ROLE OF VITAMIN C AND E ON GENOTOXICITY, HEMATOLOGICAL AND BIOCHEMICAL INVESTIGATION IN *CYPRINUS CARPIO* L. FOLLOWING ZINC OXIDE NANOPARTICLES EXPOSURE

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ABSTRACT

This study was aimed to evaluate the role of vitamins C and E on genotoxicity, biochemical and hematological indices in common carp, *Cyprinus carpio* following zinc oxide nanoparticles exposure. Zinc oxide nanoparticles were synthesized (size was < 34nm) and characterized using Fourier Transform Infrared Spectra (FTIR) and X-Ray Diffraction (XRD) analysis. About 120 common carp (weight 20.0-32.0 g) were randomly divided into 12 tanks at rate of 10 fish/tank (two (replicates/treatment); fish were fed diet as follows: Control (C) were fed basal diet; T1 fish were fed basal diet mixed with vitamins C and E (400 mg/kg); T2 and T3 fish were fed basal diet mixed with 10%and 15% ZnONPs respectively; T4 and T5 fish were fed basal diet mixed with ZnONPs 10% and 15% plus vitamins C and E (400 mg/kg dw) respectively. Post 40 d feeding trail, variable changes were registered in blood indices ("Hb content, PCV%, WBCs and RBCs numbers") in all treated groups compared to C and T1 groups. The highest DNA damage (% tail DNA using Comet assay) was seen in T2 and T3 which asserted highly significant increased (P≤0.01) compared to C and to treated groups. As well as, glutathione peroxidase (GPx) activity exhibited highly significant increase (P≤0.01) in T2 and T3 groups relative to C, T4, T1, T5 respectively. This investigation clearly proved that sub-lethal doses (10 and 15% in diet) of ZnONPs were able to induce an oxidative stress in carp fish as reflected by significantly increase of DNA damage to erythrocytes and "the combination of vitamins C and E was able to alleviate the oxidative stress generated due to exposure to ZnONPs."

Key words: Common carp-Comet- DNA damage-Glutathione peroxidase- Hematology

دور فيتامين C و E في السمية الجينيه والكيموحيوية والدمية في اسماك الكارب بعد التعرض لجسيمات الزنك النانوية

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المستخلص

هدفت الدراسة إلى تقييم دور الفيتامينات E و C في السمية الجينية والمؤشرات الكيموحيوية والدمية في اسماك الكارب الشائع بعد التعرض لجسيمات أكسيد الزنك النانوية. تم تصنيع جزيئات أكسيد الزنك النانوية ودراسة خواصها باستخدام تحليل لأطياف الأشعة تحت الحمراء (FTIR) والأشعة السينية (XRD). تم تقسيم حوالي 120 كارب شائع (وزن 20.0–32.0 غم) بشكل عشوائي إلى 12 حوض بمعدل 10 سمكة/حوض (مكررين/ معاملة)؛ تم تغذية الأسماك بالنظام الغذائي بنسبة2% على النحو التالي: تم تغذية اسماك السيطره (C) على النظام الغذائي الأساسي والمعامله الاولى (T1) تم تغذيتها على النظام الغذائي الأساسي الممزوج مع الفيتامينات E و C (400 ملغم/ كغم) تم تغذية اسماك المعاملتين الثانية والثالثة (T2 و T3) على علف ممزوج بـ 10٪ و 15٪ ZnONPs على التوالي وتم تغذية سماك المعاملتين الرابعة والخامسة (T4 و T5) على علف ممزوج بـ ZnONPs بنسبة10٪ و 15 ٪ بالإضافة إلى فيتامينات E و C(400 ملغم/كغم وزن علف) على التوالي. بعد 40 يوم من التغذية، تم تسجيل تغيرات مختلفة في المؤشرات الدمية (محتوى الهيموكلوبين وكريات الدم المضغوطه وعدد كرات الدم البيض والحمراء) في جميع المعاملات المعالجة مقارنة بالمعاملات C و T1 وقد لوحظ أعلى ضرر للحمض النووي (٪ DNA) في T2 و T3 مما أكد زيادة معنوية عالية (P≤0.01) مقارنة بالمعاملات C والمعاملات المعالجة الأخرى. علاوة على ذلك ، أظهر نشاط الكلوتاثيون بيروكسيديز (GPx) زيادة معنوية عالية (P≤0.01) في المعاملات T2 و T3 مقارنة بـ C T4، T1، T5 على التوالي. أثبت هذه الدراسه بوضوح أن التراكيز (10 و 15 ٪ في النظام الغذائي) من ZnONPs كانت قادرة على إحداث إجهاد مؤكسد في الكارب الشائع انعكس ذلك من خلال زيادة كبيرة في تلف الحمض النووي للكريات الحمراء واظهر خليط الفيتامينات C و E قدره على تخفيف الإجهاد التأكسدي الناتج عن التعرض لاوكسيد الزنك النانوي.

