

ORIGINAL RESEARCH PAPER

Intraperitoneal fipronil effects on liver histopathological, biochemistry and morphology in Caspian kutum, *Rutilus frisii kutum* (Kamenskii, 1901)

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ABSTRACT: Fipronil is a relatively new insecticide in agriculture with health and environmental effects. This is the first report studying effect of fipronil on fish administered via intraperitoneal route. Intraperitoneal LD₅₀ of fipronil in 16.3 g Caspian kutum, *Rutilus frisii kutum*, fingerlings was determined using a total of 133 fish in 19 tanks (7 fish/tank) including one control and 6 treatment groups (300, 450, 550, 650, 750, 850 mg/kg). Fish were injected intraperitoneally and monitored at 96 h. The LD₅₀ of fipronil was 632 mg/kg in Caspian kutum. Sub-lethal test doses of 10, 20, and 30% of the LD₅₀ at 96 h were used to assess the effect of fipronil on the fish's liver. The blood plasma of 90 fish were used (18 at each test dose and in controls) on days 7 and 14 for biochemistry. The hepatosomatic index (HSI) of the livers were obtained and histopathology done on the same days. Pyknosis, sinusoid dilation and vacuolization were common histological changes, and these changes became more severe in a time and dose dependent manner. This dependence was also observed for HSI and the liver biochemical test (alanine and aspartate transaminase). Liver histological alterations showed that fipronil can be a potential factor in liver carcinoma.

KEYWORDS: Caspian Sea; Fipronil; Hepatosomatic index; Intraperitoneal route; *Rutilus frisii kutum*.

INTRODUCTION

Fipronil is a relatively new insecticide with extensive uses in agricultural and urban areas. It has low to moderate solubility and affinity in water and organic carbon, respectively (Budd *et al.*, 2015). Toxicity of this phenylpyrazole insecticide results from its ability to block gamma-aminobutyric acid-gated chloride channels of neurons in the central nervous system (Casida and Durkin, 2015). Since

decades ago, many pest insects started to develop relative resistance to classic insecticides (Alyokhin *et al.*, 2008). This adaptation resulted in introduction of new insecticides, such as fipronil and neonicotinoid, to the global market. These new insecticides have some advantages over previous ones including selective toxicity to pest, systemic nature and ease of use (Simon-Delso *et al.*, 2015). The increasing use of fipronil has raised concerns for its harmful effects on human health and the environment (Tingle *et al.*, 2003). In addition to insects, fipronil has toxic effects on non-target organisms, such as aquatic

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invertebrates (Key et al., 2003), fish (Beggel et al., 2010; Aghamirkarimi et al., 2017), some reptiles (Peveling et al., 2003), birds (Kitulagodage et al., 2011) and mammals (De oliveira et al., 2012a). The acute toxicity of fipronil has been determined for these animals. Median lethal concentration (LC₅₀) determinations in different species of fish showed that fipronil has a high toxicity (100-1000 µg/L) in most fish such as rainbow trout (*Oncorhynchus mykiss*, 246 µg/L), Japanese carp (*Cyprinus carpio*, 340 µg/L), sheep head minnow (*Cyprinodon variegatus*, 130 µg/L) and a very high toxicity (<100 µg/L) in bluegill sunfish (*Lepomis macrochirus*, 83 µg/L) and Nile tilapia (*Oreochromis niloticus*, 42 µg/L) (Gibbons et al., 2015). The median lethal dose (LD₅₀) of fipronil in fish has not been studied so far. The determination of chemicals LD₅₀ in fish is a simpler, faster and less expensive than determining the LC₅₀ (Hodson et al., 1988, Hutchinson et al., 2003; Ritz, 2010). The participants in the Collaborative Workshop on Aquatic Models and 21st Century Toxicology, held at North Carolina State University on May 5-6, 2014, agreed that small fish models can be used as biological models in toxicology and have some important advantages over mammalian models if standardized protocols are prepared and used (Planchart et al., 2016). They also recognized the need for additional studies on fish toxicology and ways to obtain non-water exposure of fish to toxicants. In the area south of the Caspian Sea, pesticides, especially fipronil, are used in rice fields against striped rice stemborer. The streams containing fipronil from the farms enter the Caspian Sea (salinity ≈13 ppt) and might affect aquatic life. In addition to environmental concerns, fipronil and other pesticides might also affect public health when the fish are consumed. There are some reports about the prevalence of human gastrointestinal cancer in the Caspian Sea region (Ghadimi et al., 2007; Mohebbi et al., 2008), and a possible association between pesticides and human liver cancer (Dich et al., 1997; Soliman et al., 2010; VoPham et al., 2015). Insecticides such as fipronil are detoxified in the liver and it has been hypothesized that such insecticides contribute to liver injuries through mechanisms such as cell adhesion alterations, oxidative stress, gene toxicity, tumor stimulation, toxicity in immune and hormone systems (VoPham et al., 2017). Thus, liver alterations resulting from the intraperitoneal (i.p.) exposure to fipronil were studied.

