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Original Article

Assessment of hematological and biochemical alterations as markers in an Indian major carp *Catla catla* exposed to various concentrations of zinc oxide nanoparticles

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Abstract: Fingerlings of *Catla catla* were exposed to 1, 5, and 25 mg/L of zinc oxide nanoparticles (ZnO NPs) for 15 days, and specific hematological and biochemical parameters were evaluated to assess the toxicity. During the exposure period, red blood cell (RBC) count was found to decrease (except at the end of the 5th day in 1 mg/L) whereas white blood cell (WBC) count was found to increase in ZnO NPs treated fishes. A significantly higher hematocrit (Hct) level was recorded in fish exposed to 1 mg/L when compared with control and a higher concentration of ZnO NPs (5 and 25 mg/L). Erythrocyte indices such as mean cellular volume (MCV) and mean cellular hemoglobin (MCH) values (except at the end of 5 and 10th day at 1 and 5 mg/L exposed groups) were significantly increased. Mean cellular hemoglobin concentration (MCHC) level was found to be increased at 1 and 25 mg/L treated groups compared to 5 mg/L. Compared to the control group, plasma glucose level was increased significantly in fish exposed to 5 and 25 mg/L concentrations of ZnO NPs, while the plasma glucose level was decreased at the end of the 15th day in all the concentrations. Plasma protein level was increased at the end of the 5th day while the level of plasma protein was decreased on the 10 and 15th day. A significant increase in glutamate oxaloacetate transaminase (GOT) (except at the end of 10th day) and glutamate pyruvate transaminase (GPT) activity in gill and liver (except at the end of 10 and 15th day in gill) were noted in all the concentrations tested when compared to control groups. The results of the present study indicate that ZnO NPs at 1, 5, and 25 mg/L can alter the hematological and biochemical parameters of fish and the toxicity data may provide the ecotoxicological impact of ZnO NPs on the aquatic environment.

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Introduction

In recent years, the development of nanotechnology has led to the manufacturing of cost-effective nanomaterial's (Mansoori et al., 2008; Sarkheil et al., 2016). Among the nanoparticles, engineered nanoparticles are widely used in the field of biomedicine as cancer therapy, drug delivery, and also as antimicrobial agents (Rudramurthy and Swamy, 2018), due to their unique physical, chemical, and optical properties (Sharma et al., 2018). Furthermore, metal oxide NPs are widely used for a large variety of applications, including catalysis, sensors, electronic materials, cosmetics, sunscreens, self-cleaning coatings, textiles, and environmental remediation processes (Puzyn et al., 2011; Remya et al., 2015; Rudramurthy and Swamy, 2018; Chavali and Nikolova, 2019). However, the direct and indirect release of NPs into aquatic environments via bathing, sewage effluent (Handy and Shaw, 2007; Bundschuh et al., 2018; Mieiro et al., 2019; Kumar et al., 2020), and other engineering applications (Nagaveni et al., 2004) have increased the exposure chances of humans and other aquatic organisms (Scown et al., 2010; Remya et al., 2015; Yousef et al., 2019; Alkaladi et al., 2020). They may also enter the aquatic environment by intentional and accidental releases or via weathering of products (Scown et al., 2010).

The concentration of NPs in an aquatic environment depends on the type of the nanoparticle, which is in a range lower than ng/L to g/L (Turan et

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al., 2019). The studies on the safety and ecotoxicity of NPs are of extreme importance (Kahru et al., 2008) when compared with larger particles (Brayner et al., 2010) due to their sources, behaviour, different properties, and toxicological effects (Bundschuh et al., 2018). It has been shown that many metal oxide NPs may pose potential risks to human health and other organisms (Wu et al., 2013; Karlsson et al., 2015; Girigoswami, 2018; Kumar et al., 2020) due to their high reactivity, dissolution, and aggregation and accumulation in the environment (King-Heiden et al., 2009; Lowry et al., 2012; Song et al., 2015).

ZnO NPs nanoparticles are widely used in many applications, including sunscreen products, cosmetics, pigments, industrial coatings, plastic additives, semiconductors, textiles, ceramic manufacture, antibacterial agents (Fan and Lu, 2005; Piccinno et al., 2012; Gagné et al., 2013; Kaya et al., 2015, 2016; García-Gómez et al., 2020), wastewater treatment (Chen et al., 2004), fungicide (Theodore, 2006) and environmental remediation processes (Aitken et al., 2006) due to their antibacterial activity and absorption of ultraviolet radiation (Li et al., 2013). Moreover, ZnO NPs are used as therapeutic applications in biotechnology (George et al., 2010; Yan et al., 2011; Sruthi et al., 2018) and also used as an organic coating (García-Gómez et al., 2020). A recent study indicates that ZnO NPs are used to improve water quality and also as a feed supplement in aquaculture (Márquez et al., 2018; Onuegbu et al., 2018). Piccinno et al. (2012) has reported that the production of ZnO NPs has reached between 100 and 1000 t/year globally.

The extensive application of ZnO NPs in industrial and commercial sectors may end up in wastewaters and released into the environment (Moore, 2006; Gange et al., 2019) and has the potential to induce adverse effects on aquatic organisms such as fish (Chen et al., 2011; Zhao et al., 2013). Asharani et al. (2008) observed mortality of *D. rerio* embryos at 25-50 mg/L in a short-term study. Likewise, the LC50 value was recorded as 1.8 mg/L to *D. rerio* (Zhu et al., 2008), 2.3 mg/L to *Caenorhabditis elegans* (Wang et al., 2009), and 21.89 mg L^{-1} to *Pangasianodon hypophthalmus* (Kumar et al., 2020). Furthermore, Zhu et al. (2009) reported that the hatching rate and oxidative responses were decreased in zebrafish (*D. rerio*) exposed to ZnO NPs. In addition, recently elevation of oxidative stress in *Pangasianodon hypophthalmus* (Kumar et al., 2020), hormonal and molecular alterations in *Oreochromis niloticus* (Alkaladi et al., 2020), accumulation and histopathological alterations in *Cyprinus carpio* (Chupani et al., 2018), oxidative and biochemical alterations in *Monacha cartusiana* (Abdel-Halim et al., 2020), alterations in ALT, LDH and ALP activities in *C. carpio* (Banaee et al., 2018) and embryonic and developmental toxicity in marine fish *Mugilogobius chulae* embryos (Li et al., 2018) has been documented.

The toxicity of ZnO NPs to *D. rerio* may occur partly due to the release of zinc ions (Xiong et al., 2011). However, experiments with freshwater alga *Pseudokirchneriella subcapitata* revealed that the toxicity was solely attributed to dissolved zinc (Franklin et al., 2007). These particles are biologically inert and become toxic in their nanoparticles state (Fernandez-Cruz et al., 2013). Mostly these particles exert their toxic effects due to their particle dissolution and reactive oxygen species (ROS) (Ma et al., 2013). The potential impacts of ZnO NPs on aquatic ecosystems have attracted special attention (Wiench et al., 2009; Fernández et al., 2013; Yousef et al., 2019; Kumar et al., 2020). However, the toxicity of nanomaterials is still poorly understood with respect to aquatic ecosystems (Gottschalk et al., 2013) due to their nano-specific bio-functional properties and physicochemical transformation in the environment (Rocha et al., 2017).

Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially harmful to humans. Fish are excellent bioindicators of environmental health as they are susceptible to various types of xenobiotics. Consequently, investigating the impact of NPs on fish is an important aspect that will help to understand the effects of NPs on other organisms (Remya et al., 2015). Fish exposed to environmental pollutants exhibit a variety of physiological responses (Booth et al., 1988). There is considerable interest in

parameters related to haematological, biochemical, and enzymological parameters that have been routinely used as valuable biomarkers to assess the toxicity of any environmental contaminants on the aquatic ecosystem (Saravanan et al., 2011; Ramesh et al., 2015; Ramesh et al., 2018). Fish blood is widely used in toxicological research and environmental monitoring as a promising indicator of physiological and pathological changes of the whole body (Krishnapriya et al., 2017; Umamaheswari et al., 2019) and also reflect many diseases (Burgos-Aceves et al., 2019).

