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Haematology and melanomacrophage centers of Nile tilapia fed supplemented diet with propolis

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ABSTRACT. The objective of this study was to investigate the influence of dietary supplementation with propolis on hematology and number and area of melanomacrophage centers in spleen and kidney of tilapia. After acclimation, fish (24.7 ± 7.4 g mean weight) were distributed in 6 tanks 100 L, 6 fish per tank, in triplicate, at a temperature 24.0 ± 2.8ºC, with two treatments: Fish fed 2% propolis supplemented diet and fish fed non-supplemented diet. To monitor the evolution of the effects, two samples were collected: half of the fish from each treatment were used after 15 days and the other half after 21, composing two feeding times. After each feeding time, blood, spleen and kidney were collected. After twenty one days feeding on 2% propolis supplemented diet, fish showed a lower number of total leukocytes and lymphocytes and an increase in the total erythrocytes number. Fish fed supplemented diet presented an increased number of melanomacrophage centers. We observed hemosiderin in all spleen samples. Kidney showed no significant difference on the presence of melanomacrophage centers containing hemosiderin. Despite these changes, the fish health status was not affected. The results showed that propolis supplementation in the diet of Nile tilapia may be physiologically feasible.

Keywords: fish, blood, kidney, spleen, hemosiderin.

Hematologia e centros de melanomacrófagos de tilápia do Nilo, alimentada com dieta suplementada com própolis

RESUMO. O objetivo deste estudo foi avaliar a influência da suplementação de ração com própolis sobre os parâmetros hematológicos e os centros de melanomacrófagos no rim e baço de tilápia do Nilo. Após aclimatação, os animais (peso médio de 24,7 ± 7,4 g) foram distribuídos em seis tanques de 100 L, seis peixes em cada tanque, em triplicata, à temperatura de 24,0 ± 2,8ºC e divididos em dois tratamentos: peixes alimentados com ração suplementada com extrato de própolis 2% e peixes alimentados com ração não suplementada. Para observar a evolução dos efeitos foram realizadas duas amostragens. Foi utilizada metade dos peixes de cada tratamento após 15 dias e a outra metade após 21 para coleta de sangue, baço e rim. Após 21 dias de alimentação com ração suplementada, os peixes apresentaram menores números totais de leucócitos e linfócitos e aumento no número de eritrócitos. Aumento no número de centros de melanomacrófagos ocorreu no baço dos peixes alimentados com ração suplementada. Em todos os baços, observou-se presença de hemossiderina. Os rins não apresentaram diferença significativa quanto à presença de centros de melanomacrófagos com hemossiderina. Os resultados mostraram que a própolis é uma possibilidade fisiologicamente viável na suplementação das rações de tilápias.

Palavras-chave: peixes, sangue, rim, baço, hemossiderina.

Introduction

The major producers in aquafeed industry are greatly aware of environmentally responsible practices and they attach importance to sustainability issues during feed development (KIRON, 2012). Also coming to prominence has been the widespread implementation of biosecurity and with it an increased emphasis on health management, which has been geared toward reducing the use of drugs in disease treatment and reducing adverse effects of fish disease on fish, consumer, and environment (NOGA, 2010). Opotherapics are an alternative obtained from animal glands, other organs, tissues and secretions (ANVISA, 1978). According to Pinheiro-Filho (1998), propolis is an animal and plant product, derived from resinous substances, gummy and balsamic, collected by bees from flower buds, plants exudates and modified in the hive by adding salivary secretions and wax (PINHEIRO-FILHO, 1998). The main chemical compounds

isolated so far can be classified as: aliphatic acids and esters, aromatic acids and esters, sugar, alcohols, aldehydes, fat acids, amino acids, steroids, ketones, charconasand dihydrocharconas, flavonoids, terpenoids, proteins, B1, B2, B6, C and E vitamins, as well as several minerals (MENEZES, 2005).

With this composition, propolis has biological and pharmacological properties such as antibacterial, antifungal, antiviral, antiprotozoal, local anesthetics, anti-inflammatory and immune stimulatory (MIYAKE, 1997; SANTOS et al., 2002; SFORCIN, 2007). It has antioxidant and preservative characteristics (TALAS; GULHAN, 2009) not only improving the physiology of aquatic organisms but also contributing to the benefit of the consumer. Abd-El-Rhman (2009) analyzed the antagonistic effect between *Aeromonas hydrophila* and propolis in tilapia performance, and noticed that the best growth rate and feed conversion was obtained in the treatment containing propolis-ethanolic-extract.

