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Hepatotoxicity of herbicide Sencor in goldfish may result from induction of mild oxidative stress

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ABSTRACT

The effects of 96 h exposure to 7.14, 35.7, or 71.4 mg L^{-1} of Sencor were studied on liver and plasma parameters in goldfish, *Carassius auratus* L. Goldfish exposure to 71.4 mg L^{-1} of Sencor for 96 h resulted in a decrease in glucose concentrations in plasma and liver by 55%, but did not affect liver glycogen levels. An increase in the activity of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase (by 24–27%, 32–72%, and 87–102%, respectively) occurred in plasma of Sencor exposed goldfish, whereas in liver activities of these enzymes decreased (by 15–17%, 19%, and 20%, respectively). Lactate concentration in plasma increased by 22–36% in all treated fish groups, whereas in liver it increased by 64% only after exposure to 35.7 mg L^{-1} of Sencor. Herbicide exposure enhanced lipid peroxide levels by 49–75% and decreased activities of catalase by 46%, glutathione reductase by 25–48% and glutathione peroxidase by 21–26% suggesting development of oxidative stress in liver. The treatment induced various histological changes in goldfish liver, such as dilated sinusoids, hypertrophy and dystrophy of hepatic cells and detachment of endothelial cytoplasm with diffuse hemorrhage. The data collectively let us propose that mild oxidative stress might be responsible for the hepatotoxicity of Sencor.

1. Introduction

Triazines are a common group of herbicides that have been used extensively over the last 50 years in many countries worldwide. Metribuzin (4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one) is an asymmetrical triazine herbicide often used on vegetable crops (e.g. soybeans, potatoes and tomatoes) to control broadleaf and grassy weed species. Metribuzin acts as an inhibitor of photosynthesis by binding to a plastoquinone-binding site on the D1 protein of photosystem II complex in chloroplast thylakoid membranes [1,2].

Although beneficial to agriculture due to improved production of crops, the uncontrolled use of herbicides such as triazines results in their persistent presence in surface and ground waters potentially affecting non-target organisms such as fish [3]. In recent studies dealing with herbicide availability in surface water, metribuzin was detected (in trace amounts) in Brazil [4], Canada [5] and the Czech Republic [6].

Pesticide contamination of both surface and ground water can affect aquatic fauna and flora, as well as human health when the water is used for public consumption [7]. Aquatic organisms are directly exposed to chemicals resulting from agricultural production via surface run-off or indirectly through trophic chains. Fish have been widely used in studies of the toxicological impacts of different pollutants and their biochemical and cellular responses are extensively applied to assess potential risk related to aquatic pollution [8].

In fish, the liver plays a key role in xenobiotic detoxification through oxidation, reduction, conjugation, and hydrolysis reactions that set up toxins storage or excretion. Relatively little is known to date about the acute or chronic toxicity of metribuzin on liver of various fish species and studies to date report conflicting results. For example, Plhalova et al. [6] reported that subchronic exposure

Abbreviations: AChE, acetylcholine esterase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CP, carbonyl protein groups; G6PDH, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; H-SH, high molecular mass thiols; LDH, lactate dehydrogenase; LOOH, lipid peroxides; L-SH, low molecular mass thiols; ROS, reactive oxygen species; SOD, superoxide dismutase.

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to metribuzin caused histological lesions in hepatocytes of zebrafish (*Danio rerio*), but Velisek et al. [9] found no changes in histology of liver of common carp (*Cyprinus carpio*) exposed to metribuzin. Markers of xenobiotic metabolism, particularly total cytochrome P450 levels and activity of ethoxyresorufin-O-deethylase, were investigated in the liver of common carp after 28-day exposure to Sencor 70 WG (metribuzin 700 g kg^{-1}). The fish exposed to this herbicide at concentrations 0.25 and 2.5 mg L^{-1} showed no alterations of these markers as compared with respective controls [10]. However, Mekhed et al. [11] demonstrated increased activities of basic enzymes of gluconeogenesis (glucose-6-phosphatase and fructose-1,6-bisphosphatase) and decreased glucose levels in liver, muscle and brain of carp (*C. carpio*) exposed to Sencor for 14 days.

Hence, the available literature does not indicate the mechanisms of Sencor toxicity in aquatic organisms. Drawing on our extensive experience with the use of goldfish as a model organism to investigate effects of diverse toxicants on fish, we decided to analyze potential mechanisms for metribuzin toxicity using the goldfish model. Therefore, the present study evaluates the impact of the metribuzin-containing herbicide Sencor WG 70 on plasma and hepatic biochemical indices and the histological structure of the liver of goldfish, *Carassius auratus* L. in response to 96 h exposure to this pesticide.

2. Materials and methods

2.1. Reagents

Phenylmethylsulfonyl fluoride (PMSF), 1-chloro-2, 4-dinitrobenzene (CDNB), reduced glutathione (GSH), oxidized glutathione (GSSG), β-nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide (NAD), β-nicotinamide adenine dinucleotide reduced (NADH), glucose-6-phosphate (G6P), ethylenediamine-tetraacetic acid (EDTA), xylenol orange, cumene hydroperoxide, ferrous sulfate, 2,4-dinitrophenylhydrazine (DNPH), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), hydrogen peroxide (H_2O_2), NaCl, KH_2PO_4, NaCl, Tris(hydroxymethylaminomethane), N,N,N,N-tetramethylethylenediamine (TEMED), lactic acid, pyruvic acid, glutathione reductase from baker's yeast and lactate dehydrogenase from bovine heart, β-nicotinamide adenine dinucleotide phosphate reduced (NADPH) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Detection kits for estimation of triacylglycerol content (Liquick Cor-TG), and activities of alanine aminotransferase (Liquick Cor-ALAT) and aspartate aminotransferase (Liquick Cor-ASAT) were received from Cormay (Siedlce, Poland). Sencor 70 WG was purchased from Bayer Crop Science (Monheim am Rhein, Germany). All other reagents were of analytical grade.

