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CLINICAL SAFETY OF ZAFIRLUKAST TREATMENT DURING A FOREIGN BODY INFLAMMATORY REACTION IN NILE TILAPIAS, OREOCHROMIS NILOTICUS

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ABSTRACT

Seeking to understand the defense mechanisms of tilapia to assist in the sanitary management of fish farms, this study aimed to evaluate the clinical safety of Nile tilapia (*Oreochromis niloticus*) submitted to oral treatment with 500µg of zafirlukast/Kg of body weight during chronic inflammation, through the implantation of round glass coverslips in the subcutaneous tissue. For this study, 63 male tilapiawere randomly distributed in 9 aquariums to compose the following treatments: Control submitted to inflammatory stimulus without treatment; CT – Conventional treatment; PT – Prolonged treatment. Seven animals were evaluated per treatment in three periods: 2, 4 and 8 days post-implantation (DPI).Tilapia during foreign body reaction showed a decrease in erythrocyte counts and hematocrit values, as well as macrocytic changes, these effects were mitigated by treatment with zafirlukast. Control tilapia showed a gradual increase over time in serum values of total protein, cholesterol, and triglycerides. Serum values of ALT, AST and alkaline phosphatase of control tilapia showed an increase in the late phase of the chronic inflammatory process (8 DPI). Therefore, it was possible to conclude that tilapia treated with 500µg of zafirlukast showed attenuation of hematological changes resulting from fluid-electrolyte imbalance during a foreign body inflammation, as well as ameliorated serum biochemical disorders, demonstrating the clinical safety of oral treatment with this cys-leukotriene blocker.

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INTRODUCTION

According to SOFIA report (FAO, 2020), aquaculture production is essential for the growth of the global economy, and Brazil has enormous potential to be explored, reaching production of 758,006 tons in 2019. In intensive fish production, when outbreaks of infectious diseases occur, one of the alternatives for treatment is chemotherapy drugs during sanitary management. However, there are few drugs registered for use in fish, a fact that results in the indiscriminate use of various compounds in aquaculture (Aracati *et al*., 2021). For this reason, the study of clinical safety in fish becomes relevant, and this happens through hematological and biochemical tests, which are essential parameters to assess the physiological condition of fish (Mahmoud *et al*., 2018) against the use of pharmaceutical molecules in fish farms. Fish have a variety of specific and non-specific defense mechanisms against invading organisms (Prado *et al*., 2018, Moraes *et al*., 2018; Rodrigues-Soares *et al*., 2018). Inflammation in teleost fish can effectively reproduce the inflammatory process in mammals, demonstrating that signaling and gene expression pathways are well conserved throughout evolution (Manrique *et al*., 2015; Forn-Cuní *et al*., 2017; Manrique *et al*., 2017). These protection mechanisms are fundamental for fish, and consist of controlled and coordinated processes, seeking to minimize tissue damage and restore normal physiological conditions (Kim *et al*., 2007; Charlie-Silva *et al*., 2019). Pathophysiological studies of the foreign body type reaction in fish have become extremely relevant in aquaculture (Charlie-Silva *et al*., 2020), as it can help in the

development of therapeutic and vaccine strategies. The implantation of glass coverslips in the subcutaneous tissue of fish represents a classic model for studying chronic inflammation, resulting in macrophage accumulation and giant cell formation (Belo *et al*., 2014; Petrillo *et al*., 2017). The accumulation of these inflammatory cells derived from monocytes present in the bloodstream is evaluated as an important inflammatory event facing pathological processes (Belo *et al*., 2005; 2012a). Zafirlukast has anti-inflammatory activity by antagonizing the cysteine leukotriene receptors, CysLTR1, acting on smooth muscle contraction and preventing the increase in vascular permeability, thus reducing edema formation and diapedesis (Scott & Peters-Golden, 2013), in addition to presenting secondary anti-inflammatory activities, which appear to be particularly effective in targeting neutrophils and monocytes/macrophages (Scott & Peters-Golden, 2013). These and other secondary anti-inflammatory mechanisms of CysLTR1 antagonists have been studied in human medicine, but little is known about the blocking of this receptor and its effects in teleost fish. Inorder to understand the events and mechanisms present in the pathophysiology of chronic inflammation associated with lack of information on the clinical safety of zafirlukast in tilapia, this investigation studied the action of the cys-leukotriene antagonist through hematological and biochemical analysis during the foreign body inflammatory reaction in Nile tilapia (*Oreochromis niloticus*).

