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THE EFFECTIVENESS OF DUNALIELLA SALINA AGAINST THE DECREASE MACRONUCLEI AND MICRONUCLEI OF HYBRID GROUPE (EPINEPHELUS FUSCOGUSTATUS X EPINEPHELUS LANCEOLATUS) INFECTED BY IRIDOVIRUS

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ABSTRACT

Cultivation of hybrid groupers (*Epinephelus fuscogustatus* x *Epinephelus lanceolatus*), is one of the fish farming activities that have high economic value. Lately the existence of *Iridovirus* has become a big problem for cultivation. It was a deadly viral disease for grouper hatchery in Asia including Indonesia. Iridovirus may cause mass mortality, especially larvae up to fingerling size. The purpose of this study was to determine the effect of different level of *Dunaliella salina* against the decrease the number of micronuclei and macronuclei of hybrid grouper. As many as 180 seeds of hybrid grouper with weight 4-5 gram disperse into 15 tanks volume of 16 litres with density of 10 fish/tank. Four treatments were applied with different diets containing powder of *Dunaliella salina* of K- (0 gr kg⁻¹ diet fed), A (2 gr kg⁻¹ diet fed), B (4 gr kg⁻¹ diet fed) dan C (6 gr kg⁻¹ diet fed), with three repetition. Experimental diet was given twice for 10 days. After 10 days of treatment, the fish tested challenge with Iridovirus for 96 hours. The main parameters observed in this study were the number of micronuclei and macronuclei. Based on the research, most of the hematological parameters such as erythrocytes, Hb and HE significantly increased in *Dunaliella salina*-treated fish compared with controls (P<0.05). Micronuclei and macronuclei after challenge with *Iridovirus* in 6 mg/kg *Dunaliella salina*-treated fish was 10.57-14.58 MN/100 cells and 12.02 MAN/100 cells, which significantly decreased compared with control (P<0.05).

KEY WORDS

Hybrid grouper, *Dunaliella salina*, *Iridovirus*, *megalocytivirus*.

Iridovirus contain linear double stranded DNA genom as genetic material (Chinchar *et al.*, 2005), icosahedral symmetry with 20-sides capsid that have been infected in a fish, invertebrates and amphibians and identified from more than 20 species of fish in recent years (Hyatt *et al.*, 2000). DNA virus classified as a family Iridoviridae that contains of 5 genera, 3 of them are found in fish (Chinchar *et al.*, 2005). The complete nukleotide sequence of Iridovirus contains about 90 potential genes and contain of 112,000 base pairs (bp) in length (Kurita, 1998). Iridovirus is a representative of the genus Megalocytivirus. However Megalocytivirus is still a severe causative agent that caused mass mortalities for many spesies in both marine cultured and ornamental fish, mainly in East and South-east Asia (Jeong *et al.*, 2003, Paperna *et al.*, 2001, Sudthongkong *et al.*, 2002).

Megalocytivirus as an particular contains virusses associated with a range of diseases with clinical sign from skin lesion to systemic infection, darkening of body colouration and lethargy, anemia, petechiae of gills and enlargement of spleen (Inouye, *et al.*, 1992). The mortality rates varied from low (0,5-10%), moderate (50%) and death on 24-48 hours after the onset of sign.

One way to find out the health of fish is to observe the status of macronucleus and micronucleus. The macronucleus is a somatic nucleus (not involved in hereditary information transmission from generation to sexual generation), while the micronuclei is a small additional nucleus that is located outside the main nucleus, one form of cell nucleus due to a faulty division process and formed from an ascent or fragmentary fragment chromosome. The criteria of the observed micronuclei are less than 1/3 in diameter of the nucleus, no contact with the nucleus and the same intensity and absorption of colour.

Dunaliella salina is one of the microalgae which have the largest beta-carotene content (especially all-*trans*- β -carotene and 9- or 9'-*cis*- β -carotene) compared to other microalgae, more than 14% of dry weight (Bhattacharjee, 2016). Carotenoids in *Dunaliella salina* are a source of Vitamin A and vitamin C of 352,000 IU which can increase the immune system (Talero *et al.*, 2015). Betacaroten have been reported to be immunostimulants in fish (Gourveia *et al.* 1997, Supamattaya *et al.* 2005, Raymundo *et al.* 2005).

