Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(3): 3393 – 3410 (2025)
www.ejabf.journals.ekb.eg

The Effect of *Chlorella vulgaris* Recombinant Protein-Based Nanovaccine Formulated with Silver Nanoparticles Against *Viral Nervous Necrosis* on Hematological of Hybrid Grouper

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ARTICLE INFO

Article History:
Received: April 27, 2025
Accepted: June 24, 2025
Online: June 30, 2025

Keywords:
Hematology,
Hybrid grouper,
Nanovaccine,
Silver nanoparticles,
Viral Nervous Necrosis

ABSTRACT

The hybrid grouper *(Epinephelus fuscoguttatus* × *Epinephelus lanceolatus)*, a fast-growing and high-demand aquaculture species in Indonesia, faces significant challenges from pathogen-induced diseases particularly Viral Nervous Necrosis (VNN) despite the country's position as the world's third-largest grouper producer. Silver nanoparticles (AgNPs) are promising antiviral and antimicrobial agents in aquaculture, offering immune-boosting and pathogen-fighting benefits, though their excessive use poses risks of cytotoxicity, hematological disruption, and reduced fish health. This study investigated the effects of a recombinant nanovaccine containing silver nanoparticles (AgNPs) on the hematological profile and survival performance of the hybrid grouper challenged with VNN. Fish were vaccinated with varying doses of AgNPs (33, 66, 112µL), followed by viral exposure, and observed over a 56-day period. The administration of AgNP-based recombinant nanovaccine in the hybrid grouper resulted in significant improvements in survival (85%) and Relative Percentage Survival (76%) at the 33µL dose (T1), which also showed optimal hematological responses, including hematocrit (27.33%), monocytes (19.1%), and lymphocytes (78.3%). While platelet levels were the highest at the 112µL dose (T3), overall findings indicate that the 33µL dose was the most effective in enhancing immune response and physiological health without inducing toxicity. These results highlight the potential of low-dose AgNP nanovaccines as a promising immunostimulant in marine aquaculture, offering a promising alternative strategy for disease management in aquaculture

INTRODUCTION

One economically valuable species in marine aquaculture is the hybrid grouper (*Epinephelus fuscoguttatus x Epinephelus lanceolatus*), which is a crossbreed between

the tiger grouper and the giant grouper **(Shapawi *et al*., 2018; Chor *et al*., 2020; Yanuhar *et al*., 2024)**. However, diseases caused by pathogen infections are still a major challenge in the cantang grouper cultivation **(Dadiono *et al*., 2020)**. Moreover, the potential for hybrid species development is poised to boost the sector since these variants demonstrate faster growth rates and higher market demand, pointing toward an innovative future for grouper production in Indonesia **(Yang *et al*., 2021; Huang *et al*., 2023; Affandi & Setyono, 2024)**. According to data from the Ministry of Marine Affairs and Fisheries (KKP), grouper production reached 10,208 tons in 2019 and 3,632 tons worth 85.064,52 USD in 2020, with a cultivation trend that continues to increase. The mortality rate due to fish diseases is influenced by the type of disease, fish condition, and environment. A bad environment increases the risk of death, while a good environment decreases it. Intensification of cultivation can also trigger the emergence of diseases from viruses, bacteria, and parasites. The type of virus that often causes losses in aquaculture is Viral Nervous Necrosis (VNN) **(Galingging *et al*., 2022; Campo *et al*., 2023; Souto *et al*., 2023)**. Indonesia is recognized as a significant player in the global grouper production market, contributing approximately 17,000 tons annually, securing its position as the third-largest producer after China and Taiwan **(Putra *et al*., 2020)**.