2.2. Animals and experimental conditions

Specimens of goldfish (*Carassius auratus* L), weighing 80–100 g, were obtained commercially from a local fish farm (Halych, Ivano-Frankivsk, Ukraine) in November 2012. Fish were acclimated to laboratory conditions for 4 weeks in a 1000 L tank under natural photoperiod in aerated and dechlorinated tap water at 19.0–20.0 °C, pH 6.9–7.1, 8.1–8.6 mg L^{-1} O_2 and hardness (determined as Ca^{2+} concentration) 38–40 mg L^{-1}. Fish were fed with commercial pellets of CarpCo Excellent for Cyprinids (Koi Grower, Helmond, The Netherlands), containing 36% protein, 7% fat, 3.6% cellulose, 8.7% ash, 1% phosphorus and vitamins C, A, D_3 and E. Fish were fed during the acclimation period (4 weeks), but were fasted for 1 day prior to and during experimentation.

Experiments were carried out in 120 L glass aquaria (containing 100 L of water), in a static mode with or without the addition of the commercial herbicide Sencor 70 WG (Bayer Crop Science) which contains metribuzin (4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5-one) at a concentration of 700 g L^{-1}. Groups of seven fish were placed in aquaria with different nominal concentrations of Sencor: 7.14, 35.7 and 71.4 mg L^{-1}, which corresponds to 5, 25 and 50 mg L^{-1} of metribuzin, respectively. Animals were exposed to these conditions for 96 h (no mortality occurred during exposures). Fish in the control group were maintained in the same manner, but without addition of Sencor to the water. Aquarium water was not changed over the 96 h course to avoid stressing the animals. Levels of dissolved oxygen, temperature and pH were monitored every 24 h. After fish exposure, blood was immediately taken from caudal vessels using a syringe rinsed with 50 mM Na_2EDTA as an anticoagulant. Blood samples were centrifugated 15 min at 3000 *g* and 4 °C. Plasma was collected and used for biochemical analyses.

Fish were sacrificed by transspinal transsection without anesthesia and livers were dissected, rinsed in ice-cold 0.9% NaCl, dried by blotting on filter paper, frozen, and stored in liquid nitrogen until use.

All experiments were conducted in a strict accordance with the Ethics Committee of Precarpathian National University.

2.3. Determination of metabolic stress indices in liver and plasma

2.3.1. Glucose concentration

Aliquots of frozen liver samples were homogenized 1:10 w:v (for liver) or fresh prepared plasma mixed 2:1 v:v with 50 mM potassium phosphate buffer containing 0.09% NaN_3; and then incubated in a water bath (70 °C) for 5 min in tightly covered centrifuge tubes, followed by chilling on ice. After centrifugation (16,000 *g*, 15 min, 21 °C) of samples the thermally denatured proteins were removed and supernatants used for determination of glucose concentration by the Liquick Cor-TG (Cormay) commercial kit with spectrophotometric detection at 500 nm. Glucose concentrations in samples (mg L^{-1} or mg gwm^{-1} for plasma and liver, respectively) were estimated using a linear regression of data from a standard curve. For determination of glycogen concentration samples were first incubated with 0.5 U amyloglucosidase and then glucose concentration was assayed in the same manner. Glycogen concentration in samples was expressed in milligrams of glucose equivalents per gram wet mass of tissue (mg/gwm).

2.3.2. Total protein concentration

Protein concentration was measured by the Coomassie brilliant blue G250 method [12] using bovine serum albumin as a standard. Data are expressed as milligrams of total protein per millilitre of plasma (mg mL^{-1}) or per gram wet mass of liver (mg/gwm).

2.3.3. Triacylglycerol content

Aliquots of tissue samples were homogenized (1:10 w:v) (for liver) or mixed 1:1 v:v (for plasma) with phosphate-buffered saline (PBS) containing 0.05% Triton X100 and incubated in a boiling water bath for 15 min. Thereafter, homogenates were cooled and centrifuged 7.5 min at 5000 *g* and 21 °C. The resulting supernatants were used for determination of triacylglycerol (TAG) using commercial kit Liquick Cor-TG (Cormay) following the manufacturer's guidelines and ensuring that assays maintained a linear relationship between enzyme amount and reaction velocity. Enzyme activities were expressed as international units or miliunits per milligram soluble protein (U/mU mg $protein^{-1}$).

2.3.4. Estimation of lactate content

The concentration of lactate was measured via oxidation of lactate to pyruvate by L-lactate dehydrogenase (LDH) [13]. The amount of NADH formed was measured spectrophotometrically at 340 nm. For

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quantitative conversion of lactate to pyruvate, the medium was supplied with hydrazine, which reacts with pyruvate to produce a pyruvate hydrazone. Samples of tissue were homogenized 1:10 w:v (for liver) or mixed 1:1 v:v (for plasma) with 0.5 M perchloric acid (PCA) on ice and then centrifuged (15 min, 16,000 *g*, 4 °C). Supernatants were neutralized with 2 M KOH. The reaction mixture contained 0.5 M glycine-hydrazine buffer (pH 9.0), 2 U/mL LDH, 2 mM NAD and 50 µL of preparation. Lactate concentration in samples (µmol) was estimated using a linear regression of data from a standard curve made with 10–240 µM lactate. The concentration of lactate was expressed as micromoles of lactate per milliliter of plasma (µmol mL^{-1}) or per gram wet mass of tissue (µmol gwm^{-1}) for liver.

2.4. Determination of oxidative stress indices in goldfish liver

2.4.1. Assay of lipid peroxides

The lipid peroxide (LOOH) content was assayed by the FOX (ferrous-xylenol orange) method [14]. For that, tissue samples were homogenized (1:5, w:v) using a Potter-Elvehjem glass homogenizer in 96% cold (4 °C) ethanol and centrifuged (5000 *g*, 15 min, 4 °C). Aliquots (40 µL) of the supernatants were used for the assay as described previously [15]. The content of LOOH was expressed as nanomoles of cumene hydroperoxide equivalents per gram wet mass of tissue.

