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Acute inflammation and hematological response in Nile tilapia fed supplemented diet with natural extracts of propolis and *Aloe barbadensis*

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Abstract

This study evaluated the acute inflammatory response induced by carrageenin in the swim bladder of Nile tilapia supplemented with the mixture of natural extracts of propolis and *Aloe barbadensis* (1:1) at a concentration of 0.5%, 1% and 2% in diet during 15 days. Thirty-six fish were distributed into four treatments with three replicates: fish supplemented with 0.5% of admix of extracts of propolis and *Aloe* (1:1) injected with 500 µg carrageenin; fish supplemented with 1% of admix of extracts of propolis and *Aloe* (1:1) injected with 500 µg carrageenin; fish supplemented with 2% of admix of extracts of propolis and *Aloe* (1:1), injected with 500 µg carrageenin and unsupplemented fish injected with 500 µg carrageenin. Six hours after injection, samples of blood and exudate from the swim bladder of fish were collected. It was observed an increase in the leukocyte count in the swim bladder exudate of fish supplemented with extracts of propolis and *Aloe* injected with carrageenin. The most frequent cells were macrophages followed by granular leukocytes, thrombocytes and lymphocytes. Supplementation with propolis and *Aloe* to 0.5% caused a significant increase in the number of cells on the inflammatory focus mainly macrophages, cells responsible for the phagocytic activity in tissues, agent of innate fish immune response.

Keywords: inflammation, carrageenin, swim bladder, propolis, *Aloe*.

Inflamação aguda e resposta hematológica em tilápias do Nilo alimentadas com extratos naturais de própolis e *Aloe barbadensis* suplementados na dieta

Resumo

Este estudo avaliou a resposta inflamatória aguda induzida por carragenina na bexiga natatóriade tilápia do Nilo suplementada com a mistura dos extratos naturais de própolis e *Aloe barbadensis* (1:1), nas concentrações de 0,5%, 1% e 2% na dieta durante o período de 15 dias. Trinta e seis peixes foram distribuídos em quatro tratamentos com três repetições: peixes suplementados com 0,5% da mistura dos extratos de própolis e *Aloe* (1:1) injetados na bexiga natatória com 500 µg de carragenina; peixes suplementados com 1% da mistura dos extratos de própolis e *Aloe* (1:1) injetados na bexiga natatória com 500 µg de carragenina; peixes suplementados com 2% da mistura dos extratos de própolis e *Aloe* (1:1) injetados na bexiga natatória com 500 µg de carragenina e peixes não suplementados injetados na bexiga natatória com 500 µg de carragenina. Seis horas após as injeções foram coletadas amostras de sangue e exsudato da bexiga natatória dos peixes. Foi observado aumento na contagem de leucócitos no exsudato da bexiga natatória de peixes suplementados com os extratos de própolis e *Aloe* injetados com carragenina. As células mais frequentes foram os macrófagos seguidos pelos leucócitos granulares, trombócitos e linfócitos. A suplementação com própolis e *Aloe* a 0,5% provocou aumento significativo no número de células no foco inflamatório, principalmente dos macrófagos, células responsáveis pela atividade fagocitária nos tecidos, agente da resposta imune inata nos peixes.

Palavras-chave: inflamação, carragenina, bexiga natatória, própolis, *Aloe*.

1. Introduction

Inflammation is one of the major host defenses against all forms of aggression. By definition, according to Thomson (1983), inflammation is the vascular response of living tissues and cells to a certain attack, that may result in passive and chemical changes in attacked cells or tissues, which initiate the inflammatory process. In fishes,

the inflammatory process may be inducted by biological, chemical or physical agents (Kumar et al., 2004).

The role of the inflammatory process is to minimize irritating effects on injured tissues, and the primary response to injury is an accumulation of fluids and cells in the injured area for mobilization of immune cells such as leukocytes, macrophages and lymphocytes to the inflamed site. The rapid migration of leucocytes from microcirculation to inflammatory site is the most important characteristic of this process (Garcia-Leme, 1989). The set of fluids and cells is called exudate, and its purpose is to dilute, find, destroy and remove the irritant, leading to replacement of injured tissue portions.

