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EFFECT OF TIO_2 NANOPARTICLES ON THE HISTOLOGY OF GILLS AND LIVER OF THE FRESHWATER FISH *RASBORA DANDIA* (VALENCIENNES)

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Abstract: Nanoparticles (np) have a wide range of applications in many fields including industry to produce new type of materials with unique physio-chemical properties.TiO_2 np has a wide variety of uses in industry, as additive in food colourants and cosmetics. The aqueous environment is the ultimate recipient for np and there is uncertainty regarding the potential detrimental effects of np in aquatic biota. This paper focuses on the toxic effect of TiO_2 np on the gills and liver tissues of *Rasbora dandia* (Valenciennes). The toxic effect of np was studied by exposing the selected fish to a sub lethal concentration of 200 ppm np suspension and the effect was compared with the bulk form of TiO_2.The test fish were sacrificed and gill and liver tissues were dissected out after 24, 48, 72 and 96 hours for histopathological examination. The exposure to TiO_2 np resulted in epithelial separation, thickening and fusion of secondary gill lamellae and oedema, haemorrhage and telangiectesis in the secondary gill lamellae. The results of light microscopic studies showed that exposure to TiO_2 np affected liver structure with more pronounced changes than fishes exposed to TiO_2 bulk form. Necrosis of liver tissue was prominent in fishes exposed to TiO_2 np at 200 ppm. The degree of distortion of the gill, and liver increased with increasing period of exposure. The results clearly showed the prominent deleterious effects caused by TiO_2 np compared to its bulk form. Histopathology is thus a useful biomarker for aquatic contamination due to nanoparticles.

Key words: Nanoparticles, Ecotoxicology, Sub lethal, Titanium dioxide (TiO_2), *Rasbora*, gills, liver, histopathology.

INTRODUCTION

Nanotechnology has emerged as a recent advancement in the field of medicine, textile, pharmaceuticals, cosmetics and environmental remediation providing new engineered nano-enabled products, constituted by nanoparticles (np), with novel and unique functions that reach the market every day. These materials are increasingly being used for commercial purposes such as fillers, opacifiers, catalysts, water filtration, semiconductors, cosmetics, microelectronics etc. As the production and manufacture of these nanoscale products increases, the possibility for human exposure and unintentional release into the aquatic environment also increases eliciting an impact on the ecosystem and also to human health. Nanoparticles are materials with one or more dimensions in the nanoscale in the range of 1-100nm (Farre *et al*., 2009). A comparison study among different np showed that TiO_2 np were the most likely to enter the natural environment than nanoparticles of ZnO, Ag, Carbon nanotubes and fullerene nanoparticles (Gottschalk *et al*., 2009). Titanium dioxide, also known as titanium (IV) oxide, titania and TiO_2, is the naturally occurring oxide of titanium.TiO_2 np have been widely used in many products, such as toothpastes, sunscreens, cosmetics, food products, pharmaceuticals, nanomedical reagents and paint industries as a coloring material because of its high stability, anticorrosion, and photocatalytic properties (Long *et al*., 2007). However recent research suggest that TiO_2 np may possess higher toxic potential than their bulk counterparts (Magaye and Zhao, 2012; Khidr and Mekkawy, 2008). It is therefore important to study the effect of TiO_2 np on the organism's health as well as the pathogenic mechanisms involved. Because of its

increasing use and release into the environment, nano-sized TiO_2 could potentially provoke effects on a variety of organisms in different ecosystems. The objective of the present study was to assess the toxic effect of TiO_2 np and its bulk forms on gill and liver tissues of fish, *Rasbora dandia* (Valenciennes) exposed to sub lethal concentration at different time intervals.

Fishes have become a valuable indicator for evaluation of toxic effect of chemicals. The application of eco toxicological studies on non-mammalian vertebrates is rapidly expanding (Khidr and Mekkawy, 2008). Histopathology can be used as biomonitoring tool for assessing the degree of pollution, particularly for sublethal and chronic effects for evaluation of toxicity studies (Meyers and Hendricks, 1985).