MATERIAL AND METHODS

Fish: For this study, 63 male tilapia, belonging to the same spawn from the Aquabel farm (Porto Ferreira, São Paulo State, Brazil) were randomly distributed in 9 aquariums (100 L of water, n=7) supplied with running water without chlorine from an artesian well with a recirculation system at a flow rate of 1L/min. After being transported to the aquarium, the fish were acclimated for 15 days, the time necessary for the plasmatic concentration of cortisol and osmolarity to return for baseline levels. In the first three days of acclimatization, the animals were submitted to bath with NaCl solution at a concentration of 6.0g/L (Carneiro & Urbinati, 2001). Fish were fed with 2% of the aquarium biomass with commercial food (LAGUNA® - ocial Company). The water quality parameters were determined twice a day during the entire experimental period using pHmeter YSI-63 and oximeterYSI-55, and their values remained within the appropriate range for the well-being of tropical fish (Boyd, 1990) (dissolved oxygen = 4.07 ± 0.89 mg L^{-1}; temperature = 27.64 ± 2.05°C; pH = 7.64 ± 0.54; and conductivity = 208.29 ± 97.57µS / cm). This research was approved by the Ethics Committee on the Use of Animals from Brazil University (UB), process number 00152016.

Experimental Design: Tilapia were randomly distributed in 9 aquariums (100 L of water, n=7) to compose the following treatments: Control submitted to inflammatory stimulus without treatment with Zafirlukast; CT – Conventional treatment with oral administration of 500µg of Zafirlukast after inflammatory stimulus; PT – Prolonged treatment with oral administration of 500µg of Zafirlukast for one week before and continuously after the inflammatory stimulus. Seven animals were evaluated per treatment in three periods: 2, 4 and 8 dayspost-implantation (DPI) of the glass coverslip in the subcutaneous tissue.

Anti-inflammatory treatment: Tilapia were fed with commercial feed (LAGUNA® - Social Company, containing 32% crude protein, 7%, ethereal extract, 5% crude fiber and 12% mineral matter), and feeding was carried out twice a day (8:00 am and 5:00 pm) , with administration of 2% of the aquarium biomass. Zafirlukast (Accolate; Astra Zeneca Laboratory, UK) at a dose of 500µg/kg body weight was added to the diets from CT and PT treatments.To prepare the diets, the commercial feed was weighed in proportion to the average weight of tilapia from each tank and added 2% of vegetable oil plus the respective amount of zafirlukast (500µg) and stored in dark plastic bags, kept at 8°C, until the moment of use.The diet of control animals without Zafirlukast was mixed with 2% vegetable oil to maintain the standardization of the nutritional balance.

Fish anestesia: Tilapias were anesthetized by immersion in an aqueous solution of benzocaine in the proportion of 1: 10,000 for bacterin inoculation in the swim bladder and 1: 500 at the time of euthanasia. Benzocaine was diluted in 98 ° alcohol (0.1 g / mL), completing the volume to 1L (Wedemeyer, 1970). After experimental handling, the animals were replaced in the aquariums with continuous water flow and aeration.

