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The Effect of Hyperthermia on the Immune Response of Vannamei Shrimp Infected with IMNV

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Abstract

The purpose of this study was to determine the effect of hyperthermia on the immune response of shrimp infected by IMNV. Vannamei shrimp were challenged with IMNV and reared at temperatures 30ºC (Sk30), 31ºC (S31), 32ºC (S32) and 33ºC (S33) repectively. The temperature of shrimp without IMNV infection was 300C as control. During the study, shrimp immune response measurements were Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), Respiratory Burst (RB) activity and Phenoloksidase (PO) activity. Generally, the results showed that the response of shrimp decreased along with hyperthermic condition. Shrimp infected with IMNV, decreased immune response especially at temperatures of 32ºC and 33ºC.

Keywords: *Hyperthermia, IMNV, Immune response, Vannamei shrimp*

1. Introduction

Shrimp is the leading commodity of the Indonesian aquaculture industry. According to the Ministry of Marine Affairs and Fisheries (2020), this commodity contributed 34.83% to the value of fishery exports (KKP, 2020). Total fishery production in 2020 will reach 851.6 thousand tons. It is expected that production will reach 2 million tons in 2024 (KKP, 2022). Environmental conditions and disease incidence are challenges to increase the vannamei production (Sari et al., 2019; Palupi et al., 2022; Soedibya et al., 2023). The two factors are interrelated. Environmental changes can increase the risk of disease occurrence (Kurniawati & Pursetyo, 2021), for example in the outbreak of Infectious Myonecrosis Virus (IMNV) (Baladrat et al., 2022). The disease was reported to be triggered by drastic changes in temperature (Sunarto et al., 2016).

Infectious Myonecrosis Virus (IMNV) is a disease that has a significant effect on the production of vannamei shrimp culture (Hidayat et al., 2019). This disease was first reported in Brazil in 2002 (Lightner et al., 2004). Then in 2007, this disease was confirmed to occur in East Java, Indonesia (Senapin et al.,2007; Taukhid et al., 2010). Economic losses due to IMNV are due to persistent mortality rates and increased feed conversion ratios (Flegel et al., 2008). The mortality rate due to IMNV can reach 80%. This is strongly influenced by environmental conditions. IMNV can be found in all stages of shrimp, both larval and adult posts. However, mortality rates are more frequently reported in large shrimp (Poulos et al., 2006). IMNV can be transmitted horizontally or vertically. The

process of cannibalism in Vannamei shrimp is a way of horizontal transmission. The virus resistance without the envelope is quite good in the digestive tract. This allows the transmission of IMNV through fresh feed (Lightner et al., 2012).

Currently, the immune response of shrimp to virus infections is an interesting study. The shrimp immune system includes a humoral defense system and a cellular defense system. Humoral defense responses that are important for shrimp include melanin synthesis, blood coagulation system, and increased antimicrobial peptide (AMP) such as penaedin, anti-lipopolysaccharide (ALFs), and custin (Supungul et al., 2004). The cellular immune response of shrimp includes phagocytic activity, apoptosis, and encapsulation of pathogens or foreign bodies (Flegel et al., 2011). The immune response of shrimp to IMNV infection showed a decrease in hemocytes by up to 30%. The cause of the reduction in hemocytes has not been elucidated. IMNV has not been shown to infect hematopoietic organs. Increasing the response of the shrimp body to IMNV has been tried with the use of probiotics and synbiotics (Widanarni et al., 2020).

