

Potential toxic effects of Zirconia Oxide nanoparticles on liver and kidney factors.

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Abstract

Nanoparticles In addition to the positive aspects of tending to its toxicity in the environment is unavoidable. This research studies the effects of ZrO₂ NPs on the liver and kidney tissues as well as the activities in liver and kidney enzymes in the male rats. This study is done on 40 Wistar rat race, in 4 groups which includes on control and 3 experimental groups that are monitored daily, for the control group we give a Saline Solution and 3 other with 1ml/day nanoparticle by different doses (50,25,100 ppm) intra-peritoneally. After a 1 week period, samples were unconscious, blood sample were collected from the heart and the ALT, AST, ALP and creatinine were measured. Post-treatment tissue level of malondialdehyde as well as the activities of catalase, glutathione peroxidase and superoxide dismutase were measured in the liver. The statistical raw data was analyzed by SPSS statistical software. The significant difference ($p < 0.05$) in the levels of foregoing factors was obtained by the application of the maximum density of ZrO₂NPs (100ppm) in comparison with the control groups. The rats when exposed to a high dosage of nanoparticles reported a significant increase in MDA concentration level while significant decreases were observed in GPX, CAT and SOD activities ($P < 0.001$). In the rats which were exposed to high dosage of nanoparticles, the liver enzyme concentration was significantly increased ($p < 0.05$). The obtained results revealed the significant role of ZrO₂ as an increasing ROS generation agent and the ROS have induced the development of free radicals.

Keywords: antioxidant, enzyme, kidney, liver, zirconia nanoparticles.

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Introduction

Nanoparticles are particles that have a mean diameter and dimensions of about 10^{-9} m. These particles due to their small size have special physical, chemical, mechanical, electric and magnetic properties; for example, they freely enter the cell and can interfere in its natural process. Zirconium-oxide nanoparticles have become the most commonly used nanoparticles in different industries such as kitchen appliances and are used every day. And this has increased the necessity for the studies on their safety to use these materials. Oxide nanoparticles, due to their physicochemical properties, are extensively used as drug carriers in treatment of cancer cells in live environments. Also, these nanoparticles have many biomedical applications such as tissue regeneration, safety evaluation, detoxification of biological fluids, thermotherapy of cancer cells and etc. Oxide nanoparticles result in increase of inflammatory responses in rats treated with these nano-

particles. Studies have indicated that these nanoparticles can stop cell cycle at G1 phase. Due to their shape and size, nanoparticles can pass through physiological barriers and leave adverse effects. Our knowledge regarding the toxicity of nanoparticles is very limited. The assumption in this study is that long-terms contact with zirconium oxide nanoparticles results in disturbance in hepatic and renal systems. Aspartate aminotransferase (AST) exists in mitochondrial and cytosolic iso-zymes and is found in liver, muscle, brain and pancreas. Alanine aminotransferase is a systolic enzyme which is specific to liver. Alkaline phosphatase (ALP) is an enzyme which exists in many tissues and is released from liver and bones in high concentrations and the obstruction of biliary tract results in its serum increase. Liver cell damage results in the release of these enzymes into the blood flow. Creatinine is a characteristic enzyme of kidney which enters into the blood flow by kidney damage. The main mechanism of nanoparticles function is not known yet but difference in

their vivo and in vitro studies suggest that they can produce reactive oxygen species (ROS) and therefore they can have a role on intracellular calcium concentration, activation of transcription factors and creating change in cytokines. Oxidative stress can be considered as a response to cellular damage, Oxidative stress occurred due to nanoparticles can have several reasons, ROS can be created directly when both the oxidants and free radicals are present on the particle surface; through entering mitochondria, as different studies have shown that very small nanoparticles can enter the mitochondria and create physical damages that result in oxidative stress; activation of inflammatory cells such as macrophages and neutrophils of alveoli which are involved in the process of phagocytosis of nanoparticles. This can result in the production of reactive oxygen and nitrogen species; metal nanoparticles (iron, copper, chromium, vanadium, zirconium) can cause the production of ROS; DNA damage by ROS production as the result of the nanoparticles present can cause serious and inheritable damages to DNA; cellular oxidative stress is characterized by increase in ROS level, decrease in GSH expression and increase of lipid peroxidation. Different mechanisms are proposed for justification of the damaging actions of nanoparticles and the elevation of the ROS level is more important in this regard. Superoxide, hydrogen peroxide and other oxygen radicals can damage DNA, proteins and cell lipids directly. Studies indicated that the cells that are exposed to nanoparticles show a reduction in viability. The cell cycle of PC12 cells is stopped in phase G2/M and induces apoptosis in a dose-dependent process, here mitochondrion is a special organelle affected and the mitochondrial membrane potential is reduced. In addition, it reduces the lipid peroxide production level and the superoxide dismutase (SOD) activation level. Maybe all of the changes can be attributed to the increase in the intracellular ROS level which lowers the level of glutathione peroxide, catalase and glutathione. Recently, Gao et al concluded that magnetic nanoparticles have intrinsic peroxidase property and can catalyze H₂O₂. The aim of the present study is exploring the toxic effects of zirconium oxide nanoparticles on liver and kidney tissues and to provide necessary precautions to industry employees.

