

LEVAMISOLE MODULATES THE CELL-MEDIATED IMMUNITY OF MATRINXÃ, *Brycon amazonicus*

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ABSTRACT

The use of immunomodulators is an alternative to improve the immune system of fish and avoid the excessive use of antibiotics. Levamisole is a synthetic anthelmintic which promotes a potent immunostimulation on innate and acquired variables of fish. In order to access the levamisole effects on matrinxã, *Brycon amazonicus*, fish were fed levamisole at 500 mg kg⁻¹ of diet for seven days. After this period fish were submitted to blood collection. Immunological variables, such as leukocytes respiratory burst, total serum protein, albumin, globulin, albumin:globulin index and hematological variables, such as hematocrit, hemoglobin, red and white blood cell (WBC) and Wintrobe indexes were evaluated. The levamisole administration increased the WBC counts indicating that the immunostimulant modulates the cell-mediated immunity. The increased WBC indicate that the fish fed levamisole were in a better body condition, and probable more resistant to disease due to potential for phagocytosis activation, degranulation of neutrophilic granules, and consequently production of antibodies and immunological memory. Besides, these findings are important for news projects regarding the production of vaccines and deeper understanding of fish cell-mediated immunity.

Key words: immunostimulant; cellular immunity; innate immunity; white blood cell.

LEVAMISOLE MODULA A IMUNIDADE MEDIADA POR CÉLULAS DE MATRINXÃ, *Brycon amazonicus*

RESUMO

A utilização de imunostimulante é uma alternativa para melhorar o sistema imune de peixes e evitar o uso excessivo de antibióticos. O levamisol é um anti-helmíntico sintético que promove uma potente estimulação imunológica em peixes, aumentando variáveis inatas e adquiridas de defesa. A fim de avaliar os efeitos do levamisol sobre matrinxã, *Brycon amazonicus*, os peixes foram alimentados com 500 mg kg⁻¹ de levamisol na dieta durante sete dias. Após este período os peixes foram submetidos à colheita de sangue. Variáveis imunes, tais como a atividade respiratória de leucócitos, proteína total sérica, albumina, globulina, índice albumina:globulina e os parâmetros hematológicos, tais como hematócrito, hemograma completo, hemoglobina, número de eritrócitos e o volume corpuscular médio dos eritrócitos foram avaliados. A utilização do levamisol aumentou a contagem dos leucócitos do sangue indicando que o imunostimulante modulou a imunidade mediada por células, entretanto, nenhuma alteração foi observada nas demais variáveis. O aumento dos leucócitos do sangue indica que os peixes alimentados com levamisol estão em uma condição corporal melhor, e em um possível surto terão mais resistência às doenças e potencial para ativação da fagocitose e degranulação de neutrófilos. Além disso, os resultados são importantes para novos projetos sobre a produção de vacinas, e sobre a compreensão mais profunda da imunidade mediada por células em peixes.

Palavras-chave: imunostimulante; imunidade celular; imunidade inata; leucócitos.

INTRODUCTION

The use of immunomodulators is an alternative to improve the immune system of fish and avoid the misuse of antibiotics. Immunostimulant is a group of biological or synthetic substances that enhance the innate and acquired mechanisms of defense against disease causing agents. Among them, the levamisole is a synthetic anthelmintic commonly administered in mammals, which promotes a potent immunostimulation on

fish, increasing innate and acquired variables such as phagocytosis, cytotoxic activity of leukocyte, leukocytes respiratory burst, lytic serum proteins and antibodies production (Biller-Takahashi et al., 2015; Biller-Takahashi and Urbinati, 2014).

Levamisole is a synthetic compound from the group of imidazothiazole and their immunostimulatory effect is characterized by increased T lymphocyte proliferation, chemotaxis and phagocytic activity, and increased production of antibodies when used as a vaccine adjuvant (Biller-Takahashi et al., 2016; Misra et al. 2009). In fish, it has been found that levamisole has increased the number of circulating leukocytes, the phagocytic activity of leukocytes, the myeloperoxidase activity of neutrophil and serum lysozyme (Mulero et al., 1998; Cuesta et al., 2002; Li et al., 2006; Reich et al., 2014). However, despite these evidences, it is necessary to employ special attention in this chemical compound, since its effect is dose and time dependent, and high doses may promote an immunosuppression condition, otherwise the under dosage may not be efficient (Mulero et al., 1998).

The matrinxã, *Brycon amazonicus*, is a fish of great economic interest due to its simple reproduction and excellent growth performance (Ferraz and Gomes, 2009; Tavares-Dias et al., 2008b). However, as a result of the Brazilian aquaculture development and the intensive fish farming, there are several stressful situations, such as the capture, transport, confinement, changes in water quality among others which promote immunosuppression and diseases outbreaks (Urbinati et al., 2010; Zanuzzo et al., 2015). Conversely, little is known about the immune system of matrinxã (Ferraz and Gomes, 2009; Zanuzzo et al., 2012; Zanuzzo et al., 2015), and to our knowledge this is the first study evaluating the immunostimulant effect of the levamisole in matrinxã. The current study was conducted to search the effects of dietary levamisole on the innate immunity and hematological and biochemical variables of matrinxã.