The aim of present study was to assess the degree of histopathological alterations in the gills and liver of the selected fish on exposure to sub lethal concentrations of TiO_2 np and its bulk forms. Gills in fishes are in direct contact with xenobiotics and thus the toxic effects are usually more pronounced in gill tissues. Liver being the major organ involved in xenobiotic metabolism and excretion was considered ideal for the study.

MATERIALS AND METHODS

Rasbora dandia, a common freshwater fish in Kerala, which is very sensitive to toxicants and available throughout the year was used as the experimental fish (average length 6 –7 cm, average weight 9 g) for the present study. Fishes were collected from local ponds near Thiruvananthapuram District, Kerala, India and they were safely transported to the laboratory in well-packed polythene bags containing oxygenated water. This fish is often harvested with other smaller fish as food, locally, and for use in poultry feed. Fishes were stocked in a large tank containing dechlorinated tap water and acclimatized for two weeks before they were exposed to np. During acclimation, fishes were fed with commercial fish feed. Water (three fourths) was replenished every 24 h to maintain a healthy environment. Water quality characteristics were determined, which were as follows: temperature 27.5 ± 1.5 °c, pH 7.5 ± 0.03, dissolved oxygen 6.4 ± 0.2 mg/l.

Chemicals and Reagents

Analytical grade TiO_2 np powder (Anatase) and TiO_2 bulk form (purity 99.9% and 99.7% respectively) were used for the study. One g/L stock suspension of TiO2 np (dry powder of TiO_2) were made by dispersing the np in ultrapure (Millipore) water by sonication (bath type sonicator, 35 kHz frequency, Fisher brand FB 11010, Germany) for 30 min. Working solutions were prepared from the stock solution using dechlorinated tap water as dilution water. Working solutions were again sonicated and checked for the dispersion of particles using TEM (JEOL, Japan).Six fishes were exposed to a sublethal concentration of 200 ppm(LC_{50} 398 ppm for TiO_2 np and 570 ppm for TiO_2 bulk) of the TiO_2 np and bulk forms in 5 L glass aquarium tanks. A control was maintained without adding the toxicants.

Histopathology Studies

Fish tissues were dissected out after 24, 48, 72 and 96 hours and prepared for histopathological observations. They were fixed in bouin's fluid for 24 hours. The fixed tissues were washed in distilled water, dehydrated in graded ethanol series (30 %,50 %,70 %,80 % and 100 %) cleared in xylol, infiltrated with melting wax and embedded in paraffin at 56-58°c. Serial sections of the tissues were cut at 5µm thickness using rotary microtome with disposal blade. From the water bath sections were transferred to albumin and glycerin (1:1) coated slides and stained with haematoxylin and eosin. The stained sections were washed in running tap water, dehydrated in ethanol, cleared in xylene and mounted with DPX mountant. The stained section were examined and photographed at 400x magnification using Nickon photomicroscope. The changes in the tissues of the treated fishes were observed and compared with the control.

RESULTS

TEM analysis demonstrated that bulk and np exhibited a spherical shape and the average primary particle size of each material type was 20 nm range in diameter for nanoparticle and 200 nm size range for bulk form of TiO_2 (Fig.1 & 2). In the present study, the gills of *R. dandia* showed