الكلمات المفتاحية: الكارب الشائع–المذنب– تلف الحامض النووي الرايبوزي–انزيم الكلوتاثيون بيروكسديز–علم الدم

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INTRODUCTION

Nanoparticles (NPs) made up of metal oxide are being widely applied in a number of commercial products, expanding the "apprehensions" of their potential toxicity to human life and environmental health (3). The increasing use of metal oxide nanoparticles consequences in their discharge into aquatic ecosystem, causing detrimental influences on aquatic organisms which have drawn much special attention (29, 20, 15). ZnO NPs are strongly cytotoxic at lower concentrations (13). Genotoxicity may ascend via indirect mechanisms where NPs do not physically interrelate with the DNA molecule but with other "cellular components", such as those involved in the cell division process. Other cellular responses may be induced and give rise to genotoxicity, such as oxidative stress induction, inflammatory response, and aberrant signaling responses (34, 19, 21).The literatures on the toxicity of ZnONPs have focused on early developmental stages or on acute exposure to aquatic biomass (14). It is well known that, "Vitamins E and C are potent antioxidants and act by scavenging the free radicals or ROS and compensate the decrease in reduced glutathione" (38). Vitamin E is an important component in human diet and considered the most effective lipo-soluble antioxidant found in the biologiecal system (23). Most bony fishes are however unable to synthesize vitamin C as they lack L glunolacton oxidase enzyme to convert glucose, which makes it a vital vitamin required being provided with diet (22, 39). In general, supplying vitamin C to enhance resistance of fishes against environmental stresses has become an effective way through influencing the biochemical parameters of the blood (9). Thereby, this work was planned to shed light on the role of vitamins C and E on genotoxicity, hematological and biochemical in *C. carpio* following ZnONPs exposure.

MATERIALS AND METHODS

Synthesis and characterization of ZnONPs

ZnONPs were synthesized according to procedure of (16) using the sol_gel method. Average particles size was < 34nm. The characterization of ZnONPs was studied using FTIR and XRD analysis.

Diet preparation: In this study floating diet was obtained from Faradanah Company (Iran) applied as a basal diet. The proximate composition of a basal diet as follows: crude protein (36%), carbohydrate (29%), crude fat (9%), crude fiber (5%), moisture (10%), phosphorus (1.1%) and Ash (10%). The formulated fish feed was enriched with ZnONPs supplemented diet was grinded using a grinder and was mixed in a mixer and then added the concentrations of ZnONPs (10 and 15%) and Vitamins (C and E) needed for the experiment as mentioned in experimental design. These concentrations of ZnONPs were selected based on previous study by Taheri *et al.* (37).

Design of the experiment: A proximately of 120 common carp, *C. carpio* L. (weight 134±5.20 g; length 17.50±2.24 cm) were purchased from private cages farm in "Babylon province/Iraq". Fish were acclimatized for two weeks in glass tanks with continuous aeration (Temp: 21-23 °C; DO 7.2 mg/l and pH 7.4) before starting the experiment. Following acclimation, About 120 common carp were randomly divided into 12 tanks at rate of 10 fish/tank (two (replicates/treatment); fish were fed diet as follows: Control (C) were fed basal diet; T1 fish were fed basal diet mixed with vitamins C and E (400 mg/kg); T2 and T3 fish were fed basal diet mixed with 10%and 15% ZnONPs respectively; T4 and T5 fish were fed basal diet mixed with ZnONPs 10% and 15% plus vitamins C and E (400 mg/kg diet) respectively. All fish were fed diet twice daily at 2% body mass for 40 days. During experimental trail water were changed partially every day and water quality were recorded every day. After 40 days, four fish were sampled from each treated group; blood samples were collected from caudal vessels to detect hematological indices ("RBCs, WBCs count, Hb content, PCV value") and DNA damage using comet assay. Liver samples were collected for measuring Glutathione peroxidase (GPx) activity.

Detection of DNA damage using Comet assay: Comet assay was conducted to detect damage to DNA using erythrocytes of carp fish as defined previously by Mustafa (2011, 2012). Cells were randomly selected for the measurement of DNA breaks after ethidium

bromide staining. Scoring was performed using fluorescence microscope (Leica DMR) using "Komet 5.0 image" analysis software ("Kinetic Imaging, Ltd., UK"). A total of 100 cells were counted for each sample. The tail DNA% was applied as a dependable parameter to measure the degree of DNA strand breaks.

