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# Genotoxicity and oxidative stress analysis in the *Catla catla* treated with ZnO NPs

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**Key words:** ZnO, Nanoparticles, genotoxicity, oxidative stress.

**Abstract**

In the history of human beings nanotechnology is one of the fastest growing industries and has been referred to as next industrial revolution. Zinc oxide nanoparticle (ZnO NPs) high concentration causes genotoxicity in the aquatic animals which is mainly due to the excess production of the oxidative stress. The treatment of ZnO NPs produces micronuclei comet and oxidative stress in the dose dependant manners. In the present study, the evaluation of genotoxicity was observed by micronuclei test and comet assay. The result showed that the increase concentration of ZnO NPs increase of the frequency of micronuclei and comets significantly. High frequency of comets (22.51±0.62) and micronuclei (0.557±0.081) was recorded at 80 $mgL^{-1}$ after 28 days of treatment. This might be due to depletion of genetic repair mechanism. The oxidative stress was evaluated by observing the levels of SOD (Superoxide dismutase), CAT (Catalase), GST (Glutathione S-transferase), MDA (Malonetialdohyde content) and GSH (Reduce glutathione) after 7and 28 days of the ZnO NPs treatment. The gills and liver tissues exhibited a decrease in the activity of CAT and GST at every treatment in a dose dependent manner. However, the activity of SOD increased in response to ZnO NPs as MDA and GSH. This study concluded, ZnO NPs are genotoxic in the aquatic organisms and produce the oxidative stress at the elevated level.

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## Introduction

Recently with progress and development of nanotechnology, nanoparticles have become a burning issue to the environment especially their release in to the water bodies. Due to high rate of production, there is an increase level of discharge of nano materials into the aquatic environment through various routs. The knowledge about the interaction with living and non-living organisms of these NPs are not fully under stood yet so, there is a great concern on the risks that may be on the environment and on the human health. The previous studies show that the contamination of metals is a serious pollution problem for aquatic organisms. These accumulated heavy metals via food chain from lower to higher trophic level are absorbed by all aquatic organisms (Begum *et al.*, 2009; Tang *et al.*, 2014). Upon entrance in body, metals change the biochemistry and genetic makeup of the living organisms. These changes include DNA damage, oxidative stress and anti-oxidative defence system indicating the level of pollution in the aquatic environment (Livingstone & Black, 2003; Asghar *et al.*, 2015). Molecular, biochemical and cellular biomarkers are most commonly used as tools for the assessment of these changes (Adedeji *et al.*, 2012). In the present study, comet and micronuclei assay was used to assess the dmage to genetic materials through the exposure of Zn NPs

In the second part of the study, the oxidative stress produced by reactive oxygen species due to treatment of ZnO NPs was evaluated through the change in the level of anti-oxidant enzymes in the gills and live tissues. Reactive Oxygen Species (ROS) are produced due to oxidative metabolism of cells. These ROS are free radicals with atomic oxygen due to unpaired valence shall electrons and are highly reactive. These ROS include radicles like hydroxyl ($OH^-$), superoxide ($O^{-2}$) and hydrogen peroxide ($H_2O_2$). Due to environmental stress by metals, oxidative stress is produced due to increase in ROS level that result a large rise in cellular reduction potential. Excessive production of ROS can induce pro-inflammatory and cytotoxic effects (Nel *et al.*, 2006). The nZnO and associated released Zn(II) may ultimately lead to apoptosis (Buerki-Thurnherr *et al.*, 2013) and acute toxicity at high concentrations. It can also result in a decrease in antioxidant defence interacting with lipids, proteins and nucleic acid resulting in loss of membrane integrity, structural or functional changes in protein and genetic mutations, thus contributing to health disorders (Martin and Leibovich, 2005; Pati *et al.*, 2016). In order to neutralize the toxic effects of ROS the body utilizes antioxidant defence systems like enzymes (Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione-S-transferase (GST) and GSH-Px) and non-enzymes (Memısogullari *et al.*, 2003; Wojewoda *et al.*, 2010). However, the elevated level of oxidative stress disturbs the equilibrium between the synthesis of antioxidant enzymes and production of ROS. This imbalance can be detected by the fluctuations of individual antioxidant enzymes in the different tissues including gills and liver (Asghar *et al.*, 2015). In the present study the genotoxic effect of ZnO NPs and the level of antioxidant enzymes were recorded in response of 28 days treatment.

