

INTEGRATED TOXICOLOGICAL EVALUATION OF TITANIUM DIOXIDE NANOPARTICLES IN *LABEO ROHITA*: HEMATOLOGICAL AND HISTOLOGICAL INSIGHTS

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ABSTRACT

In the present investigation, titanium dioxide nanoparticles (TiO₂ NPs) were synthesized using suitable laboratory methods to study their toxicological impact. To understand their possible environmental effects, the freshwater fish Labeo rohita was exposed to different environmentally relevant concentrations of these nanoparticles under controlled laboratory conditions. The toxic effects were mainly evaluated through histopathological examination of important organs such as the gills and liver. Microscopic observations revealed noticeable structural changes and tissue damage in the treated fish when compared with the control group. These changes indicated that exposure to TiO₂ nanoparticles can adversely affect normal cellular structure and function. Hematological parameters were also analyzed to support the histological findings. Significant variations were recorded in blood parameters including red blood cells (RBC), white blood cells (WBC), hemoglobin content, and other related indices. The degree of alteration increased with increasing concentrations of nanoparticles, suggesting a clear dose-dependent response. In addition to cellular changes, exposed fish showed mild behavioral differences compared with untreated fish. Overall, the findings indicate that titanium dioxide nanoparticles may cause physiological and tissue-level disturbances in aquatic organisms. Therefore, continuous monitoring and careful evaluation of nanoparticle contamination in aquatic environments are important for protecting aquatic life and maintaining ecosystem balance.

Keyword: - TiO₂NPs; Freshwater fish; Nanoparticle toxicity; Environmental risk assessment

1. INTRODUCTION

The expansion of nanotechnology into sectors such as medicine, agriculture, industry, and environmental remediation has considerably elevated the production and environmental dissemination of engineered nanoparticles (ENPs). These nanoscale materials, owing to their high surface area, unique physicochemical properties, and enhanced reactivity, can interact with biological systems in unpredictable ways [1-4]. The increasing use of nanomaterials in commercial and industrial products has consequently led to their inevitable release into natural ecosystems, particularly aquatic environments, raising concerns regarding their environmental fate and ecological consequences [5-7].

In aquatic systems, the interaction of these nanomaterials with their environment is a source of concern since these nanomaterials accumulate in water bodies and may result in ecotoxicity and toxicity in aquatic organisms [8-10]. Various types of engineered nanoparticles, including metal and metal oxide nanoparticles, have been detected in aquatic ecosystems due to industrial discharge, wastewater effluents, and agricultural runoff [11,12]. These nanomaterials, for instance, such as silver (Ag), titanium dioxide (TiO₂), zinc oxide (ZnO), nickel (Ni), and

chromium oxide (Cr_2O_3), have been identified in aquatic systems and are causing concern in the field of ecotoxicology since they can induce oxidative stress, cellular damage, and organ dysfunction in aquatic animals [13-16]. Freshwater fish, particularly *Labeo rohita*, are widely used as model organisms in ecotoxicology because of their ecological relevance, commercial importance, and sensitivity to environmental contaminants [4,17,18].

Fish were considered effective bioindicators of aquatic pollution since changes in their physiological and biochemical parameters reflected environmental stress caused by toxic substances [19,20]. Recent studies have documented nanoparticle-induced behavioural disturbances, altered hematological parameters, genotoxic effects, and histopathological changes in *Labeo rohita* and other carp species following sub-chronic exposure [21,22,15,16]. For instance, experimental exposure to TiO_2 nanoparticles has demonstrated significant oxidative stress responses, immunotoxicity, and bioaccumulation in fish tissues such as liver and gills, leading to inflammation and cellular damage [13,23,4]. Similarly, assessments of nickel and chromium oxide nanoparticles have reported significant histological and hematological alterations in *Labeo rohita*, accompanied by dose-dependent reductions in key blood parameters and physiological disturbances [18,15,24].

Despite this advancing body of research, systematic evaluations of nanoparticle toxicity across multiple biological endpoints, especially integrating behavioural, hematological, and histopathological biomarkers, remain limited [16,21,20]. In addition, most studies are based on a single nanoparticle, whereas a comparison between the effects of different engineered nanoparticles after standardized exposure is required, making it difficult to perform a comprehensive ecological risk assessment for nanoparticles [11,12]. This limitation creates challenges in the development of accurate risk assessment frameworks and regulatory strategies for the release of nanoparticles in freshwater ecosystems [10,25]. Thus, the present study was designed to bridge this gap by assessing the dose dependent toxic effects of nanoparticles on *Labeo rohita* using a multi biomarker approach integrating behavioural, hematological, and histopathological parameters, which is essential for understanding nanoparticle induced systemic toxicity in aquatic organisms [23,24,26].