Haematological indices can be used as a biomarker of environmental variations and stress (Burgos-Aceves et al., 2019). The blood and its constituents may reflect many diseases; the abnormalities of erythrocytes, leukocytes, thrombocytes, and clotting factors are considered primary blood disorders. Hematological (Hb, Hct, RBC, WBC and erythrocyte indices) and biochemical (glucose and protein) parameters are widely used to monitor the impact of environmental contaminants on the aquatic organisms (Saravanan et al., 2011; Ramesh et al., 2014). Likewise, alterations in enzymological parameters (GOT and GPT) in fish have been widely used as sensitive biochemical markers for environmental contamination in an aquatic ecosystem (Sathya et al., 2012; Poopal et al., 2013; Ramesh et al., 2018).

The possible toxic mechanism of the ZnO NPs on aquatic organisms particularly in fish is more complex and needs a detailed investigation (Onuegbu et al., 2018). The knowledge on the toxic effect of NPs on the physiology of Indian major carps is meager. Moreover, the effects of ZnO NPs on the blood biochemistry of fish remain to be documented. Consequently, the main objective of this study is to determine the impact of different concentrations of ZnO NPs (1, 5 and 25 mg/L) in an Indian major carp *Catla catla* using hematological (Hb, Ht, RBC, WBC, MCH, MCV and MCHC), biochemical (plasma glucose and protein) parameters in blood, and enzymological (GOT and GPT) parameters in vital organs (gill and liver). The alteration of these parameters can be effectively used as the first indicator for environmental monitoring of nanoparticles in the aquatic ecosystem.

Materials and Methods

Experimental animal and test conditions: Fingerlings of *C. catla* were obtained from TamilNadu Fisheries Development Corporation Limited, Tamil Nadu, India, in the weight range of 4.5±0.5 g and body length of 6.5±0.5 cm. Fish were acclimatized to laboratory conditions in continuously aerated dechlorinated tap water and maintained under a photoperiod of 12-h/12-h light-dark cycle. During the acclimatization period, fish were fed twice a day with commercial pellets, and the residues and metabolic wastes were removed daily. The water quality parameters of the dechlorinated tap water used for fish culture were determined according to APHA (2005) and are as follows: temperature 27.2±1.3ºC, pH 7.1±0.08, dissolved oxygen 6.4±0.4 mg L^{-1}, and total hardness 18.2±1.5 mg L^{-1}. All experiments were performed in obedience to applicable laws and guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), with approval by the Ministry of Environment and Forests, Government of India (CPCSEA/CH/ORG/2006/2064).

Synthesis of ZnO NPs: The freshly prepared zinc acetate solution was placed in a flask. Then the sodium hydroxide solution was slowly added dropwise into the zinc acetate solution till the pH value becomes 10 along with vigorous stirring for 1½ hour and then the white colour solution was obtained. Further, the solution was kept for ½ hour under the ultrasonic condition for stabilizing the formation of particles. Finally, the solution was stored without any disturbance and the sedimentation of the particles was obtained. The sedimentation was washed out several times for removing unreacted compounds. After centrifugally, the particles are separated from the solution and then dried. Further, the sample was oxidized under annealing treatment. Finally, a white colour ZnO powder was obtained which had a little agglomeration and its size was 20 nm with spherical shape confirmed by transmission electron microscope

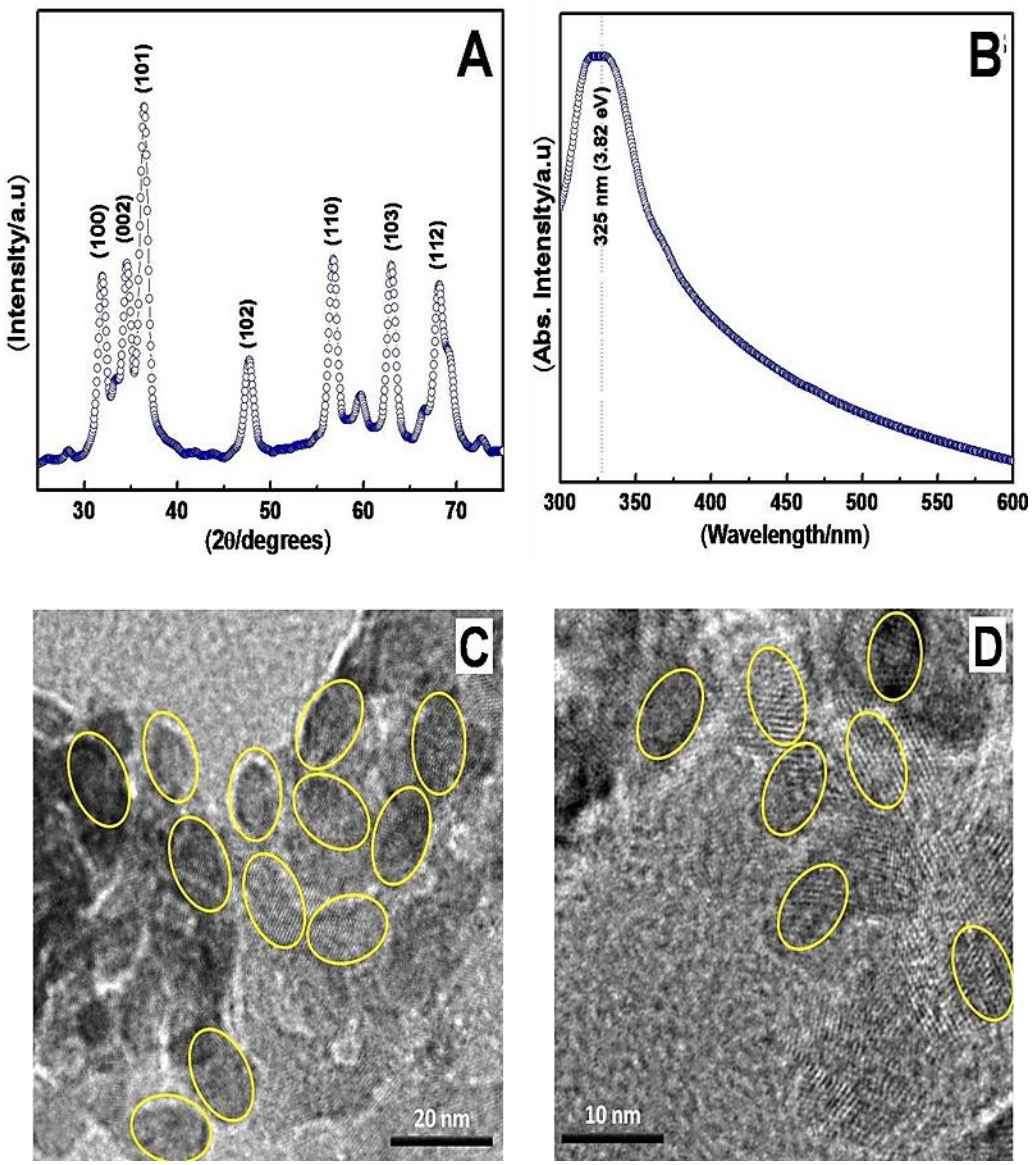

Figure 1. (A) XRD pattern, (B) UV spectra and (C), and (D) TEM images of ZnO Nanoparticles.

(TEM) technique.

