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Ameliorative effects of selenium in ZnO NPs induced oxidative stress and hematological alterations in *Catla catla*

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Abstract

Various applications of Zinc oxide nanoparticles (ZnO-NPs) can increase pollution in aquatic environments. Consequently, pollution can cause toxicity in fish as indicated by oxidative stress, hematotoxicity and changes in gill and liver histology.. Selenium is known for its antioxidant potential in scavenging the free radicals generated during ZnO NPs induced oxidative stress. This study tested the ameliorative role of selenium against ZnO NPs induced toxicity in freshwater fish *Catla catla*. Four groups of replicated fish, representing control, selenium treated, ZnO NPs treated, and ZnO NPs+Selenium treated were used in this study. The ZnO NPs (40 mgL⁻¹) were given to fish in water whereas selenium (50 µg kg⁻¹) was given as sodium selenite in feed. After 28 days of exposure, the fish specimens were processed to collect samples of blood, gills and liver. The results demonstrated that the consumption of selenium containing feeds protected the *C. catla* from ZnO NPs induced toxicity and oxidative stress. The use of selenium containing feeds appeared to have reduced the contents of Glutathione S-Transferase (GST) and Glutathione reduced (GSH), and increased the level of Catalase (CAT) and Superoxide dismutase (SOD). Furthermore, the consumption of selenium in feeds improved the hematological parameters in ZnO NPs treated fish. This study suggests that dietary selenium might be able to ameliorate ZnONPs induced toxicity in fish.

Keywords: *Catla catla*, nanoparticles, protection, selenium, toxicity, ZnO

Introduction

Water pollution is a serious problem due to the increased dumping of wastes from industries of electroplating, batteries, dyes, pigments, plastic, rubber and other chemicals [1, 2].

Metals that are indiscriminately released are accumulated in water bodies and create serious problem for aquatic organisms [3, 4], including fish [5, 6].

Zinc oxide nanoparticles (ZnO NPs) are a growing by-product of nanotechnology and their enhanced use may increase their concentration in the environment during production, processing and disposal [7]. The elevated concentration of ZnO NPs in an aquatic environment can release reactive oxygen species (ROS) that trigger oxidative stress and induce toxicity in aquatic organisms [8-17]. Excessive production of ROS can induce pro-inflammatory and cytotoxic effects [14], apoptosis [9] and oxidation of lipids, nucleic acids, and proteins. These effects can cause cancer, neurodegenerative diseases [18, 19], tissue injury [20, 21] and acute toxicity [22].

As oxidative damage can be diminished by antioxidants, research has been focused on the role of antioxidants for the treatment and prevention of relevant diseases [2, 4, 23, 24]. Antioxidants such as polyphenols, sulphur and selenium containing compounds, enzymatic antioxidants such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), and micronutrients such as vitamins C and E, have been extensively studied for their antioxidant properties [25-27].

As a vital trace element, selenium (Se) is an essential component of antioxidant enzymes like glutathione peroxidase (GPx), thioredoxin reductase (TrxR) and iodothyronine deiodinases (IDD). In fact, about 25 selenoproteins in human and at least 30 selenoproteins in mammals have been identified [28]. Collins et al. [29] stated that selenium and copper multiplexes use both metal binding and ROS scavenging ability in inhibiting oxidative stress. As a part of GPx, it can catalyse the reactions that may protect cell membrane against oxidative stress [30]. Similar positive effects were observed in fish that were exposed to heavy metals [31, 32]. Other Se compounds such as ebselen also showed neuroprotective role against stroke in embolic rabbit and humans. Significant improvement was found in the thrombolytic tissues of plasminogen activated rabbit [33]. Therefore, the present study tested the ameliorated role of Se in *C. catla* following their exposure to ZnO NPs treated water.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Aldrich and Merck (Germany). The salt of zinc acetate (99.5%), NaOH (99.5%) and tri-ethylamine (TEA) (99%) were used in the synthesis of NPs. Sodium selenite was used to provide Se for reducing the impact of ZnO NPs on fish.

ZnO NPs Synthesis

The 2.25g of aqueous zinc acetate ($\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$) (0.02 M) was dissolved in double distilled water to make the solution up to 50 ml stirring it vigorously. Then 40g of 2 M NaOH was added to this solution drop wise till the solution attained the 12 pH. Then 2ml of tri-ethylamine was added as a promoting and protecting agent. The solution was placed on magnetic stirrer (SCILOGES-MS-H-S) for 2 h. The appearance of white precipitates indicated the formation of ZnO NPs. These precipitates were washed with distilled water and then with absolute ethanol for removing all kinds of impurities. Then precipitates were filtered and dried overnight at 60 °C in the warm air incubator (JSR-JSSi-100-7) to obtain the dried Zn-NPs.

