Study of some toxicological aspects of titanium dioxide nanoparticles through oxidative stress, genotoxicity, and histopathology in tilapia, Oreochromis mossambicus

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Study of some toxicological aspects of titanium dioxide nanoparticles through oxidative stress, genotoxicity, and histopathology in tilapia, *Oreochromis mossambicus*

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Abstract

Extensive use of nanotechnology in multiple commodities is raising concern about nanotoxicity and particularly. Particularly, many studies reported the health hazardous effects of titanium dioxide nanoparticles (TiO_2-NPs). Study focuses on toxicity and accumulation of TiO_2-NPs in tilapia (*Oreochromis mossambicus*). For this purpose, Tilapia were kept in water tanks, acclimatized for fourteen days, and treated with different doses of TiO_2 nanoparticles 0, 0.5, 1.0, and 1.5 mg TiO2-NPs /L. Results revealed an increase in accumulation of TiO_2-NPs with an increase in doses. Moreover, with higher dose (1.5 mg /L) gills had maximum levels compared to muscles and liver tissues whereas other doses showed different accumulation patterns. A significantly higher concentration of catalase, glutathione, and lipid peroxidation was recorded in gills ($p<0.05$) and superoxide dismutase in the liver. Characteristics like thickening and fusion in lamellae, rupturing of filaments and hyperplasia of gills were also recorded. The phenomenon of increased necrosis and apoptosis in the liver was also noticed with increasing concentration of TiO_2-NPs along with formation of sinusoid spaces and condensed nuclear bodies. Elevated values of olive tail movement and % tail DNA were also noticed with increased concentration of TiO_2-NPs. This study concluded that TiO_2-NPs produced oxidative stress by accumulation in soft tissues and induced pathology and genotoxicity.

Keywords: Nanoparticle; toxicity; *Oreochromis mossambicus;* genotoxicity

Introduction

The structure dimensions of nanoparticles range from 1-100 nm and exist on earth for millions of years for the use of mankind (Asghar et al. 2016; Masciangioli and Zhang 2003). These particles are being engineered for various applications in industries such as drug delivery systems, chemical sensors, biosensors, optics, textiles, medical devices, electronics, and environmental remediation (Khan et al. 2015b; Khan et al. 2017a; Raza et al. 2017). However, extensive use is also responsible for aquatic habitat penetration and raising concerns of ecotoxicology (Khan et al. 2015b; Ramsden et al. 2013). The most prominent sources of these nanoparticles are colloids found in natural waters, soil, and sediments. Volcanic dust in the atmosphere is also an important source of TiO_2-NPs (Buffle, 2006 and Handy, *et al,* 2008b). Upon exposure, the concentrations of different types of nanoparticles were recorded much higher in

tissues of aquatic animals than the normal permissible concentrations (Asghar et al. 2015; Khan et al. 2018; Khan et al. 2020).

TiO_2-NPs are most extensively applicable in dyes, makeups items and sunscreens (Hao et al. 2009; Shakeel et al. 2016) and released in the natural environment through wastewater effluents where they cause gill injury, oxidative stress, changes in hematology, and plasma ion imbalance (Asghar et al. 2018; Rocco et al. 2015; Shakeel et al. 2018). Further, these NPs mostly penetrate via the skin, gills, and gastrointestinal absorption to blood and liver (Shakeel et al. 2018; Shakeel et al. 2016). therefore, the main targets to study the effect of nanoparticle exposure are gills, liver, brain, gut, and muscles (Khan et al. 2017b).

Some previous work on TiO_2-NPs toxicity showed that it produced oxidative stress and gill injury in exposed zebrafish (Griffitt et al. 2009; Tang et al. 2019). Zhu et al. (2008) also demonstrated oxidative stress caused by TiO_2-NPs with an increasing trend from lower to higher levels of dose at the developmental stage of Zebrafish *(Danio rerio)* larvae. The genotoxic potential of TiO_2-NPs has been assessed primarily through DNA breakage (Nigro et al. 2015). A significant TiO_2-NPs genotoxic effect on DNA damage was reported in zebrafish model (Rocco et al. 2015).

This study focuses on the toxicological assessment of engineered TiO_2-NPs in tilapia (*Oreochromis mossambicus*). The sub-lethal toxicological effects examined include bioaccumulation, genotoxicity, oxidative stress and histological alterations.

Materials and Methods

Characterization

TIO 2-NPs (Sigma-Aldrich, GmbH, Germany) <100 nm with 99 percent purification were in the form of nanopowder. The surface area was analyzed through scanning electron microscope (Philips, ESEM XL30). Figure 1 exhibits a spherical shape and fits the size of the nanoscale with an average of 72+12.50 nm. The photograph is taken with the magnification of 20000 and 65000 with a power source of 20 kV.

Model animal collection and acclimatization

With ethical permission, fish (weight 50.90± 0.37 g; 7.38 ± 0.19 cm length) was collected from aquaculture ponds in Patoki City, Kasur District, Pakistan. The transportation to lab was carried out in plastic bags of freshwater with continuous oxygen through the oxygen-carrying cylinder to minimize the transport stress. Before treatment, the fish were acclimatized to glass tanks environment for fourteen days.