Materials and Methods

Materials

ZrOCl₂·8H₂O, urea, CH₃OH, sulphuric acid solution were used to prepare nanoparticles. Saline, ketamine, Rat, Hematoxylin eosin, and laboratory kit (PARS AZMON. Co of IRAN) were used.

Equipment

XRD, SEM (ZIESS EM 902A), TEM (JEM-200CX), UV-visible, optical microscope (OLYMPUS CX 21

FS1) and spectrophotometer (JENWAY. England) was used.

Preparation of ZrO₂ nanoparticles

The ZrO₂ nanoparticles were prepared according to the literature. Initially, 2.58 g ZrOCl₂·8H₂O and 4.80 g urea were dissolved in 20.0 mL CH₃OH under stirring to form a colorless solution. The solution was transferred to a 20-mL Teflon-lined stainless steel autoclave, which was heated to 200 °C and maintained at that temperature for 20 h. The obtained white product was post-treated with sulphuric acid solution (0.167 mmol) and then calcined at 645 °C. The final product was analyzed by XRD, UV-visible, SEM (ZIESS EM 902A) and TEM (JEM-200CX).

Study Design

Some male wistar rats aging 2 months and weighting 250±13g were purchased from Isfahan University of Iran and kept at an ambient temperature of 25±2°C in 12h light and 12h dark cycle. The animals were quarantined for a period of two weeks before the commencement of the experiment. They were placed in a poor ventilated compartment in which the animals' cages were kept. They were freely supplied water and food. This study was carried out according to the guidelines approved by Institutional Animal Ethical Clearance (IAEC).

Experimental design

The 40 rats were divided into four groups (n=10 rats per group) as follows:

Group I- normal controlled rats receiving intraperitoneal physiological Saline.

Group II- rats treated Intraperitoneal with 1ml of ZrO₂NPs, 25ppm concentration.

Group III- rats treated Intraperitoneal with 1ml of ZrO₂NPs, 50ppm concentration.

Group IV- rats treated Intraperitoneal with 1ml of ZrO₂NPs, 100ppm concentration.

Blood sample collection

The rat did not show any symptoms of toxicity such as change in fur color, weight loss and any other symptom relevant to the morphology and behavior. Rats were anaesthetized by the Ketamine Chloride which was administered intraperitoneally. About the 8ml of the animals' blood were collected by cardiac puncture into Lithium Heparin bottles. The blood sample was centrifuged at 3000 rpm for 15 minutes in order to measure the concentration of ALP, AST, ALT and the creatinine factors then they were submitted to the Spectrophotometer and biochemical kit (Pars Azmon). *The spectrophotometer* is an instrument which is used to measure the properties of light over a specific portion of the electromagnetic spectrum typically used in spectroscopic analysis to identify the materials. The measured variable is mostly the light intensity but could also for example, be the polarization state. The independent variable is usually the wavelength

of the light or a unit directly proportional to the photon energy, such as wave number or electron volts, which has a reciprocal relationship to wavelength. A spectrometer is used in spectroscopy for producing spectral lines and measuring their wavelengths and intensities.