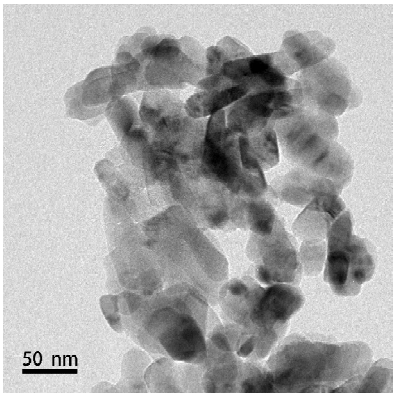

Fig. 1. TEM images of TiO_2 np

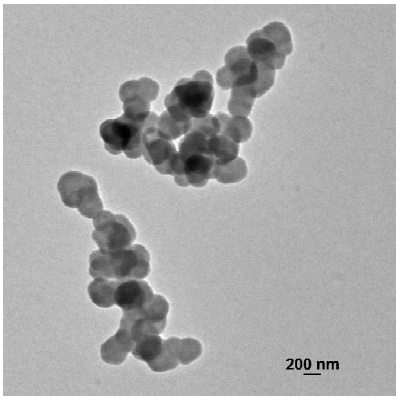

Fig. 2. TEM images of TiO_2 bulk form

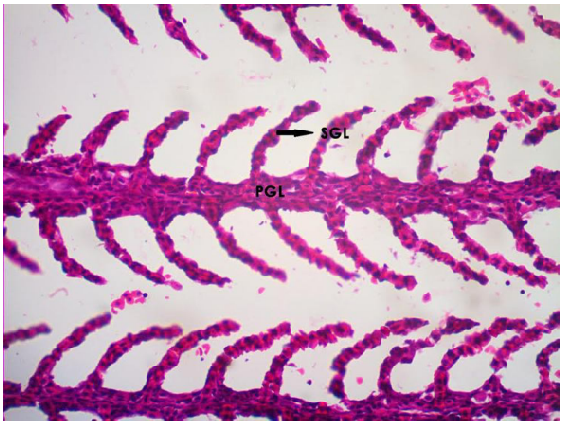

Fig. 3a. Photomicrograph of control gills of *R. dandia* showing normal gill architecture with primary gill lamellae (PGL) and secondary gill lamellae (SGL).

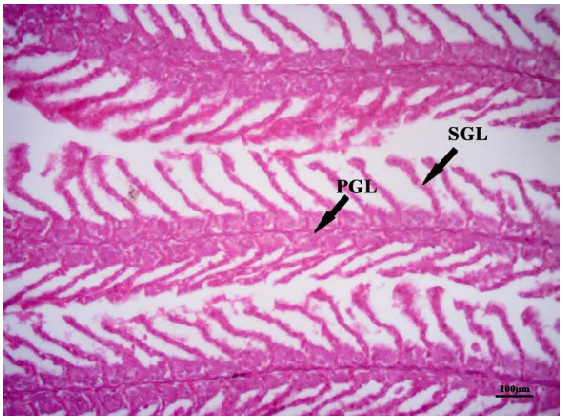

Fig. 3b. Photomicrograph of gills of *R. dandia* exposed to TiO_2 bulk form after 24 hours showing primary lamellae and secondary lamellae.

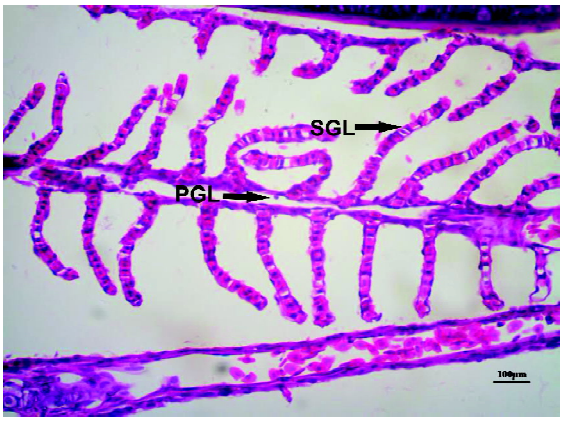

Fig. 3c. Photomicrograph of gills of *R. dandia* exposed to TiO_2 bulk form after 48hours showing PGL and SGL.

Fig. 3d. Photomicrograph of gills of *R. dandia* exposed to TiO_2 bulk form after 72hours showing haemorrhage (HE) and clubbed tips of secondary lamellae (C.SGL).

