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Hestopathological Effects of Zinc Oxide Nanoparticles on the Liver and Gills of *Oreochromis niloticus*, Protective Effect of Vitamins C and E

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The present work aimed to study the hestopathological alteration in liver and gills of *O. niloticus* exposed to Zinc oxide nanoparticles . Two hundred male *O. niloticus* were equally divided into 5 groups . Group (1) was the control. Groups (2 and 3) were exposed to 1 and 2 mgL^{-1} of ZnONPs. Meanwhile, the groups (4 and 5) were exposed to 1 and 2 mg/L of ZnONPs and treated with 500 mg of a mixture of vitamin C and E /kg diet. The obtained data indicated that, the gene expression and activities of catalase (CAT) and superoxide dismutase (SOD) were altered due to ZnONPs in a relation to the concentration and exposure time. Histopathologically, acute cell injury of hydropic degeneration and fatty change and irregular areas of coagulative necrosis were noticed in the liver in addition to congestion, telangiectasia and aneurysm, with hypertrophy and hyperplasia of the mucous cells in the gills. In presence of vitamin mixture such alterations in the antioxidant system and histology were alleviated or completely absent particularly with the low ZnONPs concentrations and short exposure time. Finally it could concluded that the supplementation of a mixture of vitamin C and E has a beneficial effect against the ZnONPs toxicity in *O. niloticus*.

Key words: ZnONPs, Tilapia nilotica, CAT, SOD, gene expression, histopathology.

Applying nanotechnology has occurred rapidly, and the use of nanoparticles (NPs) is being used in all sciences in area of chemistry, physics, medicine and biology (Oberdörster *et al* 2005; Shokouhian*et al*., 2013). Because of their high flexibility and strength, it is becoming increasingly important in modern industry (Dowling, 2004). Nanotechnologies have huge potential for solving numerous problems for example from increasing the treatment efficiency and diagnosis of various diseases to economizing materials and energy resources (Yildirimer *et al*., 2011). Among nano material ZnO is more attention due to its special properties and its fewer hazards to environmental impact. ZnO like most of nanoparticles is toxic in organisms; however the toxicity of these nanoparticles can be used for antibacterial (Xie *et al*., 2011), antifungal (Smith *et al*., 2010), antiviral (Mishra *et al*., 2011), antiprotozoa (Mortimer *et al*., 2010) and antialga (Lin and Xing, 2007).Recently, due to ecological changes and degradation of their natural spawning ground in most water body, the number and variation of fish have been decreased sharply. The scientists have consideration attention in biological study and inducing artificial spawning to prevent diminish of some valuable and endangered fish species (Yousefian *et al*., 2011). Nanoparticles (NPs) including ZnO have a

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potential environmental danger. For organisms living in the aquatic environment, there is uncertainty on exposure because of a lack of data regarding the effect of nanoparticles in behavior, physiology and bioactivity of organism in nanomaterials in the water. There are several samples of the effect of nanoparticles and its effect on fish. Dietary iron has previously been seen to cause lipid peroxidation in the liver and heart of African catfish (Baker *et al*., 1997). An increase in intracellular reactive oxygen species (ROS) was observed in zebrafish embryos exposed to nano-ZnO and implemented in some toxic effects (Hao *et al*., 2013; Brun *et al*., 2014). Effects on early life stages of fish are emerging with reports of nanometals and suggestions that the nano-forms of some metals such as ZnO NPs may be more toxic to embryos or juveniles, than the equivalent metal salt (Shaw and Handy, 2011). A significant decrease of SOD and glutathione (GSH) activity was observed in liver and brain samples taken from the adult fish, but as the exposure time increased, the adults appeared to recover from the exposure by adjusting the levels of antioxidant enzymes (Li *et al*., 2009). Histopathological and morphological changes were detected in the target organs including gills, gut, liver, kidneys, brain and muscles (Hao *et al*., 2013). Al-Bairuty *et al* (2013) showed hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills in rainbow trout. Hepatitis-like injury and cells with pyknotic nuclei in the liver; damage to the epithelium of some renal tubules and increased Bowman's space in the kidney were observed besides changes in the proportional area of muscle fibers in skeletal muscles (Yousefian and Payam, 2012).Non-enzymatic antioxidants such as ±-tocopherol (vitamin E), ascorbate (vitamin C), ²-carotene (vitamin A), flavonoids, selenium and thiol containing compounds such as glutathione (GSH) can also act to overcome the oxidative stress, being a part of total antioxidant system. Vitamin C is effectively free radical scavenger in the cell membranes in catfish (Adikwu and Deo, 2013). Moreover, the vitamin E is also essential for maintaining the integrity, function and flexibility of cell membranes, and it is an important fat soluble antioxidant. It serves to detoxify and remove reactive nitrogen species (RNS) from the body (Rasheed *et al*., 2012). The objective of this study was to assess the liver and gills of *O. niloticus* responses to ZnO-NPs so as to show their potential toxic biological responses and investigate the protective effect of vitamin C and E, employing the biochemistry and pathology.

