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Quercetin Ameliorates Zinc Oxide Nanoparticles Toxicity In Rats.

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ABSTRACT

Nanoparticles are small-scale substance with unique properties. They have many beneficial uses but their hazard use can introduce a complex health problem; so this study was designed to investigate the toxic effect of different doses of zinc oxide (ZnO) nanoparticles (NPs) and the ameliorative effect of quercetin (Qur). Sixty male albino rats was divided into six groups (10 each), first one considered as a control, second treated orally with 100mg/kg body weight (B.Wt.) of Qur, third and fourth treated by 125 mg and 300 mg/kg B.Wt. of ZnO-NPs orally for 21 days, the fifth and sixth groups were co-treated by ZnO-NPs and Qur. Serum analysis of liver enzymes showed a significant increase in ALT, AST and ALP activities and a significant increase in lipid peroxidation marker Malonaldhyde (MDA) with a significant decrease in Glutathione reduced (GSH) and Glutathion peroxidase (GPx) enzymes activity in hepatic tissue, also there were an elevation in inflammatory gene expression of cytokines (TNF-α), (IL-2) and (IL-6) after ZnO-NPs treatment especially in high doses with amelioration of that increase by Qur treatment. In the same manner histopathological examinations confirm that results. ZnO-NPs treatment ameliorated also by Qurtreatment which confirms the anti-inflammatory and antioxidant role against ZnO-NPs hepatotoxicity.
Keywords: nano- Zinc oxide, hepatotoxicity, oxidative stress, Quercetin.

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INTRODUCTION

The rapid growth of the nanotechnology industry has led to wide-scale production and application of engineered nanoparticles (NPs).Over the years, NPs have gained considerable and extensive importance because of their unique properties (size, surface area, optical characteristics and good bio compatibility) [1]. NPs are not only used in industry and medicine but are also increasingly used in various consumer products such as cosmetics, sunscreens, and food products [2]. Zinc oxide is an inorganic compound in the form of a white powder, with a unique physical and chemical properties[3]. It is part of several daily life materials such as paints, dyes, pigments, metallurgy additives, alloys, rubber, chemical fibers, ceramics, catalyst, electronics, medical diagnosis, cosmetics, sunscreens, personal care products and food additives [4]. The use of ZnO NPs, has been rising steadily, resulting in more attention being paid to their potentialtoxicity, including cytotoxic, genotoxic, and pro-inflammatory effects [5,6]. Many reports confirmed that ZnO NPs at high doses (1-5 g/kg) can cause apoptosis in murine liver cells and induce severe oxidative stress [7, 8]. Moreover, recent studies have demonstrated that ZnO-NPs are toxic to microorganisms and rodents, the release of metallic cations Zn^{2+} are the main causes of toxicity [9].

Medicinal plants has gained enormous popularity and emerged as a potential therapeutic to prevent free radical generated damage in the human body.Medicinal plants are being viewed as easily available and potent source of antioxidants as they contain a mixture of different chemical compounds that may act individually or in synergy to cure the disease and improve health especially plants contain flavonoids. Flavonoids are a class of phenolic compounds widely distributed in plants. Quercetin belongs to the family of flavonoids, which are found in many foods, including vegetables, tea, fruits, and wine, and can be absorbed by humans. Quercetin has a wide range of reported biologic effects, including antioxidant, anti-hypertensive, antimicrobial, and antiprotozoan activities [10, 11, and 12]. Quercetin, known as a natural anti-inflammatory/anti-allergy remedy, stabilizes mast cell membranes and prevents the release of histamine and other inflammatory agents in the body; it has been shown to reduce inflammatory cytokines [13].

In the light of the above mentioned, this work was designed to explore the potential protective action of Quercetin against the expected hepatotoxicity of ZnO NPs.

MATERIAL AND METHODS

Chemicals

The 27-nm ZnO-NP powders were purchased from faculty of science of BeniSuef University in EGYPT. Quercetin was obtained from Sigma-Aldrich Co. (USA).