Experiment Design

The fish were grouped in four with three replicates per group in 12 experimental water glass tanks (5 fish/aquaria). Each aquarium has dimensions of 45.72 x 60.96 x 45.72 cm with 40 liters of water. Fish were provided commercial feed consisting of crude protein (35%), fat (4%), fiber (5%) with 12% average moisture. The feed was provided twice a day at morning and evening hours.

TiO2-NPs solution (Stock solution) was prepared in Milliquat water for treatment, sonicated for 30 minutes at 35 kHz (WUC-A06H). Four doses included T0 (0 mg/L), T1 (0.5 mg/L), T2 (1.0 mg/L), and T3 (1.5 mg/L) were prepared for the treatment. The exposure of TiO2-NPs did not feed the fish to reduce the nanoparticle attachment in the food. Water from each aquarium was changed 24 hours and drain through suction pumps remaining 20 % water. The suction pumps also removed the animal waste. Then, fresh water was added to each aquarium.

On the 14th day, fish were carried in a small water container to take the samples. 3 to 4 drops of clove oil were used to anesthetize the fish. Blood samples were obtained from the dorsal aorta in EDTA vials using BD syringes. Then, Fish were dissected form liver, kidney, and muscle samples. For biochemical and oxidative stress-enzymatic and non-enzymatic analysis, samples were preserved in plastic bottles and stored at -20ºC. The tissues for histological analysis were put in Bouin's fixative.

Water Quality Parameters

pH meter (Velp Germany) was used for pH measurement. Conductivity, dissolved oxygen (DO) and temperature meter multi-function meter (JENCO, Model 3173R) respectively. p-alkalinity, absolute alkalinity, CE resilience, maximum hardness, and chlorides was measured with APHA (2005).

p-Alkalinity

took Water sample (25 ml) in conical flask with 2 drops of phenolphthalein. H_2SO_4 (0.02N) was used and the sample was titrated till the pink color started disappearing. The p-Alkalinity was recorded with the following formula in mg/l.

$$\text{p} - \text{Alkalinity} = \frac{\text{ml H2SO4 used x 1000}}{\text{water sample (ml)}}$$

Total alkalinity

Mixed indicator with 2 drops of 0.02N H2SO4 titrated sample and titrated it until the bricks red color.

$$\text{Total Alkalinity} = \frac{\text{ml H2SO4 used x 1000}}{\text{water sample (ml)}}$$

Ca-hardness

For Calcium hardness, 25 ml of water sampled was taken and added 1 ml of NOH to produce pH 12 to 13. Then Stirred the sampled with 0.1 g phenolphthalein and titrated against EDTA to express results in mg/lCaCO3.

$$\text{Ca} - \text{ Hardness} = \frac{\text{ml EDTA x 1000}}{\text{water sample (ml)}}$$

Total hardness

25 ml water sample was diluted with distilled water to make a volume of 100 ml in flask. The pH of the sample was maintained at 10-10.1 by adding 1-2 ml buffer solution. Finally added two drops of indicator solution and titrated against 0.01M EDTA solution until blue color appeared. The result was expressed in mg/l.

$$\text{Total Hardness} = \frac{\text{EDTA x 1000}}{\text{sample (ml)}}$$

Chlorides

1 ml K2CrO4 was added as an indicator in 25 ml sample. This solution is titrated with $AgNO_3$ till light red color. The results were expressed as mg/l.

$$\text{Chloride} = \frac{\text{AgNO3 (ml)x AgNO3 Normality x 35460}}{\text{water sample (ml)}}$$

Inductively Coupled Plasma Mass Spectrometry of Sample

1 g of frozen sample was mixed with 10 ml of HNO3 and 2 ml of pyruchloric acid (HClO4) to digest the sample. Then all sample was heated below 100 ℃ on a hot plate until the yellow. Then added two drops of hydrogen peroxide. 2 ml

digested sample was diluted with cold up to 50 ml, filtered (Whatman) analyzed using plasma mass spectrometry (APHA, 2005).

Biochemical Assay

For this assay, gills and liver samples were used. First both samples processed through Teflon tissue homogenizer for homogenate preparation. The samples were first washed with buffer, and then immersed in 10% 0.1 M phosphate buffer (pH 7.4), centrifuged at 4˚C for 10 min at 5000 rpm and superintendents stored in a freezer.

lipid peroxidation Estimation

LPO estimation was done through thiobarbituric acid reactive substances synthesis in homogenate liver and gills samples as (MDA) equivalents. Buege and Aust (1978) was followed for this LPO estimation

Catalase activity

CAT activities were assessed using the protocol of Claiborne (1985). Mixture for this estimation was a solution of 3 ml volume and consisted of 100 µl sample,1.90 ml potassium phosphate buffer (50 mM, pH 7.0). The reaction was initiated by 1 ml of H_2O_2 and read through a spectrophotometer at 240 nm with an interval of 30 seconds for 3 minutes.

