ORIGINAL ARTICLE

Oxidative stress parameters in fish after subchronic exposure to acetylsalicylic acid

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Abstract

OBJECTIVES: The aim of this study was to investigate the effects of subchronic exposure of juvenile development stages of zebrafish (*Danio rerio*) to acetylsalicylic acid using selected oxidative stress biomarkers.
DESIGN: Toxicity test with acetylsalicylic acid was performed according to the OECD Guideline No. 215, fish *D. rerio* aged 30 days were used. The tested concentrations were 0.004, 0.4, 40, 120 and 250 mg.L^{-1}, duration of the test was 28 days. Products of lipid peroxidation and antioxidant enzymes were determined as the markers of oxidative stress.
RESULTS: Significantly increased glutathione S-transferase activity was found in fish exposed to acetylsalicylic acid concentrations 40, 120 and 250 mg.L^{-1}. The highest values of glutathione reductase activity were found in the groups exposed to acetylsalicylic acid concentrations 0.4, 40 and 120 mg.L^{-1}. In the group exposed to acetylsalicylic acid concentrations 40 mg.L–1, catalase activity was significantly higher compared to the control group. Significantly higher glutathione peroxidase activity was found in the groups exposed to acetylsalicylic acid concentrations 0.004 and 120 mg.L^{-1}. The concentrations of TBARS were lower in fish exposed to acetylsalicylic acid at all tested concentrations compared to control.
CONCLUSION: The subchronic exposure of zebrafish to acetylsalicylic acid causes an increase in activity of antioxidant and biotransformation enzymes and a decrease in lipid peroxidation.

Abbreviations:

$ANC_{4.5}$	- acid neutralizing capacity
ASA	- acetylsalicylic acid
CAT	- catalase
COD_{Mn}	- chemical oxygen demand
GPx	- glutathione peroxidase
GR	- glutathione reductase
GST	- glutathione S-transferase
HPLC	- high performance liquid chromatography
NADPH	- nicotinamide adenine dinucleotide phosphate
NSAIDs	- non-steroidal anti-inflammatory drugs
OECD	- Organisation for economic cooperation and development
ROS	- reactive oxidative species
SEM	- standard error of the mean
STP	- sewage treatment plant
TBARS	- thiobarbituric acid reactive substances

INTRODUCTION

Pharmaceuticals are very important environmental contaminants. High amounts of drugs belonging to different medicinal classes are produced every year and applied in human and veterinary medicine. They are designed to target specific metabolic and molecular processes in humans and animals, but they often have adverse effects too. These chemicals are excreted from the body in their native form or as metabolites and enter aquatic systems through different ways. Municipal wastewater is the main source that brings human pharmaceuticals after normal use and disposal of unused medicines into the environment. Some pharmaceuticals are not readily degraded in the sewage treatment plant (STP). Therefore, residues and metabolites of these chemicals are often found in STP effluents, surface water, seawater, groundwater and sometimes even drinking water. Only little is known about ecotoxicological effects of pharmaceuticals on aquatic and terrestrial organisms and wildlife (Fent *et al.* 2006).

Acetylsalicylic acid (ASA), which belongs to nonsteroidal anti-inflammatory drugs (NSAIDs), is one of the worldwide most used pharmaceuticals in human medicine. NSAIDs are pharmaceuticals inhibiting cyclooxygenase enzyme, which is responsible for the synthesis of prostaglandins from arachidonic acid (Vane & Botting 1998). They are commonly used for the treatment of fever, pain and inflammation.

The average elimination of ASA during passage through the sewage treatment plant is up to 81%. This drug has been found in STP effluents at concentrations up to 1.5 μg.L^{-1} and in rivers and streams up to 0.34117μg.L^{-1} (Ternes 1998). Although ASA is readily biodegradable it is found in a number of river water samples (Richardson & Bowron 1985; Ternes 1998).

Recent studies showed that salicylates have negative effects on aquatic vertebrates. Salicylates modified the release of several important hormones and inhibited the response of fish to an acute stressor (van Anholt *et al.* 2003; Gravel & Vijayan 2007a, b; Gravel *et al.* 2009).

Oxidative stress is the result of an imbalance between production and elimination of free radicals. The result of this imbalance is excess of free radicals. These free radicals are necessary for some processes in organism, but are also responsible for number of degenerative processes (Davies 1995; Toro & Rodrigo 2009). The only possibility to monitor and evaluate oxidative stress is determination of the biomarkers of these processes. Biomarkers of oxidative stress are mainly products of lipid peroxidation, antioxidant and biotransformation enzymes (Di Giulio & Hinton 2008).

The aim of this study was to investigate the effects of subchronic exposure of juvenile development stages of zebrafish *(Danio rerio*) to ASA using selected oxidative stress biomarkers. As these biomarkers, activities of enzymes glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and glutathione S-transferase (GST) were used and as products of lipid peroxidation, content of thiobarbituric acid reactive substances (TBARS) were determined.