MATERIAL AND METHODS

This study was approved by the Ethics Committee on animal use of Universidade Estadual Paulista “Julio de Mesquita Filho” – UNESP (protocol number 004206/10).

A total of 180 matrinxã (117 ± 18.4 g and 19.8 ± 1.32 cm) were distributed in 18 100 L tanks (10 fish per tank, 9 tanks for each treatment) with a continuous water flow system. The water temperature and water quality variables of the tanks were assessed and stayed within the appropriate ranges: temperature 28.1 ± 0.52 °C, dissolved oxygen 5.74 ± 8.11 mg L⁻¹ (oxymeter YSI 55) 0.04 ± 0.02 NH₄ mg L⁻¹ (Nessler method) and pH 8.14 ± 0.09 (Corning pH meter).

The experiment was conducted in a completely randomized design with two treatments. One treatment received a commercial standard diet containing 28% protein and an estimated digestible energy level of 12.56 MJ kg⁻¹ (levamisole-free, FRI-RIBE, São Paulo, Brazil) used during the acclimation period and to the control group, and the second treatment received a commercial supplemented diet with levamisole at 500 mg kg⁻¹ (SIGMA, St. Louis, MO, USA), during the experimental period. The experimental

diet was prepared from a commercial standard extruded feed (levamisole-free, FRI-RIBE, São Paulo, Brazil) that was triturated and into which 500 mg kg⁻¹ of levamisole were added and then pelletized and stored under refrigeration. The feed was offered *ad libitum* in two daily meals in the acclimation and experimental periods.

Fish received the respective diets for seven days in two daily meals, then, were fasted to 24 h, anesthetized in benzocaine (0.1 g L⁻¹ of water), and two fish of each tank (18 per treatment) were submitted to blood collection by puncture of caudal vessel (around 1 mL) to assess the leukocytes respiratory burst activity, complete blood count in heparinized hole blood, and total protein, albumin in serum.

Briefly, the evaluation of the blood leukocyte respiratory burst was evaluated according to Biller-Takahashi et al. (2013), and 0.1 mL of total heparinized blood was added to 0.1 mL of 0.2% nitroblue tetrazolium (NBT, Sigma, St Louis, MO, USA). The solution was incubated for 30 min at 25 °C, then, 50 µL of the resulting suspension was added to a glass tube containing 1.0 mL N,N-dimethyl formamide (DMF, Sigma, St Louis, MO, USA) and centrifuged at 2500×g for 5 min. The optical density (OD) of the final solution was measured at 540 nm.

The hematocrit (HT), the red blood cells (RBC), the hemoglobin content (HG) and mean corpuscular volume (MCV) were determined using heparinized blood in an automatic counter (Celm CC550) (Tavares-Dias et al., 2008a). The Wintrobe indexes, the mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were derived from the primary values of HT, RBC and HG (Wintrobe, 1934). The white blood cell (WBC), thrombocyte and erythroblast counts were assessed from blood smears stained with May–Grünwald–Giemsa (Rosenfeld, 1947). The WBC was calculated using the following formula, as described by Ishikawa et al. (2008): $WBC \mu L^{-1} = (\text{leukocyte number in the smear} \times \text{erythrocyte number} \mu L^{-1}) \text{ per } 2000 \text{ erythrocytes counted in the smear}$.

The total serum protein concentrations were assessed using the Biuret method (Labtest Kit) (Reinhold, 1953), and the serum albumin concentration was determined using the bromocresol green binding colorimetric assay (Labtest Kit) (Doumas et al., 1971). The amount of globulin present in the samples was established by subtracting the albumin from the total serum protein, and the Albumin:Globulin index (A:G) was found by dividing the value of the albumin fraction by that of the globulin fraction for each tested sample.

Data was previously submitted to Bartlett analysis to verify the homogeneity of variance. Then, data was submitted to the t- test. All tests used a 5% significance level.

RESULTS

The WBC of fish fed 500 mg kg⁻¹ of levamisole in the diet was increased compared with control group (Figure 1). Regarding the immunological variables, the leukocyte respiratory burst assay detected no difference between treatments (Table 1). Equally in the hematological variables, the red blood cell (RBC), the

hematocrit, the hemoglobin, the Wintrobe indexes showed no difference between treatments (Table 1). Among the biochemical variables, the total protein, the albumin, the globulin and the A:G index showed no difference when compared with the control treatment (Table 1).