Preparation of Test Solution: The stock solution of ZnO NPs was prepared by dissolving 1 g of ZnO NPs in 1 L of distilled water and kept in an ultrasonic agitator (40 kHz frequency vibronic -250 Wt) for 1 hour to get finely dispersed particles.

Assessment of ZnO NPs toxicity: For the assessment of ZnO NPs toxicity, circular plastic tubs with 20 litres of water were taken. Then to each tub, different concentrations of the ZnO NPs (i.e. 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, and 50.0 mg/L) were added. A control tub with 20 litres of water was also taken. From the stock, ten healthy fish fingerlings were selected and introduced into each tub. The manifestation and survival time of fish was observed in each concentration. The manifestation time, the time at which fish lost their sense of balance and floated on their sides were determined. Dead fish from the experimental tubs were removed immediately. Death was indicated by the failure of the fish to respond to gentle prodding with a glass rod and cessation of opercula movement.

Assessment of ZnO NPs toxicity- a chronic study: For the present study, 1, 5, and 25 mg/L were selected based on the survival/mortality ratio and the experiment was conducted for a period of 15 days. For chronic studies, fish were divided into four main groups. Group 1 was kept in ZnO-free fresh water and

treated as the control. Various concentrations of ZnO NPs (1, 5, and 25 mg/L) were added in each glass aquaria (100-liter capacity) containing 90 L of water and grouped as 2, 3, and 4. The experiment was conducted for 15 days. Four replicates were maintained for each concentration and control group. Fish were fed ad libitum and water was replaced every 24 h in order to avoid the accumulation of faecal matter and excess feed. The concentration of ZnO NPs was renewed daily to maintain a constant concentration of the ZnO NPs after removal of the same volume of water. During the experimental period, toxic symptoms such as stress, movement, respiration, swimming, and responses to the external effects were also recorded.

Sample collection: Upon completion of the stipulated exposure period of 5, 10, and 15 days, 15 fish were randomly selected from control and ZnO NPs treated glass aquarium and sacrificed for assay. Blood was collected from control and ZnO NPs treated fish by cardiac puncture and the whole blood was used for the estimation of Hb, RBC, and WBC count. The remaining blood samples were centrifuged for 20 min at 10,000 rpm and plasma was separated for the estimation of glucose and protein. Fish were cut open, and gill and liver were removed and stored in respective plastic vials for the estimation of GOT and GPT activities. After removal of fish at various intervals of time, the volume of the control and ZnO NPs treated glass aquarium were adjusted to maintain a constant density of fish per unit volume of water.

Determination of hematological parameters: RBC and WBC were counted by haemocytometer following the method of Rusia and Sood (1992). Hb concentrations were estimated by Cyanmethaemoglobin method and Hct was determined by micro-Hct method of Nelson and Morris (1989). Erythrocyte indices, such as MCV, MCH, and MCHC, were also calculated according to standard formulas.

Determination of biochemical parameters: Plasma glucose was estimated by *O*-Toluidine method (Cooper and McDaniel, 1970) and plasma protein was done according to Lowry et al. (1951). The gill and liver were isolated from the control and ZnO NPs exposed fish and 100 mg of each tissue were weighed and homogenized with 2.5 ml of 0.25 M sucrose solution in ice-cold condition (Hogeboom et al., 1948). The homogenates were centrifuged for 20 minutes at 6000 rpm and the clear supernatant fluid was taken for the estimation of GOT and GPT activity according to 2,4-DNPH method (Reitman and Franckel, 1957).

Statistics: The results are given as means ± SEM. All data from different treatments were compared by a one-way analysis of variance (ANOVA) and statistically different treatments were identified by DMRT test ($P<0.01$ and $P<0.05$).

Results

In the present study, Hb in *C. catla* treated with 1 mg/L concentration of ZnO NPs was increased at the 5, 10, and 15th days compared to the control group (Fig. 2). However, in 5 and 25 mg/L Hb level was found to be decreased at the 10 and 15th days. The significant decrease in hemoglobin level was directly proportional to the exposure period. The Hct level was found to be increased by 1 mg/L treatment throughout the study period. In contrast to the above, the Hct level was significantly decreased ($P<0.01$) in 5 mg/L compared to that of the control group (Fig. 3). In 25 mg/L treatment a significant increase was noted on day 10, whereas on the 5 and 10th day the Hct level was decreased (Fig. 3).

RBC count was decreased in all concentrations of ZnO NPs treated fish throughout the exposure period (except in 25 mg/L at the end of the 5th day) (Fig. 4). A significant ($P<0.01$) difference was noticed in the RBC count among the concentrations, periods, and their interactions. A significant increase in WBC count was noticed in fish exposed to different concentrations of ZnO NPs compared to the control group. A maximum increase of 39.21 was noted in 1 mg/L at the end of the 10th day (Fig. 5).

MCV value was increased throughout the exposure period in *C. catla* treated with different concentrations of ZnO NPs (except in 25 and 5 mg/L at the end of 5 and 10th days, respectively) (Fig. 6). A maximum increase of 98.50 was noted in 1 mg/L at the end of the

