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CLINICAL SAFETY OF TETRACYCLINE TREATMENT IN NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

SEGURANÇA CLÍNICA DO TRATAMENTO COM TETRACICLINA EM TILAPIAS DO NILO (*Oreochromis niloticus*)

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SUMMARY

Antibiotic safety in aquaculture plays a key role in safeguarding animal and human health, reflecting the integrated vision of One Health Policies. This study assessed the clinical safety of orally administered tetracycline, incorporated into feed, in Nile tilapia (*Oreochromis niloticus*). A total of 128 fish from the same spawning were randomized by weight into 16 tanks (with 100 L of water each, n=8), comprising one control (without treatment with tetracycline) and three treatments receiving 100, 200, or 400 mg/kg body weight of tetracycline (with four tanks per treatment). Fish were sampled on days 4, 8, and 12 of treatment, and on day 16, following a four-day recovery period without tetracycline administration. Hematological, biochemical, and somatic parameters were evaluated. No significant changes were observed in hematocrit, hemoglobin, MCV, MCH, MCHC, creatinine, protein, albumin, cholesterol, triglycerides, or alkaline phosphatase. However, fish treated with 200 and 400 mg/kg showed reduced erythrocyte counts and elevated AST levels, indicating dose-dependent effects. Leukocyte profiles and organ indices remained unaffected. Overall, tetracycline was clinically safe at the tested doses, but caution is warranted for higher doses or extended treatments.

KEY-WORDS: Antibacterial. Aquaculture. Toxicity. Animal welfare. One Health. Sustainability

RESUMO

A segurança dos antibióticos na aquicultura desempenha um papel fundamental na proteção da saúde animal e humana, refletindo a visão integrada das Políticas de Saúde Única. Este estudo avaliou a segurança clínica da tetraciclina administrada por via oral, incorporada à ração, em tilápia do Nilo (*Oreochromis niloticus*). Um total de 128 peixes da mesma desova foram randomizados por peso em 16 tanques (com 100 L de água cada, n = 8), compreendendo um controle (sem tratamento com tetraciclina) e três tratamentos recebendo 100, 200 ou 400 mg/kg de peso corporal de tetraciclina (com quatro tanques por tratamento). Os peixes foram amostrados nos dias 4, 8 e 12 de tratamento e no dia 16, após um período de recuperação de quatro dias sem administração de tetraciclina. Parâmetros hematológicos, bioquímicos e somáticos foram avaliados. Não foram observadas alterações significativas no hematócrito, hemoglobina, VCM, HCM, CHCM, creatinina, proteína, albumina, colesterol, triglicerídeos ou fosfatase alcalina. No entanto, peixes tratados com 200 e 400 mg/kg apresentaram contagem reduzida de eritrócitos e níveis elevados de AST, indicando efeitos dose-dependentes. Os perfis leucocitários e os índices de órgãos permaneceram inalterados. No geral, a tetraciclina foi clinicamente segura nas doses testadas, mas recomenda-se cautela em doses mais altas ou tratamentos prolongados.

PALAVRAS-CHAVE: Antibacteriano. Aquicultura. Toxicidade. Bem-estar-animal. Saúde Única. Sustentabilidade.

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INTRODUCTION

Aquaculture has established itself as one of the most important activities for the supply of animal protein in the world. In 2020, global fish production reached approximately 178 million tons, with 88.5% destined for human consumption (FAO, 2022). Nile tilapia (Oreochromis niloticus) stands out in this scenario due to its hardiness, rapid growth and good acceptance in the market, being the main species farmed in Brazil, with more than 550 thousand tons produced in 2022 (Peixe BR, 2023).

With the increase in fish farming, the sector has faced a higher incidence of outbreaks of opportunistic infectious diseases, caused mainly by bacteria such as *Aeromonas hydrophila* and *Streptococcus agalactiae*, resulting in significant economic losses (Farias et al., 2016; 2020). Population density, environmental stress and the limitation of effective vaccines aggravate the scenario, leading to the routine use of antimicrobials as a therapeutic or prophylactic measure (Belo et al., 2012; Hossain et al., 2022; Oliveira et al., 2024).