Coverslip implant: Prior to implantation, the glass coverslips and the nylonthread for sutures were sterilized by autoclaving. Fish were anesthetized by immersion in a 1:10,000 (v:v) aqueous solution of benzocaine (Sigma Chemical Co., St. Louis, Missouri 63178, USA) for round glass coverslips implantation in the subcutaneous tissue, lateral-dorsal region caudal to the operculum (Sakabe *et al*., 2013). With a scalpel, the scales in the implant area were removed and an incision was realized. The subcutaneous tissue was divulged, and the coverslip was implanted between the skin and muscle tissue. Then the skin was sutured with simple stitches using nylon thread. After experimental handling, the animals were again placed in their respective aquariums with continuous water flow.

Hematological analysis: Seven fish per treatment (one aquarium for each treatment) were anesthetized to obtain three mL of blood samples through the caudal vessel at 2, 4 and 8 days post-implantation (DPI), which were aliquoted in two sets: one using a heparin-coated (5000 IU) needle and syringe and other without anticoagulant to obtain plasma and serum samples, respectively. The blood count was performed using a hemocytometer (Neubauer chamber) and Natt and Herrick solution (1952) (ratio 1: 100 v:v). The hematocrit was determined by the microhematocrit centrifugation technique and the hemoglobin concentration with Drabkin's reagent read at 540nm. Mean corpuscular volume (MCV)and mean corpuscular hemoglobin concentration (MCHC) were calculated from hematocrit, hemoglobin, and red blood cells (Farias *et al*., 2016).

Serum Biochemical Assessment: Blood samples from fish without anticoagulant were centrifuged at 10000rpm for 5min. at 4°C to obtain serum and determination of total protein, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, albumin, cholesterol, triglycerides using a semi-automatic biochemical analyzer (Model LabQuest® - Bioplus Company) and fish glycemia was determined using the Accu-Chek Performa device.

Statistical analysis: The experimental design for clinical safety assessment (hematological and serum biochemical analysis) was completely randomized in a 3 x 3 factorial scheme (three treatments: PT, CT and control X three evaluation periods: 2, 4 and 8 DPI). Analyzes of variance to compare the different experimental groups were performed using the GLM (General Linear Model) procedure of the SAS program, version 9.3 (Statistical Analysis Software, 2012). Significant differences ($p < 0.05$) were estimated based on Tukey's test at the 95% confidence level.

RESULTS

Hematological analysis: Control tilapia showed a significant decrease ($p<0.05$) in the percentage values of hematocrit 2 DPI compared to zafirlukast treated fish (CT) (Table 1). Control fish showed a significant increase ($p<0.05$) in MCV when compared to fish treated with Zafirlukast (PT) (Table 1). The study of inflammatory reaction evolution over time revealed that control fish showed a significant decrease ($p<0.05$) in HCMC between the second and eighth days, as well as recovery in percentage values of hematocrit in the same period (Table1).

Biochemical analysis: The analysis of hepatic cytotoxicity in tilapias implanted with coverslip revealed that control fish showed a significant increase ($P<0.05$) in serum ALT enzymatic activity when compared to groups treated with zafirlukast (PT and CT) 8DPI (Table 2).

Tabela 1. Means values and ANOVA[1] observed in the blood analysis of tilapia post-implantation