Animals

Sixty male adult albino rats weighting at the beginning of the experiment(150±20g) were randomly divided into six groups (10 rats in each). Animals were housed in groups of ten in cages at 25±0.5°C, under a 12:12 light/dark cycle, with free access to standard diets and water at libtums. Animals from all groups were kept under similar environmental conditions of temperature, illumination, acoustic noise, and ventilation, and received the determined diet during the course of the experiment in animal house of Faculty of Veterinary medicine, Zagazig University, Egypt.

EXPERIMENTAL DESIGN

Rats were divided into 6 groups (n=10).

G1: Normal healthy animals.**G2:** Animals orally administered Qur (100 mg/kg/day) [14] for three weeks.**G3, G4:** animals orally administered ZnO-NPs (125 mg/kg/day) [15]&(300 mg/kg/day) [16] respectively for three weeks.**G5:** animals orally administered (Qur 100 mg/kg/day + ZnO-NPs 125 mg/kg/day)for three weeks.**G6:** animals orally administered (Qur 100 mg/kg/day + ZnO-NPs 300 mg/kg/day) for three weeks. All groups were kept on the same condition for twenty one days under different treatments then one day after last treatment all rats are sacrificed and blood samples collected in a clean dry capped tubes.

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Blood Collection and Tissue Samples

The blood samples were about 5 ml collected without anticoagulant, left to clot at room temperature then centrifuged at 4000 rpm for 5 min. According to **Joslin**, [17] to separate serum for biochemical analysis of ALT, AST and ALP activities using kits supplied by SPINREACT Kit (Ctra. Santa Coloma, SPAIN).

Liver from all rats were collected and divided into 3 parts: as following **First part** were taken quickly after scarifying, weighted (30 mg) and washed in normal saline and immediately kept in liquid nitrogen until be used for determination of gene expression of immunologic pro-inflammatory bio-markers (Tumor Necrosis Factor alpha TNFα) , (Interleukin-6 IL-6) and (Interleukin-8 IL-8).**Second part** (1 g.) of hepatic tissue were taken, weighted, washed and kept on -20 till homogenized in distilled water using electrical homogenizer, centrifuged at 3000 r.p.m. for 15 minutes, the resulting supernatant were collected and used for determination of lipids peroxidation (Malondialdehyde MDA) according to **Satoh.** [18].antioxidant levels (reduced Glutathione GSH) was measured according the method of **Beutler et al.** [19] and antioxidant enzyme activates (Glutathione peroxidase (GPx) was determined according to Paglla and Valentine[20].**Third part** was collected from 4 rats per group preserved in 10% neutral buffered formalin, processed and stained with haematoxylin and eosin (H&E) dyes for histopathological studies using a light microscope according to **Bancroft& Gamble** [21].

Molecular determinations

Determination of TNFα, IL-2 and IL-6 enzymes gene expression. Using a semi- quantitative RT-PCR according to **Meadus**[22].The gene expression of TNFα, IL-2 and IL-6 genes were determined using RT-PCR technique. Total RNA was extracted from separated tissues using RNeasy Mini Kit (Qiagen, Cat. No.74104).First strand cDNA was synthesized using Revert Aid TM H Minus (Fermentas, life science, Pittsburgh, PA, USA). The PCR reaction was started by using SYBR® Green PCR Master Mix Catalog Number 2501130 (Master Mix) supplied by applied bio systems in a rotor gene apparatus (Biometra- Germany). The housekeeping gene β-actin was used as a constitutive control for normalization. Primers were designed as previous published [23]. Primers were provided by Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and were listed in **table (1)**. The quantitative fold's changes in mRNA expression were determined relative to the housekeeping controls (ß-actin mRNA) levels in each corresponding group and calculated using the 2-DD CT method.(2-ΔΔCT the relative Quantification level of target genes calculation).

