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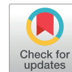

NOTE

The effect of fipronil exposure on the activity of biotransformation enzymes and histology in the liver of grass carp

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ABSTRACT: Fipronil (FPN) is an insecticide used in agriculture. This study focused on the biotransformation process and the histopathological effects of FPN in the liver of grass carp. Fish were exposed to environmental concentrations of FPN (3, 6, and 10 ug l^{-1}) for up to 14 d. The alterations in phase I and II biotransformation enzyme activity (ethoxyresorufin-O-deethylase [EROD] and glutathione-S-transferase [GST]), malondialdehyde (MDA) content, and histopathology in the liver were studied on the 1st, 3rd, 7th, and 14th days. Results showed that EROD (dose-dependent) and GST activity (time-dependent) increased. The MDA content increased in a time- and dose-dependent manner. The most common types of hepatological damage were steatosis, vein dilatation, pyknosis, and increased melanomacrophage centers, probably due to oxidative stress originating from biotransformation enzyme activity (R^2 = 0.88 for GST and MDA). The degree of tissue change (DTC) at the highest dose indicated moderate damage to the liver (R^2 = 0.82 for GST and DTC). Nevertheless, the level of EROD and GST activity and MDA content indicated complex interactions among various phase I and phase II biotransformation enzymes which should be investigated in future studies with more replications.

KEY WORDS: Fipronil · Grass carp · Liver · Biotransformation

1. INTRODUCTION

Fipronil (FPN) is a benzopyrazole insecticide frequently used in the control of agricultural and household pests through binding to γ-aminobutyric acid receptors and blocking the chloride channel (Dallarés et al. 2020). This use leads to its excessive entry into aquatic ecosystems (Wu et al. 2022) where it can cause highly toxic effects in aquatic organisms (Pisa et al. 2015). FPN has an environmental concentration lower than 10 µg l^{-1} (Bencic et al. 2013) and is mainly detoxified in the liver (Ardeshir et al. 2018). Metabolic transformation of FPN in the fish liver is accomplished by the phase I and phase II biotransformation enzyme systems (Michel et al. 2016). Ethoxyresorufin-O-deethylase (EROD) activity (CYP1A activity) can be induced by various contaminants (Whyte et al. 2000), one of which might be FPN (Wu et al. 2022).

Our previous study assessed the response of CYP1A gene expression and oxidative stress after intraperitoneal exposure of Caspian kutum fish to sublethal doses of FPN (Ardeshir et al. 2018). Sara Dallarés et al. (2020) assessed the effect of FPN on the biotransformation enzymes, including benzoxyresorufin-O-

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dealkylase (BROD) and methoxyresorufin-O-deethylase (MROD), under 2 temperature regimes. Xu et al. (2019) measured the inductive effect of FPN on the gene expression of EROD (not EROD enzyme activity) in a dose-dependent manner (not time-dependent manner). To the best of our knowledge, no study has focused on the measurement of factors dealing with biotransformation of FPN in a time- and dose-dependent manner and tried to link this event to liver histopathology. Metabolism rates for contaminants differ greatly among various fish species (van der Oost et al. 2003) following a trend with habitat depth and body size (Ribalta et al. 2015).

In the Caspian Sea region, agriculture (rice farms) and the use of pesticides, especially FPN, are common, leading to the contamination of freshwater. The grass carp *Ctenopharyngodon idella*, family Cyprinidae, is an endemic and valuable fish in the Caspian Sea region, and one of the major aquaculture species in the world (Xiong et al. 2023). It is extensively consumed by natives in this region, and is threatened by various pesticides (Vajargah et al. 2021). This study aimed to measure and track the activity of biotransformation enzymes (phase I and II) including EROD and glutathione-S-transferase (GST) and their interaction with malondialdehyde (oxidative stress) and liver tissue damage during the exposure of grass carp to different environmental concentrations of FPN for various exposure times.

2. MATERIALS AND METHODS

2.1. Fish

Immature grass carp (weighing 17 ± 2 g and 11 ± 1 cm in length) (n=48) were transferred to tanks containing 40 l groundwater, acclimated to laboratory conditions, and fed (2% of body weight) for 2 wk. The water in the tanks was changed every 3 d.

