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**Running Title:**Evaluation of waterborne zinc oxide nanoparticles (ZnO NPs) toxicity in fresh water fish, *Labeo rohita*

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**Novelty statement (4-5 points): ,**  The high exposure of humans and animals to metal oxides nanoparticles is the main topic that motivates us to conduct this present work. In this study, we evaluated acute toxicity of ZnONPs and its chronic effects on the biochemical changes in *Labeo rohita*. There is great importance for such study. First, to the best of our knowledge, this is the first record about the acute toxicity of ZnONPs in *Labeo rohita*. Second, it is important to assess the oxidative stress risk resulted from ZnONP exposure.

**Abstract:**

Concerns regarding zinc oxide nanoparticles have gained much attention due to their unique properties and widespread applications in cosmetics, electronics, sensor, communication, medicinal industry and biology that may induces an adverse impacts not only on specific ecosystem but also on human health. The 96h LC50 and lethal concentrations were investigated by using fish, *Labeo rohita* as genetic model during this study. The fish were exposed to 12 various concentrations (2-55mg/L) of ZnO-NP to determine 96-h LC50 and lethal concentations. Oxidative stress in terms of catalase, lipid peroxidation and superoxide dismutase were also determined in fish gills, muscle, liver and heart after chronic exposure of ZnO-NPs for 80 days and sampling were done on 20, 40, 60 and 80-day intervals. ZnO nanoparticles were synthesized by co-precipitation method and characterization was also done by different analytical techniques. The mean 96-h LC50 and lethal concentration were measured as 31.15 and 57.84 mg/L, respectively. Significantly decreased catalase and superoxide dismutase activity were determined in fish organs. However, level of lipid peroxidation was significantly increased in the fish organs as compared to control group. The overall results indicated that induced toxicity mechanism of ZnO-NPs in aquatic ecosystem was oxidative stress.

**Keywords:** ZnO Nanoparticles, Acute Toxicity, Catalase, Superoxide Dismutase, Lipid Peroxidation

**Introduction:**

In last decade, Nanotechnology has increased due to its wide use in biotechnology, medicine, environmental challenges, energy, and space exploration (Rosi and Mirkin, 2005; Zhang and Johnson, 2006; Francisco, 2007; Griffin *et al.*, 2008). Nanoparticles can be naturally found and synthesized by different methods and have sizes in the range from 1-100nm (Roco, 2003). Nanoparticles of metal oxide (NPs) most commonly occurred engineered nanoparticles, getting in interest for their possible harmfulness to both human beings and entire aquatic biota (Shaw and Handy, 2011) because of their excessive discharge in freshwater resource in their life cycles (synthesis, consumption, storage and movement). Exposure of NPs to aquatic organisms has revealed possible associated ecological and food chain risks and became main environmental issue (Moore, 2006).

Properties of nanoparticles (NPs) are intimately dependent on the configuration of overall size, size range distribution and composition of nanoparticles (Lee *et* *al*., 2010; Chang *et al*., 2012). The eco-toxicological data on ZnO-NPs is just rising and scanty, after TiO2 and SiO2, NPs they have the third highest worldwide production of 100-1000t/yr (Piccino *et al*., 2012). Zinc atoms discharged from ZnO nanoparticles increases the level of toxicants in exposed tissues and in other internal organs of fish, ultimately causing hazardous effects through oxidative stress system ([Morcillo](#page7) *[et al](#page7)*[., 2016](#page7); Ng *et al*., 2017). Mechanisms of nanoparticals toxicity are very complex, causes oxidative stress (Chang *et al*., 2012). Interaction between NPs and biological system may disturb homeostatic mechanism that damage the antioxidant defensive system ([Brown](#page7) *[et al](#page7)*[., 2004](#page7)), resulting in alteration of protein, DNA, lipids, carbohydrate ([Kelly](#page7) *[et al](#page7)*[.,](#page7) [1998](#page7)) and may induce the generations of reactive oxidative species (ROS) by delayed intracellular metabolism ([Long](#page7) *[et al](#page7)*[., 2006](#page7)).