Experimental conditions

Specimens of experimental *C. catla* (45±4g) were purchased from a fish hatchery (Govt of the Punjab, Faisalabad) and transported in oxygenated containers of water to the laboratory. The fish were then transferred into glass aquaria (40 liters) which were maintained at 28±2 °C and 12h:12h photoperiod. The animals were fed with a specific type of fish feed (65% Crude Protein and 10% fat) in which all feed ingredients were mixed alongside sodium selenite (50µg/kg). The fish were divided into four groups of 6 fishes each in 3 replicates. The first group served as a control group and received the same feed but without sodium selenite. The 2nd group was fed with diet containing sodium selenite. The 3rd group was exposed to 40 mg ZnO NPs /L water and fed with diet containing no sodium selenite. The 4th group was exposed to 40 mg ZnO NPs/L water and fed with the same diet which also contained sodium selenite. The experiment was conducted for 28 days and water was changed after 48 hrs.

Collection of blood sample and Haematological analysis

At the end of 28 days of the study, the fish were anesthetized by simply adding 2-6 drops of clove oil to a bucket of 1-2 litre water, when anesthesia was confirmed the blood samples were collected by cardiac puncture from each fish using 2ml syringes and gauge hypodermic needles. The point of insertion for heart puncture was ventral, midway between the anterior bases of the pectoral fins. The syringes were flushed with EDTA and the collected blood samples were transferred to EDTA tubes.

The Neubauer haemocytometer (MARIENFELD-GERMAN) was used to count white- (WBCs) and red- blood cells (RBCs) whereas Sali's haemoglobinometer (MARIENFELD-

GERMAN) was used for the calculation of haemoglobin (Hb). The value of Packed Cell Volume (PCV) was measured by the methodology of Koestner et al. [34]. The Mean Corpuscular Value (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were measured with standard formulas of Borensztein et al. [35].

The kinetic enzyme assay was used to measure aspartate aminotransferase (AST) and Alanine amino transferase (ALT) being expressed as IU/L in gills and liver tissues.

Giesma staining was done for micronuclei test of blood erythrocytes. The prepared slides were examined at 100X under the microscope (Nikon-DS-L3) equipped with digital camera (Nikon-Eclipse-Ci-L). Non-refractive, small and ovoid bodies having the same stain **were** scored as micronuclei.

The frequency of the micronuclei was calculated by dividing the number of cells having micronuclei with total number of cells and multiplying with 100. The frequency was expressed in percentage (%). Comet assay in blood samples was conducted by following the protocol of Singh et al. [36].

Enzyme Analysis in gills and liver tissues

The protocol of Payá et al. [37] was followed with modifications made by Peixoto and Pereira-Moura [38] for the estimation of Superoxide dismutase (SOD). In this methodology, the detection agent was nitrotetraazolum blue chloride (NBT). The reaction mixture consisted of 100 mM phosphate buffer of pH 7.0, 10 mM NBT and 10 mM hypoxanthine. The reaction was started by mixing 0.023 U/mol of xanthine oxidase with extracts of enzymes and incubated at 20° C. The activity of enzyme was measured by 50 % inhibition of NBT. The results were expressed as U/mg protein.

The GST activity was measured by the methodology of Habig et al. [39]. The reaction mixture was a combination of 100 mM CDNB (chloro- dinitro benzene) and 2 ml of 100 mM buffer of potassium phosphate. The reaction mixture was then mixed with 100 mM GSH and incubated at 25° C. The absorbance was then measured at 340 nm and the GST concentration was estimated as mol/ mg protein.

The method of Jollow et al. [40] was followed for the estimation of GSH in the gill and liver homogenate. The 5,5-dithio-bis-[2-nitrobenzoic acid] (DTNB) reagent was used. 500 µl of 4% sulphosalicylic acid was added to 100 µl of hemolysate. It was incubated at 4° C for 1 h. The mixture was centrifuged at 4° C for 15 minutes and 12,000 RPM. The supernatants formed was

taken out. About 0.4 ml of supernatant was mixed with 2.2 ml of 0.05 M potassium phosphate having pH 7.4 with 0.4 ml DTNB. A yellow colour appeared due to the reaction between DTNB and GSH. The absorbance was taken at 412 nm. The concentration was reported as $\mu\text{mole/mg}$ protein.

The method as described by Aebi [41] was followed for estimating the catalase (CAT) activity. About 50 μl of 50 mM solution of H_2O_2 was used as a substrate. The solution was prepared in 50 mM potassium phosphate buffer. The CAT decomposed the H_2O_2 , which was measured through spectrophotometer at 240 nm by maintaining the pH at 7.0.