Silver nanoparticles (AgNPs) are emerging as promising agents in fisheries and aquaculture due to their potent antimicrobial and antiviral properties. AgNPs can serve as effective antimicrobial agents by disrupting microbial membranes, generating reactive oxygen species (ROS), and interfering with microbial DNA replication **(Salleh *et al*., 2020)**; they are used as adjuvants—either independently or in combination with vaccines and antibiotics. Recent advancements in aquaculture have seen the introduction of various biogenic nanoparticles synthesized from natural sources. For example, research on *Mangifera indica*-mediated AgNPs has demonstrated their *in-vivo* bactericidal potential against pathogenic *Aeromonas hydrophila* in common carp (*Cirrhinus mrigala*), underscoring their effectiveness as an alternative to conventional antibiotics **(Raza *et al*., 2023)**. Additionally, marine-derived chitosan nanoparticles have been reported to improve intestinal histomorphometric characteristics and bolster immune responses in grey mullet (*Liza ramada*), highlighting the effectiveness of these materials in promoting fish welfare **(Dawood *et al*., 2020)**. Recent studies show that AgNPs are cytotoxic and genotoxic to fish, causing chromosomal damage, gill injury, and increased oxygen demand. At around 0.95ppm, AgNPs are lethal to the Nile tilapia, while silver ions ($AgNO_3$) are 314 times more toxic. Chronic exposure also impairs swimming, causes tissue damage, and disrupts metabolism, potentially lowering aquaculture productivity **(Ribeiro *et al*., 2023)**. Prolonged or high-level exposure to silver nanoparticles (AgNPs) has been shown to induce notable morphological alterations in fish blood cells, accompanied by substantial changes in key hematological parameters. Study by **Vali *et al* (2022)** reported that exposure to silver nanoparticles (AgNPs) can cause clear changes in

the shape and structure of fish blood cells, along with significant disruptions in their blood health. When fish are exposed to higher concentrations of AgNPs, levels of red and white blood cells, as well as hematocrit values, tend to drop.

These changes may indicate that the fish are experiencing impaired blood function, potentially leading to conditions such as anemia or increased oxidative stress, ultimately affecting their overall health and resilience. Exposure to silver nanoparticles (AgNPs) causes structural damage to fish gills, impairing gas exchange and increasing oxygen demand due to hypoxia. AgNPs also inhibit key liver and digestive enzymes, reducing metabolism and nutrient absorption, which leads to lower lipid and protein levels—indicators of liver and digestive dysfunction. Excessive and long-term use of AgNPs has been associated with histological damage, including liver fibrosis, kidney injury, and widespread inflammation **(Kumar *et al.*, 2018)**. While the harmful effects of AgNPs on fish organs have been documented, understanding these impacts is increasingly important due to the growing use of nanomaterials in aquaculture. This study aimed to examine the effects of a *Chlorella vulgaris* recombinant protein-based nanovaccine formulated with silver nanoparticles against VNN on the hematological parameters of hybrid grouper to better understand how these nanoparticles influence overall fish well-being.

MATERIALS AND METHODS

Ethical approval

The Research Ethics Commission, University of Brawijaya studied the research design carefully and provided ethical approval (185-KEP-UB-2024).

Material

The silver nanoparticles (AgNPs) used were obtained from China and had an average size between 1 and 10nm. The fish used were hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*), each weighing around 15 grams and measuring about 10 centimeters in length. They were sourced from a certified local hatchery along the coast of Bali, Indonesia, ensuring consistent quality and health standards.

Treatment *Chlorella vulgaris* recombinant protein-based nanovaccine formulated with silver nanoparticles

Before the treatment began, the fish were acclimated for 7 days in a clean, well-aerated seawater tank. During this period, they were fed commercial pellets twice daily, maintained at a temperature of 28 ± 3°C and a salinity of 33ppt to ensure they were in good health. Feeding was halted for 24 hours prior to the start of the experiment **(Rahman *et al.*, 2021)**.

The fish were divided into five treatment groups, each consisting of 20 individuals. The negative control group (K−) was maintained in seawater without the nanovaccine or

VNN exposure, while the positive control group (K+) was kept in seawater without the nanovaccine but was exposed to VNN. The remaining three groups were vaccinated with varying doses of an Ag/chitosan-based nanovaccine. The vaccine doses administered were 33 µL (T1), 66 µL (T2), and 112 µL (T3).

