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ANTI-INFLAMMATORY EFFECT OF CELECOXIB, A COX-2 INHIBITOR, DURING FOREIGN BODY REACTION IN TILAPIA

EFEITO ANTI-INFLAMATÓRIO DO CELECOXIBE, INIBIDOR DE COX-2, DURANTE A REAÇÃO TIPO CORPO ESTRANHO EM TILAPIA

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SUMMARY

Seeking to understand the defense mechanisms of tilapia to assist in the health management of fish farms, associated with the lack of information on the use of COX-2 inhibitors in teleost fish, this study aimed to identify important aspects involved in the pathophysiology of the chronic inflammatory reaction in Nile tilapia, *Oreochromis niloticus*, by evaluating the anti-inflammatory activity of celecoxib on the kinetics of migration and formation of gigantocytes in the foreign body response. For this purpose, 72 tilapia were used (*Oreochromis niloticus*), placed in 9 aquariums (n=8), with a capacity of 100 L of water each, consisting of the following treatments: Control - subjected to inflammatory stimulus without treatment with Celecoxib; TC - Conventional treatment with oral administration of 3mg/kg of Celecoxib after inflammatory stimulus; TP - Prolonged treatment with oral administration of 3mg/kg of Celecoxib for one week before and continuously after the inflammatory stimulus. 8 animals were evaluated per treatment in three periods, that is: 2, 4 and 8 days post-implantation (DPI) of the coverslips, with blood samples being collected to perform the differential leukocyte count in blood extensions, as well as collecting the coverslips. for cytological study. In the later phase of the inflammatory process (8DPI), a decrease in the cellular accumulation of macrophages and formation of polykaryotes was observed on the coverslips of tilapia treated with celecoxib, demonstrating the anti-inflammatory effect of this COX-2 inhibitor, associated with a significant decrease in the number of circulating monocytes in animals receiving conventional treatment. Tilapia treated with celecoxib (TC and TP) showed a significant decrease in total leukocyte count in the initial phase of the inflammatory process (2DPI), characterized by neutropenia and lymphopenia. Therefore, both treatment protocols (TC and TP) with 3mg/kg of celecoxib modulated the foreign body-type inflammatory response of tilapia by acting on the kinetics of macrophage accumulation and formation of polykaryotes on glass coverslips implanted in the subcutaneous tissue, demonstrating a correlation to the dynamics of circulating leukocytes. These results help to understand the role of prostanoids during the chronic inflammatory reaction of tilapia.

KEY-WORDS: Ciclooxigenase antagonism. Prostanoids. Inflammation. Cichlids. Teleost fish. *Oreochromis niloticus*

RESUMO

Buscando compreender os mecanismos de defesa da tilápia para auxiliar no manejo sanitário de pisciculturas, associado à carência de informações sobre o uso de inibidores de COX-2 em peixes teleósteos, este estudo teve como objetivo identificar aspectos importantes envolvidos na fisiopatologia da reação inflamatória crônica em tilápia do Nilo, *Oreochromis niloticus*, avaliando a atividade anti-inflamatória do celecoxibe sobre a cinética de migração e formação de gigantócitos na resposta de corpo estranho. Para tanto, foram utilizadas 72 tilápias, colocadas em 9 aquários (n=8), com capacidade de 100 L de água cada, constituíndo os seguintes tratamentos: Controle - submetidas ao estímulo inflamatório sem tratamento com Celecoxibe; TC - Tratamento convencional com administração oral de 3mg/kg de Celecoxibe após estímulo inflamatório; TP - Tratamento prolongado com administração oral de 3mg/kg de Celecoxibe por uma semana antes e continuamente após o estímulo inflamatório. Foram avaliados 8 animais por tratamento em três períodos, ou seja: 2, 4 e 8 dias pós-implantação (DPI) das lamínulas, sendo coletadas amostras de sangue para realização da contagem diferencial de leucócitos em extensões sanguíneas,