According to the Buege and Aust [42] lipid peroxidation is the measure of malondialdehyde (MDA) content. Here, 1 ml homogenates was mixed with 2 ml of TBA TCA- HCl. The mixture was then heated at 35°C in water bath and then cooled at room temperature, centrifuged at 4000 rpm (15 minutes). The absorbance of supernatant was recorded at 535 nm by using blank as standard. The MDA content was expressed as $\mu\text{mol/mg}$ protein.

Results

Characterization of particles

The SEM image of the ZnO nanoparticles prepared by precipitation method shows that the particles were spherical grain like and agglomerates (Fig. 1). The mean diameter of the particles was 36.77 nm. The histogram of synthesized particles specifies that the particles were in the range of 5-80 nm with concentrated frequency in the range of 5 to 40 nm (Fig. 1). Coagulation process increasingly happened through the heating practice, meanwhile the absence of separating medium between particles documented the produced particles to coalesce easily. The UV spectrophotometer showed the formation of ZnO NPs. The optical absorbance was recorded with spectrophotometer using the wave length of 200 to 700 nm. Fig. 1 shows absorbance peaks between 300 to 350 nm after 30 minutes of mixing the zinc acetate with sodium hydroxide.

The crystal structure and orientation of ZnO nanoparticles have been investigated by X-ray diffraction method using the Match Software. XRD diffraction pattern shows sharp peaks of the synthesized particles indicating their good crystallinity (Fig. 3). All XRD peaks showed hexagonal structures of particles. The average crystalline size of the particles was estimated from the XRD peaks by using the Scherrer equation. EDX pattern of the synthesized ZnO NPs showed the purity of the sample. All the peaks for the graph agreed with the standard plot (Fig.4). The average size of the ZnO NPs particles was found to be in range of 5 – 40 nm depending on the crystal growth condition. The robust signals in the Zinc section established the creation of Zn-NPs in the EDX

spectrum. Usually, Zn-NPs constantly showed a sharp optical absorbance peak roughly between 0.5 and 1 keV. The cause is superficial Plasmon resonance. Thus, the EDX spectrum confirmed the extraordinary pureness of sample without any impurity peaks (Fig. 2).

Fig. 1 Illustration of of ZnO NPs image by SEM (left) and its particles size distributon as histogram (right)

Fig. 2 UV (left) and EDX (right) spectra of ZnO NPs synthesized through co-parcipation mehtod

Fig. 3 XRD pattern of chemically synthesized ZnO NPs

Table 1 Sample purity through EDX analysis

Element	Weight%	Atomic%
O K	3.35	61.39
Ca K	0.06	0.83
Zn L	5.91	37.78
Totals	9.32	

Ameliorating role of selenium in oxidative stress

The level of CAT was found in an increasing order in both gills and liver from the ZnO NPs exposed fish as compared to the control fish. However, the use of selenium decreased the activity of CAT significantly ($p < 0.05$) to the normal level. The ZnO NPs significantly increased the activity of SOD after 28 days of treatment but the presence of selenium ameliorated the oxidative stress and reduced the level of SOD in samples of both liver and gills. The activity of GST was increased by ZnO NPs but following the use of selenium the activity of GST went back to normal. The level of lipid peroxidation increased in the ZnO NPs treated group as compared to the control group. The selenium addition significantly reduced the level of lipid peroxidation. Similarly the low level of GSH in the ZnO NPs treated samples were increased significantly due to lipid peroxidation but the treatment of selenium reduced it back to the levels approaching the normal levels (4).

Fig. 4 Ameliorated role of selenium against ZnO NPs induced oxidative stress in *C. catla* Asterisks represented statistically different values among treatments in the liver and gills ($p < 0.05$).

Ameliorating role in the hematology

High values of ALT and AST were recorded in the ZnO NPs treated group. The activity of both enzymes was significantly decreased in the selenium pre-treated fish exposed to ZnO NPs (Table 3). It was further found that the values of PCV, MCV MCH and MCHC fluctuated significantly in the ZnO NPs group as compared to the control group. However, selenium pre-treatment significantly increased the level of all parameters ameliorating the oxidative stress. The ZnO-NPs treatment significantly increased the levels of HB and RBC counts and non-significantly increased the WBC and platelet counts. However, selenium showed ameliorated role in ZnO NPs treated fish (Table 2).

Table 2 Mean ($\pm$ SD) values of hematology showing ameliorated effects of selenium on the ZnO NPs treated *C. catla*.

