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MODULATION OF GLUTATHIONE, PHOSPHOLIPIDS AND LIPID PEROXIDES LIVER CONTENTS INDUCED BY ZINC OXIDE NANOPARTICLES USING NATURAL ANTIOXIDANTS

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With increasing industrial production of engineered nanoparticles, questions have been raised about their effects on human and animals. So the objective of this study is to detect the prophylactic role of either α-lipoic acid (α-lip) or vitamin E (vit E) against the hepatotoxic effect of Zinc oxide nanoparticles (ZnO-NPs) 50-nm induced metabolic disorder. ZnO-NPs were administered orally using two doses (600 mg and 1 g/kg body weight/day for 5 consecutive days). ZnO-NPs exhibited a marked increase in serum ALT and lipid peroxide (LP) as well as total phospholipids (PL), phosphatidylcholine, phosphatidylethanolamine levels while the liver content of reduced glutathione (GHS) and sphingomyelin as well as triglycerides levels was decreased compared with normal control group. Concurrent treatment of rats with either α-lip (200 mg/kg body weight) or vit E (100 mg/kg body weight) daily for three weeks to ZnO-NPs intoxicated rats significantly alleviated most of the deviated previous biochemical parameters. It was concluded that the decline in sphingomyelin content may be considered as a novel mechanism for ZnO-NPs,it has an apoptotic action and the treatment with either the aforementioned antioxidants has a beneficial effect.

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1. Introduction

The novel physical and chemical properties of nanoparticles (NPs) make them attractive for use in medical, agricultural, industrial, manufacturing, and military sectors. Although more and more nanoparticles enter the environment with the increasing development of nanotechnology, little is known about their interactions with biological systems. The small size and large surface area endow them with an active group or intrinsic toxicity. Since nanoparticles diameter does not exceed a hundred nanometers at maximum, they are able to penetrate cells and interfere with several subcellular mechanisms [1]. Møller et al. [2] proposed that the size of nanoparticles surface area greatly increases their ability to produce reactive oxygen species (ROS). Indeed, some studies show that some nanoparticles can penetrate into cell nuclei and hence may directly interfere with the structure and function of genomic DNA [3]. Several studies have reported that inhaled or injected nanosize particles enter the systemic circulation and migrate to various organs and tissues [4] where they could accumulate and damage organs and biological systems that are especially sensitive to oxidative stress [2]. NPs interaction with cell membranes can damage the cell membrane and cause toxicity; therefore, examining interactions between NPs and cell membranes is crucial to understanding NPs toxicity mechanisms [5].

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It was reported that exposure to ZnO-NPs resulted in oxidative damage and inflammation response in vascular/lung endothelial cells [6]. Animal experiments indicated that liver, spleen, heart, pancreas, and bone were target organs of oral exposure to 20- and 120-nm ZnO-NPs (n-ZnO) [7]. Compared with the conventional toxicology, the dose of ZnO-NPs is no longer a sole factor in evaluating the toxicity of nanoparticles, but the physicochemical properties of its nanoparticles, such as size, shape, chemical composition, aggregation, high specific surface area and its solubility may play a more important role in its toxicity [8].

It was found that interaction between ZnO NPs and lipid membranes cause a significant reduction of lipids diffusion mobility, which can be explained as a result of lipid-ZnO aggregates binding, depending on ZnO concentration [9].

Phospholipids play an important role as they constitute a permeability barrier, modulate the functional properties of membrane-associated activities, provide a matrix for the assembly and function of a wide variety of catalytic processes, and act as donors during the synthesis of macromolecules [10]. Next to the naturally occurring lipids, their modification by oxidation processes via generation of ROS is very important in human physiology because oxidized free fatty acids may be converted into messenger molecules such as leukotrienes or thromboxanes [11]. Since an excess oxidatively modified PL favors inflammatory processes, and thus the development of inflammatory diseases [12]. LP in high concentrations can trigger cell death.

GSH is the major endogenous antioxidant produced by the cells, participating directly in the neutralization of free radicals and ROS, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced (active) forms [13].

Vit E (γ-tocopherol) the major lipid-soluble antioxidant in the body, protects the membranes' integrity by inhibiting lipid peroxidation and has a central role in neurological structure and function maintenance [14]. It performs its functions as an antioxidant in the glutathione peroxidase pathway [15], and it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction [16].