A shrimp's immune response is influenced by temperature. Vannamei shrimp can grow at temperatures between 7.5C-42.0ºC. The optimal temperature that supports growth depends on the size of the Vannamei shrimp. Shrimp measuring less than 5 grams grow fast at a temperature of 30ºC, while shrimps measuring more than 16 grams grow optimally at a temperature of 27ºC (Wyban et al., 1995). Hyperthermia conditions affect the incidence of viral infection in shrimp. Shrimp were resistant to Taura Syndrome Virus (TSV) strain HI94 if kept at 32ºC. However, high mortality was seen in shrimp infected with TSV strain BZ02 and reared at the same temperature (Cote, 2008). The maintenance temperature of 32ºC was able to protect shrimp from infection with White Spot Syndrome Virus (WSSV). At this temperature, viral replication is reduced and the process of apoptosis is enhanced (Granja et al.,2006; Jiang et al., 2019; Vidal et al., 2001). A temperature of 33ºC is an immunomodulator that can inhibit WSSV infection in lobsters. Lobsters experience an increase in THC and PO activity at these temperatures (Wu et al., 2014). Mortality and number of infected cells decreased significantly in Vannamei shrimp infected with WSSV and reared at 33ºC. IHHNV replication was inhibited in Vannamei shrimp reared at 32.8±1.0ºC (Montgomery et al., 2007). Based on these facts, this study was conducted to determine the effect of hyperthermia on Vannamei shrimp infected with IMNV.

2. Materials And Methods

Time and Location

The research was conducted at the Fish Health and Environmental Laboratory Serang / Balai Pengujian Kesehatan Ikan dan Lingkungan (BPKIL) Serang from January to June 2021. The tools and materials used can be seen in the Appendix.

Experimental Design

The study used a completely randomized design (CRD) which included 5 treatments with 3 replications, namely shrimp infected with IMNV and reared at 30°C (S30), shrimp infected with IMNV and reared at 31°C (S31), shrimp infected with IMNV and reared at 32oC (S32), shrimp were infected with IMNV and reared at 33°C (S33), and shrimp were not infected with IMNV and reared at 30°C (Sk30) (Cote, 2008; Wu et al., 2014; Granja et al., 2003). To increase the maintenance temperature, a heater equipped with a thermostat was used. A container in the form of 15 plastic boxes filled with 15 Vannamei shrimp each and given aeration. Parameters observed were Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), Phenoloxidase Activity and Respiratory Burst activity.

Research Procedure

Container Preparation

The maintenance container used is a plastic box with a volume of 100 litters. Containers totalling 15 units were disinfected using 50 ppm hypochlorite solution, rinsed with fresh water and dried. The container was filled with seawater with a salinity of 30 ppt and given aeration. We conduct siphoning, and water changes by 10% every day (Widanarni et al., 2016).

Test Organism

The study used white shrimp, namely L. vannamei from the Fish Health and Environmental Laboratory Serang with the shrimp measuring 9.19±0.58 g. Shrimp adapted in a rearing container for 1 week. The shrimp were treated according to the experimental design and kept for 10 days. The shrimp were fed 30% protein feed every day and as much as 5% of their biomass during adaptation and treatment. The feeding occurs at 09:00, 15:30, and 21:00.

IMNV Reinfection

We conducted this process to increase the pathogenicity and number of viruses we will use in our research. IMNV stock comes from the collection of the Fish Health and Environmental Laboratory. 0.1 ml of virus stock, then injected into Vannamei shrimp. Shrimp abdomen tissue was cleaned and the carapace was removed. The tissue was tested by qRT-PCR to determine the number of virus copies as an ingredient in the manufacture of virus inoculum (Silva et al., 2015).

Inoculum Preparation

A total of 100 grams of shrimp abdominal muscle tissue was infected with IMNV (without carapace), and crushed with 300 ml of TN Buffer (20 mM Tris–HCl and 0.4 M NaCl, pH 7.4). The tissue in TN buffer was diluted 10 times using a 2% solution of NaCl salt. The inoculum preparation was centrifuged for 25-6 minutes at 3000 rpm at 40ºC. The supernatant solution was taken, then centrifuged again for 20 minutes at a speed of 14,000 g at 40ºC. The supernatant was taken and filtered using a 0.22 m syringe filter. Then, the inoculum stock is stored in a deep freezer at -80ºC (Silva et al., 2015).

IMNV Infection Procedure

Shrimp were infected with 0.1 ml of IMNV inoculum intramuscularly. The injection was conducted in the third segment of the dorsal abdomen using a 1 ml sterile syringe with a needle size of 25 G. After the injection, the shrimp were reared by feeding 5% of the biomass. Maintenance water was changed by 10% every day.