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bem como coleta das lamínulas para estudo citológico. Na fase tardia do processo inflamatório (8DPI), observou-se diminuição do acúmulo celular de macrófagos e formação de policariontes nas lamínulas de tilápias tratadas com celecoxibe, demonstrando o efeito anti-inflamatório deste inibidor de COX-2, associado à diminuição significativa do número de monócitos circulantes nos animais que receberam tratamento convencional. As tilápias tratadas com celecoxibe (TC e TP) apresentaram diminuição significativa da contagem total de leucócitos na fase inicial do processo inflamatório (2DPI), caracterizada por neutropenia e linfopenia. Portanto, ambos os protocolos de tratamento (TC e TP) com 3mg/kg de celecoxibe modularam a resposta inflamatória do tipo corpo estranho da tilápia atuando na cinética de acúmulo de macrófagos e formação de policariontes em lamínulas de vidro implantadas no tecido subcutâneo, demonstrando correlação com a dinâmica dos leucócitos circulantes. Esses resultados ajudam a entender o papel dos prostanóides durante a reação inflamatória crônica da tilápia.

PALAVRAS-CHAVE: Antagonismo de Ciclo-oxigenase. Prostanóides. Inflamação. Ciclídeos. Peixe teleósteo. *Oreochromis niloticus*

INTRODUCTION

Inflammation is the reaction of living tissue to local aggression, occurring as a non-specific response, characterized by changes that tend to limit harmful effects to the organism, becoming a modulated process in the sense that its amplitude varies depending on the intensity of the stimulus. This phenomenon is an essential part of the individual's natural defenses (innate and adaptive immunity), in addition to showing effective participation in tissue repair (Belo et al., 2021).

In aquaculture, pathophysiological studies of the foreign body-type inflammatory reaction in fish have become quite important, as they contribute to the development of therapeutic and vaccine strategies for slow release (Charlie-Silva et al., 2020; Conde et al., 2022). The implantation of glass coverslips in the subcutaneous tissue of fish represents a classic example for evaluating chronic inflammation, resulting in the accumulation of macrophages and the formation of giant cells (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 in the face of pathological processes (Belo et al., 2012). The significance of giant cells in chronic inflammatory lesions is not completely understood. The occurrence of giant cell formation in chronic inflammatory processes in fish has been described in several infectious diseases, such as that caused by *Mycobacterium marinum* in *Piaractus mesopotamicus* (Manrique et al., 2015), *Sphaerospora renicola* in *Cyprinus carpio* (Holzer et al., 2014), *Streptococcus iniae* in reef fish in the Caribbean (Keirstead et al., 2014) and *Nocardia* spp. in *Argyrosomus regius Asso* (Elkesh et al., 2013).

Celecoxib is a non-steroidal anti-inflammatory agent (NSAID), which prevents prostaglandin synthesis by inhibiting cyclooxygenase 2 (COX-2). Most NSAIDs inhibit both forms of cyclooxygenase (COX -1 and COX-2), but celecoxib is a selective inhibitor of COX-2. It is worth noting that both COX-1 and COX-2 are involved in the conversion of arachidonic acid into prostaglandin H2, a precursor of prostaglandin and thromboxane (Cohen and Preuss, 2020). Prostaglandins are important mediators of pain and inflammation, while thromboxanes have vasoconstrictive, hypertensive and platelet activating/aggregating action (McCormack, 2011). These and other secondary anti-inflammatory mechanisms of COX-2 antagonists have been studied in human medicine, but little is known about blocking this receptor and its effects in teleost fish.

Based on the importance of the chronic inflammatory response associated with the lack of information on the use of COX-2 inhibitors, the objective was to study and identify important aspects involved in the pathophysiology of the chronic inflammatory reaction in Nile tilapia, *Oreochromis niloticus*, through the evaluation of anti-inflammatory activity of celecoxib on the kinetics of migration and formation of giant celld in the foreign body response and on the leukocyte profile of tilapia.