Fish are used as a model system to evaluate the ecological effect of nanoparticles, as they are more likely to come into contact with nanoparticles in the aquatic environment. However, the toxicological profile of most nanomaterials remains unknown, as previous reports only focused on a small number of different nanoparticles. In this context, this study aims to carry out a comparative study of Titanium dioxide (TiO_2) nanoparticles, which are synthesized in the laboratory. These nanoparticles are characterized by widely used methods, namely UV-Visible Spectroscopy, DLS, Zeta Potential, and SEM, to confirm the size, morphology, and stability of the synthesized nanoparticles. *Labeo rohita* was used as the experimental model to study qualitative as well as quantitative histopathological changes, along with hematological changes, under controlled conditions of exposure. The study aims to improve the understanding of the mechanism of nanoparticle toxicity, which will be helpful in understanding the health and physiological effects of the exposure of fish to engineered nanomaterials.

2. MATERIALS

Titanium isopropoxide (TTIP); Ethanol ($\text{C}_2\text{H}_5\text{OH}$); Deionized water; Nitric acid (HNO_3); Haematoxylin and Eosin. The reagents and solvents were of analytical grade, while the solutions were prepared using highly purified deionized water.

3. METHODOLOGY

3.1 Synthesis of TiO_2 NPs

After adding 30 mL of ethanol and 10 mL of titanium tetraisopropoxide (TTIP) to a flask, the mixture was swirled for 60 minutes at 50–60 °C to obtain a homogeneous precursor solution [27,28]. Next, 150 mL of deionized water and 3 mL of nitric acid (HNO_3) were combined, and the mixture was gradually added drop by drop to the TTIP solution while being continuously stirred at 50–60 °C to facilitate hydrolysis and condensation reactions [29,30]. To create crystalline TiO_2 nanoparticles, the final solution was heated to 100 °C for 24 h until the solvents evaporated and a gel-like residue was formed [28,31]. The obtained residue was then annealed at 600 °C for 4 hours to enhance crystallinity and phase formation of TiO_2 nanoparticles [29,30].

3.2 Characterization Techniques

To know the properties of the synthesized nanoparticles, different analytical techniques were used. To study the optical properties, the colloidal solutions were analyzed using a UV-Visible spectrophotometer (MOTRAS Scientific) in the range of 200–900 nm. To study the particle size and surface charge, dynamic light scattering and zeta potential measurements were performed using Malvern Zetasizer Nano-ZS 90 equipment. To study the detailed morphology, the nanoparticles were characterized using a field emission scanning electron microscope (FE-SEM, JEOL JSM-7600F) [15].

3.3 Toxicological analysis

For in vivo studies, fingerlings of *Labeo rohita* weighing between 12 and 17 ± 2 g were procured from NCP Aqua Pvt. Ltd., Navsari, Gujarat, India. The fish were subjected to acclimatization for 15 days prior to the experiment in dechlorinated tap water under laboratory conditions. During the acclimatization period, the fish were fed commercial pellets daily. The fish were not fed 24 hours prior to the experiment to standardize the experiment conditions and to minimize metabolic variations [15].

3.4 Behavioral Assessment

Behavioral responses of *Labeo rohita* fingerlings were carefully monitored during sub-chronic exposure to TiO₂ nanoparticles, as behavioral alterations are considered early and sensitive indicators of environmental stress in fish. During the exposure period, the fish exhibited noticeable changes in swimming patterns, including reduced activity, erratic movements, increased surface swimming, and occasional loss of equilibrium. Such behavioral disturbances may occur due to physiological stress caused by nanoparticle accumulation in vital organs such as the gills and brain, which can interfere with normal respiratory and neurological functions [23].

3.5 Histopathological Assessment and Lesion Scoring

After this, the fish were sacrificed, and the vital organs, such as the gills and liver, were removed for further histopathological analysis. These tissues were then preserved by fixing them in 10% neutral buffered formalin. After this, the tissues are dehydrated by subjecting them to a series of ethanol solutions, clearing them with xylene, and finally embedding them in paraffin wax. Thin sections with a thickness of 5 μ m are obtained by cutting the tissues with a rotary microtome. These sections are stained with hematoxylin and eosin (H&E) stain to visualize the tissues clearly. These sections are then examined under a light microscope to identify the changes. Careful attention is given to changes such as epithelial lifting and fusion occurring in the gill tissues, as well as changes such as hepatocellular degeneration, vacuolation, and necrosis occurring in the liver. Histopathological changes are a way to identify the tissue specific toxicity and damage caused by the nanoparticles. Such changes have been noted by other researchers on previous works on the toxicity of TiO₂ nanoparticles to fish [32].

