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RESEARCH ARTICLE

Effect of Engineered Nickel Oxide Nanoparticles on Antioxidant Enzymes in Freshwater Fish, *Labeo rohita*

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A B S T R A C T

Nickel oxide nanoparticles (NiO-NPs) are abundantly utilized on a large scale in different applications due to positive attributes and cause environmental pollution that may affect not only aquatic organisms but also the human beings. The co-precipitation method was used to synthesize NiO-NPs and particles were characterized through Fourier-transform infrared (FTIR) analysis, Scanning electron microscope (SEM) and X-ray diffraction (XRD). FTIR and SEM confirmed the attached functional group and crystal structure of synthesized nanoparticles, respectively. From XRD pattern, average particle size, X-ray density, lattice parameters (a, b and c) and volume of unit cell of nickel oxide nanoparticles were found to be 53.44 nm, 6.65 g/cm^3, (a =b=c=4.56 Å) and 94.81 Å^3, respectively. During this study, the acute toxicity of NiO-NPs was determined by using fish, *Labeo rohita*. The mean 96-h LC_{50} and lethal concentration were measured as 418.26 and 634.94 mg/L, respectively. The activity of catalase and superoxide dismutase was determined in fish gills and liver after chronic exposure to sub-lethal concentration of NiO-NPs for 90 days and sampling was done in 15, 30, 45, 60, 75 and 90 days. Significant time dependent variations in the activity of catalase and superoxide dismutase were determined in tissues of the gills and liver than control group during studied time interval. The overall results indicated that induced toxicity of NiO-NPs in aquatic organisms may be due to release of Ni ions from NiO-NPs and NPs induce toxicity in cells through oxidative stress under long term exposure.

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INTRODUCTION

Nanotechnology is the branch of science and engineering that produce new materials having size between 1 and 100 nm, has become one of the most likely region of engineering science (Laurent *et al*., 2010). Synthesized metal oxide nanoparticles are the most commonly used nanomaterials that have been progressively used in modern chemistry and biomedical science, consumer products and had several other applications (Rajkumar *et al*., 2016). The large applications of nanoparticles (NPs) demand the formation of these engineered particles on a larger scale in economical ways which raises the chance of entrance of nanoparticle directly or indirectly into freshwater systems (Owen and Handy, 2007). Due to their excessive release the harmful effects of NPs on the animals and the ecosystem have recently drawn much attraction. Nickel oxide nanoparticles (NiO-NPs) possess a number of particular characteristics and it is widely used in batteries, printing inks, diesel-fuel preservative, electrochromic films, magnetic materials and also act as a catalyzer in many biochemical reactions (Salimi *et al*., 2007 and Schrand *et al*., 2010). Due to various applications of NiO-NPs in many industries, it is also considered as a toxicant that adversely affect the freshwater biota included fish (Kovrižnych *et al*., 2014). The toxicity of NiO-NPs in bacteria and microalgae have also been reported (Baek and An, 2011; Gong *et al*., 2011). Nanoscale NiO is capable to cause oxidative stress associated with

respiratory diseases (Horie *et al*., 2011). Inorganic nanoparticles stimulate reactive oxygen species (ROS) that causes DNA damage, cytotoxicity and cell death in cultivated mammalian cells and *D. melanogaster* (Ahamed *et al*., 2011). Modern literature showed that one of the most important functioning of nanoparticles is the production of unnecessary ROS and oxidative stress (Nel *et al*., 2006). The *Labeo rohita* is a freshwater species related to cyprinidae family. It is the most commonly consumed fish due to their luscious taste and take up the major part of the freshwater resources of the country. Now a day, water pollution is the most important problem in freshwater ecosystems which causes adverse impacts on aquatic biota (Hamid *et al*., 2016). At the same time no activity subsists which would report the chronic effects of NiO-NPs on carps. This study was designed to investigate 96-h LC_{50} and Lethal concentration of NiO-NPs to fish. And activity of antioxidants biomarkers was checked after chronic exposure to sub-lethal concentration of NiO-NPs in *Labeo rohita* because in aquatic environments, organisms are usually exposed for a long duration (Javed, 2013).

