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Original Article

Assessing the detrimental impact of varied doses of dietary ZnO nanoparticles on *Tilapia niloticus*: Implications for fish health

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Abstract

The investigation and monitoring of exact mechanisms of induction of deleterious effects induced by various synthetic and natural compounds including nanoparticles (NPs) are crucial to lowering the toxic effects of these compounds both on public health and the environment. For this purpose, zinc oxide nanoparticles (ZnO NPs) were synthesized using the co-precipitation technique. SEM (scanning electron microscopy) showed that the synthesized NPs had formed spherical structures, and were collected as flower-shaped bundles. The calculated average crystalline size of ZnO NPs from XRD (X-ray diffraction) was 17.6nm. A total of 150 fish (*Tilapia niloticus)* were reared in cemented tanks having 200L water in different groups (T0 and T1-T3). The fish in groups T1-T3 were exposed to ZnO NPs mixed in feed @ 150mg/kg, 300mg/kg, and 450mg/kg respectively for 28 days. The results revealed significantly increased morphological and nuclear ailments in erythrocytes of fish exposed with higher doses of ZnO NPs (300mg/kg and 450mg/kg) in comparison to unexposed fish. Hematological analysis showed significantly decreased red blood cell count (RBC), hemoglobin, and lymphocytes while an increased population of white blood cell count (WBC) and neutrophil at higher doses (300mg/kg and 450mg/kg). Serum biochemistry analysis indicated significantly increased concertation of cholesterol, creatinine, urea, alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), and decreased quantity of total proteins, globulin, and albumin in fish exposed to higher doses of ZnO NPs. The results on the oxidative and antioxidant status of exposed fish unveiled a significantly increased profile of reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS), a by-product of the lipid peroxidation (LPO) process and a lower quantity of different antioxidant enzymes. The results indicated that higher doses of ZnO NPs disrupt the physiological mechanisms of fish via induction of hemato-biochemical profile and induction of oxidative stress in multiple tissues.

Keywords: Mono sex Tilapia, Haemato-biochemistry, Erythrocytes, Oxidative stress

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Introduction

During the past few decades, NPs have been widely used to develop new structures, strategies, and systems that are established in various amazing fields such as wastewater treatment, electronic devices, and agriculture due to their high surface-to-volume ratio, adsorption capacity, and their high reactivity (Jain et al., 2021; Kazmi et al., 2023; Mwafy et al., 2023; Mahmood et al., 2024). It is one of the most important emerging industries used in the developed food sector and agriculture. It has created a revolution in advancement in nanoscience to develop nano fertilizers, nano food, nanosensors, and nano pesticides (Jimayu, 2022; Elbehary et al., 2023; El-Dawy et al., 2023). This technology had a great impact on food packing, food safety, and food quality (El-Hamaky et al., 2023). With advancement, we can enhance nutrition through the production, preservation, and safe delivery of micronutrients (Sahoo et al., 2021; Shafqat et al., 2023; Younas et al., 2024). Due to the development of resistance in bacteria against antibiotics, it has been investigated that various kinds of NPs are being used in medicine as antibacterial agents for treating bacterial diseases (Al-Rashdi et al., 2023; Mansour et al., 2023). It has been found that zinc oxide NPs are also used in electrical fields in various heat resistance devices (Aslam et al., 2023; Sial et al., 2023). Despite their significant importance, these NPs induce toxic effects in multiple animals. During their production, transportation, storage, and utilization, most of these NPs leak into natural water bodies and cause adverse effects on the body of aquatic life (Kühr et al., 2018). Utilizing waste materials containing NPs can lead to toxic impacts on human health and the functioning of the normal ecosystem (Biswas and Sarkar, 2019). Hence, it is necessary to investigate risks induced by different environmental contaminants including various kinds of NPs in aquaculture. These NPs generate free radicles by damaging the antioxidant system and disturbing cellular metabolism (Shahid et al., 2023), and also exposure to these NPs in aquatic bodies revealed possible environmental issues and food chain risks (Asghar et al., 2018). The mechanisms of induction of toxic effects of NPs depend upon these nanoparticles' size, configuration, distribution, and composition (Abbasi et al., 2023).

The ZnO NPs disrupt cellular metabolism and increase oxidative stress, damaging the cellular proteins, lipids, and nucleic acid (Afifi et al., 2016; Afzal et al., 2024). ZnO NPs have distinctive properties, including eco-friendly, optical properties, and high photosensitivity (Janani et al., 2023). Moreover, during oxidative stress, NPs inhibit catalase (CAT), superoxide dismutase (SOD), and GPX activities, and increase lipid oxidation in carps (Ashraf et al., 2023). To neutralize the toxic effect of ROS, body utilize the antioxidant defence system like enzymes SOD, CAT, GPX, glutathione s-transferase, and non-enzyme glutathione vitamin E compounds.