MATERIALS AND METHODS

Preparation and characterization of ZnONPs particle

ZnONPs was obtained in the form of dispersion from Sigma-Aldrich, Steinheim, Germany (CAS Number 1314-13-2) of concentration 50 wt.% in H_2O, average particle size (APS) was <35 nm. The particle size distribution (hydrodynamic diameter) was <100 nm using dynamic light scattering (DLS) technique, pH 7±0.1(for aqueous systems) and density 1.7±0.1 g mL^{-1} at 25 °C . Suspensions of ZnONPs in a concentration of one and two mg L^{-1} were daily prepared with distilled water and dispersed with a sonicator (JL-360, Shanghai, USA) for 20 min. For characterize the ZnONPs shape and size, a small drop of aqueous ZnONPs solution was air dried by directly placing it onto a 300-mesh carbon-coated copper grid then examined under the transmission electron microscope (TEM) (JEM- 1011, JEOL, Japan) (unpublished data). The concentration of ZnONPs in the exposure solution was quantified by inductively coupled plasma mass spectrometry (ICP-MS) at zero, 12 and 24h of exposure to verify the exposure concentration are the same as the prepared concentrations (unpublished data).

Ethical statement

All procedures of the current experiment have been approved by the Committee of the Faculty of Science, North Jeddah, King Abdulaziz University, Jeddah, Saudi Arabia.

Experimental setup and acclimation of ûsh

Two hundred male of *O. niloticus*, weight 90 ± 5 g, length15 ± 3cm were obtained from Abrahem El-Solimani farms for fish, Kholes, Saudi Arabia Kingdom. Fishes were held in twenty glass aquaria (n = 10 individuals/aquarium), with100L of water (pH 7.16 ± 0.3. 0.52 mM Ca, and 0.24 mM Mg) that was changed daily, a continuous system of water aeration (Eheim Liberty 150 Bio-Espumador cartridges). Temperature was maintained at 28 ± 2 ^{o}C and dissolved oxygen, at 7.0 ± 0.5mg L^{-1}. Fishes were fed with commercial ûsh food, containing 6%

lipids, 31% proteins, 37% carbohydrates, 2.5% ûber, 1.5% total phosphorus, 12% ash, 200mg ±-tocopherol kg^{-1}, 1,700 IU vitamin D_3 kg^{-1} feed, and 10,000 IU vitamin A/kg feed. Fish were acclimatized for15 days before the beginning of the experiments.

Fish grouping and induction ZnONPs toxicity

Fishes were randomly divided into five groups, 40 fishes in each group (4 replicates). The first group was leaved as control, the 2nd and 3rd, groups were exposed to ZnONPs of one and two mg L^{-1} respectively. The 4th and 5th group were exposed to ZnONPs of one and two mg L^{-1} and treated with a mixture of vitamins C and E in a dose of 500 mg Kg^{-1} diet (250 mg of each). After seven and 15 days of the exposure, twenty fishes of each group were anesthetized on ice and killed by transaction of the spinal cord. Liver and gills were quickly removed, weighed, rinsed with ice-cold saline, frozen in liquid nitrogen, and kept at –80 °C until be used. The tissue homogenate were prepared from each individual organ (not pooled) according to Puerto *et al*. (2009), and the supernatant was used for biochemical analysis.

Biochemical Assays

Liver and gills CAT activities were determined using a kit (Cat. No. NWK-CAT01) purchased from Northwest Life Science Specialties (NWLSSTM), Vancouver, Canada, according to the manufacturer's instructions. SOD activities were determined using Cayman SOD diagnostic kit (Cat. No. 706002, Cayman, USA) according to the manufacturer's instructions.