Fishes are considered as an important indicator of freshwater quality because of their fundamental status in aquatic food web and accumulation and trophic transfer of nanoparticles is possible in all levels of marine food chains (Baker *et al*., 2014). Despite of the studies about poisoning of nanomaterials, minute information is present about metal’s bioavailability and impacts of these nanoparticles on organs of fish (Klaine *et al.*, 2008 and Handy *et al.* 2008). In Pakistan, *Labeo rohita* is consider as a most common food source due to its taste. Majority of the freshwater resources are occupied by this fish specie. Now a days, water pollution is becoming a primary concern in riverine ecosystems and underground water. Polluted water adversely affected the freshwater living organisms. *Labeo rohita* is most widely used in research works because it is capable to absorb and fix the toxic metals from the adjacent ecosystem (Hamid *et al*. 2016). It is essential to figure out permissible limits and impact of ZnO-NPs on fish. This present research was carried out to determine the acute toxicity of ZnO-NPs and its effects on antioxidant system such as LPO, SOD and CAT in selected organs of *Labeo rohita* after exposure of its 1/3rd of its respective 96-h LC50 for 80 days.

**Materials and Methods:**

**Synthesis and characterization of nanoscale ZnO**

The chemicals required for synthesis of ZnO-NPs were zinc sulfate (ZnS04.7H2O) and sodium hydroxide (NaOH). Distilled water is essential for the formation of mixture and all reagents were obtained from Merck via local distributor. In the water mixture of ZnSO4, NaOH was entered drop by drop having the ratio (1:2) with continuous mixing by magnetic stirrer and this mixing was carried out for 12 hours. The collected precipitates were well rinsed through distilled water after filtration. The precipitates were de-hydrated at 100°C in an oven Model (Shel-Lab) and agate morter was used to obtained powder form of the particles. The powder collected from this procedure was heat up for 2 hours at 500°C in furnace Model (SNOL-LHM01). After the preparation of nanoparticles, their morphology was checked by using Scanning Electron Microscope (SEM) Model (JEOL-JSM 5910). FT-IR (Fourier Transform Infrared) spectroscopy technique was used to observe the atomic configuration. XRD technique was used to determine structural properties like crystallite size of particles.

**Collection and Maintenance of Test Organism**

This research work was done in the laboratory of Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Freshwater fish, *Labeo rohita* (90-day old) were kept in cemented tanks for acclimation of 15days. After this period, the healthy group of fish having same weight and length were chosen for this research. Fish were offered with commercial pelleted feed 5% their body weight.

**Acute toxicity**

Fresh water fish, *Labeo rohita* was selected for mean lethal toxicity and lethality tests. For lethality, test concentrations of ZnO-NPs were 0, 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 mg/L. To obtain the suspensions of test, ZnO-NPs were dispersed in deionized water through ultra sonication (100W, 40 kHz) for 60 minutes immediately prior to use. Ten fish were exposed to all mentioned concentrations for 96 h in a 3.5L container with 2.5L of test solution. To maintain fixed level, all the test mixture were altered after 24 hours. Control group in trial was supplied with water without having nanoparticles. Each trial treatment was attempted triplicately under the constant conditions. All fish were not fed during testing durations to reduce the sorption of the nanoscale ZnO in solid feed and feces. During the trial, Physico-chemical parameters of water were controlled at optimum levels, temperature 29-30℃, pH, 7.5, DO, 5-7 mgL-1 and total hardness 290ppm, natural 12:12 day and night photoperiod.

**Oxidative stress biomarkers**

After calculating the acute toxicity of ZnO nanopowder *Labeo rohita* was exposed to 1/3rd of 96-h LC50 for 80 days and sampling were done after 20,40,60 and 80 days intervals, and after each period oxidative stress in terms of catalase (CAT), lipid peroxidation level (LPO) and superoxide dismutase (SOD) were assessed in the gills, muscle, heart and liver of fish. All organs of *labeo rohita* were homogenized, separately, in chilled PBS (phosphate buffer) in 1/4 ratio (weight/volume) through a homogenizer. After the homogenization, mixture were centrifuged (10,000 revolution per minute, at 4°C for 15 min.). Then transparent supernatants were selected for CAT, LPO and SOD studies.

