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Antioxidant response and serum lipid changes of *Labeo rohita* exposed to intra-peritoneal titanium dioxide nanoparticles

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Abstract
The present study was aimed to examine the toxicological effect of two sub-lethal doses of titanium dioxide nanoparticle (TiO2 NPs) injection on *Labeo rohita*. 1/50th and 1/10 th of 96 hour LD_{50} were selected as the lower and higher sub-lethal doses respectively. The exposure was for 21 days. At the end of the experiment, fishes were collected randomly, blood samples were collected, and then fishes were dissected out to collect ovary and liver. The enzymatic antioxidant activity of superoxide dismutase (SOD) and catalase (CAT) were estimated from homogenates of ovary and liver. Serum biomarkers such as cholesterol and triglycerides (TAG) were also found out. The 96hr LD_{50} value estimated was 528.3±0.002µg Kg^{-1}. The two -sub-lethal doses selected for exposure were 10.6 (lower) and 52.8 (higher) µg Kg^{-1} of TiO_2 NPs. Among the serum biomarkers, cholesterol showed a non-significant increase and TAG did not show any significant difference from the control. Hepatic SOD and CAT activities were significantly elevated with concentrations of TiO_2 NPs. No significant differences were noticed in the ovarian SOD activity. Histopathological architecture did not alter with titanium dioxide nanoparticle injection.

Keywords: TiO_2 NPs, LD_{50}, oxidative stress, *Labeo rohita*

Introduction
Titanium dioxide nanoparticles are extensively used in several industrial products, and biological fields due to their unique physicochemical properties. The entry of this nanoparticle to the ecosystem, especially the aquatic system causes severe threats to aquatic biota. Because of their small size, it can enter the body and can deposit in various tissues and create numerous health issues. Potential for photocatalytic activity and UV absorption is higher for nano-sized [1]. These properties led titanium dioxide nanoparticles (TiO_2 NPs) to a wide range of industrial applications and consumer products such as water treatment agents, self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting equipment, sunscreens and other cosmetics [2]. Eventually these nanoparticles get into the environment and finally through run-offs to aquatic systems. Aquatic environment receives substantial amounts of environmental pollutants that can be taken up by aquatic organisms [3]. Bulk form of titanium dioxide is relatively safe to mammals [4] and has been used as an inert marker in the nutritional studies of fishes [5]. Information regarding the sub-lethal toxicity of this nanoparticle in fishes is still lacking [6]. On exposure of upto 1 mgL^{-1} TiO_2 NPs by rainbow trout reported gill epithelial damage and oxidative stress [7]. The exposure of an organism to harmful stimuli enhances the synthesis of reactive oxygen species (ROS) and reactive nitrogen free radicals, which leads to oxidative stress [8]. Excess ROS can damage the antioxidant system of the body and lead to toxicity [9]. It is necessary to remove the ROS and free radicals for maintaining the normal physiological balance of the body [10]. The enzymatic and non-enzymatic antioxidant system of the biological system can neutralise the free radicals. Most important enzymatic antioxidants include Superoxide Dismutase (SOD) and catalase (CAT) [11].
Several studies reported TiO_2 NPs can deposit in the ovaries and can damage the ovarian cells and disturb the steroid hormone profile, which can affect the reproduction. Intragastric administration of 2.5, 5 and 10 mg/kg to female mice for 90 consecutive days reported a significant reduction in whole body weight, relative weight of ovary and the fertility [12].

Hou *et al*., 2009 noticed administration of TiO_2 nanoparticle inhibited follicle development and oocyte maturation in rat in-vitro [13].

Lipids are one among the vital energy store (triglycerides) and they also serve as the major components of cell membrane (cholesterol and phospholipids) [14]. Lipids functions as messengers in signal transduction pathways and molecular recognition process. So, any changes in lipid metabolism would results in impairment of these crucial pathways. Liver dysfunction and disturbance of lipid metabolism also favours elevation in these components.