## Materials and methods

*ZnO NPs treatment and samples collection*

The experimental animal *C. catla* was obtained from Punjab fish Hatchery Satiana road Faisalabad, Pakistan. The fish was first acclimatized in 40 lit aquaria for 2 weeks. The fish was then treated with 10, 20, 40, 60 and 80 $mgL^{-1}$ ZnO NPs for 28 days. The challenged fish was fed with artificial diet two times a day. The water if the aquaria were replaced with fresh water after 48 h. the photoperiod was normally 12 light and 12 h dark.

During the experimental period 7, 28 days intervals, blood samples were collected randomly, Blood was drawn from all treatments and control fishes by cardiac puncture using 2ml syringes and gauge hypodermic needles. The point of insertion for heart puncture is ventral, midway between the anterior bases of the pectoral fins. The syringe is flushed with

EDTA (Anticoagulant) and the collected blood was transferred to the EDTA tube for further haematological analysis.

*Evaluation of Genotoxicity*

Genotoxicity was evaluated through micronuclei and comet assay. For micronuclei test, a thin and evenly formed smear was formed from the blood of control and each treatment group on pre-cleaned slides, which were tagged before the smear formation. The slides were fixed in the methanol for 20 minutes and then allowed to dry. The slides were stained with 6 % Giesma stain for a period of 25 minutes. The slides were then washed to remove the excess stain and allowed to dry. The dried slides were examined under the microscope equipped with digital camera and at $100_X$. Non-refractive, small and ovoid bodies having the same stain wee score as micronuclei. The frequency of the micronuclei was calculated by dividing the number of cells having micronuclei with total number of cells and multiplying with 100. The frequency was expressed in the percentage (%). Protocol of Singh *et al.* (1988) with miner modification was followed for comet assay.

*Enzymatic Analysis of gills and river tissue*

The activities of CAT, SOD, GST, level of MDA and level of GSH was calculated form the gill and liver tissue extract. The activity of each enzyme was estimated according to following methodology.

The protocol of Payá *et al.* (1992) was followed with modifications made by Peixoto and Pereira-Moura (2008) for the measurement of SOD. In this methodology, the detection agent was nitrotetraazolium blue chloride (NBT). The reaction mixture was consisted of 100 mM buffer of phosphate having pH 7.0, 10 mM NBT and 10 mM hypoxanthine. The reaction was started by mixing 0.023 U/mol of xanthine oxidase with extracts of enzymes maintaining the temperature at 20 ºC. The activity of enzyme was measured by 50 % inhibition of NBT. The results were expressed as U/mg protein.

The activity of GST was measured by the methodology of Habig *et al.* (1974). The reaction mixture was xombination of 100 mM CDNB (chloro- dinitro benezene), 100 mM 2 ml buffer of potassium phosphate. Both, of the regants were then mixed with 100 mM GSH and then sample was added at 25 ºC the absorbance was measured at 340 nm and expressed in mol/ mg protein.

Jollow *et al.* (1974) was followed for estimation of GSH level in the gill and liver homogenate. The DTNB reagent was used. 500 µl of 4% sulphosalicylic acid was added to 100 µl of hemolysate. It was left for incubation at 4◦C for 1 h. The mixture was centrifuged at 4 C for 15 minutes and 12,000 RPM. The supernantants formed was taken out. 0.4 ml of supernatant was mixed with0.0 M 2.2 ml of potassium phosphate having pH 7.4.with 0.4 ml DTNB. A yellow colour was appeared due to reaction between DTNB and GSH. The absorbance was taken at 412 nm. The concentration is reported in terms of µmole/mg protein.

Aebi (1984) was followed for estimating the activity of CAT. About 50 mM solution of $H_2O_2$ was used as substrate. The solution was prepared in 50 mM of buffer of potassium phosphate. The CAT decomposed the H2O2, which was measured through spectrrophotomater at 240 nm wave length maintaining the pH at 7.0.

