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Toxicological Impact of Zinc Oxide Nanoparticles on Hybrid Grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*)

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ABSTRACT

The use of zinc oxide nanoparticles (ZnO-NPs) across various industries has raised concerns about their potential toxic effects on aquatic organisms, including hybrid grouper. The study aimed to evaluate the toxicological effects of ZnO-NPs exposure on hybrid grouper (±20g) through the analysis of mortality rate, blood performance, organ histopathology, and changes in immune response. The methods involved exposure of ZnO-NPs in four treatment groups (i.e., 0, 50, 100, and 200 ppm) for 96 hours, followed by analyzing the hematology, histopathology, and TNF-α, IL-1β antibody using flow cytometry. The obtained data were analyzed using ANOVA. The results indicated that ZnO-NPs significantly increased fish mortality up to 50% at 200ppm concentration. Significant changes were recorded in hematology, including a 62.96% reduction in hematocrit and a 20.28% increase in leukocyte count. Histopathological analysis showed severe intestinal tissue damage levels reaching 40.43%. Enhanced immune activity expressing TNF-α and IL-1β antibodies, which increased by 15.08% and 6.81%. The highest accumulation of ZnO-NPs was found in the gill organs, with a concentration of 2.94 ppm. This study highlights the toxic risk of ZnO-NPs to hybrid grouper, emphasizing the need for strict monitoring and the development of safety standards to support safer and more sustainable aquaculture practices.

INTRODUCTION

Aquaculture in Indonesia is an important economic sector that continues to grow rapidly, in line with increasing domestic and international market demand **(Mukti *et al*., 2020; Samara *et al*., 2024; Amin *et al*., 2025)**. According to data from the Ministry of Maritime Affairs and Fisheries (KKP), in 2022, aquaculture production was anticipated to

reach more than 16 million tons, with significant contributions coming from various marine and freshwater fish commodities. One species that has high economic value in marine aquaculture is the hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*), a cross between the tiger grouper and the kertang grouper **(Angwarmas *et al*., 2020; Khasanah *et al.*, 2020; Mahasri *et al*., 2023; Yanuhar *et al*., 2024)**. Hybrid grouper has the advantage of fast growth, resistance to changes in environmental conditions, and has a taste that is popular in the export market, especially in Asian countries **(Mahasri *et al*., 2020; Nisa *et al*., 2021)**. The value of grouper production in Indonesia in 2021 reached around 38,000 tons, with an estimated economic value of more than 79.779.050 USD, showing great potential to support the national economy through the aquaculture sector **(Zhou *et al.*, 2023)**.

Along with the development of technology, nanomaterials have begun to be utilized in various fields, including fisheries. The use of nanotechnology in the fisheries industry, especially in the form of nanoparticles, has shown great potential in increasing cultivation efficiency. Some nanomaterials that have been applied in fisheries include silver nanoparticles (Ag-NPs) **(Clark *et al.*, 2019)**, copper oxide nanoparticles (CuO-NPs) **(Aziz & Abdullah, 2023)**, titanium dioxide nanoparticles (TiO2-NPs) **(Canli *et al.*, 2018)**, and zinc oxide nanoparticles (ZnO-NPs). Ag-NPs are often used by farmers because they can prevent infections in fish and shrimp **(Vali *et al.*, 2020)**. In addition, CuO-NPs are also applied in the fisheries sector to prevent water contamination and help maintain the cleanliness of fish farming equipment **(Canli *et al.*, 2018; Aziz & Abdullah, 2023)**. Another commonly applied nanomaterial is TiO_2-NPs which have multifunctional properties such as antimicrobial activity, photocatalytic ability **(Souza *et al.*, 2019)**, and potential as a nutritional supplement **(Grasso *et al.*, 2020)**. In addition, nanotechnology products that are increasingly being used, such as ZnO-NPs. ZnO-NPs, are widely utilized because they are not only applied to control pathogenic bacteria in ponds but also as additives in fish feed, which can increase the growth and immunity of farmed animals **(Borysiewicz, 2019)**. As a microessential element, zinc (Zn) helps strengthen the immune system of fish by increasing the production of white blood cells and antioxidant enzymes that protect cells from oxidative stress **(Senapati *et al.*, 2015; Tang *et al.*, 2024)**. ZnO has environmentally friendly properties, so it is widely applied in pharmaceutical use **(Mirzaei & Darroudi, 2017)**.

The estimated production of ZnO-NPs in the world is 0.1 to 1.2 million tons **(Janani *et al.*, 2021)**. The widespread utilization of ZnO-NPs possibly enters the aquatic ecosystem through wastewater streams with an estimated concentration of 0.001–0.058µg/ l in surface waters **(Mandal *et al.*, 2024)**. The study conducted by **Geppert *et al.* (2021)** demonstrated that ZnO-NPs have low toxicity, with an EC50 value (the concentration at which 50% of cells exhibit toxic effects) exceeding 100mg/ L. This is attributed to the fact that ZnO-NPs almost completely dissolve into zinc ions (Zn^{2+}),

which likely contributes to their low particulate toxicity. **Mahjoubian *et al*. (2023b)** also reported that the LC50 value for zinc oxide nanoparticles (ZnO-NPs) following acute 96-hour exposure exceeds 100mg/ L, indicating relatively low acute toxicity to the zebrafish when compared to silver nanoparticles (Ag-NPs), exhibiting an LC50 value of 0.224mg/ L. This disparity underscores that ZnO-NPs possess a significantly higher lethal concentration threshold than many other nanoparticle types, including Ag-NPs. The reduced toxicity of ZnO-NPs may be attributed to factors such as their propensity to aggregate in aqueous environments, particle size characteristics, and their reduced reactivity due to the formation of less reactive ionic compounds.

ZnO in the form of nanoparticles also raises concerns regarding the toxicological impact on fish. Exposure to ZnO-NPs in high concentrations can interfere with the physiological functions of fish, including the respiratory, digestive, and immunological systems. Several studies have shown that exposure to ZnO-NPs in fish can cause significant changes in hematological parameters, such as decreased red blood cell counts and increased inflammatory responses, which can negatively impact fish health **(Dai *et al.*, 2020)**. Exposure to ZnO-NPs in fish can occur through various pathways, including digestion, gills, and skin (Najahi-Missaoui *et al.*, 2020). After entering the fish's body, ZnO-NPs can bioaccumulate and cause toxic effects on vital organs such as the liver, kidneys, and immune system. Chronic exposure to ZnO-NPs caused significant changes in the respiratory function of fish **(Kaya *et al.*, 2015)**. Meanwhile, a study by **Shahzad *et al*. (2019)** reported that inadvertent consumption of ZnO-NPs affected the gut microbiota and glucose balance in farmed fish. Although the toxicological effects of ZnO-NPs on organ tissues have been identified, understanding the detailed mechanisms of how these nanoparticles affect the specific functions of other organs, such as the liver and kidney, and how long-term impacts on fish growth and immunity has not yet been fully explored. Therefore, there is an urgent need to further explore the toxic effects of ZnO-NPs on fish, including their effects on fish physiology and immunology. The aim of this study was to evaluate the toxicological effects of ZnO-NPs on the hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*). Toxicology effect will be identified through analysis of mortality rate, blood performance, hystopathology and changes in immune response using flowcytometry.