The exudate is a mean for recognition of inflammatory process in tissues. In fish, the assessment of inflammatory response depends on three major factors: first is the difficulty of identification of leukocytes, that depends on the species, the second is related to ontogenetic variation in blood and inflammatory cells (Tavares-Dias and Moraes, 2004), and third kinetics of inflammatory process, which may be influenced by dietary supplementation (Moraes et al., 2003; Belo et al., 2005, 2012; Dotta et al., 2011), by the kind of inflammatory stimulous (Bozzo et al., 2007), by eicosanoids (Claudiano, 2011), by the ambiental temperature (Dias and Sinhorini, 1991; Jansson Junior and Waaler, 1967) and by circulating hormones (Martins et al., 2000; Belo et al., 2005, 2012).

Cellular response can be evaluated by inducing an inflammatory process such as injections of carrageenin, sulphated polysaccharide, classically used in the acute inflammation assays. In fish, carrageenin, thioglycolate and inactivated *Aeromonas hydrophila* were tested in the swim bladder of *Piaractus mesopotamicus* (Martins et al., 2000; Moraes et al., 2003; Bozzo et al., 2007) and *Oreochromis niloticus* (Martins et al., 2004; Dotta et al., 2011). According to Bozzo et al. (2007), thioglycolate and inactivated *Aeromonas hydrophila* induced vascular congestion, accumulation of thrombocytes followed by macrophages, granulocytes and oedema. The choice of swim bladder as local for assessment of inflammatory response is based on studies of Van Furth (1992), which showed that body cavities are an experimental model to represent the mechanisms of inflammatory response that can occur in the other animal tissues.

An efficient method to control diseases in aquaculture is strengthened fish defense mechanism by prophylactic administration of immunestimulants (Drago et al., 2000). Besides, alternatives for the use of chemicals are needed to prevent and avoid infections (Romano and Mejía, 2003). On this view, natural products must be tested to minimize the environmental degradation by indiscriminate use of chemicals.

The hypotesis of this assay was to verify whether supplemented diet with natural extracts of propolis and babosa *Aloe barbadensis* Miller may influence the acute inflammation and hematological response when carrageenin is injected in the swim bladder of Nile tilapia, a proved experimental model.

2. Material and Methods

2.1. Experimental conditions

Thirty-six juveniles Nile tilapia (22.3 ± 12.7 g mean weight and 12.4 ± 2.7 cm total lenght) from the same spawning were stored into polyethylene water tanks with a capacity of 100 L, provided with a biological filter, heater and constant aeration, maintained under the following conditions: temperature 24.0 ± 2.8 °C and dissolved oxygen 6.0 ± 0.0 mg/L (Hanna, HI 9146), pH 6.51 ± 0.43 (Alfakit, AT-350) and ammonia 0.09 ± 0.33 mg/L (Alfakit, colorimetric method). After seven days of acclimation, feeding with the experimental diets was started and continued for 15 days, to perform induction of inflammatory response to injection of 500 µg carrageenin (Marine Colloids) diluted in 0.5 ml saline solution. Fish were distributed in a completely randomizated factorial design divided in four treatments with three replicates: fish supplemented with 0.5% of admix of extracts of propolis and *Aloe* (1:1) injected with 500 µg carrageenin; fish supplemented with 1% of admix of extracts of propolis and *Aloe* (1:1) injected with 500 µg carrageenin; fish supplemented with 2% of admix of extracts of propolis and *Aloe* (1:1), injected with 500 µg carrageenin and unsupplemented fish injected with 500 µg carrageenin.

