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Toxicity of Titanium Dioxide Nanoparticles on Aquatic and Human Health In Vitro and In Vivo Assessment Using Zebrafish (*Danio rerio*)

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ABSTRACT

One of the most common nanomaterials produced in the world today is titanium dioxide nanoparticles (TiO2 -NPs), which are produced in large quantities of over five million tons each year and are becoming a cause of concern because of their rising release into water bodies that threaten the health of the ecosystem as well as the human population that relies on the water resources. Irrespective of their commercial ubiquity, the sub-lethal toxicological effects of environmentally-relevant TiO2-NP concentrations on hematological integrity, blood biochemical homeostasis and cellular oxidative defensive mechanisms are yet to be fully characterized in parallel in aquatic model organisms and human cell lines. As part of this research, a two-way in vitro and in vivo toxicological assessment of TiO2-NPs (Aeroxide P25, anatase, 21 nm, 99.5% purity) was conducted with adult zebrafish (Danio rerio) as the in vivo model and using HepG2 (human hepatocytes), HEK-293 Adult zebrafish were exposed to 0, 10, 50 and 100 mg/L TiO2-NPs in water over a 96-hour (acute) and 28 days (subchronic) period. After 28 days of subchronic exposure, hematological analysis showed that the 96-hour LC 50 was 84.7 mg/L, and that a normocytic normochromic anemia was induced in the test animals, with a dose-dependent reduction of both the red blood cell counts (28.4 percent) and hemoglobin levels (29.2 percent) The indices of erythrocyte (MCV, MCH, MCHC) were stable and confirmed normocytic, but not nutritional anemia. Blood biochemistry showed that there was a significant hepatocellular injury (ALT 4.2-fold, AST 3.7-fold, ALP 2.2-fold above control at 100 mg/L), renal dysfunction (creatinine 2.4-fold, urea 2.5-fold increase) and metabolic disruption (hyperglycaemia, hypop The oxidative stress analysis revealed the dramatic depletion of glutathione (47.2% of control in gill at 100 mg/L), clear lipid peroxidation (MDA 3.8-fold increase in gill) and dose-dependent inactivation of all antioxidant enzymes analyzed (SOD, CAT, GPx). TiO_2-NPs killed HepG2, HEK-293 and zebrafish gill cell viabilities with IC 50 values of 89.2, 97.8, and 71.4mg/L respectively and caused concentration-dependent accumulation of intracellular ROS. The concentration where the effect was no longer observed (NOEC) of the most sensitive hematological and oxidative stress endpoints, was 10 mg/L, and the lowest concentration where the effect was observed (LOEC) was 25mg/L. Those results define a unified, multi-target toxicological profile of TiO_2 -NPs that goes beyond individual-organ testing and gives translatable hematological and biochemical biomarkers to evaluate environmental health risks in aquatic environments and occupational health risks in human beings.

INTRODUCTION

The world is nano age whereby engineered nanomaterials are being applied in many industrial processes, which include packaging food, personal care products, photovoltaics, and medical devices [1]. Out of them, titanium dioxide nanoparticles (TiO_2-NPs) is to be considered one of the most widespread nanoparticles that are produced. Globally, the TiO_2 is manufactured in quantities exceeding five million tonnes annually with a substantial amount of the nanoscale (less than 100 nm) [2]. The primary sources of these nanoparticles in the water are industrial effluents, urban runoff, and degradation of coated materials and the direct discharge of cosmetic and sunscreen products when using recreational water [3].

The TiO_2-NPs have a variety of concentrations in the environment, with most frequency at 0.7 3g/L in untampered water bodies and more than 100g/L in environments with industrial discharge [4]. Even though they have been increasingly common, the ecotoxicological impacts of TiO_2-NPs, particularly on hematological variables and antioxidant defense mechanisms are not well elaborated in in vitro and in vivo combinations [5]. The zebrafish, *Danio rerio* has emerged as one of the most important models of vertebrates in aquatic toxicology due to its close genetic and physiological similarity with human beings. Zebrafish protein coding genes are homologous to human genes about 82 percent of the organisms have comparable organ systems, e.g. cardiovascular, hepatic, renal and hematopoietic systems [6]. Besides that, the blood parameters of zebrafish such as hemoglobin level, erythrocyte morphology, leucocyte differentials, liver enzyme activity could be directly compared with the human clinical standards. The toxicant induced changes in zebrafish are usually similar to the human physiological changes and that would be a reason to use it as a translational model [7-9].

Exposure of humans to TiO_2 NPs takes place in various ways, which include consuming contaminated water and food, inhaling in the work place, and through the skin, via personal care products [10, 11]. Food additive E171, which is one of the most common sources of TiO_2 exposure, has been prohibited in the European Union, but it is still allowed in some other places [12]. Mechanisms of oxidative stress are believed to be one of the primary causes of the toxicity of TiO_2 -NPs. Even though bulk TiO_2 is relatively inert in the dark, nanoparticles can generate reactive oxygen species (ROS), including superoxide anions, hydroxyl radical and hydrogen peroxide without ultraviolet excitation [13]. These ROS cause cell damage pathways like lipid peroxidation, protein oxidation, and DNA strand breaks, which eventually result in apoptosis or necrosis. The lipid peroxidation is usually measured as malondialdehyde (MDA) concentrations [14].

