CLINICAL SAFETY OF TREATMENT WITH ZILEUTON, 5-LOX INHIBITOR, DURING ACUTE INFLAMMATORY REACTION IN NILE TILAPIA *(Oreochromis niloticus)*[1]

(SEGURANÇA CLÍNICA DO TRATAMENTO COM ZILEUTON, INIBIDOR 5-LOX, DURANTE REAÇÃO INFLAMATÓRIA AGUDA EM TILÁPIA DO NILO (Oreochromis niloticus))

SUMMARY

Zileuton remains the only 5-LOX inhibitor clinically used in human medicine. Therefore, the objective of this study was to evaluate the clinical safety of treatments with 2,25 mg and 4,50 mg of zileuton/Kg^{-1} (bodyweight), administered orally in the diet, through biochemical and hematological analysis during the acute inflammatory reaction in Nile tilapia (*Oreochromis niloticus*), induced by *Aeromonas hydrophila* bacterins. The study used eighty tilapias, conditioned in 20 tanks (n=4), constituting the following treatments: T0 (control), T1 (2,25 mg zileuton) and T2 (4,50 mg zileuton), being sampled eight animals per treatment in three periods: 6, 24 and 48 hours post-inoculation, and a 10th group consisting of fish without any type of stimulus to obtain the reference values. In order to evaluate and determine the blood count and serum biochemical, it was necessary to collect blood samples. The hematology results of the tilapia treated with zileuton did not reveal alterations between tilapia subjected to different treatments and control fish (T0). The liver cytotoxicity analysis of tilapias treated with zileuton did not reveal significant ($p \geq 0,05$) alterations in AST and ALT serum enzymatic activity. The study of tilapia blood total protein showed decrease in the T1 group at 48 HPI. As the treatment time progressed, the results indicated decrease in the serum albumin levels for T2 group at 24 HPI. The determination of serum biochemichal of creatinine, cholesterol, triglycerides, and glucose did not differ statistically between treatments. The results observed in the hematological and biochemical analyzes allows to conclude that zileuton administered orally, at doses of 2,25 and 4,50 mg/Kg^{-1} (body weight) demonstrated to be clinically safe.

KEY-WORDS: Cichlids. Acute Inflammation. Neutrophils, Lipoxygenase.

RESUMO

O zileuton continua a ser o único inibidor da 5-LOX clinicamente aprovado e utilizado na medicina humana. Assim, objetivou-se avaliar a segurança clínica dos tratamentos com 2,25 e 4,50 mg de zileuton/Kg^{-1} p.v., administrado via oral na dieta, através de análises bioquímicas e hematológicas durante reação inflamatória aguda em tilápia do Nilo (*Oreochromis niloticus*), induzida por bacterinas de *Aeromonas hydrophila*. Foram utilizadas 80 tilápias, acondicionadas em 20 tanques, constituindo os seguintes tratamentos: T0 (controle), T1 (2,25 mg zileuton) e T2 (4,50 mg zileuton), sendo amostrados oito animais por tratamento em três períodos: 6, 24 e 48 horas pós-inoculação, e um 10° grupo constituído por peixes sem nenhum tipo de estímulo para obtenção dos valores de referência. Foram coletadas amostras de sangue para determinação e avaliação do hemograma e do bioquímico sérico. A avaliação hematológica das tilápias tratadas com zileuton não revelou alterações entre os peixes submetidos aos diferentes tratamentos e grupo controle. A análise de citotoxicidade hepática das tilápias tratadas com zileuton, não apresentaram alterações significativas na atividade sérica enzimática de AST e ALT. O estudo da proteína total no sangue das tilápias mostrou diminuição no grupo T1 em 48 HPI. Na evolução do tratamento ao longo do tempo, verificou-se diminuição nos níveis séricos de albumina 24 HPI no grupo T2. A determinação de bioquímica sérica de creatinina, colesterol, triglicerídeos e glicose não apresentaram diferença estatísticas entre os tratamentos. Os resultados observados nas análises hematológicas e no perfil bioquímico do sangue, permite concluir que o zileuton administrado por via oral, nas doses de 2,25 e 4,50 mg/Kg^{-1} p.v. é seguro clinicamente.

