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Research Article

Assessing the biosafety of florfenicol by histoarchitectural alterations in Nile tilapia *Oreochromis niloticus*

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Abstract

The use of antibiotics in aquaculture can have adverse effects on fish organs. While the impact of florfenicol (FFC) on the organ histology of *Oreochromis niloticus* has been studied, a comprehensive report summarizing these effects is lacking. This study aimed to evaluate the histological effects of FFC on *O. niloticus* juveniles by administering a dose of 15 mg/kg biomass/day for 10 consecutive days, which was in line with approved therapeutic guidelines. Significant alterations were observed in key organs such as the kidney, liver, intestine, and gills, while the impact on the spleen, heart, and eyes was minimal. Specifically, the kidneys exhibited renal tubular degeneration at the therapeutic dose and liver tissues documented glycogen-type vacuolation. The swollen intestinal lamina propria indicated impaired feed intake and increased biomass. Gill tissues showed epithelial hyperplasia, probably a protective response to FFC toxicity. Although minor alterations were noted in the spleen, they were considered insignificant. No abnormalities were detected in the heart or ocular tissues. This study highlighted the use of histological analysis as a valuable tool for assessing the safety of FFC in aquaculture. The findings suggested that histological alterations could serve as a preliminary assessment method for evaluating antibiotic safety in piscine research, offering insights into the organ-specific effects of FFC in *O. niloticus*.

Keywords: Biosafety, Florfenicol, Histoarchitecture, Histopathology, Tilapia

Highlights

- Appraised the alterations of florfenicol-induced histoarchitecture in different organs of *Oreochromis niloticus*
- Proposed the use of histology as a preliminary assessment tool for antibiotic safety assessment
- Unveiled the use of histology for fish health assessment

INTRODUCTION

Over the past five decades, scientific advancements have significantly deepened our understanding of aquatic ecosystems and underscored the global imperative to manage them sustainably. In 2022, global fish production reached approximately 185.4 million tonnes, with a total first-sale value of USD 472 billion (FAO, 2024). Tilapias are a popular fish raised in more than 140 countries. They are the third most farmed fish, following carps and catfish, in terms of sales and volume in international trade. About 5.3 million tons of *Oreochromis niloticus* are produced annually (FAO, 2024). However, the aquaculture industry faces ongoing challenges, particularly from significant disease outbreaks - both exotic and endemic - that threaten biosecurity, food and nutritional security, and poverty alleviation efforts (FAO, 2024). Infectious diseases pose a constant risk, leading to substantial stock losses that impact the fish supply and farmers' livelihoods. Antibiotics are commonly employed in aquaculture for therapeutic and prophylactic purposes to mitigate these risks. Florfenicol (FFC), a synthetic, broad-spectrum antibiotic, is one such agent. As a fluorinated analogue of thiamphenicol, it shares the same mechanism of action as chloramphenicol. The United States Food and Drug Administration (USFDA) has approved its use at a dose of 10-15 mg/kg biomass/day for 10 consecutive days, particularly for treating conditions like furunculosis and columnaris caused respectively by *Aeromonas salmonicida* and *Flavobacterium columnare* in freshwater fish, among others in recirculating aquaculture systems (USFDA, 2024). While studies have examined the histological effects of FFC on aquatic organisms (Shiroma *et al*., 2020; Bardhan *et al*., 2022), there is a notable gap in comprehensive research that evaluates the impact on all major organs within a single study. Previous research has often focused on individual organs, leaving the need for a more holistic

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understanding. This study aims to fill that gap by providing a detailed analysis of the histological alterations across all significant and insignificant organs, focussing on the safety of FFC administration in tropical aquaculture.

MATERIALS AND METHODS

FFC-medicated diet preparation: The maximum therapeutic dose of florfenicol for aquaculture applications is 15 mg/kg biomass/day for 10 consecutive days (USFWS, 2015; USFDA, 2024). To achieve dosages of approximately 0 and 15 mg of active ingredient/kg biomass/day, pure FFC powder (Tokyo Chemical Industry, CAS RN: 73231-34-2; Product Number: F0811-5 g; >98.0% pure) was used. The required amount of FFC was combined with vegetable oil (5 mL/kg feed) to create an emulsion, which was then top-coated onto the commercial floating pellet feed (Bardhan *et al*., 2022). The FFC-coating onto the feed pellets was facilitated by thoroughly mixing the emulsion with the feed. The control feed was prepared using the same process but without FFC. The feeds were then air-dried overnight, stored in sealable plastic containers, and kept in a cool, dark place. Feeding rates and methods were consistent for the control and FFC-fed fish during the trial.