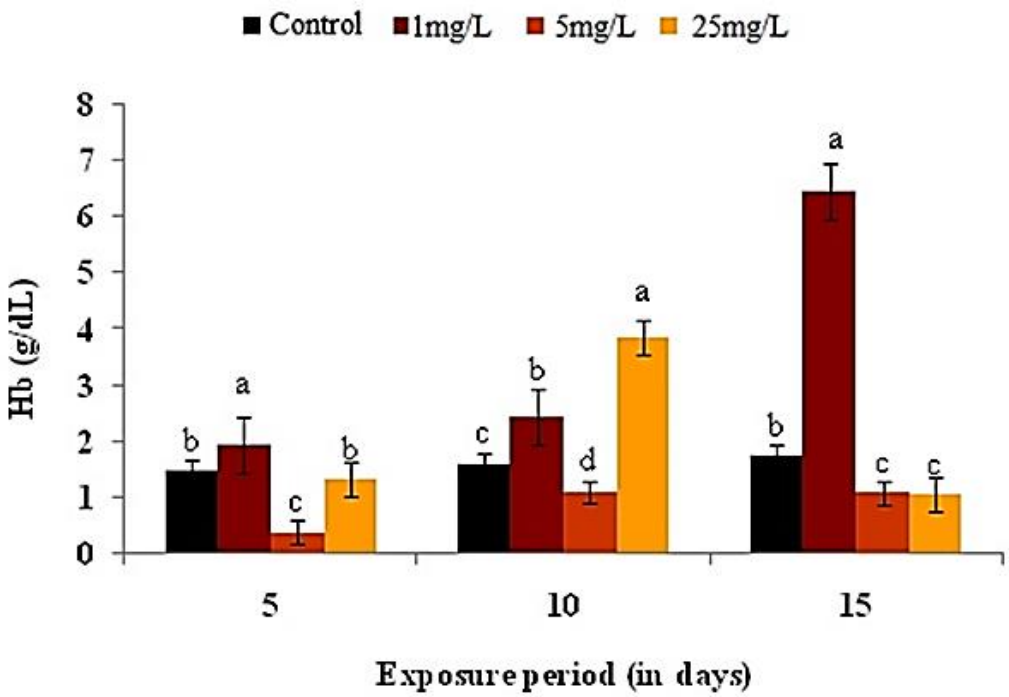

Figure 2. Hb content in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

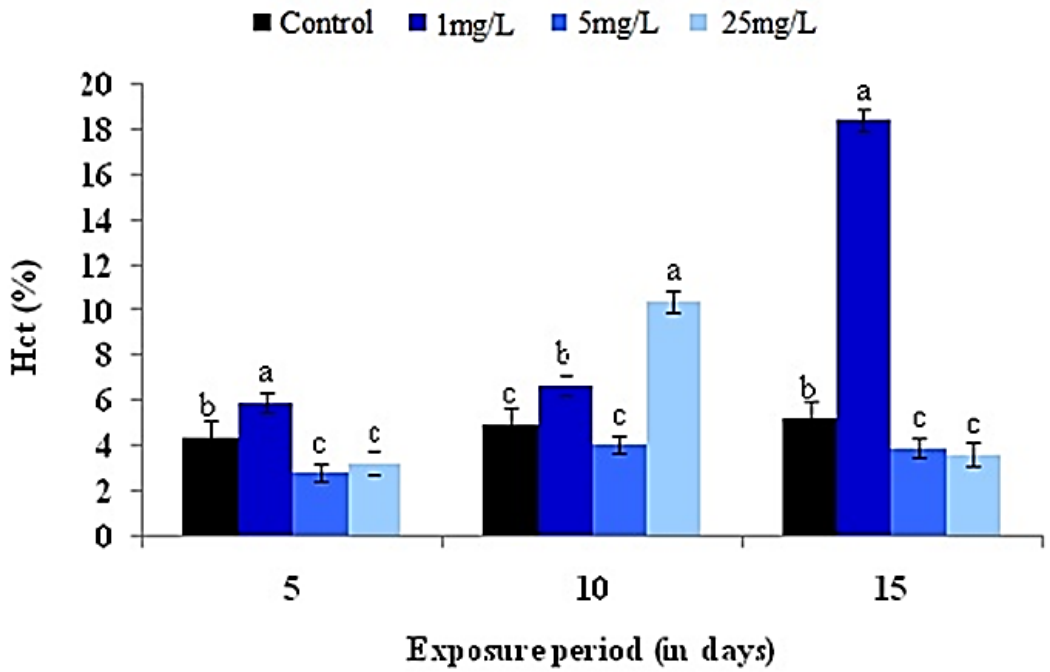

Figure 3. Hct values in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

15th day. The MCH value was found to be increased in 1 mg/L exposed fish throughout the study period. However, the MCH value was increased in fish exposed to 5 and 25 mg/L ZnO NPs on 15 and 10 days. During the study, a maximum increase was noticed in 1 mg/L at the end of the 15th day (Fig. 7). In 1 mg/L treated fish, MCHC was found to be more or less similar to the control group. However, in 5 and 25 mg/L, MCHC was increased and a maximum increase was observed in 25 mg/L at the end of the study period

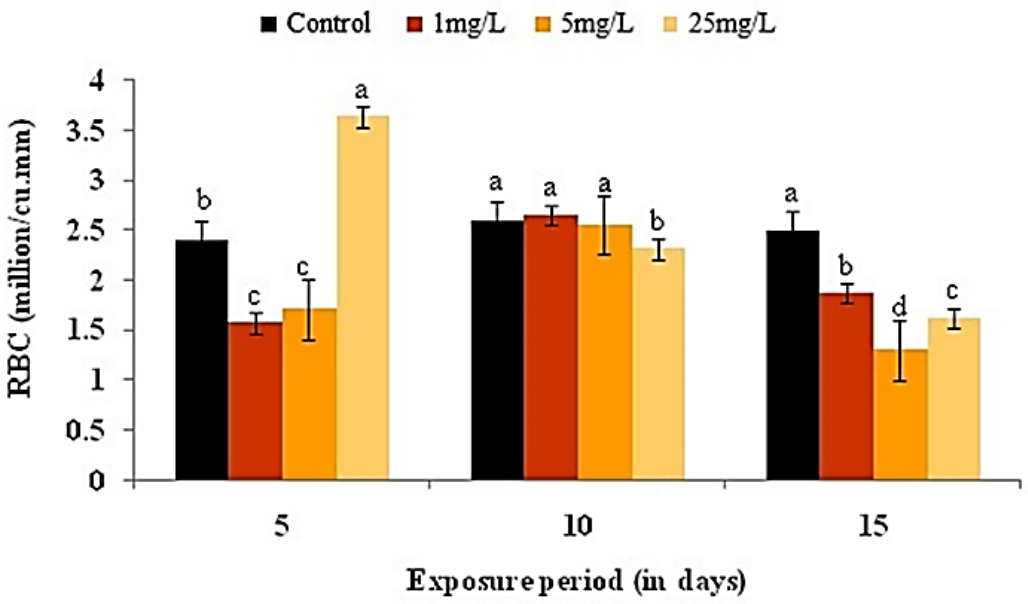

Figure 4. RBC count in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

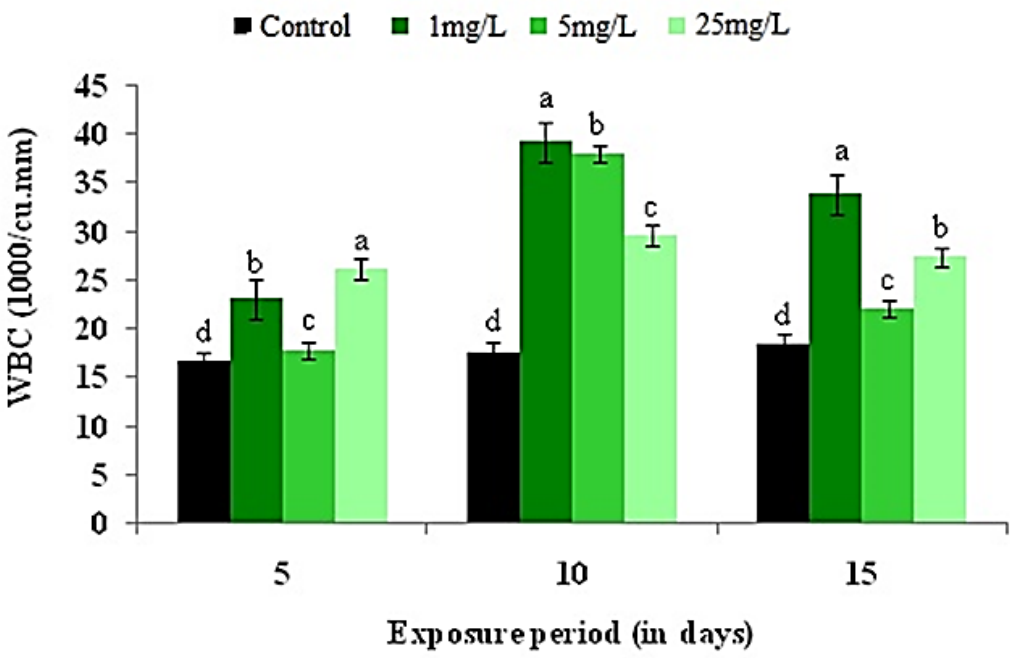

Figure 5. WBC count in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

(Fig. 8).

The plasma glucose level was elevated at all concentrations of ZnO NPs at the end of the 5th and 10th days. However, a significant decrease in plasma glucose level was noticed on day 15 when compared with their respective controls (Fig. 9). In which, the maximum increase in plasma glucose was observed in fish exposed to 25mg/L of ZnO NPs at the end of the 5th day. Plasma protein level was increased in *C. catla* exposed to all concentrations of ZnO NPs exposures on day 5 (Fig. 10). Similarly, in 5 and 25 mg/L also plasma protein was increased at the end of the 10th and 15th day, whereas in the remaining days, a significant decrease was noticed. A maximum increase of 2.01 was noted in fish exposed to 5 mg/L at the end of the 5th day.