Superoxide dismutase activity

Estimation of enzyme activities was done by Marklund and Marklund (1974) protocol and based on the ability of SOD to inhibit the auto-oxidation of pyrogallol. A mixture of sample and 2.80 ml K_2PO_4 buffets (0.05M, pH 8.2) incubated at 20 ℃ for 20 min was used for this assay. This reaction was started with 100 µl of 8 ampigravalol. The abosrance of the solution mixture was measured at 412 nm for 3 min with a 30 seconds interval and represented as per milligram of protein.

Glutathione

Estimation was done through Jollow et al. (1974). Homogenate and sulfanilic acid were taken in equal amounts, mixed well and then incubation was done at 4˚C for an hour. Centrifugation of this mixture was carried out at 12,000 rpm for 15 minutes. Then, mixed 0.4 ml supernatant with 2.2 ml of 0.1 M K_2SO_4 buffer (pH 7.4) initiated the reaction with 0.4 ml of DTNB (5.5 + -dithiobis-2-nitrobenzoic acid). Finally, the absorbance was recorded at 412 nm within 30 seconds of reaction initiation.

Histology

The liver and gills samples were processed through Humason (1979) for histological studies.

Comet Assay

Protocol of Singh *et al*. (1988) for comet assay was adopted and slide preparation. The staining of slides were done with ethidium bromide for examination under fluorescence microscope at 400X. Comet IV a Computer Software was used for scoring the comet (Chaubey, 2005).

Statistical analysis

Raw data for all studied parameters were recorded separately. Minitab (V. 17) was the computer package used for processing of data. Comparison was made through Tukey's test at 95% level and ANOVA to compare significance of means of each parameter.

Results

Physicochemical analysis

Different physicochemical parameters (temperature, TDS**,** alkalinity, total hardness, Ca-hardness, total alkalinity, and chlorides are listed in Table 1.

Table 1: Showing accumulation of Ti (µg/Kg) in the tissues of tilapia (*Oreochromis mossambicus*).

Tissues	Treatments			
	T_0 (0 mg/L)	T_1 (0.5 mg/L)	T_2 (1.0 mg/L)	T_3 (1.5mg/L)
Gills	0.07 ± 0.01^{d}	0.46 ± 0.00577^{g}	0.71 ± 0.01^{d}	4.29 ± 0.02^{a}
Liver	0.093 ± 0.02^{c}	0.41 ± 0.0153^{g}	0.62 ± 0.02^{f}	4.13 ± 0.02^{a}
Muscles	0.42 ± 0.02^{d}	0.65 ± 0.0153^{e}	3.91 ± 0.02^{b}	3.97 ± 0.4^{b}

All values described as mean ± SD ($p<0.05$)

Accumulation of TiO2 from TiO_2-NPs

With increasing doses, Ti started to accumulate in the tissues. Gills accumulated maximum compared to liver and muscles tissues. The concentration of Ti accumulation was 4.29±0.02 µg/g in gills at 1.5 mg/L dose. At a higher dose, the mean Ti concentration was 24.13 ± 0.02 µg/g and 3.97 ±0.4 µg/g in the liver and muscles. The order of concentration was gills>liver >muscles.

Oxidative Stress assay

With increasing dose, elevated activities of liver SOD were recorded in treated fish. In the control group, the value of CAT in the liver and gills was 6.73 ± 0.056 U/mg and 6.53 ± 0.058 U/mg respectively. While mean values CAT in liver and gills on the higher doses were 5.87 ± 0.15 U/mg and 11.40 ± 1.20 U/mg respectively. Fluctuation in the level of glutathione (GSH), increase in gills, and decrease in the liver, was also recorded with increasing TiO_2-NPs concentration. GSH value in liver and gills was 3.77 ± 0.058 U/mg and 2.13 ± 0.15 U/mg respectively in the control group and 1.6333 ± 0.1528 U/mg and 3.3667 ± 0.1528 U/mg in the liver and gills at a higher dose. TiO_2-NPs also raise the level of LPO (lipid peroxidation) in gills (Table 2).

GST showed different results, it was recorded higher in the treated group 2, GST in the liver (0.15 mol/mg prot) significantly ($P \leq 0.05$) was higher than gills, kidneys, and heart. In treated group 2, GSH (130.0 µmol/mg tissue) was high in gills and kidneys compared to other organs.