After vaccination, the Cantang grouper were left for 28 days to allow for immune response development. On the 28th day post-vaccination, the vaccinated fish were challenged with VNN at a concentration of 5 grams. The VNN infection was induced by introducing the virus suspension into the water where the fish were kept and by administering the virus orally. This dual infection protocol was designed to assess the effectiveness of the vaccine in protecting the fish from VNN, which can cause severe damage to the nervous system and body tissues.

Fish samples from each group were collected every 7 days until the 56th day for further analysis. Prior to blood sampling, fish were anesthetized using essential oil **(Souza *et al*., 2019)**.

Blood sampling and hematology

Blood was collected from the caudal vein to assess the fish's hematological profile, including measurements of erythrocyte count, leukocyte count, hematocrit, hemoglobin (Hb) levels, platelet count, and leukocyte differential **(Ahmed *et al*., 2020)**. According to **Pemberian *et al*. (2015)**, bood collection is carried out from the caudal vein using a 27 G × 1/2 syringe. Each fish yields approximately 0.2 to 0.3mL of blood. To prevent coagulation, the syringe is gently tilted back and forth to keep the blood mixed. The collected blood is then carefully transferred into a sterile Eppendorf tube that has been pre-rinsed with an anticoagulant. Before transferring, the needle is removed gently to avoid blood spillage. Once in the Eppendorf tube, the blood is gently mixed by inverting the tube several times to ensure even distribution with the anticoagulant.

The formula used to calculate erythrocyte count, according to **Purwanti and Sudaryono, (2014)** involves mixing anticoagulated blood with Hayem's solution using a haemocytometer pipette. After gentle mixing, two drops are discarded to remove air bubbles. A drop is placed on the haemocytometer, covered with a cover glass, and observed under a microscope at 400× magnification. Erythrocytes are counted in five small squares (four corners and one center), and the total is calculated using a standard formula.

$$N = \sum n \times 10^4 \text{ cell/mm}^3$$

Note :

N = Total erythrocyte

n = Number of erythrocytes in 5 fields of view

10^4 = dilution factor

To count leukocytes, blood is drawn up to the 0.5 mark of a red blood cell pipette and mixed with Turk's solution up to the 11 mark. The mixture is thoroughly homogenized, and the first two drops are discarded to eliminate air bubbles. A drop is then carefully placed onto a haemocytometer, covered with a cover glass, and observed under a microscope at 400× magnification. Leukocytes are then counted for analysis **(Purwanti & Sudaryono, 2014)**

$$N = \sum n \times 50 \text{ cell/mm}^3$$

Note :
N = Total leukocyte
n = Number of leukocyte infields of view

The steps for measuring hematocrit levels are as follows: First, approximately 1mL of fish blood is drawn using a 1cc syringe. The collected blood is then transferred into a microhematocrit tube until it reaches three-quarters of the tube's volume, and one end is sealed using kretoseal. Next, the blood sample is centrifuged at 6,000 rpm for 5 minutes. After centrifugation, the hematocrit level is measured using a microhematocrit reader. The formula used to calculate hematocrit using microhematocrit reader, according to **Samsisko *et al*., (2013)** is as follows:

$$\textit{Hematocrit level (\%)} = \left(\frac{n}{N}\right) \times 100$$

Note :
N = Total blood volume
n = Volume of erythrochyte

Hemoglobin levels were measured following the method described by **Alipin and Sari (2020)**. A Sahli tube was filled with 0.1 N HCl solution up to the 10 mark, corresponding to the lowest scale line on the tube, and placed between two standard color comparator tubes. A Sahli pipette was used to draw 0.02 mL of fish blood from a microtube, which was then added to the Sahli tube. The mixture was allowed to stand for 3 minutes, ensuring the pipette tip was cleaned beforehand. Distilled water was added drop by drop while stirring with a glass rod until the color of the solution matched the standard comparator. Hemoglobin levels were then read in grams per deciliter (g/dL) based on the color match.

Platelet count was determined according to the method described by **Hartika *et al.* (2014)**. A drop of fresh blood was placed on a microscope slide and spread evenly using another slide to create a thin, uniform smear, which was then air-dried. Once dry, the smear was fixed with methanol for 5 minutes, ensuring full coverage. A drop of diluted Giemsa stain was applied and allowed to sit for 20 minutes. The slide was gently rinsed with distilled water and dried completely. A small amount of immersion oil was then applied, and the slide was examined under a microscope at 1000× magnification.