Experimental biota, collection, acclimatization, and design: Healthy juvenile Nile tilapia *Oreochromis niloticus* with an average weight of 30.74±0.80 g and a length of 12.72±0.21 cm were obtained from a grow-out farm in Sonarpur, West Bengal, India (Lat 22° 27′ 50.2158″ N; Long 88° 23′ 7.4004″ E). The fish were transported to the laboratory in plastic bags filled with oxygen. Upon arrival, they were disinfected by immersion in a 2-ppm potassium permanganate solution for 3 min and then transferred to 500-L circular tanks at a density of 50 fish/tank. They were acclimatized for 15 days with constant aeration. During acclimatization, the fish were fed a commercial floating pelleted feed (2 mm diameter, Aquaxcel, Cargill, India) three times daily at 2% of their body weight (BW). This feeding rate was selected based on a preliminary study that showed 100% utilization of the feed within an hour at this rate. Fish with no signs of infection were randomly selected from the stock tanks and transferred to six separate 500-L circular tanks, with 40 fish in each tank, for an additional 7-day period (pre-dosing period) before dosing. The experimental fish were divided into two sets: set 1 (control), which received no FFC, and set 2, which received FFC at 15 mg/kg biomass/day (approved dose), with each set, in triplicates (3 tanks/set). Approximately 50% of the water was replaced every three days to remove waste and faeces. Water quality parameters were maintained at optimal levels throughout the experiment: pH ranged from 7.30 to 7.97, dissolved oxygen from 5.24 to 6.00 mg/L, water temperature from 22.00 to 26.00°C, nitrite from 0.23 to 0.55 mg/L, and nitrate from 0.25 to 0.61 mg/L.

Administration of medicated feed: The study spanned 60 days, comprising a 7-day pre-dosing, a 10-day FFC-dosing (FD), and a 43-day post-FFC-dosing (PFD) period. During the pre- and post-dosing periods, all fish were fed a control diet. During the dosing period, the control and FFC-coated diets were administered to the respective sets. The daily feed ration, equivalent to 2% BW, was divided into three equal portions and given in the morning, noon, and evening. Any uneaten feed remaining in the tank one hour after each feeding was siphoned into a pre-weighed container, dried overnight, pooled by a tank daily, and weighed. Feeding activity was visually monitored three times daily, and observations on feed consumption, behavioural changes, external changes, and mortality were recorded daily. The feed ration was adjusted according to biomass accrual and mortalities. Normal behaviours, such as swimming to the surface during feeding, aggressive feeding, and even distribution throughout the water column were noted. Additionally, external physical changes, including pigmentation and gross lesions were observed daily.

Sample collection: On days 0, 10 (end of FD), and 43 (PFD), three fish/tank were collected for sampling. Samples were taken the following day before morning feeding, except for day 0. The fish were euthanized using clove oil at 100 µL/L water, then deskinned, dissected, degutted, and washed to obtain gill, liver, kidney, intestine, spleen, heart, and eye samples. The required tissue quantities (≈ 2 g) from each replicate tank were pooled, packed in labelled polythene bags and stored at -20°C.

Histological processing and assessment: Based on the literature review, the histological assessment was documented and categorized into two groups. Group A included gill, kidney, liver, and intestinal tissues, which recorded the highest number of alterations. Group B included eye, heart, and spleen tissues that documented minimal or no alterations. This segmentation aimed to provide a comprehensive histological evaluation of less frequently studied organs and to briefly examine antibiotic-mediated alterations in these tissues. Designated tissues from *O. niloticus* juveniles were collected on day 10 of FD and day 43 of PFD from the control and FFC-dosed groups and fixed in Bouin's solution for 24 hours. Standard protocols were followed

Table 1. Histological scoring of tissue responses in florfenicol-fed *Oreochromis niloticus*

