CYP1A gene expression as a basic factor for fipronil toxicity in Caspian kutum fish

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ABSTRACT

The aim of this study was to assess the effects of fipronil insecticide on the Caspian kutum fish at different levels of biological organizations and to find possible relationship between these biomarkers. Different doses of fipronil (65, 130 and 200 mg/kg) were intraperitoneally administered to the fish for 2 weeks. After 7 and 14 days of exposure, alterations in organ-somatic index, tissue and DNA structure, oxidative stress and CYP1A gene expression in gill, liver, brain and kidney were studied. Determination of these parameters in the liver showed that the degree of tissue change (DTC), comet tail, superoxide dismutase (SOD) and relative CYP1A mRNA expression increased mostly in a time dependent manner whereas in the kidney increased mostly in a dose dependent manner. These parameters in the gill increased more in time and dose dependent manner. Apart from the changes in CYP1A expression and oxidative stress, no alterations was observed in the brain. Multiple regression analysis showed that the CYP1A had the most correlation with the organ-somatic index (R² = 0.76) and comet tail (R² = 0.89) in the liver, and with DTC (R² = 0.93) and oxidative stress (R² = 0.87) in the kidney. Generally, this study showed that CYP1A gene expression can be considered as one basic factor for fipronil toxicity in this fish. However, other possible factors also should be considered for future research.

1. Introduction

According to the global pesticide market, about 3 million tons of pesticides intentionally are released into the environment each year [1]. This wide use of pesticides can cause harmful effects on non-target organisms, especially marine organisms. Fipronil, which was produced by a French company in 1987, has raised concerns for its dangerous effects on human health and the environment [2], resulting from the increasing use in agricultural and residential zones.

Fish as the earliest class of vertebrates are extensively studied in aquatic toxicology. Moreover, scientific recommendations imply that the use of fish model in toxicology, both in ecological and biomedical studies, is a good option [3,4]. In the area south of the Caspian Sea, fipronil (under the trade name Regent®) is widely used in rice farms to kill pest insects and can reach the Caspian Sea and might threat aquatic life. Caspian white fish (Rutilus frisii kutum), classified in the cyprinidae
family, is the most popularly consumed fish in this region and cultured extensively. The life span of this species is about 9–10 years in southern part of Caspian Sea [5]. Thus, this fish was used as a biological model for studying toxicological effects of fipronil on aquatic life in this sea.

Fipronil can be exposed to fish through different routes and the most relevant route depends on the aim of the study and the physicochemical characteristics of the fipronil. Ardeshir et al. [6] compared the effects of waterborne and intraperitoneal (i.p.) routes of exposure to fipronil in the Caspian kutum and cited the advantages of i.p. route over waterborne exposure in fish. It seems that i.p. administration is the best route to study fipronil toxic effects on fish in a time/dose dependent manner. Although the mechanism of fipronil toxicity in insects has been fully determined and is related to blocking gamma-aminobutyric acid-gated chloride channels of neurons in the central nervous system [7], there is not enough information about its mechanism in vertebrates due to the existing complex interactions. However, generally, mechanism of toxicity of persistent organochlorinated pesticides might be explained through some biological reactions including binding to some receptors such as aryl hydrocarbon receptor (AhR) and induction of biotransformation enzymes such as CYP1A, oxidative stress and triggering pathological-related condition and DNA damage [8]. Thus, exposure to fipronil in fish might exert changes in biological responses at different levels of biological organization including morphological and biochemical parameters, histopathology, genotoxicity and alteration in gene expression of biotransformation enzymes. The present study was carried out to investigate these parameters in gill, liver, brain and kidney of the Caspian kutum exposed to fipronil intraperitoneally and to show correlation between biomarkers at different levels of biological organization. To assess genotoxic effect of fipronil and measure DNA strand breaks, the comet assay as a rapid and reliable technique [9,10] was used and comets were analyzed by Cellpro software. This software is able to analyze and identify thousands of biological images by advanced algorithms, and causes to save time and eliminate objective errors [11,12]. At gene level, the expression of CYP1A gene, as a member of CYP gene superfamily, was assessed. In addition to the detoxification, previous studies also demonstrated the potential function of CYP1A as a tumor suppressor [13–15]. There is a little information about effect of fipronil on fish CYP gene expression and activity. Tang et al. [16] suggested that CYP3A4 is the major isofrom responsible for fipronil oxidation in human. Afterwards, Das et al. [17] reported that fipronil can induce CYP3A4 and CYP1A1 by enhancing mRNA as well as protein expression in human hepatocytes. However, Caballero et al. [18] reported that fipronil has major effects on CYP1A activity in rat liver. In fish, CYP1A subfamily has important roles in the metabolism of exogenous chemicals, especially pesticides, and is extensively used as a biomarker to assess contamination of the aquatic environment [19–22]. Thus, in this study, gene expression of this enzyme was used as biomarker of fipronil toxicity as well.