According to the Buege and Aust (1978) lipid peroxidation is the measure of MDA content (malondialdehyde). In the methodology, 1 ml sample from homogenates was mixed with 2 ml of TBA. TCA-HCl. The mixture was then heated in a medium of water bath and then cooled, centrifuged at 4000 rpm (15 minutes). The absorbance was recorded at 535 nm using blank as standard. The MDA content was expressed as µmol/ mg protein.

**Results**

The consequences of studies showed the generation of micronuclei in erythrocytes of *C. catla* due to exposure different test concentrations and sampling

times of ZnO NPs (Fig. 1). These results were presented in Table 1. The significant increase in MN frequencies was found at all the treatments sideways with treatment durations compared to control. The lowest concentration of ZnO NPs (10 $mgL^{-1}$) treatment in fish specimen induce MN frequency of 0.053±0.023 in blood erythrocyte after 7 days which was significantly increased to 0.134±0.05 after 28 days exposure. Similar trends were observed for 20 $mgL^{-1}$ in which the MN frequency of 0.15±0.05 at 7 days increased to 0.19±0.03 after 28 days. At the highest concentration (80 $mgL^{-1}$), the MN frequency significantly increased from 0.30±0.02 at 7 days exposure to 0.557±0.081 after 28 days duration (Table-1).

**Table 1.** Frequencies (%) of Micronuclei in erythrocytes of *C. catla* exposed to ZnO NPs at different test concentrations and exposure.

| Concentration ($mgL^{-1}$) | Exposure (Days) | |
|---|---|---|
| | 7 Days | 28 days |
| Control | $0.021±0.02^{A}$ | $0.024±0.009^{A}$ |
| 10 | $0.053±0.023^{AB}$ | $0.134±0.05^{B}$ |
| 20 | $0.149±0.05^{B}$ | $0.191±0.03^{AB}$ |
| 40 | $0.213±0.21^{BC}$ | $0.283±0.049^{BC}$ |
| 60 | $0.272±0.047^{C}$ | $0.299±0.068^{C}$ |
| 80 | $0.298±0.022^{C}$ | $0.557±0.081^{AC}$ |

Values are the mean± SD of three replicates.

Values sharing the same letter in the same column significantly different at 5% level.

The single strand DNA breaks are represented in the form of percentage tail or comet formation of DNA; it increased significantly with increasing concentrations and exposure durations in the erythrocyte cells when ZnO NPs challenged fish blood was compared with control group (Table-2). The highest DNA damage in erythrocyte cells was found at 80 $mgL^{-1}$ concentration and 28 days post exposure. The DNA damage measured in terms of per-cent tail DNA was found to be increased significantly from 7.15±0.37 to 22.51±0.62 in erythrocytes of ZnO NP exposed specimens (Table 2).

**Table 2.** Tail DNA (%) in erythrocytes cells of *C. catla* exposed to different test concentrations of ZnO NPs.

| Concentration ($mgL^{-1}$) | Exposure (Days) | |
|---|---|---|
| | 7 | 28 |
| Control | $3.72±0.25^{A}$ | $4.01±0.65^{AB}$ |
| 10 | $7.15±0.37^{AB}$ | $7.46±0.76^{B}$ |
| 20 | $7.80±0.24^{AB}$ | $9.31±0.56^{BC}$ |
| 40 | $8.28±0.07^{B}$ | $11.43±0.34^{C}$ |
| 60 | $11.14±0.35^{AC}$ | $15.15±0.74^{A}$ |
| 80 | $16.24±0.71^{A}$ | $22.51±0.62^{D}$ |

Values are the mean± SD of three replicates.

Values sharing the same letter in the same column significantly different at 5% level.

*Enzymatic analysis*

Oxidative stress is the consequence of an inequality in the pro-oxidant/antioxidant level in the body homeostasis. The level of antioxidant enzymes in the ZnO-NPs treated fish tissues such as liver and gill were measured. The gills with administration of ZnO NPs produced an important decrease in the activities of antioxidant enzymes. The treated liver tissues presented the maximum changes in antioxidant enzyme activity amongst the tissues.