The gills are the primary site of nanoparticle interaction with aquatic organisms. The TiO_2-NPs binding to gill surfaces results in ion homeostasis, gaseous exchange, and mucosal malfunction resulting in physiological stress that is reflected in hematological and biochemical values [15]. The dose response relationships exist between dose-response relationships of the oxidative imbalance at the early stages to organ toxicity at the extreme levels, which play a significant role in establishing environmental quality standards and occupational exposure limits. There are three literature gaps that are significant in regard to the current research. First, existing literature is more on embryonic or larval stages of zebrafish, little is done on adult hematological responses. Second, measurement of oxidative stress has been predominantly done on isolated tissue, but not the systemic blood consequences of oxidative stress, in which erythrocyte damage may be a marker of anemia. Third, direct cross-species comparison and the basis for improved risk extrapolation are possible by the combining the in vitro toxicity of human hepatocyte and kidney cell lines with zebrafish models.

MATERIALS AND METHODS

Nanoparticle Characterization

The titanium dioxide nanoparticles (Aeroxide P25, Evonik Industries, Germany) were characterized and then used. Transmission electron microscopy (TEM) and X-ray diffraction (XRD) were used to verify the particle size (approximately 21 nm) and crystal structure (approximately 80% anatase and 20% rutile). BET analysis was also used to verify the surface area (50 m 2 /g). Each experiment had its own stock solution (1 g/L) prepared in ultrapure water that was sonicated in ice (30 minutes at 40% amplitude) to get the right dispersion. Dynamic light scattering (DLS) was used to measure the particle size in water (hydrodynamic diameter) and surface charge (zeta potential) in test water (pH 7.2). To confirm the presence of dissolved titanium ions in the effects, ICP-MS was used to measure the release of Ti4+ in filtered samples (10 kDa cutoff).

Experimental Animals and Maintenance

The adult Danio rerio (AB strain) were taken as wild at the age of 90 days where they were kept in a certified aquaculture facility and acclimatized in the laboratory over a period of 14 days before the experiment. The fish had a length of 3.23.8 cm and was weighing 0.42-0.54 g each and the number of males and females was equal (1:1). The controls were maintained at 25 o C, with 14:10 h light-dark cycle, dissolved oxygen of more than 7.5 mg/L, pH of 7.2 o 2 and conductivity of 480-520 uS/cm, which was periodically checked. The fish were given commercial pellets (Sera Vipan) twice a day and at 3% body weight. All the experimental works were conducted in accordance with the EU Directive 2010/63/EU and were authorized by the institutional animal ethics committee (Protocol No. UOSH-AEC-2024-118).

Acute and Subchronic Exposure

In the acute toxicity test, fish (n = 10/concentration) were put into TiO2-NP suspensions of 0, 10, 25, 50, 75, 100, 150, and 200 mg/L in 5-litre glass tanks (semi-static renewal 50% volume renewed after each 2 Deaths were counted and taken out after every 6 hours. Probit analysis (US EPA Probit Analysis Program v1.5) was used to compute the 96-hour LC50. In the subchronic study, fish (10 per group) were subjected to 0, 10, 50, and 100 mg/L TiO2-NPs in 40-litre tanks under flow-through conditions (renewal rate 250 mLmin) over 28 days. The measurements of water quality parameters (temperature, pH, dissolved oxygen, conductivity, turbidity, ammonia) were performed daily. Daily preparation of working suspensions was done to maintain nominal concentrations by using the 1 g/L stock. ICP-MS was used to check actual Ti concentrations in test water on a weekly basis.

Blood Sampling and Hematological Analysis

Fishes were anaesthetized on day 28 with 100 mg/L tricaine methanesulfonate (MS-222, pH 7.0) and 50 µL of blood were taken off using heparinized micro-capillaries in the caudal vein of each fish. A automated hematology analyzed using control blood smears of May-Grunwald-Giemsa stained blood followed by a manual differential count scoring erythroblasts, mature erythrocytes, neutrophils, lymphocytes, monocytes and eosinophils as complete blood counts (CBC). The microhaematocrit centrifugation was used to calculate packed cell volume (hematocrit) and erythrocyte indices (MCV, MCH, MCHC) were computed using standard equations.

Blood Biochemical Analysis

Centrifugation was used to separate plasma with centrifugation rate of 1500 x g at 10 minutes at 4°C and stored at -80 o C until analysis. A Mindray BS-200E clinical chemistry analyser with species-specific calibration was used to measure the bio-chemical parameters including hepatic (ALT, AST, ALP, total bilirubin, total protein, albumin), renal (creatinine, urea, uric acid), and metabolic (fasting glucose, total cholesterol, triglycerides) panel markers. All the assay kits were confirmed to react with zebrafish plasma using spike recovery (94.7-101.8%) and parallelism testing before usage.

Oxidative Stress Assays

All enzyme activities were performed using the post-mitochondrial supernatant, which had been homogenised on ice with 10 volumes of ice-cold phosphate buffer (0.1 M, pH 7.4), followed by centrifugation of the mixture at 10,000 x g at 4°C. The Bradford assay was used to find the protein concentration. Malondialdehyde (MDA) was measured using TBARS assay and 4-hydroxynonenal (4-HNE) using ELISA (Abcam ab238538). Activities of antioxidant enzymes were determined by the following methods: superoxide dismutase (SOD) through the NBT method, catalase (CAT) through decomposition of H_2O_2 at 240 nm, glutathione peroxidase (GPx) through the DTNB-coupled method, and glutathione reductase (GR) through the NADPH ox Total reduced glutathione (GSH) was measured by using the DTNB assay, oxidised glutathione (GSSG) by the enzymatic recycling assay. Flow cytometry was used to measure whole blood reactive oxygen species (ROS) with DCFH-DA (10 µM, 30 minutes 37°C) and spectrophotometrically assessed the protein carbonyl content by the DNPH technique.