Molecular assays and gene expressions

Liver and gills CAT and SOD genes expression were quantified using real time PCR.. Total RNA was isolated from tissue samples using the RNeasy Mini Kit Qiagen (Cat. No.74104) following the manufacturer's instructions. RNA quality was assessed as the 260/280nm absorbance ratio using NanoDrop®ND-1000 Spectrophotometer, (NanoDrop Technologies, Wilmington, Delaware USA). Then, 0.5µg of total RNA was used for production of cDNA using QIAGEN Long Range 2 Step RT-PCR Kit, (Cat. No.205920). 5 ¼L of total cDNA diluted 1:6 was mixed with 12.5 ¼L of 2x SYBR® Green PCR mix with ROX from BioRad, 6.5 ¼L of autoclaved water, and 0.5 ¼L (10 pmol/¼L) of each forward and reverse primer for the measured genes. The house keeping gene ²-actin was used as a constitutive control for normalization. Primers were designed using Primer3 software (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as per the published O. niloticus CAT, SOD and ²-actin genes sequences (JF801726, JF801727 and EU887951) of NCBI database all primers were provided by Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and are shown in table (1). PCR reactions were carried out in an AbiPrism 7300 (Applied Biosystems, USA). The RNA concentration in each sample was determined from the threshold cycle (Ct) values. The quantitative fold changes in mRNA expression were determined relative to ß-actin mRNA levels in each corresponding group and calculated using the $2^{-DD\,CT}$ method.

Histopathological Technique

Specimens from the liver and gills were collected and fixed in 10% buffered neutral formalin solution, dehydrated in gradual ethanol (70-100%), cleared in xylene, and embedded in paraffin. Five-micron thick paraffin sections were prepared and then routinely stained with hematoxylin and eosin (HE) dyes, Prussian blue stain for iron and Alcian blue stain for mucus (Bancroft and Gamble 2008) and then examined microscopically.

Statistical Analysis

The data was processed using the statistical package for social science (SPSS Inc., Chicago, IL, version 20, USA). All results were expressed as mean ± SD. The differences between the experimental and the control groups were tested for significance using One-way analysis of variance (ANOVA). Duncan's test will be used for testing the inter-grouping homogeneity. Statistical significance was set *P d"0.05*

RESULTS

Effects of ZnONPs on CAT and SOD activities and gene expression in fish tissues

Figure (1) illustrates the activities of CAT and SOD in the liver and gill tissues. The exposure of ZONPs caused a significant inhibition ($p<0.05$) in the activities of CAT and SOD in all tissues of nanoparticle-exposed groups when compared to the control fish tissues, in a time- and dose-dependent manner. Supplementations of vitamin C and E mixture with ZONPs cause a significant induction ($p<0.05$) in the CAT and SOD activities.

Meanwhile, the Figigure (2) shows the relative gene expression of antioxidant enzymes in the liver and gill tissues. The exposure of ZONPs caused a significant repression ($p<0.05$) in the relative gene expression of CAT and SOD in all tissues of nanoparticle exposed groups when compared to the control fish tissues, in a time- and dose-dependent manner. Supplementations of vitamin