On the other hand, according to Sforcin et al. (2002) it is possible to come up with the hypothesis that its complex composition may cause damage when used in large amounts. Due to the complexity of its composition, benefic reactions and some possible adverse effects, propolis provides a wide and challenging field for research and for the understanding of its effects. Evaluation of blood components helps in determining the influence of pathophysiological conditions, which contributes to the diagnosis of adverse conditions. Erythrocyte count assists in identifying anemia processes, while leukocyte quantification assists in the diagnosis of infectious processes and homeostatic imbalance (TAVARES-DIAS et al., 2009). Furthermore, histopathology may indicate exposure to contaminants (BERNET et al., 1999). Homechaudhuri and Jah (2001) reported that in carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*), kidney and spleen are the main organs related to erythropoiesis.

Activation of melanomacrophage centers in the splenic parenchyma in Nile tilapia was correlated directly to pollution (AUTHMAN et al., 2012). Lipofuscin, hemosiderin, ceroid and melanin are the constituent pigments (BALAMURUGAN et al., 2012). Hemosiderin is related to storage and reuse of iron from erythrocytes (TAVARES-DIAS; MORAES, 2004). The objective of this study was to investigate the influence of dietary supplementation with propolis on hematology and number and area of melanomacrophage centers in spleen and kidney of tilapia.

Material and methods

After acclimation for 5 days, 36 healthy juvenile *Oreochromis niloticus* (24.7 ± 7.4 g and 11.2 ± 1.2 cm) from the same spawning caught in Panama fish farming, Paulo Lopes, Santa Catarina State, were distributed in six tanks of 100 L, 6 fish per tank, in triplicate. During the test, the water quality was kept at a temperature of 24.0 ± 2.8°C, dissolved oxygen 6.0 ± 0.4 mg L^{-1} (Hanna®, HI 9146), pH 6.51 ± 0.43 (Alfakit®, AT-350) and total ammonia 0.09 ± 0.33 mg L^{-1} (Alfakit®). Two experimental groups, fish fed diets supplemented with 2% propolis extract and fish fed non-supplemented diet, were evaluated in two samples. Based on experiments performed by Abd-El-Rhman (2009) and Kaleeswaran et al. (2012) half of the fish from each treatment were used after 15 days and the other half after 21 days.

Propolis used in this trial was obtained from apiaries in the State of Santa Catarina, from *Eucalyptus grandis* shoots and *Araucaria angustifolia* resin. The extract was produced in the Laboratório de Morfogêneses e Bioquímicas Vegetais (FIT/CCA/UFSC). Based on the concentrations used by Chu (2006); Zhang et al. (2009) and Abd-El-Rhman (2009) the diet was formulated containing 2.0% propolis-extract. The extract was mixed with commercial tilapia feed, Nicoluzzi®, containing 28% crude protein, and after incorporated, the feed was dried in an oven with air circulation for 24 hours at room temperature. Fish were fed twice daily, based on 3% body weight during four weeks. During the process, the behavior of fish was observed. Before the daily feeding, the tanks were siphoned to remove excrements and food leftovers from the previous day.

For hematological analysis, animals were captured and anesthetized with benzocaine (1 g 10 L^{-1}) according to ethical procedures (Ethics Committee 23080.009240/2011-93/CEUA/UFSC) to collect 1.0 mL of blood by puncture of tail vein with syringes containing 10% EDTA (JERÔNIMO et al., 2009). An aliquot was stored and used for determination of hematocrit percentage (GOLDENFARB et al., 1971) and erythrocyte count, in Neubauer chamber, after dilution of 1:200 in a solution of sodium chloride. Blood smears were stained with May-Grunwald/Giemsa according to Rosenfeld method (1947) for total leukocyte and thrombocyte counts and differential leukocyte counts. The total numbers of thrombocytes and leukocytes in blood were calculated by the indirect method from blood smears (ISHIKAWA et al., 2008). Statistical analysis compared the means between treatments at 5% probability.

Fragments of spleen and kidney were fixed in 10% buffered formalin during 24h at room temperature and then transferred to 70% ethanol solution. The sections were subjected to classical histological procedures of dehydration, clearing and paraffin embedding. 4 μm thick sections were stained with Harris hematoxylin and eosin aqueous 1% (HOWARD et al., 2004); and Prussian blue according to Perl´s method (HOWARD et al., 2004) to identify the biological iron.