Table 1: Primers used in determination of the gene expression of the selected genes:

Gene	Primers	Target(bp)	Mimic(bp)
β-Actin	5 `-CCTGCGTCTGGACCTGGCTG3`	477	256
	5-CTCAGGAGGAGCAATGATCT-3`		
TNF-α	5 `-ACGCTCTTCTGTCTACTG-3`	592	292
	5 `-GGATGAACACGCCAGTCG-3`		
IL-2	5 `-AACAGCGCACCCACTTCAA-3`	400	292
	5 `-TTGAGATGATGCTTTGACA-3`		
IL-6	5 `-GAAATGAGAAAAGAGTTGTGC-3`	321	256
	5`-GGAAGTTGGGGTAGGAAGGAC-3`		

Statistical Analysis

The obtained data were analyzed and graphically represented using the statistical package for social science (**SPSS** Inc. Released 2007. SPSS for windows, Version 16.0. Chicago, SPSS Inc.) [24]for obtaining means and standard error. The data were analyzed using one way **ANOVA** to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparisons among the groups for testing the inter-grouping homogeneity.

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RESULTS

Result were presented as means ± SE of ten rats in each group. Values of $p < 0.05$ were regarded as statistically significant and the data are represented as mean ± SE for the absolute values or percent of controls as indicated in the vertical axis legend of figures. The statistical significance of differential findings between the experimental groups and control were determined and that are represented by symbols (a,b,c,d,e,f,g).

Table 2: Effect of ZnO NPs and/ or Qur on serum liver enzymes and hepatic tissue

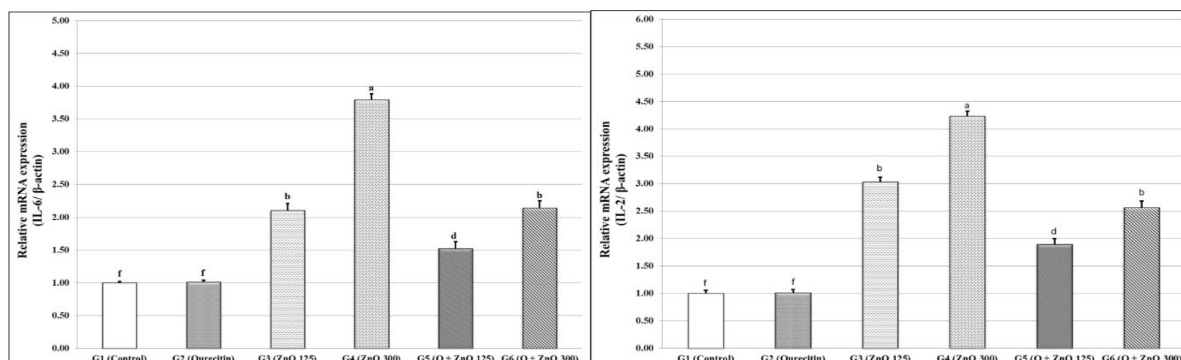
Groups	ALT u/l	AST u/l	ALP u/l	GSH mg/g.t	MDA nmol/g.t	GPX u/g.t
Group 1:(Control)	26.33 ±0.88^{g}	52.33 ±1.45^{e}	137.83 ±1.48^{f}	10.60 ±1.13^{c}	34.03 ±0.33^{d}	25.28 ±1.12^{b,c}
Group 2:(Qurecitin)	30.00 ±0.76^{f,g}	55.03 ±0.31^{e}	144.53 ±0.86^{f}	21.80 ±1.81^{a}	30.26 ±4.00^{d}	28.35 ±2.16^{a,b}
Group 3:(ZnO 125)	44.33 ±2.33^{b,c}	112.23 ±1.13^{b}	274.33 ±3.48^{c}	4.13 ±0.40^{f,g}	91.51 ±1.54^{b}	16.77 ±0.61^{e,f}
Group 4:(ZnO 300)	60.66 ±1.76^{a}	129.83 ±1.01^{a}	316.00 ±4.93^{a}	2.39 ±0.21^{g}	161.21 ±22.33^{a}	12.96 ±2.33^{f}
Group 5:(Q+ ZnO 125)	32.16 ±1.01^{e,f}	87.66 ±2.72^{d}	232.83 ±2.61^{e}	5.24 ±0.52^{e,f,g}	57.77 ±2.49^{c}	19.49 ±1.07^{d,e}
Group 6:(Q+ ZnO 300)	40.33 ±2.02^{c,d}	112.10 ±1.30^{b}	288.17 ±1.01^{b}	8.90 ±1.41^{c,d}	102.50 ±6.07^{b}	25.35 ±1.06^{b,c}

Values not sharing a common superscript letter differ significantly at $p < 0.05$.