Observation Procedure

Post Infection Shrimp were observed every hour for 10 days. Mortality and clinical symptoms are the objects of daily observation. In addition, during the study, water quality tests and hemolymph samples were taken for immune testing.

Hemolymph Collection

Hemolymph samples were taken from three shrimps for each treatment. For each shrimp, 50 µL of hemolymph was taken. Hemolymph was taken using a 0.1 ml sterile syringe containing anticoagulant (30 mM trisodium citrate, 100 mM glucose, 26 mM citric acid, 10 mM EDTANa2, and 510 mM NaCl; added NaOH to pH 6.6) the hemolymph-anticoagulant ratio was obtained by 1:1. Then, the hemolymph anticoagulant mixture was divided into 3 microtubes for Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC), Phenoloxidase (PO) testing and Respiratory Burst (RB) testing. For the preservation of the THC test, the sample was added with 4% formalin salt (0.45 M NaCl). As for the PO and RB tests, the samples were put into a cool box filled with ice.

Research Parameters

Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC)

The THC test was conducted by inserting 10 µL of the anticoagulant hemolymph mixture into the hemocytometer. Hemocytes were observed using a microscope with a magnification of 100 times.

$$\text{THC}\left(\frac{Cell}{Ml}\right) = \frac{\sum \text{observed cell}}{\sum \text{observed box}} x \text{ Dilution Factor } x\ 10^4$$

A Hemocyte differentiation test was conducted to determine the comparison of hemocyte cell types (hyaline, semi-granular, granular). The test was performed by staining 50 L of the hemocyte-anticoagulant mixture with 50 µL of trypan blue (0.4% in PBS) and 5 L of rose Bengal (1.2% in 50% ethanol) (Mangkalanan et al., 2014). After incubation, the staining solution was made for smear preparations. The estimated DHC percentage, was determined by counting 100 hemocytes using a microscope at 400 times magnification (Tampangallo et al., 2012).

$$DHC\ (\%) = \frac{\text{Certain number of hemocyte cells}}{\text{Total hemocytic cells}}\ x\ 100^{7}$$

2.4.2 Respiratory Burst (RB)

Respiratory Burst Activity is an intracellular measurement of superoxide anion (O2-). This test is based on the formation of formazan from the reduction of nitroblue tetrazolium (NBT). Measurements using a spectrophotometer at a wavelength of 630 nm (Song et al., 2003).

Phenoloxidase (PO)

A total of 50 µl hemolymph was incubated with 50 µl (1 mg/mL) trypsin for 5 minutes. Then 50 µl L-DOPA (3 mg/mL) was added and incubated for 10 minutes. PO activity was measured based on the formation of dopachrome from L- Dihydroxyphenylalanine (L-DOPA) using a spectrophotometer with a wavelength of 490 nm (Cheng et al., 2005).

Data Analysis

THC, DHC, RB, and PO test data were analyzed through One-way Analysis of Variance (ANOVA) with a 95% confidence level. Duncan's test was used as a further test to determine the effect of various treatments.

3. Results and Discussion

Total Hemocyte Count (THC)

After the IMNV challenge, the initial clinical symptoms of IMNV infection began to appear between the second and fifth days. Shrimp appetite began to decline, and visible loss of transparency of abdominal muscle tissue. The reverse transcriptase polymerase chain reaction (RT-PCR) test was then used to confirm the diagnosis of IMNV. RT-PCR results showed bands at 139 and 328 bp in IMNV-infected shrimp samples (Figure 1).