Based on a comprehensive literature review, this study aims to investigate the potential toxic impacts of ZnO nanoparticles (NPs) on mono-sex Nile Tilapia (Tilapia niloticus). The study hypothesizes that exposure to varying doses of ZnO NPs may induce significant toxic effects, including oxidative stress, nuclear alterations, and disruptions in hematological and serological parameters. The rationale for this research stems from the growing use of ZnO NPs in various industries, raising concerns about their potential environmental and biological impacts. Thus, this study seeks to provide a deeper understanding of ZnO NP toxicity in aquatic organisms, specifically fish, to assess the potential risks posed to aquatic ecosystems.

Material and Methods

Chemicals

For the synthesis of ZnO NPs, Zinc acetate dehydrate ($Zn(CHCOO)_2.2H_2O$), Sodium Hydroxide (NaOH), and ethanol [CH_3CH_2OH] were used. Various chemicals were procured from Sigma Aldrich (USA) to evaluate hematological parameters, histopathological changes, serological parameters, as well as oxidative and antioxidant enzymes. Distilled water and deionized water were used throughout the experiment.

Synthesis of ZnO NPs

ZnO NPs were synthesized using a co-precipitation method, with $Zn(CHCOO)_2.2H_2O$ and NaOH serving as the precursor chemicals. The procedure commenced by stirring a 0.1M aqueous solution of $Zn(CHCOO)_2.2H_2O$ for one h using a magnetic stirrer to ensure complete dissolution of the acetate. Similarly, a 0.8M aqueous solution of NaOH was prepared in the same manner with one h of stirring. Once the $Zn(CHCOO)_2$ was fully dissolved, the 0.8M NaOH solution was gradually added dropwise into the $Zn(CHCOO)_2$ solution under vigorous stirring at high

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speed. This addition process was conducted slowly over 45 min, ensuring the drops contacted the vessel walls. After the complete addition of NaOH, the reaction mixture was allowed to proceed for 2 h. Subsequently, the solution was left to settle overnight, and the supernatant solution was carefully decanted. The remaining solution underwent centrifugation for 10 min to separate the precipitate. The obtained ZnO NPs precipitate was washed three times with deionized water and ethanol to eliminate any residual by-products. The nanoparticles were then dried in ambient air at approximately 60°C. Throughout the drying process, the zinc hydroxide ($Zn(OH)_2$) in the precipitate underwent complete conversion into ZnO NPs following the protocol outlined by (Ghosh et al., 2022).

Sample characterization

Various methods were employed to validate the synthesis of ZnO NPs. XRD analysis, conducted using a D8 model from Advance Bruker Company, confirmed the crystalline structure of the prepared ZnO NPs within a 2θ range of 20º to 80º. The Scherrer equation, as represented in equation (1), was applied to calculate the average crystallite size of ZnO NPs:

$$D_{ave} = K\,\lambda / \beta \cos\theta \qquad (1)$$

where D denotes the crystallite size, K is a constant, λ represents the wavelength of Cu Kα X-ray radiation (0.154056 nm), β is the full width at half maximum (FWHM) of the diffraction peak, and θ is the Bragg angle (Qasim et al., 2020). Additionally, the morphology and particle size distribution of the synthesized ZnO NPs were determined using an SEM (EVOR60 Model).

Toxicity evaluation of synthesized ZnO NPs

Fish management

One hundred and fifty fish mono-sex gift tilapia, with an average size ranging from 55.1g±1 to 56.2g±1, were obtained from the Tawakal fish hatchery in district Muzaffar Garh. These samples were carefully transported in plastic bags filled with oxygen to a tilapia fish hatchery at Head Tounsa barrage, Tehsil Tounsa, District Dara Ghazi Khan. Before introduction into cemented tanks with a water capacity of 200L. All the specimens underwent treatment with $KMnO_4$ solution to eliminate external parasites. They were then acclimatized for 15 days. All experimental tanks were equipped with appropriate electric aerators to ensure sufficient oxygen supply.

Monitoring of water quality parameters

During this trial period, water quality parameters were checked regularly. The salinity measured was 0.22 ± 3 parts per thousand (ppt), the temperature was 27 ± 2°C, dissolved oxygen was 5 to 6 ppm, which was measured by a DO meter (Jenway 970), pH was 7.4, measured by a pH meter (Jenway 3510), and total dissolved substances were 202.4 mg/L. An electric aerator supplied aeration (24 h) to provide proper oxygen regularly to all experimental cemented tanks.

Feed ingredients and experimental treatment

The diet utilized in our study comprised 29.4% crude proteins, 5.8% fats, 17.2% minerals, 9.4% ash, and 4% fibers. All these components were ground into powder form and mixed with varying doses of ZnO NPs (150mg/kg, 300mg/kg, and 450mg/kg). Subsequently, 20% water was added to form a cohesive dough, which was then processed into floating feed pellets using a pelleting machine. Following the preparation of doses, 20 samples were allocated to each cemented tank, each tank having a water capacity of 200L for 28 days. The tanks were labeled as follows: C (for unexposed), T1 (150mg/kg ZnO NPs), T2 (300mg/kg ZnO NPs), and T3 (450mg/kg ZnO NPs). Pellets containing varying doses of ZnO NPs were supplied to the fish in each tank equivalent to 3% of their body weight twice daily for 28 days.