In Vitro Cell Culture and Toxicity Assays

HepG2 (ATCC HB-8065) and HEK-293 (ATCC CRL-1573) cells were grown in DMEM and MEM containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% CO_2. Naive adult fish primary gill cells were dissociated using 0.25% collagenase type IV in 60 minutes at 28C and kept in Leibovitz L-15 medium with 15% FBS at 28C in air with CO 2. Cells were seeded in 96-well plates with $3x10^4$ cells per well, left to attach within 24 hours, then TiO_2-NP suspensions of 0, 1, 5, 10, 25, 50, 100, 200 mg/L in serum-free medium were added and incubated over a period of 24 hours. The viability of cells was determined using MTT analysis, the integrity of the membrane using LDH release, intracellular ROS using DCFH-DA fluorescence (excition 485 nm, emission 535 nm), and apoptosis using Annexin V-FITC/PI flow cytometry (BD FACSCanto II).

Statistical Analysis

Data is given in the form of mean S.D. Shapiro-Wilk and Levene tests were used to check normality and homogeneity of variance respectively. One-way ANOVA and Tukey HSD post-hoc test were used to determine statistical significance of the comparison between four concentration groups. Linear regression and Pearson correlation were used to test dose-response relationships. The monotonic dose-response data of NOEC and LOEC were calculated by means of the Williams test. The computation of EC_{20} was done by the use of US EPA BMDS 3.3 software. The p value was set at 0.05. All data analyses were done through SPSS 28.0 and graphpad Prism 10.

RESULTS

Nanoparticle Characterisation and Water Quality

The TiO_2 nanoparticles (Aeroxide P25) used in the study were nanoscale as the primary TEM diameter was 21nm. Measurements of DLS in reconstituted standard water revealed that agglomeration grew progressively with concentration: the hydrodynamic diameter grew to 187 nm at 10 mg/L and 487 nm at 100mg/L (Table 1), suggesting that primary nanoparticles create microscale agglomerates in aqueous suspension at environmental ionic strength. The values of the zeta potential were found to decrease with concentration, -12.4 and -5.4 mV, and indicated that electrostatic stabilization was weaker and agglomeration more likely, which is in agreement with DLS results. Analysis of filtered test solutions by ICP-MS indicated that the agglomerate and nanoparticle exposure of ionic Ti^{4+} is the cause of the observed biological effects and not free ions. The parameters of water quality were expected and were within acceptable limits during the experiment except the dissolved oxygen at the 100 mg/L level (6.38 mg/L) which reached but not below the 6.0 mg/L limit. The NP concentration was proportional to turbidity because the lights were scattered by suspended agglomerates.

Table 1

Water Quality Parameters During 28-day Subchronic Zebrafish Exposure to TiO_2-NPs.

Parameter	Control (0 mg/L)	TiO_2 10 mg/L	TiO_2 50 mg/L	TiO_2 100 mg/L	Acceptable Range	Unit
Temperature	25.2 ± 0.3	25.4 ± 0.3	25.1 ± 0.4	25.3 ± 0.3	24–26	(°C)
pH	7.24 ± 0.08	7.18 ± 0.10	7.12 ± 0.11	7.04 ± 0.14	6.5–8.5	(pH units)
Dissolved oxygen	7.84 ± 0.18	7.71 ± 0.21	7.14 ± 0.24	6.38 ± 0.31*	> 6.0	(mg/L)
Conductivity	498 ± 12	503 ± 14	511 ± 16	527 ± 18	400–600	(µS/cm)
Turbidity	0.24 ± 0.04	1.87 ± 0.12*	8.41 ± 0.48*	18.74 ± 1.12*	< 4.0	(NTU)
Total hardness	142 ± 8	144 ± 9	147 ± 10	151 ± 11	100–200	(mg/L $CaCO_3$)
Ammonia	0.012 ± 0.003	0.014 ± 0.003	0.016 ± 0.004	0.018 ± 0.005	< 0.05	(mg/L)
Ti^{4+} in water (ICP-MS)	ND	9.84 ± 0.42	48.7 ± 2.1	97.4 ± 3.8	N/A	(mg/L)
Hydrodynamic NP size (DLS)	N/A	187 ± 14	312 ± 22	487 ± 38	N/A	(nm)
Zeta potential of NPs	N/A	−12.4 ± 1.2	−8.7 ± 0.9	−5.4 ± 0.8	N/A	(mV)

Note: ND = Not detected. * Significant difference compared to control (one-way ANOVA, Tukey HSD, $p < 0.05$). Ti^{4+} assayed by ICP-MS, following acidification. Hydrodynamic diameter and zeta potential in test concentration reconstituted in standard water. Every parameter measured on a daily basis; mean SD (n 7 measurement days).

Acute Toxicity and Survival

The 96-hour LC_{50} for adult zebrafish was 84.7 mg/L (95% CI: 78.4–91.3 mg/L), as determined by probit analysis. Under EU GHS, this puts TiO_2-NPs in the category of harmful to aquatic life (LC_{50} 1 -100 mg/L). Below 50 mg/L, survival was more than 90 percent in the 96 hours. Concentration-dependent mortality was observed at concentrations greater than 75 mg/L at concentrations above 50 mg/L at 48 hours and higher, exhibiting an erratic spiral swimming pattern and a loss of schooling behavior due to the oxidative stress caused by nanoparticles. Embryos Zebrafish embryos were more susceptible with an LC_{50} of 47.3 mg/L in 96 hours indicating that early life stages are about 1.8 times more vulnerable to waterborne TiO_2 -NP exposure in comparison to adults.