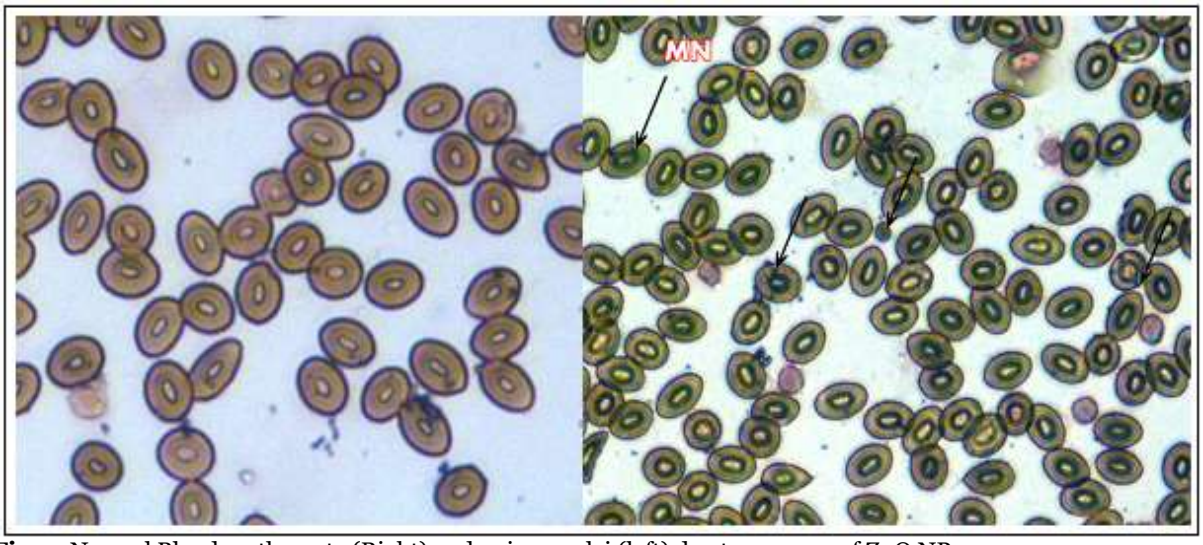


**Fig. 1.** Normal Blood erythrocyte (Right) and micronuclei (left) due to expoure of ZnO NPs.

SOD is the enzyme to contract with oxy radicals and is accountable for catalysing the dismutation of extremely reactive superoxide radical to $O_2$ and $H_2O_2$. In the current study, the SOD level of activity in the liver and gills of *C. catla* exposure to different concentration with different exposure intervals were examined (Fig.2). Exposure of 25 $mgL^{-1}$ ZnO NPs, the SOD activities of both, gill and liver tissues were enthused and presented a notable rise, which might be due to the production of new enzymes or the enrichment of pre-existing enzyme levels. But this level was decrease at higher concentrations due to oxidative stress and depilation of SOD production. However, the activity of this enzyme was found to decrease with the rise of the ZnO NPs explore time to 28 days (Fig. 2).

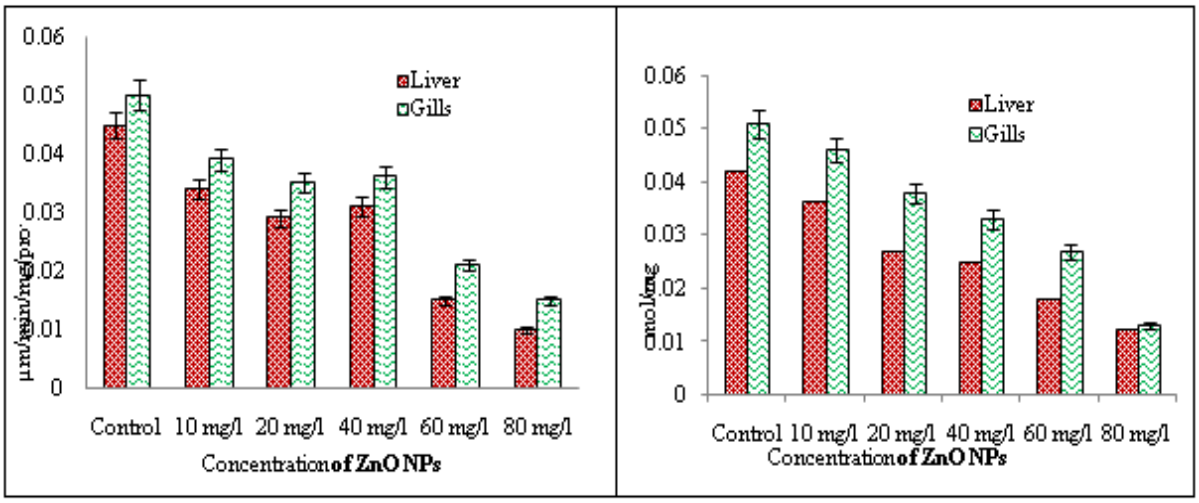


**Fig. 2.** Alter activity of Effect of ZnO NPs on the CAT activity in the liver and gills tissues of *C. catla* after 7 days.