Platelets were counted manually, and the platelet count was calculated using a standard formula.

$$PP\ (\%) = \frac{observed\ platelet\ count}{total\ number\ of\ observed\ cells} \times 100$$

Note :
PP = Platelets precentage

The differential leukocyte count was performed following the procedure outlined by **Agustina *et al.* (2023)**. Microscope slides were first sterilized using 70% alcohol. A small drop of blood was then placed on the slide, with the first and second drops discarded to avoid air bubbles. The third drop was used to prepare the smear. A second slide was positioned at a 45° angle, drawn backward to make contact with the blood, and then pushed forward to create a thin, even smear. The smear was allowed to air-dry and subsequently fixed by immersion in methanol for five minutes, followed by drying.

The fixed smear was stained by immersion in Giemsa solution for 30 minutes, then gently rinsed and dried. The stained smear was examined under a microscope at 400× magnification. Differential leukocyte counts were carried out by identifying and counting the various types of white blood cells, including lymphocytes, monocytes, basophils, eosinophils, and neutrophils. The relative percentages of each leukocyte type were calculated to determine the leukocyte profile.

$$\%\ \text{Cells} = \frac{\text{counted cells}}{100} \times 100\%$$

Survival Rate and Relative Precentage Survival

The survival rate (SR) was calculated to evaluate the viability of the test animals throughout the study period. According to **Rohaeni (2017)**, survival rate is influenced by factors such as water quality and handling practices. In addition to SR, Relative Percent Survival (RPS) was measured to provide a more accurate assessment of the impact of specific factors or conditions on fish survival. RPS is commonly used to evaluate the toxicity of chemical agents, the effects of pollutants, or environmental changes that may negatively influence fish health. According to **Ashfaq *et al.* (2019)**, RPS serves as a reliable indicator for determining the effectiveness of treatments and the resilience of fish under stress or infection. The survival rate can be calculated using the following formula

$$SR = \frac{Nt\ \times 100\%}{N0}$$

Note :

SR = Fish survival rate (%)
Nt = Number of fish at the end of maintenance
N0 = Number of fish at the beginning of maintenance

The level of protection was measured using the Relative Percentage Survival (RPS) value, calculated with the following formula **(Reynalta *et al*., 2019)**:

$$RPS = 1 - \frac{\%\ \text{Mortality of Vaccinated Fish}}{\%\ \text{Mortality of Control Fish}} \times 100$$

Notes :
RPS = Relative Percentage Survival

Data analysis

Data were analyzed using Origin version 19B, and the hematology results were compared among treatment groups by examining trends in clinically and biologically significant changes. One-way ANOVA was used to compare the five experimental groups, with the results expressed as mean values and standard deviations. Statistical significance was set at $P< 0.05$.

RESULTS

1. Survival rate and relative percentage survival

The survival rate and relative percent survival (RPS) of the hybrid grouper following administration of the *Chlorella vulgaris* recombinant protein-based nanovaccine formulated with silver nanoparticles over a 56-day period are presented in Table (1).

Table 1. Survival rate (SR) and relative precentage survival (RPS) of hybrid grouper after AgNPs vaccine treatments after 56 days

	Vaccines Treatments				
	K-	**K+**	**T1 (33 µl)**	**T2 (66 µl)**	**T3 (112 µl)**
Survival Rate (%)	45	15	85	65	60
Relative Precentage Survival (%)	45	35	76	59	53

The survival rate of the hybrid grouper (Table 1) during the maintenance period showed the highest percentage in the T1 treatment group at 85%, followed by T2 at 65%. Based on the survival rate data, these two treatments demonstrated the highest effectiveness in supporting fish survivability compared to other groups. According to **Ningrum *et al*. (2025)**, an ideal survival rate for the hybrid grouper juveniles is greater than 80%. In contrast, the lowest survival rate was recorded in the positive control group (K+), where healthy fish subjected to a challenge test on day 28 exhibited a survival rate of only 15%.