1. **Catalase activity (CAT)** The activity of catalase was observed by its potential to lessen the concentration of H2O2 at 240nm by using method of Chance and Mehaly (1977) with slight alterations.
2. **Superoxide dismutase activity (SOD)**

This activity was observed by its potential to suppress the process of photoreduction of Nitroblue tetrazole (NBT) at 560nm by using method of Giannopolitis and Ries (1977).

1. **TBARS assay**

The level of lipid peroxidation was checked by calculating TBARS contents in selected organs by using method of Gatta et al. (2000).

**Statistical analyses**

All experiment was performed in three replicates and to find out the mean lethal concentrations of particles of metals oxide on *labeo rohita*, probit analysis method was executed with 95% confidence interval (Hamilton *et al*., 1977). To determine statistical difference and similarities among different variables, ANOVA and comparison of means were done by Tukey’s/Student Newnan-Keul tests. Data were analysed statistically by using Statistix 8.1 computer software.MS excel was used to draw the graph.

**Results and Discussion**

**Characterization of ZnO-NPs**

**X-Ray Diffraction study**

To measure the size of particles, volume, solidity, manufactured nanoparticles (NPs) were observed under X-ray diffraction technique (XRD). For this method, particles in powder form was used. Powdered form of particles have lots of small crystals with indiscriminate alignment. The X-ray diffraction design of ZnO NPs is shown in Fig. 1. The average measured size was 53nm for NPs crystallites.

### FT-IR Spectroscopy (Fourier Transform Infrared) analysis

The Infrared Spectroscopy (IR) is a technique which is used to observe the phase configuration of metallic ions and also the pattern of oxygen binding. This technique is also used to denote the existence of functional group in the prepared particles. Fig. 2 represents the infrared transmission pattern of all observed samples ranged from 4000-400 cm-1 (wave number). The 3000-3500 cm-1 wide patches are allotted to O---H elongating and curved vibrations of H2O. the patches within the range of 2300-2450 cm-1 are assigned to the extending vibrations of CO2 in air. The high pitched top ranging from 1499 to 1580 cm-1 described as extending vibrations of C=O, respectively. Ultimately, the firm patch observed at 400-500 cm-1 appointed to vibrations of Zn-O (Fig. 2)

**Scanning Electron Microscope (SEM)**

This technique is important for the scanning of the samples and also provide knowledge about the size and shape of particles and growth. All the images obtained from SEM visibly demonstrate the normal size of NPs that is under the nanometer range. Fig. 3 represents that zinc oxide manufactured NPs contain clear wurtzite-hexagonal crystalline arrangement, regular distribution and all particles show well association among them.

**Acute toxicity of metals oxides nanoparticles**

The temperarure of water was controlled at 29-30°C in the course of whole study period. Dissolved oxygen and pH of water were examined (5-7mg/L and 7.5, respectively). After each 24 hours dead fishes were examined and instantly removed from the test solutions to prevent pollution in environmental condition. The toxicity of nano-scale zinc oxide in *labeo rohita* was increased as the particle concentration increased, showing dose dependency. The mean 96-h LC50 and lethal concentrationof zinc oxide nanopowder for *Labeo rohita* were calculated as 31.15 and 57.84mg/L, respectively at 95% confidence interval (Table 1). Nanoparticles show peculiar behavior in comparison with their other particles of similar chemical configuration due to its smaller size, higher reactiveness and greater surface sphere per unit mass (Wigginton *et al*., 2007). Chemical pattern and high surface reactivity of nanoparticles resulted in toxicity of aquatic system (Navarro *et al*., 2008).