MATERIALS AND METHODS

Preparation of nickel oxide nanoparticles: For synthesis of nanoscale NiO, $NiCl_2.6H_2O$ (1.17 g) and NaOH (2 g) were added in 50 ml deionized water separately. Then a solution of NaOH was added drop by drop in nickel chloride solution through burette. Then added ammonia drop by drop for pH=10 and stirred 2 hours. After that mixture was filtered and washed twice with ethanol and distilled water, then at 80℃ dried in an oven for 3 hours. After that the product was calcined at 400℃ for 2 hours to acquire NiO-NPs.

Characterization of nickel oxide nanoparticles: Atomic configuration of particles was checked by FTIR (Fourier Transform Infrared) spectroscopy. After the sample preparation of nickel oxide nanoparticles, morphology was determined by using Scanning Electron Microscope (SEM) Model (JEOL-JSM 5910). And X-Ray diffraction technique showed structural properties like crystallite size.

Experimental Fish and Acclimatization: The work was conducted in the research lab of Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Fingerlings of *Labeo rohita* were placed in cement tanks for acclimation for two weeks. Fish were fed with a commercial feed (30% Digestible protein and 3Kcal/g Digestible energy) daily in acclimation period. After this period, the healthy set of fingerlings (90-day old) of comparatively similar lengths and weights was chosen for this research. During acclimatization, stock of fish was retained at natural photoperiod and ambient temperature.

Acute toxicity test: The acute toxicity of nickel oxide nanoparticles in terms of 96-h LC_{50} and lethal concentration for *L. rohita* was checked. The control group was placed in metal free water. For the estimation of LC_{50} and lethal concentration different concentrations of nickel oxide nanoparticles were tested. Suspensions of NiO-NPs were prepared in distilled water by using a sonicator (100W, 40 kHz) for 60 minutes immediately prior to use. Ten fish were exposed to all mentioned concentrations for 96 h. The air was supplied continuously to all the glass tanks for maintenance of oxygen for fish respiration. Dead fish were instantly separated to prevent possible water quality deterioration. During the experiment, physico-chemical parameters of water like pH, temperature, total hardness, dissolved oxygen and natural 12:12 day/night photoperiod were maintained (Table 1).

Antioxidant biomarkers: After acute toxicity testing, fish were exposed to $1/5^{th}$ concentration of 96-h LC_{50} of NiO-NPs. The activity of the catalase and super oxide dismutase was checked from gills and liver of the fish. Tissues of gills and liver were homogenized in cold phosphate buffer in 1:4W/V having pH 6.5, 0.2M by using a homogenizer. After the homogenization, centrifugation of the homogenates was done for 15 minutes at 10,000rpm and 4ºC. The supernatant was stored to check enzyme activity at -4ºC while residue was removed. The catalase (CAT) and superoxide dismutase (SOD) activity were observed by using the protocol of Weydert and Cullen (2010) with minor alterations.

Statistical analysis: The data were presented as mean ± standard deviation (SD) of the three replicates. The mean lethal and lethal values with 95% confidence limits were measured by the probit analysis method. For statistical analysis of data, Analysis of Variance (ANOVA) was used, and comparison of means was done by Tukey's/Student Newnan-Keul tests.

RESULTS

Characterization of NiO-NPs

FTIR spectroscopy: The Fourier Transform Infrared Spectroscopy is a method which is utilized to determine particular functional groups and molecules in synthesized particles. This spectroscopy technique frequently used 4000 ~ 400 cm^{-1} wavenumber because this range absorbs radiations of almost all inorganic and organic ions. Fig. 1 displays the FTIR spectrum of NiO nanoparticles. The peak observed at 477 cm^{-1} and 620 cm^{-1} can be assigned to Ni-O and Ni-O-H bond, respectively. The broad absorption band at 3450 cm^{-1} showed OH modes (stretching and bending) of water (Wu *et al*., 2004). The wide absorption band around 2345 cm^{-1} can be assigned to CO_2 molecules. The peak at 1385 cm^{-1} corresponds to stretching vibrations of the intercalated C-O species (Table 2).