Data presented in **table (2)** showed that there was a significant increase in the serum ALT, AST, ALP activities and increase in the hepatic lipid peroxidation levels represented by increase in the levels of TBARS (MDA) in the groups treated with ZnONPs in a dose of (125 and 300 mg/Kg b.wt.) when compared with control group. This increase was a dose dependent. Treatment with Qurecetin in a dose of 100 mg/kg b.wt. success to ameliorate this toxic effect through the reduction of liver enzymes and MDA levels and increase in the hepatic reduced Glutathione levels (GSH) and the hepatic GPx activities in the groups co-treated with it when compared with toxic groups treated with ZnO NPs only.

Molecular results

The results showed in Figure 1 represented that, there was a significant increase in the transcriptional levels of mRNA of (IL-6), (IL-2) and (TNF-α) genes due to treatment with 125 and 300 mg/kg b.wt. of ZnONPs. Treatment with Qur. in a dose of 100 mg/kg b.wt resulted in decreasing the transcriptional level of (IL-6) gene.



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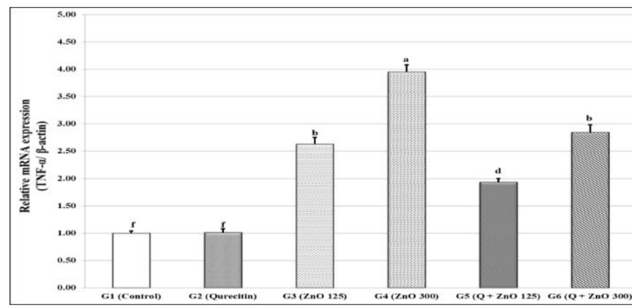

Figure 1: effect of ZnO NPs and/ or Qur. On IL-6, IL-2 and TNF-α gene expression

Histological Investigation

In the histopathological study, the liver sections of normal rats and rats treated with Quercetin revealed preserved hepatic lobular architecture and normal hepatocytes with rounded vesicular nuclei figure 2(1, 2). In ZnO-NPs groups, showed dilated congested central vein surrounded by hepatocytes with fatty change figure 2 (3, 4). In Quercetin preventive groups the section revealed that there is mild fatty change in liver cell and no cell necrosis.