Finding of LC50 concentration is most beneficial to evaluate the safety limits and tolerable limits of several toxicants (Prentera *et al*., 2004). According to previous reviews toxicity of ZnO-NPs is considered as the less studied topic in fish (Kahru *et al*., 2010). Although previous studies also showed acute lethal value always measured in mg/L not in ug/L. It is indicated that metal oxide nanoparticles which are less solvable, cause less pollution. Some nano-metals that exhibit less dissolution of metallic ions would be express lethal concentration in range of ug/L which is a major concern for those metals whose nanoparticles can easily dissolve (Shaw and Handy, 2011). In contrats to our research, Subashkumar and Selvanayagam (2014) reported 96h LC50 of nanoscale ZnO as 4.897mg/L. The difference in toxicity may be due to different physico-chemical properties of nanosacle ZnO that will affect toxicity mechanism of nanoparticles. Many researchers indicated that pollution of ZnO nanoparticles had close relation with its soluble free ions while some research articles reported that the ZnO nanoparticles was more toxic as compared to its ionic form (Franklin *et al*., 2007; Nair *et al*., 2009; Wong *et al*., 2010). Miao *et al*. (2010) inverstigated level of ZnO-NPs toxicity at various pH (7–9) and reported that ZnO-NPs showed elevated toxicity at low pH due to excess dissolution of Zn ions in exposure medium. To observe the toxicity of ZnO nanoparticles, most essential matter of concern is the solubility, temperature, salinity, and pH of particles that may change the physicochemical behvior of ZnO-NPs and the relation between the nanoparticles and the aquatic organisms.

**Oxidative stress Biomarkers**

**Catalase (CAT) activity**

CAT acticity in specified organs of *Labeo rohita* were measured after the exposure of 1/3rd of 96-hr LC50 for 20-day, 40-day, 60-day and 80-day intervals (Fig.4). Fish showed significant increase after 20-day followed by a sharp decrease at 40, 60 and 80 days in CAT activity than that of control group. Due to high concentration of ZnO-NPs, CAT became inactivate in *labeo rohita* respectively, which enhance the creation of ROS (reactive oxygen species) in fish specie. Our results demostrated that effects of ZnONP were directly proportional to the duration of exposure. Previous studies on metallic toxicity exposed that nanoparticles of metal oxide had strength to cause acute or chronic adverse impacts (Zhao *et al*., 2011). One of the most frequently studied topic of NPs toxication is Oxidative stress (Nel *et al*., 2006 and Mocan *et al*., 2010). Activity of enzymatic antioxidants can be used as biomarkers of environmental pollutants that causes changes in level of antioxidants in aquatic organisms (Borkovic´ *et al.*, 2005). Antioxidants are considered as an essential part to examine the pollution in water and prior to the occurrence of harmful impacts in fish enzymatic activities work as susceptible biochemical indicators. Abdel-Khalek (2015) suggested that CAT activities in different organs of fish disturbed due to frequent contact with metals and instability of superoxide free radicals. This result is consistant with Abdelazim *et al*. (2018) who reported significantly decreased CAT, GPx, and GST activities in Nile Tilapia after the exposure of ZnONPs. ZnO nanoparticles act as an inhibitor in Catalase activities which suggested that hydrogen peroxide (H2O2) produce through SOD was not directly eliminated via Catalase. As a result of this ROS (reactive oxygen species) increases within the cell. In freshwater biota the activity of CAT also restrain due to presence of nanoparticles such as, Gomes *et al*. (2011) observed that nanoparticles of Cu in *M. provincialis* prohibited CAT activities. Our results are also matched with Xia *et al*. (2013) who reported significantly decreased CAT activity at concentrations equal to or higher than 160 mg/L.

**Superoxide dismutase (SOD) activity**

Sub-lethal exposure of zinc oxide nanoparticlescausedsignificantly variable activity of super oxide dismutase in specified organs of *Labeo rohita (*Figure 5). Fish organs showed significant increase after 20, and 40 days followed by sharp decrease at 60 and 80 days as compared to control group in SOD activity which enhance the creation of ROS (reactive oxygen species). Superoxide dismutase is helpful in the disintegration of free radical superoxide by changing it into hydrogen peroxide (H2O2), after which at higher levels it is decayed by CAT (Arimoto *et al*., 2005). Low activity of SOD under strong stress of metals can lead to accumulation of more reactive oxygen species in animals that ultimatey results in damage of cell ([Phull](https://www.frontiersin.org/articles/10.3389/fphys.2019.00868/full%22%20%5Cl%20%22B33) *[et al](https://www.frontiersin.org/articles/10.3389/fphys.2019.00868/full%22%20%5Cl%20%22B33)*[., 2018](https://www.frontiersin.org/articles/10.3389/fphys.2019.00868/full%22%20%5Cl%20%22B33)). This is in accordance with previous study that SOD activity was significantly reduced after 100 mg/L exposure of ZnO-NPs that results in excessive ROS in cell (Zhao *et al*., 2013). This process can also be demostrated that ZnO-NPs caused ROS synthesis which the antioxidant system could not eliminate. In our present study, level of superoxide dismutase was in the following trend: liver > gills > heart > muscles, repectively. Xiong *et al*. (2011) reported that ZnO-NPs showed significantly redued SOD level in liver and increased level in gut of adult zebrafish, indicating that the nanopowders affected the SOD activity of target tissues specifically.