2.2. Experimental design and sampling

A stock solution (1 l) of FPN (1 mg l^{-1}) was prepared, and 400, 240, and 120 ml were added to 3 plastic tanks to prepare treatment groups with 10, 6, and 3 µg l^{-1} of FPN (this solution was prepared after vortexing for about 45 min). Moreover, 1 tank was left untreated as a control group (12 fish). After exposure, fish were anesthetized with MS22 (40 mg l^{-1}) and weighed. Sampling was performed on the 1st, 3rd, 7th, and 14th days. Fish were sacrificed by decapitation and the livers were dissected. One part of each liver was fixed using Bouin's solution fixation and transferred to 70% ethanol after 24 h. Other parts of the fish livers were immediately frozen in liquid nitrogen and stored at −80°C.

2.3. Liver sample preparation

Frozen fish liver samples were ground under liquid nitrogen and slowly homogenized on ice with cold homogenization buffer. Then, the homogenate sample was centrifuged 3 times at 9000 × *g* and 1°C for 20 min followed by 2 centrifugations at 105 000 × *g* and 1°C for 60 min to obtain the S9, cytosolic, and microsomal fractions. The microsomal pellets were resuspended in Tris buffer (pH 7.4) containing 20% glycerol. Cytosol protein concentrations (0.93−18.08 mg ml^{-1}) and microsomal (1.43−14.71 mg ml^{-1}) fractions were determined in each sample by the Lowry method and using bovine serum albumin as the standard (Waterborg 2009).

2.4. EROD activity

Microsomes (10 µl) were incubated for 1 h at room temperature (25°C) in a final volume of 300 µl of 100 mM phosphate buffer, pH 7.4, 0.25 mM NADPH, and 4.15 µM 7-ethoxyresorufin. Resorufin formation was determined by measuring the increase in fluorescence at 1 min intervals for 10 min (excitation: 530 nm; emission: 590 nm). The EROD activities are given as pmol per minute reaction time per mg cytosolic protein.

2.5. GST activity

The assay mixture included 0.1 mg ml^{-1} final protein concentration, 2 mM glutathione (GSH, reduced form), 100 mM phosphate buffer (pH 7.4), and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The increase in absorbance was measured by spectrophotometer for 10 min at 340 nm. GST activities are given as nmol per min reaction time per mg cytosolic protein.

2.6. Lipid peroxidation

Lipid peroxidation was quantified by measuring malondialdehyde (MDA) formation. The reaction

mixture containing 0.2 ml of liver homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 1% 2-thiobarbituric acid, 1.5 ml of 20% acetic acid buffer (pH 3.5), and 1 ml of distilled water was heated at 95°C for 60 min. After cooling, centrifugation was carried out at 5000 × *g* for 20 min. The supernatant absorbance was read at 532 nm using a UV-220 spectrophotometer. A molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used to determine the amount of MDA formed.

2.7. Liver histology

The fixed liver samples were dehydrated in an ethyl alcohol series of ascending concentrations, embedded in paraffin, and sectioned at 5 µm. The tissue sections (3 slides for each fish) were stained with hematoxylin and eosin (H&E). The degree of tissue change (DTC), which is based on the severity of lesions, was used to semi-quantitatively evaluate histological alterations in the fish livers. The alterations were classified into progressive stages of damage to the tissue: Stage I with no alteration in the normal functioning of the tissue; Stage II with more severe damage and disruption of the normal functioning of the tissue (this stage can be recognized based on observing damage such as pyknosis); and Stage III with severe and irreparable damage (damage such as necrosis) (Bernet et al. 1999). A DTC value was calculated using the formula: $DTC = (1 \times SI) + (10 \times SII) + (100 \times SIII)$, where SI, SII, and SIII correspond to the number of alterations classed as Stages I, II, and III, respectively. DTC values between 0 and 10 indicate normal functioning of the liver, values between 11 and 20 indicate slight damage, values between 21 and 50 indicate moderate damage, values between 50 and 100 indicate severe damage, and values above 100 indicate irreversible damage.

2.8. Statistical analysis

All values are presented as means ± SD from 3 samples (3 fish for each sampling time and 3 assays per fish). Multiple comparisons among groups were carried out using a 1-way ANOVA followed by the Newman-Keuls post-test. For comparison of DTC results, the Mann-Whitney test was used. Pearson's test was used to evaluate the correlation between variables. All statistical analyses were performed using Origin Pro software (Ver. 2016). Differences were considered significant when $p < 0.05$.

3. RESULTS

3.1. EROD and GST activity

EROD activity significantly increased compared to the control group, especially on the 7th and 14th days ($p < 0.001$). On the 14th day, the highest EROD activity was seen after exposure to 3 µg l^{-1} FPN, whereas the highest activity was observed after exposure to 6 and 10 µg l^{-1} FPN on the 7th day ($p < 0.001$) (Fig. 1a).