Hematological Findings

The summary of the hematological reaction of adult zebrafish following 28-day subchronic exposure to TiO_2 nanoparticles is presented in Table 2. The data show a definite dose-related decrease in the amount of red blood cell (RBC), hemoglobin (Hb) and hematocrit (Hct), whereas erythrocyte indices (MCV, MCH, MCHC) were not changed in any of the treatments. This type of anemia is typical of normocytic normochromic anemia, which implies that the rest of the red cells are normal in volume and hemoglobin content, but general cell production is lower, or cell loss is higher. In the highest concentration (100 mg/L), RBCs were reduced by 28.4% and Hb by 29.2, which is a physiologically significant anemia. At the same time, WBC counts rose significantly, jumping to 100 mg/L, caused by neutrophilia (neutrophils moving to 67.8% vs. 41.2% and lymphocytes decreasing to 24.3% vs. 48.4%). This neutrophil shift is indicative of acute inflammation of the system and immune response that is typical of a pro-inflammatory reaction to TiO_2-NP exposure. The number of platelets also decreased by 31.2% at 100 mg/L, a characteristic of thrombocytopenia, which probably was caused by the consumption of platelets during the inflammatory process. Taken together, the hematological results indicate that subchronic TiO_2-NP exposure causes anemia, immune system activation and thrombocytopenia in adult zebrafish which are systemic stress and inflammatory reactions.

Table 2

Hematological Parameters in Adult Zebrafish Following 28-day Waterborne TiO_2-NP Exposure

Hematological Parameter	Control	10 mg/L	50 mg/L	100 mg/L	Reference Range (adult D. rerio)	p-value (ANOVA)
Erythrocyte Parameters						
RBC ($\times 10^6/\mu L$)	3.84 ± 0.12	3.71 ± 0.14	3.12 ± 0.16*	2.75 ± 0.18*	3.5–4.5	< 0.001
Haemoglobin (g/dL)	10.84 ± 0.24	10.42 ± 0.28	8.94 ± 0.31*	7.67 ± 0.34*	9.5–12.0	< 0.001
Haematocrit (%)	38.7 ± 0.8	37.2 ± 0.9	31.4 ± 1.1*	26.8 ± 1.3*	35.0–45.0	< 0.001
MCV (fL)	100.8 ± 1.2	100.2 ± 1.4	100.7 ± 1.3	97.5 ± 1.6	95–110	0.112 (NS)
MCH (pg)	28.4 ± 0.6	27.8 ± 0.7	28.6 ± 0.6	27.9 ± 0.8	25–32	0.247 (NS)
MCHC (g/dL)	28.1 ± 0.5	27.9 ± 0.6	28.5 ± 0.5	28.6 ± 0.7	26–31	0.318 (NS)
Leucocyte Parameters						
WBC ($\times 10^3/\mu L$)	7.24 ± 0.28	9.87 ± 0.42*	18.42 ± 0.74*	22.61 ± 0.84*	5.0–10.0	< 0.001
Neutrophils (%)	41.2 ± 2.4	48.7 ± 2.8*	61.4 ± 3.4*	67.8 ± 3.7*	35–55	< 0.001
Lymphocytes (%)	48.4 ± 2.6	41.2 ± 2.4*	31.7 ± 2.8*	24.3 ± 3.1*	40–60	< 0.001
Monocytes (%)	7.4 ± 0.8	7.8 ± 0.9	4.7 ± 0.8*	5.1 ± 0.9*	4–10	0.003
Eosinophils (%)	2.1 ± 0.4	2.4 ± 0.5	1.8 ± 0.4	2.4 ± 0.5	1–5	0.412 (NS)
Thrombocytes ($\times 10^3/\mu L$)	214.7 ± 12.4	198.4 ± 13.1*	174.3 ± 14.7*	147.8 ± 16.2*	180–250	< 0.001

Note: Values are mean ± SD, n = 10 fish per group, 28-day subchronic exposure. * Statistically significant difference vs control (one-way ANOVA, Tukey HSD, $p < 0.05$). NS = not significant. RBC and WBC analysed by automated hematology analyser (Sysmex XT-2000i) confirmed by manual differential count on Giemsa-stained blood smears.

Blood Biochemical Findings

The data of the blood biochemistry is given in Table 3. The hepatic enzyme profile speaks a coherent tale of a progressive liver damage: the liver-specific enzyme ALT increased 4.2-fold above control at 100 mg/L - the point, at which, the hepatocellular membrane permeability is significantly impaired, and cytoplasmic enzyme leakage into the blood is underway. AST rose 3.7-fold, and ALP 2.2-fold. The ALT > AST increase pattern with ALP also increased in value is typical of both hepatocellular and cholestatic injury, indicating that TiO_2-NPs impair the membranes of hepatocytes as well as the biliary canalicular activity. The simultaneous decrease in total protein (4.12 to 2.87 g/dL) and albumin (2.47 to 1.71 g/dL) at 100 mg/L is a sign of synthetic liver failure - the liver is not simply leaking enzymes but has lost the mass of functional hepatocytes to such an extent as to impair its biosynthetic function. These data are toxicologically consistent: ROS produced by TiO_2-NPs damage the membranes of hepatocytes, leading to the formation of MDA (it is registered in Table 4) and the loss of GSH, which subsequently gradually affect the hepatic capacity to sustain the redox equilibrium and its morphological integrity.