Organ	Reaction pattern	Tissue alteration	Importance factor
Gill	Regressive changes	Epithelial lifting	1
		Fusion of secondary lamellae	1
		Curling of secondary lamellae	1
	Progressive changes	Epithelial hyperplasia	2
		Lamellar hyperplasia	2
Kidney	Regressive changes	Degeneration of renal tubule	1
		Degeneration of renal tubular epithelium	1
		Widening of lumen	1
		Shrunken glomerulus	2
	Inflammatory changes	Hydropic swelling	1
Liver	Regressive changes	Cytoplasmic degeneration	3
	Progressive changes	Cytoplasmic vacuolation	1
		Cellular hypertrophy	1
Intestine	Circulatory changes	Increased IEL numbers	1
	Regressive changes	Loss of absorptive region	2
		Epithelial degeneration	2
	Progressive changes	Swollen lamina propria	3
Spleen	Progressive changes	Increased sinusoidal space	1

IEL: Intestinal epithelial cells

for tissue processing, embedding, sectioning (5 µm), and double staining with Hematoxylin and Eosin (Roberts, 2012). Photomicrographic observations were conducted using an advanced trinocular research microscope (Olympus BX51, Japan) equipped with an SCO-LUX 16 MP camera. Images were captured and processed using Toup Tek Toup View software (Version x64, 4.11). Histological assessment and scoring were performed based on circulatory, regressive, and progressive alterations as described by Bernet *et al*. (1999). Each alteration was assigned a score of 0, 2, 4, or 6, reflecting the severity and extent of damage. An importance factor ranging from 1 to 3, depending on the pathological significance of each alteration, was also included (Table 1). Reaction index (RI) and organ index (OI) were calculated as outlined by Bernet *et al*. (1999), with higher values indicating greater severity and extent of damage.

Statistical analysis: Statistical analyses and graph preparation were conducted using OriginPro 2024. Error bars in the FFC organ index graph represented the standard error of the mean (SEM). Histological scoring data were expressed as mean ± standard deviation, and a non-parametric Kruskal-Wallis test was performed using IBM SPSS software version 22.0 (SPSS Inc., Chicago, IL) to determine significant differences, with a threshold of $P<0.05$.

RESULTS

Feed intake, mortality, and behavioural changes

The control fish exhibited consistent active feeding throughout the experiment. No abnormal behavioural changes were observed in both groups. The FFC-dosed group consumed 97% of the feed offered during the FD period, with consumption steadily increasing during the PFD period. Both groups realized 100% survival. The biomass of *O. niloticus* increased in both groups, with the highest in the control.

Histological alterations

Gill: Control gill tissues showed normal histoarchitecture. In the FFC-dosed group, circulatory changes such as aneurysms were absent. However, regressive changes like epithelial lifting, curling and fusion of secondary lamellae became evident after 10 days of FD (Fig. 1). Hyperplasia in both the epithelium and secondary lamellae was also observed. The gill tissues fully recovered after the cessation of FFC administration (Table 2).

Kidney: The control kidney tissue observed normal glomerulus and nephric tubules. The FFC-dosed kidney tissues exhibited the highest number of regressive changes, including degeneration of renal tubules and tubular epithelium, shrunken glomeruli, and lumen widening (Table 2). Hydropic swelling within the tubules indicated inflammatory alterations. Although most

Table 2. Detailed histological scoring based on reaction patterns and tissue alterations in the organs showing the highest histoalterations following oral florfenicol medication at 15 mg/kg biomass/day for 10 consecutive days

Organ	Reaction pattern	Tissue alteration	After florfenicol (Ab) diet*	Recovery
Gill	Regressive changes	Epithelial lifting	2.67±1.15	0.67±0.05
		Fusion of secondary lamellae	1.33±1.15	0.67±0.05
		Curling of secondary lamellae	1.33±1.15	0.67±0.05
	Progressive changes	Epithelial hyperplasia	6.67±2.31	1.33±1.15
		Lamellar hyperplasia	6.67±2.31	0.00±0.00
Kidney	Regressive changes	Degeneration of renal tubules	2.67±1.15	1.33±1.15
		Degeneration of renal tubular epithelium	1.33±1.15	0.00±0.00
		Widening of lumen	1.33±1.15	0.67±0.05
		Shrunken glomerulus	4.00±0.00	0.00±0.00
	Inflammatory changes	Hydropic swelling	3.33±1.15	0.67±0.05
Liver	Regressive changes	Cytoplasmic degeneration	10.00±3.46	2.00±0.31
	Progressive changes	Cellular vacuolation	2.67±1.15	0.00±0.00
		Cellular hypertrophy	0.67±1.15	0.00±0.00
Intestine	Circulatory changes	Increased IEL numbers	2.67±1.15	1.33±1.15
	Regressive changes	Loss of absorptive region	6.67±2.31	0.00±0.00
		Epithelial degeneration	1.33±1.15	1.33±0.31
	Progressive changes	Swollen lamina propria	8.00±3.46	0.00±0.00
Spleen	Progressive changes	Increased sinusoidal space	1.33±1.15	0.67±0.05
		Aggregation of MMCs	2.67±1.15	0.00±0.00