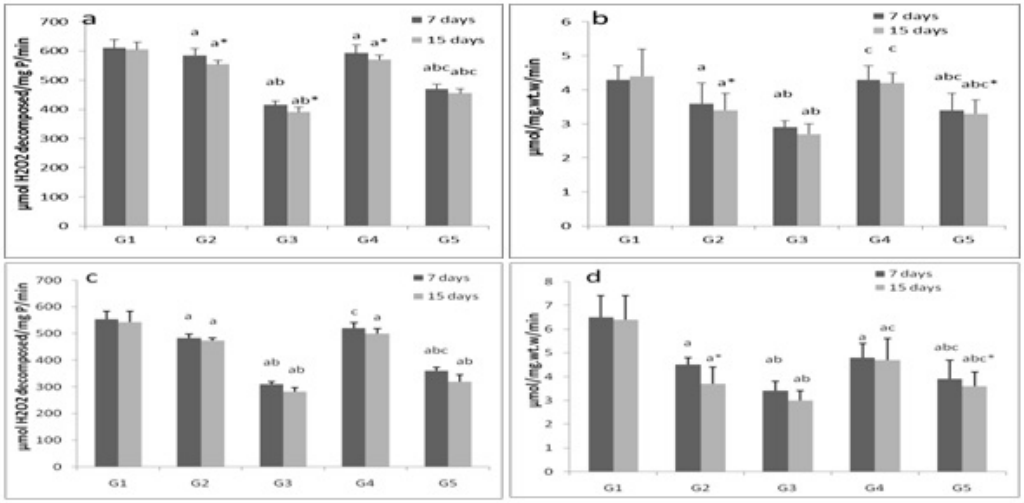

Fig. 1. The liver catalase activity (a), liver superoxide dismutase activity (b), gill catalase activity (c) and gill superoxide dismutase activity (d) in control group (G1), zinc oxide nanoparticles groups (G2 and G3) and zinc oxide nanoparticles with vitamin mixture groups (G4 and G5). Values are expressed as mean ± SD (n 20). Signiûcance levels ($p<0.05$) observed are: a = in comparison to control group, b = when 2mg ZONPs groups versus 1mg ZONPs groups are compared, c = when ZONPs + vitamins groups versus their respective ZONPs groups are compared, * = when 15 days treated groups compared with their respective 7 days treated groups.

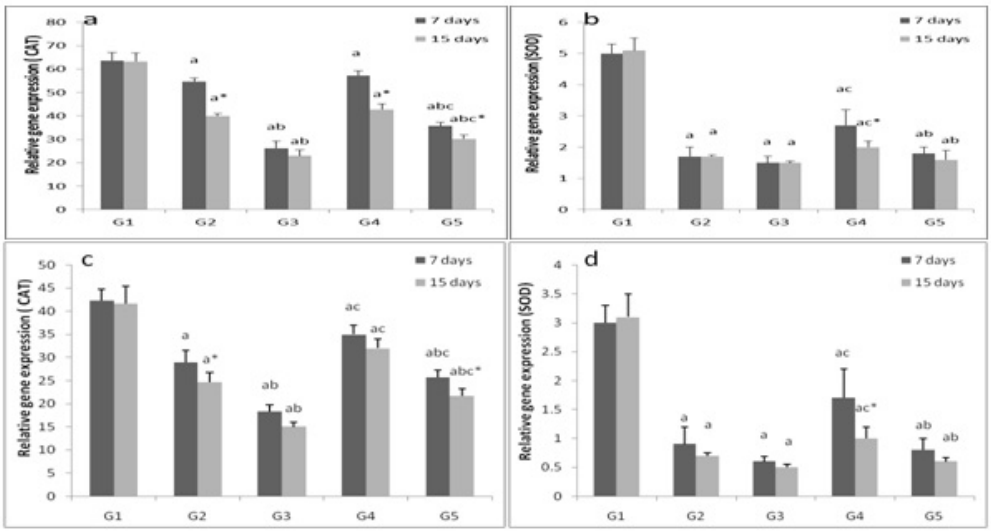

Fig. 2. Catalase relative gene expression in liver (a) and gill (c) and superoxide dismutase relative gene expression in liver (b) and gills (d), in control group (G1), zinc oxide nanoparticles groups (G2and G3) and zinc oxide nanoparticles with vitamin mixture groups (G4 and G5). Values are expressed as mean ± SD (n 5). Signiûcance levels ($p<0.05$) observed are: a = in comparison to control group, b = when 2mg ZONPs groups versus 1mg ZONPs groups are compared, c = when ZONPs + vitamins groups versus their respective ZONPs groups are compared, * = when 15 days treated groups compared with their respective 7 days treated groups.

C and E mixture with ZONPs cause a significant induction ($p<0.05$) in the antioxidant enzymes relative gene expression in all tissues as compared with the respective fish exposed to ZONPs only.

Histopathological Findings

The ZnONPs exposure induced significant pathological effects in the liver and gills in comparison with control ones (Figures 3 and 4). These pathological changes varied according to time and dose concentration. The liver of fish exposed to 1mg/L of ZnONPs (group 2) revealed mild vacuolation of microvesicular steatosis and hydropic degeneration on the 7th day (Figure 5). In addition to slight congestion of the portal blood vessels was detected on the 15th day and with no evidence of leukocyte infiltrates (Figure 6).