MATERIALS AND METHODS

Ethical approval

The Research Ethics Commission, University of Brawijaya studied the research design carefully and provided ethical approval (184-KEP-UB-2024).

Material

ZnO-NPs used in this study were purchased from Hongwu Materials, China with an average particle size of 20- 30nm. This study used the hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*) with an average weight of 20g and a body length of about 10cm. The fish were obtained from a local hatchery in the coastal area of Bali, Indonesia that has been standardized.

Acclimatization

Prior to treatment, the fish were acclimatized in a clean, well-aerated seawater tank for one week with commercial pellet feeding twice a day at a temperature of 28 ± 1°C and a salinity of 30ppt to ensure the health condition of the fish. Feeding was stopped 24 hours before the test began **(Rasheed *et al.*, 2023)**.

ZnO-NPs exposure procedure

The exposure procedure refers to the research procedure conducted by **Khan *et al.* (2022)**. ZnO weighing 0.5 grams, 1 gram and 2 grams were mixed in 500ml of seawater and were then homogenized with an ultrasonic homogenizer (AH-100D, Berkley Scientific, China) for 30 minutes. The solution was then mixed with 9.5 L of seawater to produce ZnO-NPs with concentrations of 50, 100, and 200ppm **(Khan *et al.*, 2022)**. Fish were then exposed to this suspension for 4 days (96 hours) .

Fish were divided into four treatment groups, each consisting of 6 fish. The control group was kept in seawater without ZnO-NPs, while the treatment group was exposed to ZnO-NPs suspensions at predetermined concentrations. Every 48 hours, fish samples from each group were taken for further analysis. Prior to blood sampling and dissection, fish were anesthetized with essential oils. Body weight, total length, and standard were recorded for each individual **(Ebi *et al.*, 2018)**. During the exposure period, physiological parameters such as fish mortality, swimming activity, and feeding response were recorded daily. Fish mortality was recorded every 48 hours up to 96 hours to determine the LC50 **(Sayadi *et al.*, 2022)**.

Blood sampling and hematology

Blood samples were taken from the caudal vein for analysis of fish hematological performance, including measurement of red blood cell (erythrocyte) count, hemoglobin (Hb) and hematocrit levels, white blood cell (leukocyte) count, leukocyte differential (lymphocyte and neutrophil), platelets, macronuclei, micronuclei, and erythrocyte index (mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), and then stained with gemsa solution **(Khan *et al.*, 2022; Rasheed *et al.*, 2023)**.

Histopathology

The intestine organs of hybrid grouper were taken for histopathological analysis. Refering to **Li *et al*. (2024)**, organ tissues were fixed in 10% formalin solution, followed by the process of making histological preparations, then stained with hematoxylin-eosin (HandE) for microscopic observation.

Flowcytometry analysis

Cells were isolated from organs exposed such as liver and kidneys to ZnO-NPs, then processed by trypsinization and suspended in PBS. These cells were analyzed using a Flow Cytometer (BD Biosciences, FACS Calibur model from Germany) with TNF-α and IL-1β antibody measurements (BioLegend, Inc. San Diego, USA) to determine cell size and complexity. A total of 10,000 events per sample were analyzed to measure the percentage of cells incorporating ZnO-NPs.

Data analysis

Data were analyzed using Origin version 19B, with mortality expressed as a percentage, while hematology results and other parameters were compared between treatment groups by examining trends in clinically and biologically significant changes. Comparisons among the four experimental groups were made using one-way ANOVA, and results were presented as means and standard deviations with a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

1. Fish behavior and mortality

During the 4-day exposure period (96 hours), hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*) exhibited varying responses to different concentrations of zinc oxide nanoparticles (ZnO-NPs). Fish mortality increased with higher concentrations of ZnO-NPs. In the control group (0 ppm ZnO-NPs), no mortality was observed. However, mortality rates increased significantly at concentrations of 50ppm (16.67%), 100ppm (33.33%), and 200ppm (50%). The LC50 value was determined to be 200ppm over 96 hours, marking this concentration as a critical threshold for acute toxicity. The detailed mortality data are presented in Table (1). In addition, behavioral changes such as decreased swimming activity and poor appetite were also observed in the groups exposed to higher ZnO-NPs concentrations. Fish in the 200ppm concentration group also showed symptoms of stress, such as paler body color and sluggish movement.

Table 1. Mortality percentage of death of hybrid grouper

Concentration	Number of test Animals (heads)	Death		Mortality (%)
		48 hours	96 hours	
Control	6	0	0	0
50 ppm	6	0	1	16.67
100 ppm	6	1	1	33.33
200 ppm	6	1	2	50.00

The LC50 results can be used to compare the toxic effects of ZnO-NPs with other hazardous substances and to develop mitigation strategies to protect fish health and aquatic ecosystems. A low LC50 value indicates that the substance is highly toxic, as it can cause the death of half the exposed population at a low concentration. Conversely, a high LC50 value suggests that the substance is less toxic **(Al-Kshab & Yehya, 2021; Bita *et al*., 2021)**. Determining the LC50 concentration is essential to assess the safety and tolerance limits of various toxic substances. This difference in toxicity may be due to variations in the physicochemical properties of nanoscale ZnO that affect its toxicity mechanism. The toxicity level of ZnO-NPs exposure is due to the presence of dissolved free zinc ions (Zn^{2+}). ZnO-NPs are more toxic than their ionic form due to their nanoscale properties and higher reactivity **(Ahmadi *et al*., 2020)**. ZnO-NPs show increased toxicity at low pH due to the high solubility of Zn ions in the exposure medium **(Aziz *et al.*, 2020; Al-Zahaby *et al.*, 2023)**. The increased mortality in the hybrid grouper exposed to ZnO-NPs indicates that these nanoparticles have significant toxicity potential in this species. The higher mortality rate at higher concentrations may be due to increased nanoparticle exposure causing more severe cellular and organ damage. Behavioral changes such as decreased swimming activity and poor appetite are indicators of environmental stress induced by ZnO-NPs in water.

2. Hematology analysis

The hematological characteristics of the hybrid grouper were analyzed after exposure to ZnO-NPs at various concentrations (0, 50, 100, and 200ppm) for 96 hours (Table 2 & Fig. 1.).