Alpha-Lipoic acid (1,2-dithiolane-3-pentanoic acid) is a natural occurring antioxidant compound with recognized beneficial effects in chronic inflammatory diseases such as obesity and diabetes [17]. It has been described that deficiency of this enzyme results in an overall disturbance in the antioxidant defense network, leading to increased inflammation, insulin resistance, and mitochondrial dysfunction [18]. It has an ROS scavenging activity; capacity to regenerate endogenous antioxidants such as GSH and vit C and E and metal-chelating activity [19]. It is effective in preventing hepatic oxidative stress, down-regulating the expression of hepatic pro-inflammatory cytokines, as well as inhibiting NF-κB expression [20]**.**

The aim of this work is to investigate the potential hepatoprotective role of α-lip and vit E through measuring GSH and LP liver contents as well as total PL and their components for the first time to investigate the alteration in sphingomyelin content which may hydrolyzed into ceramide and can be considered as a novel mechanism for ZnO-NPs apoptotic action.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

All chemicals used were of high analytical grade, a product of Sigma and Merck companies. Kits used for the quantitative determination of different parameters were purchased from sigma Aldrich Biogamma,Stanbio,west Germany.

2.1.2. Characterization of nanometer ZnO

The 50-nm ZnO powders were purchased from Sigma Co. (USA). The size distribution of 20- ZnO in the administration solution (1% sodium carboxymethyl cellulose) was performed using a 90Plus Particle Size Analyzer (PSA) equipped with 50-mW solid state laser operating at 659nm wavelength (Brookhaven Instrument Corp).

2.1.3. Animals and treatments

The animal experiment was performed with compliance of the local ethics committee, 90 healthy male albino rats (120-150 g.) were supplied by the Experimental Animal Center, King Saud University. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house at 20 ± 2 °C, 50–70% relative humidity and 12-h light/dark cycle. The animals were provided with commercial rat pellet diet and deionized water *ad libitum*. After one week acclimation, the rats were kept fasting overnight before treatment and randomly divided into two classes according to the dose of ZnO –nanoparticle administered to rats

Class I, consists of five groups, each of ten rats.

G1:Normal healthy animals

G2-G4 groups of animals administered orally 600mg/ Kg body weight/day [21] ZnO-NPs for 5 consecutive days and divided as follow

G2: ZnO-NPs - intoxicated animals with single oral low dose (600mg/ Kg/day)

G3: ZnO-NPs - intoxicated animals co-administered with α-lip (200mg/Kg) [7]

G4: ZnO-NPs - intoxicated animals co-administered with vit E(100mg/Kg) [7]

ClassII consists of 4 groups (G5-G7), each of ten rats, administered orally 1g/ Kg body weight / day for 5 days ZnO- NPs [21] and divided as follow

G5 : ZnO-NPs - treated animals with single oral dose (1 g/ Kg)

G6 : ZnO-NPs - intoxicated animals co-administered with α-lip (200mg/ Kg),

G7: ZnO-NPs - intoxicated animals co-administered with Vit E (100mg/ Kg),

The ZnO-NPs were suspended in 1% sodium carboxymethyl cellulose and dispersed by ultrasonic vibration for 15 min. The control group was given by 1% sodium carboxymethyl cellulose solution instead. α-lip acid and Vit E were orally administered daily for three weeks.

Three weeks later, the blood samples were collected from each animal in all groups into sterilized tubes for serum separation. Serum was separated by centrifugation at 3000 r.p.m. for 10 minutes and used for biochemical serum analysis. After blood collection, rats of each group were sacrificed under ether anesthesia and the liver samples were collected, for biochemical determination.

liver removed and rinsed with cold 0.9% sodium chloride, then homogenized in ice-cold 0.25M of sucrose solution using a Teflon homogenizer.

2.2. Methods

2.2.1. Biochemical assay of serum ALT

ALT was estimated using Stanbio kit produced by Stanbio Labs,Texas, USA.

2.2.2. Biochemical assay of liver tissue

2.2.2.1.Determination of GSH level

Liver tissue levels of acid-soluble thiols and reduced GSH were determined colorimetrically at 412 nm [22]. Homogenates were precipitated with trichloroacetic acid, and after centrifugation, supernatants were used for the estimation of protein thiols (Protein-SH) expressed as mol/g tissue.