As noted by **Salvador and Gusmao, (2025)**, survival rate serves as a key indicator in evaluating the success of treatments and aquaculture techniques. A higher survival percentage reflects a more effective intervention in minimizing fish mortality. In this context, the recombinant nanovaccine treatment combined with AgNPs adjuvant at a dose of 33µL appears to be the most optimal, significantly enhancing the survival of hybrid grouper compared to other dosage treatments.

The relative percentage survival (RPS) of hybrid grouper was the highest in the T1 group (76%), followed by T2 (59%). Higher survival rates in these groups contributed to increased RPS values. According to **Noor and Jati (2023)**, an RPS above 60% indicates an effective vaccine performance. The low RPS in T3 suggests the dosage used did not trigger a sufficient immune response, contrasting with **Hartawan *et al*. (2023)**, who stated that vaccination should enhance immunity by boosting antibody production.

2. Hematology analysis

The hematological characteristics of the hybrid grouper after *chlorella vulgaris* recombinant protein-based nanovaccine formulated with silver nanoparticles for 56 days are shown in Table (2).

Table 2. Hematology parameters of hybrid grouper after AgNPs vaccine treatments for 56 days

Hematology parameter	Vaccine Treatments				
	K-	**K+**	**T1 (33 µl)**	**T2 (66 µl)**	**T3 (112 µl)**
Erythrocyte (cells/mm^3)	$2.55 \pm 0.02 \times 10^5$	$2.16 \pm 0.13 \times 10^5$	$2.74 \pm 0.07 \times 10^5$	$2.71 \pm 0.05 \times 10^5$	$2.67 \pm 0.08 \times 10^5$
Leukocyte (cells/mm^3)	$1.26 \pm 0.03 \times 10^5$	$1.44 \pm 0.06 \times 10^5$	$1.63 \pm 0.17 \times 10^5$	$1.76 \pm 0.03 \times 10^5$	$1.59 \pm 0.16 \times 10^5$
Hematocrit (%)	22.33 ± 1.0	14.78 ± 2.8	27.33 ± 4.9	24.67 ± 2.4	23.89 ± 4.5
Hemoglobin (g/dL)	6.31 ± 0.27	4.71 ± 0.58	7.37 ± 0.77	6.78 ± 0.37	6.76 ± 0.82
Platelets (%)	22.11 ± 1.1	28.67 ± 8.0	25.11 ± 2.5	26.22 ± 3.3	28.33 ± 7.9

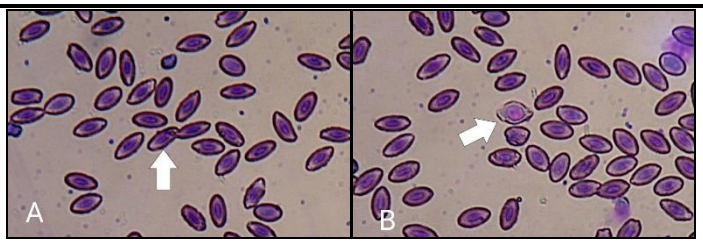

Fig. 1. Hematology profile of cantang grouper fish: **(A)** Erythrocytes and **(B)** Leukocytes

2.1 Erytrocyte

The hematology profile of red blood cells can be seen in Fig. (1). Based on the results of the hematological analysis (Table 2), the average erythrocyte count in the hybrid grouper across the treatment groups, ranging from the negative control (K−) to treatment group T3, was found to range between 2.16×10^6 cells/mm³ and 2.74×10^6 cells/mm³. Among all treatments, the highest erythrocyte count was recorded in the T1 group, reaching 2.74×10^6 cells/mm³, indicating a potentially enhanced hematopoietic response. In contrast, the lowest erythrocyte count was observed in the positive control group (K+), with a value of 2.16×10^6 cells/mm³. These values fall within the reported normal physiological range for erythrocyte counts in the hybrid grouper, which is between 1.05×10^6 and 3.00×10^6 cells/mm³ **(Yuhana *et al.*, 2019)**. The variation in erythrocyte levels among treatments may reflect the immunomodulatory or physiological effects of the AgNPs vaccine administered over the 56-day experimental period.