2.4.2. Measurement of protein carbonyls

Carbonyl groups of proteins in liver were determined as described previously [15]. Tissue samples were homogenized (1:10, w:v) in homogenization medium (50 mM potassium phosphate buffer, pH 7.0, 0.5 mM EDTA, 1 mM PMSF) and centrifuged (16,000 *g*, 15 min, 4 °C). Supernatants were removed and 0.25 mL aliquots were mixed with 0.25 mL of 40% trichloroacetic acid (TCA) (final TCA concentration 20%) and centrifuged (5000 *g*, 5 min, 20 °C). Protein carbonyl (CP) levels were measured in the resulting pellets by reaction with 2,4-dinitrophenylhydrazine (DNPH) [16]. The values were expressed as nanomoles of CP per milligram of protein.

2.4.3. Estimation of thiol-containing compounds

Free thiols were measured spectrophotometrically by the Ellman procedure with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm [17]. Using supernatants prepared as above for the CP assays, total thiol concentration was measured as described previously [18]. For determination of low-molecular mass thiols (L-SH), aliquots of supernatants were mixed with trichloroacetic acid (TCA) to reach a final TCA concentration of 10%, centrifuged (16,000 *g*, 5 min, 4 °C) to remove pelleted protein and the final supernatants were used for the assay. Thiol concentrations were expressed as micromoles of SH-groups per gram wet weight of tissue. The high-molecular mass thiol (H-SH) content was calculated by subtracting the L-SH concentration from total thiol concentration.

2.5. Assay of enzyme activities

2.5.1. Antioxidant and associated enzymes

Tissue homogenization and centrifugation was as described above for CP assays and supernatants were collected. The activities of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PDH) were measured in supernatants as described earlier [15]. The activity of lactate dehydrogenase (LDH) was assayed spectrophometrically by monitoring the change in NADH absorbance at 340 nm [19].

One unit of SOD activity was defined as the amount of enzyme (per mg protein) that inhibited quercetin oxidation reaction by 50% of maximal inhibition. Inhibition values for SOD activity were calculated using an enzyme Kinetics computer program [20]. One unit (U) of catalase, GST, GR, GPx, G6PDH and LDH activity is defined as the amount of enzyme consuming 1 µmol of substrate or generating 1 µmol of product per minute. Activities were expressed as international units (or milliunits) per milligram soluble protein (U/mU mg $protein^{-1}$).

2.5.2. Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined according to the method of Ellman et al. [21] with minor modifications and adaptation of the procedure to microplate determination. The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 0.3 mM DTNB and 10 µL supernatant. After 10 min incubation at 25 °C the mixture was supplemented with 30 µL of 75 mM acetylthiocholine iodide (final volume 0.3 mL) and reaction rate was immediately measured at 412 nm. The enzyme activity was calculated using a molar extinction coefficient of 13,600 $M^{-1}\,cm^{-1}$. One unit of AChE activity was defined as the amount of enzyme producing 1 µmol of thionitrobenzoate per minute and was expressed per mg soluble protein concentration (mU mg $protein^{-1}$).

2.5.3. Activities of aminotransferases

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the commercial kits (Cormay) following the manufacturer's guidelines. Activities of ALT and AST were measured with keeping of the linearity of the reactions, where the strongly linear relationship between enzyme amount and reaction velocity was observed. The activities of above mentioned enzymes were expressed as international units or milliunits per milligram soluble protein (U/mU mg $protein^{-1}$).

2.6. Histological examination

Liver samples of the control and treated fish were fixed in 10% neutral buffered formalin, and then the samples were processed for routine wax histological evaluation (dehydrated and embedded in paraffin). Sections of 5 µm were prepared and stained with hematoxylin and eosin stains as described by Luna [22] and Bernet et al. [23]. Histological samples of liver were prepared from four fishes from each experimental group with two replicates of each sample and the changes described were similar in all the slides that were assessed. The results and micrographs in Figure 3A–D represent the histological changes that were observed. The rest of the liver tissue of each fish was used for biochemical studies.

2.7. Statistical analysis

Statistical analysis was performed by Mynova software (version 1.3) using ANOVA followed by the Dunnett's test to compare multiple experimental treatments with the single control value. Data are presented as means ± SEM. The probability value of $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Sencor impact on plasma and liver metabolic indices

Changes in the biochemical parameters of blood are frequently used as important indices of fish response to environmental pollution [24] and are therefore crucial for understanding normal and pathological processes associated with xenobiotic impacts on fish. The biochemical status of blood reflects the course of physiological processes in the organism and may be affected by environmental stresses [25]. Furthermore, changes in blood serum parameters often correlate with metabolic changes in the liver of organisms. Under herbicide-induced stress liver can be considered as a target organ

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Table 1
The concentrations of glucose, glycogen, triglycerides (TAG), total protein, lactate and activity of lactate dehydrogenase (LDH) in plasma and liver of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h.

Parameter		Fish group			
		Control	7.14 mg L^{-1}	35.7 mg L^{-1}	71.4 mg L^{-1}
Plasma parameter	Glucose, mg mL^{-1}	1.72 ± 0.10	2.40 ± 0.24*	0.75 ± 0.07*	0.79 ± 0.08*
	TAG, mg mL^{-1}	0.73 ± 0.06	0.92 ± 0.07	0.97 ± 0.10	0.82 ± 0.05
	Total protein, mg mL^{-1}	20.0 ± 0.7	19.8 ± 1.0	24.4 ± 2.1	22.2 ± 1.1
	Lactate, μmol mL^{-1}	7.22 ± 0.37	8.81 ± 0.23*	9.12 ± 0.22*	9.83 ± 0.45*
	LDH activity, mU mg $protein^{-1}$	9.4 ± 0.8	11.7 ± 1.3	17.6 ± 2.7*	19.0 ± 3.6*
Liver parameter	Glucose, mg gwm^{-1}	6.07 ± 0.94	5.61 ± 0.79	4.14 ± 0.73	2.76 ± 0.39*
	Glycogen, mg gwm^{-1}	147 ± 8	135 ± 7	139 ± 5	135 ± 7
	TAG, mg gwm^{-1}	2.43 ± 0.11	3.87 ± 0.69	3.30 ± 0.57	2.51 ± 0.25
	Total protein, mg gwm^{-1}	34.3 ± 1.5	36.1 ± 2.2	42.1 ± 3.1	39.3 ± 2.6
	Lactate, μmol gwm^{-1}	3.43 ± 0.30	4.28 ± 0.28	5.64 ± 0.59*	3.40 ± 0.34
	LDH activity, mU mg $protein^{-1}$	8.36 ± 0.17	8.45 ± 0.49	8.57 ± 0.59	6.71 ± 0.23*

Data are presented as means ± SEM, $n = 5–7$.
* Significantly different from the control group with $P < 0.05$.

of great importance to fish, since it participates in processes such as biotransformation, conjugation and excretion of xenobiotics.