The activity of CAT reduced marginally with increasing the concentration of ZnO NPs and treatment time i.e., 7, to 28 days (Fig.3). The activities of GST decrease with the increase the contraction of the ZnO NPs compared to the control group (Fig.4). Lipid peroxidation (LPO) recognition by malondialdehyde (MDA) levels was inspected by oxidative corrosion of cell membrane lipids and has been used broadly as an indicator of oxidative stress in LPO recognition technique. In the current study, MDA substances in the liver and gill tissues were altered compared with the control.

There was significant increase in MDA level was found after 7 days of exposure of ZnO NPs (Fig. 5). It indicated that these tissues were undergoing oxidative stress, which was consistent with our results of higher concentration of ZnO NPs exhibiting more potent effects on disturbance to the antioxidant defence systems in *Catla catla*. The MDA increased and the activities of antioxidant enzymes reduced in the liver of ZnO NPs exposed *C. catla*. The outcomes proposed that the equilibrium between the oxidative and antioxidant system was wrecked in the fish. Liver comprises of extensive quantities of polyunsaturated fatty acids, which are disposed to to injury by free radicals.

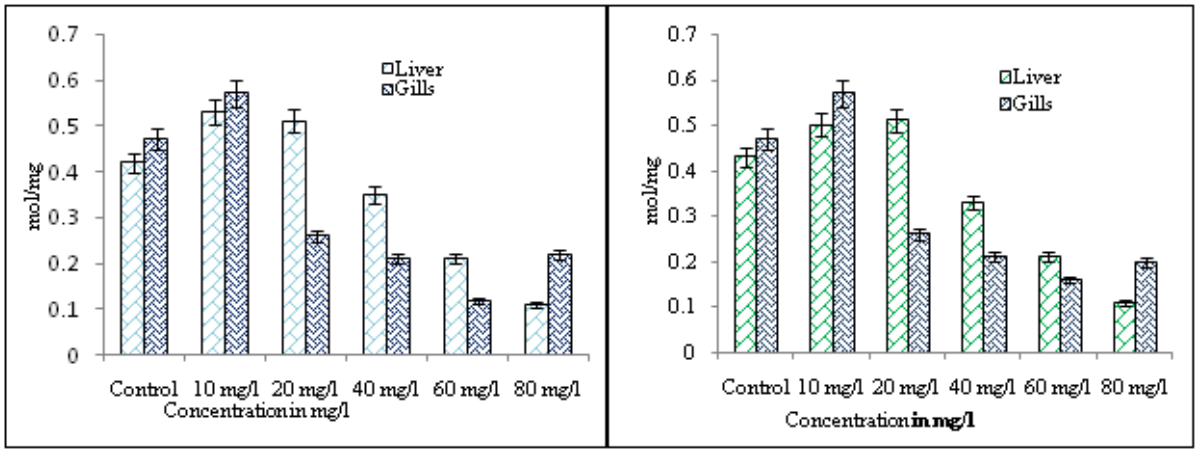


**Fig. 3.** Effect of ZnO NPs on the SOD activity in the liver and gills tissues of *C. catla* after after 7 days (Right) and 14 days (Left) treatment.

The levels of GSH increased after the 7 days of treatment with ZnO nanoparticles both in liver and gills (Fig.6). The level of GSH was found higher in the liver as compared to the gills. The activity of this enzyme increased in response to the MDA level. Additionally, nanoparticles produced more stress in the liver. Similar trends were found after the exposure of 28 days (Fig 6).