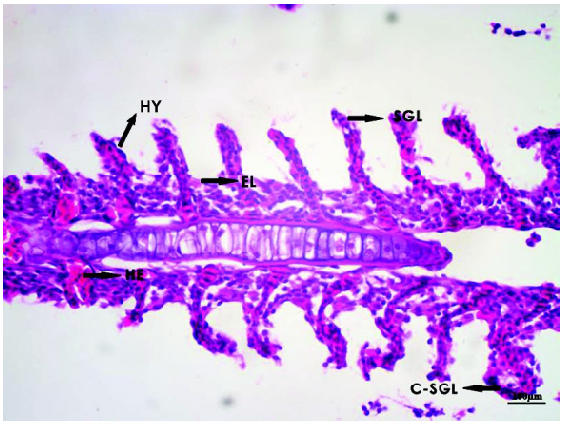

Fig. 3e. Photomicrograph of gills of *R. dandia* exposed to TiO_2 bulk form after 96 hours showing thickening of primary lamellae, epithelial lifting (EL), haemorrhage (HE) and clubbed tips of secondary lamellae (C.SGL), hyperplasia(HY)

a normal architecture of primary and secondary gill lamellae in the control (Fig.3a). Many histopathological changes were observed in the gills of fishes exposed to both TiO_2 bulk and nano forms. However, the changes were more prominent in the gills of fishes exposed to TiO_2 np. The major changes in the TiO_2 np treated fishes were architectural loss, necrosis, desquamation of epithelial layer, lamellar shortening, haemorrhage and telangiectesis (localized dialation of blood vessels) whereas haemorrhage, lamellar clubbing and hyperplasia were the notable changes in the gills exposed to TiO_2 bulk form.

There were no significant changes in the gills of *R. dandia* after 24 and 48 hours of exposure to TiO_2 bulk form (Fig.3b-c). Some degenerative changes in gill structure were observed in the gills of *R. dandia* exposed to TiO_2 bulk at 72 and 96 hours (Fig.3d-e). But gills exposed to nano form of TiO_2 showed some epithelial lesions after 48 hrs. The most common feature in TiO_2 np exposed fishes were structural changes like fusion of the primary and secondary lamellar epitheliums and proliferation in the epithelium of gill filaments and secondary lamellae, resulting in the fusion of secondary lamellae. Very conspicuous haemorrhages and telangiectesis were observed in the secondary lamellae at 72 hrs of exposure to TiO_2 np. Also degenerative and necrotic changes in the epithelium of gill filaments and secondary lamellae, and oedema in secondary lamellae accompanied with separation of their epithelium from the lamellar supporting cells were observed at 96 hours of exposure to TiO_2 np (Figs.4a-d).

Hepatocytes in the control fish were observed as polyhedral cells having a distinctive central nucleus with densely staining chromatin and a prominent nucleolus. Sinusoids were present irregularly distributed between the polygonal

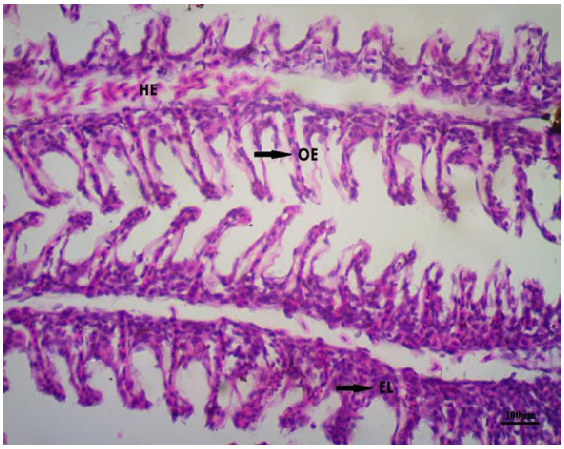

Fig. 4a. Photomicrograph of gills of *R. dandia* exposed to TiO_2 np form after 24hours showing haemorrhage (HE), epithelial lifting (EL), Oedema (OE).