2.2 Leukocyte

The hematology profile of white blood cell can be seen in Fig. (1). The leukocyte count (Table 2) in the hybrid grouper showed a general increasing trend from the negative control group (K−) up to treatment group T2. The administration of the nanovaccine led to an elevation in leukocyte levels, suggesting an immune response activation. However, a decline in leukocyte count was observed in treatment group T3. The highest leukocyte count was recorded in the T2 group at 1.76×10^5 cells/mm³, while the lowest was found in the negative control (K−) at 1.26×10^5 cells/mm³. These values indicate that the leukocyte count in certain treatment groups, particularly T2, exceeded the normal leukocyte range for fish, which is generally between 0.2×10^5 and 1.5×10^5 cells/mm^3 **(Riauwaty & Syawal, 2016)**. The increase observed following Ag-NPs administration suggests a stimulatory effect on the fish's immune system, although the subsequent decrease in T3 may reflect a potential downregulation or adaptation over time.

High leukocyte counts are typically associated with increased immune activity, playing a crucial role in the organism's defense against infectious agents. However, an overabundance of specific leukocyte types may have harmful effects on overall health. This is supported by findings from various studies that report elevated leukocyte levels in

fish exposed to toxic compounds or environmental stress, indicating that such immune responses may also be a sign of physiological distress rather than solely protective activation **(Khunrang *et al*., 2023)**.

2.3 Hemoglobin (Hb)

The hemoglobin levels observed in the hybrid grouper (Table 2) (showed a general increasing trend starting from the positive control group (K+). The average hemoglobin concentration across treatments ranged from 4.71 g/dL to 7.37 g/dL. The highest hemoglobin level was recorded in the T1 treatment group at 7.37 g/dL, while the lowest was found in the K+ group at 4.71 g/dL. These findings suggest that the administration of treatments may have positively influenced erythropoiesis and oxygen transport capacity in the fish. The normal range of hemoglobin levels in fish is approximately 5.0– 9.0g/ dL **(Maryani *et al*., 2021)**. Hemoglobin concentration in the blood is closely linked to the number of erythrocytes, a decrease in erythrocyte count typically leads to a corresponding reduction in hemoglobin levels. Measuring hemoglobin levels is essential for understanding physiological processes. A decrease in hemoglobin concentration can impair the distribution of oxygen and nutrients throughout the body, potentially leading to a reduced metabolic activity **(Liana *et al*., 2024)**.

2.4 Hematocrit levels

The hematocrit values observed in the hybrid grouper (Table 2) exhibited fluctuating trends across treatments. The lowest hematocrit level was recorded in the positive control group (K+) at 14.8%, while the highest value was observed in the Ag-NPs treatment group (T1) at 27.3%. The normal hematocrit range in fish is generally between 20 and 30%. Hematocrit levels below 20% may indicate an anemic condition and suggest a potential risk of disease infection **(Rahmaningsih *et al*., 2019)**. Hematocrit values can be influenced by various factors, including temperature, delay duration prior to analysis, centrifugation process, the type of anticoagulant used, reading errors, as well as the number, size, and shape of erythrocytes **(Yuniastutik, 2021)**.

2.5 Platelets

The platelet (thrombocyte) count in hybrid grouper (Table 2) exhibited fluctuating trends over the 56-day observation period. The lowest platelet level was recorded in the control group (K-) at 22.1%, while the highest was observed in contol group (K+) at 28.7%, and with the highest platelet level for the treatment group (T3) at 28.3%. with overall values ranging between 22.1 and 18.7%. These values fall well below the normal thrombocyte range in fish, which is typically between 20 and 30% **(Aryani & Hasibuan, 2016)**.

The increase in thrombocyte count is presumed to be a response to tissue injury or damage. Platelet plays a crucial role in localizing pathogenic invasions, thereby

preventing the spread of infection. In addition to their immunological function, thrombocytes are essential for wound closure and accelerating the blood coagulation process. These cells release the enzyme thromboplastin, which initiates a cascade leading to fibrin formation. Fibrin, produced through the polymerization of fibrinogen, is vital for establishing a stable blood clot matrix.