Table 2. Hematology parameters of hybrid grouper after ZnO-NPs treatments for 48 and 96 hours

Hematology parameter	Concentration of ZnO-NPs						
	0 ppm	50 ppm		100 ppm		200 ppm	
		48 h	96 h	48 h	96 h	48 h	96 h
Erythrocyte (cells/mm^3)	2.93 ± 0.15 × 10^4	2.75 ± 0.09 × 10^4	2.1 ± 0.20 × 10^4	1.58 ± 0.08 × 10^4	1.44 ± 0.04 × 10^4	1.15 ± 0.13 × 10^4	0.945 ± 0.01 × 10^4
Hemoglobin (g/dL)	6.3 ± 1.39	4.8 ± 0.30	4.4 ± 0.20	4.5 ± 0.3	3.7 ± 0.17	3.8 ± 0.20	3.4 ± 0.40

Hematocrit (%)	27 ± 1.73	21± 2.00	16 ± 2.00	18 ± 2.65	15 ± 2.00	16 ± 1.73	10 ± 2.65
Leukocyte (cells/mm^3)	14.2 ± 3.0 × 10^5	15.5 ± 2.65 × 10^5	15.8 ± 2.0 × 10^5	16 ± 4.36 × 10^5	16.4 ± 2.65 × 10^5	16.7 ± 1.73 × 10^5	17.3 ± 2.0 × 10^5
Neutrophil (%)	14.8 ± 0.20	19.1 ± 0.36	20.8 ± 0.44	21.1 ± 0.10	23.8 ± 0.20	22.3 ± 0.26	24.4 ± 0.26
Lymphocyte (%)	71 ± 0.40	59.5 ± 0.20	54.9 ± 0.20	56.4 ± 0.26	50 ± 0.20	50.5 ± 0.20	46 ± 0.17
Platelets (%)	22 ± 2.00	19 ± 1.73	16 ± 2.00	17 ± 2.65	15 ± 2.65	14 ± 1.00	12 ± 2.00
Macronuclei (cells/1000)	9.2 ± 0.17	10 ± 0.44	12.1 ± 0.26	12.4 ± 0.26	13.5 ± 0.20	14.1 ± 0.36	14.8 ± 0.20
Micronuclei (cells/1000)	8 ± 0.44	9.5 ± 0.26	11.6 ± 0.20	11 ± 0.62	12.5 ± 0.10	13 ± 0.26	13.9 ± 0.20
MCV (µm^3)	76 ± 5.13	92 ± 3.79	98 ± 9.17	114 ± 18.01	103 ± 11.02	141 ± 27.15	105 ± 26.86
MCH (pg)	17 ± 1.53	21 ± 3.61	23 ± 3.51	28 ± 3.21	25 ± 0.58	33 ± 5.77	35 ± 4.51
MCHC (%)	23.3 ± 4.5	25 ± 3.0	27.7 ± 2.9	25.5 ± 4.7	24.9 ± 2.3	23.9 ± 1.7	35.7 ± 10.9

Fig. 1. Hematology profile of hybrid grouper. (A) Erythrocytes; (B) Platelets; (C) Lymphocytes; (D) Basophils; (E) Monocytes; (F) Neutrophils; (G) Eosinophils; (H) Macronuclei; (I) Micronuclei.

2.1 Erythrocyte

The results showed that the number of red blood cells (erythrocytes) decreased significantly in fish exposed to ZnO-NPs (Table 2). Based on the calculation results, the average erythrocyte count in the 48-hour test ranged between $1.15x10^4$ - $2.93x10^4$ cells/mm³, while in the 96-hour test, the average erythrocyte count ranged between $0.945x10^4$ - $2.45x10^4$ cells/mm³. The highest value was found in the concentration of 0ppm at $2.93x10^4$ cells/mm³, and the lowest value was observed at a concentration of 200ppm, at $0.945x10^4$ cells/mm³. This indicates that the higher the ZnO-NPs concentration, the lower the erythrocyte count in the hybrid grouper. The range of erythrocytes generally ranges from $1.05x10^4$ - $3.00x10^4$ cells/mm^3 **(Yanuhar *et al*., 2019)**. This result showed that erythrocyte value was decreased by 67.73% from the 0ppm (2.93×10^4 cells/mm³) to the 200ppm concentration at 96 hours (0.945×10^4 cells/mm³).

The decrease in the number of red blood cells and hemoglobin levels observed in this study indicated anemia in hybrid grouper fish exposed to ZnO-NPs. This anemia is likely caused by the cytotoxic effect of ZnO-NPs which damages red blood cells, disrupting erythrocyte production in the bone marrow **(Suganthi *et al.*, 2015)**, or accelerate hemolysis **(Ng *et al.*, 2017)**. ZnO-NPs may also cause oxidative stress in red blood cells, resulting in cell membrane damage and a decrease in the number of circulating cells.

2.2 Hemoglobin (Hb) and hematocrit levels

Result of Hemoglobin (Hb) and hematocrit levels also showed a significant decrease in fish exposed to ZnO-NPs (Table 2). The average hematocrit level in the 48-hour test ranged from 16% to 27%, while in the 96-hour test, the average hematocrit level ranged from 10% to 24%. The highest hematocrit value was observed at a concentration of 0ppm, at 27%, and the lowest value was observed at a concentration of 200ppm, at 10%. This indicates that as the ZnO-NPs concentration increases, the hematocrit level in hybrid grouper decreases. The normal hematocrit range for teleost fish is between 20 and 30%, and in some marine fish species, it is approximately 42% **(Yanuhar *et al.*, 2019)**. In this research, the result of hematocrit value was decreased by 62.96% from the control (27%) to the 200ppm concentration at 96 hours (10%).

Result of Hemoglobin values showed a similar pattern of decrease, with a sharper decrease at higher ZnO-NPs concentrations (Table 2). The average hemoglobin levels in the 48-hour test ranged between 3.8– 6.3g/ dL, while in the 96-hour test, the average hemoglobin levels ranged between 3.4– 5.6g/ dL. The highest hemoglobin level was observed in the control group at 6.3 g/dL, and the lowest was found at a concentration of 200ppm at 3.4 g/dL. Normal hemoglobin levels in the fish range of 5.05 to 8.33 grams / 100ml of blood or gram /%. If the Hb level is low, it will have an impact on the low amount of oxygen in the blood **(Yanuhar *et al.*, 2019)**. This research showed that the result of hemoglobin value was decreased by 46.03% from the 0ppm (6.3 g/dL) to the 200ppm concentration at 96 hours (3.4g/ dL).

Based on the research conducted, it is known that the higher the concentration of ZnO-NPs and the longer the test time, the lower the amount of hemoglobin and hematocrit produced. Exposure to ZnO nanoparticles can cause a reduction in hematocrit levels, indicating blood dilution or the loss of erythrocytes. This may suggest that the fish experience oxygen deficiency caused by stress induced by ZnO exposure **(Burgos-Aceves *et al.*, 2019)**. The decrease in hemoglobin levels occurs due to lysis caused by the rupture of blood cells due to toxins in the blood or the so-called hemolysin **(Preedia Babu *et al.*, 2017; Singh *et al.*, 2020)**. Low hemoglobin levels are an indication of infection in fish caused by ZnO-NPs. A decrease also occurs in hematocrit levels which can indicate that fish are experiencing oxygen deprivation due to stress related to the

administration of ZnO-NPs.