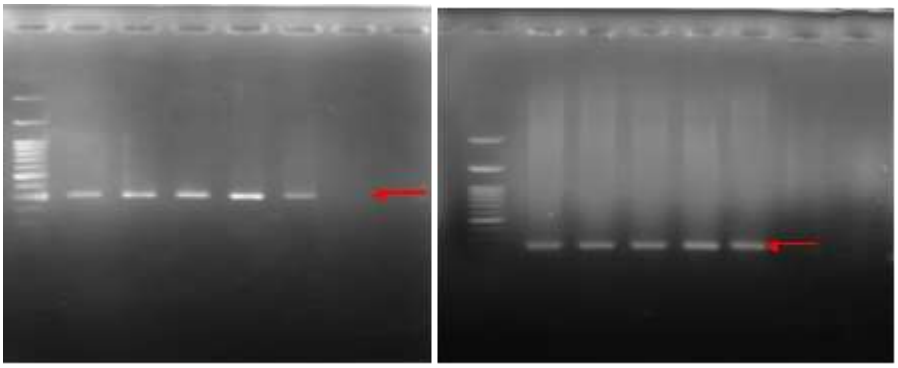

Figure 1. The results of the first step (left) and nested (right) Vannamei shrimp RT-PCR test, after the IMNV challenge test. M; marker (100bp), S30; Maintenance temperature 30C, S31; Maintenance temperature 31°C, S32; maintenance temperature 320C, S33; maintenance temperature 33°C, K (+); Positive control, K (-); negative control, SK30; negative control treatment.

Hyperthermia affects the decrease in THC values after the IMNV test. The THC value in the S33 treatment had the lowest value compared to other treatments. On the fifth day, the THC value of the S33 treatment (temperature 33°C) was significantly different ($P<0.05$) with S30, S31, S32, and SK30. The treatment of S32 was significantly different ($P<0.05$) with S30 and SK30, but not significantly different ($P<0.05$) with S31. The THC value of the S30 treatment was significantly different ($P<0.05$) from the SK30. The decrease in THC values for S30, S31, S32 and S33 when compared to Sk30 were 52.11%, 53.10%, 58.28% and 64.61% (Figure 2).

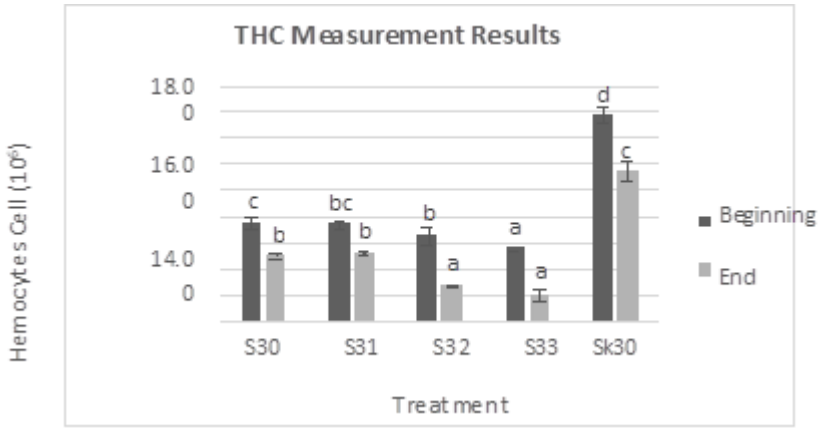

Figure 2. THC measurement results of Vannamei shrimp after the IMNV challenge test. Significant difference results (P<0.05) indicated by different letters in the same color.

On the tenth day, the THC value of S33 was significantly different (P<0.05) with S30, S31, and SK30 but not significantly different (P<0.05) with S32. While the THC value of the S31 treatment (temperature 31oC) was significantly different (P<0.05) with SK30, but not significantly different (P<0.05) with S30. The decrease in THC values for S30, S31, S32 and S33 when compared to Sk30 were 68.08%, 67.12%, 82.58% and 86.84%.

Differential Hemocyte Count (DHC)

The value of hyaline hemocytes (agranulocytes) after the IMNV test decreased significantly. The number of S33 hyaline cells had the lowest value compared to other treatments. On the fifth day, the hyaline cell values of S33 were significantly different (P<0.05) with S30, S31, and SK30, but not significantly different from S32. The decrease in the value of S30, S31, S32, and S33 hyaline cells when compared to Sk30 were 20.81%, 21.48%, 34.90%, and 38.25% (Figure 3 A).