Study parameters

Blood sampling and nuclear evaluation

Blood from each sample was extracted through a cardiac puncture with a 2-cc syringe on days 7, 14, 21, and 28th of the trial. For nuclear and morphological changes thin blood films of exposed and unexposed fish were prepared without any coagulant. All blood films on the slides were dried immediately. These dried films were fixed with alcohol and stained and Giemsa solution (Ghaffar et al., 2015). About 1400-1500 red blood cells (RBCs) on each slide were perceived by using of light microscope associated with computer-containing oil immersion lenses. Other blood was centrifuge (Hettich-EBA 20-) at 13000 rpm to separate serum for 5 min. Blood was transported in

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EDTA coted vials and serum was collected in serum cups for further analysis (Qiao et al., 2021).

Haematological analysis

The haematology parameters of each fish from the exposed and unexposed groups were determined using an automated haematology analyzer. On the day of sampling, these parameters were immediately measured within one h, after the collection of 2 mL blood into EDTA from each exposed and unexposed fish (Witeska et al., 2022).

Serological parameters

1mL serum was separated from blood and various serum biochemical profiles were determined by using commercially available kits. Different serum biomarkers of various visceral tissues including albumin (Cat; 997258), urea (Cat; 996060), cholesterol (Cat; 30183), triglycerides (Cat; 30364), and creatinine (Cat; 99108) were estimated using a commercially available kit (Canovelles; Barcelona, Spain). The quantity of ALT (Cat; 30254), and ALP (Cat; 30134) were recorded (Ghaffar et al., 2020) by using different commercially obtained kits (Analytical, Germany, and Quimica Clinica Aplicada, S.A. Spain) with the help of a chemistry analyzer (Model: Rx Monza # 328-15-1001; UK)

Enzymatic analysis

Triturated fish organ samples were utilized to assess the damage induced in term of measuring of free radicals (ROS) and TBARS (Thiobarbituric acid reactive substance: nmol/TBARS formed/mg protein/min) in gills and liver tissues, following the previously described protocol (Akram et al., 2021). R-GSH (Reduce glutathione: mmol-g^{-1} tissue), which has antioxidant properties to cope with free radicals in the body was also determined at 412 nm using the methods already described (Ghaffar et al., 2021). Biochemical markers of antioxidant enzymes such as CAT (catalase: units/min) (Akram et al., 2021; Ali et al., 2024), SOD (superoxide dismutase: units/ mg protein) (Mahmood et al., 2022), and POD (Peroxide: units/min) (Yu et al., 2023) were measured by spectrophotometer (752PC-150W) at 240 nm, 560nm, and 470 nm in the gills and liver of fish by following the protocols given in earlier research studies.

Statistical analysis

All collected data were examined with the statistical software IBM SPSS (version 20). Statistical analysis was performed by one-way analysis of variance (ANOVA). Differences were considered as significant $p \leq 0.05$. In this research data are presented as mean ± S.D. The significant difference in mean values of the exposed groups and the unexposed group was assessed with post hoc Tuckey's test.

Results and Discussions

Structure analysis of ZnO NPs

Fig 1a confirmed that the sample only contained synthesized ZnO NPs. The crystal structure of synthesized NPs was determined through XRD. Diffraction peaks of the 2θ values at 31.8, 34.4, 36.3, 47.4, 56.7, 62.84, 66.3, 67.9, 68.9 and 76.8° were assigned to the planes of (100), (022), (101), (102), (110), (103), (200), (112), (201), and (202) respectively, indicated their good crystallization and hexagonal pattern of structure. This XRD pattern of synthesized ZnO NPs matches with standards hexagonal Wurtzite crystalline ZnO NPs having JCPDS no. 00-036-1451 (Ali et al., 2023; Mahmood et al., 2022). Our study is supported by *K. Elumalai* et al (Elumalai and Velmurugan, 2015). The average calculated crystalline size from the XRD peak was 17.6 nm of ZnO NPs.