Scanning Electron Microscope (SEM): This method is essential for the sample scanning and also supply knowledge about both size and shape of particles. All the images get from SEM clearly confirm the nanometer range of NPs. Fig. 2 represents that nickel oxide NPs contain a clear crystalline arrangement, regular distribution and all particles show well association among them.

X-Ray Diffraction study: To measure the size of particles, volume, solidity, manufactured nanoparticles (NPs) were observed under X-ray diffraction technique (XRD).

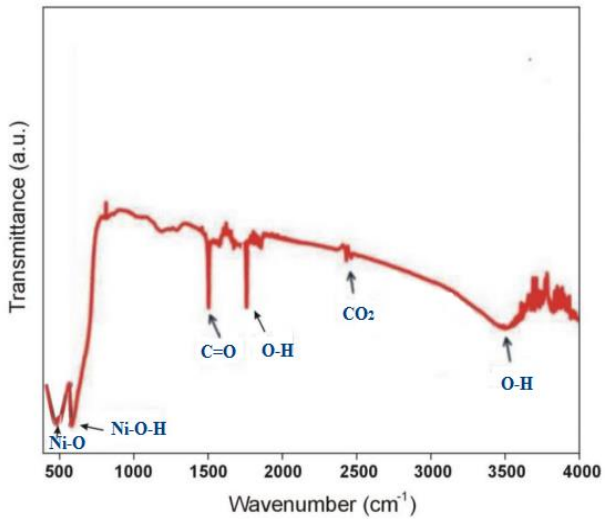

Fig 1: FTIR spectra of associated molecules in newly synthesized NiO-NPs.

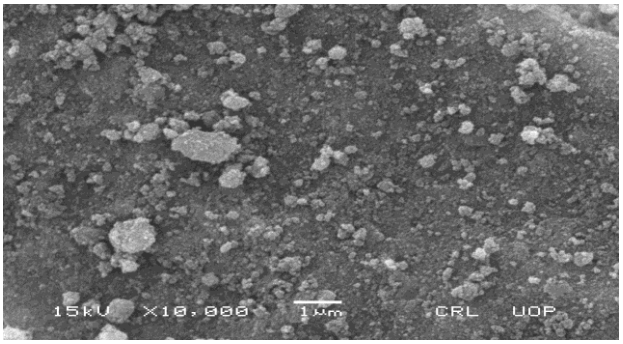

Fig. 2: Scanning electron microscope image of NiO-NPs.

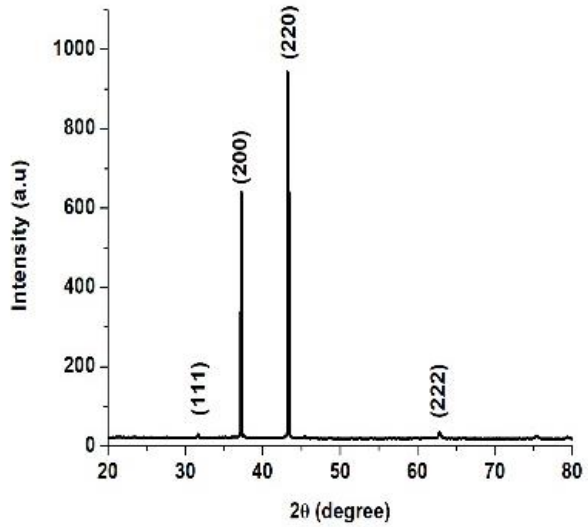

Fig. 3: X-ray diffraction Pattern of NiO-NPs.