*Scores of all organ tissue alterations differed significantly between dosing days and recovery. MMCs: Melano macrophage centres; IEL: Intestinal epithelial cells

tissues recovered after discontinuing the FFC diet, some degenerating renal tubules persisted (Fig. 1).

Liver: The liver showed a high degree of regressive changes, notably cytoplasmic degeneration, and progressive alterations such as cytoplasmic vacuolation and cellular hypertrophy (Table 2). Upon recovery, the liver tissues exhibited no abnormalities (Fig. 1).

Intestine: Progressive changes in intestinal tissues were evident, with persistent swelling of the lamina propria. Regressive changes included loss of absorptive regions and degeneration of the intestinal epithelium. An increased number of intraepithelial lymphocytes suggested circulatory changes due to FFC medication (Fig. 1). Mild persistence of elevated IEL numbers was observed during the PFD period (Table 2).

Spleen: The splenic tissues of the control group showed normal white pulp and red pulp areas. Minor alterations like increased sinusoidal space were observed after FFC medication (Table 2). Many melanomacrophage centres (MMCs) were found throughout the splenic tissues. However, recovery was complete after the withdrawal of FFC medication (Fig. 2).

Eye: The histological sections of eye tissues in the control group depicted normal architecture with a choroid body, lens fibres, pigment epithelium, photoreceptor layer, net plexiform layer, nerve fibre lining and inner limiting membrane. The FFC-dosed fish eye tissue sections were critically examined wherein the layers, outer and inner nuclear layers, and outer and inner plexiform layers showed no anomalies. The pigment epithelium in the central region of the photoreceptor layer was normal (Fig. 2). The cones and rods showed varying types throughout the photoreceptor layer in contact with the pigmented epithelium. The single and double cones in this layer were prominent throughout the tissue sections. Most of the pigment granules were completely distributed, and their colour was brown due to the presence of melanin. Deformation of the plexiform layers, generally observed during sunken eyes, was not visible. Similarly, changes in retinal pigmentation were also not observed during FFC-medication.