Table 2: Showing the oxidative and anti-oxidative enzymes estimation

Enzymes	Tissues	Treatments			
		T_0 (0 mg/L)	T_1 (0.5 mg/L)	T_2 (1.0 mg/L)	T_3 (1.5mg/L)
CAT U/mg	Gills	6.53 ± 0.058^{d}	7.7667 ± 0.1155^{c}	9.5667 ± 0.1155^{b}	11.40 ± 1.20^{a}
	Liver	6.73 ± 0.058^{d}	6.133 ± 0.1528^{e}	5.7667 ± 0.1528^{e}	5.87 ± 0.15^{e}
SOD U/mg	Gills	7.43 ± 0.1528^{h}	9.733 ± 0.1528^{f}	11.333 ± 0.153^{d}	13.733 ± 0.153^{b}
	Liver	8.8667 ± 0.0577^{g}	10.467 ± 0.115^{e}	12.133 ± 0.153^{c}	14.867 ± 0.153^{a}
GSH U/mg	Gills	2.13 ± 0.15^{e}	2.833 ± 0.1155^{c}	3.1333 ± 0.1528^{bc}	3.3667 ± 0.1528^{b}
	Liver	3.77 ± 0.058^{a}	2.667 ± 0.1528^{d}	1.8667 ± 0.1528^{ef}	1.6333 ± 0.1528^{f}
LPO nmol/mg	Gills	2.033 ± 0.1528^{d}	3.4667 ± 0.1528^{c}	3.8667 ± 0.0577^{b}	5.7667 ± 0.1155^{a}
	Liver	1.1667 ± 0.1528^{f}	1.440 ± 0.0200^{ef}	1.5233 ± 0.0208^{e}	1.6000 ± 0.0265^{e}

All values are mean ± S.D ($p<0.05$)

Histology

TiO_2-NPs induced histological alterations gills are shown in Fig.2. Fig.2 (a) shows reference control, while Fig.2 (b) to (d) indicates treated with TiO_2 nanoparticles. Fig.2 (a) expresses the normal arrangement of gill lamellae. Whereas, in reference treated with TiO_2 nanoparticles gills undergo hyperplasia, rupturing of gill filaments, gill lamellae fusion, and primary and secondary gill lamellae thickening.

The liver histology of reference treated with TiO_2 nanoparticles shows alterations in hepatic cells. Fig.3. (a) indicates liver histology of reference control whereas, Fig.3. (c) to (d) indicates apoptosis and necrosis characterized by the presence of sinusoid spaces and condensed nuclear bodies.

Comet Assay

In order to check the TiO_2-NPs potential genotoxicity and its role in damaging the DNA molecules of fish erythrocytes, an alkaline comet assay was used (Fig.4). Increased DNA damage was observed with increased concentration of TiO_2-NPs exposure. % Tail DNA in the control sample was 4.699 ± 1.955 that significantly increased in samples treated with a high dose of TiO_2-NPs (18.219 ± 0.581). T_2 (1.0 mg/L) and T_3 (1.5 mg/L) did not show any significant difference in % tail DNA. Similarly, increased dose concentration of TiO_2-NPs also elevates the level of olive tail movement. Olive tail movement in the control sample was 0.331 ± 0.053, which was much lower than samples treated with TiO_2-NPs 7.472 ± 1.249 during T_3 (1.5 mg/L) (Fig. 5 a b).

Discussion

Certain studies on the accumulation of nanomaterials in living tissues and their toxic effects have already been conducted. Ates et al. (2013) examined nanoparticle accumulation in different tissues of goldfish (*Carassius auratus*) and detected the highest accumulation in gills. Results were opposite to our research where the highest accumulation of Ti was observed in liver tissues. In a study on freshwater mussels, increased accumulation of heavy metal was noticed in soft tissues of mussels on increasing the dose of heavy metals (Sohail et al. 2016).

Lipid peroxidation is strongly associated with ROS. It produces free radicals in cellular membranes and starts the oxidation of unsaturated fatty acids. Damage to biomolecules, including DNA and proteins is destructive for lipid peroxidation at cellular or organ level (Asghar et al. 2017; Khan et al. 2015a; Khan et al. 2016; Wilhelm 1990). Membrane damage is due to an increased level of LPO as a result of oxygen reactive species ROS formation (Asghar et al. 2017). Polyunsaturated fatty acids, an important constituent of biomembranes, are highly vulnerable to peroxidation. Zhu et al. (2008) explained the impact of C_{60} on the LPO level of fathead minnow. The final results of the study suggest an increase in LPO in the brain, liver, and gills of fathead minnow. In another study by Hao et al. (2009) effect of TiO_2-NPs on the LPO level of different tissues of juvenile crap was determined. The final results of the study suggest a comparatively high level of LPO in the liver than gills. This result is opposite to our study which indicates an elevated level of LPO in the gills rather than the liver.

Further, Hamid et al. (2016) studied the role of superoxide dismutase (SOD) in regulating the concentration of superoxide radical (O_2^-) at the cellular level. SOD converting superoxide radical into H_2O_2; ultimately lowers its concentration. H_2O_2 resists the activities of different enzymes by penetrating biomembranes and is further catabolized by the CAT enzyme. Xiong et al. (2011) correlates raised CAT activity, in zebrafish gills, with TiO_2-NPs

accumulation. In a study on juvenile craps, a direct association was noticed between dose and the liver's SOD activity. In contrary to this an indirect association was observed between SOD activity and increased dose concentration in gills (Hao et al. 2009). Similarly, our study also supports raised SOD levels in the liver.

