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ORIGINAL ARTICLE

Evaluation of ibuprofen toxicity for zebrafish (*Danio rerio*) targeting on selected biomarkers of oxidative stress

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Abstract

OBJECTIVES: The aim of the study was to investigate the effects of subchronic exposure of zebrafish to ibuprofen, using selected oxidative stress parameters as a target.

DESIGN: Toxicity tests were performed on *Danio rerio* according to OECD No. 203 and No. 215. In the growth test, fish were exposed to subletal concentrations of ibuprofen (0.0001, 0.05, 1, 8, and 25 mg.L^{-1}) for 28 days. For the assessment of free radical defense in fish, the catalytic activities of glutathione reductase (GR), glutathione S-transferase (GST), glutathione peroxidase (GPx), and catalase (CAT), as well as the concentration of malondialdehyde (MDA) were measured.

RESULTS: Ibuprofen did not affect the activity of glutathione reductase and catalase. A significant (p<0.01) increase in the activity of glutathione peroxidase was found, which was proved dose-dependent (10.58 nmol NADPH per min per mg protein in the control and 20.53, 26.36, 26.89, and 45.87 nmol NADPH per min per mg protein in the ibuprofen concentrations of 0.5, 1, 8, and 25 mg.L^{-1}. An increased (p<0.05) activity of glutathione S-transferase in the highest concentration was found compared to control. Malondialdehyde levels were found significantly (p<0.01) decreased from control in the concentrations of 0.0001 and 8 mg.L^{-1}, but no dose-dependence was found.

CONCLUSION: The results suggest that ibuprofen causes the increase in the activity of some antioxidative and biotransformation enzymes in zebrafish (GPx and GST). We also found a significant decrease in lipid peroxidation in the concentrations of 0.0001 and 8 mg.L^{-1} compared to control.

Abbreviations:

CAT	- catalase
COX-1	- cyclooxygenase-1
COX-2	- cyclooxygenase-2
coxib	- cyclooxygenase inhibitor
DMSO	- dimethyl sulfoxide
GPx	- glutathione peroxidase
GR	- glutathione reductase
GSH	- reduced glutathione
GSSG	- oxidized glutathione
GST	- glutathione S-transferase
H_2O_2	- hydrogen peroxide
Hb	- hemoglobin
Hk	- hematocrit
HPLC	- high performance liquid chromatography
hsp	- heat shock protein
IB	- ibuprofen
LDL	- low density lipoproteins
LOQ	- limit of quantification
MCHC	- mean corpuscular hemoglobin concentration
MCH	- mean corpuscular hemoglobin
MCV	- mean cellular volume
MDA	- malondialdehyde
NADPH	- nicotinamide adenine dinucleotide phosphate
NSAIDs	- nonsteroidal anti-inflammatory drugs
OECD	- Organisation for Economic Co-operation and Development
PGE2	- prostaglandine 2
PGs	- prostaglandines
RBC	- red blood cells, erythrocyte count
ROS	- reactive oxygen species
SEM	- standard error of the mean
SD	- standard deviation
TBARS	- thiobarbituric acid reactive substances
WBC	- white blood cells, leukocyte count
WWTPs	- waste water treatment plants

INTRODUCTION

Ibuprofen (IB), a propanoic acid derivate, is widely used as an analgesic, antipyretic, and anti-inflammatory therapeutics. It is a non-selective cyclooxygenase (i.e. cyclooxygenase-1, COX-1, and cyclooxygenase-2, COX-2) inhibitor (coxib) known to reduce the synthesis of prostaglandins (PGs) that play a prominent role in the inflammation in both animals and humans (FitzGerald & Patrono 2001; Black & Hill 2003; Burdan *et al.* 2006; Rainsford 2009).