After exposure to 3 µg l^{-1} FPN, activity of the phase II enzyme (GST) significantly increased on the 14th day ($p = 0.045$). GST activity increased in a time-dependent manner after exposure to 6 and 10 µg l^{-1} FPN ($p = 0.019$) (Fig. 1b).

3.2. Estimating lipid peroxidation

An increase in MDA content was found for all treatment groups on the 7th and 14th days ($p < 0.001$). MDA content significantly increased in a time- ($p = 0.021$) and dose-dependent manner ($p = 0.015$) (Fig. 1c). Pearson's test showed a significant correlation between MDA content and GST activity ($R^2 = 0.88$).

3.3. Liver histopathology

The DTC values for the livers exposed to 3 and 6 µg l^{-1} FPN were $10 > DTC > 0$ and $20 > DTC > 11$, indicating normal function and mild damage to the liver, respectively (Figs. 1d & 2). However, after exposure to 10 µg l^{-1} FPN, especially on the 14th day, the DTC values were $50 > DTC > 21$, indicating moderate damage to the liver. Pearson's test showed a significant correlation between DTC and GST activity ($R^2 = 0.82$) and between DTC and MDA content ($R^2 = 0.88$).

4. DISCUSSION

In the present study, FPN significantly increased EROD activity, probably indicating that phase I biotransformation enzymes were activated to metabolize the FPN. Moreover, a concentration-dependent increase in GST activity was observed. With prolonged exposure time, the concentration of highly reactive FPN metabolites probably became elevated, which in turn may explain the time-dependent upregulation of GST activity, a key enzyme of phase II biotransformation. A previous study evaluating the effect of a 24 h exposure to sublethal concentrations (2, 4,