3.6 Haematological Responses

For the hematological analysis, the blood was collected from the caudal peduncle vein of the fish using sterile medical syringes in order to avoid any kind of stress and contamination of the blood sample. Then the blood was immediately used for the analysis without the addition of anticoagulant diluent in order to get accurate results during the analysis of the blood cells. Quantification of total red blood cells (RBC) and white blood cells (WBC) in fish was performed to know their oxygen transport ability and immunity status, respectively. Hemoglobin concentration and packed cell volume were also measured to know their circulatory ability. Erythrocyte indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated to know their erythrocyte morphology and to detect any signs of anemia or stress in fish by standardized fish hematological methodology [33].

4. RESULTS & DISCUSSION

4.1 Characterization of Prepared NPs

This is the EDX spectrum of the developed materials that gives information on the qualitative analysis of these nanoparticles and also shows the quantitative analysis whereby Cr, Cl, C, and O were detected in the samples. So, for all the samples under investigation, we have no additional information from the EDX spectra indicating that there are extra impurities present. Similar observations have been reported in recent studies, where the absence of extraneous elemental signals in EDX analysis confirmed the controlled synthesis and compositional stability of engineered nanoparticles [4,34].

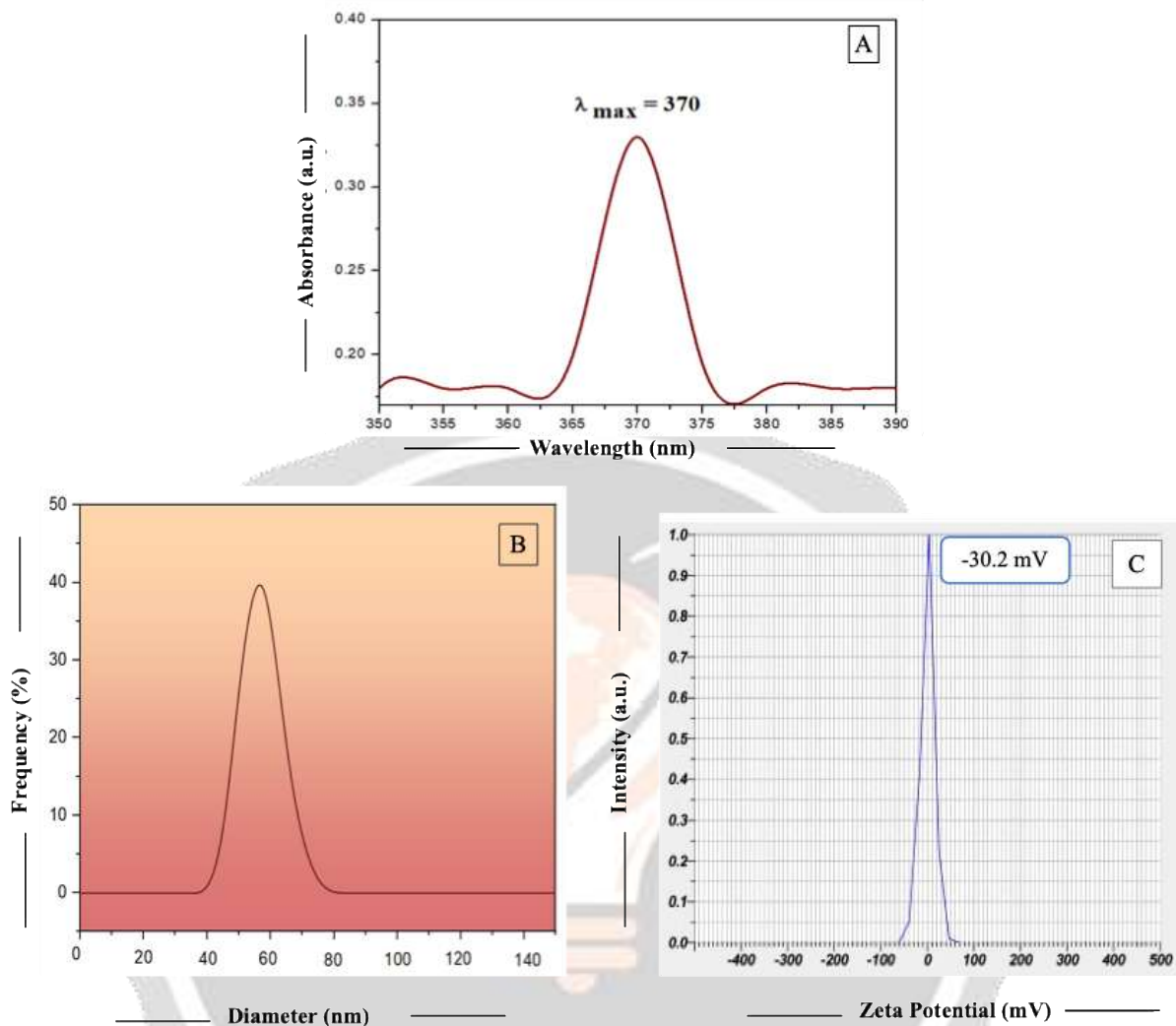


Fig - 1. Graphical representation of (A) UV-visible absorption spectra, (B) DLS (C) Zeta Potential of TiO₂ NPs

The UV-visible absorption spectrum recorded in the wavelength range of 350–390 nm. A prominent absorption peak was observed at $\lambda_{max} = 370$ nm, (**fig. 1A**) confirming the successful formation of nanoparticles. The presence of a distinct and symmetric absorption band suggests uniform particle formation and minimal aggregation in the colloidal suspension. The optical absorption behavior corresponds to characteristic electronic transitions associated with nanoscale metal oxide materials, indicating controlled synthesis and stability of the particles. The particle size distribution obtained through DLS analysis. The synthesized nanoparticles exhibited an average hydrodynamic diameter in the range of approximately 55-60 nm, (**fig. 1B**) with a relatively narrow distribution curve. This indicates moderate monodispersity and suggests that the particles remain well-dispersed in aqueous medium. The hydrodynamic size includes the core particle along with its surrounding solvation layer, reflecting the effective size under biological exposure conditions. The zeta potential profile of the nanoparticle suspension, revealing a surface charge of -30.2 mV (**fig. 1C**). This negative surface potential indicates moderate electrostatic stability and suggests a reduced tendency for rapid aggregation compared to near-neutral systems. Surface charge is a critical parameter influencing colloidal behavior, interaction with biological membranes, cellular uptake, and subsequent toxicological responses.