PGs are known to be involved in the regulation of Na^+/K^+-ATPase activity in mammals (Scherzer *et al.* 1992; Borsick *et al.* 2006; Kreydiyyeh & Markossian 2006) and there is a growing evidence of their involvement in osmoregulation in fish gills (Evans *et al.* 2005; Choe *et al.* 2006). Impaired ion regulation in ibuprofen treated fish could be mediated through the inhibition of the PGs pathway, which may modulate both cortisol production and the sodium pump activation (Gravel *et al.* 2008). The recent findings that ibuprofen is a hsp70 inducer (hsp70 is a cellular stress protein that is critical for the defense against stressor-mediated proteotoxicity in trout liver and gill) support also a distinct model of action for this drug in disturbing ion regulation (Feng *et al.* 2003; Gravel & Vijayan 2007).

Ibuprofen, belonging to one of the most important groups of pharmaceuticals worldwide, i.e. nonsteroidal anti-inflammatory drugs (NSAIDs), is one of the most widely used human medicine, administered in the amounts of hundreds of tons per year (Fent *et al.* 2006).

IB is not fully metabolized in humans and enters the sewage system. It remains in the aquatic environment and, moreover, has been recognized as one of the main pharmaceuticals present in the aquatic ecosystems (Bendz *et al.* 2005). In general, IB exhibits a relatively high (around 90%) removal rate in WWTPs (Onesios *et al.* 2009).

Continuous input of pharmaceuticals into the aquatic environment may influence the non-target organisms including fish (Fent *et al.* 2006; Ginebreda *et al.* 2010; Li *et al.* 2011).

Municipal wastewater effluents are the main water source of IB (Han *et al.* 2010; Saravanan *et al.* 2012). IB levels found in waste water treatment plants (WWTPs) effluents usually range between $\mu g.L^{-1}$ and $ng.L^{-1}$. For example, in water of the Spanish river Llobregat River, there was found IB in the concentration of $1.37\ \mu g.L^{-1}$ (Ginebreda *et al.* 2010). In all samples of surface water from Mexico City, IB has been detected in the range of $15–45\ ng.L^{-1}$. At the same time, there was found no IB in ground water samples (Félix-Cañedo *et al.* 2013).

The study of Kozisek *et al.* (2013) focused on the first large-scale assessment of pharmaceuticals in drinking water in the Czech Republic. In the initial survey of tap water from 92 major supply zones using mostly surface water, no pharmaceutical exceeded the limit of quantification ($LOQ = 0.5\ ng.L^{-1}$). In the second survey, samples were collected from the outlets of 23 WWTPs, in which IB was the most frequently found pharmaceutical (19 samples).

The previous literature clearly indicates that the toxicity of IB is varying from one species to another and even in breeds of the same species. The acute toxicity concentrations of IB appear to be in $mg.L^{-1}$ range, which is higher than the concentrations observed in natural environment (Parolini *et al.* 2011; Saravanan *et al.* 2012).

The aim of the study was to assess the effect of subchronic exposure of juvenile zebrafish (*Danio rerio*) to sublethal concentrations of ibuprofen using selected oxidative stress parameters as a target.

MATERIAL AND METHODS

Toxicity tests were performed on the model organism *Danio rerio* according to OECD guidelines no. 203 (Fish, Acute Toxicity Test) and no. 215 (Fish, Juvenile Growth Test).

Acute toxicity test

Acute toxicity test was performed according to OECD No. 203. Ibuprofen (Sigma-Aldrich, Czech Republic) was dissolved in water with the addition of dimethyl

sulfoxide as a solvent (0.1% DMSO). In the test, five ascending concentrations of ibuprofen (0.05, 1, 5, 10, and 30 mg.L^{-1}) and two control groups (the first one with water, the second one with water and solvent) were used. The test was performed in a duplicate. In the test, the total of 120 fish (*Danio rerio*) were used. Food had been withheld 72 hours before the test started. Ten juvenile fish (aged 30 days) were placed in each test aquarium. Test was performed using a semi-static method with solutions renewed every 24 hours. During the test, fish were not fed. Fish conditions and a number of dead fish were checked every 24 hours. Duration of the test was 96 hours. Water temperature, pH, and oxygen saturation were monitored every day and were as follows: temperature 23±1 °C, oxygen concentrations above 60%, and pH ranged from 8.22 to 8.67.