2. Materials and methods

2.1. Fish

One hundred Caspian white fish fingerlings (16 ± 3 g and 11 ± 2 cm) were obtained from the Shahid Rajai Fish Proliferation and Culture Center (Sari, Mazandaran Province, Iran) and exposed to fipronil in this center. Prior to the test, fish were acclimated to the treatment condition for one week, and fed powdered fishmeal until the day before fipronil exposure. Non-chlorinated well water with water-shower aeration and a semi-static system in plastic tank (200 L) was used, along with 13 h light and 11 h dark as the photoperiod.

2.2. Experimental design

Fipronil (98% purity, 50:50 racemic mixture) was bought from Moshkham Fars Chemical Company (Shiraz, Iran). Stock solutions of fipronil were made by dissolving 50, 100, 150 mg of this compound in 10 mL sunflower oil. Before the injection, the fish were anesthetized using 2-phenoxyethanol (0.2%), and their length and weight were measured. For each dose, 0.24 ± 0.04 mL of the stock solution was intraperitoneally injected into the fish using an insulin syringe based on the weight of each fish. Sub-lethal test doses of 10, 20, and 30% of LD50=96 h (65, 130, 200 mg/kg) were used according to the Lp. LD50 of fipronil in the Caspian kutum (632 mg/kg) determined in the previous.
study [6,23]. A total of 72 fish (Fig. 1) were divided into three treatment groups and one negative control group (vehicle group) and placed in 12 plastic tanks (3 replicates).

The fish were anesthetized and injected intraperitoneally with the above doses weekly (divided in two doses) for 2 weeks. Sampling was carried out on day 7th and 14th. After anesthetizing and measuring the length and the weight, the blood was collected from the caudal vein using heparinized syringes and transferred into ice-chilled vials. The vials were centrifuged (1000g, 10 min) and obtained plasma were stored at −80 °C until further use. For each sampling time, 9 fish from each group were carefully dissected and gills, kidneys, livers and brains from 3 fish were weighted (except for kidney which is not a discreet organ in fish) and fixed in Bouin’s solution. For comet assay, these organs from 3 other fish were transferred into cryotubes containing DMSO and PBS (1:9), frozen in liquid nitrogen, transported to Cellular and Molecular Biology Research Center in Babol University of Medical Science (Babol, Iran) and maintained (48 h) at −80 °C. Finally, the organs from 3 remained fish were assigned to gene expression and oxidative stress tests and stored at −80 °C after transferring into cryotube and liquid nitrogen freezing.

2.3. Histopathological tests

The fixed livers, gills, kidneys and brains were dehydrated in increasing series of ethanol (70–100% for 4 × 1 h), cleared in xylene (2 × 1 h), embedded in paraffin (2 × 2 h) and sectioned (5 μm). Deparaaffinized samples in xylene (5 min) were hydrated in decreasing series of ethanol (4 × 2 min) and stained in eosis (3 min). After washing in tap water (5 min) and rinsing in acid alcohol (2×s), the tissues were stained with hematoxylin (15 min). Finally, the stained and washed slides were again dehydrated in the series of alcohol (4 × 1 min) and cleared in xylene (5 + 6 min).

For nissl staining, paraffinized brain tissues were rinsed in xylene and hydrated in increasing series of ethanol, 70% (3 min), 90% (4 min), 100% (2 × 5 min), and washed in tap water and dH2O (5 min). The slides were stained with Cresyl violet (4 min) and washed with dH2O and transferred into 90% (10 min) and 100% ethanol (2 × 5 min). Finally, the slides were cleared in xylene (2 × 5 min) and mounted.

The gill and liver were also stained with periodic acid shiff (PAS). After deparaffinization and washing in dH2O, the slides were rinsed in 0.5% periodic acid (5 min) and hematoxylin (10 min). Washing, dehydration and clearing were next stages for slides to be mounted.

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). The histological damages were assessed semi-quantitatively by measuring the degree of tissue change (DTC), according to the procedures of Poleksic & Mitrovic Tutundzic [24].