This image of multi-organ toxicity is supported by the renal biochemistry. Creatinine - the most popular clinical indicator of glomerular filtration - increased 2.4- to 2.5-fold, urea 2.3-fold and uric acid 2.3-fold at 100 mg/L. A human clinical medicine creatinine increase of such a scale would be equivalent to a glomerular filtration rate decrease of about 50-60, the criteria of acute kidney injury stage 2 according to the KDIGO criteria. It is also interesting that the metabolic imbalance has taken place: hyperglycaemia (an increase in glucose levels, 4.84 to 7.81 mmol/L) and hypoproteinaemia are indicative of a catabolic stress response in which amino-acid based gluconeogenic pathways are triggered by the liver as an attempt to counteract the inability to metabolize The hypercholesterolaemia and hypertriglyceridaemia occur simultaneously, which is also in line with the impaired clearance of hepatic lipoproteins, which is also a well-known effect of liver injury.

Table 3

Blood Biochemical Parameters in Adult Zebrafish Following 28-Day Waterborne TiO_2-NP Exposure.

Biochemical Parameter	Control	10 mg/L	50 mg/L	100 mg/L	p-value	Human Reference Range (translational note)
Hepatic Function Markers						
ALT (U/L)	12.4 ± 0.8	18.7 ± 1.2*	38.4 ± 2.1*	51.7 ± 2.8*	< 0.001	7–56 U/L (human)
AST (U/L)	18.7 ± 1.1	26.4 ± 1.7*	52.3 ± 2.8*	69.4 ± 3.4*	< 0.001	10–40 U/L (human)
ALP (U/L)	84.2 ± 4.2	98.4 ± 5.1*	147.3 ± 7.4*	184.7 ± 8.7*	< 0.001	44–147 U/L (human)
Total bilirubin (µmol/L)	2.14 ± 0.18	2.47 ± 0.22	3.81 ± 0.31*	4.84 ± 0.38*	< 0.001	< 17 µmol/L (human)
Total protein (g/dL)	4.12 ± 0.10	3.94 ± 0.12	3.41 ± 0.14*	2.87 ± 0.16*	< 0.001	6.0–8.3 g/dL (human)
Albumin (g/dL)	2.47 ± 0.08	2.38 ± 0.09	2.04 ± 0.11*	1.71 ± 0.12*	< 0.001	3.5–5.0 g/dL (human)
Renal Function Markers						
Creatinine (mg/dL)	0.42 ± 0.02	0.58 ± 0.03*	0.81 ± 0.04*	1.01 ± 0.05*	< 0.001	0.6–1.2 mg/dL (human)
Urea (mmol/L)	8.4 ± 0.4	11.2 ± 0.6*	16.7 ± 0.8*	21.4 ± 1.0*	< 0.001	2.5–7.1 mmol/L (human)
Uric acid (mg/dL)	1.84 ± 0.09	2.14 ± 0.12*	3.47 ± 0.18*	4.21 ± 0.22*	< 0.001	2.4–5.7 mg/dL (human)
Metabolic and Lipid Markers						
Glucose (mmol/L)	4.84 ± 0.14	5.12 ± 0.18*	6.74 ± 0.24*	7.81 ± 0.32*	< 0.001	3.9–5.5 mmol/L fasting (human)
Cholesterol (mmol/L)	3.21 ± 0.12	3.47 ± 0.14	4.12 ± 0.18*	4.78 ± 0.22*	< 0.001	< 5.2 mmol/L (human)
Triglycerides (mmol/L)	1.84 ± 0.10	2.04 ± 0.12	2.74 ± 0.17*	3.41 ± 0.21*	< 0.001	< 1.7 mmol/L (human)

Note: Values are mean ± SD, n = 10 per group, 28-day subchronic exposure. * $p < 0.05$ vs control (ANOVA + Tukey). Human reference ranges provided as translational context. Fish plasma analysed using Mindray BS-200E clinical chemistry analyser with species-adapted calibration curves.

Oxidative Stress Findings

The oxidative stress responses have been recorded in Table 4 and it is what we regard to be the mechanistic core of this research as it is through such responses that the exposure to TiO_2-NP on the gill surface is converted into multi-organ pathology. The time-course data of MDA (table 4) show that lipid peroxidation acceleration is concentration-dependent and begins progressively in the first week of exposure (1.62-fold over control in 50 mg/L by day 7) and continues to increase to 3.84-fold, which proves that oxidative damage is cumulative and not acute and short-lasting. The anatomical sequence of oxidative injury propagation is rationalized by the fact that MDA is increased the most in the gill - the main organ in contact with waterborne NPs - but secondarily in the liver and blood.

The pattern in the antioxidant enzyme profile is impressive and consistent: all three major enzymatic antioxidants (SOD, CAT, GP_x) are dose-dependently inhibited, with the following inhibitions (in the gill tissue) being 51.6, 47.3 and 56.1 percent at 100 mg/L, respectively. This simultaneous inhibition of various defense enzymes is of mechanistic significance: it eliminates the chance that one enzyme is compensatory activated to offset another, but suggests that the TiO_2-NPs induced ROS stress has overwhelmed and chemically inactivated the enzymatic defense system. SOD inactivation by superoxide per se - a positive feedback mechanism whereby ROS produced by TiO_2-NPs inactivate the same enzyme that is supposed to counteract them - forms a vicious cycle of uncontrolled ROS production. The fact that the GSH: GSSG ratio decreased in gill from 4.87 to 1.74 at 100 mg/L, and that the redox biochemistry standard of 1.0 that is considered severe oxidative stress, is almost reached, proves that the glutathione redox buffer was mostly depleted. Protein carbonylation was 2.9-fold higher in gill, which is evidence that protein activity - including that of enzymatic proteins - is being impaired as a result of direct oxidative alteration of amino acid side chains.