The objective of the present study is to study the sub-lethal toxicological effects of TiO_2 NPs in maturing *Labeo rohita* by assessing serum biomarkers and enzymatic antioxidants. Ovarian disruptive effect of the same was determined in terms of alterations in histopathology.

Materials and Methods

Animal Care

Stunted female rohu of (n= 45, Mean body weight = 350 ± 6.3g) were purchased from Jayanti fish farm of Bhalabhadrapuram, Andhra Pradesh. Fishes were then acclimatised in the cement ponds of the ICAR-CIFE centre of Bhalabhadrapuram for 10 days. Same time fishes were fed with pelleted feeds of crude protein 32% and lipid 6%.

TiO_2 NPs Suspension Preparation

Titanium (IV) oxide, anatase nano powder with particle size <25 nm was purchased from Sigma-Aldrich. Stock solution of TiO_2 NPs was prepared by dispersing nanoparticle in PBS and performed ultrasonication for 20 minutes (50-60 KHz).

Determination of Acute Toxicity

Dilutions of stock solutions were prepared in PBS to calculate the 96 hr LD_{50}. Test suspensions after sonication were injected intraperitoneally to fishes. Six fishes were randomly exposed to each concentration for 96 hr in 2000 L container. The control group was injected with PBS free of nanoparticles. Each treatment was carried out in duplicate. In order to maintain water quality, fishes were not fed on the day before or during the experimental period to minimize the production of faeces, which deteriotes water quality. Mortalities were recorded intermittently and dead fishes were removed immediately. The 96 hr LD_{50} value was calculated by probit analysis.

TiO_2 NPs Exposure

There were three sets of homogenous treatment groups which were selected randomly. Weighed fishes were then transferred to cement tanks of 10,000 L (pH 7.8 and temperature 27^0C) capacity for initiating the experiment. Dosage of intraperitoneal injection of TiO_2 NPs was decided based on the 96 hr LD_{50} values. Fishes of each set were individually exposed to intraperitoneal injection of TiO_2 NPs. The three sets were respectively control (no nanoparticle exposure), 1/50 LD_{50} (low dose) and 1/10 LD_{50} (high dose). Suspensions of nanoparticle were prepared in PBS. Control group were injected only with the carrier. The exposure continued for 21 days.

Sample Collection

On completion of the experiment fishes from the cement tanks (control, 1/50 LD_{50} and 1/10 LD_{50}) were randomly selected and weighed. Fishes were anaesthetized by immersing them in 50 ppm clove oil. Blood samples were collected immediately by puncturing the caudal part and transferred the same to clean 1.5 ml eppendorf tubes. Clotted blood samples were centrifuged at 3000 g for 5 minutes (4°C) and the supernatant, serum is safely removed to other tubes for analysing the serum parameters. Fishes were then dissected and ovaries were carefully taken out. Ovaries were weighed and samples of ovaries were collected in 10% Neutral Buffered Formalin (NBF) for histological analysis. Samples of ovaries and liver were also collected in sucrose solution for antioxidant enzyme studies. Total cholesterol and triglycerides were estimated from serum using kits provided by Erba ® Diagnostic Mannheim; Transasia Bio-medicals Ltd., Solan, HP, India). Assays were done according to the protocol given by the manufacturers.

Oxidative stress enzyme

The CAT enzyme activity was assayed according to the spectrophotometric method followed by Takhara *et al*., 1960 [15], using phosphate buffer (50 mM, pH 7.0) and the reaction was started by the addition of H_2O_2 solution and the decrease in absorbance was measured at 240 nm. One unit of CAT activity was the amount of protein required to decompose H_2O_2. The SOD activity was assayed according to the method described by Miara and Frodovich, 1972 [16] based on the oxidation of epinephrine–adrenochrome by the enzyme. The activity of one unit of SOD was expressed as the amount of protein required for 50% inhibition of epinephrine auto-oxidation.