The ***gills*** were slightly congested on the 7th day and showed congestion of the lamellar blood capillaries, focal hyperplasia in the lining epithelium of the secondary lamellae (Figure 7), proliferation of mucous cells and few lymphocytes infiltrations 15th day. Few extracellular brown

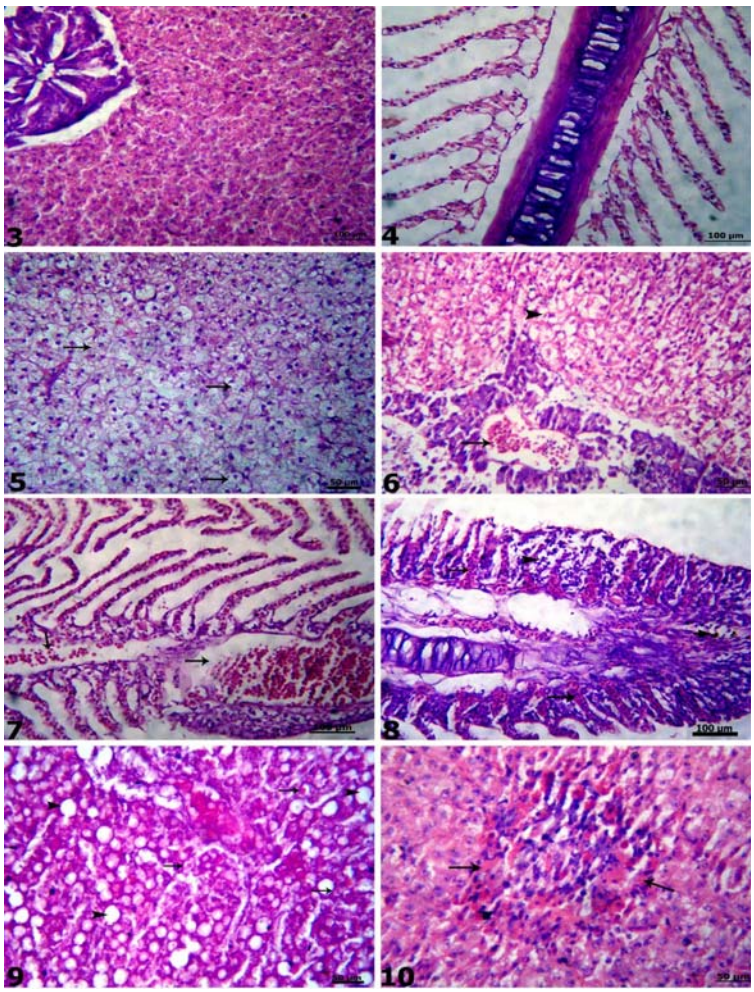

Fig. 3-10. Control group revealed normal hepatocyte, pancreatic and sinusoidal architecture (3) and gill with normal filaments (4). Group 2. exposed to 1 mg/L of ZnONPs: Liver shows mild vacuolation of microvesicular steatosis (arrows), 7 day (5), and slight congestion of the portal blood vessels (arrow), 15 day (6). ***Gill shows*** slightly congestion (arrows) on 7 day (7) and focal hyperplasia in the lining epithelium of the secondary lamellae, proliferation of mucous cells, congestion (arrows) and few lymphocytes infiltrations (arrowhead), 15 day (8). Group 3. exposed to 2 mg/L of ZnONPs: Liver shows macrovesicular steatosis and hydropic degeneration (arrowheads) besides large eosinophilic globules of Mallory bodies (arrows), 7 day (9) and area of coagulative necrosis, 15 day (arrows) (10). HE x Scale bar.

particles were observed on the tips of the primary lamellae (Figure 8).