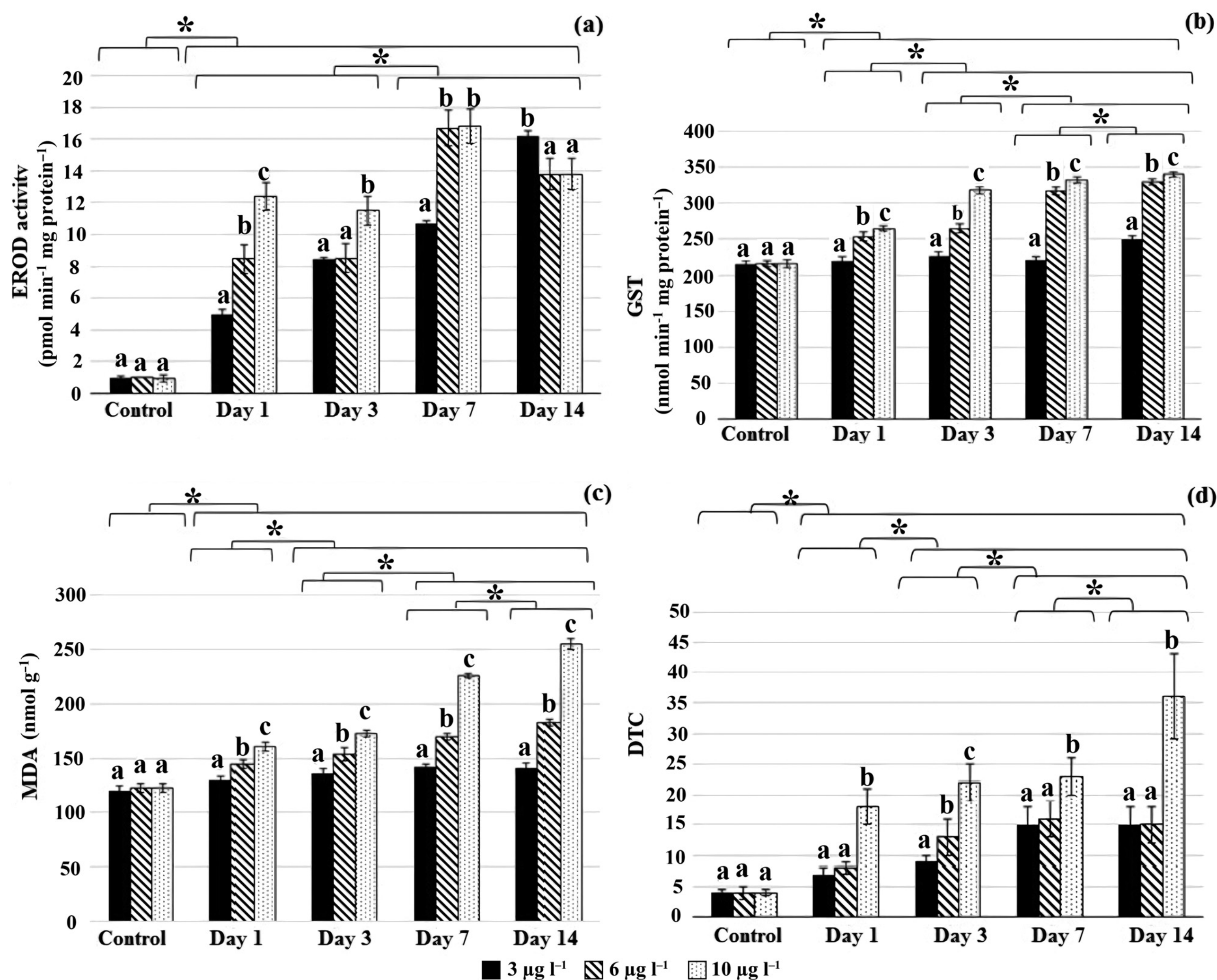

Fig. 1. (a) Ethoxyresorufin-O-deethylase (EROD) and (b) glutathione-S-transferase (GST) activity, (c) malondialdehyde (MDA) content, and (d) degree of tissue change (DTC) in the liver of *Ctenopharyngodon idella* (n = 3, each run in triplicate) exposed to environmental concentrations of FPN for 14 d. Different letters indicate significant difference between treatments on each day. *Significant differences between groups on different days

and 20 µg l^{-1}) of FPN on the biotransformation enzymes in zebrafish observed a significant increase in hepatic EROD activity, but no significant change in GST activity (Wu et al. 2014). On the other hand, Viana et al. (2022) found that GST activity in zebrafish increased after a 96 h exposure to FPN (63 µg l^{-1}). It seems that the GST response to FPN exposure may vary with time and exposure concentration as well as fish species.

In this study, we also observed that FPN exposure induced hepatic lipid oxidation as indicated by the formation of MDA. A significant correlation (R^2 = 0.88) between MDA and GST activity may indicate a relation between FPN biotransformation and oxidative stress.

After high-dose exposure to FPN, moderate damage to the fish livers was observed. The metabolic products of biotransformation of FPN were responsible for the observed liver damage (Guelfi et al. 2015). It is presumed that a mismatch in the kinetics of induction of phase I and phase II systems in the fish liver after exposure to FPN is responsible for this occurrence. This phenomenon leads to an inadequate conjugation potential resulting in the accumulation of highly reactive oxygenated metabolites in the liver and subsequent tissue damage (Shailaja & D'Silva 2003). The significant correlations between DTC and GST (R^2 = 0.82) and between DTC and MDA (R^2 = 0.88) support this suggestion. Further support comes from the mechanisms leading to the different types of liver damage observed. The