Statistical Analysis

LD_{50} values and confidence intervals were calculated using EPA Probit Analysis Program V. Results of the study were expressed as the mean ± standard error. Statistical analyses of different parameters were analyzed by using one-way analysis of variance (ANOVA) and significance of differences between control and experimental groups were assessed by Duncan multiple range test. The level of significance was accepted at 5% level ($P<0.05$). All statistical analysis was performed using SPSS, version 22, software (SPSS Inc., USA).

Results

Acute Toxicity

Along with the increasing concentration of injected nanoparticle and time the mortality rate also increased. The concentration of nanoparticle at which fifty percentage of fish death occurred (acute toxicity) was estimated using probit analysis. The 96 hr LD_{50} value estimated was 528.3 ± 0.002 µg Kg^{-1}. The two sub lethal doses selected for exposure were 10.6 (lower) and 52.8 (higher) µg Kg^{-1} of TiO_2 NPs.

Serum Biomarkers

The changes in levels of serum cholesterol and triglycerides after the nanoparticle exposure are given in Fig. 1 and 2. There was a non-significant increase in the cholesterol level of the exposed groups of fishes when compared to the control (Fig. 1). In the case of triglycerides, no significant differences were observed between control and treated fishes (Fig. 2).

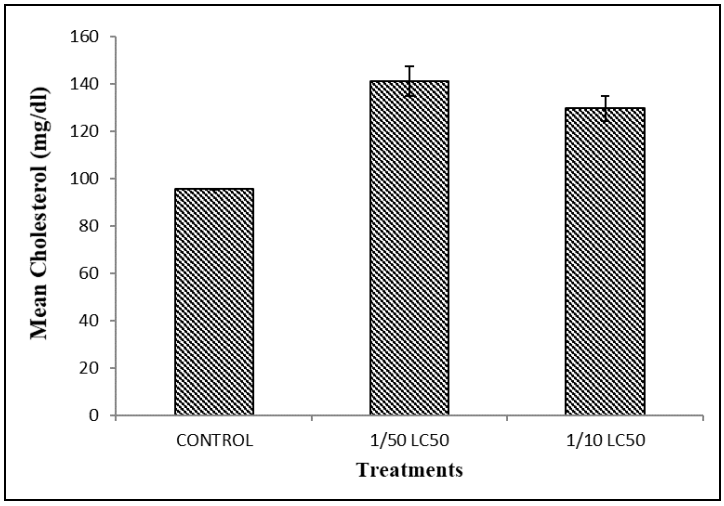


Fig 1: Mean levels of total cholesterol in the serum of *Labeo rohita* exposed to differing sub-lethal concentrations of TiO_2 NPs.

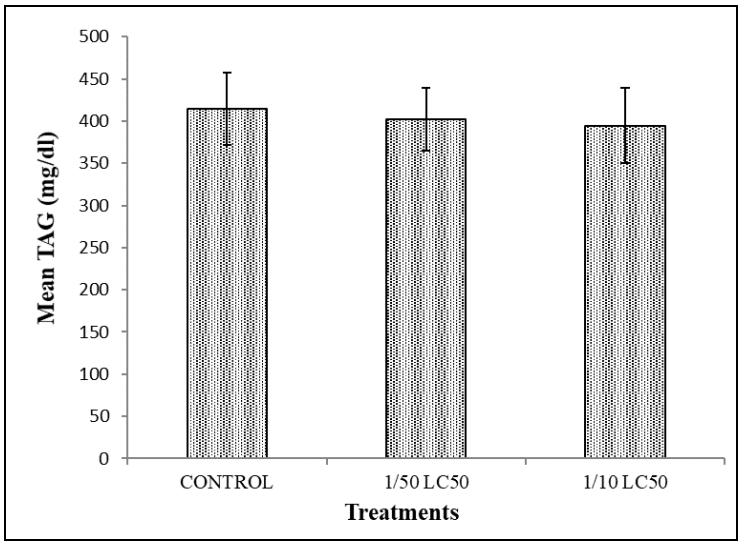


Fig 2: Mean levels of total triglycerides in the serum of *Labeo rohita* exposed to differing sub-lethal concentrations of TiO_2 NPs