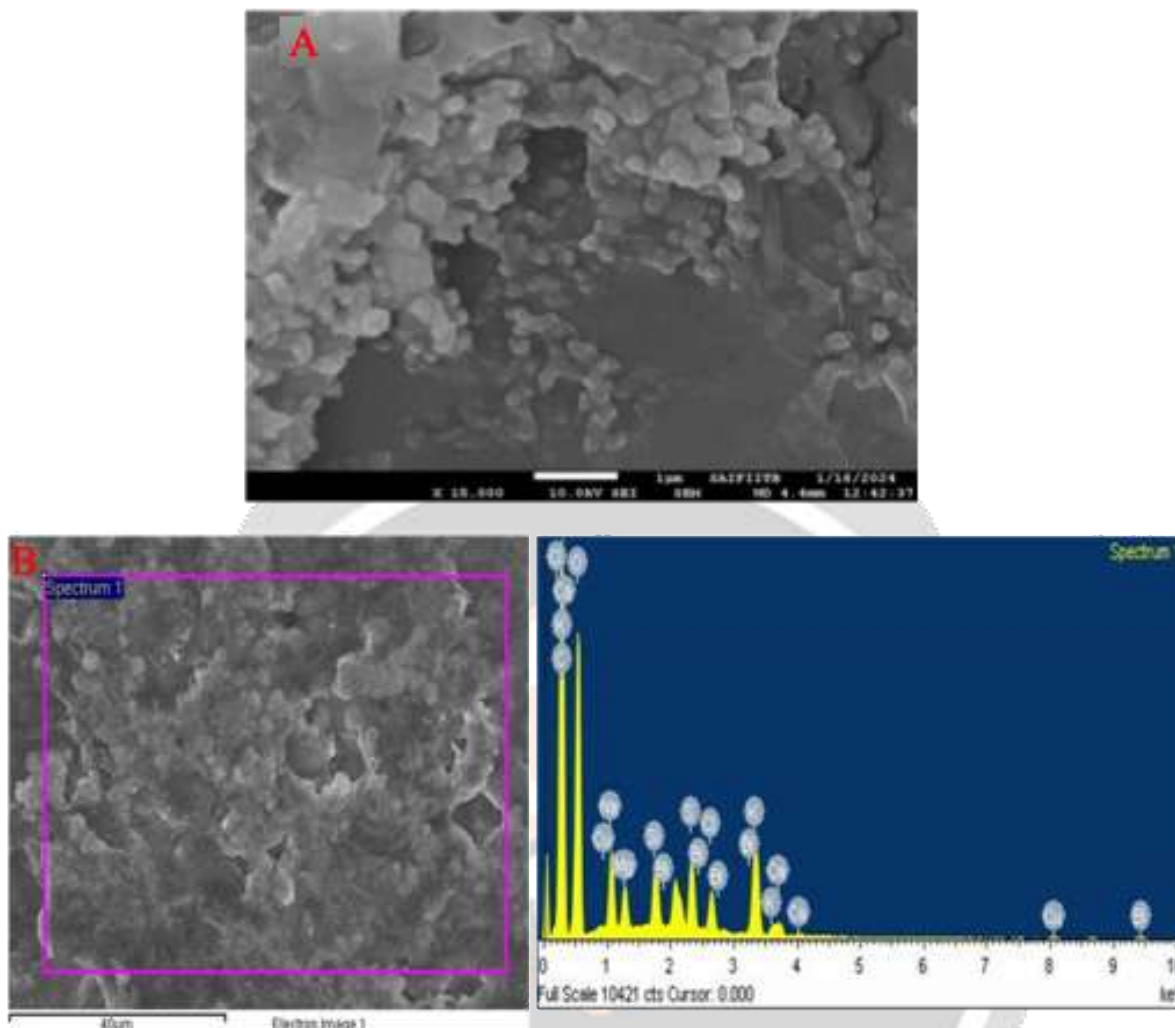


Fig - 2. Graphical representation of (A) SEM & (B) EDX of TiO₂NPs

The figure presents morphological and elemental characterization of the synthesized iron nanoparticles using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS). **Fig. 2A** shows a high-magnification SEM micrograph revealing irregularly shaped, quasi-spherical nanoparticles forming dense clusters. The particles appear fused into agglomerated networks, a common feature of iron-based nanoparticles due to magnetic interactions and high surface energy. Individual particles are distributed in the nanometer scale, and their rough surface texture suggests nucleation followed by rapid growth and partial coalescence during synthesis. **Fig. 2B** shows a lower magnification SEM image illustrating the overall surface topology and distribution of particles across the sample area, indicating relatively uniform coverage without large crystalline impurities and supporting the formation of a continuous nanoparticulate layer, along with the corresponding EDS spectrum obtained from the selected region. Prominent peaks corresponding to iron confirm the elemental composition of the nanoparticles, while minor signals from oxygen indicate surface oxidation typical of iron nanoparticles exposed to air. Additional low-intensity peaks may arise from the substrate or stabilizing agents. Collectively, these analyses confirm the successful synthesis of iron-based nanoparticles with nanoscale morphology and expected elemental composition suitable for subsequent toxicological investigations [34].

4.2 Acute toxicity tests

To determine the 96-h median lethal concentration (LC_{50}) of titanium dioxide nanoparticles (TiO_2 NPs), fingerlings of *Labeo rohita* were exposed to graded concentrations following standard probit analysis procedures. Fish were randomly assigned to experimental aquaria (10 L capacity) in groups of ten individuals per treatment, and each concentration was tested in triplicate to ensure statistical reliability. Mortality data were recorded at regular intervals, and LC_{50} values were calculated from concentration–mortality regression plots. Based on the derived LC_{50} , sub-lethal (500 mg/L) and higher exposure levels (700 mg/L) of TiO_2 NPs were selected for subsequent toxicity experiments conducted over 5 and 7 day periods. All exposure groups were maintained in separate tanks under controlled environmental conditions. Fish showing complete loss of opercular movement and absence of response to external stimuli were considered dead and were promptly removed to avoid deterioration of water quality. This approach enabled evaluation of dose-dependent toxic effects of TiO_2 nanoparticles on juvenile fish under controlled laboratory conditions[35].

4.3 Behavioral responses

During the experimental period, behavioural responses of *Labeo rohita* fingerlings exposed to titanium dioxide nanoparticles (TiO_2 NPs) were carefully observed and compared with those of the control group. Observations were recorded twice daily throughout the exposure period, focusing on swimming activity, feeding behaviour, fin movements, and social interaction. Fish in the control group displayed normal behavioural patterns, including active swimming, regular feeding, and coordinated fin movements. In contrast, TiO_2 NPs exposed fish showed noticeable alterations in behaviour. These included increased restlessness, erratic or jerky swimming, frequent surfacing with rapid opercular movements indicating respiratory stress, and abnormal vertical positioning prior to mortality. Such behavioural disturbances suggest that TiO_2 nanoparticle exposure may interfere with normal physiological functioning, leading to stress responses and impaired activity in exposed fish. These behavioural endpoints provided early indicators of nanoparticle-induced toxicity before the onset of mortality[34,36].