2.3 Leukocyte

The number of white blood cells (leukocytes) increased significantly with increasing concentration of ZnO-NPs (Table 2). The average leukocyte count in the 48-hour test ranged between $1.33x10^5$ – $1.67x10^5$ cells/mm³, while the 96-hour test showed an average leukocyte count ranging from $1.42x10^5$ – $1.71x10^5$ cells/mm³. The highest value of leukocyte count was observed at the 200ppm concentration, with $1.71x10^5$ cells/mm³, and the lowest value was in the 0ppm concentration, with $1.33x10^5$ cells/mm³. The study revealed that the higher the ZnO-NPs concentration and the longer the test duration, the higher the leukocyte count produced. The normal leukocyte range for healthy fish is between $0.5x10^5$ - $1.50x10^5$ cells/mm³ **(Bardhan *et al.*, 2024)**. In the current research, the result of leukocyte value was increased by 20.28%, from the control (1.42×10^5 cells/mm³) to the 200ppm concentration at 96 hours (1.71×10^5 cells/mm³).

The high number of leukocytes in fish is caused by an infection, prompting the fish to enhance its immune response **(Huda *et al.*, 2024)**. Leukocytes are the body's first line of defense when an infection occurs. A significant increase in the number of leukocytes in the group exposed to ZnO-NPs indicates an immune response induced by these nanoparticles. This leukocytosis can be considered as an attempt by the fish body to fight stress and damage caused by ZnO-NPs **(Sherif *et al.*, 2023; Yaqub *et al.*, 2023)**. When foreign objects enter, the fish body will signal and produce large amounts of leukocytes to provide defense against disease and infection.

2.4 Leukocyte differential

The differential performance of leukocytes, including neutrophils and lymphocytes, showed varying proportion changes among the treatment groups (Table 2). The average neutrophil values in the 48-hour test ranged between 14.8% and 22.3%. Meanwhile, the average values in the 96-hour test ranged between 11.4% and 24.4%. The highest neutrophil measurement was observed at the 200ppm concentration, with 24.4%, and the lowest was in the 0ppm concentration, at 11.4%. The increase in neutrophil count may result from the rising concentration of ZnO-NPs, which triggers a stronger immune response as indicated by the neutrophil count. The appropriate neutrophil range for fish survival is 10%-18.1% **(Palmi *et al.*, 2019)**. This research showed that the result of neutrophils value was increased by 114.04%, from the control (11.4%) to the 200ppm concentration (24.4%).

Based on lymphocyte measurements, the average lymphocyte levels in the 48-

hour test ranged from 50.5% to 71.2%, while in the 96-hour test, they ranged from 46.6 to 73.3%. The highest lymphocyte level was observed at a concentration of 0 ppm, with 73.3%, and the lowest was at a concentration of 200ppm, with 46.6%. Generally, the appropriate lymphocyte level for fish survival is between 60 and 80% **(Lulijwa *et al.*, 2019)**. The lymphocyte levels in the control group were within the normal range for fish survival, at 71.2-73.3%. However, at concentrations of 50, 100, and 200ppm, the lymphocyte levels were lower than the normal range for the hybrid grouper survival, at 46.6%-59.5%. This research showed that the result of lymphocytes value was decreased by 36.42%, from the control (73.3%) to the 200ppm concentration (46.6%).

Huda *et al.* (2024) explained that the range percentage of neutrophil cells in fish is 28%, and added that the range percentage of lymphocyte cells in fish is 74.11%. ZnO-NPs is toxic to cells that can cause decreased lymphocyte production in the immune system. Lower lymphocyte levels are an indication of toxicity or harmful effects on the fish's body. In addition to lymphocytes, eosinophil levels also decrease because the toxic effects of ZnO-NPs interfere with the normal function of eosinophils and show a negative impact on the immune system. Meanwhile, monocytes have increasing levels due to the body's immune response to fight disease infections in accordance with their role as the main phagocyte cells **(Chiu & Bharat, 2016; Guo & Dixon, 2021)**. Monocytes have a role in destroying various pathogens and diseases **(Mosquera-Murillo *et al.*, 2023)**. Basophils also have increasing levels as a response of the body to exposure to foreign substances. In addition, the increase in neutrophil levels is caused by ZnO-NPs which trigger inflammation.

2.5 Platelets

Platelet counts also showed significant changes in fish exposed to ZnO-NPs (Table 2). The average platelet levels in the 48-hour test ranged from 14% to 22%, while in the 96-hour test, they ranged from 12 to 25%. The highest platelet level was observed at 0ppm, with 25%, and the lowest was at a concentration of 200ppm, with 12%. The graph indicates that at 0ppm, the levels were still within the normal range, while at concentrations from 50ppm to 200ppm, the levels were below the normal range. The percentage of platelet count in fish normally ranges between 20- 30% **(Witeska *et al.*, 2023a)**. This research showed that the result of platelet value was decreased by 52.00%, from the 0ppm (25%) to the 200ppm concentration (12%).

This increase in platelet count may be a compensatory response to endothelial damage or oxidative stress induced by nanoparticles **(Noureen *et al.*, 2022)**. Platelets are

essential components in the wound-healing process, primarily facilitating clot formation and tissue repair. Elevated platelet percentages in fish are indicative of physiological responses to injury or hemorrhage **(Pridayem & Windarti, 2022)**. Overall, it is likely that ZnO-NPs exposure causes platelet reduction in fish, especially if the exposure is high or prolonged.

2.6 Macronuclei and micronuclei

The calculation of the number of macronuclei and micronuclei also showed significant changes in fish exposed to ZnO-NPs (Table 2). The average macronuclei levels in the 48-hour test ranged from 14.1 to 9.2 cells/1000, while in the 96-hour test, they ranged from 14.8 to 9.6 cells/1000. The highest macronuclei level was observed at a concentration of 200ppm, with 14.8 cells/1000, and the lowest was at 0ppm, with 9.2 cells/1000. These results indicate that the higher the ZnO-NPs concentration, the greater the number of macronuclei in the hybrid grouper fish **(Wang *et al.*, 2022)**.

The average micronuclei levels in the 48-hour test ranged from 8 to 13 cells/1000, while in the 96-hour test, they ranged from 9.7 to 13.9 cells/1000. The highest micronuclei level was observed at a concentration of 200 ppm, with 13.9 cells/1000, and the lowest was at 0 ppm, with 8 cells/1000. The research findings reveal that the longer the exposure duration, the higher the number of micronuclei observed. Generally, the frequency of micronuclei in fish red blood cells ranges between 0.1% and 1% of the total cells examined, equivalent to approximately 1 to 10 micronuclei per 1000 cells.