MATERIAL AND MÉTHODS

Fish and packaging

For this study, 72 male Tilapia, belonging to the same spawning farm at the Aquabel Farm (Porto Ferreira, State of São Paulo, Brazil) were randomly distributed in 9 aquariums (100 L of water, n=8) supplied with chlorine-free running water from artesian well with recirculation system with a flow rate of 1L/min. After transport to the aquarium, the fish were acclimatized for 15 days, the time necessary for plasma cortisol concentration and osmolarity to return to basal levels. In the first three days of acclimatization, the animals were subjected to a bath with a NaCl solution at a concentration of 6.0g/L (Carneiro and Urbinati, 2001). The fish were fed 2% of the aquarium's biomass with commercial food (LAGUNA® - Social Company). Water quality parameters were determined twice daily throughout the experimental period using a YSI-63 pH meter and a YSI-55 oximeter, and their values remained within the range appropriate for the welfare 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. This research was approved by the Animal Use Ethics Committee of the University of Brazil (UB), process number 0015/6.

Experimental design

Tilapia were randomly distributed in 9 aquariums (100 L of water, n = 8) to compose the following treatments: Control - subjected to inflammatory stimulus without treatment with Celecoxib; TC - Conventional treatment with oral administration of 3mg/kg of Celecoxib after inflammatory stimulus; TP - Prolonged treatment with

oral administration of 3mg/kg of Celecoxib for one week before and continuously after the inflammatory stimulus. Eight animals were evaluated per treatment in three periods: 2, 4, and 8 days post-implantation (DPI) of the glass coverslip in the subcutaneous tissue.

Treatment with anti-inflammatory Celecoxib

Tilapia were fed with commercial feed (LAGUNA® - Social Company, containing 32% crude protein, 7% ether 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's biomass. Celecoxib (Celebra®, Pfizer Pharmaceuticals LLC, Caguas – Puerto Rico) at a dose of 3mg/kg of body weight was added to the diets of TC and TP treatments. To prepare the diets, the commercial feed was weighed in proportion to the average weight of the tilapia in each tank and added 2% vegetable oil plus the respective amount of celecoxib (3mg/kg) and stored in dark plastic bags, kept at 8° C, until the moment of use. The diet of control animals without Celecoxib was mixed with 2% vegetable oil to maintain the standardization of nutritional balance.

Fish anesthesia

Tilapia were anesthetized by immersion in an aqueous solution of benzocaine at a ratio of 1:10.000 for implantation of glass coverslips in the subcutaneous tissue and 1:500 at the time of euthanasia. Benzocaine was diluted in 98° alcohol (0.1g/mL), bringing the volume to 1L (Wedemeyer, 1970). After experimental management, the animals were placed back in the aquariums with continuous water flow and aeration.

Coverslip implantation and induction of the inflammatory process

Before implantation, the glass coverslips and nylon thread for sutures were sterilized in an autoclave. The 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 implantation of round glass coverslips in the subcutaneous tissue, caudal lateral-dorsal region the operculum, with the left side being standardized for implantation (Sakabe et al., 2013). Using a scalpel, the scales in the implant area were removed and an incision was made (Figure 1A). The subcutaneous tissue was dissected and the coverslip was implanted between the skin and muscle tissue (Figure 1B). Then, the skin was sutured with simple stitches using nylon thread (Figure 1C). After experimental management, the animals were placed again in their respective aquariums with a continuous flow of water.

Figure 1 - Coverslip implantation surgery. 1A. Incision and disclosure of the subcutaneous tissue; 1B. Coverslip implantation and 1C. Single stitch suture.

Assessment of the inflammatory response

Two, four and eight days after coverslip implantation, the fish were euthanized by immersion in benzocaine diluted in water (1:500). The coverslips were carefully removed and washed with 0.9% saline solution to remove fibrin and other cells adhering to the coverslip other than macrophages and giant cells. They were then fixed in Bouin's solution for approximately 5 minutes and stained with hematoxylin-eosin. The polykaryotic cells formed were counted "in total", as well as the number of nuclei per giant cell, the number of foreign body-type and Langerhans-type giant cells to establish the correlation between them (Figure 2). These counts were carried out under an optical microscope at 400x magnification, with 5 fields per animal being counted, totaling 35 fields per treatment, according to the experimental protocol described by Belo et al. (2005).