Carbohydrates are a primary fuel source for animals and under stress conditions carbohydrate reserves may be depleted to meet energy demand [26]. In our experiments, the concentration of glucose in plasma of the control fish was 1.72 ± 0.10 mg mL^{-1} and was enhanced by 39% after 96 h fish exposure to 7.14 mg L^{-1} Sencor (Table 1). However, at higher concentrations of the herbicide, 35.7 and 71.4 mg L^{-1}, glucose concentration decreased by 56% and 54%, respectively, as compared with controls. The glucose concentration in liver of fish exposed to 71.4 mg L^{-1} Sencor was lower by 55% as compared with the control value (Table 1). The increase in plasma glucose under low herbicide concentration could be associated with an acute response to stress treatment, and the carbohydrate could be released into the bloodstream due to hormone regulated glucose mobilization from glycogen or via induction of liver glycogenolysis and gluconeogenesis under prolonged energy demand during stress conditions [27]. At the same time, lower glucose levels in both liver and plasma after exposure to high Sencor concentrations could indicate either depletion of the carbohydrate reserves of the organism or a disruption of the systems of glucose release (e.g. by enzyme or transporter inhibition) from liver tissue. Since no statistically significant decrease in glycogen level in livers of fish exposed to higher Sencor concentrations were found, the decreased glucose levels in liver and blood might result from corruption of glucose-releasing mechanisms or/and gluconeogenesis. Earlier, increased glucose concentrations in blood plasma were reported by Velisek et al. [9] in 2-year-old common carp after acute exposure to 175.1 mg L^{-1} metribuzin (250.2 mg L^{-1} Sencor 70 WG). Mekkawy et al. [28] also observed a significant increase in blood glucose concentration in *Oreochromis niloticus* and *Chrysichthyes auratus* after acute exposure to 3 mg L^{-1} of atrazine. However, chronic exposure to simazine did not change glucose concentration in blood plasma of common carp [29].

Liver glycogen is a major storage depot for carbohydrate in animals and has an important role to play in the export of hexose units for delivery to other tissues and maintenance of blood glucose homeostasis [30]. In our experiments, liver glycogen levels did not change significantly in the liver of Sencor treated fish.

Triacylglycerols and proteins also are important fuel sources for fish. In the present case, Sencor had no effect on TAG and total protein levels in either liver or plasma at any of the concentrations studied (Table 1). Some authors reported decreases in serum total protein and TAG content in triazine exposed fish [31,32]. However, Velisek et al. [29] observed no changes in TAG content in blood plasma of common carp following chronic exposure to simazine.

The data on carbohydrates, TAG and proteins collectively demonstrate that goldfish exposure to Sencor induced general mild stress. Only glucose levels were changed by the treatment, whereas neither TAG, nor protein levels were affected. It appears that Sencor exposure treatment only modified the mobilization of carbohydrates in the fish.

The activities of LDH, AST, and ALT have been used extensively as biomarkers of exposure to pollutants, including pesticides [33,34]. LDH is the terminal enzyme of glycolysis and is frequently used as a biomarker of the intensity of this metabolic process and of cell integrity under stress conditions [35]. In the latter role, because LDH is a very abundant enzyme, damage to cells can result in substantial leakage of the enzyme into the blood where it can easily be assayed. In plasma of control goldfish, LDH activity was 9.4 ± 0.8 mU mg $protein^{-1}$ and in fish exposed to 35.7 and 71.4 mg L^{-1} Sencor plasma LDH increased by 87% and 102%, respectively (Table 1). This was accompanied by an increase in plasma lactate concentrations of 22–36% in all experimental groups exposed to the herbicide in comparison with control values (7.22 ± 0.37 μmol mL^{-1}) (Table 1). The enhanced activity of plasma LDH could be associated with pesticide-induced injuries to solid tissues and a release of the enzyme into the bloodstream [36–38]. In contrast to our findings, Velisek et al. [9] observed a decrease LDH activity and lactate concentration in blood plasma of common carp treated with 175.1 mg L^{-1} metribuzin. Some authors reported no changes in lactate concentration or LDH activity in plasma of common carp after long-term exposure to simazine [29,39]. On the other hand, LDH activity decreased by 20% in liver of fish exposed to the highest concentration of Sencor (perhaps due to leakage into the plasma), whereas the level of lactate significantly increased by 64% in the 35.7 mg L^{-1} treatment group in comparison with control value (3.43 ± 0.30 μmol gwm^{-1}) (Table 1). It can be suggested that higher lactate levels and lower LDH activity resulted from impairment of function and disruption of the structure of liver due to herbicide intake.