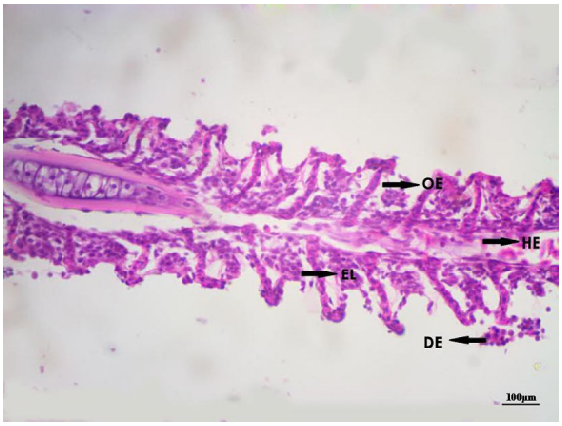

Fig. 4b. Photomicrograph of gills of *R. dandia* exposed to TiO_2 np form after 48hours showing haemorrhage (HE),epithelial lifting (EL), Oedema (OE), desquammation(DE).

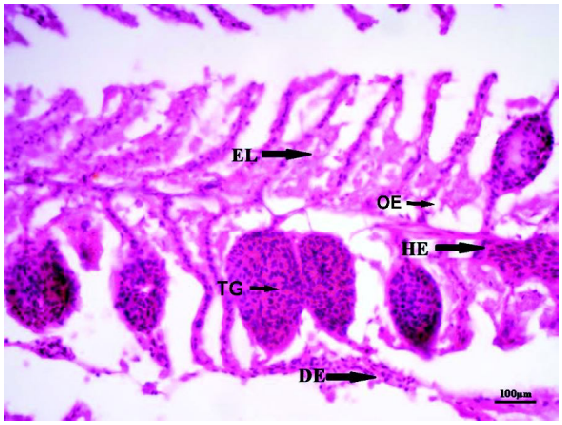

Fig. 4c. Photomicrograph of gills of *R. dandia* exposed to TiO_2 np form after 72 hours showing haemorrhage (HE) epithelial lifting (EL), Oedema(OE), desquammation (DE), telangiectesis (TG).

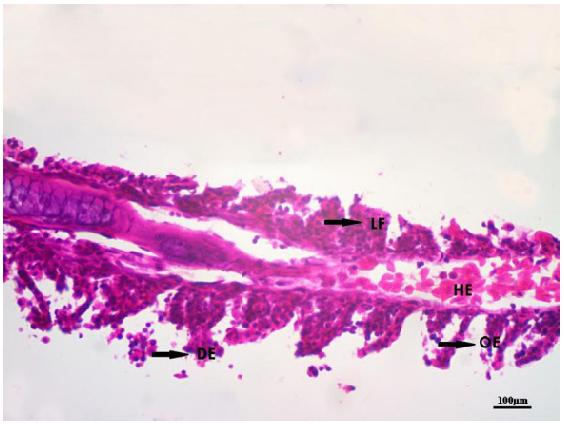

Fig. 4d. Photomicrograph of gills of *R. dandia* exposed to TiO_2 np form after 96hours showing haemorrhage (HE), Oedema(OE), desquammation(DE),lamellar fusion(LF).

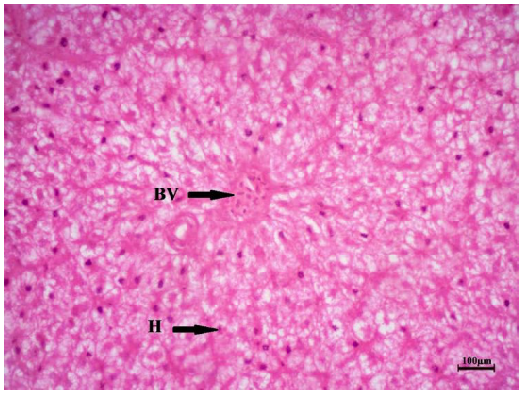

Fig. 5a. Photomicrograph of control liver of *R. dandia* showing normal architecture showing blood vessels (BV) and hepatocytes(H).