WBC Analysis

Eight fish per treatment (one aquarium for each treatment) were anesthetized to obtain two mL of blood samples through the caudal vessel at 2, 4, and 8 days post-implantation (DPI), which were aliquoted into two sets: one using heparin- coated needle and syringe (5000 IU) and another without anticoagulant to obtain plasma and serum samples, respectively. The blood count was performed using a hemocytometer (Neubauer chamber) and Natt and Herrick (1952) solution (ratio 1:100 v:v). The differential leukocyte count was performed on blood extensions with a count of 200 cells, establishing the percentage of each cell type of interest, after previously staining the extensions with May-Grünwald-Giensa-Wrigth (Belo et al., 2013).

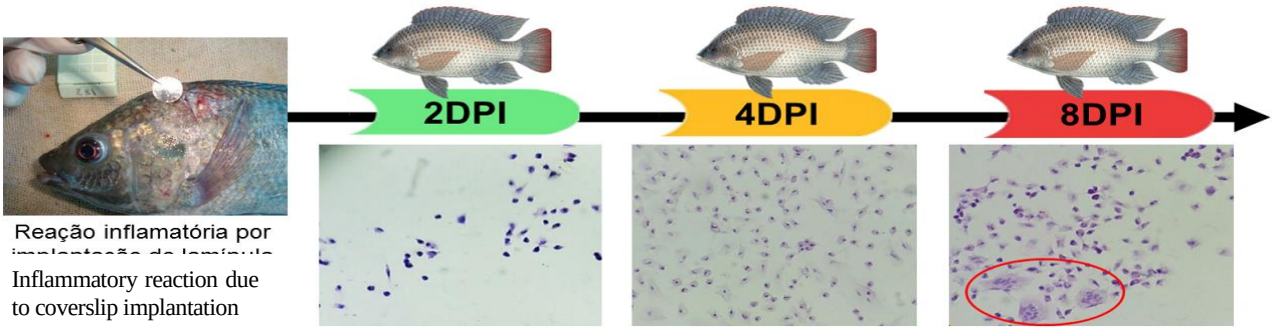

Fig of macrophages, giant cells and multinucleated Langerhans cells observed under a 400x optical microscope and stained with Hematoxylin and Eosin.

Statistical analysis

The experimental design to evaluate the inflammatory response caused by the implantation of coverslips in the subcutaneous tissue of tilapia was completely randomized in a 3x3 factorial scheme (three treatments: TP, TC and control X three evaluation periods: 2, 4 and 8 DPI). The 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 the Tukey test with a 95% confidence level.

RESULTS

Analysis of the inflammatory stimulus

In the initial phase of the inflammatory reaction, the formation of polykaryotic cells was low in all treatments, with no significant variations ($p>0.05$) occurring between treatments. The study of cell accumulation in coverslips showed that tilapia treated with Celecoxib from both therapeutic protocols (TC and TP) showed a significant ($p<0.05$) decrease in polykaryotes at 8DPI compared to fish from the control group (Figure 3). The conventional treatment (CT) animals had a significant increase ($p <0.05$) in their polykaryotes with few nuclei (2 nuclei) values at 4DPI when compared to the control and TP groups.

The study of the evolution of the inflammatory reaction over time revealed that control fish showed a significant increase ($p<0.05$) in the count of macrophages and polykaryotic cells between the second and eighth day (Figure 3), whereas in animals treated with Celecoxib (TC and TP), it was observed in the evolution of the inflammatory process a significant decrease in its values in the count of macrophages and polykaryotic cells with few nuclei between the second and eighth day, except fish from the TC treatment that showed a significant increase in polykaryotic cells with 2 nuclei 4DPI when compared to control fish and TP treatment.