The present study highlights maximum GSH level in the gills. Whereas, a decrease in GSH activity of the liver was reported. Smith et al. (2007) also reported parallel results while working on the treatment of rainbow trout with single-walled carbon nanotubes. They examined decreased GSH activity in liver tissues of rainbow trout. Ramsden et al. (2013) in another study treat embryos of zebrafish with nanoparticles of TiO_2 and noted elevated levels of GSH in liver tissues. GSH plays a crucial role in cell protection from the highly reactive hydroxyl group. GSH reduces the hydroxyl group and because of this oxidative stress mediating property of GSH, it becomes essential to regulate the level of antioxidant and oxidative enzymes (Ahmad et al. 2019; Hamid et al. 2016; Khan et al. 2016).

Gills being direct contact with the aquatic environment are considered the primary site for gaseous exchange (Guan and Lin 2004). This explained that the osmoregulatory functions of fish were disturbed and it was in severe stress. Different hepatic alterations, specifically liver cell apoptosis, are also caused by TiO_2-NPs (Rahman et al. 2002). To determine the possible association between hepatic apoptosis and TiO_2-NPs, liver tissues of fish were exposed to titanium dioxide. Smith et al. (2007) in their study observed the same hepatic apoptotic phenomenon. In another study by Hao et al. (2009) on juvenile carp same results were reported.

TiO_2-NPs role in damaging the erythrocytes of fish (*O. mossambicus*) has been proved by comet assay. Increased exposure to elevated TiO_2-NPs concentration significantly raises the level of olive tail movement and % tail DNA. Maximum genotoxic action of TiO_2-NPs was observed during the 14^{th} day of study at an increased dose concentration of T_3 (1.5 mg/L). Vignardi et al. (2015) studied the genotoxic activities of nano TiO_2 in m*Trachinotus carolinus*; a marine fish and reported similar results. Shakeel et al. (2018) demonstrated the increased toxicity of bulk TiO_2, in marine mussel's (*Mytilus galloprovincialis*) hemocytes. This study also detected the genotoxic effects of TiO_2 and its role in damaging the DNA molecule. A previous study contradicts our results and reports lower level damage to DNA at increased dose concentration (Sohail et al. 2016). In an experiment on European sea bass (*Dicetrarchus labrax*), showed that TiO_2-NPs have more potential of inducing genotoxicity (Nigro et al. 2015). Effect of TiO_2-NPs elevated dose concentration in zebrafish (*Danio rerio*) is also examined in a study that inferred the role of TiO_2-NPs in DNA damage and apoptosis (Rocco et al. 2015).

Conclusion

Lethal and toxic effects of TiO_2 nanoparticles, on tilapia (*O. mossambicus*), are examined in this study. TiO_2-NPs exposure within the range of 0.5-1.5 mg/L is not lethal to the tilapia. Because of this reason variety of changes were observed during this experiment. The liver shows the highest vulnerability to Ti toxicity during TiO_2-NPs exposure. Increased accumulation of Ti in liver tissues results in elevated LPO level, decrease in the antioxidant enzyme, and pathological modifications in tissues. Titanium dioxide also results in RBCs genotoxicity of fish which ultimately leads to different abnormalities including olive tail movement or %tail breakage.

Declarations

Ethics approval and consent to participate

Approval was taken from ethical review board of the University before start of the study.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KS, MNK, and FJ analyzed, interpreted, and formatted the data. ASC, CA, MKAK, and MSK performed the histopathology and genotoxicity examination and write the manuscript. The final manuscript was read and approved by all authors.