In response to these outbreaks, the use of antimicrobials in health management has become a common practice. However, the lack of drugs officially registered by the World Organization for Animal Health (OIE) for use in fish contributes to the indiscriminate use of unlicensed substances, without adequate support for safety and efficacy (Oliveira et al., 2024). Therefore, it is essential to carry out studies that evaluate the safety of new molecules in fish, using hematological and biochemical parameters to monitor the health and physiological condition of the animals (Pultz et al., 2024; Araujo et al., 2024).

Tetracyclines, a class of broad-spectrum antibiotics, have been widely used in the treatment of bacterial infections in fish due to their efficacy against several Gram-positive and Gram-negative pathogens (Li et al., 2023). Repeated or inappropriate use of this antibiotic can result in the presence of residues in tissues, induction of bacterial resistance, and potential toxic or immunosuppressive effects in treated animals (Furushita et al., 2003; Fang et al., 2020; Zanuzzo et al., 2022a, 2022b).

Given the scarcity of drugs registered for specific use in fish and the risk associated with the empirical use of antimicrobials, it is essential to conduct clinical studies to evaluate the safety of compounds such as tetracycline in Nile tilapia, using hematological and biochemical parameters as indicators of health status and physiological homeostasis (Mahmoud et al., 2018; Oliveira et al., 2021). Such studies are essential to ensure effective and sustainable health management, ensuring both animal welfare and the quality of the final product and environmental preservation.

MATERIAL AND METHODS

Fish and packaging

For the clinical safety study of tetracycline, 128 Nile tilapia (± 100 g) from the same spawning (Sales Oliveira farm/SP, Brazil) were were randomized by weight into 16 tanks (100 liters of water each, n=8) filled with chlorine-free water. After being transported to the appropriate tanks, the fish were acclimated for 15 days, the time required for plasma cortisol concentration and osmolarity to return to baseline levels. During the first three days of acclimation, the animals were bathed in a NaCl solution at a concentration of 6.0 g/L (Carneiro and Urbinati, 2001). The animals received commercial extruded feed with 36% crude protein (Nutripiscis® - Presence Company), constituting the basal diet. The fish were fed twice a day, at 8:00 a.m. and 4:00 p.m., corresponding to 2% of the tank biomass. Water quality parameters were determined twice a day throughout the experimental period using a YSI-63 pH meter and a Y-55 oximeter, and their values remained within the range suitable for the welfare of tropical fish (Boyd, 1990) (dissolved oxygen = 5.07 ± 0.89 mg L^{-1}; temperature = 28.64 ± 2.05°C; pH = 7.37 ± 0.54; and conductivity = 197.29 ± 97.57µS/cm). The experimental procedures were approved by the Ethics Committee on Animal Use (CEUA) of UNESP/FCAV, under protocol no. 3237/2024.

Experimental design

The fish were randomized by weight into 16 tanks (100 L of water, n = 8) to compose the following treatments: control group (not treated with tetracycline); groups treated with 100, 200 and 400 mg of tetracycline /kg of body weight. Eight animals were sampled per treatment in 4 periods, that is, 4, 8 and 12 days after treatment with antibiotics and a group that was treated for 12 days with the drug and after that was treated only with commercial feed until the 16th day, being called the recovery period. Blood samples were collected for determination of blood count and biochemistry, in addition to organs such as spleen, liver and kidneys (cranial and caudal) for somatic evaluation.

Table 1 - Distribution of tilapia in different treatments.

Treatments	**Days after starting treatment with Tetracycline**			**Recovery period**
	4	**8**	**12**	**16**
*Control (not treated with Tetracycline)	N=8	N=8	N=8	N=8
Treated with 100 mg/kg b.w. of Tetracycline	N=8	N=8	N=8	N=8
Treated with 200 mg/kg b.w. of Tetracycline	N=8	N=8	N=8	N=8
Treated with 400 mg/kg b.w. of Tetracycline	N=8	N=8	N=8	N=8

* Control group, although there was no administration of tetracycline, 1% carboxymethyl cellulose (CMC) was used, homogenized in the feed, in order to standardize the diets and maintain nutritional balance.