2.2.2.2. Determination of lipid peroxides

The LP concentration was determined by the method measuring the amount of thiobarbituric acid (TBA) reactivity by the amount of malondialdehyde (MDA) formed during acid hydrolysis of the LP compound. Thiobarbituric acid-reactive substances

(TBARS) in liver were measured using a modification of the method of Uchiyama and Mihara [23]. The absorbance was measured spectrophotometrically at 532nm and quantified as nanomoles of malondialdehyde (MDA)/g wet tissue.

2.2.2.3. Determination of phospholipid

The method reported by Folch et al. [24] was modified to extract PL from biological samples. Briefly, tissue homogenate was transferred to a graduated glass tube. Subsequently, chloroform– methanol (2:1, v/v) was added to the glass tube at twice the volume as that of the used samples. It was then strongly oscillated for 1 min and centrifuged at 2500 g for 10 min. After

centrifugation the supernatant was discarded, but the boundary layer was not. The methanol–water solution (1:1, v/v) was added to the glass tube at a quarter of the volume as that of the subnatant. It was also subsequently oscillated strongly for 1 min and centrifuged at 2500 g for 10 min. The supernatant and the boundary layer were then discarded. Finally, the subnatant was transferred to another glass tube, dried under a stream of the nitrogen, and stored at –20°C. The extracted PL was dissolved in a mobile phase solvent containing 20% chloroform before HPLC analysis.

2.2.2.4. Determination of triglycerides

Liver content of triglycerides was determined using Kit from Randox Company Chemical CO, it was measured in mg/dL

2.3. Statistical Analysis

Data were expressed as means ± SEM. The results were analyzed statistically by One-way analysis of variance (ANOVA) using SPSS (Statistical Package for the Social Sciences, version 16.0.1, Chicago, IL) software. Individual treatment means were compared post hoc by the Scheffé test. The level of significance was set at; $p<0.05$ (slightly significant); $p < 0.01$ (significant) and $p < 0.001$(highly significant). Statistical analysis was performed using Graph pad Instat 3 software Inc, San Diego, CA, USA.

3. Results

The level of serum ALT was significantly increased in rats administered either low or high repeated doses of ZnO-NPs for 5 consecutive days (Fig 1). Co-administration of either vit E or α-α-lip significantly attenuated ZnO-NPs induced elevation of serum ALT.

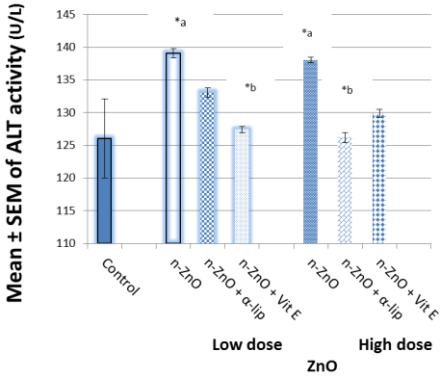

*Fig. 1. ALT activity (U/L) in the serum of control and different treated groups * p<0.05 was considered slightly significant; Data were expressed as mean ± SEM; n=10.a,b: Significantly different from control and n-ZnO -treated group.*

The intake of α-lip or vit E along with ZnONPs ingestion markedly increased GSH level which was decreased in a ZnO-NPs administered group versus intoxicated animals (Fig 2A).

Treatment by either the antioxidant agents significantly reduced the elevation of the liver content of LP in ZnO-NPs- intoxicated rats (Fig 2B) while such treatment significantly ameliorated the highly significant depletion in triglycerides liver contents compared with control group ($p<0.05$) (Fig 2C).