4.4 Histological responses

In the histopathology of **gills** lamellar epithelium proliferation and aneurism were observed after 5 days of exposure at lower concentrations of TiO_2 , fig. 3(a). After exposure at higher concentration disruption of the cartilaginous core was distinguished along with marginal channel, fig. 3(c). Epithelial shorting, necrosis and disruption of cartilaginous core were seen at lower concentrations, fig. 3(b) exposure for 7 days whereas, exposure at high concentrations showed in fig. 3(d) dilated and clubbed epithelial, necrosis and hypertrophy. **In the liver**, cytoplasmic vacuolation, fusion and infiltrating of erythrocytes, start in lower concentration exposure to TiO_2 within 5 days, fig. 3(e). Nuclear alteration and lymphocyte infiltration were seen in higher concentration exposure, fig. 3(g). When fingerlings were treated for 7 days, at a lower concentration than hepatolysis micro centre and vacuoles were noted, fig. 3(f), while at higher concentrations alterations like cytoplasmic vacuolation, melano-macrophage centre and necrotic liver tissue were prominent, fig. 3(h). These changes are often interpreted as protective responses to reduce the entry of toxicants; however, they can impair respiratory efficiency and ionic regulation in fish. Similar gill alterations following TiO_2 nanoparticle exposure have been widely reported and are associated with oxidative stress and nanoparticle deposition on gill surfaces [23]. In the liver, which plays a central role in detoxification and metabolism, notable histological changes such as hepatocellular degeneration, cytoplasmic vacuolation, sinusoidal dilation, and necrosis were observed. These alterations suggest disrupted metabolic activity and cellular damage due to the accumulation of nanoparticles and the generation of reactive oxygen species (ROS). Previous studies have demonstrated that TiO_2 nanoparticles can induce oxidative stress-mediated damage in hepatic tissues, leading to impaired liver function in fish [20]. Overall, the observed histopathological changes in both gill and liver tissues highlight the toxic potential of TiO_2 nanoparticles and support their use as reliable biomarkers in assessing nanoparticle-induced stress in aquatic organisms [21,24].