PALAVRAS-CHAVE: Ciclídios. Inflamação Aguda. Neutrófilos. Lipoxigenase.

INTRODUCTION

According to the FAO report “State of World Fisheries and Aquaculture” (SOFIA), it is estimated that total fish production is expected to increase to 204 million tons in 2030, a 15% increase compared to 2018, with the participation of aquaculture growing from the current 46% (FA0, 2020).

Tilapia progressively consolidates as the most cultivated species in Brazil, reaching the position of fourth largest producer in the world. In 2021, 534.005 tons were produced in the country, representing an increase of 9.8% over the previous year's performance (486,155 t). With this result, tilapia participated with 63.5% of the national production of farmed fish and the species is present in all regions of the country (Peixe BR, 2022). Compared to other animal species, teleost fish have several advantages and can replace experimental models using rodents. It can provide additional information when used as a model for research into new drugs and vaccines (ARACATI et al., 2021; CHARLIE-SILVA et al., 2020).

Etiological agents that apparently cause little damage to fish populations in their natural habitat are capable of becoming precursor agents of diseases of great economic relevance when subjected to rearing conditions (BELO et al., 2013). Thus, poor sanitary management favors the emergence of diseases caused by opportunistic aquatic microorganisms, such as *Aeromonas hydrophila* (MARTENINGHE et al., 2008), an agent responsible for causing ulcerative lesions, gastroenteritis, dissemination to various organs causing septicemia and an systemic inflammatory response in freshwater fish, causing losses in its production and quality (REQUE et al., 2010).

Seeking to minimize tissue damage and restore normal physiological conditions, teleost fish have a variety of innate and adaptive defense mechanisms against invading organisms, which are fundamental for the maintenance of integrity, and constitute controlled and highly coordinated processes (BELO et al., 2005).

In the pathogenesis of inflammation, leukotrienes (LTs) constitute a family of lipid mediators with a fundamental role. These are immunocompetent cells, including mast cells, eosinophils, neutrophils, monocytes, and basophils that are activated via the lipoxygenase, resulting from the release of arachidonic acid from cell membrane phospholipids by phospholipase A2 and donated by 5-lipoxygenase activating protein (FLAP) to 5-lipoxygenase (CAPRA et al., 2015). LTs are involved in the pathogenesis of inflammatory diseases, therefore, LT inhibitors or antagonists represents an important therapeutic advance in the treatment of inflammatory diseases (WOSZCZEK et al., 2010). According to Arts & Kohler (2009), teleost fish have the same leukotriene production, and as reported by these authors, these eicosanoids have a role more neuroendocrine in inflammation, particularly with regard to leukocyte activity, which the lipoxygenase activates these cells.

With the increase in the number of indications for anti-LT therapies, 5-LOX inhibitor drugs become increasingly important (KAKULARAM et al., 2022). Thus, zileuton, a benzothiophene N-hydroxyurea, is the only drug approved and available to inhibit 5-lipoxygenase (5-LOX), acting in inflammatory diseases by suppressing LT biosynthesis (PETERS-GOLDEN & HENDERSON, 2007), being a compound that belongs to the class of inhibitors of the iron-binders type of 5-LOX, that not only blocks the active site of the enzyme, but also has reducing properties (ROSSI et al., 2010). It is currently available for prescription as an anti-asthmatic drug in the US (Zyflo®) (ORAFAIE, 2020).