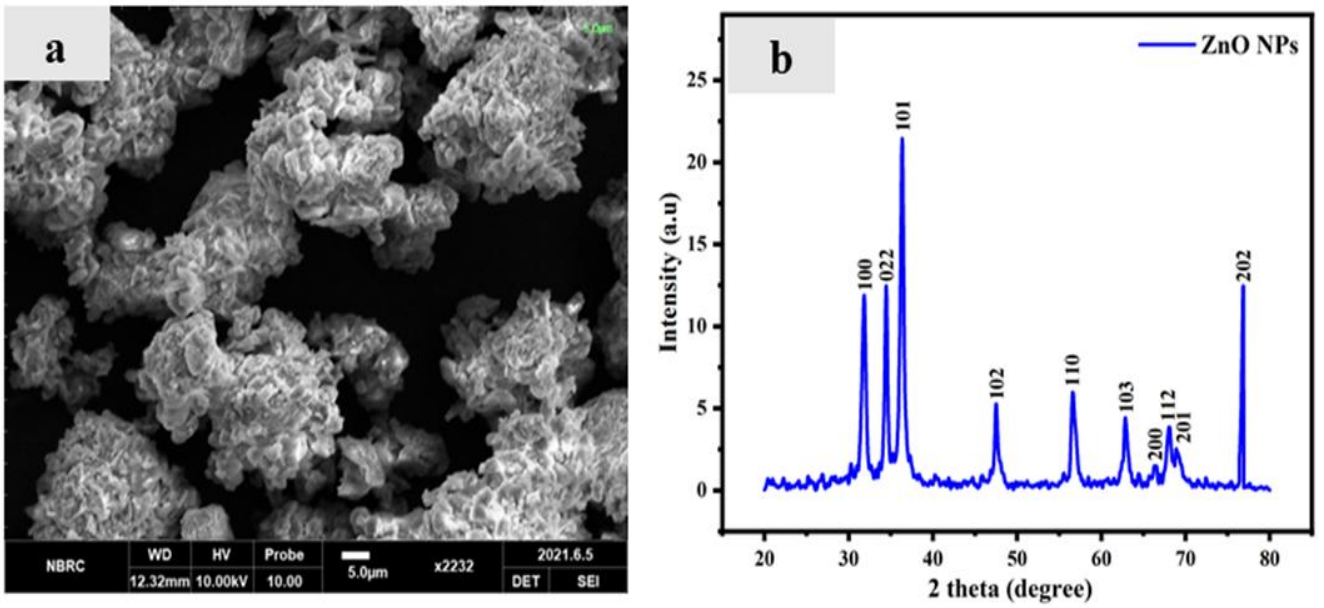

Figure-1: (a) The image of SEM exhibits of morphology of ZnO NPs (b) XRD shows structural properties of ZnO NPs.

Morphology analysis of ZnO NPs

Morphological structures of the ZnO nanoparticles were observed by using SEM. In this zinc acetate used as an originator, the ZnO molecules grow gradually to form spherical structures, and collect as flower-shaped bundles (Fig.1b) (Qasim et al., 2020). SEM images were perceived in different magnification series such as 5 µm–200 nm which show the presence of spherical formed nanoparticles with a mean average diameter of 70 nm.

Biological analysis

Nuclear changes and morphology in red blood Cells

The changes in the percentage rate of nuclear and morphological in RBC of fish exposed to variable doses of ZnO NPs indicated a significantly increased frequency of morphological abnormalities. According to these results percentile rates of erythrocytes with a broken nucleus, pear-shaped erythrocytes, leptocytes, spherocytes, macrocytes, erythrocytes with a lobed nucleus, erythrocytes with micronucleus, erythrocyte with a blabbed nucleus, erythrocyte with a vacuolated nucleus, and erythrocyte with nuclear remnants, were significantly higher in fish of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs as compared to the unexposed group at days 14, 21 and 28 of an experiment that is presented in Fig 2. These results can be compared with (Rajkumar et al., 2022) work on freshwater fish *Cyprinus carpio* to investigate the potential adverse effects. Previous research has demonstrated that erythrocytes serve as excellent indicators of oxidative stress exhibiting a wide range of nuclear and morphological changes in animals exposed to various toxic substances (Ghaffar et al., 2018; Hussain et al., 2014; Raza et al., 2022). The heightened intracellular production of ROS is believed to underline these alterations in the cellular body. In our study, the elevated percentage of erythrocytes exhibiting micronuclei and lobed nuclei could be linked to an overactivity of caspase-activated DNase. This enzyme plays a role in cleaving cytoskeletal proteins, nuclear proteins, aneuploid proteins, and causing oxidative damage to mitochondria (Raza et al., 2022).