Every 48 hours, water samples were collected for the measurement of a real concentration of ibuprofen in water. The concentrations of the test substance were measured using HPLC with photometric detection over 90% of nominal value.

The results of the acute toxicity test were used for the determination of a concentration range used in a subchronic toxicity test.

Subchronic toxicity test

Ibuprofen subchronic toxicity test was performed according to OECD No. 215. The test was conducted using a 30-day-old *Danio rerio*. Fish were acclimatized before the test.

In each aquarium, ibuprofen was dissolved in the water using DMSO as a solvent in volume of 2 µl.L^{-1}. Fish were exposed to five sublethal concentrations, i.e. 0.0001 (an environmental concentration), 0.05, 1, 8, and 25 mg.L^{-1} (all in a duplicate). Two groups were control, the first one with water, the second one with water and solvent (DMSO). The total of 360 fish was used in the test. The fish were randomly distributed into 30 liter glass aquaria, 30 specimens in each aquarium.

The experiment was conducted in a flow-through system with a test solution renewal every twelve hours. Fish were fed with dried *Artemia salina* without shells at 8% of body weight per day. The food ration was based on an initial fish weight and recalculated after 14 days of the test. During the test, water condition parameters were monitored at 24 hr intervals. Water quality values were as follows: temperature 23±1 °C, oxygen saturation above 60% (ranged between 70% and 91%), pH 8.61–8.88. Duration of the test was 28 days.

Water samples were collected each 7th day to measure real concentrations of ibuprofen in water.

Fish sampling and homogenization

At the end of the subchronic test, fish were euthanized by carbon dioxide. Body weight and length of each fish were recorded. Fish were frozen and stored at –85 °C until analysis. During the analysis, whole body samples were weighed and homogenized (1:10 w/v) using a phosphate buffer (pH 7.2). The homogenate was then divided into two parts. First one was used for the measurement of thiobarbituric acid reactive substances (TBARS), the second was centrifuged (10,500 *g* at 4 °C for 20 min), an obtained supernatant fraction was then used for the determination of glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx), and catalase (CAT) catalytic activities.

Measurement of oxidative stress parameters

The total catalytic activity of glutathione S-transferase (GST) was determined by the measurement of the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione (GSH) at 340 nm (Habig *et al.* 1974). The specific activity was expressed as nmol of formed product per min per mg of protein.

The catalytic activity of glutathione peroxidase (GPx) and glutathione reductase (GR) was determined spectrophotometrically at 340 nm by catalysis conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) for the consumption of NADPH (Carlberg & Mannervik 1975). The specific activities were expressed as nmol of NADPH consumption per min per mg of protein (Carlberg & Mannervik 1975).

For the assessment of GST and GR catalytic activities, the concentration of proteins was determined by Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, St. Louis, MO), bovine serum albumin was used as a standard (Smith *et al.* 1985).

The catalytic activity of catalase was determined by a spectrophotometrical measurement of H_2O_2 breakdown at 240 nm. The specific activity was expressed as µmol of decomposed H_2O_2 per min per mg of protein (Aebi 1984).

To determine a lipid peroxidation in fish samples, malondialdehyde (MDA) was measured by the TBARS assay at 535 nm described by Lushchak *et al.* (2005). The concentration of MDA was expressed in the units of nmol per gram of tissue wet weight.

All spectrophotometric measurements were performed using the Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc., USA).

Determination of ibuprofen concentration in water

Measurement of IB concentrations in water samples was performed using a high performance liquid chromatography (HPLC) with photometric detection. Samples were filtered through 0.45 µm nylon filter (Millipore, Billerica, MA) and used for analysis. The sample volume injected into the HPLC system was 10 µL. IB was separated by an isocratic elution method with acetonitrile/water 70/30 (v/v) on a 150×4.6 mm, 5 µm Zorbax Eclipse XBD-C18 column (Agilent Technologies, Santa Clara, CA). The mobile phase flow rate was 1 mL.min^{-1}, column temperature was 35 °C, and UV detection was performed at 220 nm. The chromatographic analysis was accomplished by means of Alliance

2695 chromatographic system (Waters, Milford, MA) with a PDA 2996 photodiode array detector (Waters, Milford, MA). IB was purchased from Sigma-Aldrich (St. Louis, MO). All solvents used in the measurement were HPLC-grade purity (Chromservis, Ltd., CZ). A detection limit for IB was 150 ng.mL^{-1}. The coefficient of variation was 4.5%.