Zileuton has a high affinity to FLAP, which expression is necessary for the biosynthesis of LTs by 5-LOX. The development of these new LT inhibitor drugs represents an alternative to corticosteroid therapy (ROBINSON et al., 2001). However, this drug exhibits liver toxicity, so its clinical use is limited by the necessity to monitor serum levels of liver enzymes, resulting in a direct toxic effect on liver tissue, not showing a correlation with the inhibitory effect of 5-LOX (STEINHILBER & HOFMANN, 2014). It is known that zileuton is effective in

preventing the formation of TLs and it is used to inhibit the pathophysiological effects of them (TLs) and other 5-lipoxygenase products in animals and humans (CARTER et al., 1991). However, the knowledge about blocking the synthesis of LTs and their effects in teleost fish is little, therefore, this investigation studied experimentally and identified the innocuousness of treatment with zileuton in Nile tilapia (*Oreochromis niloticus*), through biochemical evaluation and hematological analysis.

MATERIAL AND METHODS

Fishes

Eighty tilapia (*O. niloticus*) were used, weighing approximately 30 grams (g), placed in 20 tanks (n=4), with a capacity of 100 liters (L) of water each, supplied with chlorine free running water, coming from an artesian well with a flow of 1 L/min. After placement to the tanks, the fishes were acclimatized during one week, period of time necessary for the plasma cortisol concentration and osmolarity return to baseline levels. Until de 3rd day of acclimatization, NaCl was added at a concentration of 6,0 g/L in each tank, favoring the hydroelectrolytic balance of the fishes (CARNEIRO & URBINATI, 2001). Water quality was determined twice daily (at the feeding time), temperature and dissolved oxygen concentration, measured by the YSI device, model 55, and pH and electrical conductivity by the YSI device, model 63. All experimental procedures were approved by the Animal Ethics Committee of Universidade Brasil (protocol 18-19/028- CEUA).

Experimental Design

Tilapia were randomly distributed in 20 tanks (100L of water, n=4) to constitute the repetitions of the different treatments: T0 (control), T1 (treatment with 2,25 mg/kg^{-1} of alive weight of zileuton) and T2 (treatment with 4,50 mg/kg^{-1} of zileuton alive weight), being

sampled 8 animals (2 tanks) per treatment in three periods, that is: 6, 24 and 48 hours post-inoculation (HPI) of bacterin *A. hydrophila*, and a 10th group consisting of 2 tanks (n=8) of fish without any type of stimulus to obtain the reference values (physiological standard).

Feed Standardization with the Addition of Zileuton

Tilapia were fed twice a day (8am and 5pm), administered 2% of the biomass of the tanks with commercial basal diet (Fri Acqua Growth Tilapias), containing 32% of crude protein. In the diets of animals from treatments T1 and T2 were added 5-LOX inhibitor, zileuton (PubChem CID 60490), which was acquired from Cayman Chemical® (ZYFLO CR®, Laboratory Chiesi, USA), distributed by Interprise USA Corporation, at a dose of 2,25 and 4,50 mg/kg^{-1} alive weight. For the preparation of the diets, the tilapia were individually weighted and an average was calculated for the ration administration. Right after, the commercial ration was weighed in proportion to the average weight per kilogram of tilapia from each tank and 2% of vegetable oil was added plus the respective amounts of zileuton, being kept at -20ºC until the moment of use, as recommended by the drug's manufacturer. Fishes from treatments T1 and T2 were fed with this diet for one week before inoculation of the bacterin.

Anesthesia of Fish

Fish were pre-anesthetized by immersion in a benzocaine aqueous solution in the proportion of 1:100.000, anesthetized at 1:10.000 to inoculate the bacterin in the swim bladder. Initially, benzocaine was diluted in 98° alcohol (0,1 g/mL), completing the volume to 1L (WEDEMEYER, 1970). After the experimental handling of bacterin inoculation, the animals were placed again in the tanks with continuous water flow and aeration.