Histological analysis of the spleen, on the basis of the quantification of melanomacrophage centers (MMC) was done by stereology. The quantification of the area occupied by the melanomacrophage centers in relation to the spleen tissue, as well as their number was investigated by a quantitative analysis for morphometric studies using Weibel´s point count technique (1963). Using a Weibel graticule (WEIBEL, 1963; LOWE; MOORE, 1985) coupled to the microscope objective and using the 40 x lens, 10 fields were randomly chosen per slide per animal. On each field, the number of melanomacrophage centers was verified. The size of the MMC was identified by the points below each of the 42 intersection points present on the graticule. In each animal, the counting done on the 10 random chosen fields were measured (42 x 10), in a total of 420 points maximum, per cut per animal, adapted from Garcia and Magalhães (2008). The volume fraction of each organ was calculated according to the formula of Lowe and Moore (1985). With the aid of STATISTICA software, data were analyzed for homoscedasticity (Levene test) and normality (Kolmogorov-Smirnovo) and subjected to analysis of variance ANOVA. Tukey´s test was used to compare the means.

The analysis for the presence or absence of melanomacrophage centers containing hemosiderin in the kidney was performed by examining the slides stained with Prussian blue according to Perl´s method (HOWARD et al., 2004). Because these are prevalence data, Fisher's exact test at 5% probability was used, comparing the results between the supplemented and non-supplemented animals and between sampling days, 15 and 21 days after feeding.

Results

After 15 feeding days, there were no changes in the hematological parameters between animals fed non-supplemented and 2% propolis-supplemented diets. After 21 days of feeding, the fish supplemented with 2% propolis presented lower ($p < 0.05$) numbers of leukocytes and lymphocytes (Table 1).

Taking into consideration the feeding time, we observed that fish fed diets supplemented with 2% propolis showed an increase ($p < 0.05$) in the number of total erythrocytes, leukocytes and thrombocytes after 21 days of feeding. Hemolysis was observed on the blood smears.

After 15 days, fish fed propolis-supplemented diet had a higher number of melanomacrophage centers compared to non-supplemented animals ($p < 0.05$) (Figure 1). On the other hand, animals supplemented with propolis showed no significant difference ($p > 0.05$) in the number of melanomacrophage center 21 days after feeding compared to non-supplemented animals. The other comparisons between animals fed non-supplemented and supplemented diets were not significantly different ($p > 0.05$) (Table 2).

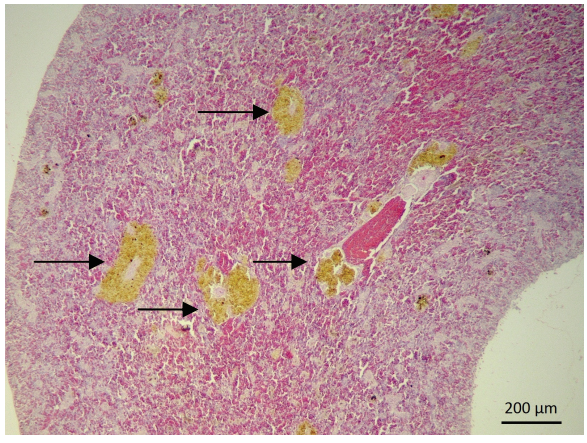

Figure 1. Melanomacrophage centers (arrow) in spleen stained with Harris hematoxylin and eosin aqueous 1% (HOWARD et al., 2004) of Nile tilapia fed with 2% propolis supplemented diet.

Table 1. Hematological parameters (± standard error) of Nile tilapia fed diet supplemented with 2% propolis and non-supplemented for 15 and 21 days.

Parameters	15 days		21 days	
	2% propolis	Non-supplemented	2% propolis	Non-supplemented
Hematocrit (%)	29.0 ± 0.5^{aA}	31.2 ± 0.4^{aA}	36.7 ± 0.3^{aA}	34.9 ± 0.7^{aA}
Erythrocytes (x $10^6 \mu L^{-1}$)	1.59 ± 0.1^{aA}	1.58 ± 0.1^{aA}	3.9 ± 0.2^{aB}	3.43 ± 0.1^{aB}
Leukocytes (x $10^3 \mu L^{-1}$)	55.7 ± 4.1^{aA}	54.4 ± 1.9^{aA}	70.4 ± 8.3^{aB}	88.4 ± 4.2^{bB}
Thrombocytes (x $10^3 \mu L^{-1}$)	24.6 ± 2.8^{aA}	22.9 ± 1.2^{aA}	$40.3 \pm 8,2^{aB}$	38.1 ± 9.6^{aB}
Lymphocytes (x $10^3 \mu L^{-1}$)	49.3 ± 3.3^{aA}	44.1 ± 1.7^{aA}	57.1 ± 4.6^{aA}	80.5 ± 3.8^{bB}
Neutrophils (x $10^3 \mu L^{-1}$)	3.6 ± 0.3^{aA}	6.5 ± 0.7^{aA}	9.1 ± 0.6^{aA}	4.0 ± 0.1^{aA}
Monocytes (x $10^3 \mu L^{-1}$)	2.7 ± 0.6^{aA}	4.1 ± 0.4^{aA}	4.1 ± 0.2^{aA}	3.8 ± 0.3^{aA}