The increase in the number of these cells indicates genetic damage and increased genotoxic activity due to exposure to these nanoparticles. The increased levels of macronuclei are caused by free radicals in the fish's body which results in DNA damage and spindle thread dysfunction **(Xu *et al.*, 2020)**. Additionally, free radicals can trigger the formation of macronuclei, which are cellular abnormalities induced by the effects of free radicals. Meanwhile, the higher levels of micronuclei can indicate that the fish is experiencing physiological dysfunction. Based on the results obtained, exposure to ZnO-NPs exceeding normal limits and increasing in concentration due to high pollution in a water body leads to a higher micronuclei count. Conversely, the lower the number of micronuclei in the blood, the lower the level of pollution in the water body.

2.7 Erythrocyte index

Erythrocyte index including MCV, MCH, and MCHC showed varying proportion changes among treatment groups (Table 2). The average MCV (Mean corpuscular volume) in the 48-hour test ranged from 76 to 139μm³, while in the 96-hour test, it ranged from 76 to 105μm³. The highest MCV value was observed at a concentration of 200ppm, with 139μm³, and the lowest at 50ppm with 76 μm³. Based on the graph, it can be seen

that MCV increases with higher treatment concentrations. Generally, MCV in aquatic fish ranges between 150– 350µm³ **(Acar *et al.*, 2019)**. This research showed that the result of MCV value was decreased by 24.46%, from the control (139µm³ at 48 hours) to the 200ppm concentration at 96 hours (105µm³).

The average MCH (Mean corpuscular hemoglobin) in the 48-hour test ranged from 17 to 33pg, while in the 96-hour test, it ranged from 20 to 35pg. The highest MCH value was observed at a concentration of 200ppm, with 35pg, and the lowest at 50ppm, with 17pg. Based on the graph, it is evident that MCH increases with higher treatment concentrations. Generally, the average MCH range in fish is 30– 100pg **(Arnaudov & Arnaudova, 2022)**. This research showed that the result of MCH value was increased by 22.73%, from the control (20pg) to the 200ppm concentration at 96 hours (35pg).

The average MCHC (Mean corpuscular hemoglobin concentration) in the 48-hour test ranged from 23.3 to 25%, while in the 96-hour test, it ranged from 23.3 to 34%. The highest MCHC value was observed at a concentration of 200ppm, with 34%, and the lowest MCHC value was at 50ppm, with 22.8%. MCHC values indicate that the hemoglobin concentration is within the volume of red blood cells and serves as a health indicator. Higher MCHC values indicate higher hemoglobin content in the blood **(Docan *et al.*, 2018)**. MCHC values are categorized into three levels: low (<33%), normal (33%–36%), and high (>36%) **(Witeska *et al.*, 2023b; Tang *et al.*, 2024)**. This research showed that the result of MCH value was increased by 45.92%, from the lowest at 50ppm (22.8%) to the highest at 200ppm (34.0%).

Exposure to ZnO-NPs in fish can have varying effects depending on the concentration and duration of exposure. ZnO-NPs can act as an effective photocatalyst in degrading harmful substances but at high concentrations can be toxic to fish causing damage to their biological systems, including the circulatory system. These findings align with previous studies, suggesting that ZnO-NPs can disrupt cellular homeostasis, leading to compromised health and immune functionality in fish **(Bojarski *et al.*, 2021; Witeska *et al.*, 2022)**. The MCV value will be high if the hematocrit value is high and the number of erythrocytes is less. The high MCV value indicates that the size of the erythrocyte cells is larger. The MCH value is influenced by the hemoglobin level and the number of erythrocytes in the blood circulation. Higher doses of ZnO-NPs and longer exposure times can increase MCH values. A drastic increase in MCHC values with high doses can cause damage or changes to the hematology system. MCHC values can indicate the concentration of hemoglobin in the volume of erythrocytes which can be used as an indicator of health.

3. Histopatology analysis of fish

3.1 Necrotic damage

The observations indicate that necrotic damage occurred in the hybrid grouper fish at every concentration level. Necrosis reflects a condition of decreased tissue activity, characterized by the sequential loss of parts of cells within a tissue, ultimately leading to cell death in a short period **(Wallig & Janovitz, 2022)**. The necrotic damage observed in the intestine tissue of hybrid grouper fish can be seen in Fig. (2).

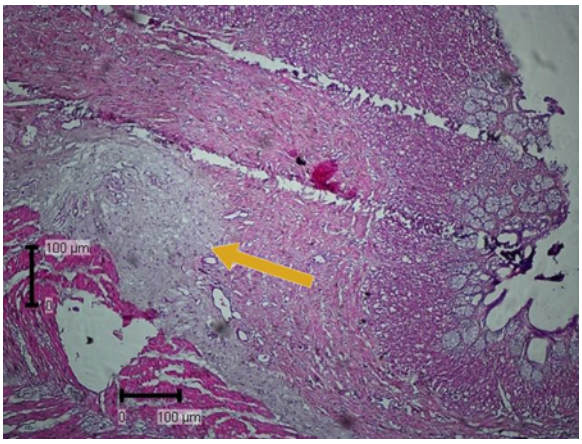

Fig. 2. Representation of necrotic lesions in the intestinal tissue of hybrid grouper observed under 100x magnification

The results showed that necrotic damage to the instine tissue increased in fish exposed to ZnO-NPs (Fig. 3). Observations conducted on the 48-hour exposure revealed that the treatments with 0, 50, and 100ppm concentrations exhibited low or mild levels of damage, categorized as score 1, with percentages ranging from 0 to 13.03%. In contrast, the 200ppm concentration displayed moderate damage, categorized as score 2, with a damage level of 15.67%. In the 96-hour exposure, 0 and 50ppm concentrations maintained low or mild damage levels, also categorized as score 1, with percentages ranging from 0 to 9.03%. However, the 100ppm concentration demonstrated moderate damage, categorized as score 2, with a damage level of 16.35%, while the 200ppm concentration showed severe damage, categorized as score 3, with a damage level of 27.92%.

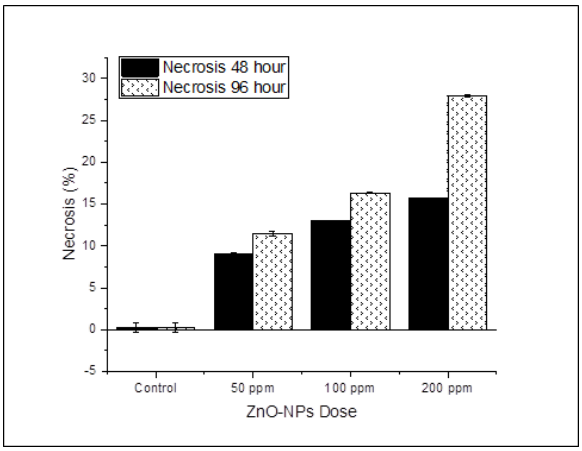

Fig. 3. Percentage necrosis damage in intestine organ of hybrid grouper after ZnO-NPs treatments for 48 and 96 hours

Necrosis damage due to toxic exposure, such as heavy metal ZnO-NPs necrosis damage indicates that toxic exposure from heavy metal ZnO-NPs will cause damage to tissue structure in fish. As a result of necrosis damage or cell death due to the relationship with necrosis **(Suganthi *et al.*, 2015)**, if necrosis damage occurs continuously it will cause cell death, because cells lose the ability to repair existing damage. The observed damage to the intestine of hybrid groupers may be attributed to various factors, including the presence of toxic substances such as heavy metals entering the fish's body. These substances can inhibit or disrupt tissue functionality, which is subsequently identified through histological changes, such as necrosis in cells **(Duan *et al.*, 2023)**.

