium dioxide) smaller than 100 nm, which aggregate in larger particles, suggest that most NPs are indeed stopped by alveolar macrophages [51, 52]. Hence, minute concentrations of NPs with size smaller than 100 nm can have a higher probability of translocation to the circulatory system and organs (and produce damage) than high concentrations of the same particles, which are likely to form aggregates, and which will be stopped from translocation by macrophage. As demonstrated in results, plasma levels of Zn were significantly higher in lower dose of ZNP-treated rats. Overall, our results highlight the need for caution during the use of ZNP to prevent health impacts. We demonstrated that ZNP exhibited toxicity at lower concentrations. Thus future nanotoxicology research needs to be focused on importance of dose metrics rather following the conventional methods while conducting in vivo experiments.

#### ACKNOWLEDGMENTS

This paper is part of M. Sc thesis for Zahra Jozi and was supported by a Grant (CMRC-108) from the research council of the Ahvaz Jundishapur University of Medical Sciences in 2013.

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**How to cite this article:**

Mansouri E, Khorsandi L, Orazizadeh M, Jozi Z . Dose-dependent hepatotoxicity effects of Zinc oxide nanoparticles. *Nanomed. J.*, 2015; 2(4): 273-282.