Statistical analysis

Oxidative stress biomarkers were tested for normal distribution using the Shapiro-Wilk test. After testing for homogeneity of variance across groups, an analysis of variance (one-way ANOVA) was used. The differences among test groups were assessed with the Tukey-HSD test with $p<0.05$ as the level of significance.

Ethical statement

All experimental procedures were approved by the institutional committee and performed in a compliance with institutional guidelines and national legislation (Act No. 246/1992 Coll., on the Protection of Animals Against Cruelty, as amended).

RESULTS

Mortality of the fish in the subchronic test and the concentration of ibuprofen

In subchronic toxicity test, mortality of juvenile fish was found less than 7% in all experimental and control groups. In the control group, mortality did not exceed 4%, which is in an agreement with validation criteria of the juvenile growth test.

Analyzed concentrations of ibuprofen in water were found above 80% of the nominal concentrations in the course of acute and subchronic test.

Fish weight and length

At the end of subchronic toxicity test, all fish were weighed and their length was measured. Statistical analysis of somatic parameters was then performed. Final weight of the fish ranged between 94.6 and 105.0 mg in all groups.

As for fish length, a significant ($p<0.01$) decrease was found in fish exposed to 25 mg.L^{-1} of ibuprofen (20.3 mm) compared to control (22.1 mm).

Effect of ibuprofen on glutathione reductase (GR) activity

The activities of glutathione reductase in fish exposed to ibuprofen at the concentrations of 0.0001, 0.05, 1, 8, and 25 mg.L^{-1} were found 10.22**,** 7.76, 8.32, 8.02, and 8.32 nmol per min per mg protein, respectively. In the control group, the enzyme activity was found 9.16 nmol per min per mg protein. GR activity slightly (non-significantly) decreased in fish exposed to 0.05, 1, 8, and 25 mg.L^{-1} of ibuprofen when compared to control (Figure 1). No significant shift was determined in the activity of GR.

Effect of ibuprofen on glutathione peroxidase (GPx) activity

A significant ($p<0.01$) increase in the activity of glutathione peroxidase was found in all tested groups (except the environmental concentration, i.e. 0.0001 mg.L^{-1}) when compared to the control (10.58 nmol per min per mg protein). GPx activity in fish exposed to ibuprofen was found 14.68, 20.53, 26.36, 26.89, and 45.87 nmol per min per mg protein at the concentrations of 0.0001, 0.05, 1, 8, and 25 mg.L^{-1}, respectively (Figure 2).

Effect of ibuprofen on glutathione S-transferase (GST) activity

When compared to the control (52.18 nmol per min per mg protein), a slight increase in glutathione S-transferase activity was found in fish exposed to ibuprofen at the concentrations of 0.0001, 0.05, 1, and 8 mg.L^{-1} (55.53, 56.58, 57.11, and 55.81 nmol.min^{-1}.mg^{-1} protein, respectively). A significant ($p<0.05$) increase was

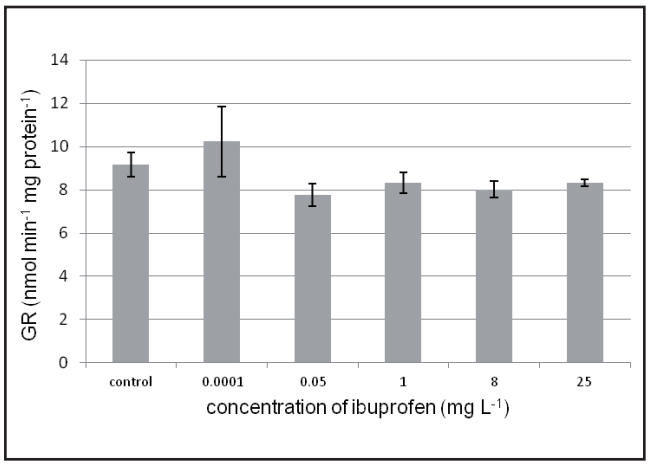

Fig. 1. Glutathione reductase (GR) activity in zebrafish exposed to ibuprofen (means ± SEM).