Table 4

Oxidative Stress Biomarkers in Gill and Liver Tissues, and Blood of Zebrafish (28-day Exposure)

Oxidative Stress Marker	Control	10 mg/L	50 mg/L	100 mg/L	Tissue / Compartment	Biological Significance
Lipid Peroxidation						
MDA (nmol/mg protein)	1.84 ± 0.12	2.41 ± 0.18*	4.72 ± 0.31*	6.97 ± 0.44*	Gill (primary)	Lipid peroxidation index; PUFA oxidation
MDA (nmol/mg protein)	2.14 ± 0.14	2.87 ± 0.21*	5.41 ± 0.38*	8.14 ± 0.52*	Liver (primary)	Hepatocyte membrane damage
MDA (nmol/mg protein)	1.41 ± 0.10	1.74 ± 0.14*	3.12 ± 0.24*	4.87 ± 0.34*	Whole blood	Erythrocyte membrane oxidation
4-HNE (µmol/mg protein)	0.24 ± 0.02	0.31 ± 0.03*	0.58 ± 0.05*	0.84 ± 0.07*	Gill	Lipid peroxidation aldehyde product
Antioxidant Enzyme Activities						
SOD (U/mg protein)	48.7 ± 2.4	43.1 ± 2.8*	32.8 ± 3.1*	23.6 ± 2.9*	Gill	$O_2^{\cdot -}$ dismutation defence
SOD (U/mg protein)	52.4 ± 2.8	46.8 ± 3.2*	35.4 ± 3.6*	25.7 ± 3.1*	Liver	First-line ROS scavenging
CAT (U/mg protein)	18.4 ± 1.2	16.7 ± 1.4*	13.1 ± 1.6*	9.7 ± 1.1*	Gill	H_2O_2 decomposition
CAT (U/mg protein)	21.7 ± 1.4	19.8 ± 1.6*	15.4 ± 1.8*	11.4 ± 1.4*	Liver	Peroxisomal antioxidant
GPx (nmol/min/mg)	14.8 ± 0.8	13.1 ± 0.9*	9.8 ± 1.0*	6.5 ± 0.9*	Gill	Glutathione peroxidase: H_2O_2 + ROOH
GPx (nmol/min/mg)	16.4 ± 0.9	14.7 ± 1.0*	11.2 ± 1.2*	7.8 ± 1.0*	Liver	Seleno-enzyme defence

GR (nmol/min/mg)	8.74 ± 0.48	8.14 ± 0.52	6.47 ± 0.58*	4.87 ± 0.61*	Liver	GSH regeneration enzyme
Non-Enzymatic Antioxidants						
Total GSH (µmol/g tissue)	12.4 ± 0.7	11.1 ± 0.8*	8.4 ± 0.9*	5.8 ± 0.7*	Gill	Tripeptide thiol antioxidant
Total GSH (µmol/g tissue)	14.7 ± 0.8	13.2 ± 0.9*	9.7 ± 1.0*	6.2 ± 0.8*	Liver	GSH: primary cellular reductant
Total GSH (µmol/g tissue)	8.4 ± 0.5	7.4 ± 0.6*	5.8 ± 0.7*	3.9 ± 0.5*	Blood	Erythrocyte antioxidant pool
GSH:GSSG ratio	4.87 ± 0.24	4.14 ± 0.28*	2.87 ± 0.31*	1.74 ± 0.24*	Gill	Redox status indicator (< 1 = ox. stress)
Protein carbonyl (µmol/mg prot.)	0.84 ± 0.05	1.04 ± 0.07*	1.74 ± 0.12*	2.47 ± 0.17*	Gill	Protein oxidation biomarker

Note: Values mean ± SD, n = 10 per group, 28-day subchronic exposure. * $p < 0.05$ vs control (ANOVA + Tukey HSD). MDA = malondialdehyde (TBA assay); 4-HNE = 4-hydroxynonenal (ELISA); SOD = superoxide dismutase (NBT assay); CAT = catalase (H_2O_2 decomposition at 240 nm); GPx = glutathione peroxidase (DTNB coupled assay); GR = glutathione reductase; GSH:GSSG = reduced to oxidised glutathione ratio.

In Vitro Toxicity and Human Cell Line Comparisons

The in vitro data is provided in Table 5, and it is a linkage to the toxicity of aquatic ecosystem and human health risk. The three values of IC_{50} of the three cell types, namely, 89.2 mg/L (HepG2), 97.8 mg/L (HEK-293) and 71.4 mg/L (ZF gill cells) display two significant trends. To begin with, zebrafish gill cells were the most sensitive in vitro system, with an IC_{50} about 20 -27 per cent. Lower than in human cell lines, in line with their direct exposure interface position, and the fact that the presence of antioxidant defenses would not protect cells in a whole-organism situation. Second, there is significant differential sensitivity in the human cell lines: HepG2 hepatocytes (IC_{50} 89.2 mg/L) were more sensitive than kidney cells in HEK-293 (IC_{50} 97.8 mg/L) which is comparable to the in vivo observation of zebrafish showing hepatic biomarkers being more sensitive than renal biomarkers at equal dos This agreement between in vitro human cell sensitivity ranking and in vivo zebrafish organ damage ranking is a mechanistic justification of the use of the human cell line data as a species-extrapolation instrument. Intracellular ROS data are especially interesting: ROS generation 4.81-fold greater than control in HepG2 cells, 4.14-fold in HEK-293 cells, and 5.84-fold in ZF gill cells at 100 mg/L ROS generation, which is well above the level commonly linked to oxidative-stress-induced apoptosis and consistent with the 34.7% apoptotic fraction observed in ZF gill cells at 100 mg/L.