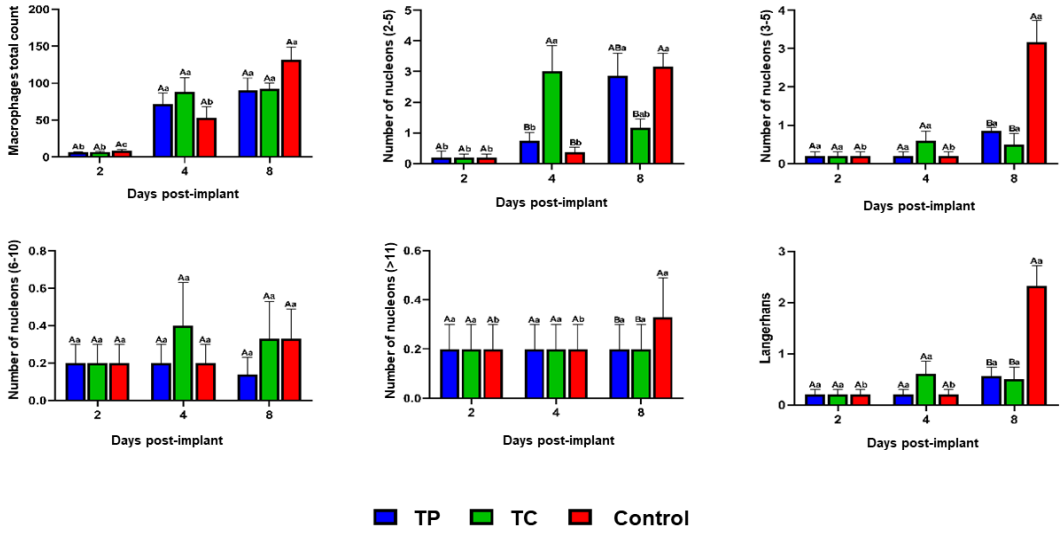

Figure 3 - Mean values 1 (±SD) and ANOVA 2 of the count of inflammatory cells present on glass coverslips during a foreign body reaction in tilapia. 1 Means (n = 7) followed by the same letter do not differ from each other for Tukey ($p < 0.05$). 2 Statistical analysis of variance represented by capital letters compares the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between different experimental days.

Hematological analysis

The results observed in the white blood cell count of tilapia during foreign body inflammation showed a significant increase ($p<0.05$) in the total number of leukocytes characterized by an increase in neutrophil and lymphocyte counts in control tilapia. Furthermore, in the late phase of inflammation (8DPI), a decrease in monocytes was observed in animals in the TC group compared to TP (Figure 4). Fish from prolonged treatment (PT) with Celecoxib showed an increase in erythrocyte count compared to conventional treatment (CT) 4DPI (Figure 4). Tilapia from the TC group showed an increase in neutrophil and thrombocyte counts, as well as a decrease in monocytes 8DPI when compared to fish from prolonged treatment (TP (Figure 4).

Over time, a significant increase ($p<0.05$) in circulating erythrocytes, total number of leukocytes and lymphocytes was observed in animals in the 4DPI control group, however, with the evolution of the foreign body-type chronic inflammatory process, there was a decrease in 8DPI counts (Figure 4). Animals subjected to conventional treatment (CT) with Celecoxib achieved an increase in erythrocytes, neutrophils and thrombocytes over time, with their peak at 8DPI (Figure 4). It was observed in the evolution of the inflammatory reaction over time that the animals in the TP group showed a significant increase ($P>0.05$) in the number of monocytes at 8DPI (Figure 4).