Aminotransferases are sensitive indicators used to assess and monitor the degree of liver cell inflammation and necrosis, which results in the release of these enzymes into the bloodstream due to increased permeability or breakdown of cell membranes [38]. In our experiments the plasma activities of aspartate (AST) and alanine aminotransferases (ALT) increased in plasma of Sencor treated fish. The control values for AST and ALT activities were 4.25 ± 0.10 U mg $protein^{-1}$ and 279 ± 24 mU mg $protein^{-1}$, respectively (Fig. 1). Exposure to the herbicide at concentrations 37.5 and 71.4 mg L^{-1} lead to increased AST activity by 24% and 27%, respectively (Fig. 1A) in fish plasma, whereas ALT activity increased in all treatment groups by 32–72% (Fig. 1B). Enhanced plasma aminotransferase activities

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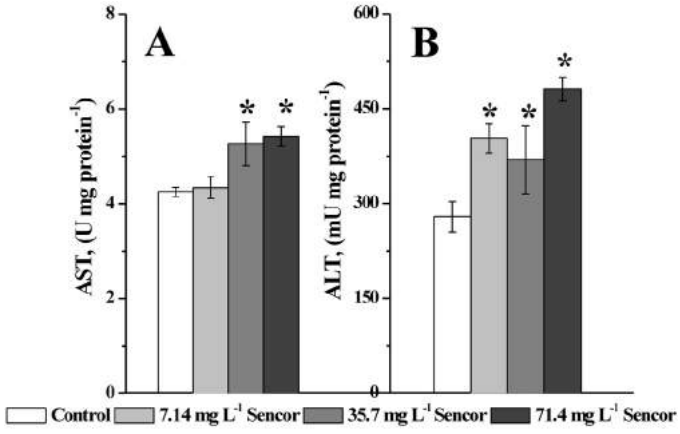

Fig. 1. The activities of aspartate (A) and (B) alanine (B) aminotransferases in plasma of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h. Data are presented as means ± SEM, $n = 5–7$. *Significantly different from the control group with $P < 0.05$.

could result from impaired functions, injury of hepatic cells and release of the enzymes into the bloodstream [38]. These data showed a negative correlation with the activities of aminotransferases in liver. Exposure of fish to Sencor at concentrations of 37.5 and 71.4 mg L^{-1} decreased AST activity in liver by 15% and 17%, respectively, as compared with control values (85.1 ± 3.0 U mg $protein^{-1}$) (Fig. 2A). Similarly, ALT activity decreased by 19% in liver of fish exposed to the 71.4 mg L^{-1} concentration of the herbicide, as compared with the control (52.7 ± 2.1 mU mg $protein^{-1}$) (Fig. 2B). Previously, Velisek et al. [9] observed no changes in AST and ALT activities in plasma of common carp after acute exposure to 175.1 mg L^{-1} metribuzin. Also, Velisek et al. [29] reported a decrease in ALT activity and no change in AST activity in blood plasma of common carp after long-term exposure to simazine. Other studies report similar findings in the aminotransferase activities in liver when investigating the effects of pesticides on various animals [40,41].

In concert, the data presented in this section clearly show that goldfish treatment by Sencor induced mild stress. However, it seems that even this sort of the stress could injure solid tissues and liver is the first candidate to be affected under these conditions. Therefore, we next decided to examine the integrity of this organ.

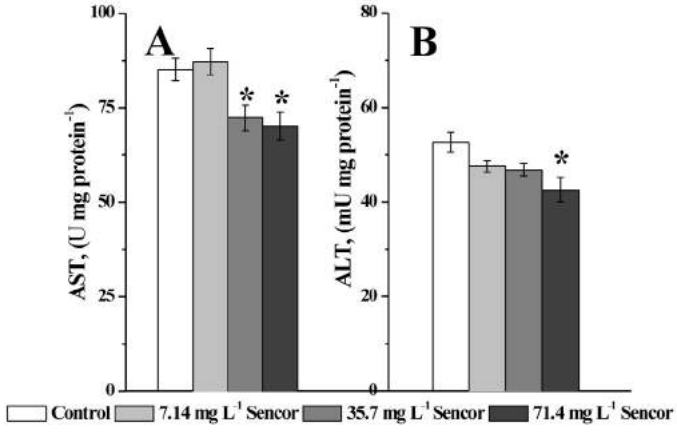

Fig. 2. The activities of aspartate (A) and alanine (B) aminotransferases in liver of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h. Other information as in Fig. 1.

3.2. Histological alterations

Fish liver is functionally similar to that in mammals and plays a vital role in many metabolic activities including detoxification processes. However, the localization of detoxification activities into this one organ may lead to injuries to hepatocytes which can be seen by histological investigation [42]. Therefore the assessment/evaluation of the hepatic histology is frequently applied to reveal the impact of contaminants on organisms including aquatic animals [43,44].

The histological assessment of the liver tissue showed histological changes in the liver cells in fish exposed to all concentrations of Sencor. Morphological changes were observed and showed severe degenerative injury to liver hepatocytes with increasing Sencor levels. In the control group acentric nuclei and fatty vacuolization were observed (Fig. 3A). Exposure for 96 h to 7.14 mg L^{-1} Sencor altered liver histology resulting in visible small-granulated hepatocytes with dystrophic lesions (Fig. 3B). Even greater changes occurred with the higher Sencor exposures. Hypertrophy in liver cells was observed along with an increased number of dilated sinusoids and damage to the vascular endothelium with subsequent hemorrhage at Sencor concentrations of 35.7 and 71.4 mg L^{-1} (Fig. 3C, D). The histological changes in liver of Sencor-exposed fish could be associated with pesticide metabolism. This fits well with the alterations to biochemical indices in liver and plasma of the fish, described earlier. For example, significantly increased activities of LDH, AST and ALT in plasma and decreased activities of these enzymes in liver of Sencor exposed fish suggest damage to hepatocytes and release of enzymes into the bloodstream. Similarly, Plhalova et al. [6] reported changes in hepatic histology (vacuolar degeneration of hepatocytes) in *Danio rerio* after exposure to metribuzin at a concentration of 53 mg L^{-1} for 28 days. Mela et al. [45] recently showed that exposure of neotropical catfish (*Rhamdia quelen*) to atrazine led to necrotic processes and enhanced the number of melanomacrophages (pigmented cells) in hepatic tissue. Pathological changes in the livers of yolk sac larvae of gilthead seabream (*Sparus aurata*) after 72 h exposure to a commercial formulation containing simazine (4.5 mg L^{-1}) were also demonstrated [46]. Banaee et al. [47] observed necrosis with occasional diffuse vacuolar degeneration of other hepatocytes, cytoplasmic vacuolation and hypertrophy of hepatocytes in gourami fish (*Trichogaster trichopterus*) after exposure to paraquat. However, Velisek et al. [9] did not find histological changes in liver of common carp after exposure to metribuzin for 96 h, but later Velisek et al. [48] reported histological changes in the liver (diffused steatosis with the loss of cellular shape and the presence of lipid inclusions in hepatic cells) in common carp after a 28-day exposure to 40 μg L^{-1} of terbutryn. Taken together, these results demonstrate different effects of herbicides on hepatic tissues depending on fish species, pesticide concentrations and exposure time.