Measurement of Malondialdehyde Levels in Liver

Once finished, the animals were anesthetized with ketamine. Liver was dissected from the body, rinsed with cold saline solution weighted after dryness promptly, homogenized (10%) subsequently and centrifuged separately with tris buffer for 2 minutes with the homogenizer device at 5000rpm speed. In order to prevent the elimination of enzymes and proteins, all the above steps were conducted at 4° c centrifugation, the clear supernatant solution was removed and the bottom sediment was discarded and the clear supernatant solution was used for measurement. Measuring the levels of Malondialdehyde was based on the method of thiobarbituric acid (TBA) reaction and carried out at the boiling point temperature. In this experiment, the MDA or Malondialdehyde-like materials reacted with Thiobarbituric acid and provided a pink color at the maximum absorbance which was at 532 nm. The reaction was carried out at pH=2-3⁰ and temperate of 90⁰ c for 15 minutes. After cooling the sample, the absorbance level was observed. To this end, 150 µl of the centrifuged sample were taken and the amount of 1.5cc of Trichloroacetic acid and 1.5 cc of TBARS were added and then we put all the samples and standard tubes with different dilutions into Binary hot water for 80 min to facilitate the reaction. The solution was centrifuged at 3000 rpm for 10 min and the absorbance was read in a spectrophotometer at a $\lambda = 532$ nm. Standard curves were prepared based on the dilution of tetraetoxyp propane and the obtained absorbance peaks of samples were adapted on the standard curves.

Measurement of Superoxide Dismutase Enzyme Activity

The SOD activity was measured according to the inhibiting reductions of Nitroblueterazolium by Exanthin-Xanthin oxidase system as the producer of superoxide. In this experiment, the solutions consisted of Xanthine, Xanthine Oxidase were applied in the Potassium Phosphate Buffer and Nitroblueterazolium. The absorbance of each sample was read every 30 second for 5 min. To gain the level of inhibition by SOD enzyme, the obtained data was concluded from the corresponding formula on the basis of set commercial kit. The activity of enzyme was measured by the level of inhibition adapted on the standard curve in terms of u/mg protein.

Measurement of Catalase Enzyme Activity

The activity of catalase was assayed according to the method of Abie (20). Briefly 0.01 mml/mml of the Ethanol was added to a definite mass of tissue homogenate

and was incubated in ice for half an hour. And Triton X-100, 10% was added to solution with the final concentration of 1%. This solution was used to measure the activity of enzyme. The enzyme reaction was initiated by the addition of 30mM H₂O₂ to extract sample tissue homogenate in Buffer Phosphate Sodium 50mM with pH=7.0. Then, the absorbance was measured at 40nm within 3 minutes and the enzyme activity was measured in terms of u/mg protein.

Measurement of Glutathione Peroxidase Enzyme Activity

The activity of GPX was assayed according to the Rotac and et al. method. Glutathione Peroxidase oxidized Glutathione in tissue, homogenates that results in the retrieve of hydrogen peroxide to water simultaneously. This reaction was stopped after 10 minutes by Tri-chloroacetic acid and remaining glutathione was reactivated by DNTB solution and it led to the formation of colored compounds 420nm, that can be measured with a spectrophotometer. Reagent mixture consists of 0.2ml of ethylene diamine tetra acetate (EDTA) 0.8mM, 0.1ml of Sodium oxide 10mM, 0.1ml of Hydrogen-peroxide 2.5mM, 0.2ml homogenate. The mixture was incubated at 37° c for 10 min. The reaction was stopped by adding 0.5ml of 10% Tri-chloroacetic acid and the tubes were centrifuged for 15 min in 2000Rpm. 3ml of Disodium hydrogen 0.8 mM, and 0.1ml DTNB of 0.4% were added to supernatant solution and the resulting color was measured at 420nm accordingly. Glutathione Peroxidase activity was expressed as µ moles of Glutathione oxidized/min/mg protein.

Histological studies of the experimental animals

The vital organs (i.e., liver and kidney) were dissected separately from the rats in all groups. They were fixed with a 10% of formalin neutral buffer solution. The obtained sections were stained by Hematoxylin and Eosin procedure and examined under the light microscope. Their photomicrographs were obtained.

Statistical Analysis

Obtained data were stored by use of SPSS software and then delivered to EXCELL program, and edited and then data was excluded from ANOVA table from SPSS programs. The results were offered by average and standard deviation. According to normal distribution of data for comparison of enzyme results in each group before and after investigation we use ANOVA test by repeated measurement and for comparison of groups in each periodic time we used ANOVA test and Dunnett test and we consider less significant surface from 0.05.

Results

The ZrO₂ nanoparticles Diffracted by X-Ray

The XRD pattern Fig. 1 for ZrO₂ nanoparticles, the absorption of diffraction peaks are occurred at 2θ values. The peaks that are prominent have been employed for the

estimation of sample grain size, through Scherrer equation $D = K\lambda / (\beta \cos \theta)$ where K is constant (0.9), λ is the wavelength ($\lambda = 1.5418 \text{ \AA}$) ($\text{Cu K}\alpha$), β is the full width at the half-maximum of the line and θ is the angle of diffraction. The estimated grain size employing the relative intensity peak for ZrO_2 nanoparticles was found to be

20nm and the addition in sharpness of XRD peaks showed that particles have crystalline nature. All the various peaks in figure 1 are related to ZrO_2 nanoparticles and linked to Joint Committee for Powder Diffraction Studies.