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Figures

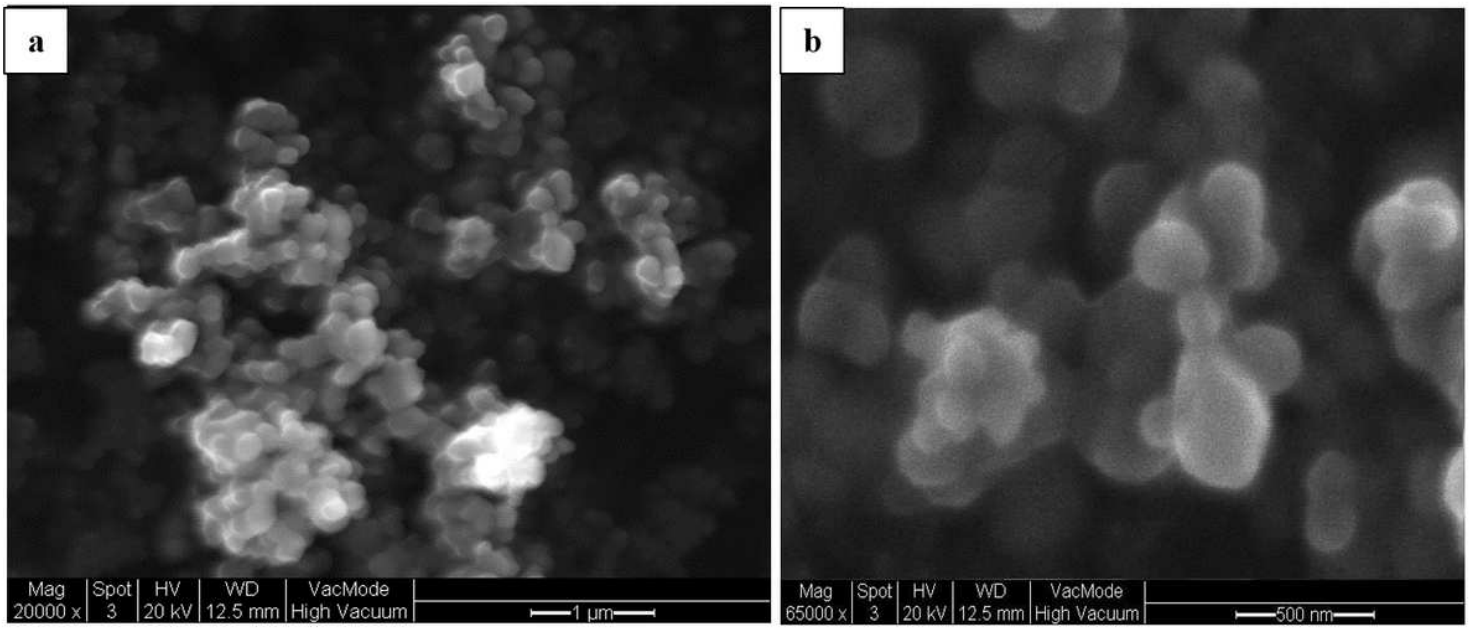

Figure 1

(a) and (b) showing the ESEM images of titanium dioxide nanoparticles (TiO2-NPs) at 20000 and 65000 magnifications

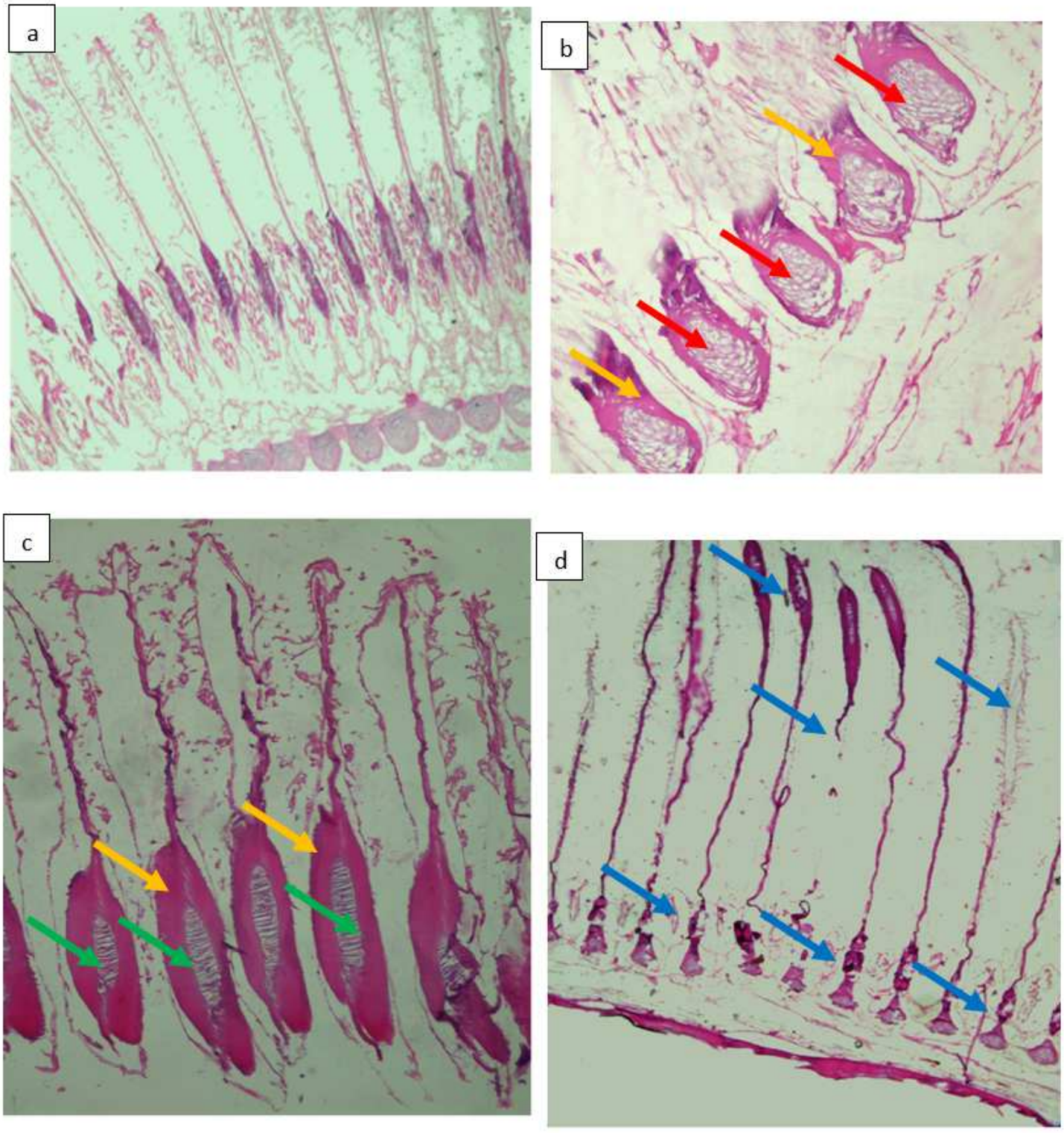

Figure 2

a-d Section of the gill of exposed fish. (a) The gill of control fish showing normal arrangement of primary and secondary gill lamellae. (c) to (d) The reference treated gill were showing hyperplasia (red arrows), fusion of gill lamellae (yellow arrows), rupturing of gill filaments (blue arrows) and thickening of primary and secondary gill lamellae (green arrows).