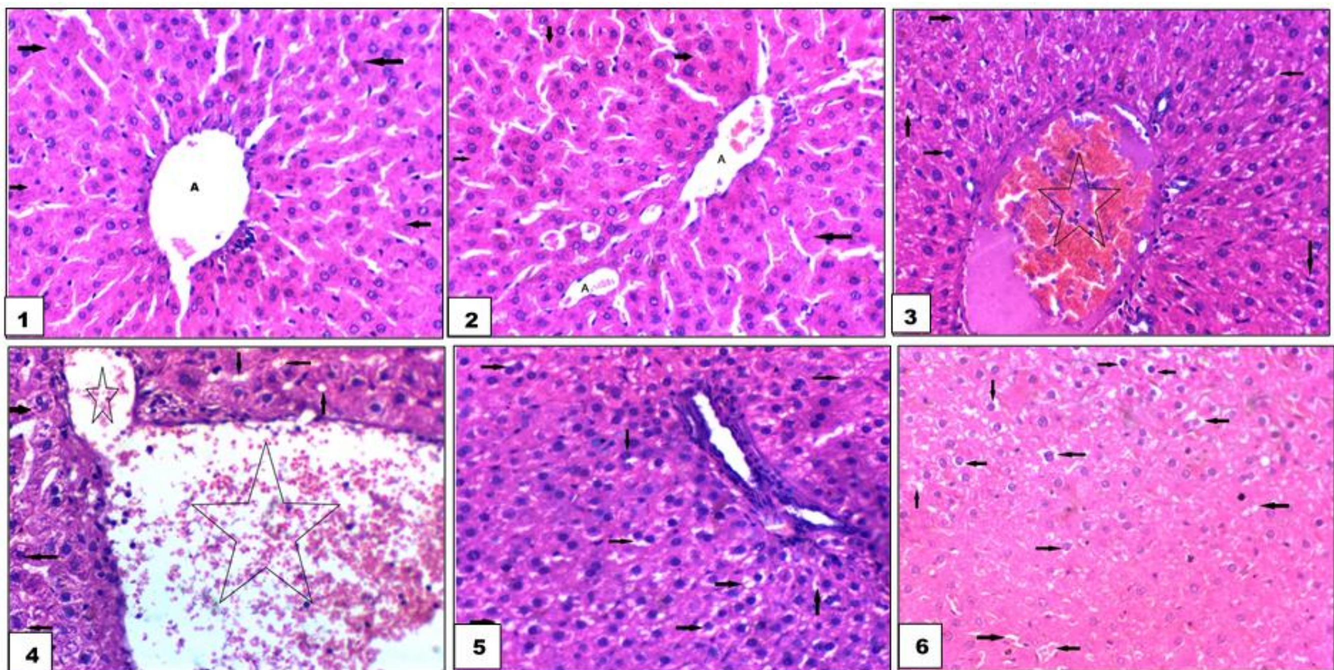

Figure 2: Histopathology of hepatic cells: 1) control 2) 100 mg/kg bwQur 3) 125 mg/kg bwZnO-NPs 4) 300 mg/kg bwZnO-NPs 5) 125 mg/kg bwZnO-NPs + 100 mg/kg bwQur 6) 300 mg/kg bwZnO-NPs + 100 mg/kg bwQur.

DISCUSSION

The present data suggests that ZnO-NPs can cause liver function impairment via elevation of liver function enzymes activities including ALT and AST and ALP. And these results seem to be conceivable with that obtained by Sharma et al. [25] and Fazilati [26]. The reversing of hepatotoxic effect induced by Zinc oxide NPs, herewith observed after treatment with Quercetin which evaluated by significant decreasing in liver marker ALT, AST and ALP serum proteins comparing with Zinc oxide NPs only treated group. The present results revealed that treatment with Quercetin after ZnO-NPs administration was able to normalize the activities of liver enzymes biomarker. These demonstrated by significant decrease in ALT, AST and ALP as compared with ZnO-NPs treated group **(table2)**.Serum aminotransferases (ALT & AST) are cytosolic enzymes of hepatocytes; an increase in their activities reflecting an increase in the plasma membrane permeability of hepatocyte which

in turn associated with cell death, ALT and AST indicate status of liver cells, ALP further demonstrates the performance and biliary Hungarian injuries, especially Hungarian extra hepatic [27, 28]. In the present study, the toxicity effect of Zinc oxide NPs were investigated on ALT, ALP and AST enzymes in male Rat. Due to their small size, NPs can translocate from these entry portals into the circulatory and lymphatic systems, and ultimately to body tissues and organs [29].When the NPs are accumulated in a tissue, may be absorbed into the cells or not to be absorbed. If these particles are absorbed, the finally replacement in cell lysosomes or cell cytoplasm will depend on the characteristics of NPs. If the NPs are located in the cytoplasm, the presence of some coarse grain material can cause direct damage or cell death is caused by these interactions [21].ALT and AST were located in cell and ALP was located in cell membrane. In effect the loss of liver cells, these enzymes are released in the blood. Therefore, increases of these enzymes are a sign of liver cells damage[26].Qur, is known as an anti-inflammatory/anti-allergy natural remedy, where it stabilizes mast cell membranes and prevents the release of histamine and other inflammatory agents in the body [30].