3.2 Edema damage

The observations indicated the occurrence of edema damage in hybrid grouper at all concentrations. Edema is a condition characterized by the abnormal accumulation of fluid within body cavities or interstitial spaces, resulting in swelling **(Eiras, 2008)**. The edema damage observed in the intestine tissue of hybrid grouper fish can be seen in Fig. (4).

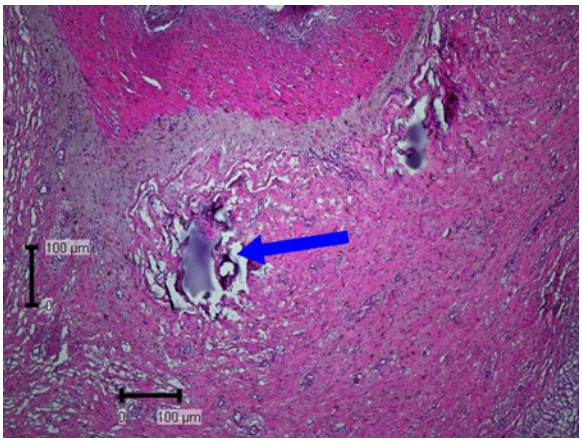

Fig. 4. Representation of edema lesions in the intestinal tissue of hybrid grouper observed under 100x magnification

The percentage of edema damage also showed a significant increase in fish exposed to ZnO-NPs (Fig. 5). At 48 hours, the observations showed no damage (damage score 0) in the 0ppm concentration group, as no tissue damage was identified. In fish exposed to a concentration of 50ppm, the damage level was categorized as moderate, with a damage score of 2 and an average of 15.30%. Similarly, at concentrations of 100 and 200ppm, moderate damage was observed, with damage scores of 2 and averages of 18.18 and 25.7%, respectively. At 96 hours, the 0ppm concentration group continued to show no tissue damage, maintaining a damage score of 0. For the 50ppm concentration, the damage progressed to a severe level, with a damage score of 3 and an average of 34.00%. In the 100 and 200 ppm groups, severe damage was also observed, with damage scores of 3 and averages of 37.08 and 40.43%, respectively. The severity of the damage increased with both time and concentration.

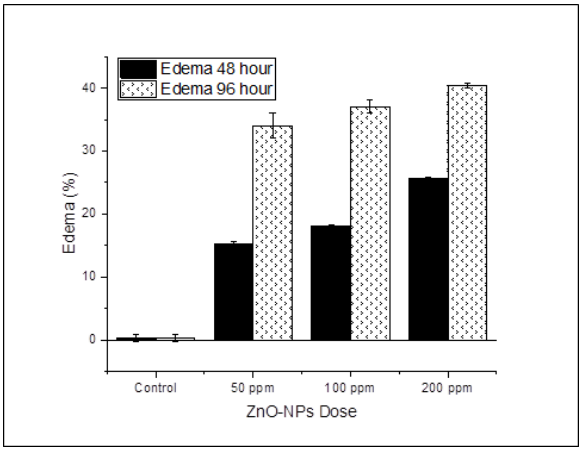

Fig. 5. Percentage edema damage in intestine organ of hybrid grouper after ZnO-NPs treatments for 48 and 96 hours

The edema observed in the intestine of hybrid grouper is likely due to exposure to toxic substances or heavy metals, such as ZnO-NPs, at higher concentrations. These exposures can disrupt digestive system functions and physiological responses in the intestine **(Chupani *et al.*, 2018)**. Higher concentrations of heavy metals accelerate and worsen tissue damage. Therefore, the duration of exposure and the concentration of heavy metals are critical factors contributing to edema formation and tissue damage in the intestine of hybrid grouper.

3.3 Hemorrhagic damage

The observations indicated that hemorrhagic damage was found in hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*) across all treatments. Hemorrhage is classified as moderate liver damage, characterized by the rupture of blood vessels and

the presence of blood in abnormal locations **(Fahmi *et al.*, 2019)**. The hemorrhagic damage observed in the intestine tissue of hybrid grouper fish can be seen in Fig. (6).

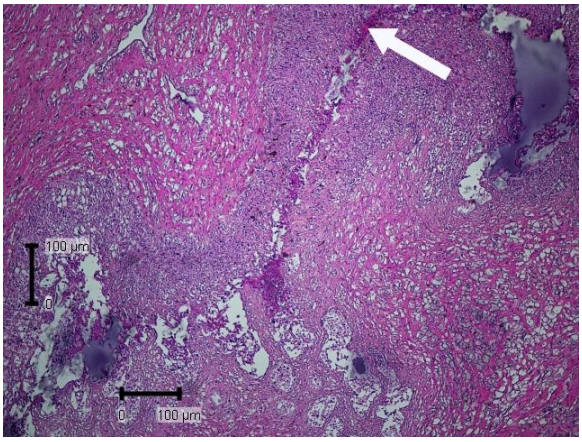

Fig. 6. Representation of hemorrhagic lesions in the intestinal tissue of hybrid grouper observed under 100x magnification

The amount of hemorrhagic damage increased significantly with increasing ZnO-NPs concentration (Fig. 7). The observations at 48 hours indicated that the control group showed no damage, with a score of 0, as no damage was detected. At a concentration of 50 ppm, mild damage was observed, with a score of 1, showing an average of 0.6%. Similarly, at concentrations of 100 and 200 ppm, mild damage was observed with scores of 0, averaging 5.13 and 8.07%, respectively. At 96 hours, the control group again showed no damage, with a score of 0, as no abnormalities were found. At a concentration of 50ppm, mild damage persisted, with a score of 1, averaging 3.02%. At 100 and 200 ppm, mild damage was observed, with scores of 0, averaging 12.05 and 12.26%, respectively.

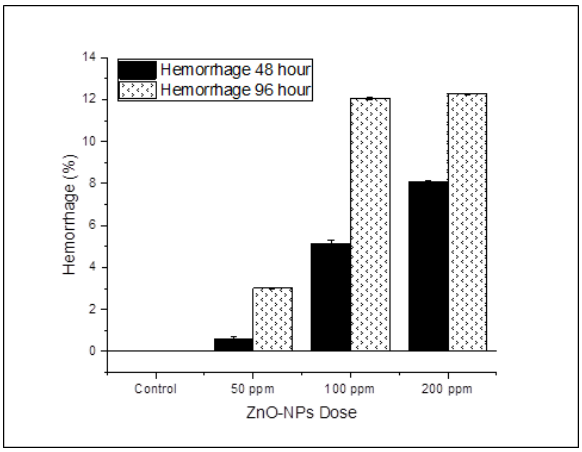

Fig. 7. Percentage hemorrhagic damage in intestine organ of hybrid grouper after ZnO-NPs treatments for 48 and 96 hours

The occurrence of hemorrhage in the fish was observed in all treatment groups. Hemorrhage is a form of moderate tissue damage where blood vessels rupture, causing blood to accumulate in abnormal locations. This condition can disrupt the blood supply to epithelial cells, leading to damage of the villi. As a result, the absorption of nutrients is impaired, which may cause the fish to suffer from nutritional deficiencies.