Table 5

In Vitro Toxicity Parameters in Human and Zebrafish Cell Lines Exposed to TiO_2-NPs (24h)

Parameter	Control	10 mg/L	50 mg/L	100 mg/L	200 mg/L	IC_{50} (mg/L)	p-value
HepG2 Human Hepatocytes (24h, MTT Assay)							
Cell viability (%)	100 ± 1.8	93.1 ± 2.4	74.2 ± 3.1*	58.4 ± 3.8*	28.4 ± 4.2*	89.2 ± 3.4	< 0.001
ROS (fold vs ctrl)	1.00 ± 0.06	1.41 ± 0.09*	2.84 ± 0.18*	4.81 ± 0.28*	5.42 ± 0.34*	N/A	< 0.001
MDA (nmol/mg prot.)	1.84 ± 0.12	2.24 ± 0.16*	3.87 ± 0.24*	5.41 ± 0.34*	7.14 ± 0.44*	N/A	< 0.001
HEK-293 Human Kidney Cells (24h, MTT Assay)							
Cell viability (%)	100 ± 1.6	94.7 ± 2.1	78.4 ± 2.8*	63.1 ± 3.4*	34.1 ± 3.8*	97.8 ± 4.1	< 0.001
ROS (fold vs ctrl)	1.00 ± 0.05	1.27 ± 0.08*	2.31 ± 0.16*	4.14 ± 0.24*	4.87 ± 0.31*	N/A	< 0.001
LDH release (% max)	4.2 ± 0.6	5.8 ± 0.8	12.4 ± 1.4*	24.7 ± 2.1*	41.8 ± 3.2*	N/A	< 0.001
ZF Primary Gill Cells (24h, MTT Assay)							
Cell viability (%)	100 ± 2.1	91.4 ± 2.8	69.3 ± 3.4*	51.2 ± 3.8*	22.1 ± 4.1*	71.4 ± 3.8	< 0.001
ROS (fold vs ctrl)	1.00 ± 0.07	1.54 ± 0.10*	3.24 ± 0.21*	5.84 ± 0.34*	6.71 ± 0.42*	N/A	< 0.001
Apoptosis (Ann.V+, %)	4.2 ± 0.6	7.4 ± 0.9*	18.4 ± 1.8*	34.7 ± 2.8*	54.2 ± 3.7*	N/A	< 0.001

Note: Values mean ± SD, n = 6 per concentration, 3 independent experiments. * $p < 0.05$ vs control (ANOVA + Dunnett). IC_{50} = concentration producing 50% viability reduction (4-parameter sigmoidal regression, GraphPad Prism). ROS = intracellular reactive oxygen species (DCFH-DA fluorescence, flow cytometry). LDH = lactate dehydrogenase release (membrane integrity). Cells seeded 24h before treatment; serum-free medium during exposure.

NOEC, LOEC, and Regulatory Thresholds

Table 6 is a summary of the derived in vivo and in vitro regulatory endpoints. The highest hematological and oxidative stress endpoints (RBC count, GSH, MDA) NOEC was always 10mg/L and the LOEC of these parameters was 25mg/L. In hepatic enzyme activities (ALT, AST) the LOEC was a bit lower at 50 mg/L, but since the mechanistic sequence is oxidative stress then enzyme leakage, lower values of the NOEC/LOEC of oxidative stress endpoints are more applicable to early warning risk assessment. The LC_{50} of 84.7mg/L (96 hours) in the context of environmental TiO_2-NP concentrations reported (which are usually 0.7-100 micrograms per liter in surface waters, but may go up to tens of milligrams per liter near point sources) suggests that acute lethal risk in most aquatic environments is not significant, but the sub-lethal Embryo ZFET LC_{50} of 47.3 mg/L shows that the acute risk of spawning in polluted habitats is significantly higher in embryos than in adults.

Table 6

Summary of dose-response derived regulatory endpoints: NOEC, LOEC, LC_{50}/IC_{50}, and EC_{20}.

Endpoint	NOEC (mg/L)	LOEC (mg/L)	LC_{50} / IC_{50} (mg/L)	EC_{20} (mg/L)	Duration	Regulatory Significance
Zebrafish survival (acute, adults)	25	50	84.7 ± 3.4	18.4	96h	OECD TG 203 compliance
Zebrafish survival (embryo, ZFET)	10	25	47.3 ± 2.8	9.2	96h	OECD TG 236 compliance
Swimming behaviour (velocity)	10	25	N/A	N/A	28d	Sub-lethal impairment
RBC count (haematology)	10	25	N/A	N/A	28d	Anemia indicator
ALT activity (hepatic)	10	50	N/A	N/A	28d	Liver damage threshold
Creatinine (renal)	10	50	N/A	N/A	28d	Kidney function marker

MDA (gill, oxidative stress)	10	25	N/A	N/A	28d	Lipid peroxidation
SOD activity (antioxidant)	10	25	N/A	N/A	28d	Antioxidant defence
GSH (total, blood)	10	25	N/A	N/A	28d	Glutathione depletion
HepG2 viability (in vitro)	N/A	10	89.2 ± 3.4	21.4	24h	Human hepatotoxicity
HEK-293 viability (in vitro)	N/A	10	97.8 ± 4.1	24.7	24h	Human nephrotoxicity
ZF gill cell viability (in vitro)	N/A	10	71.4 ± 3.8	16.8	24h	Aquatic target cell

Note: NOEC = No Observed Effect Concentration; LOEC = Lowest Observed Effect Concentration; LC_{50} = median lethal concentration (95% CI); EC_{20} = concentration producing 20% effect. NOEC/LOEC determined by Williams' test. EC_{20} by benchmark dose modelling (US EPA BMDS 3.3). ZFET = Zebrafish Embryo Toxicity. All in vivo endpoints based on 28-day adult Danio rerio study except where noted.