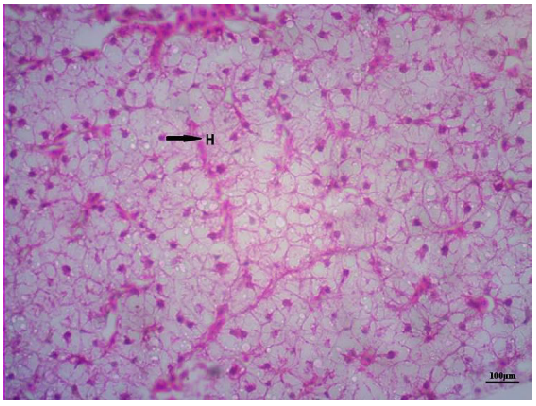

Fig. 5b. Photomicrograph of liver of *R. dandia* exposed to TiO_2 bulk form after 24 hours showing hepatocytes(H).

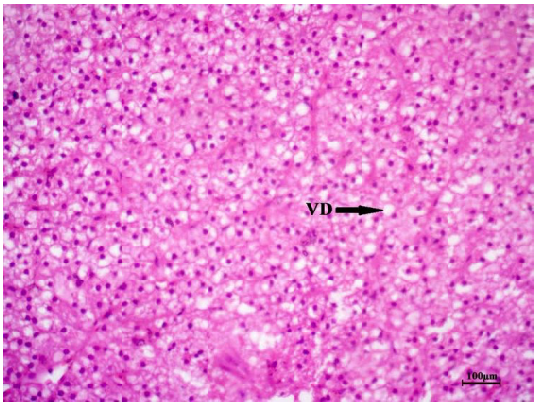

Fig. 5c. Photomicrograph of liver of *R. dandia* exposed to TiO_2 bulk form after 48 hours showing vacuolar degeneration(VD).

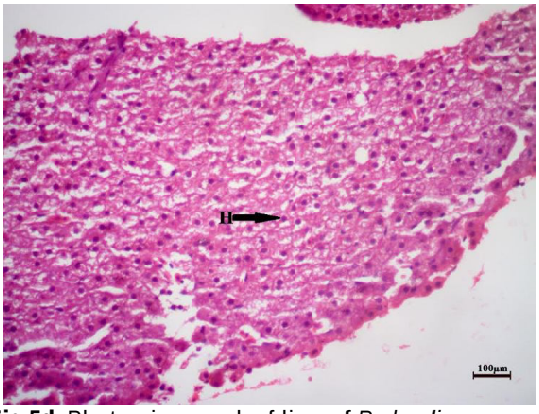

Fig.5d. Photomicrograph of liver of *R. dandia* exposed to TiO_2 bulk form after 72 hours showing hepatocytes.

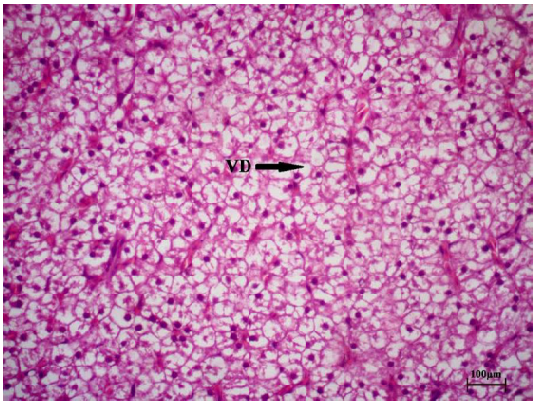

Fig. 5e. Photomicrograph of liver of *R. dandia* exposed to TiO_2 bulk form after 96 hours showing hepatocytes with vascular degeneration (VD).

hepatocytes and are lined by endothelial cells with a prominent nuclei (Fig.5a). Liver tissue exposed to TiO_2 bulk form for four different time periods did not produce any significant histopathological alterations (Fig.5b-e). Vascular degeneration was one of the notable features in TiO_2 bulk exposed fishes. In fishes exposed to TiO_2np, haemorrhage and vacuolization of hepatocytes were observed at 72 hours. At 96 hours of exposure to TiO_2 np, significant changes were noted in the hepatocytes including pyknotic nuclei, focal necrosis, narrowing of sinusoids, irregular shaped nucleus and cytoplasmic degeneration. (Fig. 6a-d).