Caspian kutum (*Rutilus frisii kutum*), belonging to the cyprinidae family, is the most consumed and popular fish in this region and cultured extensively. Thus, this species was used as a model to determine median lethal dose of fipronil in fish and studying liver histological, biochemical and morphological alterations. This study has been carried out in Shahid Rajai Fish Proliferation Center and Babol University of Medical Sciences in 2016-17.

MATERIALS AND METHODS

Fish

Two hundred and fifty Caspian kutum fingerlings (mean body weight: 16 ± 3 g) were purchased from the Shahid Rajai Fish Proliferation and Culture Center (Sari, Mazandaran Province, Iran). The fish were randomly distributed into groups without regard to their sex. Fish were acclimated for 2 wk prior to the test, and fed commercial fish food until the day before fipronil exposure.

Determination of 96 h LD₅₀ value for fipronil

Nineteen plastic tanks (1000 L capacity) including a negative control tank (no replicate) and treatment tanks with water-shower aeration and a semi-static system were used. Non-chlorinated well water with characteristics showed in Table 1 was used. The photoperiod was 13 h light and 11 h dark. The volume of water used in each tank was about 200 L.

Preparation and injection of fipronil solution

Fipronil (98% purity, 50:50 racemic mixture) was purchased from the Moshkfam Fars Chemical Co. (Shiraz, Iran). Stock solutions of fipronil were prepared in 6 amber glass vials containing 5cc sunflower oil and 108, 162, 198, 234, 270, 306 mg fipronil and one glass vial containing only 5cc sunflower oil. To dissolve fipronil in the oil, the stocks

Table 1: Characteristics of groundwater used for the experiment

Parameter	Value
pH	6.9 ± 0.3
Dissolved oxygen (mg/L)	7.8 ± 0.5
Temperature (°C)	18.1 ± 0.8
Total hardness (mg/L)	394 ± 7
Total dissolved solid (mg/L)	445 ± 15
EC (µs/cm)	856 ± 20
Nitrate (mg/L)	0.9 ± 0.3
Nitrite (mg/L)	0.009 ± 0.003
Bicarbonate (mg/L)	405 ± 14

were vortexed for 30 min. Before the injection, the fish were anesthetized using 0.1% 2-phenoxyethanol, and weighed. For each treatment, 0.25 ± 0.05 cc of the standard solution was intraperitoneally injected into the fish using an insulin syringe based on the weight of each fish as described below.

LD₅₀ determination

There were 6 treatment groups with three replicates and 7 fish in each group. After some experimental tests to estimate the lethal dose range, fipronil was intraperitoneally injected into the fish at 300, 450, 550, 650, 750, 850 mg/kg of fish weight. The fish were monitored for 96 h (4 d) for any mortality and then sacrificed for histopathological tests.

Experimental design

Sub-lethal test dose of 10, 20, and 30% of LD₅₀-96 h (65, 130, 200 mg/kg) were used to assess the effect of fipronil on Caspian kutum livers. A total of 90 fish were used (18 at each test dose and then the controls) and were placed in 15 plastic tanks. The fish were anesthetized as previously described and injected intraperitoneally with the above doses in half dose twice a week. Sampling was carried out on days 7 and 14 (Table 2).

Liver biochemical and morphological assays

On day 7 and 14, 9 fish for each dose were anesthetized. The blood was collected from the caudal vein using heparinized syringes and transferred into ice-chilled vials. The vials were centrifuged ($1000 \times g$, 10 min) and obtained plasma were stored at $-80^\circ C$ until further use. The livers were carefully dissected, weighed and fixed in Bouin's solution.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were assessed according to the methods proposed by the International Federation for Clinical Chemistry as modified for a commercially available kit (Pars Azmoon Co., Tehran, Iran) and using an Auto-analyzer (Response 910, Dia

Sys, Holzheim, Germany) at 340 nm and $37^\circ C$.