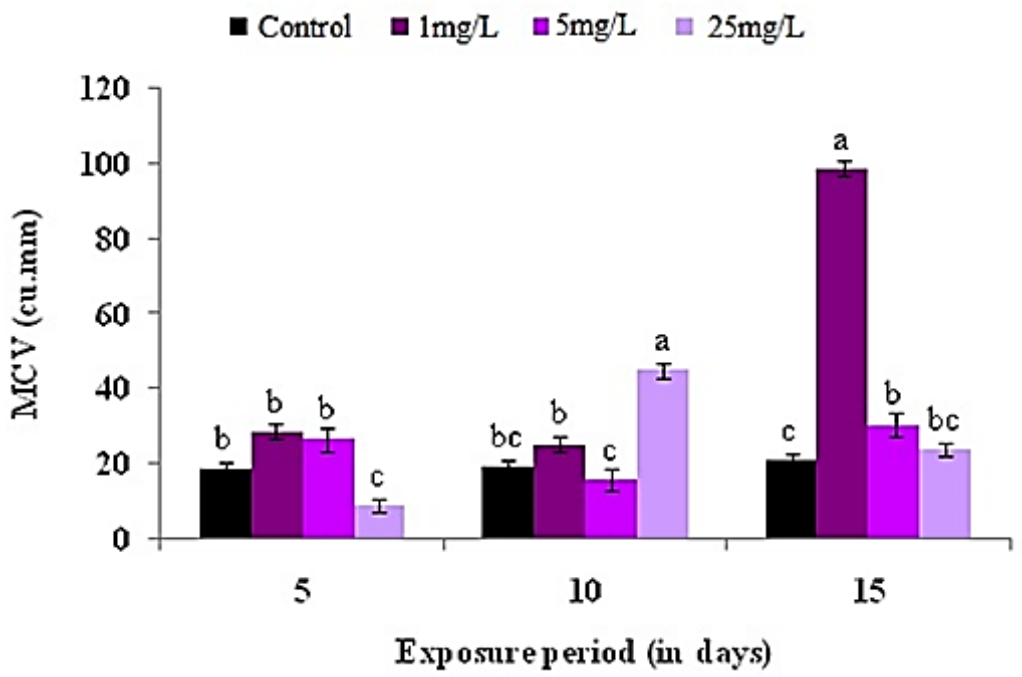

Figure 6. MCV values in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

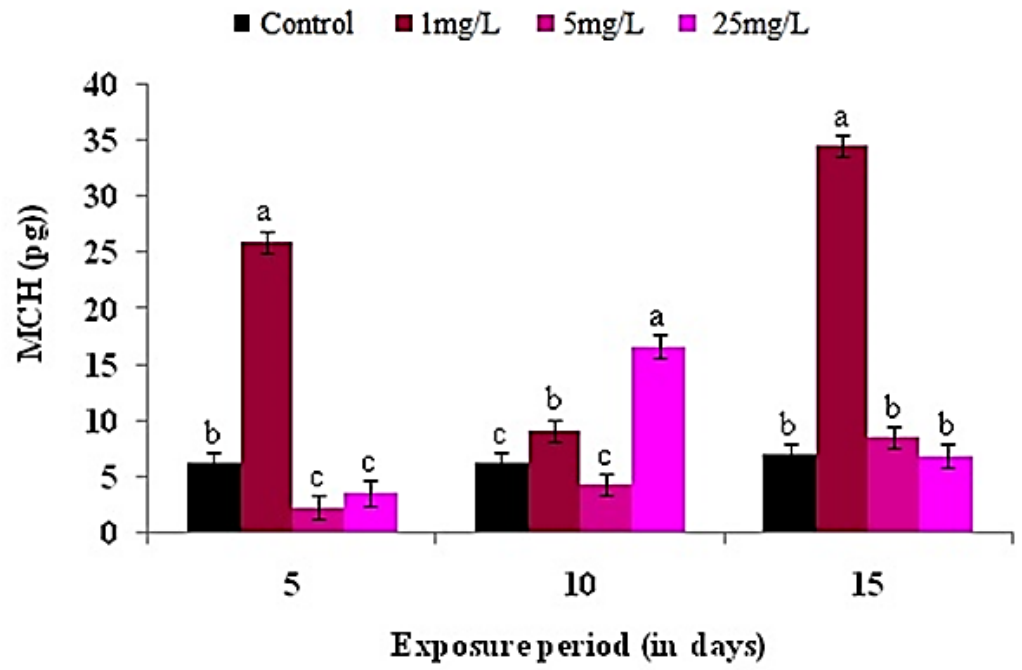

Figure 7. MCH values in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

Alterations in the GOT activity in the gill and liver of the fish *C. catla* exposed to various concentrations (1, 5, and 25 mg/L) of ZnO NPs for 15 days (Fig. 11). The GOT activity was increased in the gills and liver except in 1 mg/L at the end of the 10th day. A maximum increase of 53.72 and 77.14 was noticed in the gill and liver of fish exposed to 25mg/L. In gill, GPT activity was elevated in all concentrations of ZnO NPs exposed fish at the end of the 5th day when compared with the control groups, whereas a mixed trend was noticed in the remaining concentrations and days. The GPT was increased in the liver

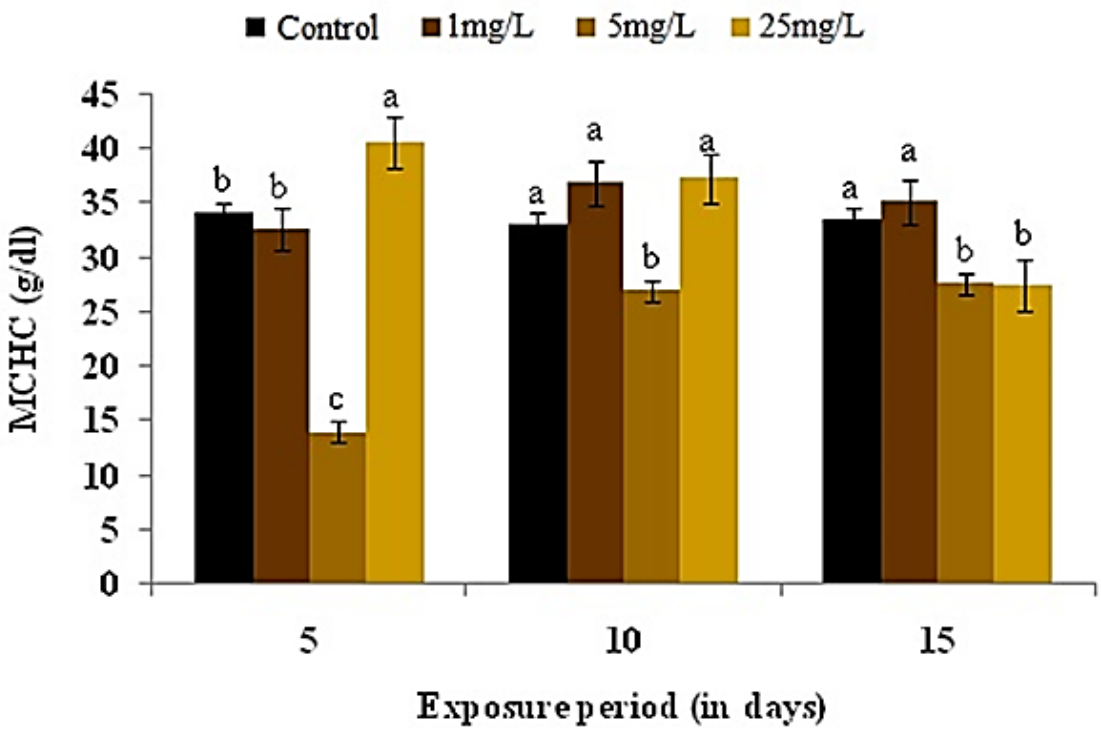

Figure 8. MCHC values in *Catla catla* treated with different concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