2.4. Biochemical analysis

Superoxide dismutase (SOD) and catalase (CAT) activity were also measured by using commercial available kits (ZellBio GmbH, Germany). Briefly, for each organ, the tissue was homogenized in PBS (1 mg: 100 mL), centrifuged at 5000g for 20 min. The supernatant was separated and used to make the sample and blank solutions using R1 (buffer), R2 (H2O2/O2− as substrate), R3 (chromogen, used only in sample solution) and R4 (diluent). After mixing (exactly 60 s for CAT reagents in a 96 wells microplate, the absorbance was read (at time 0 and 2 min for SOD) by ELISA reader (Awareness, Stat fax-2100, USA) at 450 nm and SOD and CAT activity were measured according to the following formulas:

\[
\text{SOD activity} \ (\text{U/mL}) = \frac{OD_{(\text{blank})} - OD_{(\text{sample})}}{19} \times 60; \quad \text{CAT activity} \ (\text{U/mL}) = (\text{OD}_{(\text{blank})} - \text{OD}_{(\text{sample})}) \times 45.16
\]

2.5. Comet assay

Alkaline version of comet assay was used to measure DNA damage along with modifications. To prepare lysing solution, NaCl (14.6 g), EDTA (7.3 g), Tris base (0.12 g) and NaOH (0.8 g) were dissolved in dH2O (100 mL) with final pH of 10. Before using the lysing solution, triton X100 (1 mL) and DMSO (1 mL) was added to the solution. To make electrophoresis buffer, 15 mL NaOH (0.4 g/mL) and 0.25 mL EDTA (0.7 g/mL) were added to 500 mL dH2O with pH 13. For neutralization buffer, 100 mL Tris base solution (0.048 g/mL) with pH 7.5 was used.

After thawing and discarding stabilizer solution, the organ was homogenized in PBS using a fine scissors, centrifuged (142g) for 10 min at 4 °C. Pellet dispersed in PBS (1 mL) was centrifuged again (93g) for 7 min at 4 °C. The obtained pellet along with PBS (1 mL) was gently vortexed, passed through a 100 μm nylon cell strainer (BD; Franklin Lakes, NJ) and kept on ice until transferring to slides. For preparing comet slides, conventional slides were dipped in hot normal melting agarose (1%) and dried overnight at room temperature. Then, 50 mL low melting agarose (75%) was prepared and kept warm in a water bath (37 °C). The melted agarose solution (300 μL) was mixed with the prepared liver cells (100 μL) in a microtube and 200 μL of mixed product was distributed on the agarose covered slides. Coverslips were gently removed after the slides were cooled (15 min) in a refrigerator. The slides were transferred into Schieferdecker jar, covered with aluminum foil, containing lysing solution and kept for 12–18 h at 4 °C. Then, electrophoresis was carried out (in darkness) by electrophoresis buffer (500 mL) for 25 min (300 mA and 20 V). Comets were washed in neutralization buffer (15 min), fixed in ethanol (100%) and dried at room temperature. For staining the comets, fluorescence and Giemsa staining were used. SyberGreen (1 μL stain + 10 mL TE buffer) and Giemsa (50%) solutions were exposed to slides for 7 (at 4 °C) and 4 min respectively. The slides stained with SyberGreen were observed and photographed by an Axioscop-40 FL fluorescent microscope (Carl Zeiss, Jena, Germany) equipped with a MRC-5 CCD camera. To analyze the comets, Cellprofiler® software 2.0 (revision: 10997 for Windows) was downloaded from “http://www.cellprofiler.org” website and used. There is a special pipeline that the software performs analysis on comets which is also downloadable from the site. This analysis was based on comet morphology (number of pixels in each comet), and the pipeline was regulated in the range of 25 pixel (comet with no damage) and 200 pixel (comet with severe damage) [12]. The computer used for this analysis involved 1.5 GHz Pentium Intel processor and 4 GB RAM with Windows 10.

2.6. Analysis of CYP1A gene expression

RNA was isolated by RNA extraction kit (YT9065, Yeka Tajhiz Azma, Iran) according to the manufacturer’s instructions. Briefly, after homogenization and mixing of thawed tissues (30 mg) in nuclease free tubes containing RB buffer (350 μL) and beta-mercaptoethanol (3.5 μL), the mixture was passed through filter column and centrifuged (18,000 g for 2 min). The supernatant was mixed with ethanol (70%), passed through RB mini column and centrifuged (1 min). To eliminate DNA contamination, washing buffer 1 (250 μL) was added to the column and centrifuged (1 min) and 60 μL RNase free DNase I solution (Roche) was exposed (15 min) to the column membrane. After washing the column twice and drying, ddH2O was added to the column and centrifuged (1 min).