164 **Obtaining *Aeromonas hydrophila* bacterin and experimental inoculation**

165 Isolates of *A. hydrophila* were provided by LAPOA (Laboratory of Aquatic Organisms
166 Pathology), CAUNESP. The bacterial mass was obtained by centrifugation (4000 rpm, 4ºC,
167 during 20 minutes), after three successive washes with sterile Phosphate-bufferid saline (PBS)
168 solution (pH 7.4) to completely remove the cultivation medium and then it was suspended again
169 in 100 mL of PBS. Bacterin concentration was adjusted to 1.0 x 10^9 cells mL^{-1}. For inactivation
170 0,5% formaldehyde (volume/volume) was added to the bacterial suspension, which remained
171 in constant agitation at ambient temperature, then kept at 40ºC for 24 hours. Appropriate alcohol
172 antisepsis were performed before the procedure and later some scales were removed from each
173 animal to facilitate inoculation of the bacterin. Right after, 0,5 mL of the inoculum was
174 administered into the tilapia swim bladder with sterile material. Eight animals per treatment
175 were evaluated in three periods: 6, 24 and 48 hours after inoculation of the bacterin.

176

177 **Hematological evaluation**

178 Eight fish per treatment (2 tanks for each treatment) were anesthetized to obtain blood
179 samples by puncturing the caudal vessel at 6, 24 and 48 hours post-inoculation (HPI), which
180 were aliquoted into two sets: one using a needle and syringe coated with heparin (5000 IU) and
181 another without anticoagulant to obtain plasma and serum samples, respectively. The counting
182 of red blood cells was performed in a Neubauer chamber, using the solution of Natt and Herrick
183 (1952) with diluent in the proportion of 1:100 (v.v). The determination of hematocrit percentage
184 was realized in a microcentrifuge and the amount of circulating hemoglobin using Drabkin's
185 reagent for reading at a wavelength of 540nm. Mean corpuscular volume (MCV) and mean
186 corpuscular hemoglobin concentration (CHCM) were calculated from hematocrit, hemoglobin
187 and red blood cells (Farias et al., 2016).

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Serum Biochemical Evaluation

Serum aliquots were intended for determination and evaluation of serum biochemical of alkaline phosphatase, cholesterol, triglycerides, creatinine, albumin, total protein, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) through enzymatic and colorimetric determination in a semi-automatic biochemical analyzer. (LabQuest Model – Bioplus®).

Statistical Analysis

The results were analyzed statistically by the factorial scheme 3 X 3 (three treatments with anti-inflammatory and three different times), "Split Plot Design", using the GLM (General Linear Model) procedure of the SAS (Statistical Analysis Software, 2012) program, version 9.3. The analysis of variance of the means was determined by the Tukey test ($P<0,05$), according to Snedecor & Cochran (1984).

RESULTS

Hematological Analysis

The hematological evaluation of the tilapia treated with zileuton and inoculated with *A. hydrophila* did not reveal significant changes ($P>0,05$) between the fishes submitted to the different treatments and the control group in the percentage of hematocrit, in the values of erythrocytes and circulating hemoglobin, as well as in the values of VCM and CHCM (Figure 1).