Lowercase letters indicate significant differences between the supplemented and non-supplemented animals and uppercase letters indicate significant differences between feeding times, by analysis of variance (ANOVA) ($p < 0.05$).

Table 2. Biometric analysis of spleen (± standard error) of Nile tilapia fed with 2% propolis supplemented diet and with non-supplemented after 15 and 21 days.

Parameters	2% propolis		Non-supplemented	
	15 days	21 days	15 days	21 days
Number	10.8 ± 1.3^{bA}	9.8 ± 0.7 ^{aA}	6.7 ± 0.8 ^{aA}	10.8 ± 1.6 ^{aB}
Size	4.9 ± 0.6 ^{aA}	5.0 ± 0.6 ^{aA}	4.8 ± 0.8 ^{aA}	4.0 ± 0.3^{aA}
STV (%)	95.0 ± 0.6 ^{aA}	94.9 ± 0.6 ^{aA}	95.3 ± 0.8 ^{aA}	96.0 ± 0.3^{aA}

Lowercase letters indicate significant differences between the supplemented and non-supplemented animals and uppercase letters indicate significant differences between feeding times, by analysis of variance (ANOVA) ($p < 0.05$). STV: spleen total volume.

The presence of hemosiderin was found in all spleens stained with Perl (Figure 2). The same was not observed in the kidneys. Kidneys stained with Perl exhibited no significant difference ($p > 0.05$) for the presence or absence of hemosiderin (Table 3).

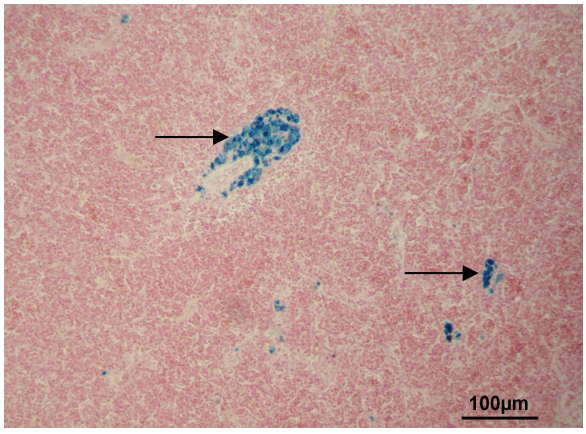

Figure 2. Melanomacrophage centers containing hemosiderin (arrows) in spleen stained with Prussian blue according to Perl´s method (HOWARD et al., 2004) of Nile tilapia fed with 2% propolis supplemented diet.

Table 3. Percentage of melanomacrophage centers (MMC) containing hemosiderin in the kidney of Nile tilapia fed diet supplemented with 2% propolis and non-supplemented for 15 and 21 days.

Parameter	15 days		21 days	
	2% propolis	Non-supplemented	2% propolis	Non-supplemented
MMC hemossiderin	88.9 ^{aA}	62.5 ^{aA}	62.5 ^{aA}	66.7 ^{aA}

Lowercase letters indicate significant differences between the supplemented and non-supplemented animals and uppercase letters indicate significant differences between feeding times, by Fisher's exact test ($p < 0.05$).

Discussion

The reduction in the total number of leukocytes after 21 days in fish fed 2% propolis probably occurred because the number of lymphocytes was also lower, since there was no significant difference in the other leukocyte cells. In this study, propolis did not cause any reaction that could consequently increase the number of lymphocyte cells in the blood. The diet supplemented with propolis was well accepted by the fish. While feeding, they appeared to have eaten to satiation.