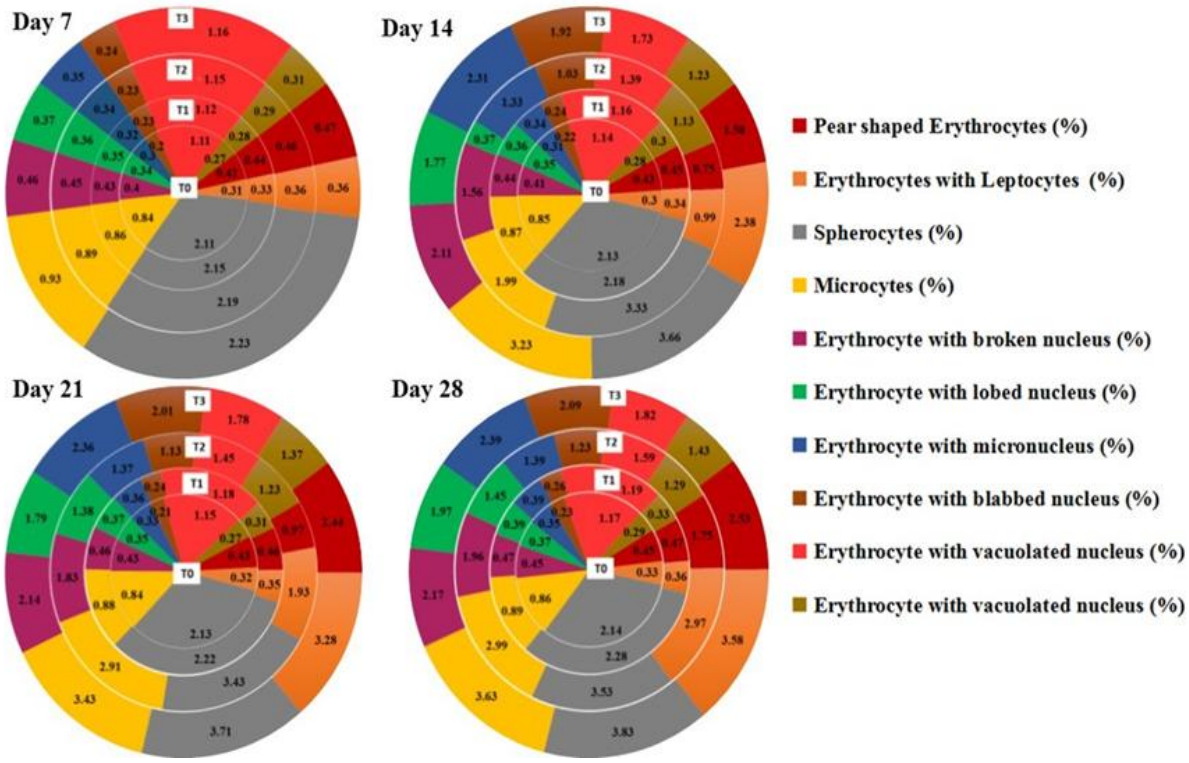

Figure-2: The photograph illustrates the comparisons of various morphological and nuclear alterations in fish erythrocytes at different sampling day of experiment.

Hematological alterations

The results on different blood parameters including the erythrocyte count, leukocyte counts, hemoglobin, monocyte, lymphocyte, and neutrophil population showed a significant difference in fish of exposed groups compared to the unexposed group as shown in Fig 3. This study indicates that the lymphocytes, erythrocytes, monocytes, and hemoglobin decline significantly in fish of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs at days 21 and 28 of the experiment. Leukocyte counts and Neutrophils were elevated significantly in fish of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs on days 14, 21, and 28 of the experiment. These results indicated that fish became anemic and vulnerable to diseases. Earlier studies were similar to our results, presented by (Naz et al., 2021; Sivakumar et al., 2019). According to the above results, RBCs and hemoglobin decreased causing anaemic conditions while WBCs decreased condition causing less resistance against pathogens. Similar results were reported by (Asghar et al., 2018).

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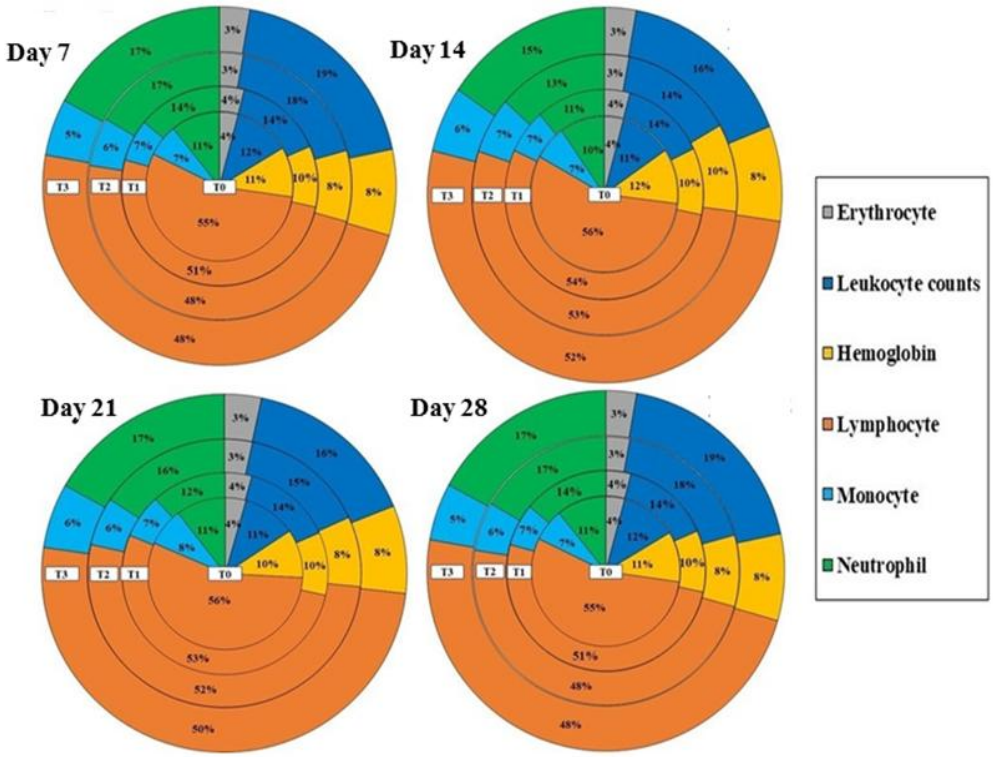

Figure-3: The photograph shows the comparisons of different hematological attributes at different intervals of the fish experiment.