2.2. Supplemented diet preparation

After fish biomass calculation fish were fed at 3% body weight with commercial diet supplemented or not with natural extracts. Propolis and *A. barbadensis* were added to the ration in the amounts of 1:1 to get a final concentration of 0.5; 1 and 2% of the total quantity of offered food per day. The extracts of propolis and babosa were diluted in 50% alcohol before diet mixture.

2.3. Injection and collection of exudate cells

After feeding period of 15 days, fish were anesthetized with clove oil at a concentration of 100 mg.L^{-1} to be injected with carrageenin in the swim bladder, according to Dotta et al. (2011). Six hours after injections they were euthanized with clove oil for collection of blood and swim bladder exudate cells (Ethic Committee nº 23080.009240/2011-93/CEUA/UFSC). After opening the abdominal cavity, the swim bladder was ruptured and washed with 0.5 ml of phosphate buffer solution (PBS) with a drop of 0.001 ml of 5% EDTA. Briefly, with the aid of pipete the content was collected, maintained in ice for total leucocyte count. Afterthat, the exudate was centrifuged at 150 G for 10 min, the supernatant discharged to use the pellet for smears that were stained with Giemsa for 10 min according to Dotta et al. (2011) to determine differential cell count.

2.4. Hematological parameters

Blood samples were collected from the caudal vein using a syringe containing a drop of 10% EDTA solution (Ethic Committee nº 23080.009240/2011-93/CEUA/UFSC) to measure hematocrit (Goldenfarb et al., 1971), red blood cells count in a Neubauer chamber, total counts of leukocytes and thrombocytes and differential count of leukocytes. Blood smears in duplicate were stained with a lood combination of Giemsa/May-Grünwald (Rosenfeld, 1947). The smears were used for differential leukocyte count and the total count of thrombocytes and leukocytes (Martins et al., 2004).

2.5. Statistical analysis

Data was submited to factorial analysis of variance (ANOVA) using Statsoft. Bartlett test was used to verify the homoscedasticity while Tukey test was used to compare means. Data transformations were used according to pertinence.

3. Results

Hematological parameters as hematocrit and number of red blood cells did not show significant difference (P>0.05) among the treatments (Table1). In the circulating blood, there was a reduction in the number of total leukocytes in fish fed 1% and 2% of the mixtures of extracts. On the other hand, fish fed 0.5% and unsupplemented fish did not differ in the number of total leukocyes (Table 1). The differential leukocyte count in circulating blood (Figure 1) showed changes between treatments. The inflammatory response evaluated from the swim bladder exudate (Table 1) revealed a significant increase in total leukocyte count in fish supplemented with 0.5% extract mixture in the diet, compared to the other treatments, especially in relation to non-supplemented animals.

In differential count of exudate cells, lymphocytes were the most abundant cells, followed by thrombocytes, granulocytes and macrophages (Figures 1 and 2).

Table 1. Mean values and standard deviation of hematological parameters in circulating blood and total leukocytes count in swim bladder exudate of *Oreochromis niloticus* fed supplemented diet with propolis and *Aloe* (1:1) in different concentrations.

	Circulating blood			Exudate
Treatments	**Hematocrit (%)**	**Erythrocytes ($x10^6.\mu L^{-1}$)**	**Total leukocytes ($x10^3.\mu L^{-1}$)**	**Total leukocytes ($x10^6.\mu L^{-1}$)**
0.5% + IC	20.88 ± 7.11a	2.32 ± 0.66a	34.79 ± 17.47b	0.65± 0.90c
1% + IC	23.0 ± 5.32a	2.41 ± 0.26a	29.47 ± 16.59a	0.23 ± 0.48b
2% + IC	23.60 ± 5.85a	2.11 ± 0.37a	30.33 ± 10.59a	0.35 ± 0.36b
NS + IC	24.28 ± 5.58a	2.23 ± 0.48a	33.46 ± 11.09b	0.08 ± 0.01a

Lowercase letters indicate a significant difference between treatments (p<0.05). Injected with carrageenin (IC) and non-supplemented (NS).