Abd - El - Rhman (2009) observed a higher number of monocytes in fish fed 1% propolis, different from that observed in this study, in which there was no significant difference between treatments. According to Clauss et al. (2008), the production of monocytes is suggestive of inflammatory response in teleost fish. The absence of increase in monocyte number with the use of propolis in this test was expected because the inflammation response was not stimulated. Monocytes are transiting blood cells (LORENZI, 1999) and they are able to leave the peripheral blood and migrate into the tissues, where they become macrophages. In higher teleosts machophages are organized into centers, which occur primarily in hematopoietic tissues (AGIUS, 1980). Even with no change in monocyte count, histomorphometric analysis revealed a greater number of melanomacrophage centers in the spleen of fish fed propolis, after 15 days, compared to non-supplemented ones. The melanomacrophage centers have important physiological functions such as erythrocyte storage (QUESADA et al., 1990), catabolism of erythroid tissue (MESSEGUER et al., 1994) and reuse of substances involved in hematopoiesis (TSUJII; SENO, 1990), such as iron from hemolysis, which was observed on blood smears stained with May-Grunwald/Giemsa.

Increase in number, size and/or pigment content of macrophages in fish with impaired health and under stressful conditions (ELSTON et al., 1997), or in response to environmental contaminants (NOWAK; KINGSFORD, 2003) has already been observed. Changes in melanomacrophage centers have been considered as reliable biomarker (FOURNIER et al., 2001) and sensitive biomarkers for immune toxicology (BALAMURUGAN et al., 2012). Increase in the number of melanomacrophage centers caused by propolis could have been the result of stress. However, despite the number of melanomacrophage centers in fish fed diet supplemented with 2% propolis have been higher than that of non-supplemented animals after 15 days, in 21 days this number became statistically similar, with no significant difference. These results suggest that the use of propolis affects the number of melanomacrophage centers and possibly the organism has get adapted during treatment through its physiologic tools to reach homeostasis.

The staining obtained by Perl´s method showed that most melanomacrophage centers were involved in phagocytosis of biological iron that came from erythrocytes hemoglobin. This process occurs in small quantities in healthy fish. However, the excessive accumulation in organs reveals a pathological state (WOLKE et al., 1985). In agreement with Gabrielle and McMillan (1984), this iron could possibly be reused for synthesis of new erythrocytes.

Despite the large amount of biological iron found in the spleen, there was no significant difference in erythrocyte count between treatments after 21 days. According to Junqueira and Carneiro (1999), spleen is the most important organ of erythrocyte breakdown because of its amount of phagocytic cells. The function of erythrocytes is to transport oxygen and carbon dioxide, which is carried by hemoglobin.

It is worth emphasizing that in attempt to establish hematological values for fish rearing in Brazil, Tavares-Dias et al. (2009) used healthy tilapia, not taking into consideration outliers, and determined the reference, minimum and maximum values. The reference range found by these authors was 1.120 - 1.715 x 10^6 μL^{-1}erythrocytes and the maximum value of 2.42 x 10^6 μL^{-1}. The number of red blood cells found in this study is within the reference range for fish caught 15 days after feeding, but at 21 days erythrocyte values were higher than the maximum reference value for fish fed propolis-supplemented diet.

Regarding the kidney, no significant differences were detected for melanomacrophage centers containing hemosiderin between fish fed supplemented and non-supplemented diet. The same result was obtained when time of feeding was compared. The amount of fish that showed hemosiderin may not have been influenced by propolis, in this organ. According to Thiyagarajah et al. (1998), hemosiderosis usually occurs as a result of excessive erythrocyte breakdown in vertebrates and the product remaining, hemosiderin, can be found in the spleen hematopoietic tissue.

In this way, Estrada and Garcia (1991) stated that hemosiderosis suggests anemia in fish due to erythrocytes breakdown. Nevertheless, in this study even with large amount of hemosiderin in kidney and some in spleen, the quantity of red blood cells remained high. It may have occurred reduction of erythrocyte lifespan, triggering phagocytosis of erythrocytes that were no longer able to transport oxygen and stimulating the production of new erythrocytes.

Conclusion

Propolis in concentration of 2% showed differences in hematological and histological parameters. The presence of hemosiderin in melanomacrophage centers was not affected by feeding.

Despite the fact of such alterations, the fish health state was not affected. The results showed that propolis supplementation in the diet of Nile tilapia may be physiologically feasible.

Furthermore, due to its antiviral, anti-inflammatory, antioxidant, antiparasitic properties and also the results that show that propolis promotes higher growth rate and feed conversion, its use as a preventive measure and to increase productivity in aquaculture should be taken into consideration.

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