However, the fish exposed to 2 mg/L of ZnONPs (group 3) showed macrovesicular steatosis and hydropic degeneration besides large eosinophilic globules of Mallory bodies in the liver (Figure 9), 7th day. Few eosinophilic particles were seen in the cytoplasm of some hepatocytes besides coagulative necrosis of individual hepatocytes represented by pyknosis or disappearance of their nuclei was also observed. Meanwhile, the liver at 15 days revealed small area of coagulative necrosis (Figure 10). In addition to diffuse periportal hydropic degeneration was noticed (Figure 11) and the cytoplasm of these hepatocytes showed

These pigmented cells were negative for Prussian blue reaction. The other hepatocytes revealed enlarged nuclei (karyomegaly) and contained brownish pigmented particles. Congestion of hepatic blood vessels and sinusoids, focal hemorrhage and round cells infiltrations in the portal areas were visualized (Figure 13). The

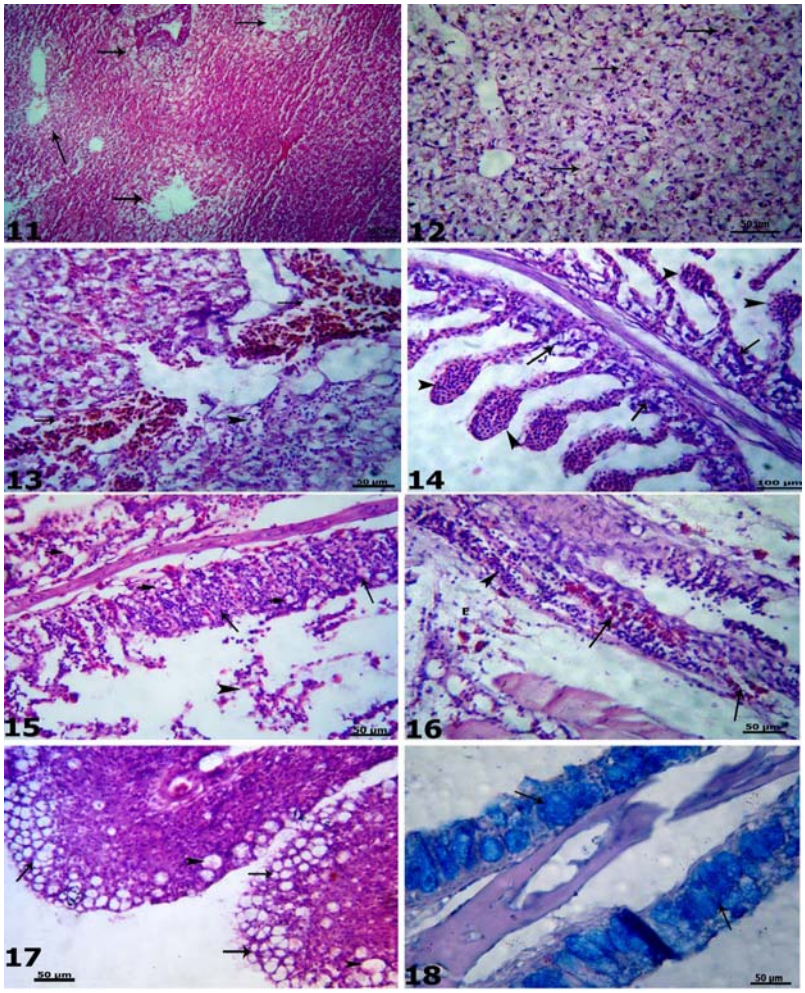

Fig. 11-18. Group 3; exposed to 2 mg/L of ZnONPs, 15 day: Liver shows diffuse periportal hydropic degeneration (arrows) (11), eosinophilic or slightly brown particles in the cytoplasm (arrows) (12) and portal area with congestion, hemorrhage (arrows) and round cells infiltrations (arrowhead) (13). Gill shows congestion and aneurysm (arrowhead) (14), proliferation, desquamation and fusion of the epithelial covering of the secondary lamellae (arrowhead) and lymphocytes infiltration (arrows) (15), sloughing in the epithelium and focally replaced by lymphocytes (arrowhead) and eosinophilic granular particles (arrows) (16), hypertrophy and hyperplasia of the mucous cells (arrows) which contained granular eosinophilic particles (arrowheads) (17) and stained blue by Alcian blue stain (arrows) (18). HE x Scale bar.

gills revealed congestion and telangiectasia of the branchial blood capillaries on the 7th day. The congestion became severe with aneurysm on the 15th day (Figure 14). Focal proliferation, desquamation and fusion of the epithelial covering of the secondary lamellae were noticed with numerous leukocytes infiltration (Figure 15). Sometimes, the gill filaments and its covering epithelium were sloughed and focally replaced by lymphocytes and eosinophilic granular particles (Figure 16). Hypertrophy and hyperplasia of the mucous cells were seen particularly at the tips of the filaments and contained granular eosinophilic particles (Figure 17). The mucus inside these cells was stained blue by Alcian blue stain (Figure 18). The gill arches, especially at the base of the gill filaments showed congested blood vessels, focal hemorrhage and edema (Figure 19).