Histopathological tests

The fixed liver were rinsed in a graded series of ethanol to be dehydrated, cleared in xylene, embedded in paraffin, sectioned at a thickness of 5 μm and stained with hematoxylin and eosin (H & E). Three random sections per fish tissue were observed under the light microscope (Olympus Co., Tokyo, Japan) and photographed using a Microscope Camera Eyepiece (Dino-Lit Premier AM7023; AnMo Electronics Corporation, Taiwan).

Statistical methods

Data analysis was done using MedCalc (ver. 16.8.4) statistical software (Microsoft Partner, Korea). The acute toxic effect of fipronil on the Caspian kutum was determined by the use of Finney's probit analysis. Sigma Plot ver. 11 software (Systat Software, Inc., CA, USA) was used for statistical analysis. Independent-sample t- tests were used for comparison of biochemical components. For multiple comparisons, one-way ANOVA test followed by Student-Newman-Keuls test was used. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Clinical signs

About 12 h after i.p. injection, fish showed clinical signs of semi-circular swimming behavior. In high dose treatments, the fish showed muscle shivering. In some cases, darkening and swelling on the dorsal side were also observed. The dead fish showed erected pectoral fins, larger livers (increased liver weight) and color change of the gallbladder bile along with more reddish kidney.

Intraperitoneal median lethal dose of fipronil

No mortality was recorded during the acclimation period except for the first day of the acclimation with 4 mortalities and in the control group. During

Table 2. Experimental design for fish exposure to fipronil and tissue sampling

Groups	Day 0 (Injection)	Day 7 (first sampling)	Day 14 (second sampling)
Control (18 fish)	No injection	9 fish	9 fish
Solvent control (18 fish)	i.p. oil injection	9 fish	9 fish
65 mg/kg (18 fish)	i.p. fipronil injection	9 fish	9 fish
130 mg/kg (18 fish)	i.p. fipronil injection	9 fish	9 fish
200 mg/kg (18 fish)	i.p. fipronil injection	9 fish	9 fish

the exposure period, the fish were considered dead if they did not have any movement. Most mortalities happened on the first day (Table 3) in the treatment groups. No mortality was observed in the negative controls. The LD₅₀ was calculated as 632 mg fipronil/kg fish (95% Confidence Interval (CI) = 585 – 682) (Table 4 and Fig. 1).

Fish were injected intraperitoneally with different doses of fipronil. LD50 was calculated as 632 mg/kg. Confidence interval (95%) curves are also shown in the Fig. 1.

This is the first study to determine i.p. LD₅₀ of fipronil in fish. Trophic (feeding), waterborne exposure, and i.p. injection are the common routes for

administration of xenobiotics in fish. The preferred route of administration depends on the purpose of the study and physicochemical properties of the toxicants. Median lethal concentration (LC₅₀) and dose (LD₅₀) have been widely used to determine acute toxicity of toxicants in aquatic and terrestrial animals, respectively. Waterborne administration has advantages such as simulating environmental exposure, not requiring anesthesia and less handling of fish and relatively more absorption rate constant for contaminants. Compared to waterborne (w.b.) exposure, evaluating i.p. exposure to fipronil in fish has some advantages. Although both LD₅₀ and LC₅₀ estimate expressed toxicity, LD₅₀ can be a closer estimate of the inherent

Table 3: Daily numbers of dead Caspian kutum after injection with different doses of fipronil

Dose (mg/kg)	Number of dead fish by day				
	Day 1	Day 2	Day 3	Day 4	Decapitation on day 5
300	0	0	0	0	21
450	5	0	0	0	16
550	7	0	0	0	14
650	8	0	0	3	10
750	9	0	0	3	9
850	15	0	3	3	0
Total	44	0	3	9	70

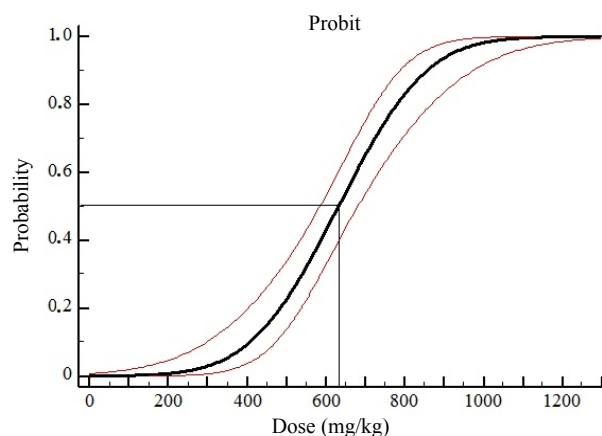


Fig. 1: Fipronil 96-h predicted mortality – dose response curve for Caspian kutum fingerlings based on parameter estimates from the probit analysis

Table 4: Dose-response values after i.p. injection into Caspian kutum (degree of freedom was 1, P<0.05).