**Discussion**

Fishes are frequently used as sentry organisms for eco-toxicological studies since they play numeral roles in the trophic web, accrue toxic ingredients and retort to little concentration of mutagens and carcinogens (Çavaş & Ergene-Gözükara, 2005). The use of fish biomarkers as catalogues of the properties of pollution, are of cumulative importance and can permit initial recognition of environmental glitches in marine or fresh water ecosystem (Van der Oost *et al.*, 2003). Genotoxicity can occur due to numerous physico-chemical mediators that result in an extensive diversity of potential injuries to the genetic material, vacillating from numerous DNA adducts to single and double-strand breakages, DNA-DNA and protein DNA cross-links or even chromosomal damage (Sancar *et al.*, 2004; Zhu *et al.*, 2009b; Cavalcanti *et al.*, 2010).

The results showed ZnO NPs also induced genotoxic and mutagenic effects in *C. catla* exposed with different concentrations and exposure time. The combined use of assays (both) suggested the testing of the genotoxic compounds to understand the basic mechanisms which induce mutagenicity (Van Goethem *et al.*, 1997; Ali *et al.*, 2008). Hence, frequencies of micronuclei and comet assay were studied in the present study for toxicity and genotoxicity studies of ZnO NPs on the challenged fish.

Micronuclei assay actually detects injuries of the damage cells that survive at least up to one mitotic division. It is the result of formation of laggards

excluded from the nucleus in the process of mitosis (Kumari *et al.*, 2011). It also measures chromosome breakage or chromosome loss due to toxic metabolites (Kirsch-Volders *et al.*, 2003). The Zno NPs increased the frequency of micronuclei formation in dose dependent manner. The results of micronuclei (MN) induction in erythrocytes of *C. catla* in this study revealed that at the lowest concentration of ZnO NPs (10 $mgL^{-1}$) treatment induced MN frequency of 0.05±0.02 in erythrocyte at 7 days, significantly increased to 0.13±0.05 after 28 days exposure. Similar trends were also observed at 20 $mgL^{-1}$ in the MN frequency was 0.15±0.05 at 7 days and increased to 0.19±0.03 after 28 days. At the high concentration (80 $mgL^{-1}$), the MN frequency was increased significantly from 0.3±0.02 at 7 days treatment to 0.56±0.08 after 28 days duration. The micronuclei induction in erythrocytes was highest at the high test concentration of 80 mg $L^{-1}$. Therefore, the present study concluded that ZnO NPs were the main cause of induction of micronuclei in challenged fish.

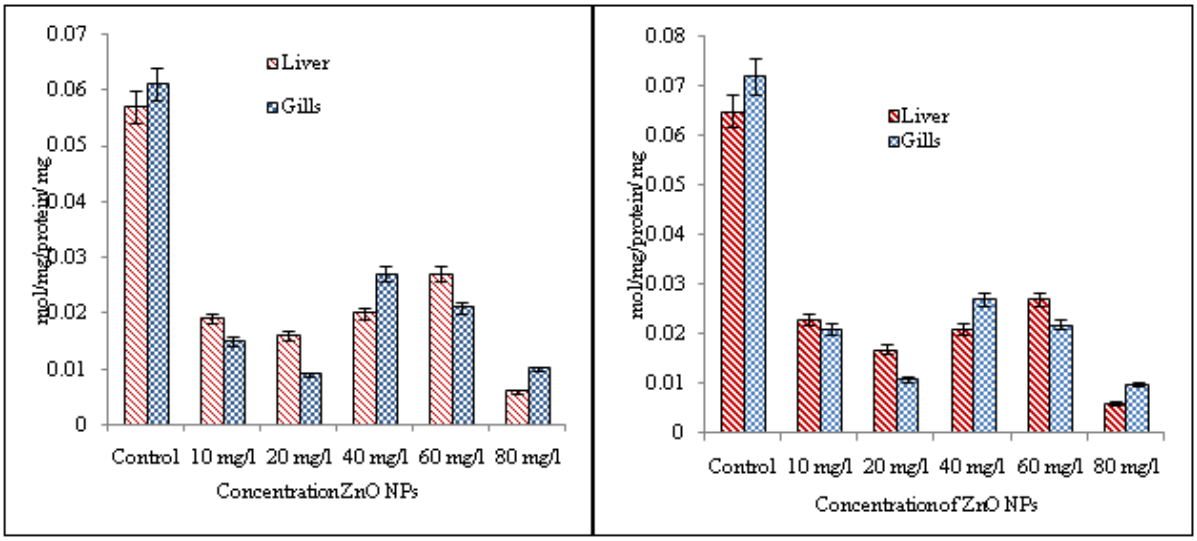


**Fig. 4.** The change in GST activity in the liver and gills tissues of *C. catla* after after 7 days (Right) and 14 days (Left) treatment.