Our results revealed that increasing the dose of ZnO NPs lymphocytes were counted as low while neutrophils and monocytes were measured as high. The above study indicates that with increasing the concentration of ZnO NPs fish become less resistant against diseases. Lymphocyte count was measured as low as compared to normal lymphopenia. It is a suitable marker of xenobiotic substance deficiency and immune system (Banaee et al., 2019). The number of monocytes and neutrophils increased by increasing the concentration of zinc oxide NPs compared to control-indicated infectious problems in fish bodies. Variations in leucocyte counts are known as indicators of certain infectious diseases, dysfunction in hematological tissues, and xenobiotics (Khabbazi et al., 2015). Such hemoglobin variations were also observed by (Faiz et al., 2015) and (Abdel-Khalek et al., 2016).

Serological alteration

Acute toxicity was observed concerning the kidney and liver through serum analysis including, cholesterol, triglyceride, proteins, globulin, urea, albumin, creatinine, ALP, AST, and ALT levels that indicated significant differences in fish of exposed groups as compared to the unexposed group. The study showed that the proteins, globulin, and albumin, were decline significantly in fish of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs at days 14, 21, and 28 of an experiment that is shown in Fig. 4. Whereas cholesterol, triglycerides, urea, creatinine, AST, ALP, and ALT levels were elevated significantly in fish of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs at days 14, 21 and 28 of experiment are shown in Fig 4. In another work, earlier results were similar to our study (Taheri et al., 2017; Hussain et al., 2017). Moreover, prior studies have indicated that elevated levels of various serum parameters and TBARS lipid may stem from deterioration of hepatic cells, and hypoxic situations affecting different tissues of body (Hussain et al., 2020; Liu et al., 2021;Sun et al., 2021). Increasing the dose of zinc (Zn) in the diet may increase the cholesterol level in the plasma. An increased level of creatinine in plasma is an index of kidney damage because it is produced from muscles in the blood and then excreted through the kidney from the body of the fish (Hussain et al., 2017; Hussain et al., 2020; Aslam et al., 2023). According to another work Supplementation of ZnO NPs (15mg/Kg) for about 21

days causes increased creatinine levels in fish plasma and results in kidney damage. High levels of ALP, ALT, and AST enzymes in blood circulation are an important cause of liver damage (Alkaladi et al., 2015). These findings align with another study where the activity of AST significantly increased in fish plasma fed with 10mg/kg and 15mg/kg ZnO NPs compared to the unexposed group fed with 5mg/kg ZnO NPs. This suggests that oral supplementation of more than 5mg/kg of ZnO NPs elevates the AST activity in plasma, potentially indicating liver damage, as reported in previous research (Banaee et al., 2019). Moreover, such supplementation can induce oxidative stress, as highlighted by Dekani et al. (Dekani et al., 2019).

However, at variable doses of ZnO NPs great alteration in urea level. With increasing the dose of ZnO NPs albumin and total proteins were gradually increased in the body of fish as compared to the exposed group. But according to previous work supplementation of ZnO NPs in the diet of the fish had no significant effects on albumin and total proteins (Sial et al., 2023)