Period	Treatments[3]	Hematocrit (%)	Erythrocyte (10^6/mm^3)	MCV[5] (fL)	MCHC[5] (g/dL)	Hemoglobin (g/dL)
2 DAYS	PT	19.83 ± 0.44 ABa	1.80 ± 0.12 Aa	126.04 ± 6.78 Aa	23.16 ± 2.96 Aab	5.05 ± 0.53 Aa
	CT	22.71 ± 0.88 Aab	1.62 ± 0.05 Aa	133.51 ± 5.92 Aa	20.99 ± 0.41 Aa	4.60 ± 0.28 Aa
	Control	17.66 ± 0.42 Bb	1.34 ± 0.08 Aa	139.48 ± 4.89 Aa	23.62 ± 1.01 Aa	4.32 ± 0.18 Aa
4 DAYS	PT	19.57 ± 0.35 Aa	1.77 ± 0.13 Aa	115.51 ± 7.92 Aa	25.44 ± 0.89 Aa	5.00 ± 0.25 Aa
	CT	20.00 ± 0.76 Ab	1.38 ± 0.08 Aa	150.85 ± 12.40 Aa	24.74 ± 0.51 Aa	4.80 ± 0.10 Aa
	Control	21.42 ± 0.40 Aab	1.53 ± 0.03 Aa	134.63 ± 2.55 Aa	23.39 ± 0.70 Aa	5.00 ± 0.13 Aa
8 DAYS	PT	22.87 ± 1.27 Aa	1.85 ± 0.15 Aa	116.41 ± 9.25 Ba	16.81 ± 0.25 Ab	4.11 ± 0.38 Aa
	CT	27.00 ± 0.71 Aa	1.94 ± 0.03 Aa	143.62 ± 4.63 ABa	19.86 ± 0.52 Aa	5.33 ± 0.12 Aa
	Control	25.42 ± 1.27 Aa	1.52 ± 0.09 Aa	169.15 ± 4.72 Aa	15.73 ± 0.80 Ab	3.95 ± 0.19 Aa
Treatment		4.74 *	6.15 **	8.44 **	0.37 NS	1.27 NS
Time		23.94 **	2.69 NS	1.19 NS	17.75 **	1.18 NS
Treatmente X Time		3.15 *	1.88 NS	2.21 NS	1.49 NS	2.04 NS
C.V.[4]		12.25	18.97	17.81	18.53	21.08

[1]Means (n=7) followed by the same letter do not differ by Tukey test (P<0.05).
[2]Analysis of statistical variance represented by capital letters compares in the column the different treatments within each experimental day, lowercase letters compare the evolution of each treatment on the different experimental days.
[3]Control- implemented and untreated fish; CT- Conventional Treatment with zafirlukast; PT- Prolonged treatment.
[4]CV- Coefficient of Variation (%); NS - Not significant; *significant for P<0.05; **Significant for P<0.01.
[5]MCV – Mean corpuscular volume; HCMC – Mean corpuscular hemoglobin concentration.

Tabela2. Means values (±SD) and ANOVA[1] observed in the biochemistry analysis of AST, ALT and Alkaline phosphatase of tilapia during foreign body reaction

Period	Treatments[3]	AST[5] (U/L)	ALT[5] (U/L)	Alkaline phosphatase (U/L)
2 DAYS	PT	69.00 ± 7.45 Aa	28.20 ± 1.24 Aa	19.57 ± 2.88 Aa
	CT	87.00 ± 23.43 Aa	31.57 ± 5.97 Aa	20.71 ± 2.04 Aa
	Control	35.66 ± 7.35 Aa	23.66 ± 4.37 Ab	18.28 ± 2.47 Ab
4 DAYS	PT	32.28 ± 5.74 Aa	13.33 ± 0.84 Aa	29.14 ± 1.67 Aa
	CT	70.71 ± 12.62 Aa	18.50 ± 1.32 Aa	31.20 ± 1.09 Aa
	Control	81.00 ± 5.62 Aa	29.42 ± 2.67 Aab	30,00 ± 1.51 Aab
8 DAYS	PT	57.12 ± 11.10 Aa	21.37 ± 3.05 Ba	29.50 ± 2.65 Aa
	CT	89.25 ± 12.75 Aa	23.14 ± 1.46 Ba	28.37 ± 1.93 Aa
	Control	115.14 ± 26.84 Aa	56.42 ± 13.25 Aa	30.28 ± 2.04 Aa
Treatments		2.41 NS	4.32 *	0.06 NS
Time		2.01 NS	2.97 NS	14.92 **
Treatment X Time		2.13 NS	2.98 *	0.23 NS
C.V.[4]		66.05	62.67	26.49