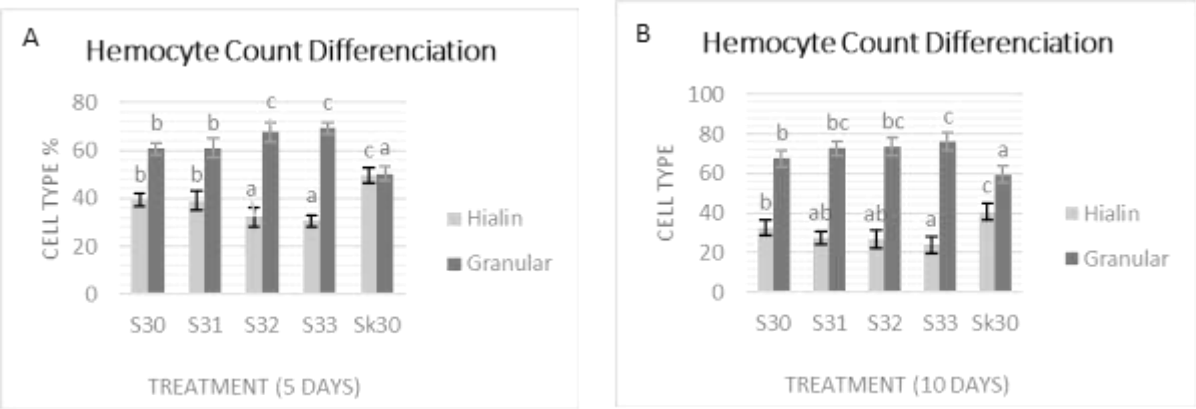

Figure 3. DHC measurement results of Vannamei shrimp on the fifth (A) and tenth (B) days after the IMNV challenge test. Significant difference results (P<0.05) indicated by different letters in the same color.

On the tenth day, the number of S33 hyaline cells was significantly different (P<0.05) with S30 and SK30 but not significantly different (P<0.05) with S31 and S32. On the tenth day, the value of S33 hyaline cells was significantly different (P<0.05) with S30 and SK30, but not significantly different from S31 and S32 (Figure 3B). The decrease in the value of S30, S31, S32, and S33 hyaline cells when compared to Sk30 were 34.23%, 44.97%, 46.31%, and 51.68%.

Phenoloxidase Activity

Based on the results, the value of PO activity on the tenth day was lower than on the fifth day in the treatment and control. On the fifth day, the PO value of the S33 treatment had the lowest PO activity compared to other treatments. On the fifth day, the PO value of S33 treatment was significantly

different (P<0.05) with S30 and SK30, but not significantly different (P<0.05) with S31 and S32. The PO value of the S30 treatment was significantly different (P<0.05) with SK30, but not significantly different (P<0.05) with S31 and S32. The decrease in the value of PO activities for S30, S31, S32, and S33 when compared to Sk30 were 30.88%, 40.63%, 47.69%, and 49.71%.

On the tenth day, S33 treatment was significantly different (P<0.05) with S30, S31 and SK30, but not significantly different (P<0.05) with S32. The PO value of S30 treatment (P<0.05) was significantly different from SK30, but not significantly different from S31 (P<0.05). On the fifth and tenth day, the PO value of S33 treatment was significantly different (P<0.05) with S30 and SK30, but not significantly different from S32 (Figure 4). The decrease in the value of PO activities for S30, S31, S32 and S33 when compared to Sk30 were 53.30%, 66.37%, 65.96%, and 73.85%.

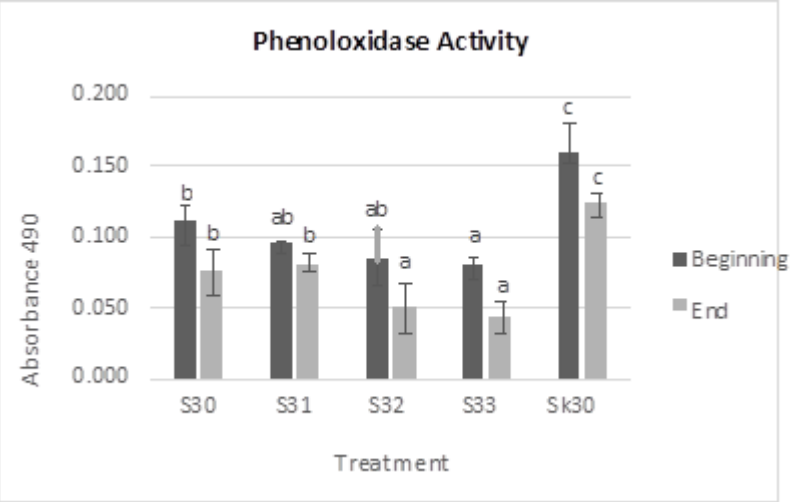

Figure 4: PO activity measurement of Vannamei shrimp after the IMNV challenge test. Significant difference results (P<0.05) indicated by different letters in the same color.