The reported lesions with ZnONPs exposure were alleviated by supplementation of vitamin C and E with a high dose (2 mg) and long exposure time (15 days) and completely absent with a low dose (1 mg) and short exposure time (7days). The examined organs of group (4) which exposed to 1mg/L of ZnONPs was nearly normal except for dilated hepatic sinusoids (Figure 20) and congestion of the gill-blood vessels at the 15th day. Meanwhile in group (5) exposed to 2 mg/L of ZnONPs, slight hydropic degeneration in the hepatocytes and slight congestion in the gills were visualized at 7th day. On the 15th day, the ***liver*** showed intact hepatocyte architecture and moderate swelling in the hepatocytes with cleared cytoplasm or contained eosinophilic globules (Figure 21). The congestion and the brown pigments were completely disappeared. The ***gills*** showed slight congestion and proliferation of the mucous cells (Figure 22).

DISCUSSION

In the present study, the most prevalent biomarkers of ZnONPs toxicity is reduced CAT and SOD activities and gene expression in the liver and gill tissues . The latter could explicate the mechanism of tissue alterations in the liver and gills through the histopathological analysis. The aforementioned finding suggested that fish exposed to ZnONPs had suffered oxidative stress (Hao *et al* 2013). When, the vitamin C and E included to the diet with ZnONPs exposure, these CAT and SOD activities and gene expression were upregulated toward the control levels. Such results could explain the hazard effects of ZnONPs

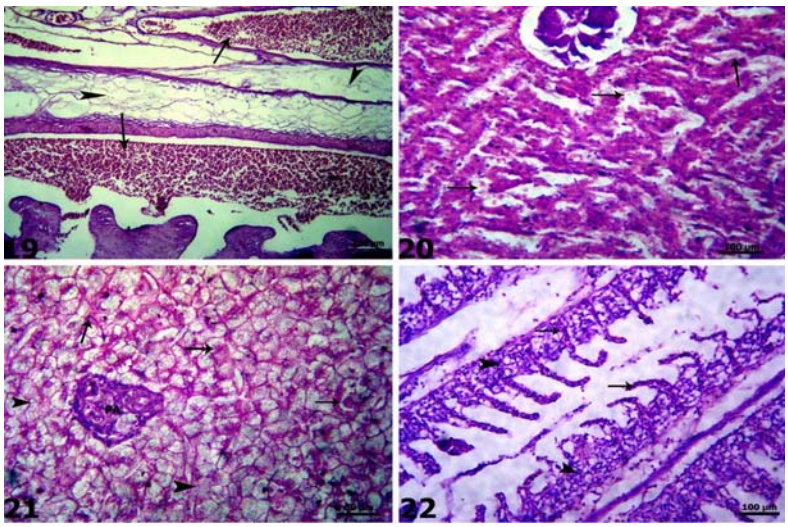

Fig. 19-22. Group 3; exposed to 2 mg/L of ZnONPs, 15 day: Gill arch shows congested blood vessels (arrows), focal hemorrhage and edema (arrowheads) (19). Group 4; exposed to 1 mg/L of ZnONPs and supplemented with vitamin C and E shows liver with dilated hepatic sinusoids (arrows).Group 5; exposed to 2 mg/L of ZnONPs and supplemented with vitamin C and E shows liver with intact hepatocyte architecture and moderate swelling in the hepatocytes (arrows) containing few eosinophilic globules (arrowheads) (21) and gill with slight congestion (arrows) and proliferation of the mucous cells (arrowheads) (22). HE x Scale bar.