[1] Means (n = 7) followed by the same letter do not differ by Tukey test (P < 0.05);
[2] Analysis of statistical variance represented by capital letters compare in the column of different treatments within each experimental day, lowercase letters compare the evolution of each treatment on different experimental days;
[3] P.T. – prolonged treatment with zafirlukast; C.T. - Conventional treatment with zafirlukast; Control - implanted but not treated with zafirlukast;
[4] CV- Coefficient of Variation (%); NS- Not significant; * - significant for P<0.05; **- Significant for P<0.01
[5] AST- A spartate aminotransferase; ALT- Alanine aminotransferase.
Tabela3. Means values (±SD) and ANOVA1 observed in the biochemistry analysis of creatinine, total protein, cholesterol, triglycerides, albumin, and glucose of tilapia during foreign body reaction.

Control fish showed a significant increase (P<0.05) in serum alkaline phosphatase and ALT values between periods 2 and 8 DPI, as well as a non-significant increase (P>0.05) in serum AST values (Table2). The serum biochemical study of renal and hepatic functionality showed an increase in blood creatinine values in the initialphase (2DPI) of the inflammatory reaction in all fish (Table 3), these findings were statistically significant (p<0.05) in tilapia treated with zafirlukast (PT). Control fish showed a significant decrease (p<0.05) in serum total protein levels in the initial phase 2 DPI and recovery in the later phase 8 DPI. There were no significant variations (p>0.05) in circulating values of total protein in fish treated with zafirlukast (Table 3). Control fish showed a significant increase in serum levels of cholesterol and triglycerides 8 DPI (Table 3), while treated fish did not show significant changes (p>0.05) in serum values of these lipids. The blood glucose study did not reveal significant changes (p>0.05) between the different treatments. However, increases in 4DPI blood glucose values are observed in animals treated with zafirlukast CT (Table 3).

Correlation analysis: Correlation analyzes between hematological and biochemical measurements in the CTtreatment revealed 69.37% (p=0.014; Fig 1A) and 57.50% (p=0.010; Fig. 1B) significant negative correlations when comparing hemoglobin with creatinine and ALT, respectively. When hematocrit and creatinine were correlated, the control groups presented -57.27% (p=0.010; Fig 1C). However, the correlation between hematocrit and glucose showed a significant result 71.84% (p=0.0004) for control group (Fig. 1D).

DISCUSSION

In the initial phase of inflammation, the hematological study of tilapia showed a decrease in red blood cell counts and hematocrit values in control fish, these effects were mitigated by treatment with zafirlukast. Changes in fluid-electrolyte balance may be due to stressful stimuli (Moonsen *et al*., 1999). Belo *et al*. (2014) observed in pacus during chronic inflammation a significant increase in the