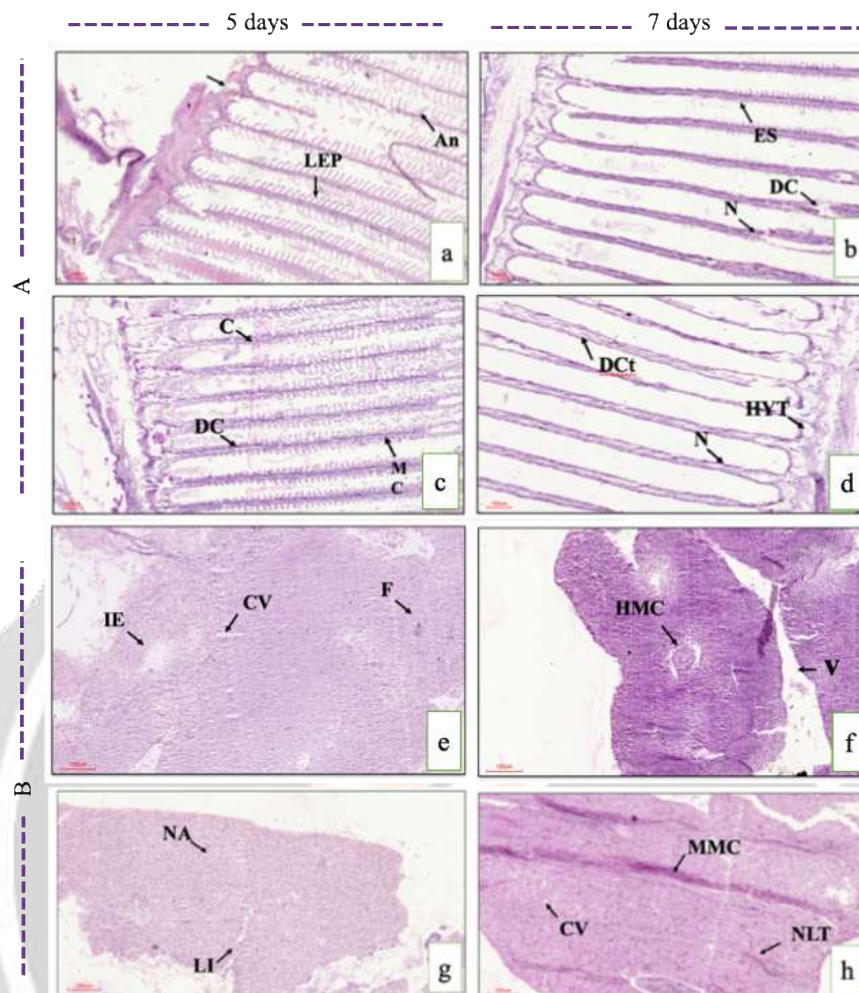


Fig - 3. Representative histological changes in *L. rohita* after exposure of TiO_2NPs
A: Gill a: 5days, b: 7days (lower concentration); c: 5days, d: 7days (higher concentration)
B: Liver e: 5days, f: 7days (lower concentration); g: 5days, h: 7days (higher concentration)
Gill: LEP: Lamellar epithelium proliferation I, An: Aneurism, ES: Epithelial shorting, N: Necrosis, DC: Disruption of cartilaginous core, C: Collapse, DC: Disruption of cartilaginous core III, MC: Marginal channel, DCt: Dilated and clubbed, N: Necrosis, HVT: Hypertrophy.
Liver: CV: Cytoplasmic vacuolation, F: Fusion, IE: Infiltrating of erythrocytes, V: Vacuolated II, HMC: Hepatolysis micro centres, NA: Nuclear alteration, LI: Lymphocytes infiltration, CV: Cytoplasmic vacuolation, MMC: Melano macrophage centre, NLT: Necrotic liver tissue.

4.5 Haematological responses

The hematological profile of *Labeo rohita* fingerlings exposed to titanium dioxide nanoparticles (TiO_2 NPs) was compared with that of the control group to evaluate physiological stress. The analyzed parameters included RBC, WBC, Hb, PCV, MCV, MCH, and MCHC, and the results were presented in graphical form.