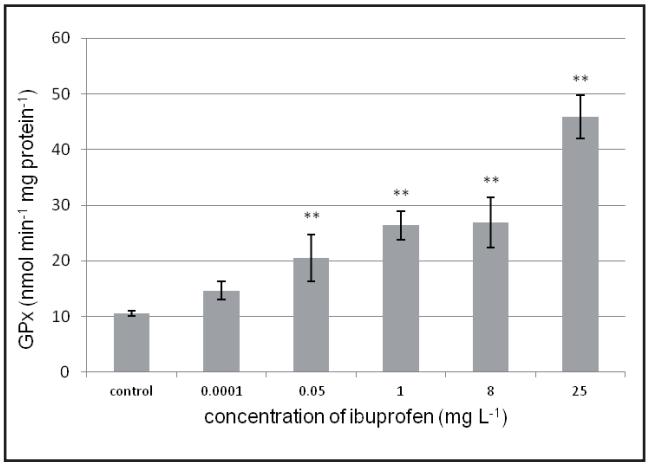

Fig. 2. Glutathione peroxidase (GPx) activity in zebrafish exposed to ibuprofen (means ± SEM).

found in the group exposed to ibuprofen in the concentration of 25 mg.L^{-1} (61.67 nmol.min^{-1}.mg^{-1} protein) compared to the control (Figure 3).

Effect of ibuprofen on catalase (CAT) activity

When compared to the control group, no significant differences in catalase activity were found in all tested concentrations. In our study, ibuprofen did not affect the activity of catalase (Figure 4).

Effect of ibuprofen on lipid peroxidation

A significant (p<0.01) decrease in malondialdehyde (MDA) level was found in the concentrations of 0.0001 mg.L^{-1} (29.49 nmol per gram of tissue wet weight) and 8 mg.L^{-1} (27.92 nmol.g^{-1} of tissue wet weight) compared to control group (40.81 nmol.g^{-1} of tissue wet weight). In the concentrations of 0.05, 1, and 25 mg.L^{-1}, malondialdehyde levels were found 33.37, 35.13, and 33.29 nmol.g^{-1} of tissue wet weight. In general, the decrease was not found dose-dependent (Figure 5).

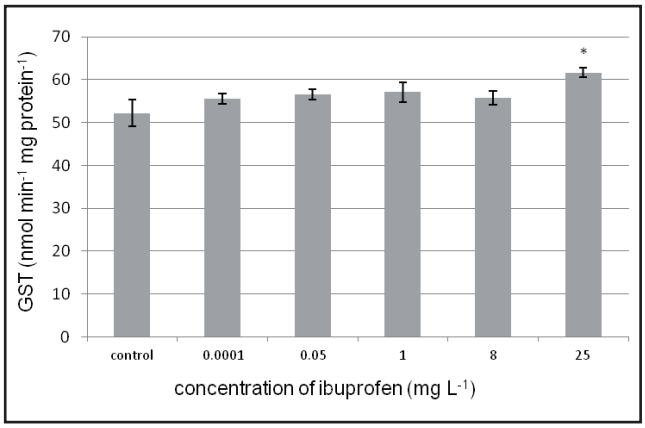

Fig. 3. Glutathione S-transferase (GST) activity in zebrafish exposed to ibuprofen (means ± SEM).