Determination of glutathione peroxidase (GPx): GPx activity (ng/l) was determined spectrophotometrically using a commercial kit (Sigma, UK) according to enclosed booklet.

Hematological indices: All blood indices were carried out according to Mustafa, (24). The RBCs and WBCs count were determined by using hemocytometer and special diluting fluid (Deices fluid). For fish blood Hemoglobin content was detected by using "the cyanmethemoglobin colorimetric method after centrifugation." Packed cell volume (PCV) % was measured "by using microhematocrit tubes method".

Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to detect the influence of altered factors in study parameters. "One way analysis of variance (ANOVA) was used to compare between means". Least significant difference –LSD test was applied and P value less than 0.01was considered significant different.

RESULTS AND DISCUSSION

Characterization of ZnONPs

Fourier Transform Infrared (FTIR) Spectra Analysis: FTIR examination showed the infrared spectrum of pure zinc oxide nanoparticles. The vibrations of ZnO bond are found to be lying at 495.67 per cm, the peaks lying from 3200 to 3600 per cm, are representing the functional groups corresponding to C-H stretching mode and O-H stretching mode respectively (Figure 1). Presence of O-H group represents the presence of water molecules on the surface of ZnO nanoparticles. These functional groups resulted from the synthesis of (ZnO NPs) are in agreement with **Swati and Mahendra** (36).The band (415cm-^{1})can be observed, which is due to the rubber vibration of the (ZnO NPs) group (8).

X-Ray Diffraction (XRD) analysis

Figure 2 showed the XRD values for ZnO NPs at each of (20= 31.8°,34.5°,36.3°,47.6°,56,6°,52.9°,66.4°,67.9 °,69.1°), which is identical to the previous studies (35,7). When calculating the average size of crystals which was calculated by the Debye–Scherrer equation for the top three (36.33°,34.5°,31.84°) peaks of zinc oxide nanoparticles (ZnO NPs) it was found that they are equal to (25.61,19.75,25,14 nm) respectively.