Effects of betacaroten on aquatic animals are various: enhance the larval survival and growth (Amar *et al.* 2004), increase the performance of brood stock (Supamattaya *et al.* 2005) and larvae (Wyban *et al.* 1997), increase fish hematological parameters (Nakano *et al.*, 1995) and improve the immune system from various diseases (Amar *et al.* 2001; Supamattaya *et al.* 2005).

MATERIALS AND METHODS OF RESEARCH

Dunaliella salina seedlings used as much as 10 liters with a density of 35 x 10⁵ cells/ml which were then cultured in tank on 350 liters of concrete with an initial density of 10 x 10⁴ cell/ml. Harvesting was carried out on day 7 with a density of 47 x 10⁵ cells/ml. A total of 350 liters of culture can produce 53.9 grams of dry weight. Furthermore *Dunaliella salina* is mashed into a powder.

Preparation of *Dunaliella Salina* Powder. *Dunaliella salina* is filtered to obtain biomass in the form of paste then are flattened on a tray and dried for about 4 days. The dried paste is smoothed until it changes into powder. A total of 350 liters of *Dunaliella salina* culture produced 53.9 grams of powder.

Feed Repelletting. The amount of *Dunaliella salina* powder used is 0 gr, 2 gr, 4 gr and 6 gr added in 1 kg of refined pellet feed. Then added enough water and adhesive material by 1% of the total feed. After all components are evenly mixed, the feed are reprinted using molds, and dried using an oven for 2 days at 40 ° C until they are completely dry. Feed is given to the hybrid grouper *adlibitum* for 10 days.

Iridovirus Isolation. Spleen on hybrid grouper crushed and homogenized using PBS solution with a ratio 1: 5. Homogenization was carried out by centrifugation at 3.000 rpm for 15 minutes to separate the pellets from the supernatant. The supernatant obtained was filtered and stored in appendorf at a temperature of -80 ° C.

Challange the Iridovirus

The hybrid grouper used is obtained from BBAP Situbodo with weight 4-5 gram. After the hybrid grouper was acclimatized for 7 days and given food containing *Dunaliella salina* for 10 days, it was soaked in water containing *Iridovirus*. The dose used is 1 ml of viral isolate for 10 liters of water.

Measurement of erythrocyte, hemoglobine, hematocrite, macronuclei and micronuclei. Total erythrocytes measurement follows the steps: blood sample is sucked with a thoma pipette until a scale of 0.5 is then sucked up with a hayem solution to a scale of 101. After being homogenized for 3-5 minutes, the first drop is then removed and the next drop is inserted into the haemocytometer. The total calculation of erythrocytes was carried out in 5 sightings of the haemocytometer.

Hemoglobin levels can be measured by filling a valid tube with 0.1 N HCl up to number 10. Subsequently placed a linear tube between 2 tubes of standard colour. 0.2 ml of fish blood was put into a valid tube, then distilled water and stirred until the colour was exactly the same as the standard colour. Hemoglobin levels are read by looking at the surface of the liquid and matched with numbers on a yellow scale. Hemoglobin levels are expressed in g%.

Hematocrit levels are expressed as% of the volume of blood cell solids that can be measured by inserting a blood sample into a microhematocrit tube up to 3/4 of the tube. Then clogged the end of the tube (marked in red) with critoseal. Then centrifuged for 15 minutes at a speed of 12,000 rpm. Hematocrit levels can be calculated by the following formula:

Calculation of the number of macronuclei and micronuclei according to Kousar and Javed (2015) is done by placing one drop of blood on the object glass and making a smear.

After drying, it was rinsed with methanol, stained using 10% giemsa for 20 minutes and rinsed with distilled water and dried.

RESULTS AND DISCUSSION

The results of the effect of *Dunaliella salina* on hybrid grouper were shown in Fig. 1. Based on Figure 1, the mean of erythrocytes at a dose of 6 g kg⁻¹ of feed is the highest erythrocytes which is 6,29±0,017cells/mm³ and significantly affected compared to other treatments (P/0.05). The high number of erythrocytes is thought to be because carotenoids in *Dunaliella salina* can induce limfomyeloid tissue in the kidneys to produce the enzyme erythropoietin so that lymphomyeloid tissue can produce more red blood cells. Carotenoid compounds contained in *Dunaliella salina* can function as red blood cell enhancers, antioxidants, antibacterials and enhance immunity and substitute for damaged cells (Fretes, et al., 2012). Erythropoietin or EPO is a glycoprotein hormone released by peritubular capillary wall cells in the kidneys and serves to control erythrocyte production (Soldatov, 2005).