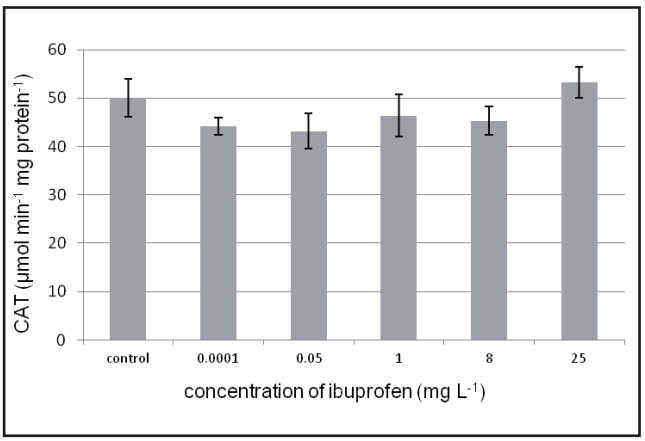

Fig. 4. Catalase (CAT) activity in zebrafish exposed to ibuprofen (means ± SEM).

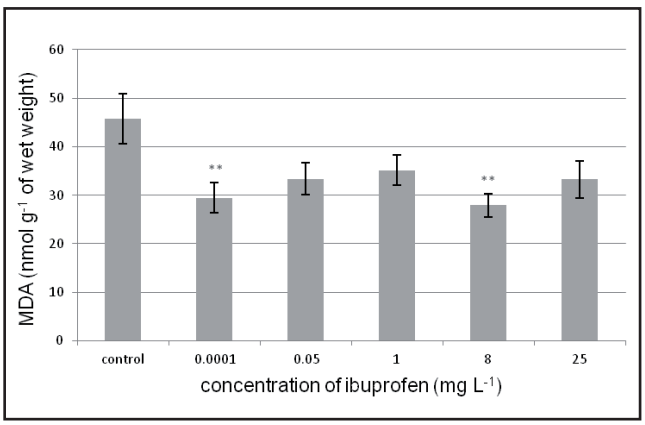

Fig. 5. Malondialdehyde (MDA) concentrations in zebrafish exposed to ibuprofen (means ± SEM).

DISCUSSION

Antioxidants help prevent cellular damage caused by metabolically- and environmentally-produced reactive oxygen species (ROS). Antioxidants can be enzymes or molecules. Among important antioxidant enzymes glutatione reductase belongs, which regenerates a reduced glutathione used as a direct scavenger of ROS or as a substrate for the antioxidant enzyme, glutathione peroxidase. GPx transforms hydroperoxides to hydroxyl compounds using a reduced glutathione as a substrate. Glutathione S-transferase is the phase II detoxifying enzyme. Catalase (CAT) catalyzes the transformation of H_2O_2 to water and oxygen (Speers-Roesch & Ballantyne 2005).

An increase in antioxidant enzymes activities contributes to the elimination of ROS. Oxidative stress will occur, if the activities of the antioxidant defense system decrease and/or ROS production increases (Zhang *et al.* 2004).

There was not found any significant effect (p>0.05) of ibuprofen on glutathione reductase (GR) catalytic activity in our experiment. Gonzalez-Rey & Bebianno (2012) assessed IB potential effect as an oxidative stress inducer in mussel *Mytilus galloprovincialis*. Over the first week of exposure, GR activity increased linearly, reaching 2.8-fold higher activity than control (p<0.01). After two weeks of exposure, GR activity decreased (about 2.4-fold), reaching a value not different from control. In the study of Blahova *et al.* (2013), the activity of GR increased significantly in experimental groups of zebrafish after 28-day exposure to atrazine at the concentrations of 0.0003 and 0.03 mg.L^{-1}. Hostovsky *et al.* (2012) found a significant increase in GR activity in embryo-larval stages of common carp (*Cyprinus carpio*) exposed for 30 days to 0.52 mg.L^{-1} of terbuthylazine and 0.9, 4, and 14 mg.L^{-1} of metribuzin compared to control.

In our study, there was found a significant increase (p<0.01) in the activity of glutatione peroxidase (GPx) in zebrafish exposed to ibuprofen at the concentrations of 0.05, 1, 8, and 25 mg.L^{-1}. It can be explained by the

presence of oxidative substances in cells, which may cause an elevation of antioxidant enzymes as a defense mechanism (Li *et al.* 2010). Similarly, Blahova *et al.* (2013) found an increase in GPx activity in zebrafish (*Danio rerio*) exposed to atrazine at the concentrations of 0.0003, 0.003, and 0.03 mg.L^{-1}. Environmentally relevant concentration of IB (0.0001 mg.L^{-1}) did not cause an increase in the activity of GPx in our experiment.