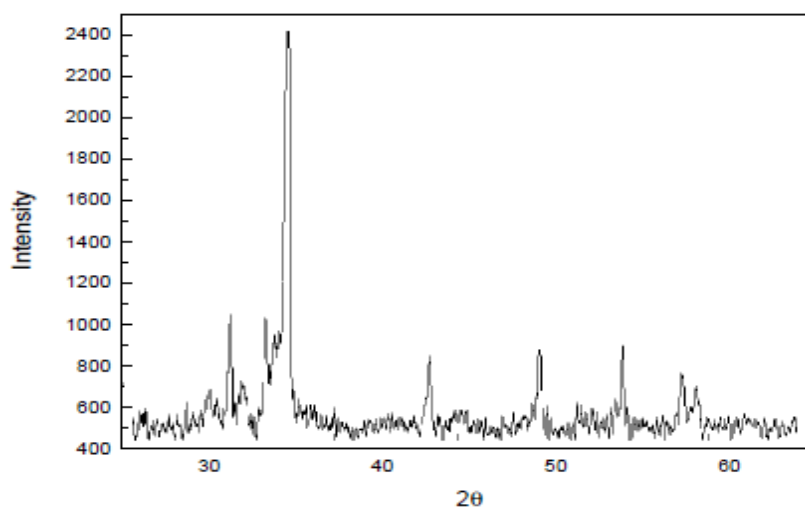


Figure 1. XRD pattern for ZrO_2 nanoparticles

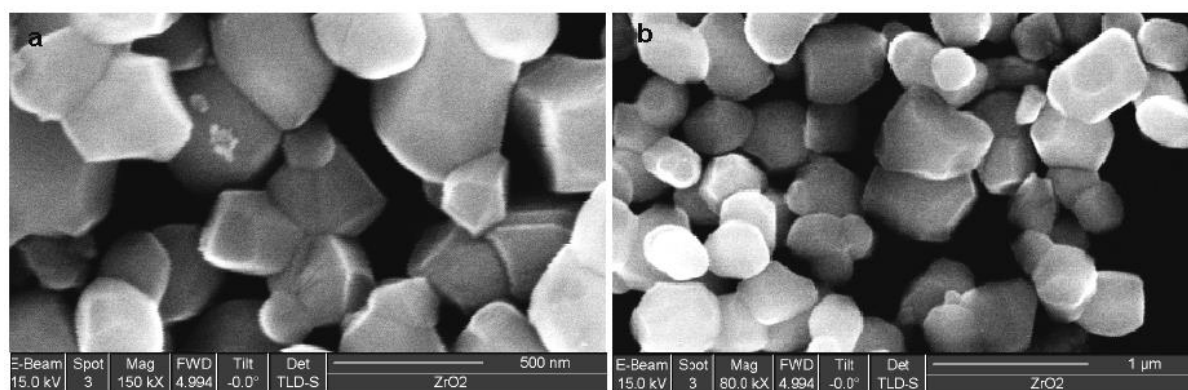


Figure 2. TEM picture of ZrO_2 nanoparticles (a) the size of the taken image was 500 nm and it was magnified by 150 KX and (b), the size of the taken image was 1 μm and it was magnified by 80 KX

ZrO_2 Nanoparticles Electron Microscopic study

Surface attributes are important elements and it is a known fact that the characteristics of a high number of materials and many devices' performance to a great extent are depended on their surface attributes. In figure 2 (a & b) regular pictures of ZrO_2 Nanoparticles can be observed. The two sections of the picture were taken at 150 kV by TEM microscopy. Part (a) of Figure 2 was magnified by 150 KX; however, figure 2, part (b) was magnified by 80 KX. The mean diameter of the synthesized ZrO_2 nanoparticles is around 20nm, and includes a very narrow particle distribution.

Serum Analysis

The enzymes such as ALT, AST, and ALP are responsible for the well-functioning of the liver and any dam-

age induced to the liver rising from the use of NPs' conditions may lead to the appearance of these enzymes within the blood stream. Thus, the effects of ZrO_2 NPs on the level of different metabolic enzymes result in the optimum function of the liver. The serum was analyzed and the significant effects of NPs upon the liver damage are shown in (figure3 (a, b, c)). The ALT, ALP, and AST enzymes showed some significant elevated levels in the treated group in comparison with the control group ($p < 0.05$).