The various haematology after treatments of some herbal immunostimulants in fish was reported (Harikrishnan et al. 2003; Alishahi et al. 2010). Nakano et al. (1995) reported that carotenoids-treated increased hematological parameters of fish, dietary carotenoids also influenced blood components. Supplementation of dried algal cells caused retardation of growth in black tiger shrimp (Liao et al. 1993) and striped jack (Watanabe et al. 1990).

Figure 1 – The average of hybrid grouper erythrocyte, hemoglobine and hematocrite. A, B, and C fish groups fed with 2, 4 and mg kg⁻¹ *Dunaliella salina*, respectively. K diet free from *Dunaliella salina*. Mean ± SD from 10 fish. Groups with different letter on SD bar were significantly different (P<0.05). Supplementation of the feed with 6 gr kg⁻¹ *Dunaliella Salina* for 10 days significantly (P<0.05) increases the erythrocytes, Hb and HE of Hybrid grouper

The highest hybrid grouper hemoglobin level was also obtained at a dose of 6 g kg⁻¹ of feed (Figure 2), which was 6.40 ±0,43 g% and significantly affected compared to other treatments (P<0.05). The amount of hemoglobin is generally directly proportional to the number of erythrocytes (Lagler, et al., 1977).

Hematocrit (HE) values and red blood cell counts are general indicators used to determine fish health and describe abnormalities caused by viruses. Hematocrit examination results can also be used as a benchmark to determine fish health. Based on the results of the study, it was found that the highest hematocrit was at a dose of 6 g/kg of feed, and significantly affected compared to other treatments ($P < 0.05$). which was $27,33 \pm 0,57$ %.

After challenging tests with iridovirus, showed hybrid grouper were not given *Dunaliella salina* had more micronuclei than those given *Dunaliella salina*. The direct impact due to viral infection is the increase in ROS (Ractive Oxygen Species). In addition to the innate immune system which is an important factor, ROS is also beneficial for hosts, which can limit viral replication. However, ROS that is too high can cause damage to the cells, causing inflammation (Hui-Wem et al., 2017).

The average of micronuclei of hybrid grouper fish after being challenged with *Iridovirus* ranged between 10.57-14.58 MN/100 cells. Micronuclei is a abnormality in red blood cells that are formed from chromosomal fragments (chromosomal faults) that experience lag in the anaphase phase and fail to divide in cell division (Figure 3a). The disorder is caused by damage to the spindle thread. After arriving at the stage of cell division, the chromosome fragments form a small nucleus called micronuclei. Sayed, *et al.* (2017) states that if the amount of free radicals is too much, it can cause core DNA damage and spindle thread disfunction.

Based on Figure 2 The dose of 6 g/kg of feed is the best treatment with the lowest number of micronuclei compared to other treatments, it was $10,57 \pm 1,09$. In this research, micronuclei significantly decrease in all *Dunaliella salina*-treated groups ($P < 0.05$). The higher the dose of *Dunaliella* given, the less the number of micronuclei found. Beta-carotene in *Dunaliella salina* serves as an antioxidant capable of suppressing free radicals at the onset of macrophage stimulation and avoiding too much ROS.

Figure 2 – The average of hybrid grouper macronuclei and micronuclei

Figure 3 – Morphology of hybrid grouper macronuclei and micronuclei: (a) micronuclei, (b) macronuclei

Similar to micronuclei, the highest number of macronuclei in hybrid groupers infected by Iridovirus can be found in fish without *Dunaliella salina*. The least amount of macronuclei was found in hybrid grouper which was given *Dunaliella salina* at a dose of 6 g kg⁻¹ of feed, which was 12,02±0,83 cells, and significantly decrease compared to other treatments (P<0.05).

The effectiveness of carotenoid source in fish, in terms function of physiological and deposition is species-specific. The same pathways for the metabolism of carotenoids of fish species not possess; therefore, In fish tissues there is no universal transformation of carotenoids (Kop and Durmaz 2008).

CONCLUSION

Dunaliella salina has an effect on the number erythrocytes, Hb, HE, macronuclei and micronuclei of hybrid groupers infected by Iridovirus. The use of *Dunaliella salina* powder as much as 6 g kg⁻¹ of feed is the effective dose which can reduce the number of macronuclei and micronuclei. However, the optimal dose and mechanism of *Dunaliella salina* is still not completely clear, we suggest to do further studies o dose addition and mechanism of *Dunaliella salina* in as a function of immunostimulant for viruses.

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