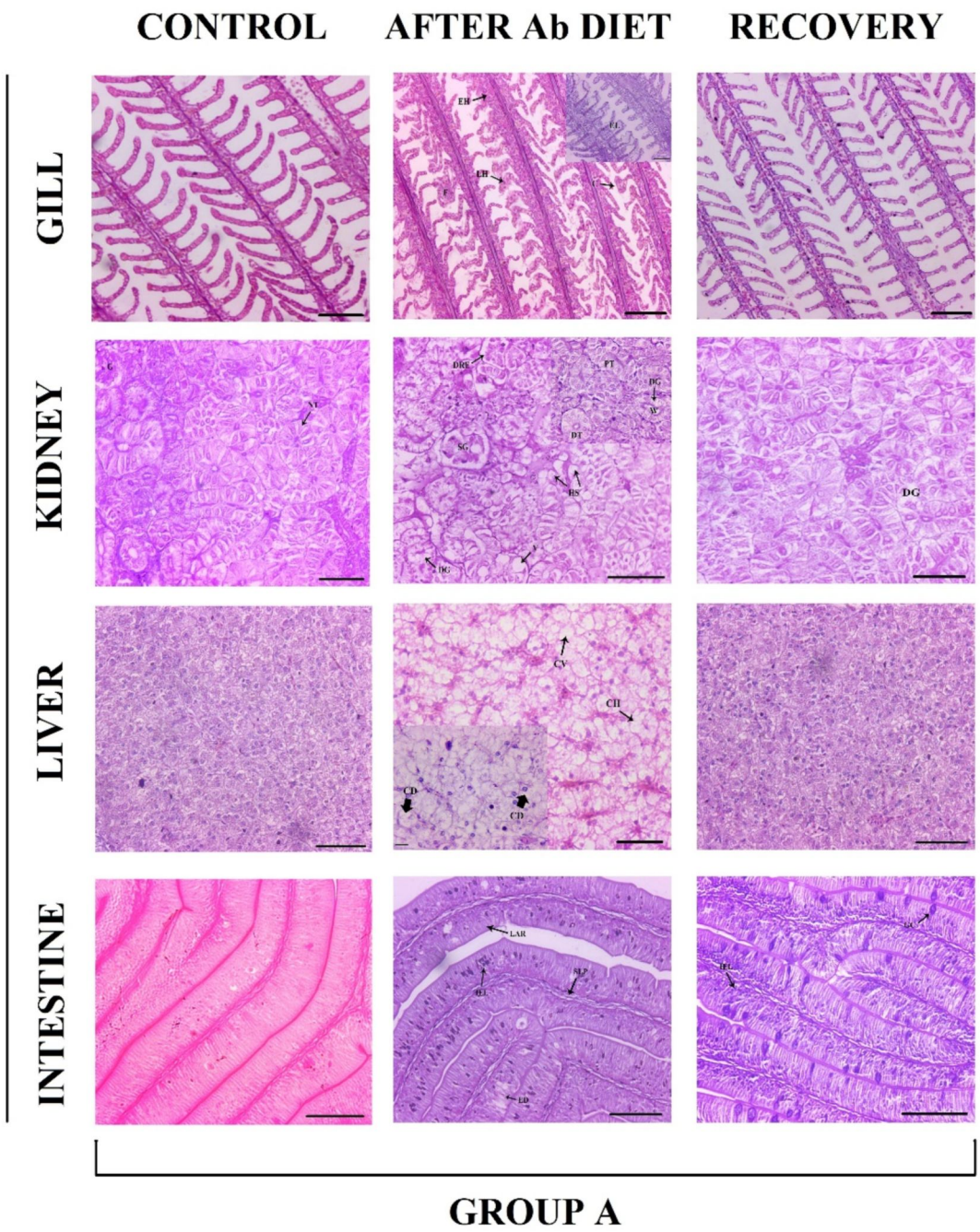

Fig.1. Histological alterations in the gill, kidney, liver, and intestine (group A) of *Oreochormis niloticus* juveniles after florfenicol administration at 15 mg/kg biomass/day for 10 consecutive days. Scale: 50 µm. Normal glomerulus (G) and nephric tubules (NT), epithelial lifting (EL; inset), curling (C) and fusion of secondary lamellae (F), hyperplasia in the epithelium (EH) and secondary lamellae (LH), degeneration of renal tubules (DT) tubular epithelium (DRE), shrunken glomeruli (SG), lumen widening (W), hydropic swelling (HS), cytoplasmic degeneration (CD; inset), cytoplasmic vacuolation (CV), cellular hypertrophy (CH), swelling of the lamina propria (SLP), loss of absorptive regions (LAR), degeneration of the intestinal epithelium (ED) and intraepithelial lymphocytes (IELs).