Histology is one of the most commonly used biomarkers to evaluate toxicity in aquatic organisms exposed to pollutants, especially for vital organs such as digestion **(Shahzad *et al.*, 2019)**. Histopathological damage in fish intestines showed that ZnO-NPs caused significant tissue damage, including hepatocyte necrosis and vacuolar degeneration, indicating serious metabolic disorders due to ZnO-NPs accumulation and increased oxidative stress. ZnO-NPs damaged cellular integrity and triggered inflammation, consistent with previous findings on oxidative stress induced by metal nanoparticles **(Suganthi *et al.*, 2015; Rajkumar *et al.*, 2022)**. Epithelial cell degeneration and goblet cell atrophy impaired mucus secretion and intestinal protection, increased susceptibility to pathogens, and reduced nutrient absorption efficiency. Metal nanoparticles are known to produce oxidative stress, which damages cell membranes, DNA, and cellular components, leading to tissue damage and inflammation.

4. Flow cytogram results

4.1 TNF-α antibodies

In flow cytometry testing, TNF-α and IL-1β of fluorescently labeled monoclonal antibodies used to detect and measure the expression of specific cytokines on the surface or inside cells. TNF-α is a pro-inflammatory cytokine that is important in immune responses and inflammation. TNF-α is produced by various types of cells, including immune cells such as macrophages, and is involved in regulating immune responses and inflammation. The relative levels of TNF-α are a parameter that can be used to determine the increase in activation of T lymphocytes which play a role in adaptive immunity **(Railean & Buszewski, 2022)**. Analysis of the percentage of cells expressing TNF-α antibodies from various ZnO-NPs contents of 0, 50, 100, and 200ppm shows a comparison of the relative levels of TNF-α as shown in Fig. (8).

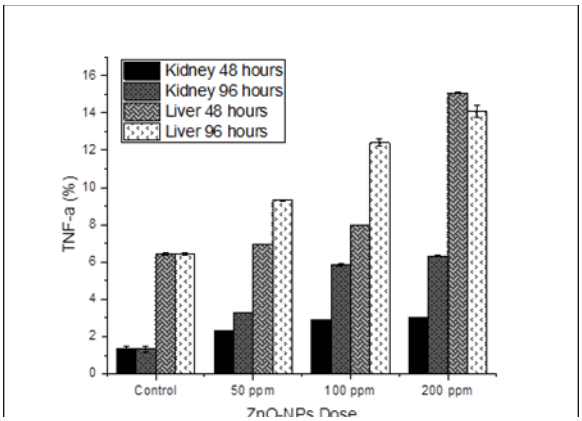

Fig. 8. Percentage of T cells expressing TNF-α after ZnO-NPs treatments for 48 and 96 hours

Prior to the exposure, the relative levels of TNF- α antibodies in the kidney organs were 1.32%. The results for 48-hour exposure showed that the relative levels of TNF-α antibodies in the kidney organs reached their highest at 3.04% at a ZnO-NPs concentration of 200ppm, while the lowest levels were observed in the control group at 1.32%. After 96 hours of exposure, the kidney organs exhibited the highest relative levels of TNF-α antibodies at 6.32% at a ZnO-NPs concentration of 200ppm, with the lowest levels recorded at 3.29% at a ZnO-NPs concentration of 50ppm. This research showed that the result of TNF-α antibodies value in kidney was increased by 378.79%, from the control (1.32%) to 200ppm at 96 hours (6.32%).

In the liver organs prior to the exposure, the relative levels of TNF- α antibodies in the liver organs were 6.42%. The highest relative levels of TNF-α antibodies were recorded at 15.08% at a ZnO-NPs concentration of 200ppm, and the lowest levels were found in the control group at 6.42%. After 96 hours of exposure, the highest levels were observed at 14.1% at a ZnO-NPs concentration of 200ppm, while the lowest levels were found at 9.31% at a ZnO-NPs concentration of 50ppm. This research showed that the result of TNF-α antibodies value in liver was increased by 134.11%, from the control (6.42%) to 200ppm at 96 hours (15.08%).

While this activation demonstrates an initial protective mechanism, prolonged exposure at high concentrations likely exacerbates oxidative stress and inflammation, contributing to cellular damage. The findings demonstrate that exposure to ZnO-NPs enhances the activation of T lymphocytes, which differentiate into T cells. This process boosts the immune response by increasing the production of TNF-α antibodies, particularly at higher ZnO-NPs concentrations and longer exposure durations. This suggests a concentration- and time-dependent response of the immune system to ZnO-NPs exposure.

4.2 IL-1β antibodies

IL-1β is another proinflammatory cytokine that is also important in responding to infection and injury and regulating the inflammatory response. This cytokine is produced by various immune cells, such as macrophages, and is involved in significant inflammatory processes. The results of the analysis of the percentage of cells expressing IL-1β antibodies from various control treatments, 50ppm of ZnO-NPs, 100ppm ZnO-NPs, and 200ppm ZnO-NPs to show the comparison, a diagram of the relative levels of IL-1β T cells was made as in Fig. (9).

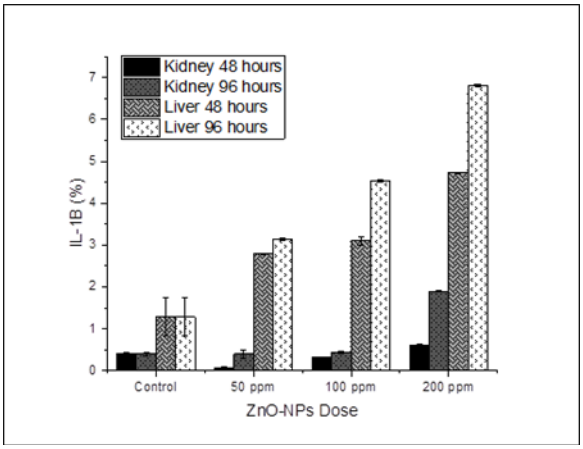

Fig. 9. Percentage of T cells expressing IL-1β after ZnO-NPs treatments for 48 and 96 hours

The results of the analysis of the percentage of T cells in the kidney and liver organs based on Fig. (9) obtained the relative levels of T cells expressing IL-1β antibodies. Prior to the exposure, the relative levels of IL-1β antibodies in the kidney organs were the lowest at 0.045% at a ZnO-NPs concentration of 0ppm. After 48 hours of exposure, the highest relative level of IL-1β antibodies was observed at 0.61% in the kidney organs at a ZnO-NPs concentration of 200ppm, while the lowest level after exposure was 0.06% at a ZnO-NPs concentration of 50ppm. Following 96 hours of exposure, the kidney organs exhibited the lowest relative levels of IL-1β antibodies at 0.4% at a ZnO-NPs concentration of 50 ppm. The highest levels were recorded at 1.89% at a ZnO-NPs concentration of 200ppm. This research showed that the result of IL-1β antibodies value in kidney was increased by 410%, from the control (0.045%) to 200ppm at 96 hours (1.89%).