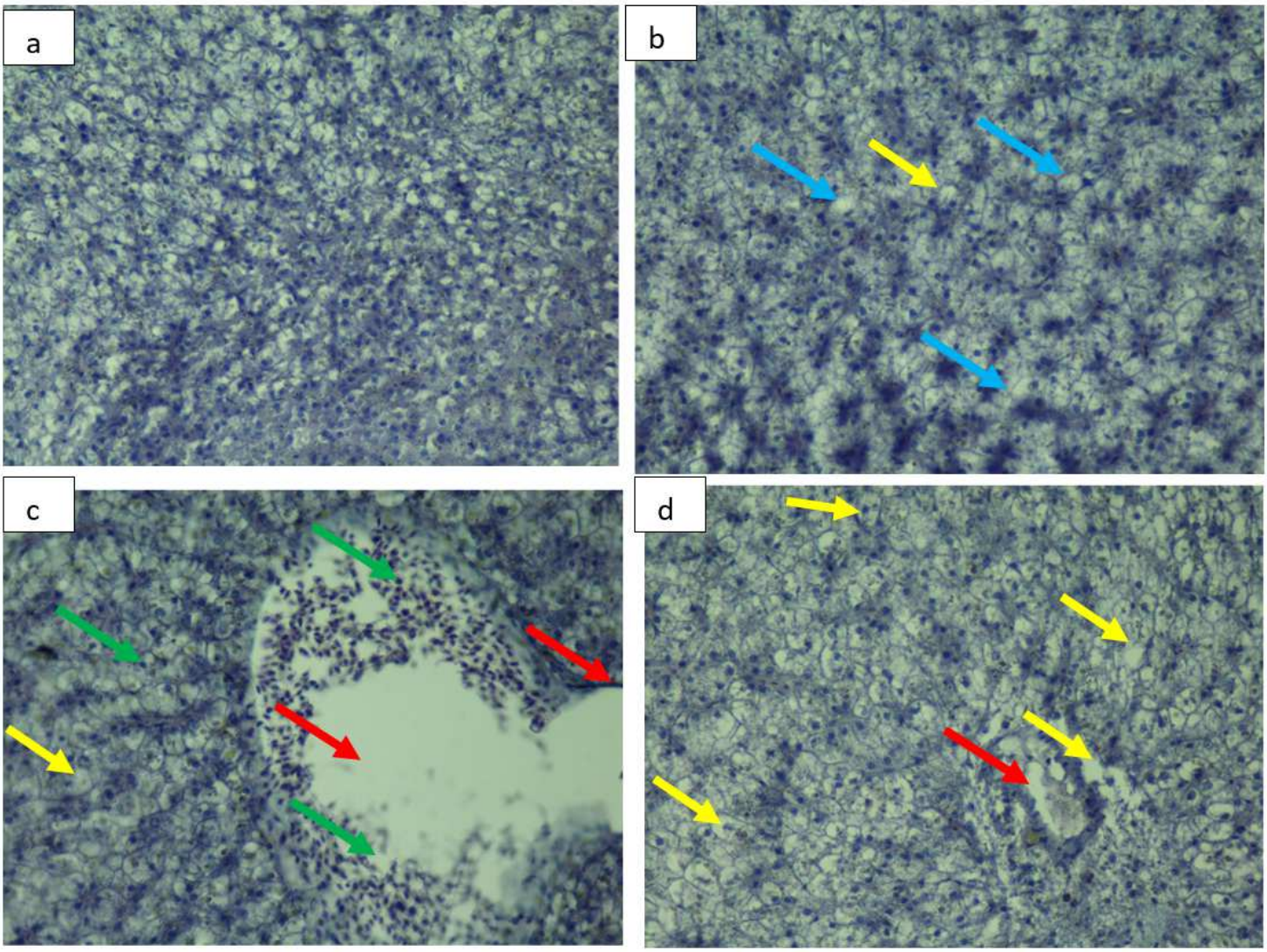

Figure 3

a-d Section of reference and treated fish liver. (a) The liver of control fish showing normal arrangement and distribution of hepatocytes. (c) to (d) Reference treated liver showing necrosis (blue arrows) and apoptosis (yellow arrows) with condensed nuclear bodies (green arrows) as indicated by black arrows, sinusoid spaces in relation to parenchyma (red arrows).