Probability	Dose (mg/kg)	95% Confidence interval	
LD ₁₀	407.9	298.2	472.8
LD ₂₀	485	403.6	537.6
LD ₅₀	632.4	585.4	681.5
LD ₈₀	779.9	724.6	867.9
LD ₉₀	857	788.6	974.1
LD ₉₉	1040	934.1	1230

toxicity and is determined based on a whole-body dose (mg/kg) and not concentration (mg/L) (Hodson *et al.*, 1988). Moreover, toxicological studies, such as those for determining the detoxification mechanisms in fish can be more accurately extrapolated to terrestrial mammals based on LD₅₀. The other reason for determination of LD₅₀ of fipronil in fish is due to its low/moderate water solubility (Aajoud *et al.*, 2003) which makes it difficult to directly and exactly find measure the fipronil dose response relationship. In addition, photolysis can transform fipronil into its metabolites (Bobe *et al.*, 1998; Simon-Delso *et al.*, 2015), which are more toxic than the primitive compound. Therefore, the LD₅₀ is only for fipronil. On the other hand, to measure LC₅₀ with larger fish

needs larger amounts of fipronil and water in a non-static system, which is not good economically and environmentally.

Some factors affecting toxicity of insecticides include species, weight of tested fish, the water quality characteristics (Altinok *et al.*, 2006), purity of the fipronil, and route of exposure (intraperitoneal or waterborne exposure). In this study, all factors were the same (including the mean of fish weights ± SD) between the test and the control group.

In addition to the dose and duration of exposure, the route of administration can significantly affect the degree of toxicity (Driver *et al.*, 1991; Pickford *et al.*, 2003). According to the US Environmental Protection Agency (EPA, 2012), LD₅₀ measured in this study for

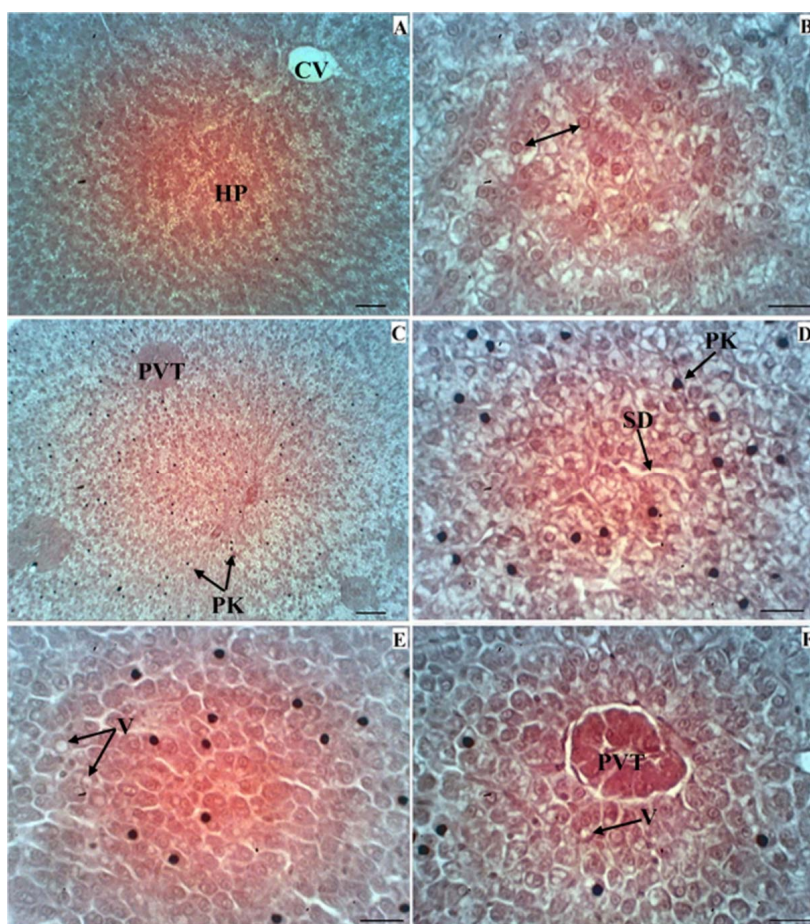


Fig. 2: Transverse sections showing the histopathology of livers with fipronil exposures in the first week of sampling. A ($\times 725$) and B ($\times 2900$) Paranchymal structure of hepatocytes in the control group with the central vein (CV) and normal nuclei. C ($\times 725$) and D ($\times 2900$) Pyknotic nuclei (PK) and sinusoid dilation (SD) in hepatocytes along with pancreatic-venous tracts (PVT) in the 65 mg/kg group. E ($\times 2900$) and F ($\times 2900$) Vacuolization (V) in hepatocytes in the 130 and 200 mg/kg groups, respectively. H&E, scale bar: 0.05 mm (A and C) and 0.02 mm (B, D, E and F).