Experimental diet

The commercial pelleted feed, containing crude protein 36%, moisture 12%, ether extract 70 g/kg, mineral matter 140 g/kg, crude fiber 50 g/kg, calcium 25 g/kg, phosphorus 8 g/kg and vitamin C 350 mg/kg (Nutripiscis® - Presence Company) was used to compose the experimental diets of tilapia. Feeding will be carried out twice a day (8 am and 5 pm), with administration of 2% of the biomass of the tanks. To prepare the diets, the feed was weighed daily in proportion to the average weight of the tilapia in each tank.

Tetracycline (Tetracycline hydrochloride: Prati Donaduzzi) was then added at doses of 100, 200, and 400 mg/kg bw and homogenized in 1% CMC (carboxymethyl cellulose). To standardize the diets and achieve nutritional balance, 1% CMC was added to the diet of the control group.

Fish anesthesia

Tilapia were anesthetized by immersion in an aqueous solution of benzocaine at a ratio of 1:10,000 for blood collection and 1:500 at the time of euthanasia. Benzocaine was diluted in 98° alcohol (0.1 g/mL), completing the volume to 1 L (Wedemeyer, 1970). Initially, pre-anesthesia was performed, in which the water level of the tanks was lowered to a volume of 10 L by adding 0.1 g of benzocaine already diluted in 98° alcohol. Soon after, each fish was transferred to a container containing 1 L of water with 0.1 g of benzocaine; both procedures were performed under aeration to minimize stress caused by handling. As soon as the operculum stopped moving, the fish was removed and blood collected. Finally, the animal was transferred to another container containing 0.5g of benzocaine diluted in 1L of water for euthanasia.

Blood collection and hematological analysis

Eight fish per treatment (one tank for each treatment) were anesthetized and three mL of blood samples were collected from the caudal vessel of each at 4, 8 and 12 days after treatment (DPT) and during the recovery period (16th day). These samples were aliquoted into two sets: a syringe coated with heparin (5000 IU) and one without anticoagulant, to obtain plasma and serum, respectively. During the exchange of syringes (with and without heparin), the needle was not removed from the vessel to avoid blood loss. The hemogram was performed using a hemocytometer (Neubauer chamber) and Natt and Herrick's solution (1952) in a 1:100 v:v ratio. Hematocrit was determined by the microhematocrit centrifugation technique. And circulating hemoglobin, using Rabkin's reagent for reading at a wavelength of 540 nm and the mean corpuscular volume (MCV) values were obtained by calculating MCV = (HT/HE)*100 and mean corpuscular hemoglobin concentration (MCHC) by calculating MCHC = (HG/HT)*100. Differential leukocyte counts were performed on blood smears with 200 cell counts, establishing the percentage of each cell type of interest, after prior staining of the smears with May-Grünwald Giensa Wright (Belo et al., 2013).

Serum biochemical evaluation

Blood samples from fish without anticoagulant were centrifuged at 10,000 rpm for 10 minutes to obtain serum and determine alkaline phosphatase (AP), aspartate aminotransferase (AST), creatinine, albumin, total protein, cholesterol and triglycerides, using a semi-automatic biochemical analyzer (Model LabQuest® - Bioplus Company) and the fish's blood glucose was determined using the Accu-Chek Performa device.

Morphometric evaluation of organs

After 4, 8 and 12 days of treatment and recovery period (16th day), the tilapia were euthanized by immersion in an aqueous solution of benzocaine (1:500) until the anesthetic plane deepened and complete loss of opercular movements. They were then weighed and dissected by a longitudinal ventral cut, from the anus to the operculum; another from the anus to the head following the lateral line and a third passing through the pectoral fin. This dissection allowed a wide view of all organs. For morphometric evaluation according to Weibel et al. (1969), the liver, spleen and kidneys (caudal and cranial) of the tilapia were collected, which were subsequently weighed to express the hepatic, renal and splenic somatic index, calculated by the formula: Somatic index = organ weight *X* 100/body weight.

Statistical analysis

The experimental design for clinical safety assessment was completely randomized in a 4 x 4 factorial scheme (four treatments: 100, 200, 400 and control X four evaluation periods: 4, 8 and 12 DPT and 16th - recovery period). Analyses 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 at the 95% confidence level.