DISCUSSION

The administration of levamisole for seven days stimulated the cell-mediated immunity in matrinxã, so this compound could be an alternative as vaccine adjuvant in fish (Misra et al. 2009). The innate immune system is the first line of body defense, and

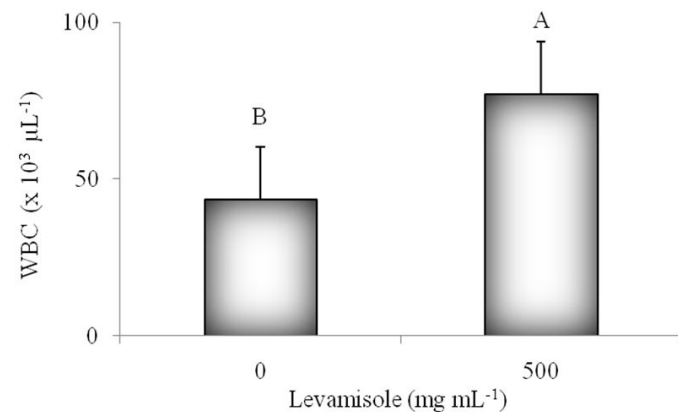


Figure 1. White blood cell (WBC) of matrinxã, *Brycon amazonicus*, fed levamisole for seven days. Bars with different capital letters denote significant differences (calculated $T = 3.462$; $P = 0.0030$). Values are means \pm standard error.

the recognition of invaders, including living microorganism or vaccine antigens, leads to the activation and proliferation of WBC. The well known WBC receptors, the toll like receptor, when stimulated will provide the intracellular and extracellular antimicrobial defenses against invading pathogens (Rieger and Barreda, 2011; Rocha et al., 2018). In this study, the dietary levamisole promoted the increase of WBC of matrinxã and the same profile was observed by Siwicki (1989) in *Cyprinus carpio* fed levamisole.

The WBC encloses some leukocytes that are able to perform phagocytosis, a process that generates large quantities of superoxide and nitric oxide (reactive oxygen species - ROS) and consequently lead to the formation of highly toxic compounds in order to destroy microorganism, however these substances are nonspecific and may also act on the host molecules (Rieger and Barreda, 2011; Biller-Takahashi et al., 2013). In the current study, the levamisole administration stimulated the production of cell-mediated immunity, but no increase on ROS production was detected as observed on the leukocyte respiratory burst assay. The oxidative compounds are also deleterious to the host organism, and its release occurs when the pathway is triggered, mainly in microbiological presence, however, in this study there was no such challenge, and probably there was no release of ROS to avoid potential oxidative stress due to oxidants over-production (Biller-Takahashi et al., 2015; Stuart and Ezekowitz, 2005). The same profile was observed by Mulero et al. (1998) on phagocytes of *Sparus aurata*, and by Ispir and Yonar (2007) on phagocytes of *Oncorhynchus mykiss* in several levamisole concentrations.

The levamisole has been widely applied in both human and veterinary medicine (Renoux, 1980), and the administration as a vaccine adjuvant has been applied, and there is a known enhancement of the immunization efficiency (Jeney and Anderson,

Table 1. Immunological, hematological and biochemical variables (means \pm standard error) of matrinxã, *Brycon amazonicus*, fed levamisole for seven days.

	Levamisole (mg kg ⁻¹ diet)		T calculated	P value
	0	500		
BURST (DO)	0.43 \pm 0.01	0.45 \pm 0.03	1.060	0.3041
RBC (x 10 ⁶ µL ⁻¹)	2.68 \pm 0.13	2.92 \pm 0.16	1.157	0.2629
Hematocrit (%)	28.5 \pm 1.52	30.4 \pm 1.64	0.901	0.3805
Hemoglobin (g dL ⁻¹)	13.4 \pm 2.20	15.0 \pm 2.8	1.447	0.1658
MCV (fl)	106 \pm 1.54	106 \pm 0.86	0.490	0.6314
MCHC (g dL ⁻¹)	47.1 \pm 3.45	49.9 \pm 2.56	0.985	0.4256
MCH (pg cell ⁻¹)	49.1 \pm 1.49	51.2 \pm 2.33	0.875	0.2582
Erythroblast (x10 ³ µL ⁻¹)	38.3 \pm 7.08	57.3 \pm 9.45	1.610	0.1265
Thrombocyte (x10 ³ µL ⁻¹)	22.2 \pm 3.15	27.9 \pm 3.59	1.206	0.2438
Protein (g dL ⁻¹)	3.37 \pm 0.14	3.60 \pm 0.10	1.348	0.1963
Albumin (g dL ⁻¹)	1.11 \pm 0.06	1.15 \pm 0.06	0.470	0.6441
Globulin (g dL ⁻¹)	2.30 \pm 0.09	2.48 \pm 0.07	1.527	0.1465
A:G index	0.47 \pm 0.02	0.44 \pm 0.01	1.221	0.2381

Values are means \pm standard error. BURST = Leukocyte respiratory burst activity; RBC = red blood cell; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; MCH = mean corpuscular hemoglobin; A:G = Albumin:Globulin.