fipronil (632 mg/kg) would have a slight toxicity (501-2000 mg/kg) while previous LC_{50} determinations in different fish showed that fipronil has a high to very high toxicity (Tingle et al., 2003). After i.p. injection, fipronil is absorbed into the bloodstream, transferred directly to the liver with potential detoxification while in w.b. exposure, fipronil is distributed to other organs before potential detoxification in the liver.

Histological changes

The livers of *Rutilus frisii kutum* include the exocrine pancreatic tissue (hepatopancreas). The liver histology of this species showed the sponge-like structure of the parenchyma which was basically made up of polygonal hepatocytes and cords of hepatocytes that encompass sinusoidal portal blood (Fig. 2). For the first sampling (the 7th day of exposure), pyknosis,

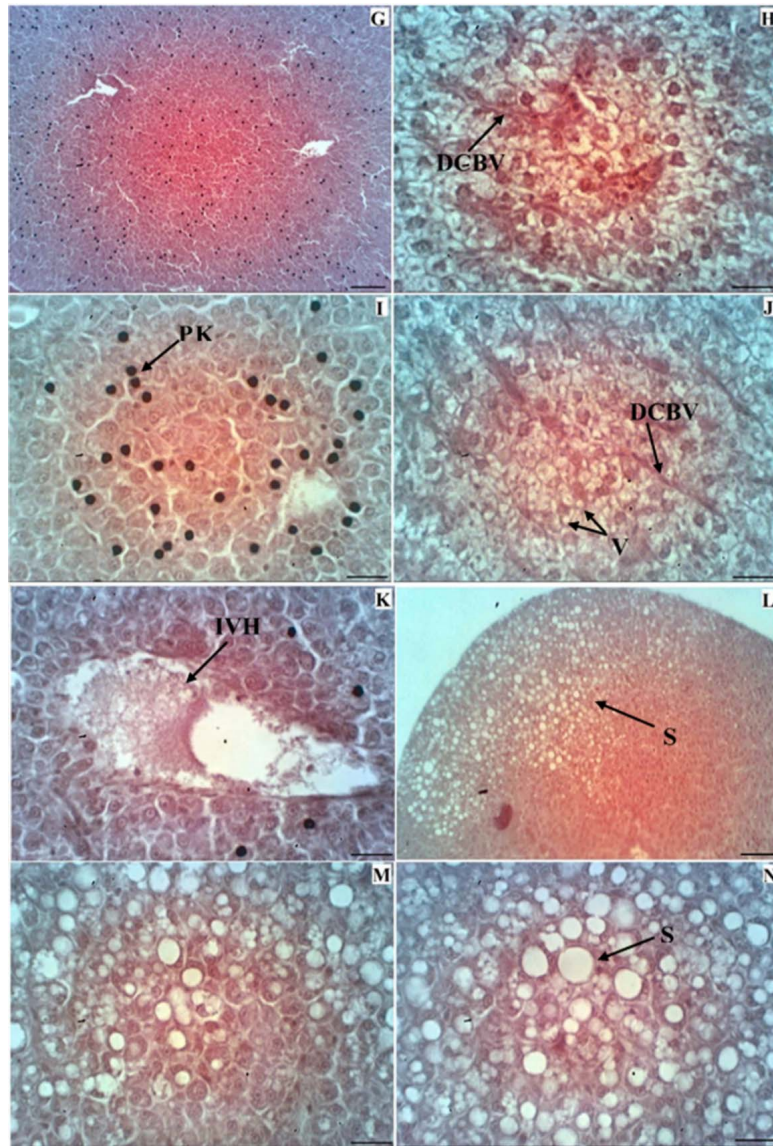


Fig. 3: Transverse section showing histopathology of liver on fipronil exposures in the second week of sampling. G ($\times 725$), H ($\times 2900$), I ($\times 2900$) Dilation and congestion in blood sinusoids (DCBV) in the 65 mg/kg group. J ($\times 2900$), K ($\times 2900$), L ($\times 725$), M ($\times 2900$) Intravascular haemolysis in blood vessels (IVH) and steatosis (S) in the 130 mg/kg group. N ($\times 2900$) Extensive steatosis (S) in the 200 mg/kg group. H&E, scale bar: 0.05 mm (G and L) and 0.02 mm (H, I, J, K, M, N).

sinusoid dilation and vacuolization were observed. For the second sampling, these alterations were more severe (Fig. 3). Liver histological changes in fipronil exposed fish are shown in Table 5.