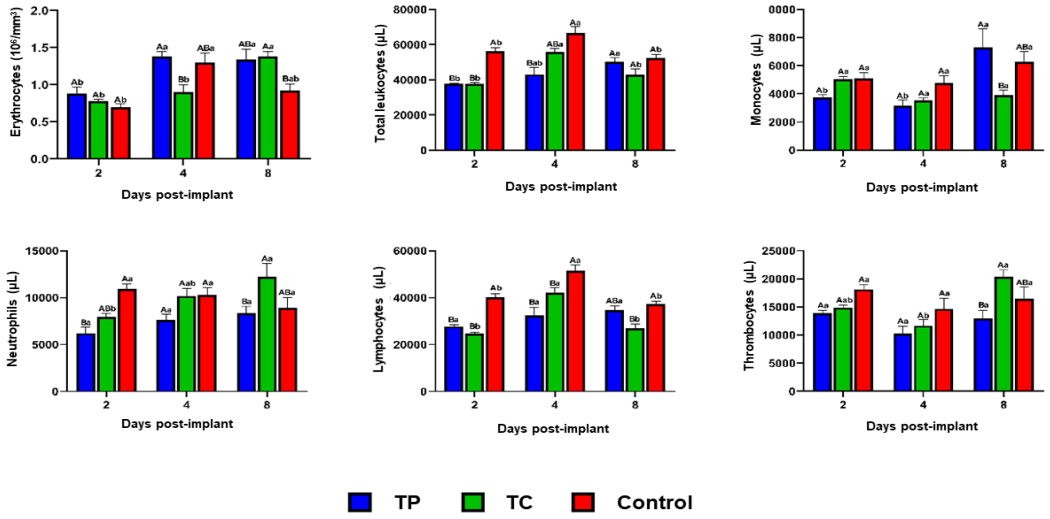

Figure 4 - Mean values 1 (±SD) and ANOVA 2 of leukocyte count in tilapia during foreign body inflammation. 1 Means (n = 7) followed by the same letter do not differ from each other for Tukey ($p < 0.05$). 2 Statistical analysis of variance represented by capital letters compares the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between different experimental days.

DISCUSSION

The classic model of implanting glass coverslips in the subcutaneous tissue of fish results in the accumulation of macrophages and the formation of giant cells on the coverslips, allowing the evaluation and quantification of their participation during the foreign body type reaction (Petric et al., 2003 a, b). In this study, the kinetics of macrophage accumulation was clearly observed, being low in the initial phase of inflammation (2DPI) and increasing throughout the study, as well as in the final phase, a significant increase in the formation of polykaryotic cells was observed, including with a high number of nuclei. , corroborating the observations described by Sakabe et al. (2013) who studied the participation of omega 3 and 6 fatty acids in the foreign body response of tilapia, these authors described the same evolution in the inflammatory reaction of these cichlids.

In the later phase of the inflammatory process (8DPI), a decrease in the cellular accumulation of macrophages and the formation of polykaryotic cells was observed on the coverslips of tilapia treated with celecoxib, demonstrating the anti-inflammatory effect of this COX-2 inhibitor. Such a change was also observed by Belo et al. (2005 and 2012) who found a similar effect in reducing the accumulation of macrophages and formation of giant cells in pacus with high levels of cortisol, resulting from the glucocorticoid effect of this steroid. The significance of giant cells in chronic inflammatory lesions is not completely understood. They are formed by the fusion of macrophage membranes, being dependent on the recruitment of monocytes from the blood, which in turn are required for reserve compartments, and on the rate of granuloma renewal (Ryan and Spector, 1970). Birman and Mariano (1985) demonstrated that the formation of giant cells depends on the nature of the inflammatory process, corroborating the idea of a rate of macrophage renewal within the lesion.

The decrease in the accumulation of macrophages and formation of giant cells observed in tilapia treated with celecoxib suggests the hypothesis that this COX-2 blocker has decreased the production of prostanoids, important mediators of inflammation as they act as potent vasodilatory agents and potentiate the mechanism of

increased vascular permeability (Suleyman et al., 2004). Therefore, the anti-inflammatory action of celecoxib would have resulted in a decrease in diapedesis and cell accumulation in tilapia. Furthermore, selective COX-2 inhibitor can reduce leukocyte recruitment, an effect observed in several experimental models of inflammation (Menezes et al., 2008). This fact may have possibly also contributed to the reduction in tilapia diapedesis, by acting on chemotactic mechanisms. A similar effect was observed by Charlie-Silva et al, (2020) who studied the modulating activity of cyclophosphamide on the chronic foreign body-type inflammatory reaction in tilapia, resulting in the suppression of the cellular response in the inflamed focus, since fish treated with this agent alkylating agent showed a decrease in the accumulation of macrophages and the formation of giant cells on the glass coverslip. For Luster et al. (2005) controlling this cell migration would be an effective strategy for the treatment of chronic inflammatory diseases.