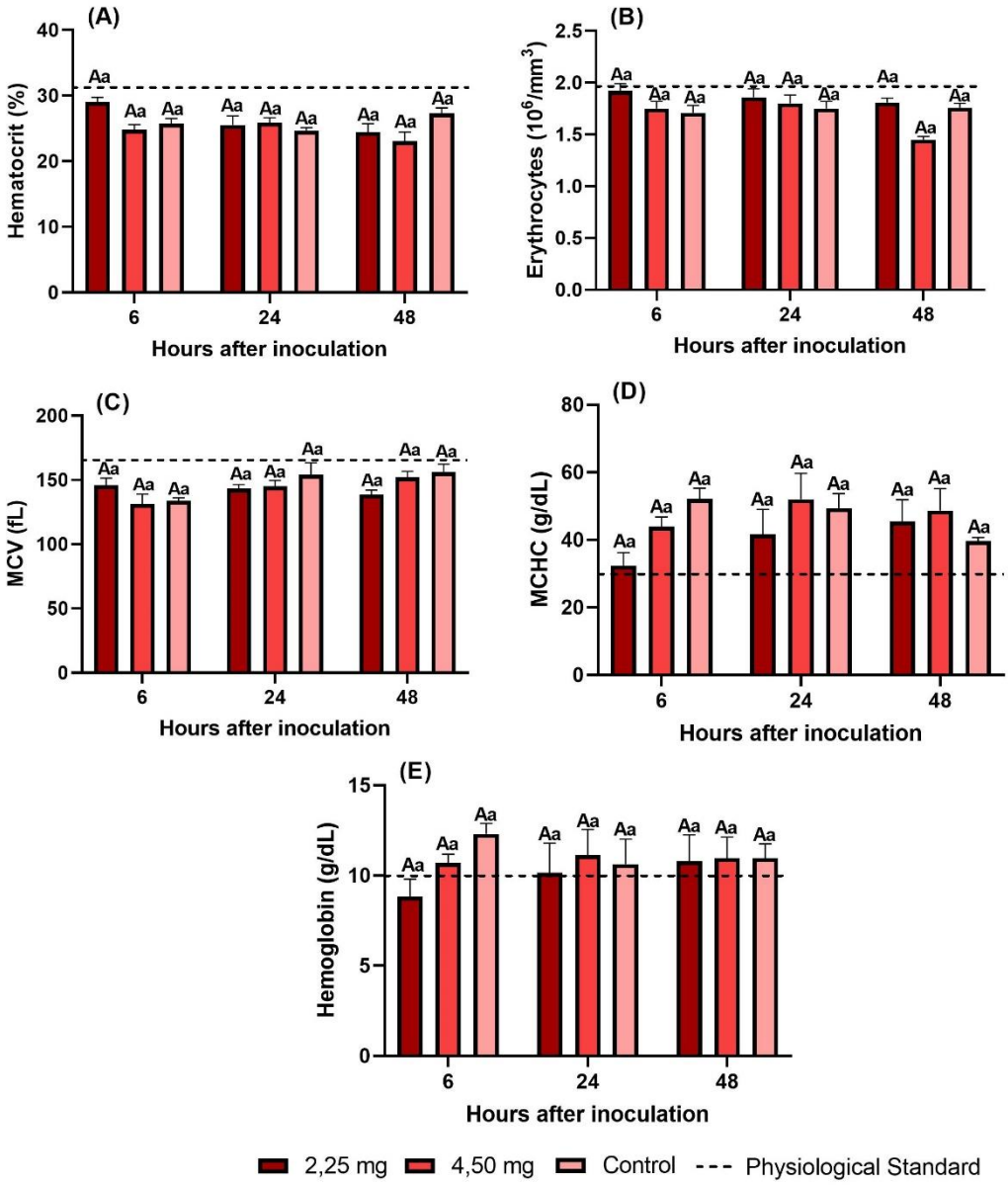

Figure 1. Erythrocyte parameters (Mean ± Pattern error) of Nile tilapia (*O. niloticus*) treated with zileuton 2,25 mg and 4,50 mg/kg^{-1} of alive weight, collected 6, 24 and 48 hours after challenge with bacterin *A . hydrophila* in swim bladder. A: Hematocrit; B: Erythrocyte; C: Mean Corpuscular Volume (MCV); D: Mean Corpuscular Hemoglobin Concentration (MCHC); E: Hemoglobin. Treated with 2,25 mg of zileuton; Treated with 4,50 mg of zileuton; The control was inoculated and untreated. Capital letters compare groups at the determined time. Lowercase letters compare the same group over the experimental period.

Serum Biochemical Analysis

The hepatic cytotoxicity analysis of tilapia treated with zileuton did not suggest significant alterations in the serum enzymatic activity of AST and ALT in fishes submitted to

the different treatments (P>0.05). Serum alkaline phosphatase levels from the 4,50 mg zileuton treatment increased at the onset of aerocystitis (6 HPI) compared to the 2,25 mg treatment (P<0.05). In contrast, the values observed at 24 and 48 HPI were similar to those of the control group and treated with 2,25 mg (Figure 2).