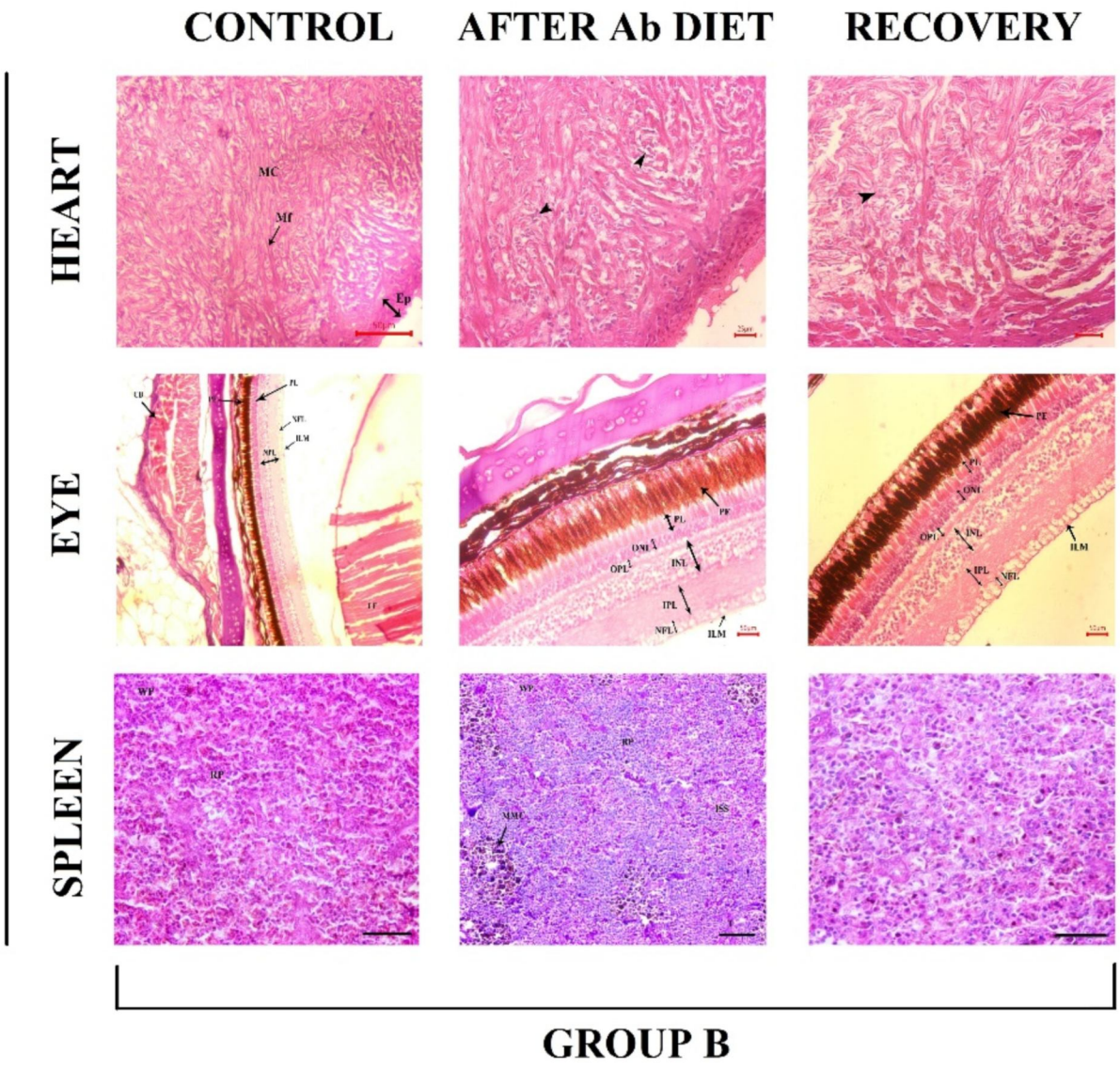

Fig. 2. Histological alterations in the heart, eye, and spleen (group B) of *Oreochormis niloticus* juveniles after florfenicol administration at 15 mg/kg biomass/day for 10 consecutive days. Scale: 50 µm. Normal white pulp (WP), red pulp (RP), choroid body (CB), lens fibres (LF), pigment epithelium (PE), photoreceptor layer (PL), net plexiform layer (NPL), nerve fibre lining (NFL), inner limiting membrane (ILM), outer and inner nuclear layers (ONL and INL), outer and inner plexiform layers (OPL and IPL), epicardium (Ep), myocardium (MC), myocardial filaments (Mf),multi-nucleated filaments (arrowheads), increased sinusoidal space (ISS), and melanomacrophage centres (MMCs).

Heart: The control group displayed normal architecture of cardiac muscles with multiple nuclei (Fig. 2). Epicardium, myocardium, and myocardial filaments were visible. Following FD, no alterations of the cardiac striated muscles or blood vessel dilation/congestion were observed. The myocardium of both dosed and post-dosed fish showed good numbers of multi-nucleated filaments.

The enumeration of organ indices (OI) led to a comprehensive understanding of the histological alterations in the piscine organs showing the spleen, kidney, liver, gill, and intestine in an increasing hierarchy of tissue alterations in the therapeutic dose group (Fig. 3).