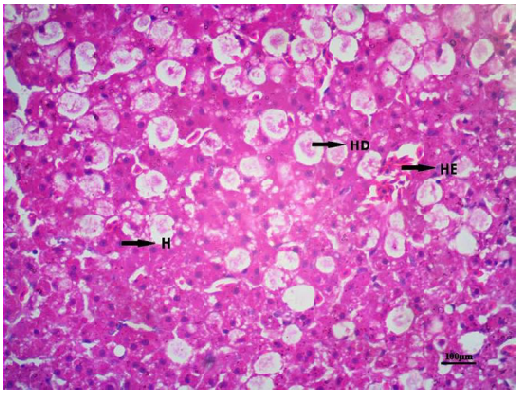

Fig.6a. Photomicrograph of liver of *R. dandia* exposed to TiO_2 np form after 24 hours showing hepatocytes(H), Haemorhage(HE), Hyaline degeneration(HD)

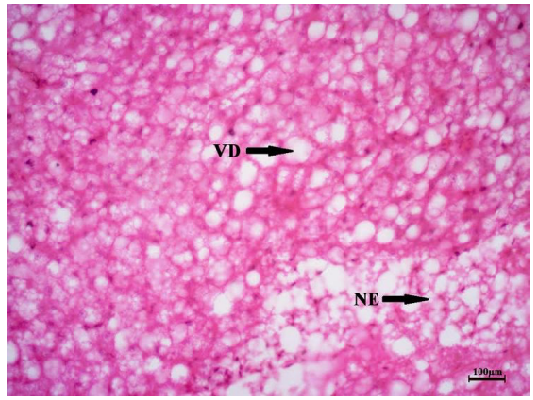

Fig. 6b. Photomicrograph of liver of *R. dandia* exposed to TiO_2 np form after 48 hours showing vascular degeneration (,VD) and necrosis(NE).

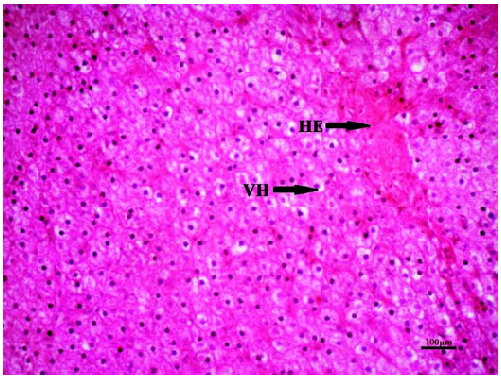

Fig. 6c. Photomicrograph of liver of *R. dandia* exposed to TiO_2 np form after 72 hours showing haemorrhage (HE), Vacuolization of hepatocytes (VH).

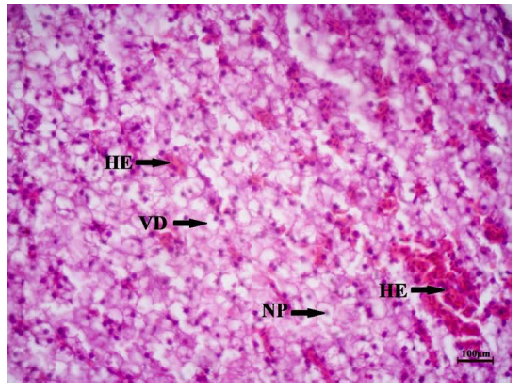

Fig. 6d. Photomicrograph of liver of *R. dandia* exposed to TiO_2 np form after 96 hours showing nuclear pyknosis (NP), haemorrhage (HE), vascular degeneration(VD).