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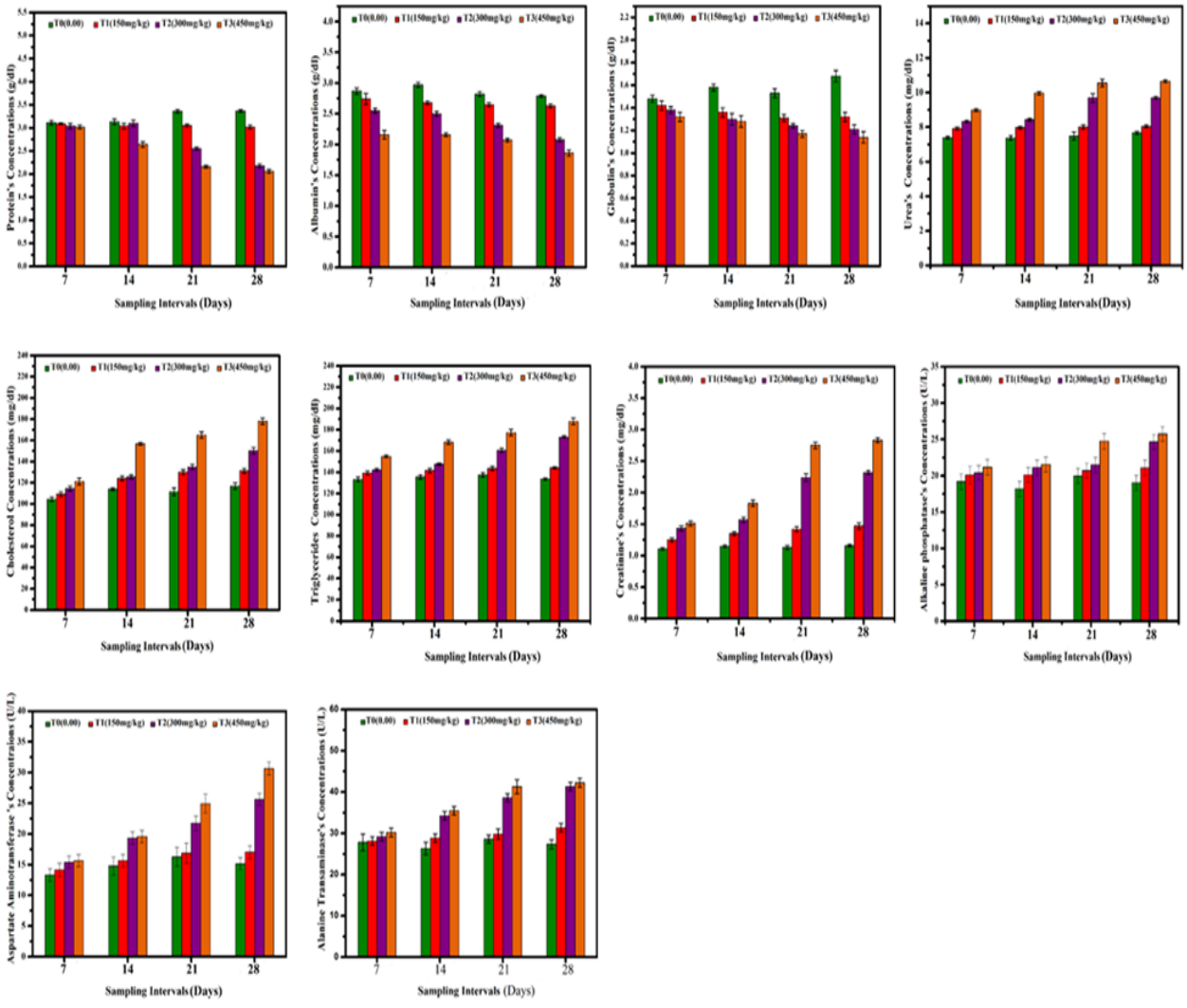

Figure-4: Photograph shows the comparisons of different serum biochemical attributes of fish at different intervals of the experiment.

Enzymatic analysis

Liver: At variable doses of ZnO NPs, significant alterations were observed in the quantity of antioxidant enzymes in the liver of exposed fish. According to these results, GST, SOD, CAT, and R-GSH were decline significantly in fish of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs at days 14, 21, and 28 of the experiment in comparison to unexposed group that is shown in Fig 5.

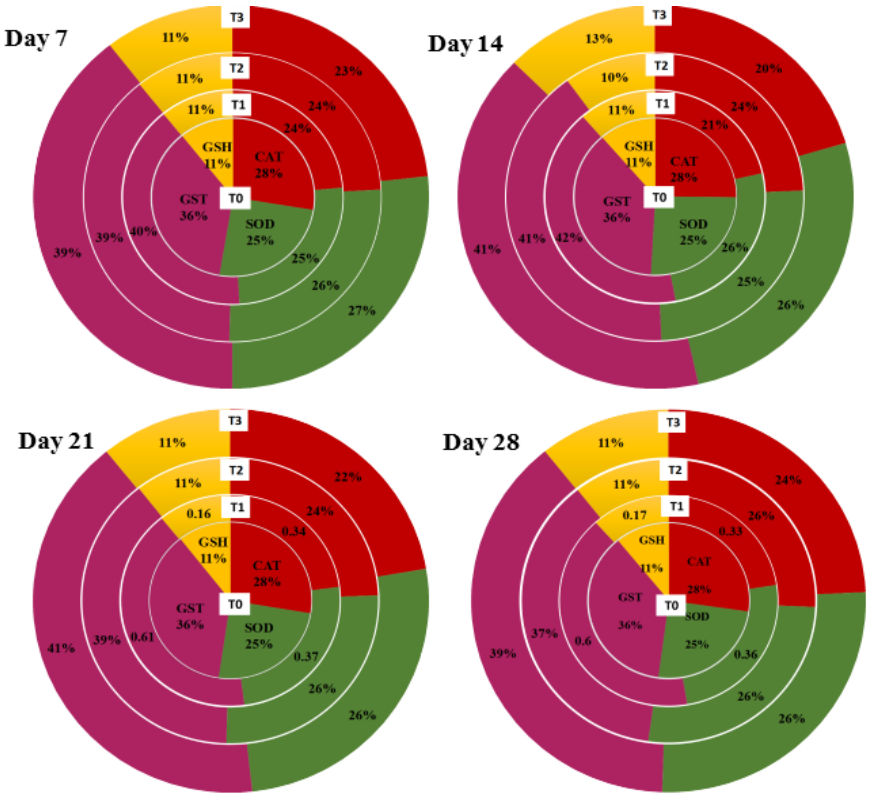

Figure-5: The Photograph shows the comparisons of different antioxidant biomarkers of the liver of fish at different intervals of the experiment.