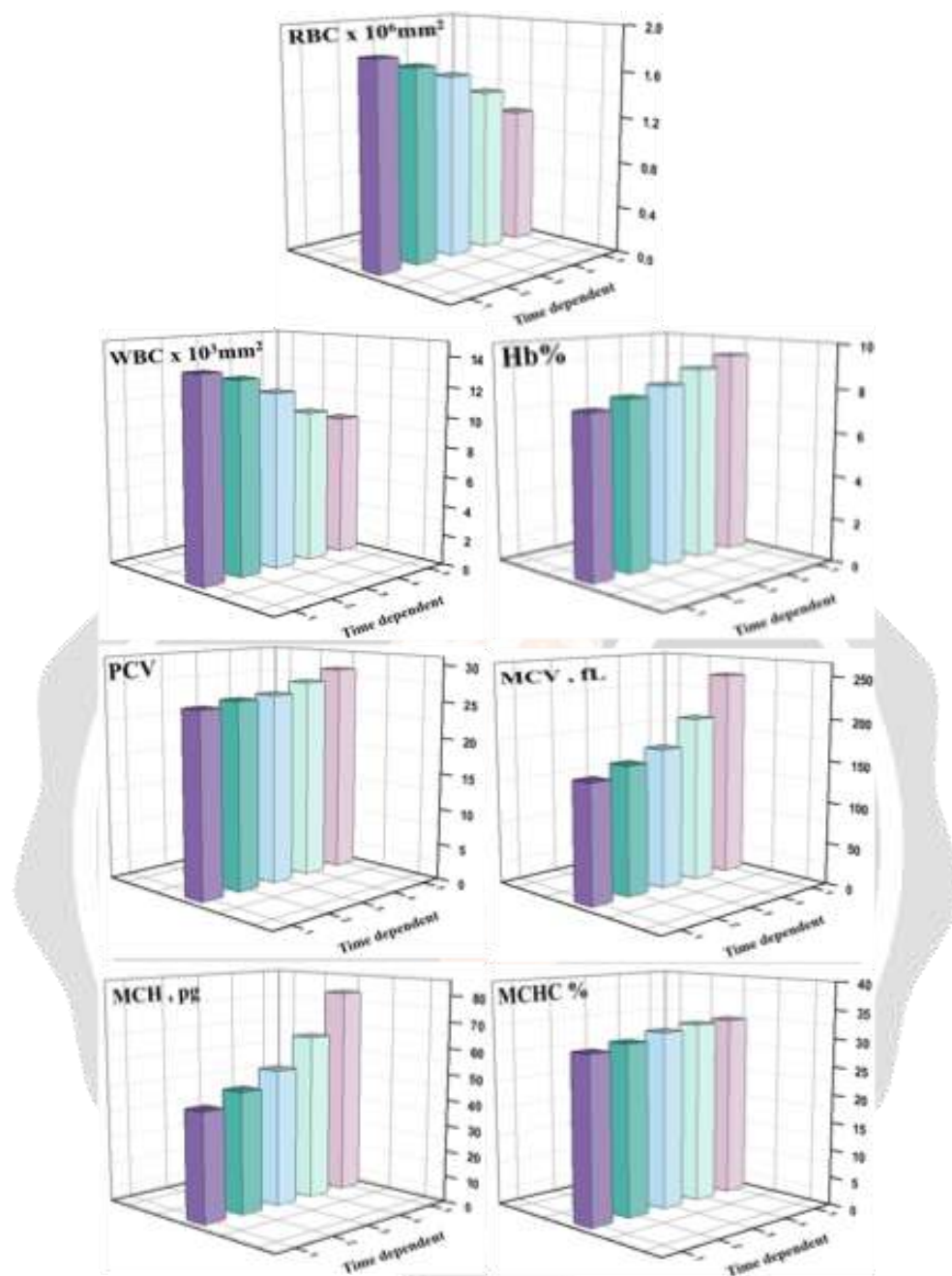


Figure 4. : Haematological parameters of *L. rohita* exposed to TiO₂ NPs as a function of time(days) (■ control, TiO₂ NPs : ■ 5 days LC, ■ 7 days LC, ■ 5 days HC, ■ 5 days HC).

A noticeable reduction in red blood cell count was observed in TiO₂ NPs treated fish compared with the control [23,34]. After 5 days of exposure, RBC values decreased from the control level ($1.78 \pm 0.01 \times 10^6$ cells/mm³) to $1.68 \pm 0.02 \times 10^6$ cells/mm³ in the low-concentration group and $1.39 \pm 0.03 \times 10^6$ cells/mm³ in the high-concentration group. A similar decline was recorded after 7 days, with values of $1.57 \pm 0.03 \times 10^6$ cells/mm³ and $1.17 \pm 0.02 \times 10^6$ cells/mm³ in low- and high-concentration treatments, respectively. White blood cell counts also showed a reduction in exposed fish, indicating possible suppression of immune function. After 5 days, WBC values declined from $13.4 \pm 0.2 \times 10^6$ cells/mm³ in the control to $12.8 \pm 0.2 \times 10^6$ cells/mm³ and $9.4 \pm 0.3 \times 10^6$ cells/mm³ in low- and high-dose groups, respectively. After 7 days, WBC values further decreased to $11.7 \pm 0.3 \times$

10^6 cells/mm³ and $9.4 \pm 0.3 \times 10^6$ cells/mm³. Hemoglobin concentration showed moderate fluctuation but generally declined relative to the control, suggesting reduced oxygen-carrying capacity. PCV values also indicated mild decreases under TiO₂ exposure, reflecting possible anemia like conditions [18,24]. In contrast, MCV, MCH, and MCHC values showed gradual increases with exposure duration, likely due to compensatory changes in erythrocyte size and hemoglobin content in response to reduced RBC counts. Overall, these hematological alterations indicate that TiO₂ nanoparticle exposure can disrupt normal blood physiology in *Labeo rohita*, reflecting stress-induced metabolic imbalance and impaired oxygen transport. Such changes provide useful biomarkers for assessing nanoparticle toxicity in freshwater fish [34].