Histological alterations may result from direct toxic effects, while others are defense strategies against toxicants (Bhagwant and Elahee, 2002). Livers are the most important organ for detoxification and biotransformation of toxicants (Van der Oost *et al.*, 2003). Some of the alterations observed such as sinusoid dilations and cytoplasmic vacuolations might result from circulatory system disturbances (Ferreira *et al.*, 2012). Another alteration was congestion in the blood sinusoids, a process that leads to more blood cells sooner in the liver, which facilitates migration of the white blood cells that aid in removal of foreign substances (De Oliveira *et al.*, 2012b). Vacuolization and occupation of extensive area of the cytoplasm may result in peripheral nuclei and consequently nuclear atrophy. This study also showed that fipronil (130 and 200 mg/kg) stimulates steatosis in the fish hepatocyte after 2 wk. This change along with hypertrophy can be related to endoplasmic reticulum changes (Ferreira *et al.*, 2012). Metabolization, inactivation and detoxification of many compounds are done by this organelle (De Oliveira *et al.*, 2012b). Fipronil injection through i.p. route can lead to hypertrophy of this organelle and consequently hyperactivity of its enzymes contributing in detoxification of fipronil as well as in lipid synthesis. The control group showed some vacuolizations that might be due to the use of oil as a carrier. However, healthy hepatocyte normally show some vacuoles. The occurrence of steatosis in the liver of mice and rats administrating fipronil has been reported (De Oliveira *et al.*, 2012b; Ferreira *et al.*, 2012). Trauner *et al.* (2010) reported that if steatosis persists and worsened, it could lead

to fibrosis, cirrhosis and carcinoma. Qureshi *et al.* (2016) observed hemorrhagic liver, hyperplasia and nuclear hypertrophy, ceroid formation, contraction of central veins, cytoplasmic shrinkage and eccentric nuclei and tissue congestion in common carp after waterborne exposure to fipronil (400 µg/L) for 96 h. In the present study, pyknosis was the most obvious damage seen in the liver and is the initial step toward necrosis or apoptosis. It seems that livers will show more damages after chronic exposure to fipronil, as reported by Mossa *et al.* (2015) for the livers of male albino rats after sub-chronic exposure (45 d) to fipronil. Fipronil could induce apoptotic cell death by activation of some enzymes contributing to the apoptotic process such as caspases 3 and 7 (Das *et al.*, 2006; Ferreira *et al.*, 2012).

Morphological and biochemical alterations

The results showed that liver morphological and biochemical alterations increased with increasing i.p. fipronil (Figs. 3 to 6). There was no significant difference in weight of fish between control and treatment groups after 7 and 14 days of exposure to fipronil (data not shown). Previous studies showed that liver weight increases as a result of exposure to pollutants. However, there is no report to assess effect of different concentrations/doses of fipronil on HSI.

The highest HSI of the Caspian kutum exposed to fipronil (200 mg/kg, wk 2) may be due to an increase in liver fat content (steatosis). The stimulatory effect of fipronil on vitellogenin (Vtg) production, however, and the subsequent increase in liver weight should also be considered. Recent study showed that fipronil could induce elevation in hepatic Vtg and estrogen receptor α (ER α) gene expression in male Japanese quail (Khalil *et al.*, 2017). In addition, Beggel *et al.* (2012) reported highly significant elevation in the Vtg

Table 5: Impact of intraperitoneal exposure to fipronil in livers of Caspian kutum for 7 and 14 days.