To control the quality of extracted RNA, 2 μL loading buffer (Bromophenol blue + Sucrose + H2O) and the RNA (5 μL) was loaded on 1% electrophoresis gel (agarose + TBE) and electrophoresed (30 min at 85 V). To quantify the RNA concentration and its purity,
Nanodrop spectrophotometer (Thermo Scientific NanoDrop 2000, USA) was used (at 230, 260 and 280 nm wave lengths).

cDNA was also synthesized by a commercial available kit (Thermo Scientific, K1622). Briefly, after mixing and centrifuging random hexamer primer and the RNA and placing at 65 °C (5 min), RiboLock RNase Inhibitor (1 μL) was added (on ice). Then, dNTP (2 μL), Reaction Buffer (4 μL), RevertAid RT (1 μL) were added. The reaction was carried out in thermocycler (Peq STAR, Spain) (5 min at 25 °C, 1 h at 42 °C, 5 min at 70 °C and unlimited at 20 °C).

There was no report about CYP1A mRNA sequence for Rutilus frisii kutum and, consequently, qPCR primers for this fish were designed, based on the CYP1A mRNA sequences for relative species (Rutilus rutilus: 253 bp, Pinephales promelas: 390 bp, Hypophthalmichthys molitrix: 902 bp, Carassius auratus:2537 bp) that are available in National Center for Biotechnology Information (NCBI). The sequences of designed primers were forward: 5′-AATCGTCAATGACCTCTTCGG-3′ (21 bp, GC%: 47.6 and Tm: 59.4 °C) and reverse: 5′-ATCTGGGATAGGACACTAGA TAG-3′ (24 bp, GC%: 45.8% and Tm: 63.5 °C) for target gene (89 bp). The reference gene (94 bp) was ribosomal protein L8 of roach [25] with the following temperature program: 94 °C (3 min), 40 cycles of denaturation (95 °C for 5 s), 40 cycles of denaturation (95 °C for 5 s), and annealing/extending (58 °C for 20 s, the gradient temperature for each sample) cited above (30 s), 72 °C (60 s), 72 °C (7 min), 4 °C (5 min). After amplification, PCR products was electrophoresed on agarose gel (2%).

To choose the best temperature for the annealing reaction, RT-qPCR was carried out at gradient temperature for target [53, 54.4, 55.9, 57.3, 58.7, 60.1, 61.1, 63 °C] and reference [55, 56.4, 57.9, 59.3, 60.7, 62.1, 63.6, 65 °C] genes with the following temperature program: 94 °C (5 min), 35 cycles at 94 °C (30 s), the gradient temperature (one temperature for each sample) cited above (30 s), 72 °C (60 s), 72 °C (7 min), 4 °C (5 min). After amplification, PCR products was electrophoresed (85 V and 30 min) and qualified on agarose gel (2%).

To perform RT-qPCR, 10 μL qPCR Master Mix containing SyberGreen (YT2551, Yekta Tajhiz Azma, Iran), forward and reverse primers (0.4 μL), cDNA (1 μL), ROX dye (0.4 μL) and nuclease free H2O (20 μL final volumes) were prepared for each treated sample, reference gene and blank samples (without cDNA). All reactions (triplicates) were run on the ABI 7300 (Applied Biosystems, USA) using the following condition: initial denaturation (95 °C for 2 min), 40 cycles of denaturation (95 °C for 5 s), and annealing/extending (58 °C for 20 s, the best temperature determined for annealing). The q-PCR products were electrophoresed for qualification. Relative expression of CYP1A gene was calculated using the comparative CT (2−ΔΔCT) method [26].

### 2.7. Statistical methods

SPSS ver. 24 software (IBM corp., USA) was used for statistical analysis. The Mann–Whitney test was used for comparison of DTC results. For comparisons with more than two groups, One Way ANOVA was used along with Tukey test. Multiple regression analysis was used for finding correlation between biomarkers. The significance level was set at P < 0.05.

### 3. Results

During acclimation, 5 fish were died and replaced with new ones. No mortality was observed during treatment.

#### 3.1. Morphological alterations

Measuring length and weight of the Caspian white fish in different groups after 7 and 14 days showed no significant change compared to the control (P < 0.05). This result was also observed for condition factor (CF) (data not shown). However, this factor showed significant decrease in 200 mg/kg fipronil after 14 days (200 mg/kg (2)). The results showed that fipronil had significant effect on liver-somatic index in time and dose dependent manner (Fig. 2) and no significant effect on brain-somatic index. This effect was observed in the gill after 2 weeks.