The effects of NPs on kidney damage are shown in the figure4. The level of symptomatic creatinine developed by the renal function has also shown a significant increase in comparison with the control group ($p < 0.05$) that shown in the figure 4.

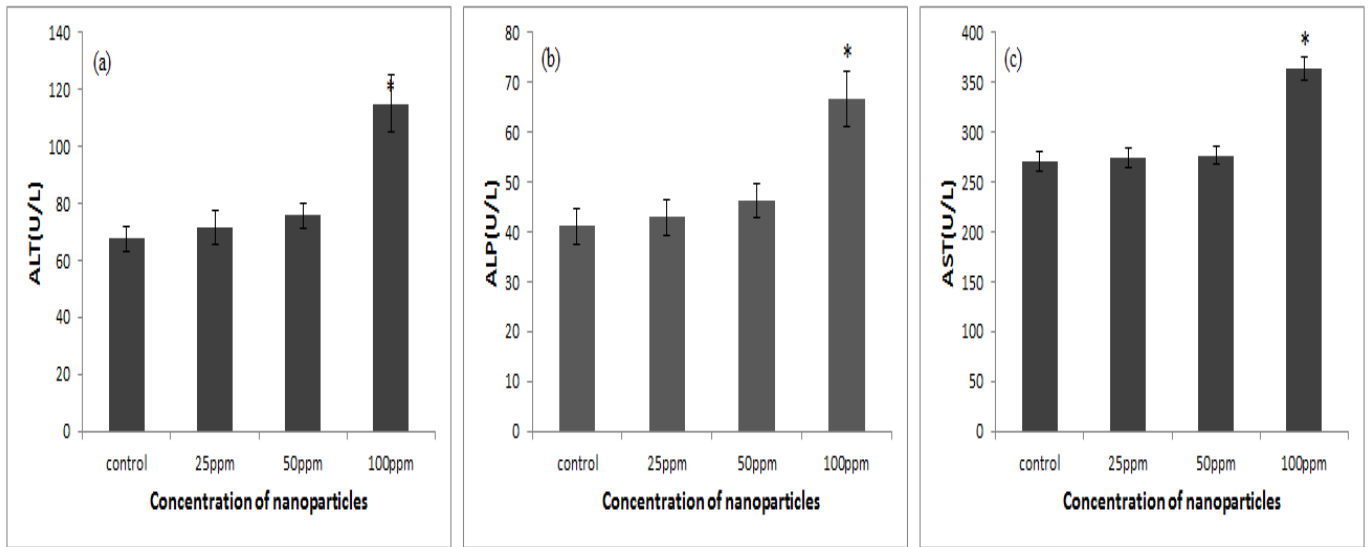


Figure 3. Serum concentrations of blood factor in experimental groups were treated by different doses of zirconia oxide nanoparticles. Data are presented as the MEAN±SD of 3 independent experiments.*p<0.05 vs. control, **p<0.01 vs. control. (a): effect of ZrO₂ on ALT, (b): effect of ZrO₂ on ALP, (c): effect of ZrO₂ on AST.