Gills: Antioxidant enzymes such as GST, SOD, CAT, and R-GSH were significantly declined in the gills of groups (T2 and T3) exposed to 300mg/kg and 450mg/kg ZnO NPs at days 14, 21, and 28 of the experiment in comparison to the unexposed group as shown in Fig 6. Similar results were presented by (Saad et al., 2023) on fish (*Oreochromis niloticus*) that indicate increased levels of such enzymatic activities as antioxidant biomarkers to compensate for the oxidative stress (Asala et al., 2022) of ZnO NPs. Previously, different studies have recorded that significantly elevated contents of oxidative stress parameters and declined values of antioxidant parameters in the visceral tissues of fish are linked with each other (Ghazanfar et al., 2018; Ghaffar et al., 2019) to our study. Furthermore, earlier studies have reported decline levels of antioxidant parameters in visceral organs of catfish (Qiang et al., 2019), R-GSH in the liver of Delta smelt (Jin et al., 2018), and Jundiara fish, as well as decreased values of SOD and R-GSH in goldfish's plasma due to exposure to various toxicants (Wang et al., 2022). Furthermore, noticeable depletion of antioxidant enzymes and increased contents of oxidative stress profile could also be related to induction of pathological lesions in multiple tissues (Ghaffar et al., 2015; Ghaffar et al., 2020).

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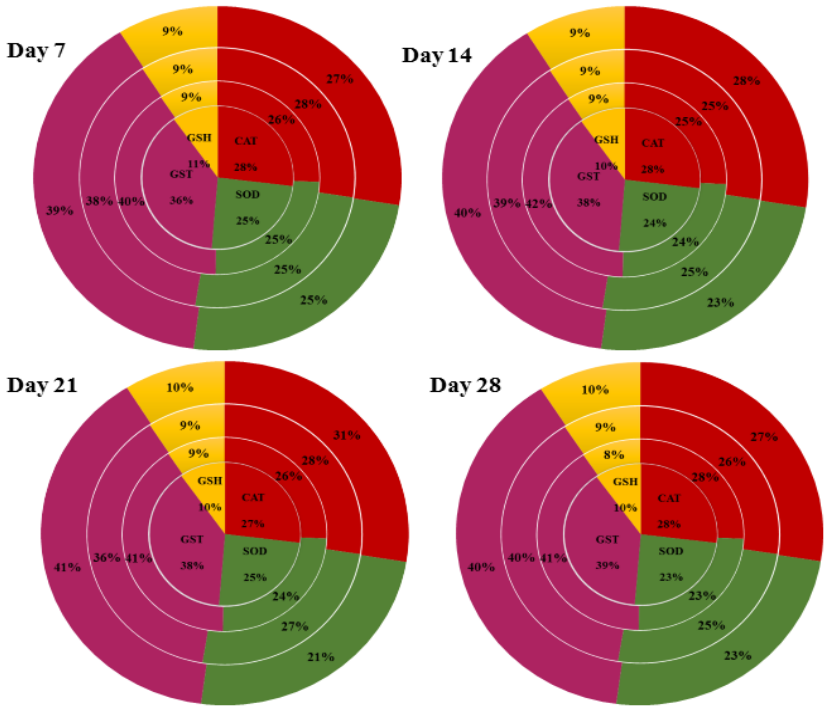

Figure-6: The Photograph shows the comparisons of different antioxidant biomarkers of the gills of fish at different intervals of the experiment.

Conclusion

This study demonstrates that the co-precipitate synthesis of ZnO NPs, characterized by XRD and SEM techniques, exhibit significant toxic effects on blood and various visceral organs of fish.The higher concentrations ZnO NPs have produced significant alteration in morphological and nuclear changes in erythrocytes, hematological parameters, serological profile and enzymatic activities of mono-sex fish and these variations may be taken as wide-ranging biomarkers in the area of Nanotoxicology. Histopathological analyses further highlight the detrimental effects on tissue integrity, particularly in the liver and gills of fish. However, the toxicity of ZnO NPs could be judged through the application of a wide range of concentrations. In general, these toxicological calculation methods are used to identify and maintain the adverse health effects of the products with these NPs.

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Conflict of Interest: None.
Source of Funding: None.

Ethical Approval Statement

This study was approved by Ethics Committee of Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Multan, Pakistan (Approval No. 12/PAB/2023).

Data Availability Statement

The relevant data is provided in the paper. However, any other relevant data/information regarding the research would be provided upon inquiry by the readers.

Contribution of Authors

Iqbal R: Designed the experiment.
Khan SR & Khalid M: Conducted trial and collected data.
Ahsan MA, Akram R, Hussain S, Rehan S, Ali A, Rehman MMU & Mammadov A: Contributed to article write up, data analysis and preparations of initial draft.
All authors read and approved the final draft.

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