Histological Analysis

The histological alterations observed in the ovary of control and nanoparticle exposed *Labeo rohita* are shown in Fig. 3, 4 and 5 respectively. The ovary of control fish depicted normal ovarian histology with oocytes in different stages of development. No histopathological changes were observed (Fig. 3). Some signs of histopathological alterations were observed in the ovary of lower sub-lethal dose exposed fish. An irregularity in the shape of maturing and mature ova were seen. Less number of pre-vitellogenic ova was found. Higher sublethal dose injected fishes also shown a little histopathological effect in the ovary. Irregularity in the shape of the ova was the change observed here also.

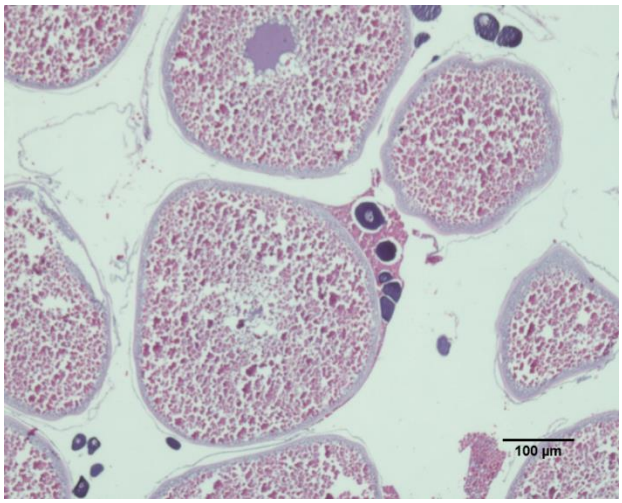
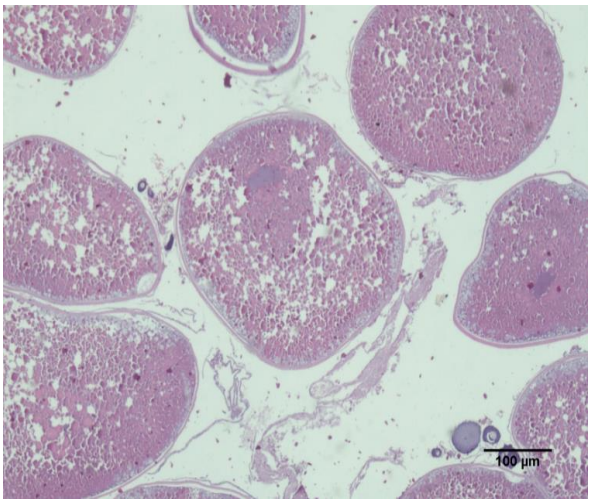


Fig 3



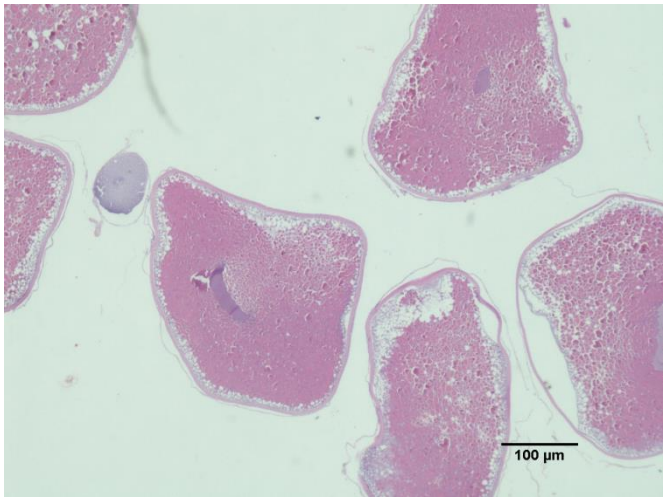


Fig 4 **Fig 5**

Fig 3-5: Ovarian morphology of *Labeo rohita* after 21 days of exposure to 0 (Fig. 3), 10.6 (Fig. 4) and 52.8 (Fig. 5) µg Kg^{-1} of TiO_2 NPs.