In the liver organs, the relative levels of IL-1β antibodies before exposure were 1.28% in the control group. After 48 hours of exposure, the highest relative level was found at 4.72% at a ZnO-NPs concentration of 200ppm, while the lowest level after exposure was 2.78% at a ZnO-NPs concentration of 50ppm. After 96 hours of exposure,

the lowest relative levels of IL-1β antibodies were observed at 3.14% at a ZnO-NPs concentration of 50ppm. The highest levels were found at 6.81% at a ZnO-NPs concentration of 200ppm. This research showed that the result of IL-1β antibodies value in liver was increased by 431.25%, from the control (1.28%) to 200ppm at 96 hours (6.81%).

The increased expression of TNF-α and IL-1β suggests that ZnO-NPs trigger an inflammatory response in fish. These cytokines play an important role in modulating the immune response, and their increased production suggests the activation of immune cells in response to toxic exposure. This is consistent with previous studies showing that nanoparticle exposure can increase the secretion of pro-inflammatory cytokines, which can potentially lead to chronic inflammation if not addressed **(Gholinejad *et al.*, 2019; Sakr *et al.*, 2021)**. The increased number of cells expressing TNF-α and IL-1β indicates that the fish immune system not only responds to ZnO-NPs exposure, but may also lead to long-term negative effects, such as autoimmune diseases or more severe immune system disorders.

ZnO-NPs can stimulate antibody production at low doses due to their role in supporting the immune system **(Abinaya *et al.*, 2023)**. However, at high doses, ZnO-NPs trigger oxidative stress by generating excessive reactive oxygen species (ROS), which damage lipids, proteins, and DNA **(Koner *et al.*, 2021)**. ROS accumulation leads to mitochondrial dysfunction, membrane damage, and harm to vital tissues such as the liver, kidneys, and gills **(Diab *et al.*, 2022)**, which are critical for metabolism and respiration in *Epinephelus fuscoguttatus x Epinephelus lanceolatus* (Hybrid grouper). This damage disrupts bodily homeostasis, ultimately causing mortality, highlighting their immunostimulatory effects at low doses and high toxicity at elevated levels.

5. Nanoparticle levels in organs

The results of the analysis of heavy metals expressing ZnO-NPs from various control treatments, 50ppm ZnO-NPs, 100ppm ZnO-NPs, and 200ppm ZnO-NPs to show the comparison, a diagram of heavy metals in the intestine and gill organs was made, as illustrated in Fig. (10).

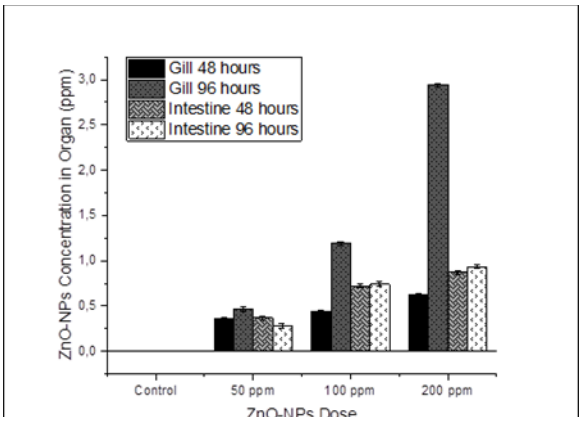

Fig. 10. Results of ZnO-NPs exposure in the intestine and gill organs of hybrid grouper after ZnO-NPs treatments for 48 and 96 hours

The results of the analysis of heavy metals in the intestine and gill organs based on data presented in Fig. (10) obtained the relative levels of heavy metals expressing ZnO-NPs in the gills and intestines. Prior to the exposure, the control group (0ppm) exhibited no detectable heavy metal levels in the intestine organs. After 48 hours of exposure, the highest relative level of heavy metals in the intestine organs was observed at 0.87ppm for the ZnO-NPs concentration of 200ppm. The lowest level was 0.37ppm at a concentration of 50ppm. Following 96 hours of exposure, the highest relative level of heavy metals in the intestine organs was recorded at 0.93ppm at 200ppm ZnO-NPs. The lowest level was observed at 0.28ppm at a concentration of 50ppm. This research showed that the result of ZnO-NPs value in intestine was increased by 215.79%, from 0ppm (0%) to the 200ppm concentration at 96 hours (0.93ppm).

In the gill organs, no heavy metals were detected in the control group. After 48 hours of exposure, the highest relative level of heavy metals was 0.62 ppm at a ZnO-NPs concentration of 200ppm, while the lowest level was 0.36ppm at a concentration of 50 ppm. After 96 hours of exposure, the gill organs showed the highest relative level of heavy metals at 2.94ppm for a ZnO-NPs concentration of 200ppm, whereas the lowest level was 0.47 ppm at 50ppm. This research showed that the result of ZnO-NPs value in gill was increased by 525.53%, from 0ppm (0%) to the 200ppm concentration at 96 hours (2.94ppm).

Measurement of Zn levels after 96 hours of exposure showed an increase in Zn accumulation in accordance with the concentration of ZnO-NPs exposure, with the highest accumulation correlating with the lowest mortality rate, indicating the essential nature of Zn **(Mahjoubian *et al.*, 2023a)**. This accumulation highlights the gills as a primary site of nanoparticle interaction, with implications for respiratory efficiency and detoxification processes. Lower accumulation in the intestine still indicates potential for digestive disruption. The gills had higher ZnO-NPs accumulation compared to the

intestine, indicating its role as the main detoxification organ, which is important for the respiratory function and ionic balance of fish **(de Campos *et al.*, 2019)**. The accumulation of ZnO-NPs in the intestine, although lower, still has the potential to damage tissues and affect digestion, indicating the toxic impact of ZnO-NPs on overall health.

CONCLUSION

In conclusion, this study demonstrates that exposure to zinc oxide nanoparticles (ZnO-NPs) can adversely affect the hybrid grouper by causing hematological alterations, tissue damage in vital organs, and heightened immune responses indicative of stress. These findings underscore the importance of monitoring and regulating the concentration of ZnO-NPs in aquaculture environments to safeguard fish health and to maintain production efficiency. Looking ahead, further research should focus on optimizing application doses and investigating alternative nanoparticle formulations that minimize harmful impacts on fish physiology and the surrounding ecosystem. Additionally, developing clear regulatory guidelines and refining best practices will be crucial in promoting safer, more sustainable use of nanomaterials in aquaculture.

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