Table 1: Physico-chemical parameters used for the experiments

Parameters	Unit	Mean	Analysis Method
Dissolved Oxygen	mg/L	6.0	Oxygen meter
Ammonia	mg/L	0.41	Titrimetric method
Electrical Conductivity	µSiemens/cm	520.32	Conductivity meter
Temperature	°C	29.30	Temperature meter
Total Hardness	mg/L	225	Titration method
PH	_	7.3	pH meter
Sodium	mg/L	300.45	Spectrophotometry
Calcium	mg/L	21.18	Spectrophotometry
Magnesium	mg/L	40.21	Spectrophotometry
Nitrates	mg/L	16	Spectrophotometry

Table 2: The data of different peaks recorded in FTIR of NiO-NPs samples

Wavenumber (cm^{-1})	Peak Assignment
477	Ni-O bond
620	Ni-O-H bond
1385	Asymmetric stretching of C=O
2345	CO_2 molecules in air
3450	O–H bond

Table 3: 96h acute toxicity of NiO-NPs (mg/L) for *Labeo rohita*

MeO-NPs	LC_{50}	95%CI (LCL-UCL)	Lethal conc.	95%CI (LCL-UPL)	Pearson goodness of fit tests: Chi-Square	DF	P-value
NiO-NPs	418.26	381.63-451.75	634.94	584.25-714.79	6.813	15	0.963

CI, confidence interval (mg/L); LCL, lower confidence limit (mg/L); UCL, upper confidence interval (mg/L); Lethal Conc., lethal concentrations (mg/L); DF, degree of freedom.

For this method, particles in powder form were used. Powdered form of particles has lots of small crystals with indiscriminate alignment. The X-ray diffraction design of NiO NPs is shown in Fig. 3. XRD patterns clearly display the diffraction peaks of the (1 1 1), (2 0 0), (2 2 0) and (2 2 2) planes that demonstrate that the NiO-NPs are in single phase. The average size (D) was 53.44nm, measured by the Debye-Scherrer formula. Lattice parameters (a, b and c), X-ray density and volume of unit cell of NiO nanoparticles were a=b=c=4.56 A^o, 6.65 g/cm^3 and 94.81 $(A^o)^3$, respectively.

Acute toxicity of NiO-NPs: The temperature, dissolved oxygen and pH of water was maintained during the study period. After every 24 hours, dead fish were examined and instantly removed from the test solutions to prevent pollution in environmental condition. The toxicity of nanoscale nickel oxide in *Labeo rohita* was increased as the particle concentration increased, showing dose dependent response (Fig. 4). The mean 96-h LC_{50} and lethal concentration of nickel oxide nanopowder for *Labeo rohita* were calculated as 418.26 and 634.94 mg/L, respectively at 95% confidence interval (Table 3).

Catalase (CAT) activity: CAT activity in gills and liver of *Labeo rohita* was measured after the exposure to sub-lethal dose (1/5th of 96-hr LC_{50}) for 15-day, 30-day, 45-day, 60-day, 75-day and 90-day intervals (Fig. 5). Fish showed significant decrease in CAT activity than that of the control group. Due to the high concentration of NiO-NPs, CAT became inactivate, which enhance the creation of ROS (reactive oxygen species) in fish.

Superoxide dismutase (SOD) activity: After sub-lethal exposure, nanoscale nickel oxide caused significantly variable activity of super oxide dismutase in gills and liver of *Labeo rohita (*Fig. 6). Tissues of gills showed significant increase after 15, 30, 45 days followed by a sharp decrease in 60, 75 and 90 days as compared to control group which enhance the creation of ROS (reactive oxygen species). The liver showed significant increase after 15, 30, 45 and 60 days followed by a sharp decrease at 75 and 90 days as compared to control group.