Groups	RBC ($\times 10^6/\text{mm}^3$)	WBC ($1000/\text{mm}^3$)	Platelet counts ($\times 10^6/\text{L}$)	Hb (g/dl)
Control	2.81 $\pm$ 0.12 ^C	7730 $\pm$ 281 ^C	281,000 $\pm$ 1001 ^B	4.61 $\pm$ 0.17 ^C
Selenium	2.85 $\pm$ 0.19 ^C	7834 $\pm$ 281 ^C	285,000 $\pm$ 1202 ^A	4.49 $\pm$ 0.79 ^C
ZnO NPs	3.87 $\pm$ 0.17 ^A	9354 $\pm$ 371 ^A	190,327 $\pm$ 582 ^C	7.22 $\pm$ 0.12 ^A
ZnO NPs+ Se.	2.99 $\pm$ 0.04 ^B	7977 $\pm$ 637 ^B	191,633 $\pm$ 730 ^D	6.82 $\pm$ 0.11 ^B

Values are the mean $\pm$ SD of six replicates.

Values sharing the different letters in the same column were significantly different at 5% level of probability or $p < 0.05$.

Table 3 Mean ($\pm$ SD) values ALT and AST showing ameliorated effects of selenium on the of ZnO NPs treated *C. catla*

Groups	ALT (IU/L)	AST (IU/L)
Control	9.90 $\pm$ 1.92 ^C	27.32 $\pm$ 1.96 ^D
Selenium	12.81 $\pm$ 1.27 ^C	43.78 $\pm$ 2.37 ^{BC}
ZnO NPs	50.16 $\pm$ 3.47 ^A	58.75 $\pm$ 5.57 ^A
ZnO NPs+ Se.	26.32 $\pm$ 2.15 ^B	40.15 $\pm$ 2.95 ^B

Values are the mean $\pm$ SD of six replicates

Table 4 Mean ($\pm$ SD) values of blood parameters showing ameliorated effects of selenium on theZnO NPs treated *C. catla*

Groups	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Control	33.44 $\pm$ 5.34 ^C	83.30 $\pm$ 12.44 ^C	27.07 $\pm$ 4.32 ^A	31.13 $\pm$ 3.38 ^A
Selenium	32.14 $\pm$ 2.54 ^C	82.50 $\pm$ 10.14 ^C	25.11 $\pm$ 2.31 ^A	27.53 $\pm$ 2.44 ^B
ZnO NPs	38.65 $\pm$ 2.15 ^A	97.81 $\pm$ 4.55 ^A	15.54 $\pm$ 2.65 ^C	16.72 $\pm$ 1.29 ^D
ZnO NPs+ Se	35.23 $\pm$ 1.34 ^B	90.55 $\pm$ 5.34 ^B	19.77 $\pm$ 2.48 ^B	19.38 $\pm$ 1.49 ^C

Values are the mean $\pm$ SD of six replicatesValues sharing the different letters in the same column were significantly different at 5% level of probability or $p < 0.05$ *Ameliorating role in genotoxicity*

The ZnO NPs treatment increased the frequency of micro nuclei significantly from the control group. Selenium pre-treatment reduced the frequency of micronuclei in ZnO NPs treated fish showing the genoprotective role. Furthermore, the ZnO NPs treatment significantly increased the frequency of comet due to its toxicity to erythrocytes. However the frequency was significantly reduced in the selenium pre-treated group (Table 5).

Table 5 Mean ($\pm$ SD) frequencies (%) of micronuclei and comet showing the ameliorated effects of selenium on in the ZnO NPs treated *C. catla*

Groups	Frequency (%)	
	Micronuclei	Tail DNA
Control	1.04 $\pm$ 0.9 ^C	2.01 $\pm$ 0.65 ^C
Selenium	0.94 $\pm$ 0.5 ^C	2.56 $\pm$ 0.76 ^C
ZnO NPs	6.23 $\pm$ 0.49 ^A	11.83 $\pm$ 1.14 ^A
ZnO NPs+ Se	3.57 $\pm$ 0.81 ^B	8.18 $\pm$ 0.06 ^B

Values are the mean $\pm$ SD of three replicatesValues sharing the different letters in the same column were significantly different at $p < 0.05$.

Discussion

The ZnO NPs were synthesized by the precipitation technique, in which 0.02 M aqueous Zn (CH₃COO)₂·2H₂O was dissolved in distilled water to which about 50 ml of 2.0 M NaOH was added to increase pH 12. The triethylamine was used as a promoting and protecting agent that resulted in white precipitate in 2 h which showed the after completion conversion into nanoparticles. The precipitate was prewashed and then dried for 12 h at 60 °C for complete conversion and formation of ZnO NPs from Zn (OH)₂. Controlled parameters in this process were the concentration of the reagents, the rate of addition of substrates, and the reaction temperatures.