Parameter	Solvent control	Fipronil dose (mg/kg)- first sampling			Fipronil dose (mg/kg)- second sampling		
		65	130	200	65	130	200
Pyknotic nuclei	-	+	+	++	+++	+++	+++
Vacuolation	+	+	+	++	++	+++	+++
Dilation and congestion in blood sinusoids	-	+	+	+	+++	+++	+++
Intravascular haemolysis in blood vessels	-	-	-	+	+	++	+++
Sinusoids dilation	-	+	+	+	++	++	++
Steatosis	-	-	+	+	+	+++	+++
Hypertrophy	-	+	+	+	++	++	++

(-) None, (+) mild, (++) moderate, (+++) severe

Fipronil effects on Caspian kutum liver

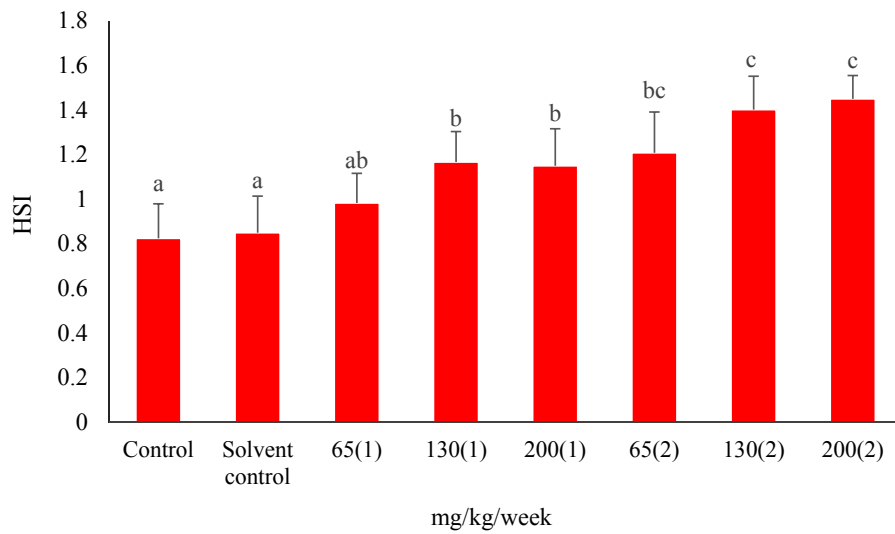


Fig. 4: Mean hepatosomatic index (HSI, mean \pm standard error) of the Caspian kutum after 7 (1) and 14 (2) days of exposure to fipronil

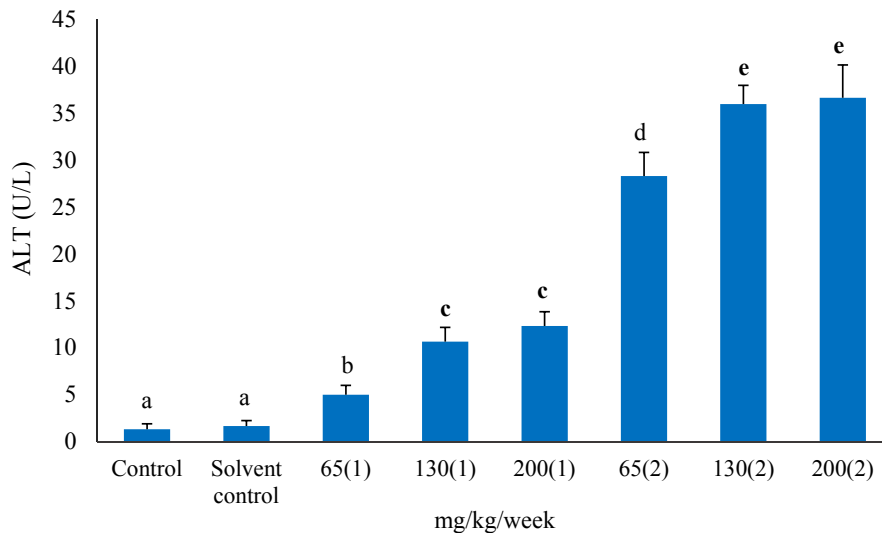


Fig. 5: Means \pm SD for alanine transaminase (ALT) (unit/L) in the Caspian kutum exposed to fipronil for 7 (1) and 14 (2) days

gene expression in larval fathead minnow exposed to fipronil for 24 h.

AST and ALT participate in amino acids catabolism and biosynthesis, detoxification processes, metabolism and biosynthesis of energetic macromolecules (Seven *et al.*, 2004), and are used as biomarkers for liver injury. Whereas the AST is a mitochondrial enzyme found in various tissues, ALT can be found in high levels in the liver (Schiff *et al.*, 2007). The results showed that ALT

and AST levels increased as fipronil dose and time increased so that the highest levels of these enzymes were found in the fish treated with 200 mg/kg after 2 wk. However, there was no significant difference in ALT levels in groups treated with 130 and 200 mg/kg. The histology showed that fipronil exposure could lead to histopathological changes in a dose and time dependent manner. Thus, the increased levels of ALT and AST could be due to liver histological changes