RESULTS

Hematological analysis

In the hematological evaluation of tilapia treated with tetracycline (Figure 1), no significant changes ($P>0.05$) were observed in hematocrit, MCV, MCH, MCHC and hemoglobin values. However, over time, it was observed that animals treated with 200 and 400 mg presented a decrease in the levels of circulating erythrocytes.

Leukogram analysis

In the leukocyte evaluation (Figure 2), there were no significant changes ($P \geq 0.05$) in the overall leukocyte count between the different treatments. However, over time there was a decrease in monocytes in animals treated with 400 mg and in lymphocytes in the treatment with 200 mg, but they returned to normal levels after 16 days.

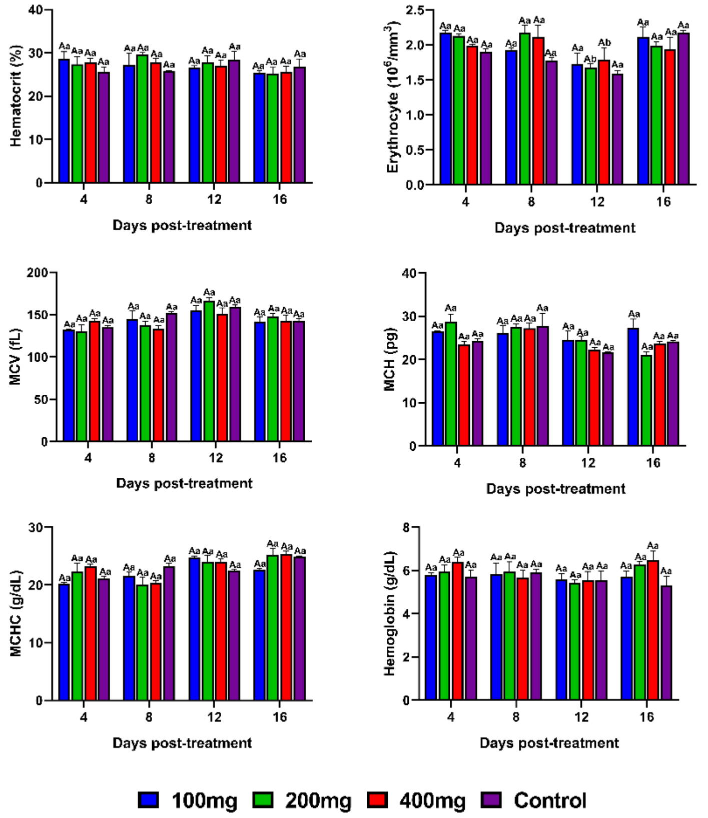

Figure 1 - Hematological analysis of tilapia treated with tetracycline. Means (n = 8) followed by the same letter do not differ by Tukey's test ($P < 0.05$). Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between the different experimental days.