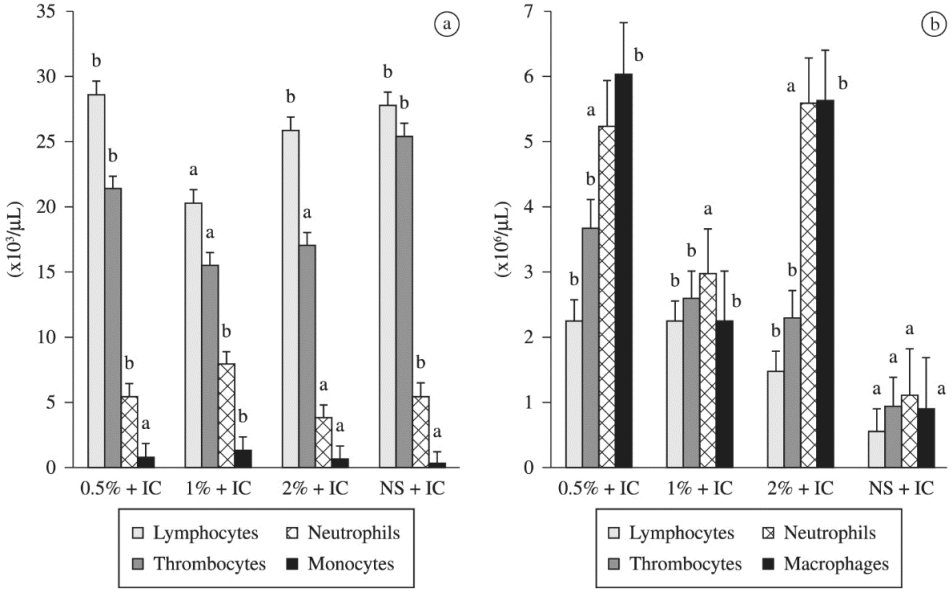

Figure 1. Mean values of total leukocyte and thrombocyte counts in circulating blood (a) and inflammatory exudate (b) of *Oreochromis niloricus* fed supplemented diet with propolis and *Aloe* (1:1) in different concentrations. Lowercase letters indicate a significant difference between treatments (p<0.05). Injected with carrageenin (IC) and non-supplemented (NS).

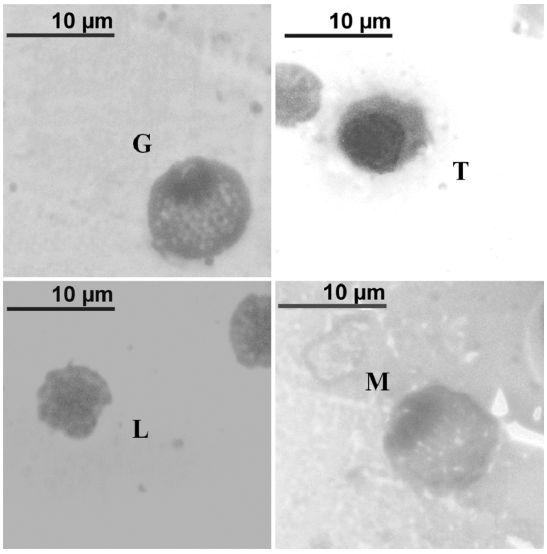

Figure 2. Exudate cells of *Oreochromis niloticus* fed supplemented diet with propolis and *Aloe* (1:1) in different concentrations. Granulocytes (G), thrombocytes (T), lymphocytes (L) and macrophages (M).

4. Discussion

Carrageenin injection caused accumulation of inflammatory cells into the swim bladder of Nile tilapia, as reported by Dotta et al. (2011). The circulating blood cells that migrate into tissues to participate in inflammatory response include thrombocytes, lymphocytes, macrophages and granulocytes, corroborating previous studies. (Bozzo et al., 2007; Reque et al., 2010; Salvador et al., 2012; Claudiano, 2011).