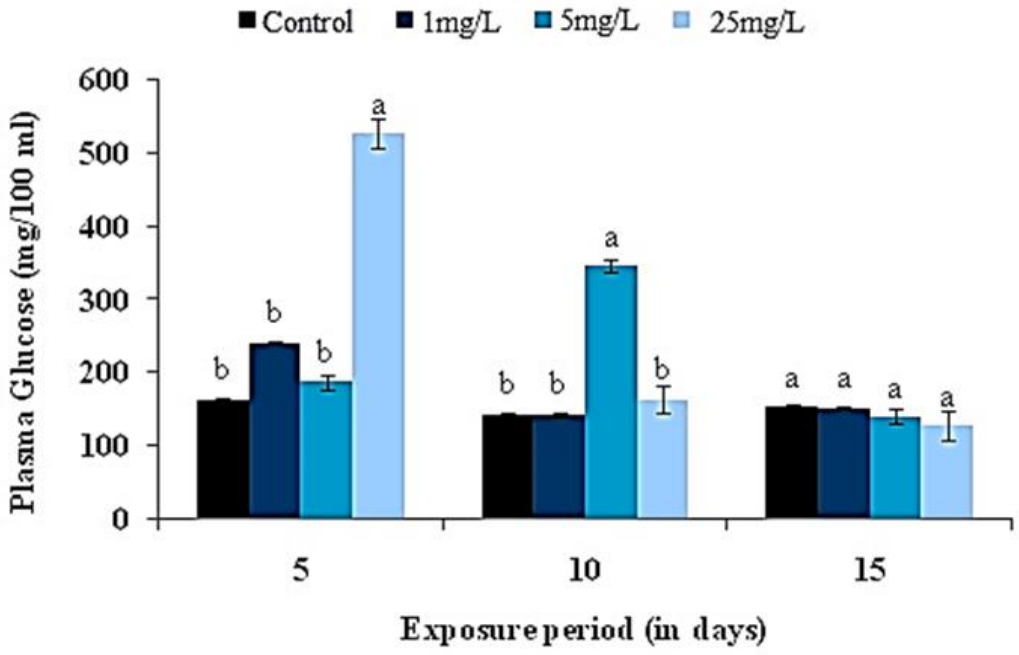

Figure 9. Plasma glucose level in *Catla catla* treated with nominal concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

of *C. catla* exposed to ZnO NPs (Fig. 12).

A significant (P<0.01) difference in Hb, Hct, RBC, WBC, MCV, MCH, MCHC, glucose, protein, GOT, and GPT values were observed among the concentrations, days, and their interactions between concentrations and days.

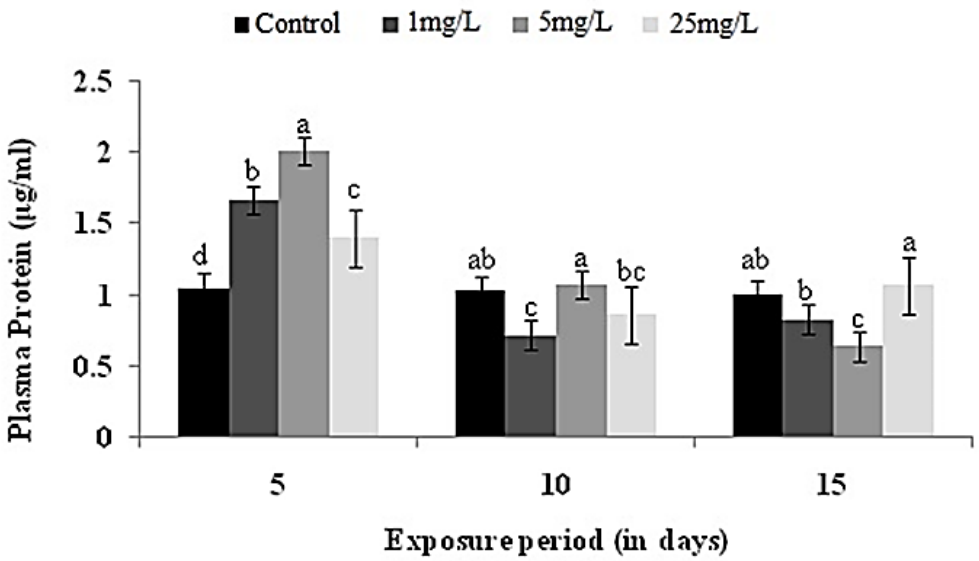

Figure 10. Plasma protein level in *Catla catla* treated with nominal concentrations of ZnO NPs (1, 5 and 25 mg L-*l*). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

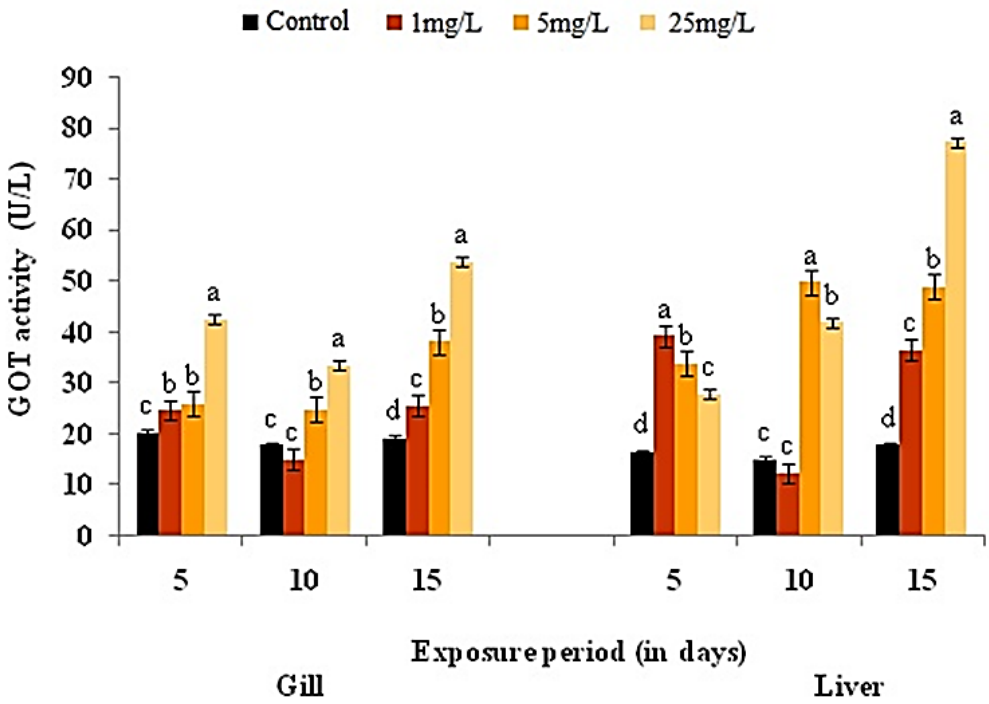

Figure 11. GOT activity in gill and liver of *Catla catla* treated with nominal concentrations of ZnO NPs (1, 5 and 25 mg L^{-l}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

Discussions

The toxicity of various NPs to aquatic organisms (from bacteria to fish) underlined the potential risk of NP exposure to aquatic life in the water column and sediment compartments (Blaise et al., 2008). The fate of NPs in the water bodies is influenced by the surface coating changes, diffusion, and homo– and hetero-aggregation (Rocha et al., 2017). Furthermore, they may exist as stable colloidal suspensions of single particles or become agglomerated, aggregated, or fused (Nowack and Bucheli, 2007), may affect their interactions with biological systems (Braz-Mota et al., 2018).