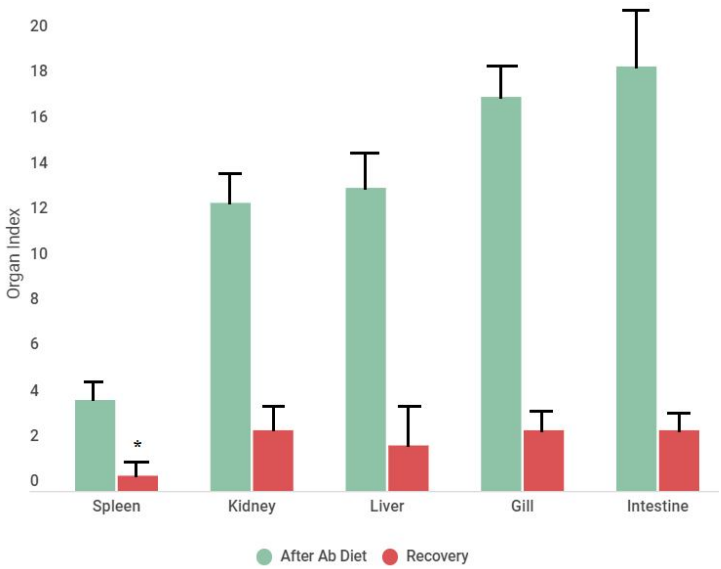

Fig. 3. Comparison of organ index (OI) among those depicting the maximum number of histoalterations following florfenicol medication at 15 mg/kg biomass/day for 10 consecutive days and consequently observing a 43-day recovery period. The organ indices during the florfenicol (Ab) diet administration differed significantly from each other (P<0.05). The spleen organ index during recovery differed significantly (P<0.05) from other organ indices.

DISCUSSION

For numerous reasons, histopathological examinations have long been regarded as credible biomarkers of stress in fish (van Dyk, 2003; de Oliveira and Narcisco, 2013). The current study aimed at utilizing histology as a diagnostic tool for assessing the safety of drug usage in fish. Henceforth, this study produces a pioneering effort to characterize the safety of FFC in healthy *O. niloticus* juveniles using histology. Kidneys are particularly vulnerable to toxic injury since they are exposed to blood plasma directly through the open fenestrae of their glomerular capillaries (Rodrigues *et al*., 2019). The persistence of tubular degeneration at a moderate level, even on day 43 PED, implied the nephrotoxic potential of FFC at the therapeutic dose, which might lead to tissue necrosis (Yun *et al*., 2023). Further, the FFC-nephrotoxicity could also be justified by the glomerulopathy and dilated Bowman's space as well as the widened lumen. The occurrence of hydropic swelling and vacuolation in renal tubules with intact nuclei has also been reported previously (Gaikowski *et al*., 2013). The prominence of degeneration of renal epithelium signified the role of FFC in the loss of structural integrity. The widening of the lumen hinted at the excessive flow of filtrate and reabsorption. Apart from the renal tubular degeneration, the qualitative scores of all other aberrations obtained on day 43 PED were considered normal with <5% of tissue damage. These observations hinted at the good tolerability of *O. niloticus* to FFC at the maximum therapeutic dose and that it can be safely used.

There was an increased incidence of glycogen-type vacuolation in the liver of FFC-fed fish, which was severe. The glycogen-type vacuolation can cause an increase in the size of hepatocytes leading to cellular hypertrophy, which was profoundly recorded in this study. The increased vacuolation of hepatocytes is a signal of a degenerative process (Mallik *et al*., 2023). It resulted in changes to cell membrane integrity (Guo *et al*., 2023). The current study's high rate of cytoplasmic vacuolization could indicate a mismatch between the rate of substance synthesis in parenchymal cells and the rate of their release into the systemic circulation (Kalra *et al*., 2018). Cellular hypertrophy reduced significantly, whereas cellular degeneration and cytoplasmic vacuolation reduced insignificantly on day 43 PED.