#### 3.2. Histological alterations

Histopathological observations on liver, gill, kidney and brain are shown in Figs. 3–5 and Table 1. Hematoxylin & eosin and nissl stainings showed that these doses of fipronil have no effects on brain histology after 14 days of exposure. The liver showed some damages including pyknosis, hypertrophy, sinusoid dilatation, steatosis and glycogen deposition (Fig. 5). The DTC measured for liver showed slight damage after 14 days exposure to fipronil (11 ≤ DTC ≤ 20). Degenerative fatty vacuolization and hydropic glycogen droplets as plasma alterations increased obviously in 200 mg/kg after 14 days exposure. DTC measured (Fig. 6) showed that histological alterations in the liver is mostly dependent on exposure time. Intraperitoneal exposure to fipronil also caused some damages in the gills including fusion, hypertrophy and hyperplasia. Although DTC (0 ≤ DTC ≤ 10) showed normal performance of the gill, there were significant difference between control and treatment groups. Periodic acid shift (PAS) staining showed some histological alterations in cellular levels such as shrinkage of blood spaces and dilation of pillar cells (Fig. 3C and D). Degeneration in renal tubules, sinusoid dilatation, structural alterations and hemolysis were the most important damages observed in the kidney. These damages were more obvious in maximum dose whereas exposure time had less effect on toxicity of fipronil in this organ.

#### 3.3. Oxidative stress condition

Oxidative stress condition of the four organs are shown in Fig. 7. The results showed that following exposure to 3 doses of fipronil for 7 and 14 days, superoxide dismutase activity increased and catalase activity decreased in these organs. However, these changes in the brain were only observed after 2 weeks exposure to 200 mg/kg fipronil. SOD and CAT activity alterations in the kidney were more dose-dependent manner and liver showed these alterations as time-dependent manner. However, this time dependent manner in the liver was not very obvious. It seems that these alterations in gill were in both time and dose-dependent manner.
dependent manners.

3.4. DNA damage

Fluorescence and Giemsa staining for comet assay are shown in Fig. 8. According to the quality of the pictures, since fluorescence staining shows comets more obvious and, therefore, was used in analysis by Cellprofiler® software. This software detected comets in treatment and also control groups. The results were shown based on comet morphology and number of pixels (amount of DNA damage) involving each comet. The number of detected comets was different among groups and generally, amount of DNA damage increased after exposure to doses of fipronil. Fig. 9 compares tail comets in the four organs from the fish exposed to fipronil in time and dose dependent manner. Apart from brain with no significant difference, in the other remained organs, there were significant difference (P < 0.05) in tail comets between control and treatment groups. However, there was no significant difference among treatment groups in tail comets from the kidney. The time dependence of the liver damage from fipronil exposure was also observed for comet tails and results showed that fipronil causes more DNA damage in this organ.

3.5. CYP1A gene expression

Quality assessment of RNA extracted from the organs showed obvious 28S, 18S and 5 s rRNA bands on agarose gel (figure not shown). Quantity assessment of RNA resulted from Nanodrop spectrophotometer showed A260/280: 2.09–2.20 and A260/230: 1.5–1.8, implying suitable purity of the RNA. Dissociation curve showed high specificity for primers. RT-qPCR products, including reference (RPL8) and target (CYP1A) genes, qualified on agarose gel, showed only one band around 100 bp (Fig. 10). The results of relative CYP1A mRNA expression in the organs showed significant difference between all treatment and control groups (P < 0.05), and increased in time and dose dependent manner. However, it seems that these alterations in the kidney is more dose dependent and in the liver is more time dependent.