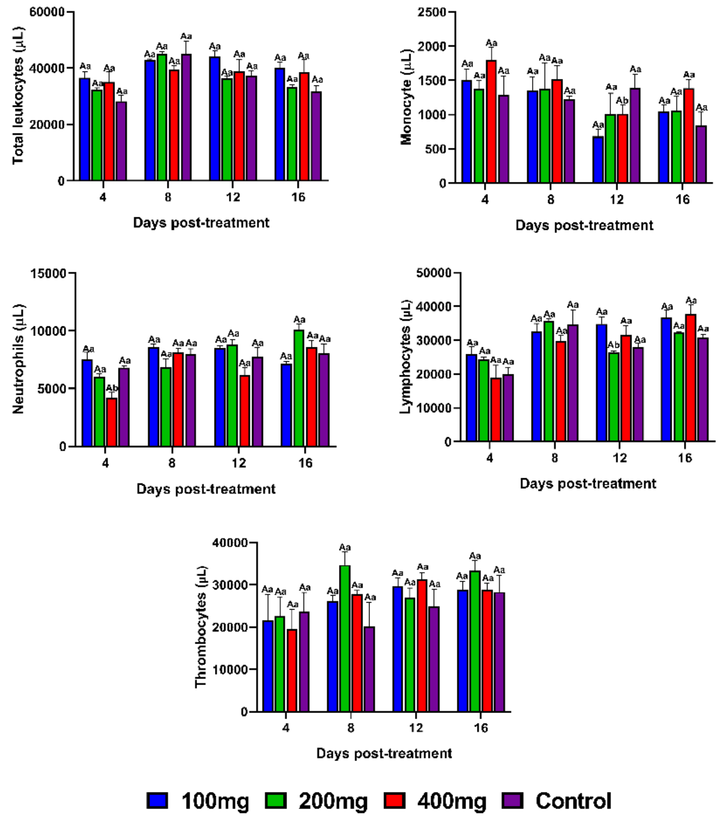

Figure 2 - Analysis of leukocytes from tilapia treated with tetracycline. Means (n = 8) followed by the same letter do not differ by Tukey's test ($P < 0.05$). Capital letters compare the different treatments on each experimental day, lowercase letters compare the evolution of each treatment between the different experimental days.

Serum biochemical analysis

In the analysis of hepatic cytotoxicity of tilapia treated with tetracycline (Figure 3), no significant changes in serum alkaline phosphatase enzyme activity were observed. However, serum AST levels were significantly higher (P < 0.05) in animals treated with 200 and 400 mg of tetracycline in the longest period (12 days), compared to animals treated with the lowest dose (100 mg) and the control group. This result was also observed over time, in which treatment with 200 and 400 mg increased their values in the later period.

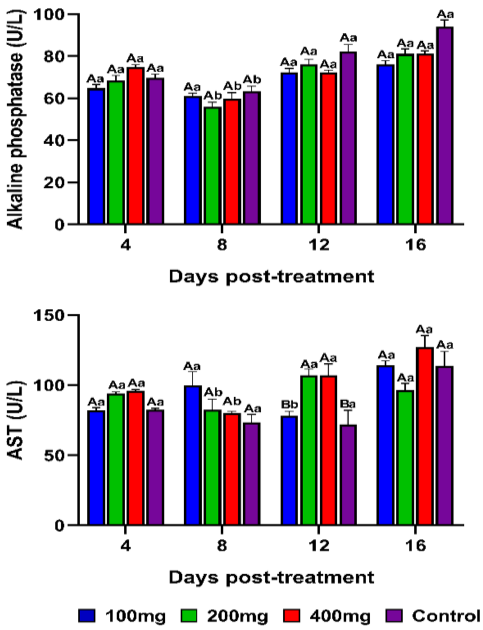

Figure 3 - Analysis of alkaline phosphatase and aspartate aminotransferase (AST) in tilapia treated with tetracycline. Means (n = 8) followed by the same letter do not differ by Tukey's test (P < 0.05). Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between the different experimental days.

In the evaluation of liver function during tetracycline treatment (Figure 4), no significant changes were observed in serum values of creatinine, total protein, cholesterol, triglycerides and albumin.

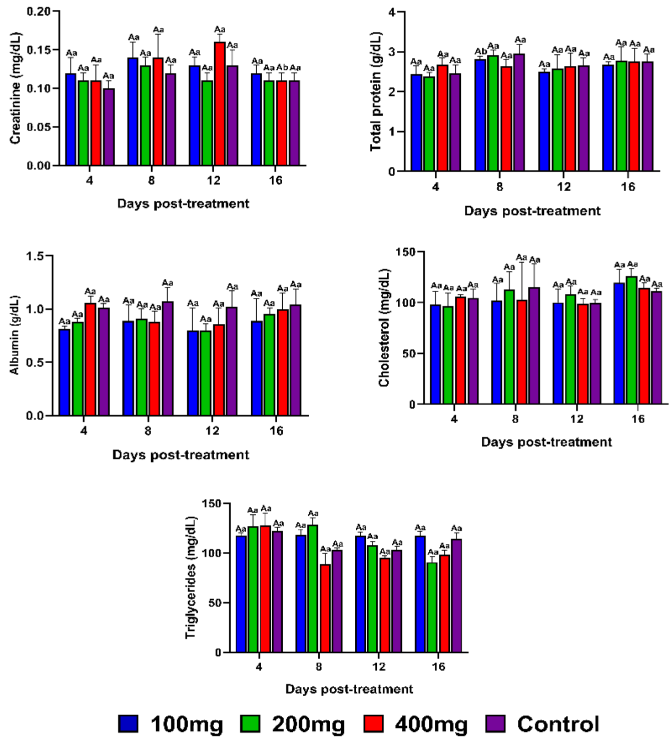

Figure 4 - Analysis of creatinine, total protein, albumin, cholesterol and triglycerides of tilapia treated with tetracycline. Means (n = 8) followed by the same letter do not differ by Tukey's test (P < 0.05). Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between the different experimental days.