Oxidative Stress Markers

The SOD activity of the liver showed a significant increase along with the increase in concentration of nanoparticle (Fig. 6). Whereas the SOD activity in the ovary of injected fish did not show any significant alterations from the control (Fig. 7). Hepatic catalase activity was remarkably high after exposing the fish to higher sub-lethal dose and a slight elevation (a non-significant) was noticed in the lower sub lethal exposed rohu when compared to control fish (Fig. 8). Ovarian catalase activity was unable to estimate.

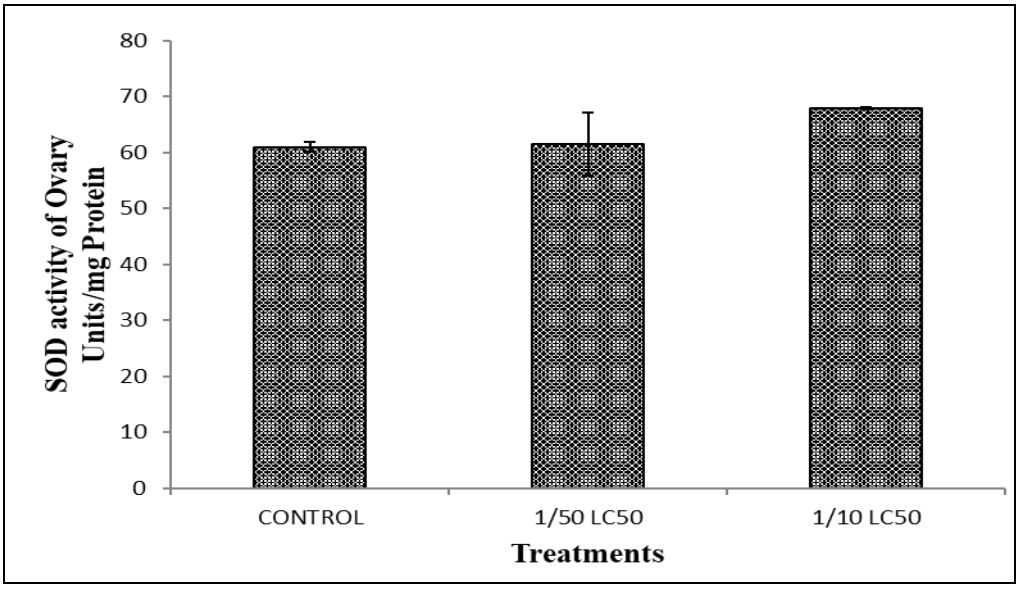


Fig 6: Mean values of ovarian SOD activity of *Labeo rohita* exposed to differing sub-lethal concentrations of TiO_2 NPs