4. Discussion

This study was going to find relationship between CYP1A mRNA expression, as one possible basic occurrence for fipronil toxicity, and DNA damage, biochemical, histological and morphological alterations in the Caspian kutum fish. Many previous studies have only used CYP1A gene expression as a sensitive biomarker in toxicology and environmental monitoring assessment [27–30]. Induction of CYP1A is mediated through the binding of the ligands, such as halogenated aromatic hydrocarbon (HAHs) and polycyclic aromatic hydrocarbons (PAHs) to a cytosolic aryl hydrocarbon receptor (AhR), and this induction is considered as a biomarker of AhR agonist exposure in fish [31,32]. Regarding either structural similarity between fipronil and these ligands or fipronil induction of CYP1A, fipronil could be potentially an AhR binder. After binding fipronil to AhR and formation of heterodimer including AhR-fipronil subunit and AhR nuclear translocator protein, the active heterodimer acts as CYP1A gene transcription factor and increases mRNA, protein and catalytic activity of CYP1A. Each of these steps can be used to measure CYP1A in fish [16], and thus CYP1A gene expression was used in this study. In addition to detoxification of fipronil, activity of CYP1A in phase I biotransformation reactions may also resulted in generation of reactive oxygen species and reactive intermediate compounds. However, oxidative stress can be resulted from activity of different enzymes. In this study, CYP1A gene expression and oxidative stress had good correlation in the organs.
**Fig. 4.** Histological alterations in the brain and kidney of the Capian white fish intraperitoneally exposed to fipronil. A (nissl staining) and B (H&E): normal structure of the brain from fish exposed to fipronil (200 mg/kg). Normal granular layer and purkinje cells (A) and perikaryons (B) are also shown. C (H&E): Normal structure of the kidney from control group. D (H&E) and E (H&E) show some changes in structure of proximal tubule and degeneration in fish exposed to 200 mg/kg fipronil for 14 days.

**Table 1**
The frequency (F) of histopathological alterations in *Rutilus frisi kutum* after 7 and 14 days exposure to 65, 130 and 200 mg/kg fipronil. Absent (F = 0), rare (F = 1), low frequency (F = 2), frequent (F = 3) and very frequency (F = 4).

| Tissue       | Tissue alterations                  | The first week (F 0–4) | The second week (F 0–4) | control (F 0–4) | stage |
|--------------|------------------------------------|------------------------|-------------------------|-----------------|-------|
|              |                                     | 65         | 130   | 200 | 65     | 130   | 200 |
| Gill         | Epithelial lifting                  | 1          | 1     | 2   | 2      | 3     | 3    | 1   | 1   |
|              | Hypertrophy                         | 1          | 1     | 1   | 2      | 2     | 2    | 0   | 1   |
|              | Hyperplasia                         | 0          | 0     | 1   | 1      | 2     | 2    | 0   | 1   |
|              | Deletion                            | 0          | 0     | 1   | 1      | 2     | 2    | 0   | 1   |
|              | Fusion                              | 1          | 1     | 1   | 2      | 2     | 2    | 0   | 1   |
|              | Structure alteration                | 2          | 2     | 2   | 2      | 2     | 2    | 0   | 1   |
| Liver        | Pyknosis                            | 2          | 2     | 3   | 4      | 4     | 4    | 0   | II  |
|              | Vacuolation                         | 1          | 1     | 2   | 2      | 4     | 4    | 1   | 1   |
|              | Congestion in blood sinusoids       | 1          | 1     | 1   | 3      | 3     | 3    | 0   | 1   |
|              | Intravascular haemolysis            | 0          | 0     | 1   | 1      | 2     | 3    | 0   | 1   |
|              | Sinusoids dilation                  | 1          | 1     | 2   | 3      | 3     | 3    | 0   | 1   |
|              | Steatosis                           | 0          | 1     | 2   | 2      | 4     | 4    | 0   | 1   |
|              | Hypertrophy                         | 1          | 1     | 1   | 2      | 2     | 2    | 0   | 1   |
|              | Glycogen deposition                 | 2          | 2     | 3   | 3      | 3     | 3    | 1   | 1   |
| Kidney       | Hemolysis                           | 0          | 1     | 2   | 1      | 1     | 2    | 0   | 1   |
|              | Degeneration in renal tubules       | 0          | 0     | 1   | 0      | 0     | 2    | 0   | II  |
|              | Thrombosis                          | 1          | 1     | 2   | 1      | 2     | 2    | 0   | 1   |
|              | Structure changes in renal tubules  | 1          | 1     | 2   | 1      | 2     | 2    | 0   | 1   |
|              | Sinusoids dilation                  | 0          | 1     | 2   | 1      | 2     | 2    | 0   | 1   |
| Brain        |                                     | --         | 0     | 0    | 0      | 0     | 0    | 0   | --  |
Fig. 5. Liver histopathology of the Capian white fish intraperitoneally exposed to fipronil. A (PAS) and B (H&E) show normal structure of the liver from control group. C (H&E) shows extensive steatosis in the liver of fish exposed to fipronil (200 mg/kg) for 14 days. D (H&E): Pyknosis (PK) and sinusoid dilation (SD) are shown. E and F (PAS) show deposition of glycogen in the liver of fish exposed to fipronil (200 mg/kg) for 7 and 14 days.