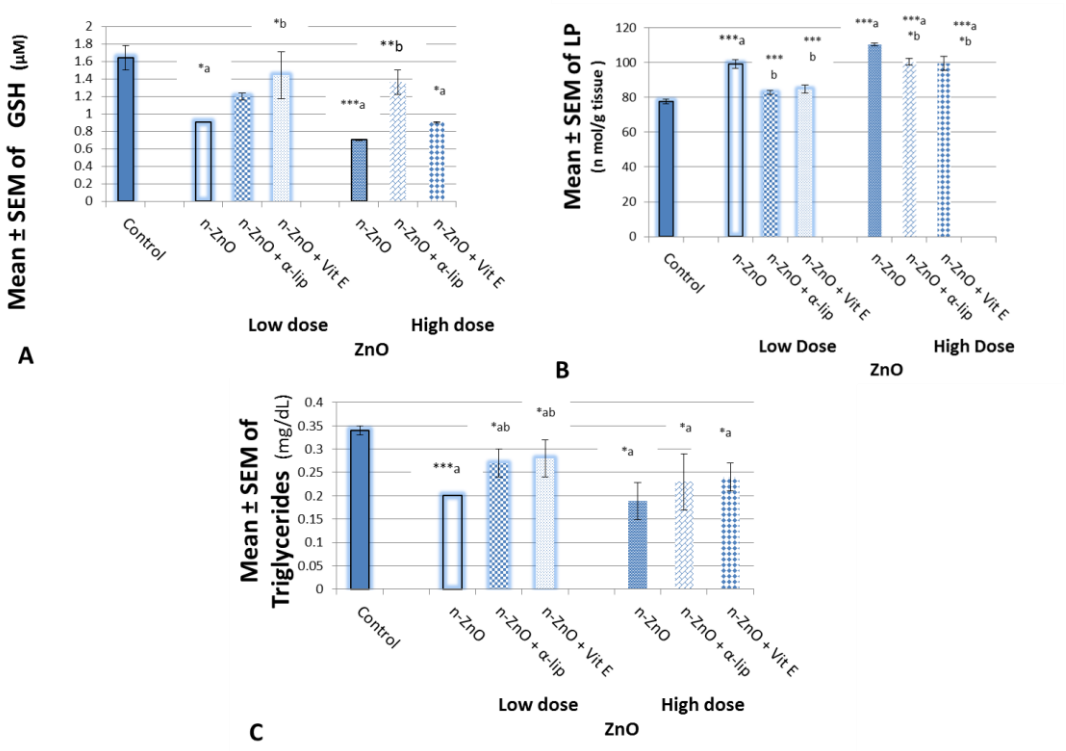

*Fig. 2. Liver content of A: Glutathione B: Lipid peroxide C: Triglycerides of control and different treated groups ***P < 0.001, * p<0.05 were considered highly significant and slightly significant; respectively; Data were expressed as mean ± SEM; n=10.a,b: Significantly different from control and n-ZnO -treated group. ab: Significantly different from both control and n-ZnO -treated group*

Table (1) presents total PL, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin (SPM) in livers of ZnO-NPs-intoxicated rats with low and high doses as well as different treated groups.

PL content in the liver of ZnO-NPs- intoxicated rats after the treatment of either the low or high dose showed a highly significant increase at (P<0.001), co-administration of the pre-mentioned antioxidants significantly decreased such elevation.

Ingestion of the low dose of ZnO-NPs significantly decreased SP content compared with control group (p<0.05), while the high dose depleted the SP content at (p<0.001), such depletion was highly significant decreased after the co-administration of either vit E or α- lip compared with the control group (p<0.001).

Table 1. Total phospholipids (PL), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin(SPM) in livers of ZnO-NPs-intoxicated rats with low and high doses in different treated groups.

	Groups	PL	PE	PS	PC	SP
	Control	1. 12±0.035	0.34±0.021	0.24 ±0.08	0.227 ±0.03	0.385±0.01
	n-ZnO	1.47±0.045 ***a	0.33±0.05	0.26±0.02	0.214 ±0.05	0.316 ±0.02 *a
Low Dose	n-ZnO + α-lip	1.32±0.031 **a	0.39±0.09	0.31± 0.06	0.209±0.03	0.345 ±0.02
	n-ZnO + Vit E	1.22±0.092 ***b	0.41 ±0.08	0.36 ±0.055 *a	0.219±0.07	0. 406±0.03 **b
	n-ZnO	1.65±0.066 ***a	0.37±0.05	0.25±0.06	0.209 ±0.05	0.247 ±0.03 ***a
High Dose	n-ZnO + α-lip	1.32±0.052 ***ab	0.34±0.04	0.23± 0.04	0.221±0.033	0.285 ±0.04 ***a
	n-ZnO + Vit E	1.28±0.022 *a/***b	0.33±0.05	0.24±0.05	0.223±0.06	0. 298±0.02 ***a

Values are expressed as mean ± SD. ***p≤0.001, ** p ≤0.01, *p ≤0.05 were considered highly significant, significant and slightly significant; respectively; a: Compared to Control group, b: Compared to ZnO-NPs group. ab: compared to both control and n-ZnO - treated groups.