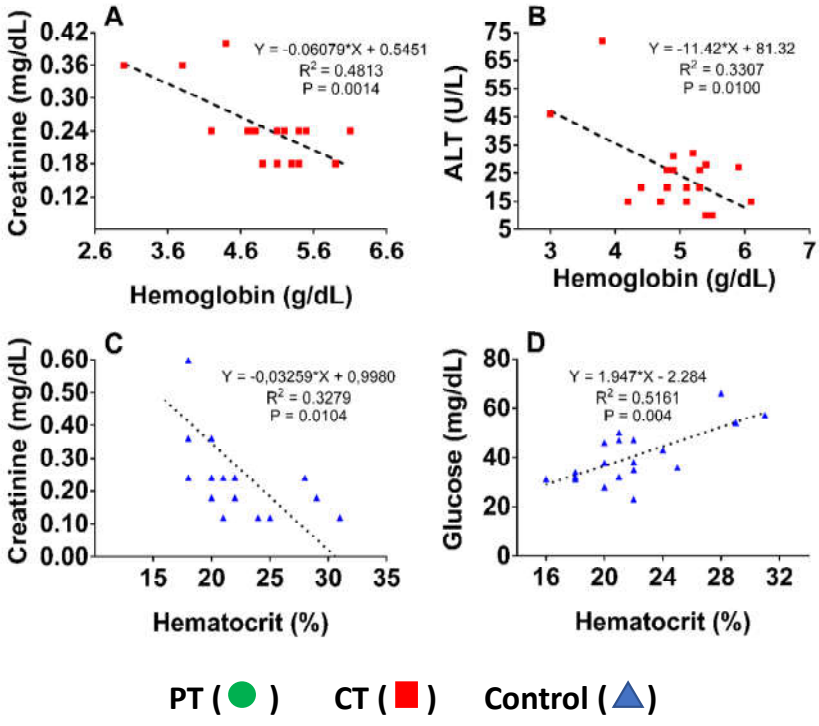

Figure 1. Correlation analysis between biochemical and hematological parameters of control animals, prolonged treatment (PT) and conventional treatment (CT) with 500 µg zafirlukast

Tabela 4. Means values (±SD) and ANOVA1 observed in the biochemistry analysis of creatinine, total protein, cholesterol, triglycerides, albumin, and glucose of tilapia during foreign body reaction

Period	Treatments[3]	Creatinine (mg/dL)	Total protein (g/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	Albumin (g/dL)	Glucose (mg/dL)
2 DAYS	PT	0.74 ± 0.35 Aa	3.30 ± 0.08 Aa	152.33 ± 8.82 Aa	165.33 ± 14.74 Aa	1.22 ± 0.03 Aa	47.00 ± 2.14 Aa
	CT	0.30 ± 0.03 Aa	3.48 ± 0.10 Aa	170.00 ± 2.39 Aa	131.14 ± 20.06 Aa	1.23 ± 0.02 Aa	31.83 ± 1.27 Ab
	Control	0.52 ± 0.10 Aa	3.22 ± 0.03 Ab	155.71 ± 6.25 Ab	122.83 ± 9.01 Ab	1.15 ± 0.04 Aa	32.16 ± 0.48 Aa
4 DAYS	PT	0.22 ± 0.01 Ab	3.70 ± 0.03 Aa	213.00 ± 16.85 Aa	285.33 ± 47.41 Aa	1.33 ± 0.02 Aa	44.85 ± 2.32 Aa
	CT	0.24 ± 0.00 Aa	3.48 ± 0.08 Aa	189.14 ± 16.35 Aa	276.85 ± 55.55 Aa	1.23 ± 0.03 Aa	55.28 ± 4.36 Aa
	Control	0.20 ± 0.01 Aa	3.70 ± 0.12 Aab	205.57 ± 16.12 Aab	196.83 ± 42.00 Aab	1.33 ± 0.11 Aa	38.14 ± 2.75 Aa
8 DAYS	PT	0.19 ± 0.01 Ab	3.41 ± 0.08 Aa	178.12 ± 10.49 Aa	359.50 ± 70.09 Aa	1.12 ± 0.03 Aa	33.33 ± 2.77 Aa
	CT	0.19 ± 0.01 Aa	3.56 ± 0.10 Aa	163.87 ± 8.26 Aa	169.00 ± 24.85 Aa	1.15 ± 0.04 Aa	46.00 ± 2.27 Aab
	Control	0.18 ± 0.02 Aa	3.87 ± 0.12 Aa	237.71 ± 27.63 Aa	486.00 ± 126.89 Aa	1.31 ± 0.05 Aa	46.66 ± 4.66 Aa
Treatments		1.34 NS	0.78 NS	1.62 NS	1.17 NS	0.63 NS	1.31 NS
Time		8.99 **	4.98 *	4.61 *	5.80 **	2.25 NS	3.98 *
Treatment X Time		1.49 NS	2.38 NS	1.98 NS	2.21 NS	1.25 NS	5.66 **