The next section will focus on potential reasons leading to liver injury. Oxidative stress is commonly believed to induce these effects [42]. Reactive oxygen species (ROS) at elevated levels may cause membrane damage making them leaky and leading to cell apoptosis and necrosis.

3.3. Oxidative stress indices in liver

Different pesticides, including herbicides, may induce oxidative stress in aquatic organisms via several mechanisms. The induction of oxidative stress is usually monitored by measuring products of ROS-induced modification of cellular constituents, such as ROS-modified lipids and proteins, as well as low and high molecular mass antioxidants [49]. In our experiment the levels of CP and LOOH in the liver of control fish were 3.93 ± 0.41 nmol mg $protein^{-1}$ and 20.3 ± 2.2 nmol gwm^{-1}, respectively (Fig. 4). Fish exposure to Sencor led to reductions in CP levels by 34–60% as compared with

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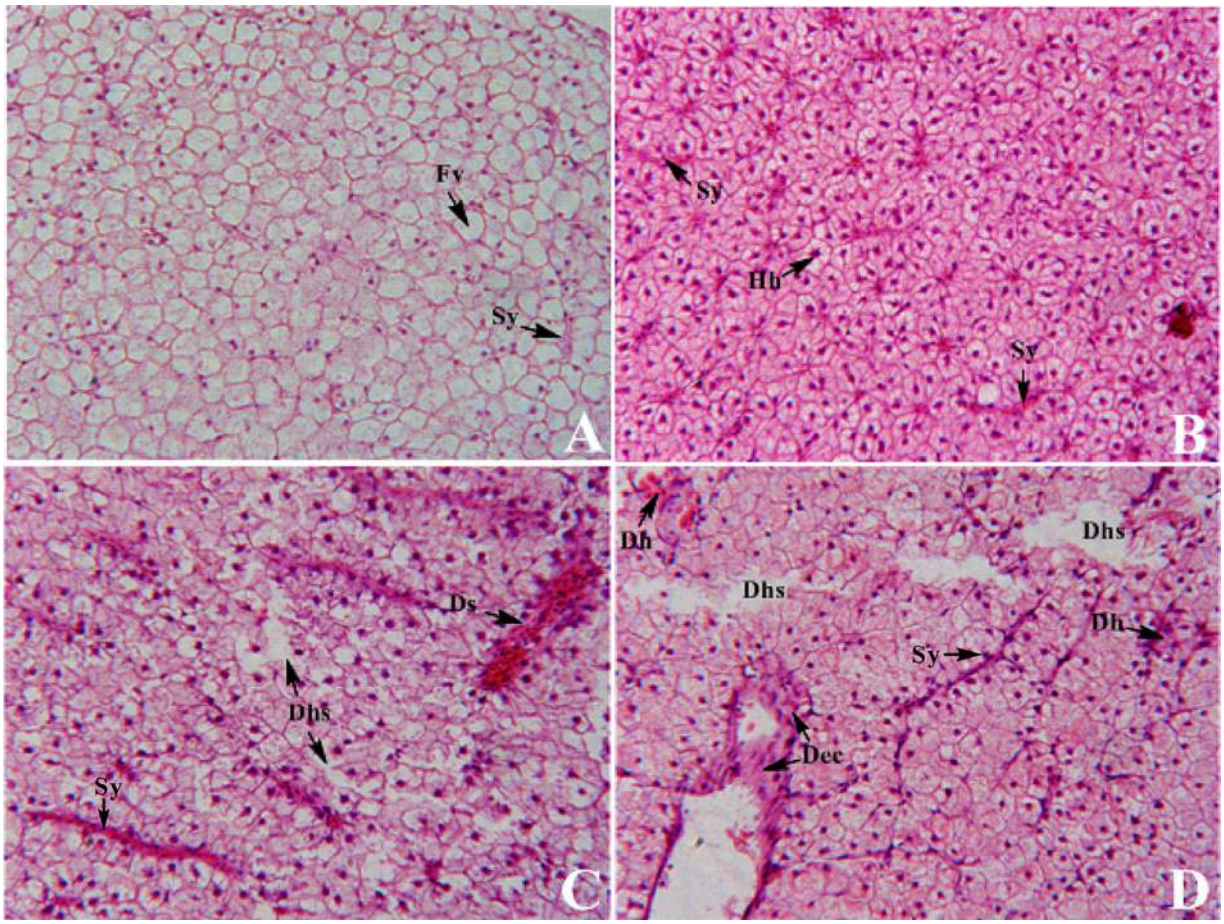

Fig. 3. Light micrographs of sections through liver of goldfish (*C. auratus* L.) showing histological structure of the control group (A), and animals treated with 7.14 (B), 35.7 (C) or 71.5 (D) mg L^{-1} of Sencor for 96 h. Samples were stained with hematoxylin and eosin and photomicrographs were taken using 200 × magnification. Fv, fatty vacuolization; Sy, sinusoids; Ds, dilated sinusoids; Hh, hypertrophy of hepatic cells; Dhs, dystrophy of hepatic cells; Dec, detachment of endothelial cytoplasm; Dh, detachment of endothelial cytoplasm with diffuse hemorrhage. These are representative pictures of sections prepared from multiple fish – at least four animals were tested per control or experimental groups.

controls. By contrast, the levels of LOOH increased by 49% and 75% in liver from the 35.7 and 71.4 mg L^{-1} of Sencor treatment groups, respectively. Increases in lipid peroxide contents indicate oxidative damage to lipids. These data correspond well to our previous studies which demonstrated stimulation of lipid peroxidation in goldfish liver by pesticides such as Tattoo [50] and Roundup [51]. However, lower CP levels in Sencor-treated animals was unexpected and theoretically could be related to enhanced proteolytic degradation of ROS-modified proteins or stimulated synthesis *de novo* of hepatic proteins which could result in "dilution" of damaged proteins. Finally, this could be associated in some way with selective elimination of ROS-modified proteins due to disintegration of hepatocytes.