The present results suggested that ZnO-NPs significantly decreased the activities of GPx and concentration of non-enzymatic GSH and enhanced Lipid Peroxide(LPO) in the hepatic tissues indicating ZnO-NPs-induced oxidative damage. In contrast, a marked increase in the antioxidant enzyme activities was seen when rats were treated with Quercetin after ZnO-NPs administration. However, oxidative stress can occur as a result of either increased ROS generation and/or decreased antioxidant enzyme system comprising SOD, catalase and GSH. These antioxidant enzymes protect the cell against cytotoxic ROS. GSH use thiol-reducing power of glutathione to reduce oxidized lipids and protein targets of ROS. Under ineffective antioxidant enzyme status, lipid peroxidation in the cellular and subcellular membranes is the inevitable outcome of ROS injury [31]. Reduced glutathione (GSH) is considered as a sensitive oxidative stress marker because it helps to maintain the integrity of mitochondria and cell membrane. Its compromised level in the cells may deteriorate the membrane permeability and risks the cellular defense against ROS resulting in oxidative injuries [32]. Many glutathione based antioxidant enzymes and proteins are important to maintain redox status of the cells. All these enzymes utilize glutathione (GSH) in the reactions they catalyze which may lead to depletion of GSH in the living system in the condition of oxidative stress. This may be the possible reason for decreased level of GSH and its regenerating enzymes Glutathione peroxidase (GPx) in target organs in ZnO NPs treated animals.

Xia et al. [33] reported that ZnO NPs induce generation of reactive oxygen species which can lead to cell death when the antioxidative capacity of the cell is exceeded. Glutathione, a ubiquitous and abundant antioxidant cellular tripeptide, was found to be strongly depleted after exposure to ZnO NPs. Superoxide dismutase is specialized to convert the highly toxic superoxide radical to less toxic H_2O_2. The catalase enzyme reduces H_2O_2 to H_2O. More production of intracellular reactive oxygen species and more membrane lipid peroxidation in cells exposed to ZnO NPs along with depletion of their antioxidant components suggest that oxidative stress might be a primary mechanism for the toxicity of ZnO NPs. According to the present results treatment with Quercetin afforded a significant increase in antioxidant enzymes GSH and GPx level and significant decrease in lipid peroxidation (MDA) level comparing with ZnO-NPs only groups **(taple2)**.

Low and high doses of ZnO NPs significantly elevated inflammatory cytokine levels, including TNF α-, IL-6, and IL-2 in hepatic tissue compared with the normal control group. In this study, Quercetin shows desired protective activity as the cytokine levels were reduced compared with the ZnONP treated group with alleviation in IL-2, IL-6 and TNF-α **(figure 1)**.ZnO nanoparticles elicit a pronounced inflammatory response while the later nanoparticles are cytotoxic and lead to considerable cell death at higher concentration [34].TNF-α is one of the most common inflammatory injurious chemokine immunological markers increased in response to different metal oxide NPS-toxicity including ZnO[35, 36].The up-regulation of this cytokine triggers the production of other cytokines as IL-6, that end with inflammatory liver injury [37].

Previous studies showed that Qur reduced inflammatory pain by inhibiting oxidative stress and cytokine production in rats, suggesting that Qur possesses anti-inflammatory effects in vivo [38].

CONCLUSION

From this study, we concluded that ZnO nanoparticles 125 mg/kg and 300 mg/kg showed toxicity, in preventive therapy with Quercetin 100 mg/kg showed protective activity. Quercetin exerts its anti-inflammatory effects against ZnO nanoparticles induced toxicity by altering the levels/expressions of TNF-α, IL-2, IL-6. In our study the results support the ZnO nanoparticle toxicity was reduced by bioflavonoid. We

conclude that the development of nanotechnology and the study of nanotoxicology have increased our awareness of environmental particulate pollution generated from natural and anthropogenic sources, and hope that this new awareness will lead to significant reductions in human exposure to these potentially toxic materials.

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