percentage values of hematocrit associated with high levels of circulating cortisol by high stocking density. According to these authors, endogenous cortisol present glucocorticoid and mineralocorticoid activity, such fact influence the fluid-electrolyte balance. With the evolution of the inflammatory reaction, tilapia showed macrocytic changes with a marked increase in the mean corpuscular volume of red blood cells, probably the fluid-electrolyte imbalance resulted in the influx of fluids into the intracellular compartment. These events were ameliorated by treatment with zafirlukast. On the other hand, Aracati *et al*. (2021) observed side effects of 500 µg zafirlukast treatment in fish during acute aerocystitisby bacterins of *Aeromonas hydrophila*, characterized by microcytosis. The difference between both studies may be associated with the types of inflammatory stimulus between them, one evaluated in the acute phase by the presence of bacterial antigenic epitopes, and the other in the chronic phase associated to inert stimulus determined by the presence of the glass coverslip in the subcutaneous tissue.

Hematological and biochemical tests are essential parameters used as part of the assessment of fish health (Mahmoud *et al*., 2018). Several studies have demonstrated the hepatotoxicity of chronic treatments with zafirlukast (Reinus *et al*., 2000; Scheen, 2001; Marcy *et al*., 2004). Although serum biochemical values in the present study are within previously recorded reference ranges for tilapia under normal health conditions (Hrubec *et al*., 2000; Mauel *et al*., 2007). Control tilapia showed a gradual increase over time in serum values of total protein, alkaline phosphatase, cholesterol, triglycerides, and ALT in relation to animals treated with Zafirlukast. These findings suggest that the anti-inflammatory effect of this cis-leukotriene blocker attenuated the serum biochemical disturbances during the tilapia's foreign body type inflammatory reaction. The functionality of liver tissues can be determined by the concentration of substances synthesized in the liver or belonging to specific metabolic processes, such as albumin, urea, glucose, cholesterol, triglycerides, and others (Suja *et al*., 2004; Shih *et al*., 2005; Belo *et al*., 2012b). Serum values of ALT, AST and alkaline phosphatase of control tilapia showed an increase in the late phase of the chronic inflammatory process (8 DPI). These effects were mitigated by treatment with zafirlukast, corroborating the findings of El-Dessouki *et al*. (2018) who used tamoxifen to induce liver damage aiming to investigate the hepatoprotective effects of Zafirlukast in female Wistar rats for 10 days. These authors observed a significant decrease in plasma ALT activity compared to the control group, demonstrating the hepatoprotective effect of this anti-inflammatory. Furthermore, these authors highlighted antioxidant effects associated with treatment with this Cys-leukotriene antagonist. On the other hand, Reinus *et al*. (2000) and Actis *et al*. (2001) reported severe liver alterations associated with an increase in humans ALT submitted to prolonged treatment with zafirlukast.

The importance of assessing liver function in clinical safety tests is noteworthy, mainly because this organ is the most important site for metabolizing drugs and chemicals (El-Sayed *et al*., 2007). In addition, renal evaluation in clinical and toxicology studies has the same importance sincethis organ present significant excretory function in fish (Yildirim *et al*., 2006; Abdel-Daim *et al*., 2015).In this context, tilapia submitted to treatment with Zafirlukast for a prolonged period showed a reduction in creatinine values over time. These findings correspond to the results presented by Hagar *et al*. (2012) in which the anti-inflammatory action of zafirlukast significantly reduced the severity of acute ischemic renal failure in rats, resulting in a significant reduction in serum creatinine levels compared to untreated animals. According to Moraes *et al*. (2018), the reduction in plasma creatinine in mammals may be due to excessive diuresis. In freshwaterfish, this behavior can be explained by the osmoregulatory control mechanism, when these animals are in stress situations there is an imbalance in the ionic exchanges resulting in greater absorption of water from the environment (Takei *et al*., 2016) and the fish organism in an attempt to eliminate the liquid increases diuresis and consequently the elimination of creatinine. Therefore, it was possible to conclude that tilapia treated with 500µg ofzafirlukast showed attenuation of hematological changes resulting from fluid-electrolyte imbalance during a foreign bodyinflammation, as well as ameliorated serum biochemical disorders, demonstrating the clinical safety of oral treatment with this cys-leukotriene blocker.

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