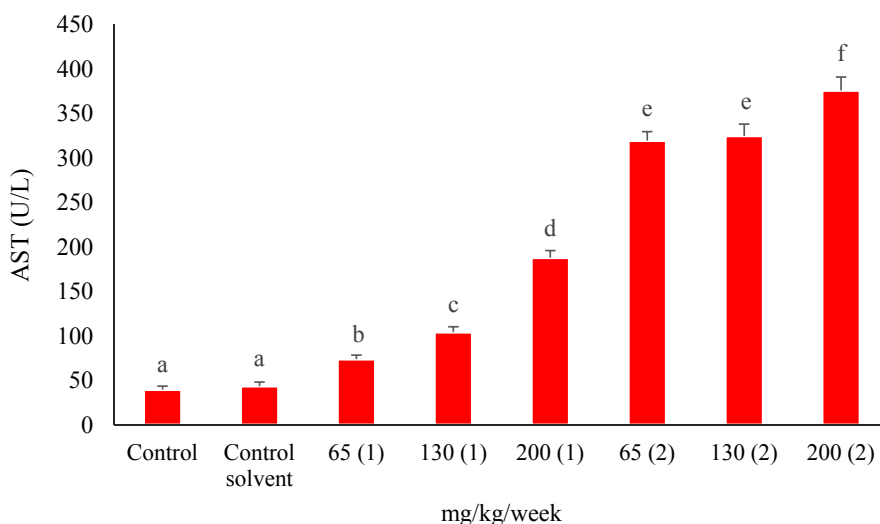


Fig. 6: Means \pm SD of the levels of aspartate aminotransferase (AST) (unit/L) in the Caspian kutum exposed to fipronil for 7 (1) and 14 (2) days

or injury to other organs (results not shown), which resulted in cytosolic leakage of these enzymes into the blood (Bhattacharya *et al.*, 2008). Moreover, previous study showed that stress could induce elevation of ALT and AST levels for energy production through catabolism of amino acids (De Smet and Blust, 2001). Gupta *et al.* (2014) reported that *Cyprinus carpio* fry exposed to sublethal concentrations of fipronil (142 μ g/L) showed significant increases in AST and no significant change in ALT. Recently, significant increases in ALT and AST in male rats treated with three concentrations of fipronil (0.1, 1 and 10 mg/L) in drinking water for 45 days has been reported by Mossa *et al.* (2015). The current results showed that liver histological, biochemical and morphological characteristics of Caspian kutum exposed to various doses of fipronil changes in a dose and time dependent manner, and it seems that this dependence is more marked in the liver biochemical characteristics (ALT and AST).

CONCLUSION

This study showed that fipronil has slight toxicity in Caspian kutum when administered intraperitoneally. Liver histological, biochemical and morphological alterations in the fish exposed to various dose (65, 130, 200 mg/kg) of fipronil showed that these characteristics change in a time and dose dependent manner so that the most alterations were observed

at 200 mg/kg after 2 weeks. Steatosis was the most obvious histological alteration and those livers were the heaviest observed, and it seems that fipronil can be a potential factor in carcinoma. Among these characteristics, liver biochemical alterations seems to have more correlation with fipronil dose and time of exposure suggesting that they could be used as potential biomarker in Caspian kutum exposed to fipronil intraperitoneally.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

ABBREVIATIONS

<i>ALT</i>	Alanine transaminase
<i>AST</i>	Aspartate transaminase

<i>CV</i>	Central vein
<i>CI</i>	Confidence Interval
<i>d</i>	Day
<i>DCBV</i>	Dilation and congestion in blood sinusoids
<i>ERα</i>	Estrogen receptor α
<i>h</i>	Hour
<i>H & E</i>	Hematoxylin and eosin
<i>HP</i>	Hepatocyte
<i>HSI</i>	Hepatosomatic index
<i>i.p.</i>	Intraperitoneal
<i>IVH</i>	Intravascular haemolysis in blood vessels
<i>LD₅₀</i>	Median lethal dose
<i>LC₅₀</i>	Median lethal concentration
<i>mg</i>	Milligram
<i>min</i>	Minute
<i>PK</i>	Pyknotic nuclei
<i>ppt</i>	Part per billion
<i>PVT</i>	Pancreatic-venous tracts
<i>S</i>	Steatosis
<i>SD</i>	Sinusoid dilation
<i>U/L</i>	International unit/ Liter
<i>V</i>	Vacuolization
<i>Vtg</i>	Vitellogenin
<i>w.b.</i>	Waterborne
<i>Wk</i>	Week
<i>kg</i>	Kilogram
<i>μg</i>	Microgram

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