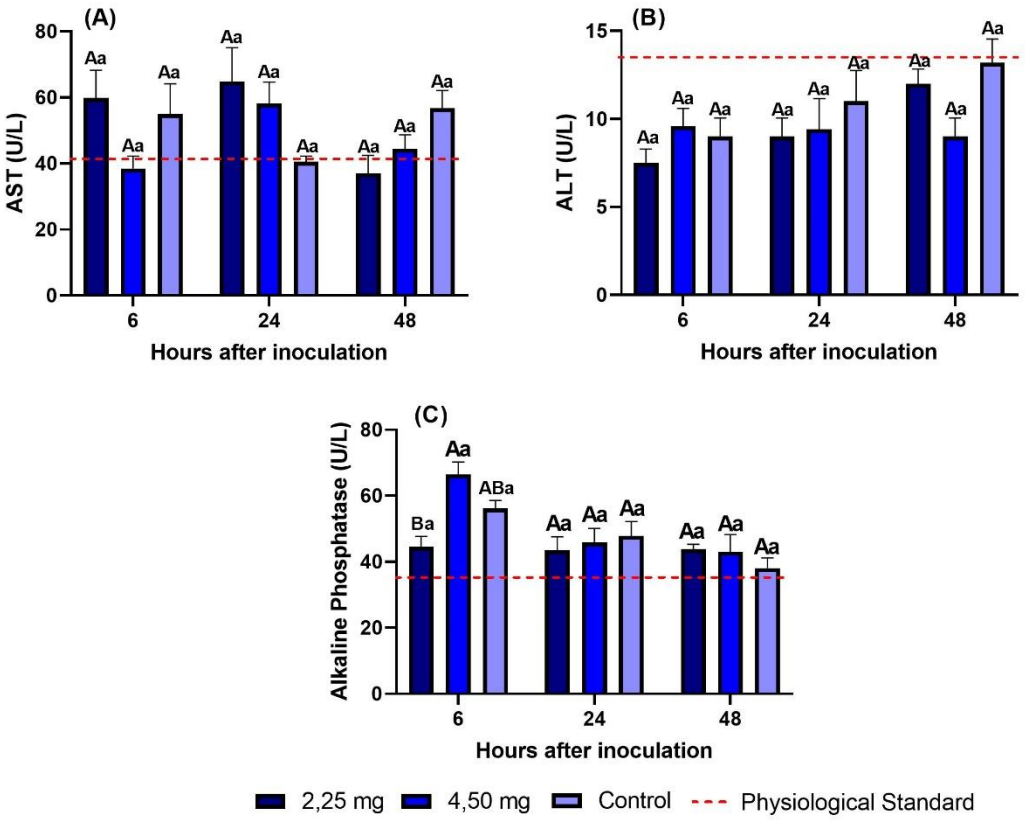

Figure 2. Mean values (± Pattern error) observed in the analysis of serum enzymatic activity of A: Aspartate aminotransferase (AST); B: Alanine aminotransferase (ALT); C: Alkaline phosphatase (AF) in tilapia submitted to different treatments during aerocystitis induced by *A. hydrophila* bacterins. Treated with 2,25 mg of zileuton; Treated with 4,50 mg of zileuton; The control was inoculated and untreated. Capital letters compare groups at the determined time. Lowercase letters compare the same group over the experimental period.

The serum values of creatinine, total protein, cholesterol, triglycerides, albumin and glucose are shown in figure 3. The study of total protein in tilapia's blood showed a significant decrease (P<0.05) in fish treated with 2,25 mg of zileuton 48 HPI, when compared to animals treated with 4,50 mg of zileuton. In the evolution of the treatment over time, it was possible to identify a significant decrease in serum levels of albumin 24 HPI in fish treated with 4,50 mg

of zileuton. The determination of serum biochemistry of creatinine, cholesterol, triglycerides and glucose did not show statistical difference between treatments.