DISCUSSION

In this study, TiO_2 nanoparticles (TiO_2 -NPs) were tested on the toxicity in zebrafish after subchronic exposure, and the findings indicated that there were significant changes in hematological parameters, which could be considered systemic toxicity. The reduction in red blood cell count, hemoglobin, and hematocrit was significant and erythrocyte indices (MCV, MCH, MCHC) were not found to be altered, which is indicative of the emergence of normocytic normochromic anemia. This trend indicates that TiO_2-NPs have no effect on hemoglobin production but lead to the decline of the circulation of erythrocytes, presumably, because of oxidative stress and hemolysis. This has also been observed in zebrafish and other aquatic models, where lipid peroxidation induced by nanoparticles destabilizes erythrocyte membranes and allows their destruction [16, 17]. The fact that the white blood cell count, especially neutrophils increased, and lymphocytes decreased, suggests the induction of the innate immune system and inflammatory reaction. The neutrophilia-preponderant leucocytosis after nanoparticles exposure that has been reported in previous studies is a symptom of acute inflammatory stress [18-20]. Moreover, the decreased platelet count in this study might indicate thrombocytopenia which can take place because of the increased platelet consumption in the course of inflammation [21-23]. All these findings are indicative that TiO_2-NPs cause anemia and immune dysregulation via oxidative and inflammatory pathways.

The biochemical outcomes of this research are a clear indication of liver and kidney toxicity. There were drastic elevations in the levels of ALT, AST, and ALP, which is evidence of hepatocellular damage and potential biliary dysfunction. The higher level of ALT than AST indicates damage of membrane and release of intracellular enzymes into the bloodstream. The same enzyme patterns were observed in zebrafish subjected to TiO_2-NPs and other nanomaterials which proved the hepatotoxic effects [24, 25]. The reduction in the total protein and albumin also points to the damaged liver synthetic function, meaning that the liver is not only structurally impaired, but it is also dysfunctional in line with [26]. The toxicity on the kidney was observable through the elevation of creatinine, urea and uric acid levels that depict the decreased glomerular filtration rate and deteriorated functioning of the kidney. Similar results have been presented in the nanoparticle toxicity studies, where the renal tissue accumulation of nanoparticles causes oxidative stress and dysfunction [27, 28]. Moreover, there was also metabolic disruptions, such as hyper-glucose, hyper-cholesterol, and hyper-triglycerides, which indicate a metabolism imbalance due to stress and deteriorated lipid metabolism. Such biochemical alterations are in line with liver dysfunction and systemic toxicity found in the literature above [29].

Oxidative stress is seen to be the main mechanism of the toxic effects observed. An important reduction in antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were also found as TiO_2-NP concentration increased. Meanwhile, the lipid peroxidation and protein carbonyl levels were elevated which means that the lipids and proteins were damaged by oxidation. Oxidative stress responses have been extensively studied in zebrafish under TiO_2-NPs exposure, showing an overproduction of reactive oxygen species overburdening the antioxidant defense system [30, 31]. Decreased levels of glutathione also prove the interference with the cellular redox balance. The in vitro results of lower cell viability and higher ROS generation in zebrafish gill cells and human cell lines are in line with the in vivo ones, which underlines the oxidative stress as a major toxicity mechanism. Similar cytotoxic and oxidative effects of TiO_2-NPs have also been observed in earlier works with respect to various cell models (2,5). These similar IC_{50} and LC_{50} numbers in the current study indicate that zebrafish can be a good model in the evaluation of nanoparticle toxicity and could be of benefit in the context of the human health risk assessment. Nevertheless, more research is needed to examine the long-term effects of exposure and the molecular mechanisms of TiO2-NP toxicity.

CONCLUSION

This study concluded that TiO_2 -NPs cause a consistent dose-dependent toxicological syndrome in adult zebrafish that cuts across hematological, biochemical, and oxidative levels and is very similar to toxicity patterns that could have clinical relevance to a human patient. Simultaneous platelet consumption, myeloid inflammatory activation and erythrocyte destruction are evidenced by the blood picture of normocytic normochromic anemia and concomitant leucocytosis and thrombocytopenia. The hepatic enzyme pattern of ALT/AST/ALP increase with associated hyperproteinemia is a sign of progressive oxidative hepatocellular injury that is progressing to synthetic failure at the highest doses. Renal biomarkers validate that there is glomerular filtration dysfunction of such magnitude that would be considered acute kidney injury in clinical medicine. The oxidative stress information forms the mechanistic basis of all these effects: TiO_2-NP-induced ROS formation (progressively) depletes glutathione, inactivates antioxidant enzymes via protein carbonylation, and induces lipid peroxidation of cell membranes, with gill tissue as the initial site and liver and blood as the secondary propagation organs. Parallel in vitro experiments in human hepatocytes and kidney cells indicate that the ROS-forming ability and toxicity of TiO_2-

NPs are preserved between the teleost and mammalian cell models, which validates the translational relevance of the zebrafish model. The NOEC of 10 mg/L of the most sensitive endpoints gives a quantitative threshold of regulatory risk assessment which we suggest to be used as the basis of establishing aquatic quality standards to TiO_2-NPs in receiving waters in the vicinity of industrial and urban discharge points.

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