Respiratory Burst (RB) Activity

The infection of IMNV and hyperthermia resulted in a simultaneous decrease in RB activity. The value of RB activity on the tenth day was lower than on the fifth day both in the treatment and control. S33 treatment had the lowest RB activity compared to other treatments. On the fifth day, the RB value of S33 treatment was significantly different (P<0.05) with S30 and SK30, but not significantly different from S31 and S32. The activity value of RB S30 was significantly different (P<0.05) with Sk30, but not significantly different (P<0.05) with S31. Decrease in the value of RB activity S30, S31, S32 and S33 when compared to Sk30 were 23.75%, 37.09%, 49.26% and 52.73% (Figure 5).

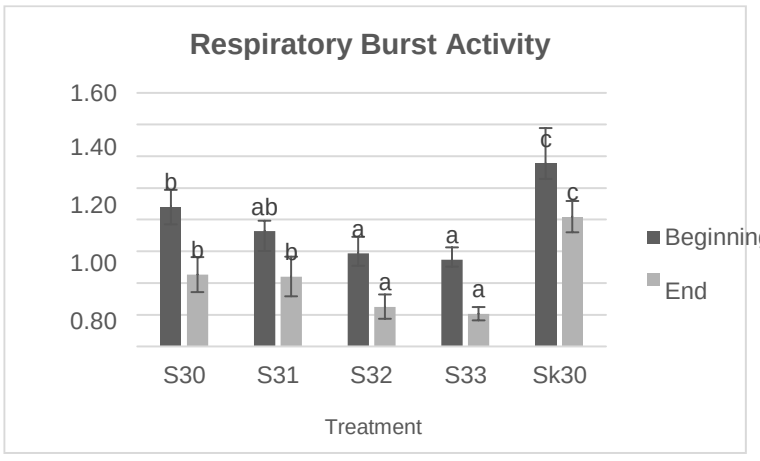

Figure 5. Results of measurement of RB activity of Vannamei shrimp after the IMNV challenge test. Significant difference results (P<0.05) indicated by different letters in the same color.

On the tenth day, the RB activity value of the S33 treatment was significantly different (P<0.05) with the S30 and Sk30 treatments, but not significantly different (P<0.05) with S32. The RB activity value

of the S30 treatment was significantly different (P<0.05) with Sk30 but not significantly different (P<0.05) with S31. The results showed that there was an effect of hyperthermia on the THC value of Vannamei shrimp by the IMNV challenge test. Hyperthermia causes the body's immune response to IMNV infection to decrease significantly. The decrease in THC values in this study ranged from 52.11% - 86.84%. The THC value decreased most significantly in the S33 treatment. The value of THC varies in each individual or depends on physiological conditions and environmental changes (Le Moullac et al., 2000; Mahasri & Sari., 2018). Hamocyte abundance is also influenced by the circulatory system (open/closed), sex, molting, growth, and reproductive and nutritional status (Owens & O'Neill). The decrease in THC values can reach 30% after natural IMNV infection (Costa et al., 2009). The decrease in THC values was reported to reach 40% in shrimp that experienced drastic temperature changes (Le Moullac et al., 2000). In the incidence of infectious diseases, the decrease in THC is influenced by the infiltration of hemocytes in the tissue, decreased production of hemocytes by hematopoietic organs, or apoptotic processes. Hemocyte infiltration in the tissue is a response to an inflammatory reaction. In the event of IMNV, many hemocytes infiltrates were found in the necrotic abdominal muscle tissue (Andrade et al., 2008). The migration of hemocytes from the circulatory system to the tissues indicates that the body's defense system is working (Smith et al., 2003; Mahasri et al., 2018).