Fig. 6. The degree of tissue change (DTC) mean ± SD for gill, liver and kidney from fish exposed i.p. to fipronil (65, 130 and 200 mg/kg/week) for either 1 week (1) or 2 weeks (2). Different letters show significant difference between groups for all organs.
(Fig. 12) so that this correlation was the most in the kidney ($R^2 = 0.87$) and the least in the liver ($R^2 = 0.56$). Probably, other factors are more effective to generate oxidative stress in the liver compared to the other three organs.

### 4.1. Morphological and histological alterations

Organ-somatic indices along with condition factor were studied for assessment of fish general health. In this study, increasing HSI in time and dose dependent manner is related to liver weight (no significant difference in fish total body weight). Histological observations showed that increasing glycogen deposition and fatty degeneration were also in time and dose dependent manner and can be a reason for increasing HSI. The liver is an important and active organ in xenobiotics detoxification, and increasing liver weight is a sign of fenpropir toxicity. Probably, the most important reason for increasing HSI is the increase of CYP450 synthesis and liver hypertrophy, as observed in this study. Ferreria et al. [33] reported that the liver hypertrophy in mice exposed to fenpyrion is explainable by increasing the number of rough endoplasmic reticulum and the role of this organelle in fenpyrion metabolism through CYP450 synthesis. As shown in Fig. 12, there is relatively high correlation ($R^2 = 0.76$) between CYP1A mRNA expression in the liver and HSI, and this relation confirms the role of CYP1A in increasing HSI in time and dose dependent manner. The other organs also showed the role of CYP1A in alteration of organ-somatic index. Apart from the kidney, the gill-somatic index increased in relatively time and dose dependent manner (probably due to hypertrophy and hyperplasia observed in histological study) and showed good correlation ($R^2 = 0.7$) with the CYP1A expression. Finally, the brain with no significant alteration in brain-somatic index did not show any correlation ($R^2 = 0.07$) with the brain CYP1A expression. Moreover, the increase of CYP1A expression in the brain is lower than the other organs. There is no report about the effects of fenpyrion on fish gill and brain-somatic indices. In accordance with this result, Badgajar et al. [34] reported that the mice orally exposed to fenpyrion showed no significant change ($P > 0.05$) in brain-somatic index.

Histological damages, observed in this study, also showed that most of these alterations directly or indirectly can be explainable by CYP1A gene expression. For the liver, although the regression analysis for degree of tissue change (DTC) in treatment groups showed lower correlation between this parameter and CYP1A gene expression ($R^2 = 0.59$) and oxidative stress ($R^2 = 0.5$) compared to that for the kidney ($R^2 = 0.93$; $R^2 = 0.83$), it seems that kind of tissue changes implied more relationship to CYP1A and oxidative stress. Blood congestion and sinusoid dilation resulted from more blood flow to the liver to support this organ for detoxification activity catalyzed by enzymes (e.g. CYP1A) [35]. Pyknosis is intense nuclear condensation and final step of all apoptosis and necrosis, found in almost all dying cells and only occurs in stress condition (e.g. oxidative stress) [36]. However, the mechanism of pyknosis has remained unknown so far [37]. In this study, this damage was only observed in the liver which had the highest level of oxidative stress and CYP1A gene expression, and it seems that this organ is more susceptible to this damage. Previous studies reported this damage in mice liver cells and rat liver exposed to fenpyrion [38,39]. Liver steatosis may resulted from different biological alterations at molecular level and there is not enough information about molecular basis of this damage [40], especially in fish. Videla et al. [41] suggested that oxidative stress and consequently depletion of long chain polyunsaturated fatty acid (LCPUFA) may cause steatosis. In other words, LCPUFA have key role in gene regulation of triacylglycerol export and
Fig. 8. Comet assay results for the liver cells from the Caspian kutum exposed i.p. to fipronil. A: Control group. Comets for liver cells from the fish exposed to fipronil (200 mg/kg) for 7 days (B) and 14 days (C, D, E and F). A–E and F were stained by SyberGreen and Giemsa dyes, respectively. G: Analysis of comets by CellProfiler software, identification of comet tail based on regulations on pipeline (25–200 pixels). Comets with similar tails are shown by the same color.