(Shokouhian *et al*., 2013) and the protective role of vitamin C and E which completely reversed such toxicity (Rasheed *et al*., 2012). Concerning the levels of CAT and SOD activity in the liver and gill tissues, the results showed significant inhibition of such activities when compared to the control. The previous findings suggested that the lipid peroxidation and ROS were in a high status, particularly at 2 mg /L exposure for 15 days. CAT and SOD were important to eliminate reactive oxygen species and to assist the aerobic organisms in defense against oxidative stress: superoxide dismutase (SOD) breaks down O^{-2} to H2O2 and O2 and catalase (CAT) inactivates H2O2 and interrupts the formation of toxic intermediates besides the alkyl hydroperoxide reductase (AHP) destroys toxic hydroperoxide intermediates and repairs damage to molecules caused by oxidation (Han *et al*., 2011). Moreover, these activities were upregulated by supplementation of vitamin C and E towards the control levels. Such elevation could explain the antioxidant role of vitamin C and E to minimize the lipid peroxidation and production of ROS (Rasheed *et al*., 2012; Adikwu and Deo, 2013). Also these findings were coincided with previous studies revealed that vitamin E plays a major role in reducing inflammation as well as cleansing the body of free radicals. As vitamin E supplements lowered IL6 and mitochondrial and membrane damage dramatically in ZnONPs toxicity (Al Attar, 2011). A number of genes/proteins that play critical roles in protecting cells from different stresses, especially oxidative stress, have been identified (Polisak, 2011). Analysis of ZnONPs modulated stress gene expression showed that the transcription levels of two oxidative stress genes (CAT and SOD) in the liver and gills were significantly repressed, particularly with 2 mg/L ZnONPs exposure for 15 days. While these genes expression was upregulated with vitamin C and E supplementation. They significantly increased in the supplemented groups in comparison with the ZnONPs exposure.

The pathological findings were confirmed that the liver and gills are considered to the primary target organs of ZnONPs toxicity. These findings were milder with the low concentration (1 mg/L) and short duration of exposure and became severe with the high concentration and long duration. The lesions in the liver varied from microvesicular steatosis and mild hydropic degeneration with slight congestion with 1 mg concentration to coagulative necrosis, macrovesicular steatosis, hemorrhage and inflammatory responses represented by lymphocytes infiltrations besides eosinophilic or brown ZnONPs accumulation in the cytoplasm were visualized. Such alterations may be due to either through attachment to the surface of cells and stimulation of phospholipase A2 and C which hydrolyze the lipid in the cellular membrane into fatty acids such as arachidonic acids and its metabolites to produce prostaglandins and leukotrienes. The latter are mediated the inflammatory responses (Jones *et al*., 1997). Similar findings were also obtained by Yousefian and Payam (2012) and Al-Bairuty *et al* (2013). The elevated level of ROS influx (as biochemical results) is implicated in abnormal endothelial activation, redox signaling, and cellular injury (Birukov, 2009), all involved in the pathogenesis of vascular disorders including inflammation, ischemia and edema (Boueiz and Hassoun, 2009). The gills were the first organ in contact with water containing ZnONPs and they also revealed significant lesions. The latter were represented by congestion and telangiectasia of the branchial blood capillaries in low concentration and short duration. Aneurysm, proliferation, desquamation and fusion of the epithelial covering of the secondary lamellae and numerous leukocytes infiltration were recorded with a high concentration and long duration. Hypertrophy and hyperplasia of the mucous cells was the prominent feature of ZnONPs toxicity on the gills with congestion, hemorrhage and edema in the gill arches. The aforementioned lesions could reflect the effects of ROS (induced by ZnONPs exposure) on the blood vessel-endothelium and increases its permeability. The increase in the permeability is the main cause of hemorrhage and edema appeared in the gills besides the congestion (Han *et al* 2011.,). Almost all these findings are in accordance to those obtained by Al-Bairuty *et al* (2013). The reported lesions with ZnONPs exposure were alleviated by supplementation of vitamin C and E with a high dose (2 mg) and long exposure time (15 days) and completely absent with a low dose (1 mg) and short exposure time (7days). These findings are coincided with our biochemical findings showed induction of CAT and SOD gene expression and elevation of the enzyme activities.

These findings were similar to those obtained by Rasheed *et al* (2012) who showed decline in the ZnONPs toxicity (biochemical and histopathological analysis) in rats by ±-lipoic acid and vitamin E treatment.

CONCLUSION

The supplementation of a mixture of vitamin C and E has a beneficial effect against the ZnONPs toxicity through reduction of the oxidative stress and the histopathological alterations in the liver and gills of *O. niloticus*.

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