4. Discussion

Engineered nanoparticles enter the body by inhalation [25],ingestion [26]. In order to protect ourselves from their (potentially) harmful effects and use them beneficially, their interactions with living beings should be well studied as well as the underlying biochemical and biophysical mechanisms should be better understood [27].

Previous reports showed that nanoparticles can interact with erythrocytes resulting in shape transformations [28] and lysis [29], induce platelet activation, aggregation and adhesion [30], and increase the risk of thromboembolic disorders [31].

Previous studies reported that exposure to ZnONPs results in the generation of excess ROS in zebrafish embryos resulting in the up- and down-regulation of several antioxidant enzymes based on the concentration and duration of nanoparticle exposure [32]. Changes in the expression of antioxidant genes are the initial mechanisms that act to adjust antioxidant levels to protect the cells [33]; hence, NPs can hinder cellular transcription and translation machinery and released metal ions may also lead to cytoplasmic mRNA degradation by interacting with mRNA-stabilizing proteins [34] which contain metal responsive domains. NPs can interact with cellular signal molecules, which lead to signaling cascade activation [35] and induce DNA damage and cell death.

The current study revealed that either the two doses of ZnO-NPs (600 mg/kg/day and 1 g/kg/day for 5 days) induced liver damage documented by the elevation of serum ALT compared with the control group; these findings coincide with that of Yang et al. [36], implying cellular leakage and loss of the functional integrity of cell membranes in the liver [7]. This finding reflects that liver is one of the target organs of such NPs toxicity. Wang et al. [37] reported that high dietary zinc caused liver toxicity in mice and resulted in an increase in the activity of ALT in liver homogenate of mice. Co-administration of either α-lip or vit E to ZnO-NPs intoxicated rats with either low or high doses, reduced serum ALT level compared with intoxicated rats. This may indicate that the used agents act as effective hepatoprotective against liver dysfunction caused by nanomaterials toxicity. The hepatoprotective effect of either α-lip or vit E against hepatic tissue injury was previously documented [38].

ROS can initiate an increase in lipid peroxidation, and an impairment of both enzymatic and non-enzymatic antioxidant defense systems of brain tissues [39].Previous studies suggested that ZnO NPs induced DNA damage may be related to lipid peroxidation and oxidative stress [40].It caused dramatic increases in the apoptosis marker caspase 3 malondialdehyde (MDA, a major product of lipid peroxidation) and serum pro-inflammatory biomarkers including, TNF-α, and C-reactive protein (CRP) as well as in serum vascular endothelial growth factor (VEGF) (angiogenic factor). While, it decreased antioxidant enzyme, glutathione peroxidase (GPx), it also caused oxidative DNA damage in kidneys of intoxicated rats [41].In the current study hepatic GSH levels were significantly reduced after ZnO-NPs treatment this is in agreeing with that of [36] while treatment with either α-lip or vit E increased GSH content in liver compared with ZnO-NPs intoxicated rats. This was attributed to the antioxidant properties of both agents. Previous investigations reported that vit E supplementation protects against oxidative damage caused by different pathological conditions through inhibition of ROS production [42-43]. Additionally, α-lip has the ability to correct thiol deficiency within cells [44] increase the de novo synthesis of GHS [45] and enhance GSH synthesis in normal animals [46]. Moreover, α-lip can maintain high levels of vit C and participate in vit E-recycling, thus complementing some of the functions of GSH [47]. These data augment the current results in which either of the used antioxidants significantly decreased liver damage due to ZnO-NPs by synergistically increasing cellular levels of GSH (Figure 2 A). Sharma et al. [48] indicated that ZnO NPs-induced cytotoxicity was concentration- and time-dependent.