The increase in micronuclei correspondingly ropes that the test chemicals (ZnO NPs) were lactogenic and are proficient of producing different sorts of chromosomal anomalies. Numerous hypotheses advocated to account for the lactogenic or genotoxic effects, comprising the foundation of adduct or impairment at the level of DNA and chromosomes. DNA destructive agents like ZnO NPs have the ability to root genomic instability, which is a prompting factor in carcinogenesis. Therefore, vigilant nursing and further description of their systemic toxicity, genotoxicity and carcinogenicity is also vital (Zhou, 2015).

The comet assay, also known as the single cell gel electrophoresis (SCGE) assay is a method for the straight imagining of DNA damage in discrete cells. Singh *et al.* (1988) presented the electrophoresis in alkaline (pH >13) circumstances for discovering DNA damage in single cells. At alkaline conditions, DNA migration is related to incidence of strand disruptions (single or double strand), SSB allied with incomplete deletion of repair sites, and alkali-labile sites (ALS). The alkaline form of comet assay had additional success since it permits the recognition of an extensive range of damages, and in fact nearly all genotoxic mediators persuade more SSB and ALS than DSB (Tice *et al.*, 2000). The comet assay has been deliberated as a subtle, quick and consistent method of quantitatively assessing DNA damage in both prokaryotic and eukaryotic cell (Tice *et al.*, 2000; Andem *et al.*, 2013). It is progressively being

employed in testing of materials such as industrial chemicals, biocides, agrochemicals, food additives and pharmaceuticals for genotoxicity challenging (Brendler-Schwaab *et al.*, 2005; Kumaravel and Jha, 2006; Olive and Banáth, 2006). The assay is preferred on other cytogenetic approaches castoff for the finding the DNA damage (Buschini *et al.*, 2004; Olive and Banáth, 2006) as it is adapted for sensing extensive diversity of DNA damage such as DNA single strand breaks (Maluf, 2004).

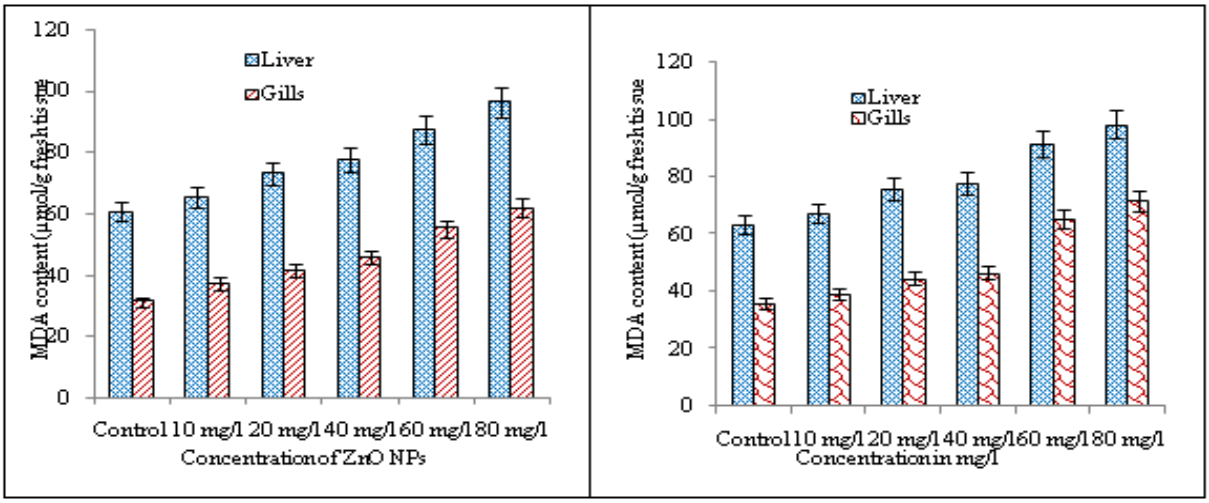


**Fig. 5.** The Change in the level of lipid peroxidation in the liver and gills tissues of *C. catla* after 7 days (Right) and 14 days (Left) treatment.