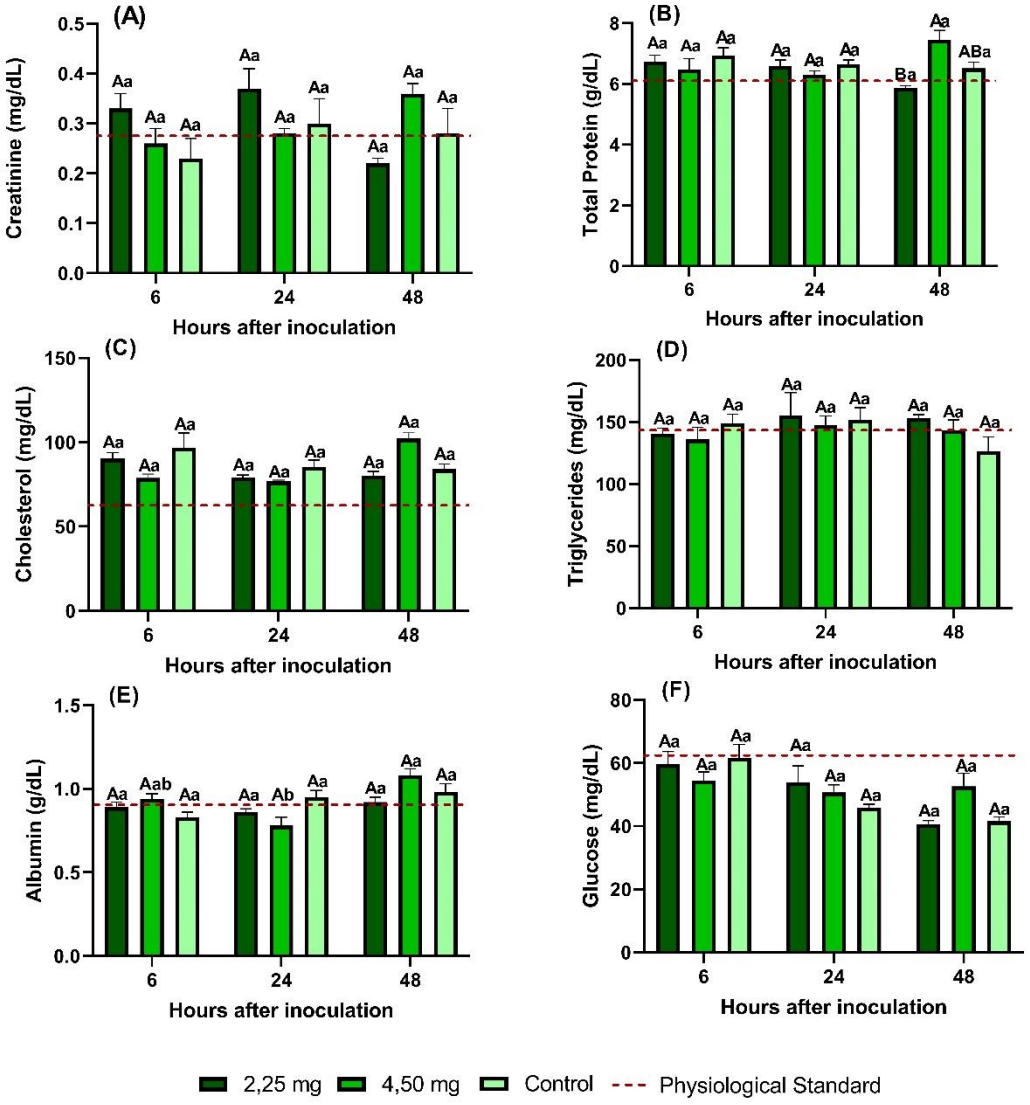

Figure 3. Mean values (± Pattern error) observed in the analysis of serum biochemistry of: A: Creatinine; B: Total Protein; C: Cholesterol; D: Triglycerides; E: Albumin; F: Glucose in tilapia submitted to different treatments during aerocystitis induced by *A. hydrophila* bacterins. Treated with 2,25 mg of zileuton; Treated with 4,50 mg of zileuton; The control was inoculated and untreated. Capital letters compare groups at the determined time. Lowercase letters compare the same group over the experimental period.