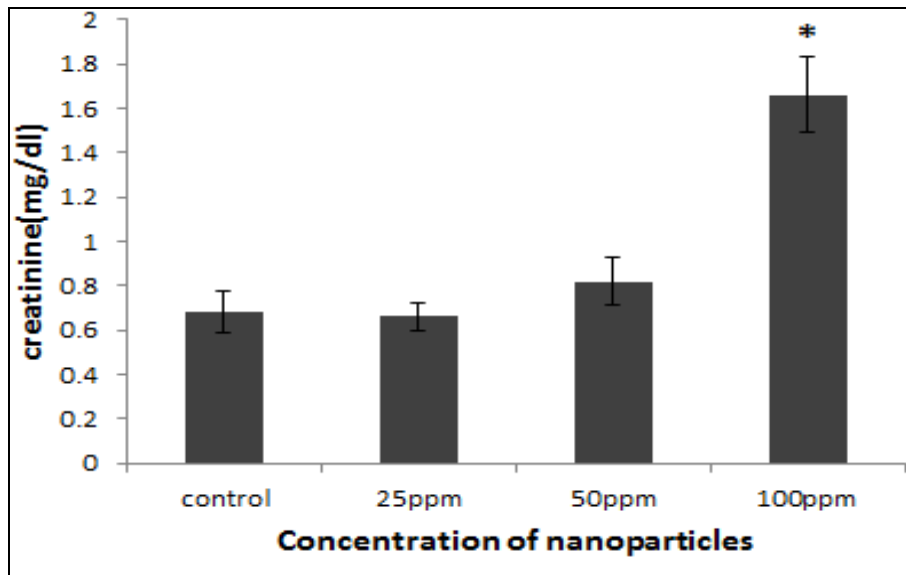


Figure 4. Serum concentrations of blood factor in experimental groups were treated by different doses of zirconia oxide nanoparticles. Data are presented as the mean ± SD of 3 independent experiments.*p<0.05 vs. control, **p<0.01 vs. control. Effects of ZrO₂ on creatinine.

Effects of ZrO₂ NPs on the antioxidant system

The Catalase, Glutathione Peroxidase and Superoxide Dismutase which are considered as the primary antioxidants are responsible for the direct elimination of generated ROS. A significant decrease in the level of enzymes received by the treatment groups respectively (see figure 5 (d, e, f), showed a significant difference with the control

group (p<0.05). Oxidative stress are generated due to imbalance state of the antioxidant enzymes and induces the generation of free radicals and free radicals cause the generation of apoptosis in turn. In the MDA, a significant increase in the level of enzymes in the treatment groups respectively showed a significant difference with the control group (p<0.05) (see figure 5 (g).

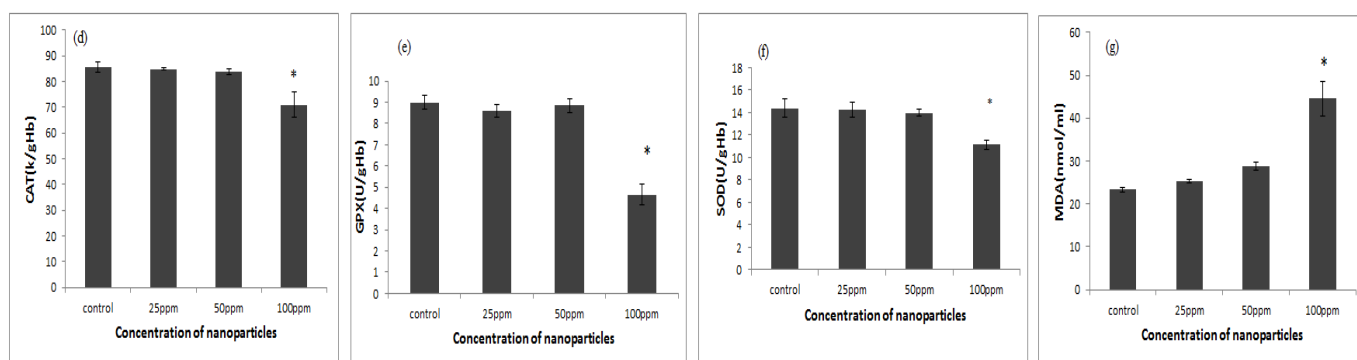


Figure 5. Serum concentrations of blood factor in experimental groups were treated by different doses of zirconia oxide nanoparticles. Data are presented as the mean \pm SD of 3 independent experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control. (d): effect of ZrO₂ on CAT, (e): effect of ZrO₂ on GPX, (f): effect of ZrO₂ on SOD, (g): effect of ZrO₂ on MDA

Histo-pathological Studies

Histological analysis of liver and kidney tissues was carried out in order to examine the potency of ZrO₂NPs to the tissue damage. The liver of the control rats showed a normal hepatic architecture of portal triad and central vein (figure6 (a)). The rats treated with ZrO₂NPs showed a significant difference in inflammation of the liver (table 1) attributed to the fourth group that were

received NPs with 100ppm concentration (figure6 (b)). The controlled kidney sections showed normal renal cortex and glomerular tufts (figure7 (c)). The rats treated with ZrO₂NPs showed a significant difference (table 1) in kidney congestion and in destruction of the glomerular capsule space attributed to the fourth group that were received NPs with 100ppm concentration (figure7 (d, e)).