3. Leukocyte differentials analysis

The leukocyte differentials analysis of the hybrid grouper after *chlorella vulgaris* recombinant protein-based nanovaccine formulated with silver nanoparticles for 56 days is presented in Table (3).

Table 3. Hematology parameters of hybrid grouper after AgNPs vaccine treatments for 56 days

Leukocyte Differential	Vaccine Treatments				
	K-	K+	Treatment 1 (33 µl)	Treatment 2 (66 µl)	Treatment 3 (112 µl)
Monocyte (%)	14.2 ± 1.2	22 ± 4.7	19.1 ± 4.1	19 ± 4	16.7 ± 2.1
Lymphocyte (%)	66.6 ± 2.1	76 ± 9.1	78.3 ± 8.2	76.6 ± 6.3	75.2 ± 6.9
Basophil (%)	0.011 ± 0	0.189 ± 0.2	0.133 ± 0.2	0.1 ± 0.2	0.111 ± 0.2

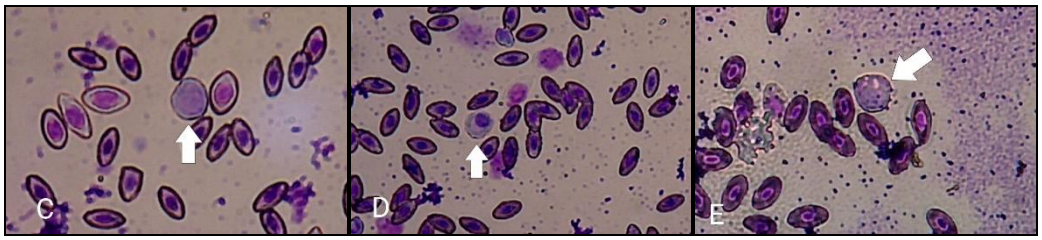

Fig. 2. Leukocyte differentials of cantang grouper fish: (C) Lymphocyte, (D) Monocyte, and (E) Basophil

3.1 Monocyte

The leukocyte differentials of monocyte can be seen in Fig. (2). Monocyte counts in the hybrid grouper (Table 3) showed fluctuating patterns over the 56-day study period. The lowest monocyte percentage was recorded in the negative control group (K−) at 14.2%, while the highest was observed in the Ag-NPs vaccination treatment group (T1) at 19.1%. Interestingly, the positive control group (K+) also exhibited a notably high monocyte count, reaching 22%. Overall, the monocyte values ranged between 14.2 and 22%, with the normal physiological range for fish reported to be between 9.3 and 21.0% **(Anshar *et al.*, 2024)**. The results of the ANOVA analysis indicate that there is a

statistically significant difference in monocyte among the treatment groups receiving different vaccine doses (α = 95%, *P* = 0.00051; n = 20).

3.2 Lymphocyte

The leukocyte differentials of lymphocyte can be seen in Fig. (2). Lymphocyte counts in hybrid grouper (Table 3) exhibited an overall increasing trend throughout the 56-day experimental period. The highest lymphocyte percentage was recorded in the Ag-NPs vaccine treatment group (T1) at 78.3%, followed by the positive control group (K+) at 76%, while the lowest value was observed in the negative control group (K−) at 66.6%. Across all treatment groups, lymphocyte levels ranged from 66.6 to 78.3%, falling within the normal physiological range for fish, which is typically between 60 and 80%.

The increase in lymphocyte count is closely associated with the development of immune responses. Lymphocytes can proliferate and differentiate into effector and memory cells. Cytoplasmic granule-containing T lymphocytes function in lysing antigens, whereas B lymphocytes are responsible for antibody production **(Udlhi *et al*., 2023)**. Additionally, B lymphocytes stimulate T cells to promote the activation of phagocytic cells such as monocytes and neutrophils, which work collectively to combat pathogenic infections. The results of the ANOVA analysis indicate that there is a statistically significant difference in lymphocyte among the treatment groups receiving different vaccine doses (α = 95%, *P* = 0.0087; n = 20).