DISCUSSION

Blood parameters are important criteria to show physiological changes in fishes and can provide essential information for disease diagnosis and prognosis (FAZIO, 2019). Tilapia treated with zileuton did not show any alteration between the different concentrations of the drug administered, as well as revealed no change over time regarding the number of erythrocytes, hemoglobin concentration, hematocrit percentage, such as MCV and CHCM calculations, demonstrating the clinical safety of this drug. These results are in agreement with Moraes (2017), in which there was no difference between the hematological parameters in a study of the clinical safety of amoxicillin for the treatment of streptococcosis in Nile tilapia.

The biochemical profile of blood helps to predict the physiological disturbances that may occur in organisms due to pathological or chemical stress (BHARTI & RASOOL, 2021). The administration of 2,25 and 4,50 mg of zileuton/Kg^{-1} in the diet did not result in changes in the serum enzymatic activity of ALT, AST, creatinine, cholesterol, triglycerides and glucose, suggesting that zileuton did not cause damage in cytotoxicity and on liver functionality. Such facts corroborate with Aracati et al. (2021) who reported an improvement in the biochemical profile of tilapia supplemented with astaxanthin during *A. hydrophila* infection. Crow et al. (2001) also used oral zileuton at a dose of 2 mg/kg^{-1} in dogs diagnosed with canine atopic dermatitis and did not observe hepatic changes. According to Abdel-Daim et al. (2020) and Selim et al. (2014) severe hepatic changes were related, associated with increase of AST, ALT and FA in Nile tilapia fed with contaminated feed by aflatoxin B1.

The determination of total protein concentration in plasma and its fractions is of great clinical importance, since its plasma concentration is responsible for the colloid osmotic pressure of this body fluid (MELO et al., 2009). Tilapia treated with 2,25 mg of zileuton showed a decrease in total protein in the blood 48 HPI, similar to what was observed by Garcia (2009) in which total protein levels were also reduced in *Piaractus mesopotamicus* after challenge with

A. hydrophila. Literature results reveal that fish affected by both bacteria and parasites presented a reduction in blood protein levels (BOON et al., 1990). Among the factors that lead to a reduction in plasma protein levels, there is a greater demand for this nutrient for the replacement of damaged and injured tissues in inflammatory processes, in which vascular permeability is increased and there is extravasation of protein to the extravascular spaces, with consequent loss of this protein in these places (KANEKO, 1989).

Albumin is the most abundant serum protein produced by the liver (MOSHAGE et al., 1987). Serum albumin levels showed a significant decrease in the treatment with 4,50 mg of zileuton 24 HPI, this result is in agreement with those presented by Charlie-Silva et al. (2019) in which they showed lower plasma concentrations at 6 and 24 HPI by *A. hydrophila* in tilapia. Albumin is considered a negative acute phase protein, in other words, during the acute phase response the serum values of this protein decrease in detriment of the increase of other proteins considered positive, as they undergo an increase in circulating values (GABAY & KUSHNER, 1999). A hypothesis to explain the decrease of those during inflammation would be the metabolic deviation for the synthesis of proteins considered positive.

Thus, tilapia treated with the 5-lipoxygenase inhibitor (5-LOX) did not show changes in hematological parameters and did not significantly alter the circulating values of AST, ALT, creatinine, triglycerides, cholesterol and glucose, demonstrating the clinical safety of the treatment, as it does not compromise the tilapia's hepatic and renal functionality, closely with the non-observance of behavioral changes and clinical signs. Allowing, this way, the conclusion that zileuton administered orally at doses of 2,25 and 4,50 mg/kg $^{-1}$ (alive weight) it is clinically safe.

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