Similar methods have been used to precipitate ZnO NPs from aqueous solutions of zinc chloride and zinc acetate[43]. Hong et al. [44] used a controlled precipitation method of precipitating zinc oxide with zinc acetate (Zn (CH₃COO)₂·H₂O) and ammonium carbonate (NH₄)₂CO₃. The solutions were then ultimately dosed into aqueous solution of polyethylene glycol a vigorously mixed and average molecular mass of 10,000.

The UV spectrophotometer showed the formation of ZnO-NPs from Zn (CH₃COO)₂·H₂O measurement of absorption spectrum within the wavelength of 200 to 700 nm. A unique peak absorbance between 300 to 350 nm after the 30 minutes of vigorous mixing of zinc acetate with sodium hydroxide for 2 hours at room temperature and for 2 hours at 150 °C showed the formation of ZnO-NPs [45].

The sharp diffraction peaks of the synthesized particles indicate their good crystalline features. The diffraction peaks in the XRD patterns of ZnO NPs confirmed the spherical structure. No characteristic peaks were observed other than for the ZnO NPs, which indicated the purity of the ZnO NPs sample. The elemental compositions of the synthesized ZnO NPs determined by energy dispersive X-ray diffraction showed no impurity peaks in the EDX. The average size of the synthesised ZnO NPs ranged between 5 to 100 nm with mean diameter of 36.77 nm estimated from XRD peaks by using the Scherrer formula or equation. The SEM images of the synthesized ZnO NPs showed that the morphology of the synthesized ZnO NPs were uniform and spherical in shape and were similar to the results discussed by Abdullah and Okuyama [46].

The main mechanism of NPs toxicity is oxidative stress. ZnO-NPs triggered oxidative stress in *C catla* [22]. This stress causes typical changes in the activity of SOD, CAT and GST as well as the levels of GSH and MDA [47]. The activities of SOD, CAT and GST increased in a dose

dependent manner and their level increased significantly in comparison to the control ($p < 0.05$). These results are in agreement with the findings of Kaya et al. [48] who found similar effects of ZnO NPs in tilapia. Furthermore, the ZnO NPs treatment also increased the lipid peroxidation (LPO) and GSH in the treated fish which was similar to the findings of Hao and Chen [49] for *Cyprinus carpio*.

In the present investigation, Selenium was used as an antioxidant to study the ameliorative impact against ZnO NPs toxicity. The results revealed that the activities of CAT, SOD and GST were altered by using selenium for 28 days in ZnO NPs treated *C. catla*. It was reported that excessive free radical production being involved in the depletion of antioxidant system and lipid peroxidation resulted in the cellular membrane degeneration and alterations in DNA, liver and gill tissues, while selenium showed significant effects in lowering the oxidative stress [50]. The superoxide anion as well as alkoxyl and peroxy ions might deactivate antioxidant enzymes including catalase and decrease the efficiency of cells to guard against free radical injury [51]. As an antioxidant, selenium helps fight against the free radicals and plays a role in the recovery of the antioxidant system [52]. Selenium is a cofactor of GSH and helps the enzymes to overcome the oxidative stress [53]. The treatment of the selenium also increases the activity of CAT [54], GST [55] and SOD [56]. Further, it also reduces the lipid peroxidation [57, 58].

The metal pollution also affects the liver of target organisms [59]. The main cause of liver injury is the oxidative stress [60]. The elevated levels of AST and ALT were the most common biomarkers used in the metal induced liver damage tests [61]. In the present study, significantly increased levels of AST and ALT were detected in the serum of ZnO-NPs treated fish due to its damaging effects on the liver. However, the selenium treatment reduced the activities of both enzymes showing the protective effects against ZnO NPs toxicity. Similar protection was recorded by Bitiren et al. [62] against carbon tetrachloride toxicity in rats. Further, the ZnO NPs treatment induced DNA damage in the form of comet and micronuclei. The treatment of the selenium actively reduced the damage to the genetic materials [63, 64].

Conclusions

It appeared that ZnO NPs treatment was able to induce oxidative stress, haematological alterations and DNA damage in fishes living in the pre-arranged ZnO NPs contaminated environment. Interestingly, the selenium treatment showed an ameliorative effect on haematology and antioxidant systems in response to elevated level of oxidants.

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Conflicts of Interest: The authors declare no conflict of interest.

Ethical Approval

All procedures performed in this study involving vertebrates like fish were approved by the research ethical committee and the animal care and use committee of the Government College University Faisalabad, Pakistan.

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