**TBARS assay:**

The levels of TBARS as an effective symbol to detect lipid peroxidation have been used during this research. Chronic exposure of ZnO-NPs showed significant variability in induction of TBARS level in body organs of *Labeo rohita*. Level of TBARS increased significantly with increase of exposure duration than control (Fig. 6). In the same line of our data, Abdelazim *et al*. (2018) reported more lipid peroxidation in the muscles of treated Nile Tilapia after ZnO nanopowder exposure. Benavides *et al*. (2016) and Nadhman *et al*. (2016) also tested high level of lipid peroxidation under the exposure of ZnO nanopowder and under other nanopowder exposure (Wang *et al*., 2016). High liver MDA in common carp after chronic exposure of metals was reported by Vinodhini and Narayanan (2009). More MDA contents in selected organs of carp after 50 mg/l ZnO NPs for 10 and 14 days, showed the oxidative stress of ZnO nanopowder in cells of fish (Hao and Chen, 2012). Rise in Lipid peroxidation level may be attributed to the change in mechanism of antioxidant system to inhibit the available radicals formation (Kim *et al*., 2010). The measurement of Lipid peroxidation supply a comparative measurement of the all possible toxicants to drive oxidative harm (Vlahogianni *et al*., 2007). Abdel-Khalek (2015) investigated significant rise in both gills and liver lipid peroxidation level after Zn bulk and nanoparticles exposure after different durations. It has been mostly recognized that free radicals that produced due to stress is a damaging element, which results in high lipid peroxidation and inactivation of different enzymes (Valko *et al*., 2004).

**Conclusion:**

Acute toxicity of ZnO nanopowder to *Labeo rohita* was determined at 96h (LC50) as 31.15 mg/L. Zinc oxide nanoparticles disturbed the functions of many enzymes that provide defence due to excess of ROS (reactive oxygen species). Due to exposure of 1/3rd of 96-h LC50 of ZnO nanopowder, reactivity of LPO, CAT and SOD in *labeo rohita* were significantly fluctuating and showed tissue specific response. The well-known functions of synthesized metal oxide nanoparticles develop an interest towards the protection of freshwater biota and mankind. It is necessary to promote this work to understand the impacts of different ecological factors on pollution of nanoparticles and a distinct mechanism of toxicity.

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**Statement of conflict of interest**

Authors have declared no conflict of interest.

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**Fig. 1: X-ray diffraction Pattern of nanoscale ZnO**



**Fig. 2. FTIR spectra of associated with attached molecules with newly synthesized ZnO-NPs.**



**Fig. 3: Scanning electron microscopy (SEM) result of ZnO nanoparticles**

**Table 1: 96h acute toxicity of ZnO-NPs (mg/L) for *Labeo rohita***

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fish species** | **MeO-NPs** | **LC50** | **95%CI** | **Lethal****conc.** | **95%CI** | **Pearson goodness of fit tests** |
|  |  |  | **(LCL-UCL)** |  | **(LCL-UPL)** | **Chi-Square** | **DF** | **P-value** |
| *Labeo rohita* | ZnO-NPs | 31.15 | 26.29-35.31 | 57.84 | 51.39-68.73 | 3.31045 | 11 | 0.986 |

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

Fig. 4. Effect of Zno-NPs on catalase activity (UmL-1) in different organs of *Labeo rohita*. Values are means of three replications and are given with standard deviations.

Fig. 5. Effect of ZnO-NPs on superoxide dismutase activty (UmL-1) in different organs of *Labeo rohita.* Values are means of three replications and are given with standard deviations.

Fig. 6. Changes in TBARS level (mg/g) in different organs of *Labeo rohita*. Values are means of three replications and are given with standard deviations.