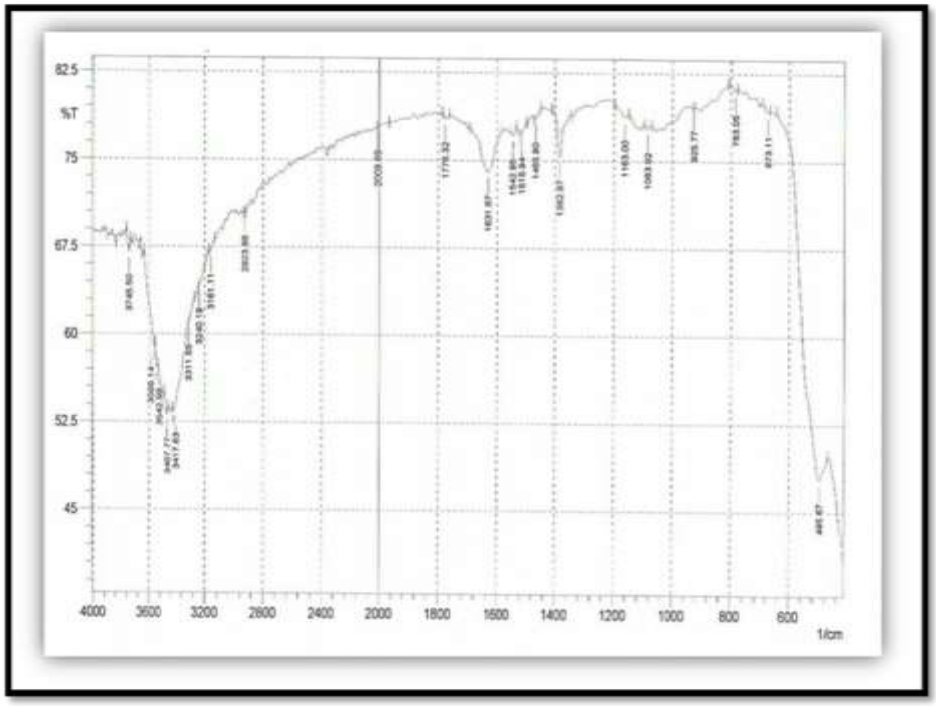

Figure 1. FTIR spectrum of zinc oxide nanoparticles

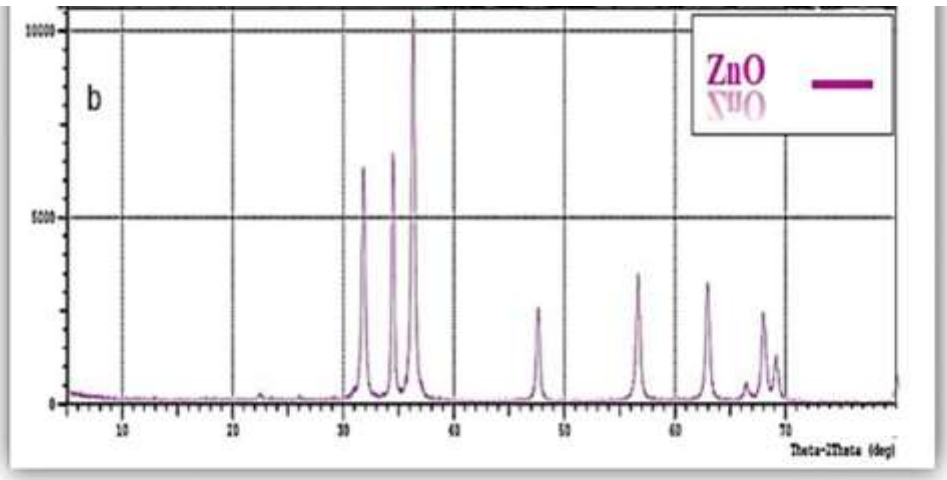

Figure 2. X-ray diffraction (XRD) spectra of Zinc oxide nanoparticles

Detection of DNA damage using Comet assay: In the present study post 40 days to dietary supplement with different concentrations of ZnO NPs plus vitamin C and E, DNA damage (as presented in % tail DNA) was relatively low and acceptable in control and vitamins groups compared to ZnO NPs groups. The highest level of DNA damage was registered in T3. DNA strand breaks highly significantly increase (P≤0.01) in all ZnO NPs groups (T2, T3, T4 and T5) compared to control and to T1 groups. But DNA damage was significantly decrease in T4 and T5 relative to T2 and T3 suggesting the protective role of Vit C and E E to fight the oxidative damage results from ZnONPs exposure (Table 1). The alkaline comet assay was previously confirmed as an initial indicator of general, nonspecific DNA damage/genotoxicity and an robust biomarker for environmental checking (11, 24, 26). It has great potential to estimate DNA damage in fish because neither metaphases nor knowledge of the chromosome numbers are required (4). As it involves the analysis of single cells, inter-cell variability in responses may be also studied. Few studies are available about the genotoxicity of ZnO-NPs towards aquatic organisms. Our results suggest that ZnO-NPs have the potential to induce DNA damage in *C. carpio*. "It may be the result of ROS generation which reacted with DNA purines and pyrimidines bases and damaged them." Al-Rudainy *et al.* (2) observed the same results in freshwater snail, *Lymnaea luteola* where ZnO-NPs damaged the DNA. Our findings are similar to Shahzad *et al.* (33) whom asserted that the high level of ZnO-NPs induced DNA damage in tilapia, *Oreochromis mossambicu* using comet assay. Similarly, Alkaladi *et al.* (1) reported that ZnO NPs induced cytotoxicity and genotoxicity in *Oreochromis niloticus* exposed to zinc oxide nanoparticles. **Measurement of Glutathione peroxidase (GPx) activity:** Results of GPx activity displayed that there was highly significant increase (P≤0.01) in T2 and T3 groups, compared to C, T4, T1,T5 respectively. But there were no significant differences between T2 and T3 (Table 2). Glutathione peroxidase (GPx), which is important in the defense mechanisms against reactive oxygen toxicity, has been reported to be present in the muscles of various fish species (28). "GPx catalysis the reduction of H_2O_2 derived from oxidative metabolism as well as peroxides from oxidation of lipids and is considered the most effective enzyme against lipid peroxidation" (40). Its activity is considered complementary to catalase activity, being especially suited for hydroperoxide detoxification at low substrate concentrations (12). Nakano et al. (28) strongly supported the result of GPx of this study, who reported that the GPx activity is located in various fish organs, and its activity increased significantly during toxicity. These findings propose that the increase in the enzyme activity could be owing to highly reactive oxygen species (ROS) induced from ZnOPNs exposure. Some studies exhibited a far higher acute toxicity of nano-ZnO to aquatic organisms such as zebrafish, daphnia, algae etc. (41).