The glycemia assessment (Figure 5) revealed a decrease after 12 days of treatment in all groups, except for the group treated with 400 mg of tetracycline, which did not show a decrease, remaining high throughout the evaluation period.

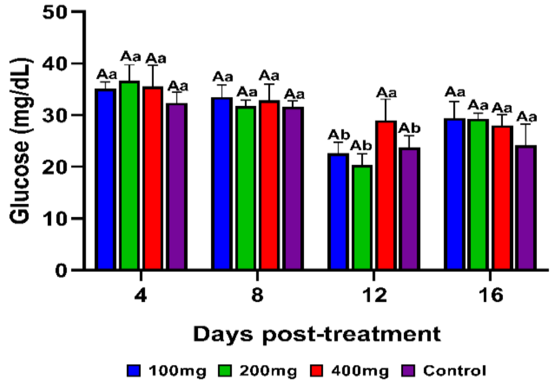

Figure 5 - Analysis of glycemia in tilapia treated with tetracycline. Means (n = 8) followed by the same letter do not differ by Tukey's test ($P < 0.05$). Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between the different experimental days.

Somatic analysis

In the somatic analysis of weight, liver, spleen and kidney, no significant changes were observed in the animals of the different treatments and the control group (Figure 6).

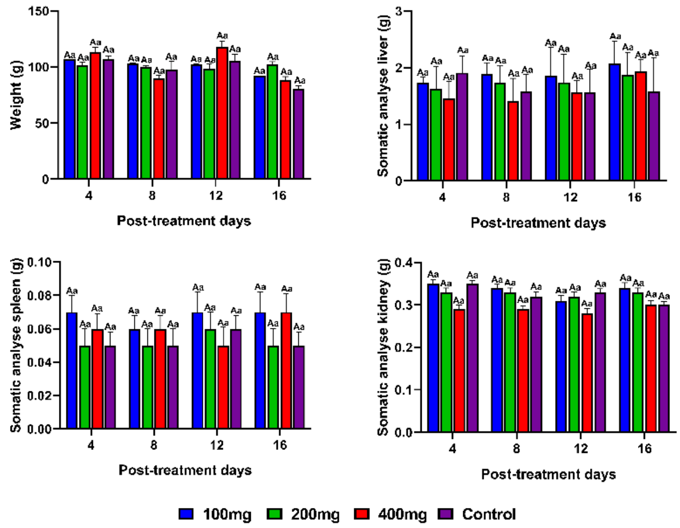

Figure 6 - Somatic evaluation of the spleen, liver and kidney of tilapia treated with tetracycline. Hepatic somatic index, splenic somatic index and renal somatic index. Means (n = 8) followed by the same letter do not differ by Tukey's test ($P < 0.05$). Capital letters compare the different treatments within each experimental day, lowercase letters compare the evolution of each treatment between the different experimental days.

DISCUSSION

In outbreak conditions, the administration of drugs in aquaculture is a fundamental measure for disease control, despite the scarcity of products registered for use in fish, which may favor the indiscriminate use of substances (Carraschi et al., 2017; Charlie-Silva et al., 2020). In this context, clinical safety studies become essential, and hematological and biochemical parameters stand out as important tools for assessing the physiological condition of fish exposed to different pharmacological agents (Oliveira, et al., 2022).