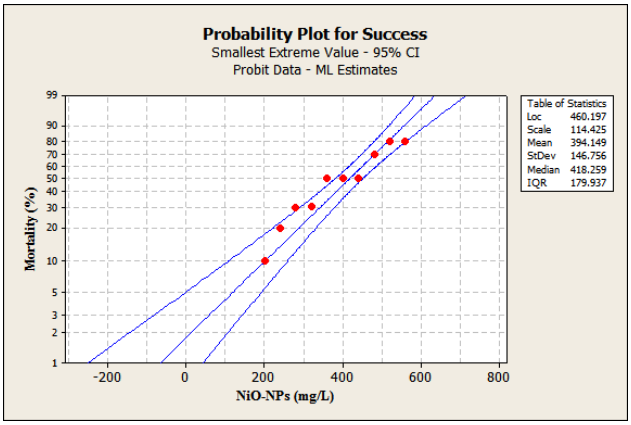

Fig 4: Probability plot of *L. rohita* for success at 95% CI.

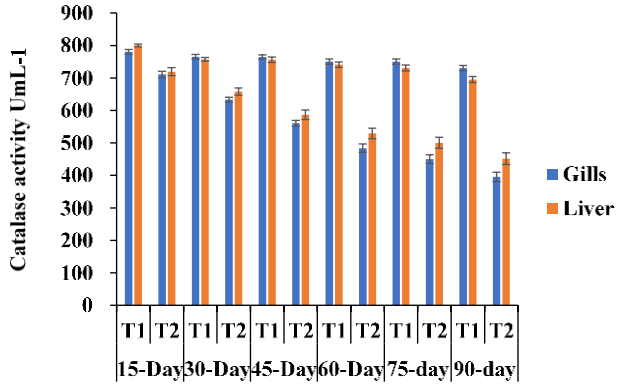

Fig 5: Effect of NiO-NPs on catalase activity (UmL-1) in gills and liver of *Labeo rohita.* Values are means of three replications and are given with standard deviation. T1=Control, T2=Treated.

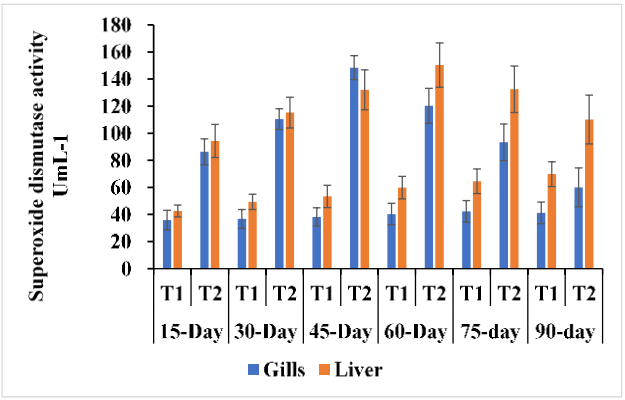

Fig 6: Effect of NiO-NPs on superoxide dismutase activity (UmL-1) in gills and liver of *Labeo rohita.* Values are means of three replications and are given with standard deviation. T1=Control, T2=Treated.