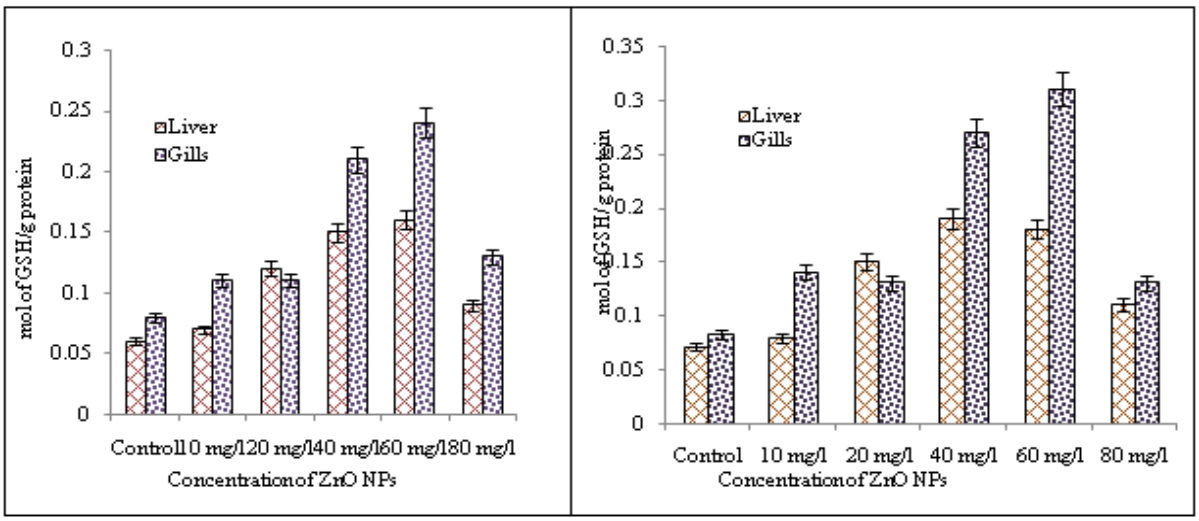


**Fig. 6.** The Change in the level of GSH in the liver and gills tissues of *C. catla* after 7 days (Right) and 14 days (Left) treatment.

In the present study the alkaline comet assay was used for assessing the entire DNA strand breaks in the erythrocyte cells of challenged *C. catla* ZnO-NPs. The data obtained showed that the frequencies of % tail DNA damage for all concentrations of ZnO NPs were significantly higher than control groups. The highest damage of erythrocyte cells was found at 80 $mgL^{-1}$ treatment group and 28 days exposure time. The DNA damage measured in the form of per-cent tail DNA increased significantly from 7.15±0.37 to 22.51±0.62 (10 to 80 $mgL^{-1}$) in erythrocytes of ZnO NPs exposed experimental fish (Table 2).

The main mechanism of NPs toxicity is oxidative stress. ZnO-NPs triggered oxidative stress in *C catla*, the experimental fish. Due to oxidative stress there

are typical changes in the activity of SOD, CAT and GST as well as in the levels of GSH and MDA. The activities of enzyme SOD increases dose dependently and their level increases significantly in comparison to the control (p value ≤0.05), while the activity of CAT and GST decreases dose dependently. The activities of GST decrease with the increase the contraction of the ZnO NPs compared to the control group (Fig.4). this decrease in the activities of the GST might be due to the depletion of the antioxidant system of the ZnO-NPs treated fish.

The oxidative corrosion of lipid in cell membrane is called LPO are used as biomarker of oxidative stress and can be assessed by calculating the level of MDA. This above accretion of MDA can damage cells and activates apoptosis. The present study also presented a noteworthy rise in the activity of MDA at the higher concentration of the ZnO NPs. GST activity decreased gradually and significantly as a result of increasing concentrations. It is concluded from the present study that ZnO NPs high concentration causes genotoxicity in the aquatic animals which is mainly due to the excess production of the oxidative stress. The treatment of ZnO NPs produces micronuclei comet and oxidative stress in the dose dependant manners. This study is mainly focused on the toxicity of ZnO NPs and the mechanism of the toxicity is still unknown and topic of the future studies.

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