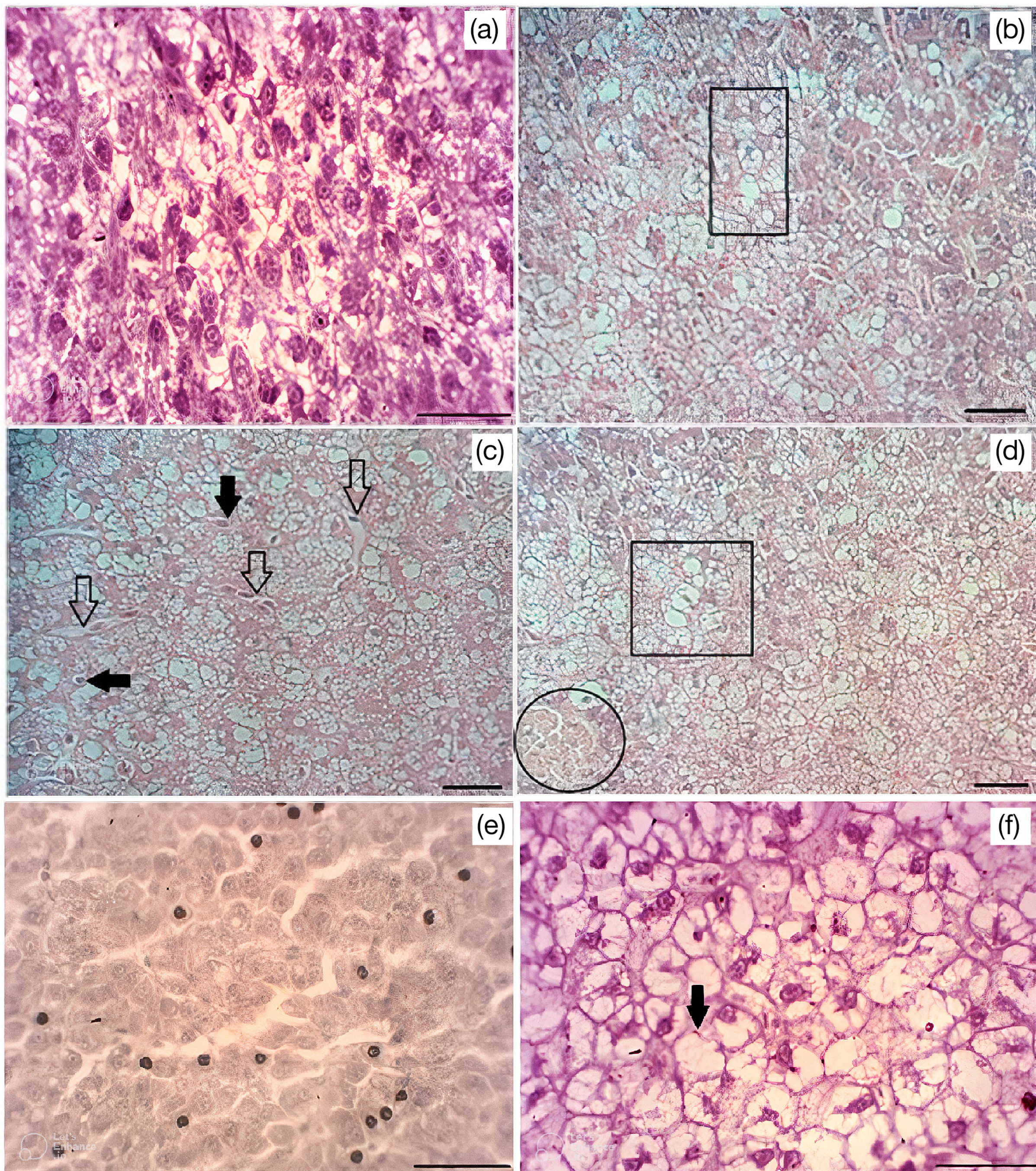

Fig. 2. H&E staining of fish (*Ctenopharyngodon idella*) liver (n = 3, each run in triplicate) exposed to FPN. (a) Control with normal cells; (b) liver from the treatment group in which fat-containing vacuoles (hepatic steatosis) are evident rectangle; (c) liver from the treatment group in which vein dilatation (outline arrow) and pyknosis (black arrow) are evident; (d) liver of the treatment group in which melanomacrophages (circle) and hepatic steatosis (square) are evident; (e) hepatic pyknosis; (f) hepatic steatosis (black arrow). Scale bars = 0.02 mm

increased formation of melanomacrophage centers observed in the present study is mainly due to oxidative stress (Ardeshir et al. 2022). Previous studies have shown that the biotransformation of toxicants leads to the generation of reactive oxygen species (ROS) in the liver and the increased formation of melanin is a strategy to neutralize these (Mohamed et al. 2019). On the other hand, the formation of lipofuscin is due to re-

peated cycles of lysosomal uptake and the release of macromolecules oxidized by the oxygen-derived free radicals in the Fenton reaction (Jung et al. 2007). In addition, the formation of hemosiderin is dependent on the discharge of erythrocytes to the surrounding tissues. This discharge results from lesions caused by toxicants (Paunescu et al. 2010). The pyknosis observed in the present study is the intense nuclear condensation that occurs during the final step of all apoptosis and some necrosis, and can only be seen in stress conditions. Previous studies have shown that this damage only occurs in livers exposed to the highest levels of oxidative stress and CYP1A gene expression (Ardeshir et al. 2018). Another type of liver damage, steatosis, is seen when the liver is under oxidative stress leading to depletion of long chain polyunsaturated fatty acids (LCPUFA) (Videla et al. 2004). Therefore, the observed difference in sensitivity to FPN-mediated liver lesions is likely due to differences in hepatic biotransformation phase activities and oxidative stress.

5. CONCLUSION

The liver biotransformation enzymes in grass carp were highly active after exposure to environmental concentrations of FPN, and exposure to this insecticide led to hepatic toxicity in the fish, as shown by liver damage. However, this response differed based on the exposure concentration and time, indicating more complex interactions between the phase I and phase II biotransformation enzymes, which should be investigated in future studies with more replications.

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