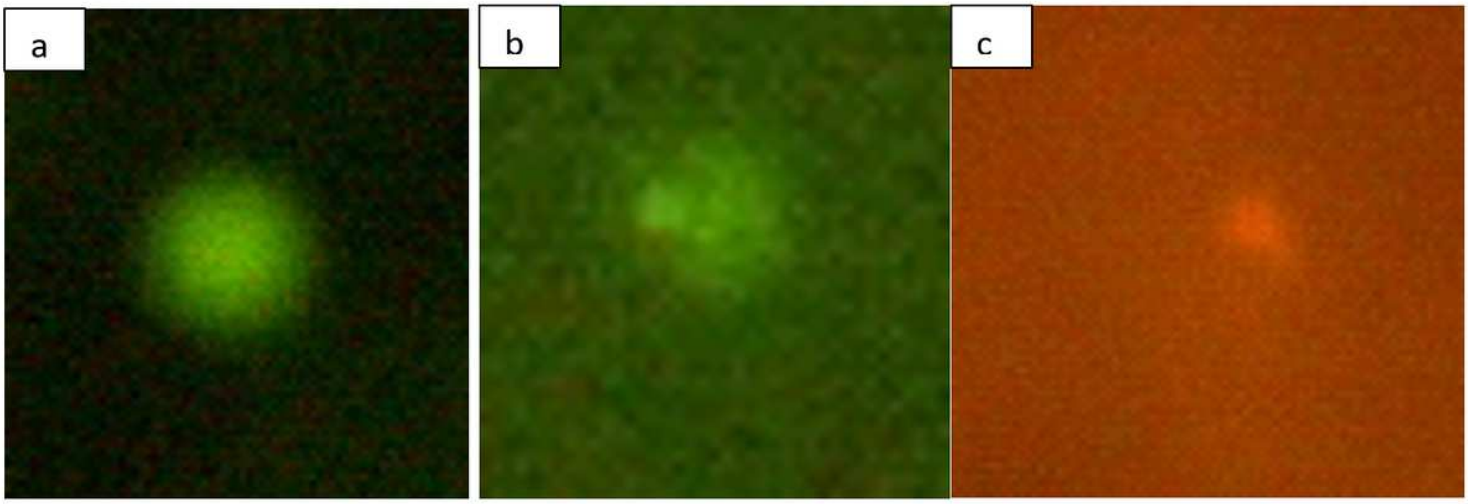

Figure 4

Evaluation of genotoxicity of TiO2-NPs in erythrocytes through Comet assay (a) normal erythrocytes (b) slightly damage erythrocyte (c) Large comet cell

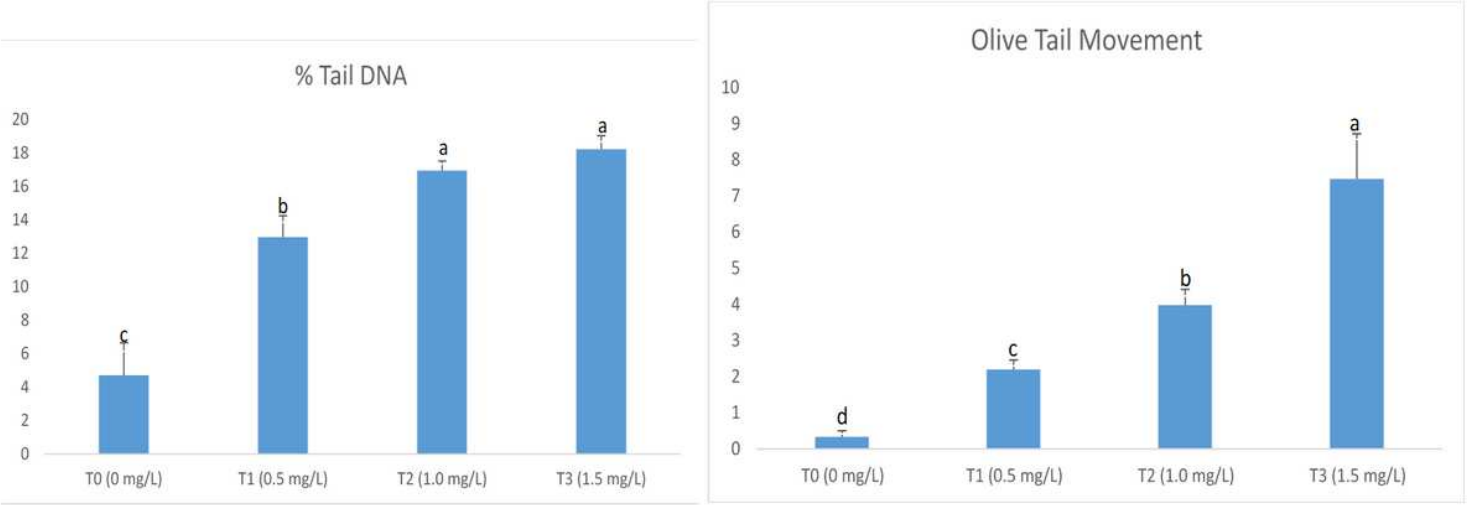

Figure 5

Genotoxicity of TiO2-NPs in erythrocytes. (a) Comets percentage of tail DNA and (b) olive tail movement Data are expressed as mean ± S.D ($p<0.05$) (N=10)