Fig. 9. Comparison of comet tails in treatment groups after 7 and 14 days exposure to fipronil. Different letters show significant difference \( (P < 0.05) \) between groups for each organ so that A, B, C and D for liver, a, b and c for gill, a” for brain, and A” and B” for kidney.
fatty acid oxidation through activation of peroxisome proliferator-activated receptor (PPAR); and thus oxidative stress and lipid peroxidation resulted in LCPUFA depletion and consequently steatosis. In our study, with increasing oxidative stress, fatty vacuolization also increased, and this result (with ignoring differences between mice and fish) supports the suggestion cited above. Moreover, Ross and Pawlina [42] suggested that drug-induced steatosis is related to the increase in the activity of smooth endoplasmic reticulum and its enzymes due to their role in detoxification as well as lipid synthesis. In contrast to our expectation, glycogen deposition also increased in time and dose dependent manner and it is probably related to fipronil effect on mechanisms contributing in synthesis of glycogenolytic enzymes. In agreement with this result, De Oliveira et al. [43] reported the increase of glycogen deposition in mice liver cells after 7 days exposure to fipronil. Comparison of DTCs in different doses of fipronil after 7 and 14 days exposure showed that this parameter in the liver was more related to time exposure. Ardeshir et al. [6] observed no alteration in DTC of the liver in the Caspian kutum after 4 days exposure to sublethal doses/concentrations of fipronil. Histological alterations in the kidney was only observed in the renal tubule and not in glomeruli and hematopoietic tissue. Interestingly, previous studies on immunohistochemical localization of CYP1A in the fish kidney showed that this enzyme had the most distribution in the renal tubular epithelium and the least distribution in glomeruli and hematopoietic tissue [44,45]. The increase of DTC in this organ in dose dependent manner is probably related to concentrating fipronil and its metabolites in the renal tubule. Fusion of gill secondary lamella may resulted from simple adherence of adjacent lamella to each other or through epithelial hypertrophy and hyperplasia. Histological observations in this study and previous study [6,23] showed that this damage mostly resulted from the last case, and as cited above in the liver, this damage (the most obvious damage in the gill) is probably related to the detoxification. Moreover, PAS staining (Fig. 3) showed some alterations in the condition of pillar cells and blood spaces so that these cells were contracted and blood spaces decreased compared to the control. This alteration might be a mechanism to decrease entering blood containing fipronil and its metabolite into the gill epithelium [46]. Previous studies showed that pillar cells and epithelium of the gill have the most level of CYP1A activity [44,45]. No damage was observed in the brain which had the least alteration in oxidative stress and CYP1A gene expression (Figs. 7 and 11).

4.2. DNA damage

To the best of our knowledge, this is the first report of genotoxic effect of fipronil on fish. The comet assay, as a method of DNA analysis to measure genotoxicity, is efficient and sensitive way for different pollutants and marine species [47]. DNA damage can be resulted from either direct effect on DNA and genome structures or damage to lysosomes and consequently releasing DNAase into cytoplasm [48]. In the present study, alkaline method and Cellprofiler’s software were used to measure and analyze the amount of DNA migration out of nucleus due to probable double and single-strand breaks and alkalilabile sites. According to regression analysis (Fig. 12) for comet results, the highest correlation between comet and CYP1A (R² = 0.89) was in the liver, and oxidative stress in the gill (R² = 0.9). Surprisingly, the comet had more correlation with CYP1A gene expression (R² = 0.69) in the liver. However, in the other organs this result was not observed. Previous studies suggested that fipronil generate genotoxic damage more through oxidative stress [48-50]. The regression analysis showed that genotoxicity of fipronil is more related to CYP1A gene expression in the liver. This organ had the most increase of CYP1A gene expression with increasing fipronil dose and time of exposure. Afterwards, kidney had high increase of CYP1A gene expression, and correlation between comet and CYP1A and oxidative stress were similar in this organ. There is no report about direct effect of fipronil metabolite (fipronil-desulfanyl and fipronil-sulfone), with higher toxicity compared to fipronil, on DNA. However, Lovinskaya et al. [50] suggested that the increase in DNA damage in the mice liver cells 24 h after injection of fipronil compared to 6 h is probably due to the metabolism of fipronil and the formation of fipronil sulfone in these cells. Thus, this result might be related to CYP1A bio-transformation activity, and fipronil metabolite might generate DNA damage directly. This case probably needs some more studies.
5. Conclusion

The results demonstrate that the degree of tissue change and alterations in organ-somatic index and DNA structure in the Caspian kutum fish exposed to fipronil have mostly high correlation with CYP1A gene expression and oxidative stress and it seems that CYP1A has important role in generating toxicity of fipronil. However, some other factors may also contribute in this toxicity and should be considered for
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