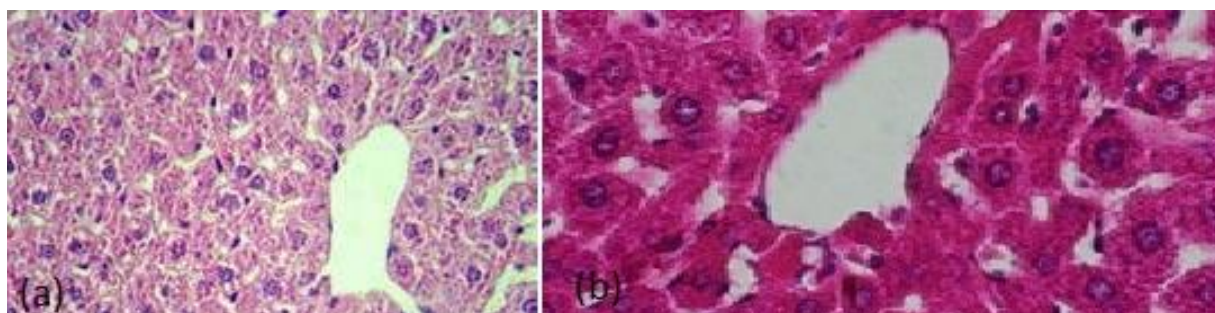


Figure 6. Histopathologic effect of ZrO₂NPs on liver of rat, H & E staining, magnification 400 xs.

A: Normal liver of rat as a control type.

B: Morphometric changes after treatment by high dose dosage of ZrO₂ NPs. Inflamed liver of rat following to high dosage treatments by ZrO₂ NPs.

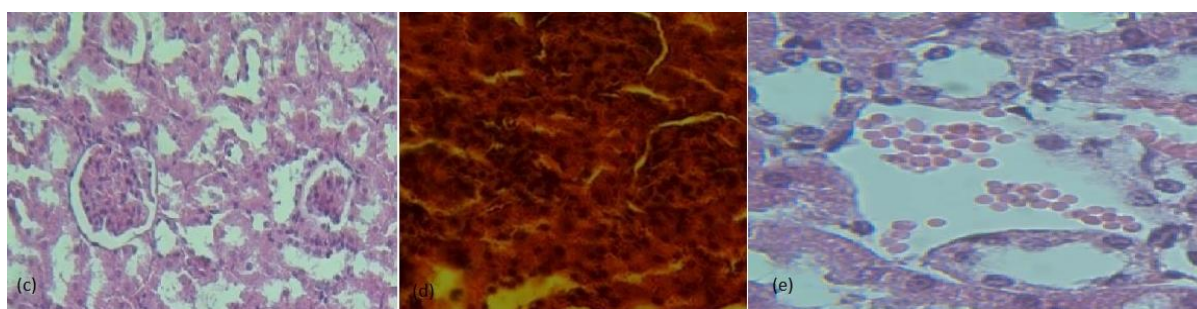


Figure 7. Histopathologic effect of ZrO₂NPs on kidney of rat, H & E staining, magnification 400x

C: Normal kidney of rat as a control type.

D: Morphometric changes after treatment by high dosage of ZrO₂ NPs. Glomerular damage of rat after high dosage treatment of ZrO₂NPs.

E: Morphometric changes after treatment by high dosage of zirconium Oxide nanoparticles kidney congestion of rat after high dosage treatment of ZrO₂ NPs.

Table 1. Effects of zirconia oxide nanoparticles over the damage of liver and kidney. Data are presented as the mean \pm SD of 3 independent experiments. * $p < 0.05$ vs. control, ** $p < 0.01$ vs. control. Grade0: no damage, grade1: middle damage, grade2: moderate damage, grade3: high damage.

		N	Mean	St. Deviation	Std. error	P value
Inflammation of liver	Control	10	0.0000	0.0000	0.0000	P=0.016*
	25ppm	10	0.2000	0.44721	0.2000	
	50ppm	10	0.4000	0.54772	0.24495	
	100ppm	10	1.0000	0.70711	0.31623	
Glomerular damage	Control	10	0.0000	0.0000	0.0000	P=0.003**
	25ppm	10	0.4000	0.54772	0.24495	
	50ppm	10	1.0000	0.70711	0.31623	
	100ppm	10	1.6000	0.89443	0.4000	
Kidney congestion	Control	10	0.0000	0.0000	0.0000	P=0.022*
	25ppm	10	0.2000	0.44721	0.2000	
	50ppm	10	0.2000	0.44721	0.2000	
	100ppm	10	1.2000	1.09545	0.48990	