3.3 Basophil

The leukocyte differentials of basophile can be seen in the image above (Fig. 2). Basophil counts in the hybrid grouper (Table 3) showed fluctuating trends over the 56-day experimental period. The highest basophil percentage was observed in the positive control group (K+) at 0.18%, followed by the Ag-NPs vaccine treatment group (T1) at 0.13%, while the lowest value was recorded in the negative control group (K−) at 0.01%. Overall, basophil counts ranged from 0.01 to 0.18%, with the normal reference range for fish reported to be between 0.10 and 3.88%.

Basophils represent the least abundant type of leukocyte in fish. Their limited presence is due to their relatively minor role in immune defense, as fish rely more heavily on other immune cells such as neutrophils, monocytes, and lymphocytes to combat infections **(Maulaningrum *et al*., 2024)**.

4. One-way ANOVA analysis for hematology parameters

The ANOVA results showed that several parameters showed statistically significant differences among treatments (*P*< 0.05) (Table 4), such as total erythrocytes, total leukocytes, hemoglobin level, hematocrit level, thrombocyte, monocyte, lymphocyte, and basophil.

Table 4. ANOVA analysis for hematology parameters

Parameter	F Value	Sig. (*P*-value)	Description
Erythrocyte	85.538	4.71	Not significantly different
Leukocyte	27.30	5.728	Not significantly different
Hematocri	1.28	0.045	Significantly different
Hemoglobin	25.22	1.77	Not significantly different
Platelets	2.22	0.043	Significantly different
Monocyte	6.28	0.0005	Significantly different
Lymphocyte	3.929	0.0087	Significantly different
Basophil	1.021	0.408	Not significantly different

The ANOVA results presented in Table (4) provide a statistical evaluation of the differences in hematological parameters among treatment groups. A significant difference was observed in hematocrit levels (F= 1.28, *P*= 0.045), indicating that the treatments had a measurable effect on this parameter. Similarly, statistically significant differences were found in platelet counts (F= 2.22, *P*= 0.043), monocyte percentages (F= 6.28, *P*= 0.0005), and lymphocyte percentages (F= 3.929, *P*= 0.0087), suggesting that these components of the immune response were influenced by the administered treatments. Conversely, erythrocyte counts (F= 85.538, *P*= 4.71), leukocyte counts (F= 27.30, *P*= 5.728), hemoglobin concentrations (F= 25.22, *P*= 1.77), and basophil percentages (F= 1.021, *P*= 0.408) did not exhibit statistically significant differences, indicating that these variables were not significantly affected by the treatments. Overall, the findings highlight that specific hematological and immunological indicators responded to the treatments, while others remained unchanged.

CONCLUSION

The administration of AgNP-based recombinant nanovaccine in the hybrid grouper demonstrated significant improvements in both survival rates and hematological parameters. The 33 µL dose (T1) was found to be the most effective, resulting in the highest survival rate (85%) and relative percentage survival (76%), with the optimal hematology such as the most effective nanovaccine on hematocrite levels are in the 33µL dose (T1) with 27.33%, while platelets with the most effective nanovaccine levels are in the 112µL dose (T3). The highest monocyte percentage, recorded at 19.1%, was observed in Treatment 1 (T1), indicating that the nanovaccine at a dose of 33µL was the most effective in stimulating monocyte response. The highest lymphocyte percentage, recorded at 78.3%, was found in Treatment 1 (T1), suggesting that the nanovaccine at a dose of 33 µL was the most effective in enhancing lymphocyte response. These findings suggest that low-dose AgNP nanovaccine can effectively stimulate the immune system, enhance disease resistance against VNN infection, and support the physiological health of the hybrid grouper without inducing toxicity. However, higher doses showed reduced

effectiveness, indicating the importance of precise dosage optimization. The results confirm the potential of AgNP-based nanovaccines as an innovative and efficient immunostimulant in marine aquaculture.

ACKNOWLEDGEMENT

This research was funded by the Directorate of Research and Community Service (DRTPM) of Brawijaya University through the Research Ecosystem Strengthening for Professors 2024 program, under Research Contract Number: 00144.23/UN10.A0501/B/PT.01.03.2/2024.

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