The liver is regarded as one of the central metabolic organs in the body, regulating and maintaining lipid homeostasis [49]. Nonenzymatic molecular modifications induced by reactive carbonyl species (RCS) generated by peroxidation of membrane PL acyl chains play a causal role in the aging process. Most of the biological effects of RCS, mainly α, β-unsaturated aldehydes, dialdehydes, and keto-aldehydes, are due to their capacity to react with cellular constituents, forming advanced lipoxidation end products (ALEs). Compared to reactive oxygen and nitrogen species, lipid-derived RCS are stable and can diffuse within or even escape from the cell and attack targets far from the site of formation. Therefore, these soluble reactive intermediates, precursors of ALEs, behave as mediators and propagators of oxidative stress and cellular and tissue damage. The consequent loss-of-function and structural integrity of modified biomolecules can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage [10]. It was reported that treated *E. coli* cells exhibited triple membrane disorganization and increased membrane permeability following exposure to ZnO-NPs, which was internalized and accumulated in cell membranes [50]. The reduction in triglyceride level seen in the present study may also be attributed to the unavailability of free fatty acids for triglyceride production in the liver as has been reported by [51]. On the other hand, PL contents of liver in ZnO-NPs treated animals increased significantly. It is well known that the synthesis of triglycerides in the liver is achieved by transfer of an acyl group to a diglyceride molecule which is the product of phosphatidate phosphohydrolase enzyme. When a fatty acid is limited, diglyceride molecules so produced are diverted to PL synthesis. This explains why the reduction in triglyceride levels is accompanied by the elevation of PL contents of the liver in the present study.

It was reported that ZnO-NPs could cause membrane rupture and blood cells and PLvesicles are convenient systems for the study of membrane properties and their interactions with various substances [52].

Ceramide, which is considered to serve as the second messenger, is mainly generated by hydrolysis of the membrane sphingophospholipid (sphingomyelin (SM) through the action of sphingomyelinase (SMase neutral sphingomyelinase (N-SMase) in the generation of the proapoptotic ceramide. It plays an important regulator of cell growth, senescence, and apoptosis [53]. The activation of sphingomyelinase and the generation of ceramide has been proposed to mediate TNF-α-induced NF-κB activation through its second messenger ceramide. Ceramide may also be a Sphingolipid breakdown products are now being recognized as important players in apoptosis [54].

It was reported that the signal transduction pathways triggering apoptotic mechanisms after ischemia/reperfusion may involve TNF- α secretion, ceramide generation, and initiation of

lipid peroxidation. TNF- α is known to induce free radical production. Accumulation of TNF and increase of sphingomyelinase activity during the development of ischemic/reperfusion injury coincided with an increase in the content of lipid peroxidation products (conjugated dienes) and DNA degradation detected by gel electrophoresis [55]. Superoxide radicals are used as signaling molecules within the sphingomyelin pathway. This suggests the existence of cross-talk between the oxidation system and the sphingomyelin cycle in cells, which may have important implications for the initial phase and subsequent development of post-ischemic injury [55].

It has recently been demonstrated that administration of antioxidant therapy such as Vit E and or α-Lip significantly decreased the increase in serum pro-inflammatory biomarkers level including TNF-α, IL-6, and CRP. Moreover, either of the two agents successfully alleviated the alteration in nitric oxide (NO) and VEGF in the serum of ZnO-NPs intoxicated group [56]. The modulatory role of either α-lip or vit E in sphingomyelin level in the present study which may attribute to the apoptotic action of ZnO-NPs are in agreement with those of Al- Rasheed et al. [57] who proved that either α-lip or vit E is a good hepatoprotective agent against ZnO-NPs toxicity because they ameliorated metabolic disorders related to liver damage and modulated serum ALT and glucose levels as well as the pro-inflammatory biomarkers NO, TNF-α, IL-6, CRP . Reduced GSH level was decreased while caspase3 level was elevated in liver tissues of ZnO-NPs treated group compared with intoxicated one also ZnO-NPs induced hepatic oxidative DNA damage.

5. Conclusion

It was concluded that the decline in sphingomyelin content may consider as a novel mechanism for ZnO-NPs through hydrolyzing it into ceramide which has an apoptotic action. Co-administration vit E and α-lip acid supplementation showed a hepatoprotective effect against zinc-oxide nanoparticle hepatoxicity. So the use of the aforementioned antioxidants may be used as a candidate for liver protection and therapy.

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