The Hemocyte circulation process plays an important role in protecting shrimp from infection. Hemocytes play a role in immunity through the pathogen recognition process, phagocytosis, melanization, and cytotoxicity (Nkuba et al., 2023). Morphologically, shrimp hemocytes are classified into hyaline cells (agranulocytes), semigranulocytes (SGC), and granulocytes (GC). Some experts classify hemocytes into agranulocytes and granulocytes. In this study, both cell types decreased significantly after IMNV infection. The most significant decrease occurred in the S32 and S33 treatments. The types of hemocytes are important in the immobilization and destruction process of pathogens. Hyaline hemocytes is a young hemocytes. In the infection process, hyaline cells turn into granular hemocytes to respond to pathogens that enter the shrimp body. Hyaline hemocytes play a role in the absorption of pathogens and foreign particles. In addition, these cells are involved in the coagulation process. SGC plays a role in the early detection of pathogens. GC contains proPO, peroxinectin, and crustin (antimicrobial) (Soderhall & Cerenius, 1992). The number of granular cells in the circulatory system may also decrease. This is due to the degranulation process of granular hemocyte cells in lymphoid organs. In the event of viral infection, accumulation of Lymphoid Organ Spheroid (LOS) was reported. This is the response of the shrimp body to viral infection (Van de Braak et al., 2002). LOS accumulation is associated with the degranulation of granular hemocyte cells containing the virus. In LOS, inclusion bodies were found in shrimp infected with IMNV (Tang et al., 2005).

Measurement of PO activity is an important parameter used to evaluate the health status of shrimp (Maggioni et al., 2004). In this study, PO activity decreased by the IMNV challenge test. The S33 treatment experienced the most significant decrease in PO activity compared to other treatments. Shrimp infected with the disease can experience decreased immunity with a marked decrease in PO activity (Cerenius et al., 2010). Decreased PO activity has been reported to occur 24 hours after infection (Mathew et al., 2007). The value of PO activity is almost always directly proportional to the value of THC. Hemocytes release PO into the hemolymph in the form of proPO (inactive proenzyme). Under normal conditions, the increase in the amount of proPO was accompanied by an increase in the number of hemocytes (Smith et al., 2003). ProPO activation begins with the introduction of microbial-specific molecules called pathogen-associated molecular patterns (PAMPs) by pathogen-recognition receptors (PRPs). This process triggers serine proteinase (PRPs) activity which can activate proPO zymogen to become a PO enzyme. PO enzymes can initiate the formation of melanin from quinones that can destroy pathogens. PO activity is related to phagocytosis, encapsulation, and melanization of foreign bodies (Amparyup et al., 2013; Wiradana et al., 2019). Pathogens that enter the shrimp body will be destroyed through PO activity. In addition, this will trigger antibacterial activation by antimicrobial peptides (AMPs) such as pentamidine, crustin, and anti-lipopolysaccharides factor (ALFs) (Tassanakajon, 2013).

The results of the S31, S32 and S33 treatments showed a significant decrease in RB activity after the IMNV challenge test. RB activity is the process of destroying pathogens or foreign particles through phagocytosis (Yeh et al., 2009). The release of degradative enzymes into phagosomes leads to the destruction of pathogens as a result of the production of reactive oxygen intermediate (ROI) or respiratory burst (RB). Hemocyte count results are related to RB activity. The decrease in RB activity is directly proportional to the decrease in the number of hemocytes due to migration to pathogen-infected tissues. The value of RB activity was determined by measuring the amount of superoxide anion (O2-) formed by hemocytes. In addition to superoxide anion, RB activity produces hydrogen peroxide (H2O2). Hydrogen peroxide will turn into hypochlorous acid (HClO) The product of RB activity is toxic to pathogens (Rodriguez & Le Moullac, 2000).

4. Conclusion

Hyperthermia affects the immune response of Vannamei shrimp by IMNV challenge test. The value of THC, hyaline cells, PO activity, and RB activity decreased in shrimp reared at 30°C, 31°C, 32°C, and 33°C. Shrimp infected with IMNV, their immune response decreased significantly, especially at temperatures of 32°C and 33°C.

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Conflict of interest

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

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