In the present study, a considerable level of

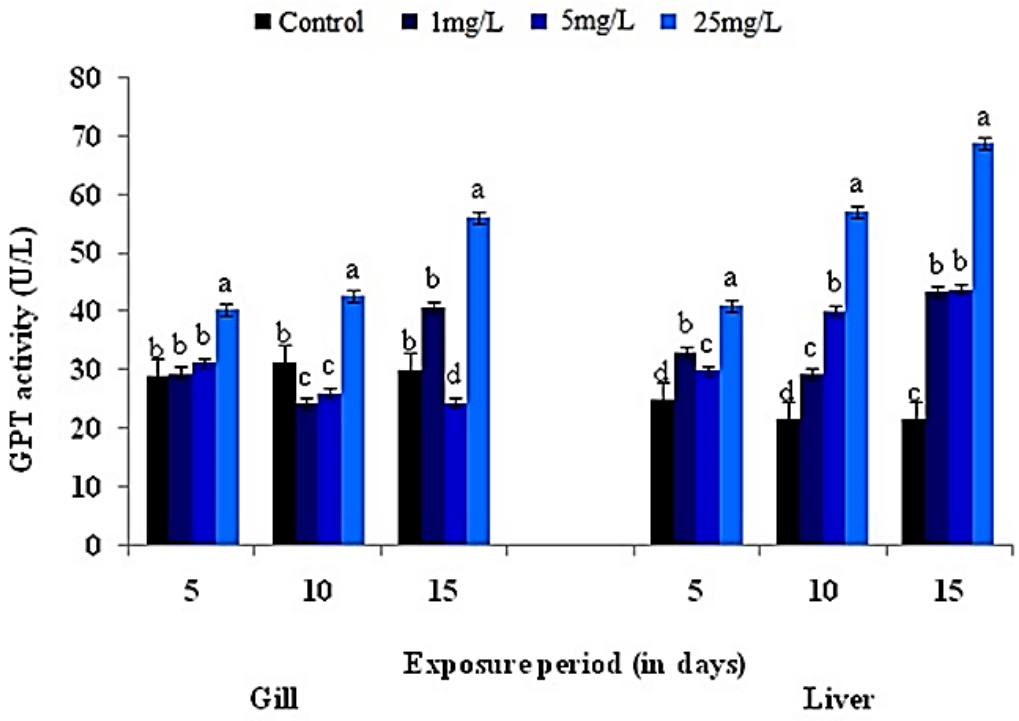

Figure 12. GPT activity in gill and liver of *Catla catla* treated with nominal concentrations of ZnO NPs (1, 5 and 25 mg L^{-1}). Means in the bars followed by common letters for the experiment are not significantly different (P<0.05) according to DMRT.

mortality was observed at 1, 5, and 25 mg/L of ZnO NPs at the end of 15 days. Also, the fish showed some abnormal behavioural responses, such as decreased passive movements at the end of the experiment. Similar behavioural changes such as fast and altered movement, mucus from body parts, and hyperactivation were observed in *Pangasianodon hypophthalmu* exposed to zinc nanoparticles (Kumar et al., 2020). Likewise, inactivity and escaping behaviors were observed in fish *Oreochromis niloticus* treated with ZnNPs, which indicates the physiological and toxicological disturbances of ZnNPs on fish (Kaya et al., 2017).

The toxicity of ZnO NPs may be due to the number and size of the aggregates in the solution (Fernández et al., 2013). They also reported that the ZnO-NPs were more toxic than bulk ZnO and Zn^{2+}ions. Similar to ZnO-NPs, Ag-NPs and Cu-NPs were also found to be more toxic than the metal salt, particularly in the early life stages of organisms due to the crossing of the chorion (Shaw and Handy, 2011). It is often expected that the smaller size, inherent properties, and surface chemistry of the compounds cause stronger toxicity (Ali et al., 2011; Alkaladi et al., 2020). Furthermore, NPs can cross the cell membrane and may accumulate in the cell organelles (Srikanth et al., 2016). Uptake of particles of different sizes via the gastrointestinal tract can also lead to different toxicological effects (Böckmann et al., 2000). Moreover, cell organelles such as the endoplasmic reticulum, lysosomes, and Golgi apparatus may also play a major role in digesting and neutralizing the nanoparticles and their effects (Fernández et al., 2013).

In the present study, the observed mortality of fish exposed to 1, 5, and 25 mg/L of ZnO NPs might have resulted from the size and entry of these particles through chorion. The amount of penetration of NPs is related to organ, age, and other agents (Medina et al., 2009). Solubilization of ionic zinc or dissolved Zn^{2+} and particle-induced generation of reactive oxygen species may be the important mechanism for the toxicity of nano-ZnO (Zhao et al., 2013; Li et al., 2013; Ma et al., 2013). Furthermore, ZnNPs in an aquatic environment are likely to dissolve into free Zn ions (Zn^{2+}) that are toxic to most of the cells (Plum et

al., 2010; Ates et al., 2013; Abdel-Halim et al., 2020). However, cellular oxidative stress response may be the main toxic mechanism for the toxicity of nano-ZnO (Hao et al., 2013). Nanoparticles may penetrate the mucous layer and bind with mucoproteins due to their surface charge and electrostatic properties, thus becoming entrapped (Handy et al., 2008). In the present study also, excessive mucous production was noticed upon exposure to various concentrations of ZnO NPs. These nanoZnO particles are easily bio-accumulated by aquatic organisms, wherein they elicit toxic effects (Zhao et al., 2013; Kaya et al., 2016). Nanoparticles may induce oxidative stress which may be responsible for DNA damage, membrane disruption, and cell death (Li et al., 2008; Gagné et al., 2012; Morcillo et al., 2016).

Analysis of blood parameters has been extensively used as an important tool for determining the physiological and pathological conditions of the fish. ZnO NPs caused hematological disturbances in this study. Notably, ZnO-NPs at the end of the experiment showed a statistically significant decrease in Hct and Hb contents. Similar to our findings, Smith et al. (2007) found a significant decrease in the Hct and Hb in rainbow trout exposed to carbon nanotubes. In our study, the significant decrease of Hb and Hct might have resulted from the interference of the hemometabolism or disturbance in fluid volume balance produced by ZnO NPs. It has been reported that the circulatory system plays an important role in the distribution of nanoparticles in the fish body (Jovanović and Palić, 2012). Furthermore, the accumulation of ZnO NPs in the gill may cause structural damage and hemolysis which leads to a decrease in RBC count. The significant increase in Hb level and RBC count in the present investigation might have resulted from a hypoxic condition due to ZnO NPs stress. Increases in Hct values were associated with osmotic shifts; as the pH of the blood decreased, RBC swelled and plasma volume decreased. A significant increase in RBC, Hb, and Hct has also been observed in African catfish fed with ZnO nanoparticles as a feed supplement (Onuegbu et al., 2018).

WBCs are involved in the control of immunological function and the changes in WBC counts after exposure to various toxicants may indicate a decrease in nonspecific immunity of the fish (Saravanan et al., 2011). Increase in WBC count in fishes exposed to chronic and lethal doses indicates leucocytosis. Stimulation of lymphopoiesis and/or enhanced release of lymphocytes from lymphomyeloid tissue under toxic stress may lead to an increase in WBC number (El-Sayed et al., 2007; Alkaladi et al., 2015; Umamaheswari et al., 2019). Furthermore, a significant increase of WBC count may be due to an adaptation to meet the stressful condition of the fish. The removal of cell debris due to tissue damage may be another reason for the increased level of WBC count. In the present investigation, the significant increase in WBC might have resulted from stimulation of the immune system and to protect the fish against ZnO NPs toxicity.