1993). In the present study, it was possible to observe the potential of the levamisole as vaccine adjuvant in fish, due to the increased WBC of matrinxã, and its function on the cell-mediated immunity that is essential for antibody development (Korytář et al., 2013). Furthermore, the cell-mediated defense is especially important for the protection against viral infections (Utke et al., 2008).

The increased WBC observed in this study may also indicate a better body condition and possibly greater disease resistance, with a possible better potential for activation of phagocytosis, degranulation of neutrophil granules in front of a biological challenge (Faurshou and Borregaard, 2003; Biller-Takahashi and Urbinati, 2014). So, it was possible to observe that levamisole has shown an immunostimulant role for fish. Kajita et al. (1990) have found improvement in nonspecific immune response such as increased counts of “natural killer” cells, in the phagocytic activity, in the hemolytic activity of the complement and in the serum bactericidal activity after levamisole injection in *Oncorhynchus mykiss*, additionally, after challenge with virulent strains of *Vibrio anguillarum*, a higher resistance against the disease were observed.

The levamisole administration in matrinxã did not influence the energetic metabolism, showed by the lack of effect on red blood cell and Wintrobe indexes. The lack of influence of the levamisole on the hematocrit percentage was also observed by Li et al. (2006) in the hybrid *Morone chrysops* × *Morone saxatilis*; by Sahoo and Mukherjee (2002) in *Labeo rohita*; by Ispir and Dorucu (2005) in *Oncorhynchus mykiss*; and by Sado et al. (2010) in *Piaractus mesopotamicus*. Studies with Atlantic salmon did not show a good effect of levamisole (Morrison et al., 2000; Morrison et al., 2001).

The body status can be assessed by hematological variables, such as erythrocytes and hemoglobin, that are responsible for gas exchange in the body to respond to the energy demand in adverse situations, such as stressful condition or disease outbreak (Costa et al., 2014; Fazio, 2019; Ispir and Dorucu, 2005; Li et al., 2006; Sado et al., 2010). Matrinxã fed levamisole showed no variation in the hemoglobin and MCV measured values, so that, levamisole prompt no impact on body's anemia and the response to energy demand, since hemoglobin and MCV are hematological variables that assess those physiological features (Fazio, 2019; Ranzani-Paiva and Silva-Souza, 2004). In this study, there was no alteration on hematological variables, nevertheless fish were not submitted a bacterial or stressful challenges that could increase energy demand, as a result it was not possible to evaluate the ability of matrinxã blood cells in this experimental protocol.

The short time period of administration of this study did not modulate the humoral immune system. Proteins are important to maintain oncotic pressure, essential for the homeostasis of body fluids, in addition to transport components among body tissues. The two major groups of proteins are albumin and globulins (Schell and Blumberg, 1977). The total protein levels in serum encloses different protein components such as albumin and globulin. In the current study, the total protein concentrations showed no alteration by treatment, and consequently the albumin and globulin levels were also not affected.

Albumin is the most abundant serum proteins, Misra et al. (2009) have found increased concentrations of albumin of *Labeo rohita*, after 15 days of feeding with levamisole, the same result was observed by Maqsood et al. (2009) in *C. carpio* fed the immunostimulant. Sado et al. (2010) observed decreased total protein of *Piaractus mesopotamicus* after 30 days of levamisole administration. Conversely, the globulins are proteins found in the serum, among them are the immunoglobulins and other components responsible for the organism defense (Maqsood et al., 2009). Maqsood et al. (2009) and Misra et al. (2009) also found in *C. carpio* and *Labeo rohita*, respectively, increased globulin levels after long-term administration of levamisole. According to these findings and the observed lack of levamisole effects on serum proteins, we conclude that the humoral immune stimulation requires longer administration in this species.

There was no mortality during the experiment or signs of toxicity in matrinxã. Factors such as time and route of administration are important to the efficiency on the organisms responses (Sakai, 1999), even though, it was possible to observe the immunostimulant role of the levamisole on cell-mediated immunity of matrinxã.

CONCLUSION

The administration of the levamisole at 500 mg kg⁻¹ in the diet for seven days in matrinxã increased white blood cell counts, indicating a modulation on cell-mediated immunity, and probably leading to a better body condition and greater disease resistance. The levamisole effect is dose and time dependent, and perhaps higher doses or longer administration may promote a more expressive result. However, in order to enhance this issue, appropriate techniques should be applied to determine the completely cellular composition of the blood after levamisole administration, such as the development of monoclonal antibodies for leukocytes subsets recognition by specific surface markers.

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