Fish gills have a role in respiration, osmotic and ionic balance, acid-base management, nitrogenous waste excretion, and neurotransmission modulation (Rodrigues *et al*., 2017). Depending on the degree and duration of exposure/administration, their delicate structure normally responds to stressors via non-specific structural modifications. Drug-induced alterations, particularly epithelial hyperplasia, mucous cell hypertrophy, and pillar cell hypertrophy, can result in an increased distance between water and blood, compromising respiratory performance (Sayed *et al*., 2012). Excessive epithelial hyperplasia and subsequent lamellar fusion corroborated the observations of Gaikowski *et al*. (2013) following FFC treatment. The

other aberrations like epithelial lifting, lamellar fusion, and changes in tissue architecture causing secondary lamellae curling can be interpreted as an adaptation defence mechanism in fish similar to the earlier study (Rodrigues *et al*., 2019).

The current study's histopathological findings revealed the harmful effects of FFC on various intestinal layers. A striking observation was the increase in the intraepithelial lymphocyte-like cells (IELs) and goblet cells and a decrease in enterocytes in the epithelial region surrounding the laminar propria. Enterocytes have a clear role in digestion by ensuring the uptake of ions, water, nutrients, vitamins, and the absorption of unconjugated bile salts (Ali *et al*., 2021). The epithelial cells are crucial for intestinal absorption (Ali *et al*., 2021) and the observations of necrotized absorptive region and loss of absorptive vacuoles corroborate the aforesaid statement. The significantly increased intensity of swollen laminar propria on day 10 of FD may be directly related to the toxic effects of FFC, either as an allergen or a toxicant, preceding the development of anorexia. The record of reduced feed intake in the FFC-fed group also supported the statement. The alterations in the intestinal histoarchitecture led to faulty absorption and biomass reduction in the current study. However, on day 43 PED most alterations were significantly reduced. The documented scores suggested a reasonable recovery in intestinal tissues of *O. niloticus* at the therapeutic dose.

The splenotoxicity, as observed in histological sections of the FFC-dosed group, was quite meagre. Although increased sinusoidal space and the MMCs were observed during the dosing period, they disappeared completely after recovery. Increased sinusoidal space and the development of macrophage aggregates have often been linked with several toxicants and pharmaceuticals (Khan and Chetty, 2016; Sales *et al*., 2017) as a protective mechanism of the body. Increased sinusoidal space has been correlated with vacuolations in the spleen, and these aberrations are precedents of splenic infarctions or necrosis (Khan and Chetty, 2016). However, the approved dosing produced no such architectures, and henceforth, splenotoxicity was less imminent following FD.

The heart and optic tissues exhibited no anomalies and, therefore, were not included in the discussion. The epicardium remained uniform throughout the dosing period, with no observed abnormalities. Additionally, the centrally located nuclei within the cardiac filaments appeared normal. Although minor anomalies, such as the infiltration of inflammatory cells in the epicardium, were noted, these were not deemed significant as they were observed in only one section. Similarly, the optic tissues in the control eye sections displayed normal histoarchitecture. Consequently, it was determined that FFC administration did not affect the heart and eyes in tilapia, suggesting that FFC-induced cardiotoxicity and ocular toxicity are rare. These findings indicated that it may not be necessary to include these aspects in future amphenicol safety studies.

While previous research has thoroughly examined the effects of FFC medication on *O. niloticus*, this study uniquely focused on the histological alterations in all major organs following antibiotic treatment. By providing a detailed histoarchitectural analysis, our study offered a novel approach to assessing the biosafety of antibiotics in aquaculture, which has not been previously reported. The observed histological changes highlighted the potential of using histological analysis as a primary tool for evaluating the safety of antibiotic use in aquaculture. This approach not only deepens our understanding of the impacts of antibiotics like FFC but also introduces a valuable method for future biosafety or risk assessments in aquaculture practices.

Conflict of interest: The authors have disclosed no conflicts of interest in the study.

Author's contribution: AB: Data and literature collection, data formulation, writing; TJA: Data and literature collection, reviewing and editing, data curation; PKP: Funding acquisition and methodology validation.

Data availability statement: All the data supporting the research findings have been presented in this paper. The corresponding author is willing to provide the raw data upon reasonable request.

Ethical statement: The experimental protocols for the All-India Network Project on Fish Health were endorsed by the Indian Council of Agricultural Research, New Delhi (CIBA/AINP-FH/2015-16). All procedures adhered to the relevant guidelines established by the Government of India, as outlined in CCSEA (2021).

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