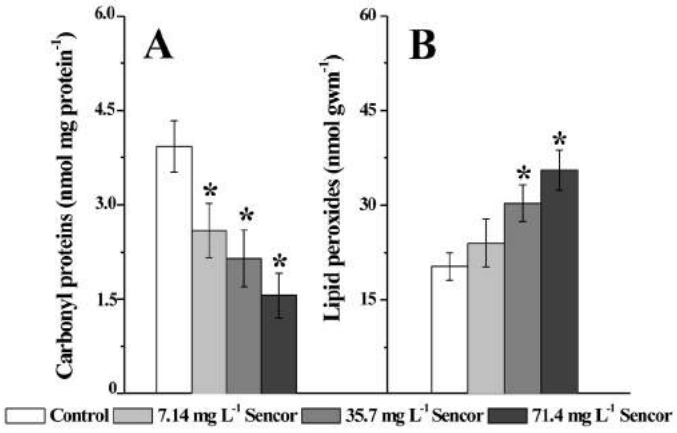

Fig. 4. Changes in the levels of indices of oxidative stress (A) carbonyl proteins, CP and (B) lipid peroxides, LOOH in liver of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h. Other information as in Fig. 1.

Cellular free thiols are usually classified in two groups, low molecular mass and high molecular mass components (L-SH and H-SH, respectively). The first group is mainly represented by glutathione (GSH), and the second one by protein thiols. Glutathione is mainly used for detoxification of ROS either via direct interaction with them, or by serving as a cofactor for antioxidant enzymes (glutathione peroxidases and glutathione-S-transferases) as well as for conjugation with xenobiotics [52]. Oxidative stress induced by xenobiotics may deplete GSH reserves [42,49]. In our experiments, Sencor did not affect the level of H-SH in the liver of goldfish (Table 2). However, the level of L-SH decreased by 28% in liver of fish exposed to 71.4 mg L^{-1} of Sencor as compared with the control value (1.97 ± 0.10 μmol gwm^{-1}) (Table 2). These results show the existence of efficient defense mechanisms which are capable of preventing some modifications of hepatic proteins and supporting detoxification of the herbicide. Similar results were obtained in our previous investigations with other herbicides diethyldithiocarbamate and Roundup [51,53].

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Table 2
The concentrations of high molecular mass (H-SH), low molecular mass (L-SH) thiols (μmol gwm^{-1}), and activities of acetylcholinesterase (AChE) (mU mg protein^{-1}) in liver of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h.

Parameter	Fish group			
	Control	7.14 mg L^{-1}	35.7 mg L^{-1}	71.4 mg L^{-1}
H-SH	4.39 ± 0.20	4.09 ± 0.36	4.66 ± 0.25	4.62 ± 0.39
L-SH	1.97 ± 0.10	1.75 ± 0.07	1.79 ± 0.12	1.42 ± 0.08*
AChE	17.0 ± 0.9	18.4 ± 1.1	14.9 ± 0.6	16.2 ± 0.4

Data are presented as means ± SEM, $n = 5–7$.
* Significantly different from the control group with $P < 0.05$.

3.4. Sencor-induced changes in the activities of liver antioxidant and associated enzymes

Inhibition of acetylcholine esterase (AChE) serves as a biomarker of environmental pollution with pesticides [54]. However, in our experiments goldfish exposure to Sencor at any concentration used did not change AChE activity in liver (Table 2).

Living cells possess enzymatic and non-enzymatic antioxidant defense systems capable of neutralizing ROS. The enzymatic antioxidant system includes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), glucose-6-phosphate dehydrogenase, etc. [49]. These enzymes form the group of high molecular mass antioxidants that act in concert with low molecular mass antioxidants under resting conditions to sustain a steady state ROS level. Under oxidative stress conditions antioxidants help cells to cope with enhanced ROS levels resulting from an imbalance between ROS generation and elimination [49,55,56].

Superoxide dismutase plays a critical role in the protection against ROS. Goldfish exposure for 96 h to 35.7 mg L^{-1} of Sencor led to higher SOD activity (by 56%) in liver as compared with controls (Fig. 5A). Similarly, other authors reported increased SOD activity in liver of zebrafish (*Danio rerio*), bluegill sunfish (*Lepomis macrochirus*) and freshwater fish (*Channa punctatus*) after atrazine exposure [57–59]. Velisek et al. [60] also observed increased SOD activity in liver of common carp after long-term exposure to terbutryn.

Goldfish exposure to 71.4 mg L^{-1} of Sencor decreased catalase activity by 46% as compared with the control group (49.3 ± 4.5 U mg protein^{-1}) (Fig. 5B). Inhibition of catalase could result from enzyme inactivation by superoxide anion radicals [61]. Previously,

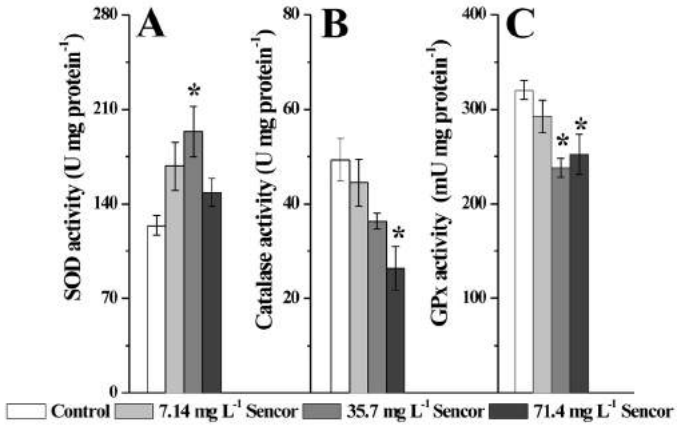

Fig. 5. The activities of antioxidant enzymes: (A) superoxide dismutase, SOD, (B) catalase, and (C) glutathione peroxidase, GPx in liver of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h. Other information as in Fig. 1.