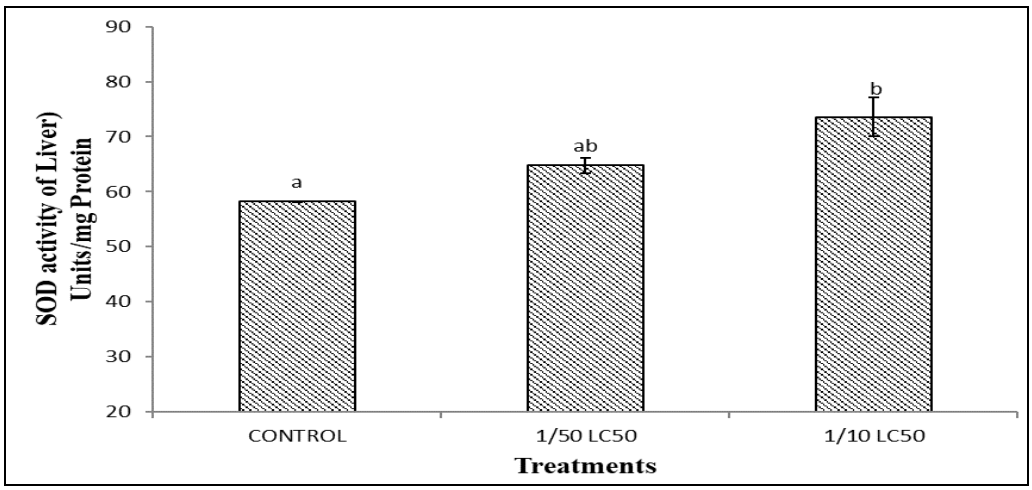


Fig 7: Mean values of liver SOD activity of *Labeo rohita* exposed to differing sub-lethal concentrations of TiO_2 NPs

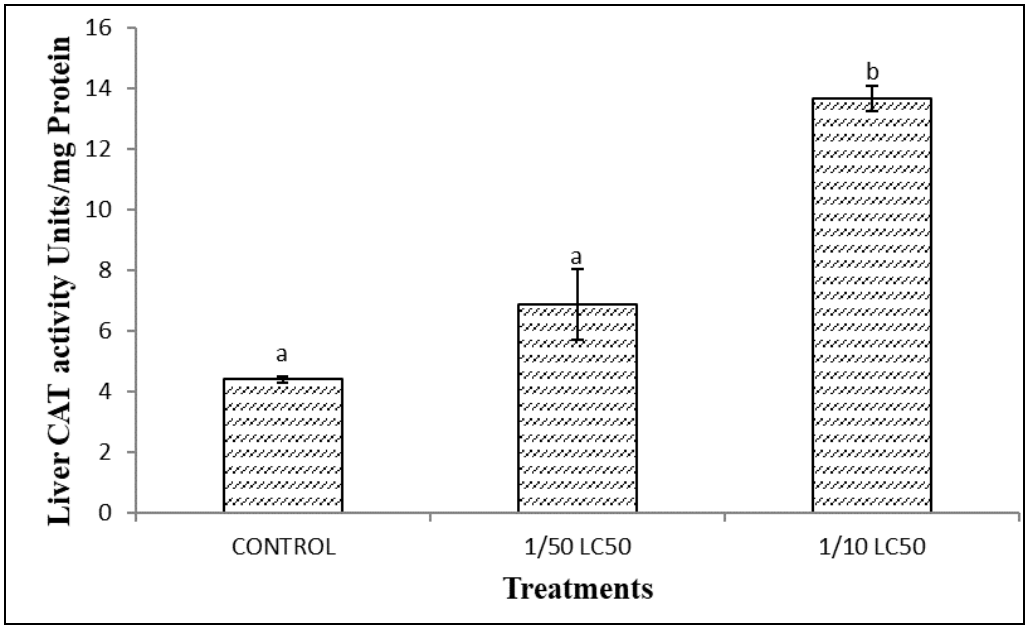


Fig 8: Mean values of hepatic catalase activity of *Labeo rohita* exposed to differing sub-lethal concentrations of TiO_2 NPs

Discussion

In toxicity studies, determination of LD_{50} or LC50 has to be done as a primary step [17]. Most acute toxicity studies were conducted on the basis of LC_{50} values. LC50 determines that particular concentration of the exposing chemical at which 50% of death occurs within a short time period, usually 96 hour [18]. Again it is determined by the factors such as age, size, and condition of the testing organism and also on the experimental conditions for the same species and toxicant [19]. The 96hr LD_{50} value for *Labeo rohita* females used in the study was 528.3 ± 0.002µg Kg^{-1}.