The results observed in the white blood cells of tilapia during foreign body-type inflammation revealed a significant decrease in the total number of leukocytes characterized by neutropenia and lymphopenia in tilapia treated with Celecoxib (TC and TP) in the initial phase of the inflammatory process when compared to animals in the control group. These results corroborate the findings of Feng et al., (2008) in humans with osteoarthritis treated with Rofecoxib, who showed a decrease in the number of lymphocytes and neutrophils. Charlie-Silva et al., (2020) observed a decrease in the number of blood leukocytes in tilapia treated with cyclophosphamide during a foreign body-type inflammatory reaction. Similar leukocyte changes were observed in pacus during chronic inflammation, but these characids showed more prominent effects when fed diets lacking in vitamin E (Belo et al., 2014).

According to Belo et al. (2005), the macrophages present in the inflamed focus and fixed on the coverslips are derived from circulating monocytes after diapedesis. Tilapia subjected to conventional treatment showed a significant decrease in the number of circulating monocytes, associated with a decrease mainly in the formation of giant cells on the coverslips in the later phase (8DPI). These results demonstrate the anti-inflammatory effect of celecoxib on chronic inflammation in tilapia. It is worth noting that the conventional treatment protocol with this COX-2 inhibitor resulted in an initial increase in the kinetics of cell accumulation on the coverslip (4DPI), but without correlation with the values of circulating monocytes. Meanwhile, fish subjected to prolonged treatment showed a significant increase in monocyte counts, at the expense of a significant reduction in circulating thrombocytes (8DPI). Little is known about the role of thrombocytes in chronic foreign body inflammation, but according to Tavares-Dias et al. (2008), thrombocytes from teleost fish not only play a relevant role in hemostatic mechanisms but also effectively participate in defense responses. Conde et al. (2022) studied the vaccine response of biopolymers implanted in the subcutaneous tissue of tilapia and found a significant increase in circulating thrombocyte values over the six months studied. For these authors, fibroblasts and thrombocytes participate in the synthesis of granulation tissue that will support the formation of the capsule around the foreign body. Thrombocytes express MHCI and MHCII, being able to process intracellular antigens and present them to effector cells, in addition to producing immunoregulatory cytokines and chemokines, such as interleukin 1β (Köllner et al., 2004; Jaros et al., 2013; Obirikorang et al., 2019). High thrombocyte counts have been described in the acute phase of teleost fish exudate by different types of inflammatory stimuli (Moraes et al., 2018; Prado et al. 2018; Rodrigues-Soares et al., 2018; Charlie-Silva et al., 2019; Aracati et al., 2022).

Animals submitted to prolonged treatment (PT) with 4DPI and animals undergoing conventional treatment (CT) with 8DPI showed an increase in erythrocyte counts. The increase in circulating erythrocyte values may be the result of changes in the liquid electrolyte balance of fish, which would result in hemoconcentration, as cardiovascular and renal changes associated with the use of celecoxib have been reported in mammals (Whelton et a., 2000; Ahmad et al., 2002; Drożdżal et al., 2021). The polycythemia observed in tilapia treated with celecoxib corroborates the findings of Aracati et al. (2021) and Oliveira et al. (2021) who described similar effects in tilapia treated with zafirlukast, which acts as a Cys-leukotriene blocker, during the acute and chronic inflammatory reaction, respectively. Xu et al. (2015) reported a similar effect with a significant increase in erythrocyte counts in cachectic mice treated with celecoxib.

CONCLUSIONS

Both treatment protocols (TC and TP) with 3mg/kg of celecoxib modulated the foreign body-type inflammatory response of tilapia by acting on the kinetics of macrophage accumulation and formation of polykaryotes on glass coverslips implanted in the subcutaneous tissue, demonstrating a correlation with the dynamics of circulating leukocytes and helping to understand the role of prostanoids during the chronic inflammatory reaction of tilapia.

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