In the present hematological evaluation, tilapia treated with different doses of tetracycline did not present significant changes in hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and hemoglobin values, indicating maintenance of erythrocyte parameters even after exposure to different doses of the drug. This finding corroborates the study by Oliveira et al. (2022), in which they evaluated the clinical safety of doxycycline and observed no changes between the different concentrations of the drug. However, a progressive reduction in the number of circulating erythrocytes was observed at doses of 200 and 400 mg, which may reflect a possible cumulative or dose-dependent action of tetracycline. Previous studies have shown that antibiotics of the tetracycline class, such as oxytetracycline and doxycycline, can induce mild or transient toxic effects in fish, depending on the dose, exposure time and species analyzed (Kondera et al., 2020; Maklakova et al., 2011; Oliveira et al., 2022).

Furthermore, stressors, such as prolonged exposure to drugs or experimental conditions, can affect fish physiology, leading to discrete but clinically relevant changes in erythrocyte profiles (Ramesh et al., 2018). It is worth mentioning that, in studies with common carp (Cyprinus carpio) treated with oxytetracycline, Dobšíková et al. (2013) reported variations in erythrocyte parameters depending on the dose and duration of treatment, reinforcing the idea that the hematological effects of tetracyclines are modulated by multiple factors, including drug pharmacokinetics and metabolism of the target species.

The total leukocyte count did not show relevant changes between the different groups, corroborating previous studies that suggest low immunotoxicity associated with the use of tetracyclines (Popal et al., 2017; Do Huu et al., 2016). However, transient variations were recorded over time, with a reduction in the number of monocytes in the group treated with 400 mg and lymphocytes in the 200 mg group, both reversible at the end of the experimental period (16 days), suggesting a temporary modulation of the cellular immune response, possibly influenced by homeostatic or pharmacokinetic regulation mechanisms of the drug (Maklakova et al., 2011).

In the liver evaluation, it was observed that serum AST levels increased significantly in fish treated with 200 and 400 mg of tetracycline after 12 days of treatment, indicating potential transient dose-dependent and/or exposure-time related hepatic cytotoxicity. This finding is in agreement with previous studies with tetracyclines, which associate increased AST with lipid peroxidation and hepatocellular injury caused by reactive metabolites of the drug (Oyeniran et al., 2021; Aracati et al., 2021; Moraes et al., 2017). On the other hand, alkaline phosphatase (AP) did not undergo significant changes, suggesting that the observed liver damage was more associated with the integrity of hepatocyte membranes than with flow bile activity and cholestasis.

Serum levels of creatinine, total protein, cholesterol, triglycerides, and albumin did not show significant differences in all groups, suggesting that treatment with tetracycline, even at the highest doses, did not significantly compromise the liver and kidney functions. However, there was a decrease in blood glucose levels after 12 days of treatment in all groups, except in the group treated with 400 mg of tetracycline, which maintained high blood glucose levels. The maintenance of hyperglycemia in this group may be associated with a state of chronic physiological stress, with activation of the hypothalamic-pituitary axis and release of catecholamines and glucocorticoids, which promote glycogenolysis and neoglucogenesis (Ramesh et al., 2018). Similar results were described by Costa et al. (2022) in tilapia subjected to inflammatory stimuli, reinforcing that tetracycline in high doses can act as a stressor.

Somatic analysis of organs (liver, kidney and spleen) and weight did not show significant changes between the different treatments, corroborating previous studies evaluating the safety of antibiotics such as amoxicillin, oxytetracycline and doxycycline, which did not observe relevant somatic changes even after prolonged use (Dobšíková et al., 2013; Oliveira et al., 2022). These results corroborate the results of erythrocyte, leukocyte and biochemical studies of tilapia, suggesting that the drug did not compromise liver and kidney functions.

All fish in this study, regardless of the treatment received, did not present behavioral and/or clinical changes such as erratic swimming, lethargy, increased opercular beat, skin lesions or loss of appetite during the twelve-day treatment with tetracycline. Therefore, the results obtained in this study demonstrate that tetracycline, when administered orally to Nile tilapia, presents a relatively adequate clinical safety profile, especially at lower doses. The hematological, immunological and biochemical changes observed at doses of 200 and 400 mg/kg are mostly transient and reversible, although they indicate the need for caution regarding prolonged use or at high concentrations. These findings contribute to the understanding of the effects of tetracycline in aquaculture and reinforce the importance of physiological monitoring during pharmacological treatments in fish.

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