Serum biomarkers such as cholesterol and triglycerides are important indicators of fish health. The results of this study say TiO_2 NSs injection elevated the total serum cholesterol level non-significantly from the control. Canli *et al*., 2018 couldn't find any statistical difference in total cholesterol level in *Oreochromis niloticus* after exposing to TiO_2 NPs [20]. But Javed *et al*., 2017 reported increase in cholesterol level after exposing to heavy metal [11]. After synthesis cholesterol are transported from their site of synthesis to other tissues for ther further utilization either by process of oxidation or through gradual instauration of lipid molecules, which also elevates the cholesterol levels of serum or blood [21]. Hepatic tissue damage and any disturbance in the normal body lipid metabolism elevate serum cholesterol. Lipids are the major constituent of the plasma membrane, which maintains the fluidity of the same. Disintegration of the lipid bilayer contributes to the increase of serum lipid components, which may be another possible reason for their elevated levels. Triglycerides (TAG) depicted a non-significant change in the present study. Even though a slight reduction was there after 21 days. Stress increases energy demands and is met by mobilising triglycerides, hence saving as energy depots. Decrease in TAG can also be correlated with the use of TAG in membrane biosynthesis [22, 23]. Results of Canli *et al*., 2018 [20] is consistent with our results.

TiO_2 NPs disturbed the normal architecture of the ovarian tissue, which shows the role of the nanoparticle as an ovarian disruptor. No extreme defects were observed. Comparatively similar results were observed when zebra fish (*Danio rerio*) were exposed to 0.1 and 1.0mg/L of TiO_2 NPs for a shorter period of time. Female gonads showed a normal spread of oocyte development in all exposed groups [6]. But Wang *et al*., 2011 reported chronic exposure to 0.1 mg/L of TiO_2-NPs can significantly impair zebra fish reproduction in females, which is due to the increase in exposure time [24].

SOD and CAT can be considered as sensitive biomarkers of pollution in aquatic system [11]. SOD is the first enzyme among them to deal with oxyradicals and is responsible for catalysing the dismutation of highly superoxide anion radical $O_2^{\cdot-}$ to O_2 and H_2O_2 [25]. CAT is a marker enzyme of peroxisome which promotes the decomposition of H_2O_2 to O_2 and H_2O, thus protecting the cell from the deleterious effect of H_2O_2 [26]. In the present study, fluctuations in the SOD and CAT activity of liver and ovary as an effect of TiO_2 NPs exposure were estimated. Lower and higher sub-acute injections increased the SOD activity of liver while no significant changes were noted in the ovarian SOD activity. Similar to our results injection of 3.12 and 6.25 mg/Kg copper nanoparticles in rats showed no significant change in the SOD activity of ovary [27]. This projects the active role of liver in regulating redox metabolism. Findings of [11] supports our results, who got increasing trend for SOD and CAT in *Channa punctatus* exposed to heavy metal waste water. The increase in activity of these enzymes may be due to the synthesis of new enzymes in order to nullify the deleterious effects of different oxyradicals. But [25] reported a statistically significant reduction in SOD and CAT activity upon exposure of 100 and 200 mg/L TiO_2 NPs by juvenile *Cyprinus carpio*, suggesting the excess utilisation of the same in order to mitigate oxidative stress.

Conclusion

The present study demonstrates that the sub-lethal exposure of titanium dioxide nanoparticle can interfere with the normal physiology of the fish in or another way. The nanoparticle disturbed the normal lipid profile. Alterations in the level of cholesterol and triglycerides affect various biological processes including membrane fluidity, signal transduction etc. Enzymatic antioxidants SOD and CAT activities were increased in liver in order to mitigate the oxidative stress induced by the nanoparticle. This shows the entry of TiO_2 NPs to the water bodies pose potential risk to aquatic biota.

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