The exposure of zebrafish to ibuprofen caused a slight (non-significant) increase in glutathione S-transferase (GST) activity in all tested groups compared to control. A significant (p<0.05) increase was found only in the fish exposed to 25 mg.L^{-1} of ibuprofen. Wiegand *et al.* (2001) reported the increase in GST activity in zebrafish exposed to atrazine in the concentrations up to 5 mg.L^{-1}. The higher concentrations caused a decrease in GST activity. Similar trends were seen in the study of Blahová *et al.* (2013) where GST activity was non-significantly increased in fish exposed to atrazine at the range of 0.0003–0.03 mg.L^{-1} compared to control group, while atrazine at 0.09 mg.L^{-1} significantly reduced GST activity. This decrease might indicate that high concentrations of atrazine lead to a down-regulation of the detoxifying system. The study of Hostovsky *et al.* (2012) showed that subchronic concentrations of the pesticide terbuthylazine non-significantly increased GST activity in embryo-larval stages of common carp. The exposure of common carp embryo-larval stages to a herbicide metribuzin caused a significant increase in GST activity in all tested concentrations (up to 90% at 32 mg.L^{-1}) compared to the control group.

In our experiment, IB did not affect catalase (CAT) activity in zebrafish after 28 days of exposure. In the study of Gonzalez-Rey & Bebianno (2012), CAT activity in the digestive gland of exposed mussels *Mytilus galloprovincialis* was about 2-fold higher on the 3rd and 7th day when compared to control (p<0.05), decreasing to control levels after two weeks (p>0.05).

Malondialdehyde (MDA), one of the major terminal products of lipid peroxidation, appears to be a potential biomarker of this process. MDA concentration sharply increases in cells exposed to oxidative stress (Slaninova *et al.* 2009). We found a decrease in MDA concentration in all investigated groups. A significant decrease (p<0.01) was found in fish exposed to 0.0001 and 8 mg.L^{-1} ibuprofen compared to control. It seems that ibuprofen protects lipids against peroxidation in zebrafish. Lipid peroxidation levels of mussels (*Mytilus galloprovincialis*) were significantly enhanced by ibuprofen presence over the first week of exposure indicating damage derived from oxidative stress. This was followed by a significant decrease in lipid peroxidation by the end of the two weeks (Gonzalez-Rey & Bebianno 2012).

In the investigation of Saravanan *et al.* (2012), Indian major carp (*Cirrhinus mrigala*) was exposed to ibuprofen. In a sublethal treatment, a significant decrease in erythrocyte count (RBC), mean corpuscular hemoglobin concentration (MCHC), and plasma protein levels were observed throughout the study period. In contrast, hemoglobin (Hb), hematocrit (Hk), mean cellular volume (MCV), mean corpuscular hemoglobin (MCH), leukocyte count (WBC) as well as plasma glucose and alanine transaminase levels were increased in the study. A mixed trend was noticed in aspartate aminoferase activity.

The study of Gonzalez-Rey & Bebianno (2012) highlights the higher and more progressive impact of IB as an endocrine disruptor than as a short-term ROS-generator.

Other studies reveal the importance of ibuprofen in inhibiting the synthesis of prostaglandine 2 (PGE2) in the gill tissue of bluntnose minnow (*Pimephales notatus*). Bhandari & Venables (2011) estimated the IC50 of ibuprofen for the inhibition of PGE2 production in the gill tissue of bluntnose minnows to be 0.4μM based on fresh tissue weight.

Further studies of different tissues under conditions of field exposure to the full range of NSAIDs occurring in WWTP receiving streams will be required to reveal the potential inhibitory role of NSAIDs for eicosanoid production in an ecologically realistic context (Bhandari & Venables 2011).

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