Hematological indices

Following 40 days of feeding trail with ZnONPs, WBCs and RBCs count recorded considerable variations in all treated groups

relative to T 1 and C groups. In contrast, Hb content and PCV % registered highly significant increase (P<0.01) in C and T1 groups comparison to all treated groups (T2, T3, T4 and T5). The lowest Hb content and PCV % was noticed in T3 which registered significant decreased relative to other groups (Table 3). In all vertebrates involving fish, the leucocyte decrease or increase in response to different stressors such as infections and pollutants (30). Our results recorded significant decrease in WBCs count in all group of fish fed ZnO supplemented diet as compared to C and T1 groups. Like this results, other researchers also registered the decrease in WBCs count in Clarias and "Heteroclarias" species (31,17) in response to Zn exposure. This decrease ("leucopoenia") in the present work or previous studies might either be the result of bioaccumulation of Zn in different tissues that cause toxicity and effect on cell production from spleen (10) or owing to an increased level of corticosteroid hormones (5). Because these hormones are necessary for prevention and healing of inflammation. Decreased of Hb content a signal of the status or size of the erythrocytes and "reflects an abnormal or normal cell division" during erythropoiesis (43) which shows that "the erythrocytes have shrunk, either due to hypoxia or a microcytic anemia and oxygen carrying capacity of blood was declined in metal-exposed *C. carpio* due to the reduction of RBC count and Hb content" (32). Additionally, erythrocyte count founded to be significant affected by ZnO NPs toxicity especially in T3 and T5 which cause significant decrease in erythrocyte count and that proposed due to disorder in the erythropoiesis. This outcome established by Chahardeh *et al.* (6). Similar results are also registered by Kori-Siakpere *et al.* (17) whom noticed that in freshwater fish, *Heteroclarias spp* .From our opinion, the increase in RBC count in T4 and T5 this improvement could be owing to the role of vitamin E and C which decreased the oxidative stress. Also, the protective efficiency of vit C+E was clearly on the group treated with vit E+C represented by increase in erythrocyte count. In the current study, the enhancement effects for the hematological parameters observed in groups treated with vit E+C are in agreement with Chahardeh, *et al.* (6).

Conclusions

From the results of this study the mixture of vitamin C and E with ZnONPs has moderately infleunces on DNA damage this was clearly in T4 and T5. The activity of GPx in T4 and T5 started to return to normal as control in carp fish fed ZnONPs plus vitamin E and C mixture. This confirms the ability of both vitamin C and E to fight the oxidative damage results from ZnONPs exposure. It suggest that before applications of ZnONPs in various industrial sectors, the toxic potential must be carefully assessed and the effluents are also to be processed before it gets entered into the environment to protect both the aquatic biomes as well as human health.

Table 1. Results of DNA damage (% Tail DNA) in erythrocytes of *C. carpio* supplemented with different concentrations of ZnONPs in diet plus Vit C+E for 40 days

Groups	Mean ± SE of (%Tail DNA)
C	15.61 ±2.20 d
T1	16.93 ±2.72 d
T2	50.38 ±3.82 b
T3	58.73 ±2.47 a
T4	30.48 ±1.96 c
T5	34.51 ±3.26 c
LSD value	7.651 **

Means having with the different letters in the same column differed significantly at P≤0.01, n=4.

Table .2 Results of GPx activity (ng/ml) of *C. carpio* supplemented with different concentrations of ZnONPs in diet plus Vit C+E for 40 days

Groups	Means ±SE GPx activity (ng/ml)
C	1.47±0.18 c
T1	4.60±0.86 b
T2	8.41±1.13 a
T3	7.49±0.61 a
T4	2.65±0.14 c
T5	5.09±071 b
LSD value	1.755 *

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Means±SE having with the different letters in the same column differed significantly at P≤0.01. n=4.

Table 3. Hematological indices of *C. carpio* supplemented with different concentrations of ZnO NPs in diet plus Vit C+E for 40 days

	Mean ± SE			
Groups	WBC (10^3/µl)	HB(g/dl)	PCV (%)	RBC(10^6/µl)
C	16.89±0.08 a	7.29 ±0.21 ab	23.31±0.59 b	3.31 ±0.16 b
T1	16.85±0.12 a	7.77 ±0.42 a	27.47±1.77 a	3.37 ±0.12 ab
T2	16.05±0.05 b	6.06 ±0.21 cd	19.51±0.32 c	3.31 ±0.22 b
T3	16.06±0.05 b	4.82 ±0.04 e	15.48±0.11 d	2.82 ±0.03 c
T4	16.17±0.06 b	6.62 ±0.11 bc	20.88±0.33bc	3.74 ±0.02 a
T5	16.03±0.04 b	5.78 ±0.04 d	18.05±0.21cd	2.32 ±0.02 d
LSD value	0.286 **	0.829 **	3.221 **	0.402 **

Means having with the different letters in same column indicated significantly different ** (P≤0.01), n=4.

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