Discussion

The main finding of this study was the significant increase ($p < 0.05$) in ALP, ALT, AST levels by the administration of 100ppm ZrO₂NPs as compared to the normal group. An initial step in detecting the liver damage is to run a simple blood test to determine the presence of certain liver enzymes in blood. Under normal circumstances, these enzymes reside within the cells of the liver. But when the liver is damaged, these enzymes are spilled into the blood stream. The ALT, AST and ALP are available in liver cells in normal conditions; however, in case of damaging to the cell, they transmit into the serum. The absorbed Nano silver bound to the plasma proteins results in its destruction in certain organs such as liver, or causes damage to the mitochondria and declines the Glutathione level. Other results of this study provided a significant increase ($p < 0.05$) in creatinine level with 100 ppm ZrO₂NPs concentration as compared to the normal group. An initial step in detecting the kidney damage is running a simple blood test to determine the presence of certain kidney enzymes in the blood. The level of symptomatic creatinine attributed to the renal function was also increased significantly in the treated groups in comparison with the Control group. In the analysis associated with the molecular mechanisms of the Anti-oxidative effects of ZrO₂NPs in high concentrations showed that they induce the oxidative stress in rat and the effects of ZrO₂NPs on GPX and the CAT levels in treated rats were investigated simultaneously. As oxidative stress is due to the increase in the formation of free radicals, it results in damage to the chemical elements of the cell such as protein and lipid structures which in turn results in release of some enzymes into the blood and in this regard, The levels of some tissue enzymes related to liver such as Aspartate Aminotransferase and Alanine Aminotransferase are increased in the blood. Oxidative stress in humans is the result of imbalance in the situation of some antioxidants.

In most cases, it finally results in oxidative damage. It seems that reactive oxygen species are produced in every tissue through different mechanisms. Nanotechnology researchers have become familiar with extensive aspects of nanoparticle applications that may have a big role in medicine, prevention and treatment of diseases and production of drugs. Dewey et al (2009) showed that zinc nanoparticles are able to protect the integrated structure of cell membrane against oxidative damage of free radicals, increase the level of antioxidant enzymes and reduce Malondialdehyde level; while in this study gold nanoparticles have resulted in the increase of free radicals production. Using gold nanoparticles equal to 2.5 mg/kg in intraperitoneal injection in rats, Selvaraj Barath ManiKanth et al (2010) concluded that gold nanoparticles result in reduction of catalase and glutathione peroxide in healthy and diabetic rats. Many studies in laboratory environment have shown the toxicity of zinc oxide. For example, in 2009 Cory Hanley proved that zinc oxide nanoparticles in culture medium result in the production of reactive oxygen species (ROS) and then result in oxidative damage, cell inflammation and death and zinc oxide decomposition in cell culture medium. Also, intracellular ROS has a significant relationship with survival and the level of lactate dehydrogenase. In terms of increase in the level of lactate Dehydrogenase and cell death, the results of the present study are compatible with the results of the study by Cory Hanley. The study of zinc oxide antibacterial effects and the measurement of zinc spin resonance in 2010 by Venubabu Thati indicated that aqueous suspensions of zinc oxide small nanoparticles results in the production and increase in the activity of special oxygen and significant increase of oxidative stress. In terms of cell damage, the observations of the present study are compatible with the study of Venubabu Thati. The use of magnesium oxide nanoparticles in Wistar rats showed that magnesium oxide nanoparticles result in oxidative stress in rats by reducing the antioxidant capacity and result in

sub-acute toxicity in them by reducing superoxide dismutase and catalase and the observations of this study are compatible with those results. The use of silver nanoparticles at 100 and 200 ppm doses results in the increase of catalase enzyme and with the increase in the level of the used dose, the amount of this enzyme increases too and it was concluded that silver nanoparticles result in oxidative stress and inducing antioxidant enzymes. The use of copper nanoparticles at the dose of less than 50 nm resulted in the reduction of superoxide dismutase and catalase and this damage was increased gradually in one day and one week after intra-pulmonary injection. In a study by Liu et al, the researchers showed that the use of copper oxide nanoparticles results in the increase of superoxide dismutase and catalase secretion. Oxidative stress is increased with the toxicity of nanoparticles and the increase in the production of ROS and oxidative stress can be one of the sign of the toxicity of nanoparticles.

Conclusion

The obtained results reveal the significant role of ZrO₂ as an increasing ROS generation agent and the ROS induces the generation of free radicals in turn. Those free radicals cause damage to different tissues. Following, to damage to the liver, its enzymes may leak into the blood stream excessively.

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