DISCUSSION

Characterization of nanomaterials is particularly important to a full understanding of toxicity mechanisms and effects in a biological system. Proprieties such as size, chemical composition, surface area, shape, solubility and aggregation contribute for nano toxicity. In the present study, toxicant used for the study was TiO_2 of 20 nm size range (np) and the other with 200 nm size range (bulk form).The size ranges were confirmed using TEM analysis. The dispersion of particles was also satisfactory which was assessed by TEM analysis of the water sample. The gill tissue of *R. dandia* used as control showed a normal arrangement of primary and secondary gill lamellae, consisting of numerous gill filaments with two rows of secondary lamellae perpendicular to each filament. Primary lamella were lined by squamous epithelium composed of non differentiated cells. Between the secondary lamellae, the primary lamellae were lined by a thick stratified epithelium. This region contained numerous mucus pavement cells, chloride cells and below the epithelium were lamellar blood sinuses.

Histopathological analysis is a method frequently used to analyse the changes in the target tissue in the aquatic organisms exposed to pollutants (Bernet *et al*., 1999).These are reliable and sensitive indicators of the health status of fishes in the aquatic system and is identified as meaningful indicators of cellular responses to pollutant induced stress. Two types of gill injuries were reported in fishes exposed to xenobiotics, pathological effect resulting from defense response, including hyperplasia of the gill filaments epithelium, oedema of gill lamellae and the second type is the direct injury, including necrosis and shedding of gill epithelium (Richmonds and Dutta, 1989; Fanta *et al.,* 2003; Cengiz and Unlu, 2006).

In the present study, fishes when exposed to bulk form of TiO_2 resulted in clubbed tips and hyperplasia of gill lamellae and epithelial lifting showing defense response of fish to the xenobiotic. Fishes when exposed to TiO_2 np resulted in oedema and haemorrhage after 24 and 48 hours. With increase in time period of TiO_2 np exposure, lamellar fusion, necrosis and disruption in the filaments were observed. Rajkumar and Rahman (2012) observed histopathological conditions in 50 mg/l silver np treated fish gill such as mild congested blood vessels, fused primary lamellae and hyperplasic branchial arch. Exposure to np were reported to cause hyperplasia of gill filaments epithelium and oedema of gill lamellae of carp (Hao *et al.,* 2009), zebra fish (Griffitt *et al.,* 2007; Chen *et al.,* 2011) and rainbow trout (Federici *et al.,* 2007). Farmen *et al.,* (2012) reported necrosis of gill lamellae at high concentrations (100 µg/L) to juvenile Atlantic salmon exposed to Ag nanoparticles. These observations are in accordance with the present observations for TiO_2np. One of the prominent observations made in the present study was lamellar telangiectesis (localized dialation of blood vessel). Lamellar telangiectesis of the secondary lamellae results from the collapse of the pillar cell system and breakdown of vascular integrity with a release of large quantities of blood that push the lamellar epithelium outward (Alazemi *et al*., 1996).

The liver of teleosts function as an important organ in maintaining the internal homeostasis and there by the metabolism of xenobiotics (Chambers and Yarbrough, 1976). Liver is susceptible to damage by toxic agents as it is the site of detoxification of all types of toxic substances (Racicot *et al*., 1975; Soufy *et al*., 2007).The functional integrity of the liver in fish can be affected by xenobiotics (Gingerich, 1982). Hao *et al.,* (2009) reported that the exposure to TiO2 np caused cellular pathologies to liver with increase in time period of TiO2 np exposure which agrees with the present study. In the present study, some pathologies in liver tissue including cell oedema, nuclei deformation, and vacuolar degeneration were observed in bulk treated groups, but cell architecture was not altered. However in fishes exposed to TiO_2 np, many other pathological observations were recorded such as pyknotic nucleus, focal necrosis, narrowing of sinusoids, irregular shaped nucleus and cytoplasmic degeneration.

CONCLUSION

The histopathological changes observed in gills and liver tissues when treated with nano and bulk forms were distinct. The degenerative changes were more pronounced in gill tissue compared to liver as these tissues are in direct contact with the aquatic environment. Compared to the bulk form of TiO_2, the nanoparticles produced much drastic changes in the gill architecture. The histopathological conditions demonstrate the increased adverse effects of exposure to TiO_2 np compared to its bulk form. Thus the present study shows that TiO_2np is highly toxic to *R. dandia* compared to the slight toxic effects of its bulk form.

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