5. CONCLUSIONS

The present investigation demonstrated that exposure to titanium dioxide nanoparticles (TiO₂ NPs) can induce measurable toxic effects in *Labeo rohita* fingerlings under laboratory conditions. Acute toxicity analysis showed a clear dose dependent response, while behavioural observations revealed early signs of stress such as restlessness, erratic swimming, increased surface respiration, and abnormal positioning before mortality. These behavioural changes were supported by evidence of physiological disturbance, indicating that TiO₂ nanoparticles can interfere with normal metabolic and respiratory functions in exposed fish. The study also suggests that the toxicity of TiO₂ nanoparticles is influenced by their physicochemical characteristics, including particle size, aggregation behaviour, and surface properties, which determine their interaction with biological tissues. Oxidative stress is likely to be a major mechanism underlying toxicity, leading to cellular damage and impairment of organ function. Although mortality occurred only at higher concentrations, sub lethal exposure produced significant behavioural and physiological alterations, highlighting the ecological risk posed by nanoparticle contamination. Overall, the findings emphasize the need for careful monitoring of nanoparticle discharge into aquatic environments and for establishing safe exposure limits. Further long-term studies on growth, reproduction, histopathology, and bioaccumulation in *Labeo rohita* and other freshwater species are recommended to better understand the environmental consequences of TiO₂ nanoparticle pollution.

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7. CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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