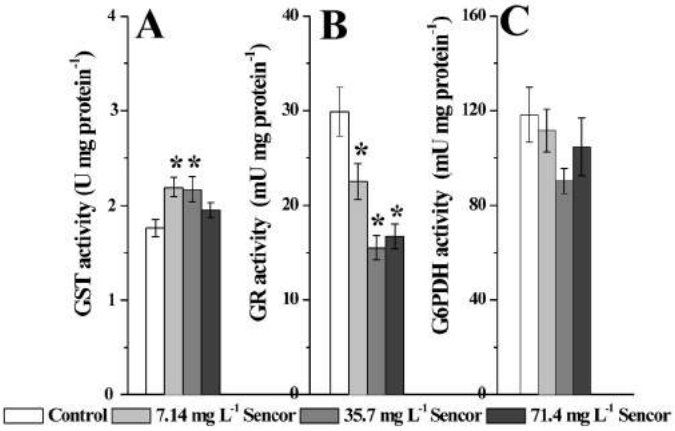

Fig. 6. The activities of antioxidant-associated enzymes, (A) glutathione-S-transferase, GST, (B) glutathione reductase, GR and (C) glucose-6-phosphate dehydrogenase, G6PDH in liver of goldfish, exposed to control conditions or 7.14, 35.7 and 71.4 mg L^{-1} of Sencor for 96 h. Other information as in Fig. 1.

it was shown that exposure to 10 and 100 μg L^{-1} of atrazine increased mRNA transcript levels of SOD and catalase in liver [57,62]. Ballesteros et al. [63] observed a significant decrease in catalase activity in liver of the one-sided livebearer (*Jenynsia multidentata*) exposed to endosulfan. On the contrary, Jin et al. [57] reported increased catalase activity in liver of zebrafish after 14 day exposure to atrazine at a concentration of 1 mg L^{-1}. Stara et al. [64] also observed increased catalase activity in liver of common carp (*Cyprinus carpio* L.) after 14 and 28 days exposure to simazine, while after 60 days catalase activity decreased. No changes of catalase activity were observed by Elia and colleagues [59] in liver of bluegill sunfish after exposure to different concentrations of Sencor. Overall, then, the data indicate a variable response by catalase to herbicide challenge and resolving these conflicting studies will require focused studies that examine the many potential parameters that could affect catalase activity.

Glutathione peroxidase catalyzes the GSH-dependent reduction of many peroxides, including H_2O_2 and LOOH, thereby protecting cells from ROS-induced damage [52]. In the liver of control fish, GPx activity was 320 ± 10 mU mg protein^{-1}. It decreased by 26% and 21% due to exposure to Sencor at concentrations 35.7 and 71.4 mg L^{-1}, respectively (Fig. 5C). A decrease in GPx activity might reflect a failure of the antioxidant system or could be related to enhanced $O_2^{\cdot -}$ production in liver of fish exposed to pesticides [63,65]. Similarly, Stara et al. [64] observed decreased GPx activity in liver of common carp after 28 or 60 days of simazine exposure. Ballesteros et al. [63] also found lower GPx activity in liver of fish exposed to 1.4 mg L^{-1} endosulfan for 24 h.

The group of glutathione-S-transferases consists of at least eight isoenzymes which catalyze GSH conjugation with electrophiles or operate as non-selenium peroxidases acting on lipid peroxides [52]. Generally, studies of glutathione-S-transferases focus on GSH conjugation with electrophiles, as we do in this work. It has been demonstrated that GST activity can be altered in polluted locations, and that the presence of organic contaminants may increase its activity [66]. After 96 h exposure to 7.14 and 35.7 mg L^{-1} of Sencor GST activity increased by 24% as compared with the control group (1.76 ± 0.09 U mg protein^{-1}) (Fig. 6A). These results may indicate enhanced liver potential for biotransformation of the xenobiotic Sencor in response to pesticide challenge, indicating activation of defense mechanisms. Similarly, Elia et al. [58] observed increased GST activity in liver of bluegill sunfish after exposure to atrazine at a concentration of 6 and 9 mg L^{-1}. However, Velisek et al. [29] observed no changes in GST activity in liver of common carp after

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long-term exposure to simazine at different concentrations. Some authors reported decrease in GST activity after exposure to atrazine in various fish organs [67,68].

Glutathione reductase is an enzyme that plays an important role in cellular antioxidant protection because it catalyzes the conversion of oxidized glutathione back to the reduced state [52]. Hence, GR activity is a potential biochemical marker of oxidative stress development in animals [69]. In our experiments, the activity of GR in liver decreased by 25, 48 and 44% at 7.14, 35.7, or 71.4 mg L^{-1} of Sencor, respectively, as compared with controls (29.9 ± 2.6 mU mg protein^{-1}) (Fig. 6B). These changes could result in GSH depletion in liver, as was seen in Table 2 for L-SH, while GSH synthesis alone cannot support redox status of cells [70]. Similarly, Velisek et al. [60] reported decreased GR activity in liver of common carp after 90-day exposure to terbutryn at environmentally realistic concentrations. On the contrary, Nwani et al. [59] reported that GR activity increased significantly in the liver of *Channa punctatus* exposed to 10.6 mg L^{-1} of atrazine. No significant difference in GR activity was found in liver of common carp and bluegill sunfish after chronic and acute exposure to simazine and atrazine [58,64].

Glucose-6-phosphate dehydrogenase is the first enzyme in the pentose phosphate pathway. It carries out the oxidation of glucose-6-phosphate and synthesizes NADPH in a coupled reaction. One of the primary roles of NADPH is participation in the GR-catalyzed reduction of oxidized glutathione to maintain GSH/GSSG balance at proper levels [49,71]. In our investigation, there was no significant difference of G6PDH activity in liver of control and Sencor-treated fish (Fig. 6C).

4. Conclusions

The work reported here clearly demonstrates that short-term exposure of goldfish to the metribuzin-containing herbicide, Sencor, affected blood plasma and liver parameters collectively reflecting development of general stress. These changes corresponded with histological changes to liver tissue. Inspection of the parameters of oxidative stress in liver indicates that the histological changes could be induced by stimulation of ROS-related processes.

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