DISCUSSION

The use of nanoproducts in various industries has elevated the demand for a comprehensive inquiry of their risky consequences in living organisms because metallic nanoparticles liberate into the surroundings may have harmful effects on fish and other aquatic animals. The poisonous effects of nanoparticles on the different animals depend upon many factors, including their size, shape, morphology and properties (chemical, structural aggregation and surface) (Nam *et al*., 2019). These components affect significant the interactions between the target tissues and NPs (Xiang *et al*., 2018). Nanoparticles have high a density to volume ratio that supply large surface areas to intensify the responsiveness of nanoparticles with different biological organs. Moreover, NPs can have various levels of agglomeration, ion-dissolution and dispersion in the solutions, which consequently results in different level of toxicity (Raza *et al*., 2016). Determination of the median lethal concentration is highly helpful in the assessment of tolerance levels or riskless levels of a certain pollutant (Prentera *et al*., 2004). Therefore, it has ecological and biological significance (Kumar *et al*., 2016). It is clear from the consequences that the metal level has a direct impression on the LC_{50} values of the respective fish. Various studies have explained specie-specific sensitivity of freshwater fish species after exposure to different toxicants. Present work shows acute toxicity of NiO-NPs for *Labeo rohita* as 408.241 mg/L. Boran and Şaffak (2018) showed acute lethal toxicity (LC_{50}) in their study of Ni-NPs on zebrafish (larval) at 122.2 mg/L for 96-hour while Ni-NPs showed no mortality to zebrafish (adult) even at higher levels > 400 gm/L for 96 hour. The NiO-NPs toxicity differ for various organisms, showing the higher toxicity for algae (the 72-h EC50 = 32.28 mg/L; Gong *et al*., 2011). Microorganisms such as *E. coli, B. subtilis,* and *S. aureus* are fewer sensitive to NiO-NPs (the EC_{50} value=121-160 mg/L; Baek and An, 2011). In contrast to mammalian animals, NiO-NPs are more poisonous for fish than other mammal. It is clear from the previous results that the level of metals has a nonstop or direct impact on the LC_{50} values of the respective aquatic vertebrate. That's why, the outcomes of our and earlier reports indicate that exposure period and lethal concentrations may differ in different species. The discrimination in acute toxicity may be due to the changes in both quality of water and species used in the trial. The sensitivity of different species to a particular metal is a primary factor for LC_{50} phase. In the ecosystem, Fish that are more sensitive to the one metal may be less or even not sensitive to the toxicity of some other metal at the more level of that metal. Unlike, a pollutant that is very harmful for a fish variety at minimum levels may be less or even non-toxic to other species at the same or even higher levels (Sadeghi and Peery, 2018). The earlier research work showed that Ni-NPs created severe toxicity through the release of Ni(II) (Kanold *et al*. 2016).

Oxidative stress is result of an instability between the antioxidant defence system and creation of reactive oxygen species (ROS) in living animals. Superoxide dismutase and catalase (SOD–CAT) system are main signals of oxidative stress and provide defenses against oxygen toxicity (Li *et al*., 2011). The results of the present investigation indicated that NiO-NPs significantly induced SOD activity up to 45 days in gills and after that inhibitory effects of SOD activity were observed as time increases. The liver showed significant increase after 15, 30, 45 and 60 days followed by a sharp decrease at 75 and 90 days as compared to control group in SOD activity. Aziz *et al*., 2020 observed time dependent change in enzymatic activity after chronic exposure to nanoparticles. As nanoparticles are capable to create ROS in the liver, may point that tissue damage and oxitaive stress were evoked by nanomaterials through increased ROS level (Long *et al*., 2006).

The metallic NPs can induce an oxidative stress in various organs of animals. The level of ROS toxicity in different organs can be function-specific as well as nanoparticle structure-specific depending upon the quality of metal ions liberation (Zou *et al*., 2017; Bouallegui *et al*., 2018). Free radicals generated by stress has also been reported by Wise *et al*. (2010) as the contributive agent for disintegrating biomolecules and cell membranes. In our work, the treated fish gills and liver showed significant variations in SOD and CAT activities. The inhibition in enzyme activity is considered to be caused by nanoparticle evoked oxidative stress.

Conclusions: Acute toxicity (96h LC_{50}) of NiO-NPs to *Labeo rohita* was investigated as 418.26 mg/L. Our study demonstrated that NiO-NPs induce toxicity in fish, which is likely to be occurred by production of ROS and leading to larger inhibition of antioxidant enzymes activities in organs. That's why the liberation of NiO-NPs into the submerged ecosystems may pose hazards to aquatic life.

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Authors contribution: SA* executed the research. SA supervised and planned the research. HA helped in synthesis of nanoparticles. FL helped in laboratory work and write up of article.

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