In the present study, the significant increase of MCV and MCH indicates the swelling of red blood cells or an increase in the erythrocyte volume due to the toxic stress of ZnO NPs. Whereas the significant decrease in MCV and MCH may be due to compensating for impaired oxygen uptake due to gill damage caused by ZnO NPs as suggested by Ghalazy (1992). Suganthi et al. (2019) reported that the decrease in Hb and MCV value in ZnO NPs exposed *O. mossambicus* may be due to hypoxic conditions caused by ZnO NPs which results in shrunken or ruptured erythrocytes. The observed low concentration of MCHC during this study period might have resulted from a decrease in Hb synthesis due to the toxic action of NPs. However, no significant alterations in hematological parameters were observed in *Cyprinus carpio* exposed to zinc oxide nanoparticles (Chupani et al., 2018). In general, the alterations of hematological parameters indicate a generalized immune response to the toxicant (Ramesh et al., 2018).

Environmental contaminants such as nanoparticles in aquatic media alter the physiological status of aquatic organisms which results in significant changes in biochemical parameters. The hatching rate of

M. chulae was decreased when exposed to 25 mg/L of ZnO NPs which may be due to spinal bending caused by nano-ZnO exposure (Li et al., 2018). ZnO NPs at low concentration may cause damage to the connective tissues of *O. mossambicus*, which results in alterations in haemato-immunological parameters (Suganthi et al., 2019). Likewise, cytotoxic effects and changes in the glutathione S-transferase activity and total glutathione content have been observed in fish cell lines (RTG-2, RTH-149, and RTL-W1) exposed to ZnO NPs (Fernández et al., 2013).

Since plasma glucose is regulated by complex interactions of hormones such as glucagons and cortisol (Agrahari et al., 2007), the changes in blood glucose level due to stress showing both a rise (Li et al., 2011; Ramesh et al., 2014) and a fall (El-Sayed et al., 2007; Lavanya et al., 2011; Umamehaswari et al., 2019). Elevation of the blood glucose level, induced by corticoids and catecholamines, is a well-known secondary response to stress in fish (Vuorinen et al., 2003). It has been reported that the increased blood glucose is usually observed in fish under undesirable conditions and it helps the animal by providing energy substrates to vital organs to cope with the increased energy demand (Banaee et al., 2008; Saravanan et al., 2011). However, Min and Kang (2008) suggested that increased plasma glucose levels may be a response to respiratory insufficiency due to stress. In this study, plasma glucose was significantly higher in fish exposed to different concentrations of ZnO NPs which may be due to the high utilization of glucose to meet the metabolic demands caused by ZnO NPs. The increase in serum glucose level in *O. niloticus* exposed to zinc nanoparticles is an indicator of depletion of glycogen reserves and glycolysis capacity as a result of a decrease in certain enzymes under stressful conditions (Kaya et al., 2017).

Fish under stress may also mobilize protein to meet the energy requirements needed to sustain increased physiological activity (Martinez et al., 2004). The chronic exposure of various concentrations of ZnO NPs may cause hepatocellular damage (because of NPs are usually concentrated in the liver (Ali et al., 2011) which leads to an increase in the plasma protein level. Also, it indicates physiological adaption to overcome stress induced by the ZnO NPs. The decrease in plasma protein may be due to kidney disorder (albuminuria), liver cirrhosis, or nephrosis or might be due to alteration in enzymatic activity involved in protein biosynthesis (Lavanya et al., 2011). A significant decrease in serum total protein level in *O. mossambicus* exposed to ZnO nanoparticles may be due to hypoalbuminemia (Alkaladi et al., 2015). However, no significant change in protein level was observed in *C. carpio* fed with Zn nanoparticles (Banaee et al., 2019a). In the present study, the observed decline in protein level during 1 and 25 mg/L at the end of the 10^{th} day and 1 and 5 mg/L at 15^{th}-day exposure to ZnO NPs treatments might have resulted in liver cirrhosis due to the influence of ZnO NPs. The impacts of ZnO NPs on the biochemical parameters of fish can help to understand the mechanism and mode of action of ZnO NPs.

Fish respond to environmental pollutants by altering and adapting their metabolic functions. The gill and liver are sensitive indicators of aquatic pollution, and alterations in their enzyme levels may be used in the evaluation of the health of fish (Pacheco and Santos, 2002). Transaminases play an important role in protein and carbohydrate metabolism, and any change in these metabolisms may cause significant alterations in GOT and GPT activities. Hence, any alterations in AST and ALT activities of fish may serve as suitable biomarkers in the detection of tissue and organ damage (Venkateswara Rao, 2006; Ramesh et al., 2018). In recent years, evidence is now emerging that some nanometals can affect the gill in similar ways to dissolved metals (Shaw and Handy, 2011). These are the most target organs for the accumulation of xenobiotics and cause pathological alterations in fish. ZnO NPs are easily bioaccumulated in aquatic organisms and cause adverse effects on the physiology of the organisms (Onuegbu et al., 2018). In addition to this, Amutha and Subramanian (2009) reported that GOT and GPT activity was significantly increased in the gill and liver of *O. mossambicus* exposed to ZnO nanoparticles. Abdel-Halim et al.

(2020) reported an increase in AST and ALT activities in snails treated with zinc nanoparticles. Hao et al. (2013) stated that the liver and gill might be the target organ with exposure to ZnONPs.

In the present study, the significant increase in GOT and GPT activity in the gill and liver of *C. catla* indicates that the organism tries to mitigate the stress by the increased rate of metabolism. The enzyme GOT is present in all animal tissues and plays a vital role in gluconeogenesis in hepatocytes (Ellinger et al., 2011) and the synthesis of neurotransmitters and the neuroglial pathway in the nervous system (Wang and Chen, 2018). Banaee et al. (2019b) showed that increased activity of GOT activity in fish *C. carpio* exposed to cadmium and microplastic particles might indicate the oxidative stress and tissue damage caused by the toxicants. The increase in GPT activity could be an indicator of increased activity of this enzyme in the blood due to injury of the cell membrane (Banaee et al., 2019b, c) which may affect transamination reaction, and the biosynthesis of non-essential amino acids (Banaee et al., 2020).

Significant elevation of AST and ALT activities in *P. hypophthalmu* (Kumar et al., 2020), in common carp, exposed to ZnNPs (Lee et al., 2014) and in tilapia (Younis et al., 2012) exposed to zinc nanoparticles indicates the bioaccumulation of zinc and the tissue injury caused by zinc particles. Similarly, Farkas et al. (2004) reported that the elevation of ALT and AST might be due to significant damage to muscle, intestinal and hepatic injury. The decreased activities of GOT and GPT indicate a disturbance in the structure and integrity of cell organelles (Saravanan et al., 2011). The inhibition of Na^+/ K^+-ATPase activity, oxidative stress, respiratory disturbances, and organ pathology due to the impact of nanomaterials in fish indicates that nanometals may interfere with, and/or stimulate stress responses in fish (Shaw and Handy, 2011). In general, the alteration of these parameters could be caused due to zinc nanoparticles induced production of reactive oxygen species (ROS) and oxidative stress (Chen et al., 2014; Onuegbu et al., 2018) and also direct interaction with biological targets (Ma and Diamond, 2013).

Conclusion

Our results show that all parameters measured in this study were significantly affected by the different concentrations of ZnO NPs in the Indian major carp *C. catla.* The toxicity observed in this study may be attributed to the combined effect of both Zn^{2+} and ZnO NPs. Furthermore, ZnO NPs may induce oxidative stress resulting in alterations in the physiological responses of fish. Moreover, the accumulation of ZnO NPs in the gill and liver may affect the structure and function, resulting in a change in AST and ALT activity. From the results, it is concluded that the ZnO NPs at the tested level can be potentially harmful to fish and other aquatic organisms, and alterations of these parameters may be taken as potential biomarkers in the field of (nano) toxicology. Furthermore, the long-term effects of ZnO NPs on these parameters along with other parameters, need to be investigated in future studies.

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