In this assay, macrophages were the most frequent cells, followed by neutrophils, thrombocytes and lymphocytes in fish supplemented with 0.5% and 2% of extracts mixture and injected with carrageenin. It should be noted that in studies of Bozzo et al. (2007) the authors utilized inactivated *Aeromonas hydrophila* to induce inflammation in *Piaractus mesopotamicus*, and the second most frequent cells were lymphocytes. This may be related to the kind of irritant agent, the bacterium. Martins et al. (2009) observed that tilapia injected with 1×10^6 colony forming units *Enterococcus*/ml showed higher number of thrombocytes. Thrombocytes are important cells of hemostasis also involved in the organic defense as reported in previous studies (Ellis et al., 1976; Penha et al., 1996; Matushima and Mariano, 1996; Martins, et al., 2000). studies (Ellis et al., 1976; Penha et al., 1996; Matushima and Mariano, 1996; Martins, et al., 2000). The importance of thrombocytes as a phogocytic-cell has been reported in birds, *Gallus gallus domesticus* (Grecchi et al., 1980; Kajigaya et al., 1985), bullfrog, *Lithobathes catesbeianus* (Ishida et al., 1985; Dias and Sinhorini, 1991; Penha et al., 1996) and tilapia (Suzuki, 1986; Matushima and Mariano, 1996). Similarly to the present results, Reque et al. (2010) observed increased number of thrombocytes accompanied by lowered number of macrophages, neutrophils and lymphocytes in the inflammatory exudates of tilapia fed *Saccharomyces cerevisae*.

Zanuzzo (2010) evaluated the respiratory burst of macrophages in *Brycon amazonicus,* kept in water containing *A. vera*, and observed an increase in the respiratory activity of leucocytes . These findings indicate that *A. vera* can be reacted with the neutrophil membrane receptors by stimulating the ROS (reactive oxygen species) production. According to Jorgensen and Robertsen (1995), an increase in the ROS production could be considered as indicator of non-specific immune system activation. Zhang et al. (2009), observed significant increase in the respiratory burst, phagocytosis and lyzozyme activity in *O. niloticus* when fed propolis extracts The presence of a great number of phagocytic cells found in this assay suggests that this fish supplemented with the extracts of propolis and babosa showed increased acute inflammation. "In vitro" tests with inflammatory cells from circulating blood are needed, such as conducted by Zhang et al. (2009) and Zanuzzo (2010) to evaluate the phagocytic activity of these cells.

In agreement with the findings of Reque et al. (2010) in tilapia fed supplemented diet with *Saccaromyces cerevisiae*, the acute inflammation induced by *A. hydrophila* did not show alterations in hematocrit.

Hematological parameters assessments may be important indicators of fish health status (Martins et al., 2008). Similarly, Cuesta et al. (2005) have related increased number of monocyte-macrophages and granulocytes phagocytosis from the blood of *Sparus aurata* fed supplemented diet with propolis. On the other hand, a gradated increase in leucocyte accumulation on the inflammatory site induced by *Escherichia coli* injection was reported for tilapia with the highest migration activity 24 hours after injection (Matsuyama and Iida, 1999).

The use of vegetal extracts on fish food has been responsible for enhancement of the immune system not only in the specific response but also in the non-specific as shown in *Catla catla* fed diet supplemented with *Achyranthes aspera* (Rao Y and Chakrabarti, 2005).

To state precisely the real effectiveness of immunestimulants supplementation in fish, careful analysis is needed (Zhang et al., 2009). With the evaluation of inflammatory response in the swim bladder of tilapia, there was a significant increase in cell accumulation to the site of injection of carrageenin, and increased number of macrophages in fish supplemented with 0.5% of propolis and *Aloe* extracts mixture.

In addition, studies must be encouraged to analysis the feasibility of the use of natural extracts in fish farm to minimize the economic losses caused by infectious or parasitic diseases.

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