MATERIALS AND METHODS

Subchronic toxicity test

Toxicity test with acetylsalicylic acid was performed on fish *Danio rerio*, which is one of the model organisms widely used in toxicity testing of various environmental contaminants (Dave & Xiu 1991; Ferrari *et al.* 2003; Lawrence 2007; Plhalova *et al.* 2011).

Toxicity test was performed on *D. rerio* at the age of 30 days, according to OECD Guideline No. 215 Fish, Juvenile Growth Test. Experimental fish were exposed to a range of sublethal concentrations of ASA (Sigma-Aldrich, Czech Republic; chemical purity – 99.0%) in water. These concentrations were 0.004, 0.4, 40, 120 and 250 mg.L^{-1}, the duration of the test was 28 days.

The fish were randomly distributed into 30 L glass aquaria, 47 specimens per each. The experiment was conducted in a flow-through system, and the test solutions were replaced twice a day. Each concentration was performed in duplicate. The control group was with dilution water only. The average beginning weight of fish used in the experiment was 0.0272 g. Fish were fed with dried *Artemia salina* without nutshells in amount of 8% of their body weight per day, the food ration was based on initial fish weight and was recalculated after 14 days. During the test, conditions were checked at 24-h intervals and the number of dead fish was recorded for each concentration. At the end of the test, fish were weighed and their tank-average specific growth rates determined. Food was withheld from the fish 24 h prior to weighing. The mean values for water quality were temperature 25±1 °C, oxygen saturation above 60% (ranging from 73% to 94%), pH from 7.81 to 8.23. Tank-average specific growth rates were calculated using the following formula according to OECD method No 215:

$$r = \frac{\overline{\log_e W_2} - \overline{\log_e W_1}}{t_2 - t_1} * 100$$

r – tank-average specific growth rate

W_1, W_2 – weights of a particular fish at times t_1 and t_2 respectively

$\overline{\log_e W_1}$ – average of the logarithms of the values W_1 for the fish in the tank at the start of the study period

$\overline{\log_e W_2}$ – average of the logarithms of the values W_2 for the fish in the tank at the end of the study period

t_1, t_2 – time (days) at the start and end of the study period

Fish sampling and homogenization

At the end of the test, the fish were euthanized, immediately frozen, and stored at –85 °C until analyses. Whole body samples were weighed and homogenized (1:10 w/v) using phosphate buffer (pH=7.2). The homogenate was divided into two portions, the first one for the measurement of TBARS and the second one was centrifuged (10,500 *g*, 4 °C, 20 min) to obtain supernatant fraction for the determination of activities of GST, GPx, GR and CAT.

Measurement of detoxifying enzymes and oxidative stress parameters

The activity of GST was determined by measuring the conjugation of 1-chloro-2.4-dinitrobenzene with reduced glutathione at 340 nm (Habig *et al.* 1974). The specific activity was expressed as the nmol of the formed product per min per mg of protein. The catalytic concentration of GR was determined spectrophotometrically by measuring oxidation of the nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm (Carlberg & Mannervik 1975). The activity of GPx was calculated from the rate of NADPH oxidation by the reaction with GR at 340 nm (Flohe & Gunzler 1984). The specific activity of GR and GPx was expressed as the nmol of NADPH consumption per min per mg of protein. The activity of CAT was determined spectrophotometrically by measuring H_2O_2 breakdown at 240 nm. The specific activity was expressed as the μmol of decomposed H_2O_2 per min per mg of protein (Aebi 1984).

Protein concentrations were determined by Bicinchoninic Acid Protein Essay Kit (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard (Smith *et al.* 1985). To check lipid peroxidation, malondialdehyde was measured by the TBARS method at 535 nm (Lushchak *et al.* 2005). The concentration is expressed as nmol of TBARS per gram of tissue wet weight.

All spectrophotometric measurements of oxidative stress parameters were performed using the Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific Inc.).

Statistical analysis

Data were subjected to Kruskal-Wallis one-way ANOVA and subsequently to Dunn test in order to assess the statistical significance of differences in tank-average fish specific growth rates between test groups with different concentrations and those of the control groups.

Oxidative stress markers were tested for the normal distribution using the Shapiro-Wilk test. Data of TBARS, GR, GPx were log-transformed to improve the homogeneity of variance. These values together with data of CAT were subjected to one-way ANOVA and subsequently to Dunnett test to reveal the differences in determined parameters between tested groups and control groups. The data for GST were subjected to Kruskal-Wallis one-way ANOVA, which was applied to the differences in determined parameters between tested groups and control groups. Individual differences between the means were tested successively using Dunnett test and $p<0.01$, $p<0.05$ were chosen as the levels of significance.

Determination of acetylsalicylic acid

ASA determination in water samples was performed by 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. Acetylsalicylic acid was separated by an isocratic elution method with acetonitrile/water 50/50 (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 235 nm. The chromatographic analysis was accomplished by means of Alliance 2695 chromatographic system (Waters, Milford, MA) with the PDA 2996 photodiode array detector (Waters, Milford, MA). ASA standard was purchased from Sigma-Aldrich (St. Louis, MO). All solvents were of HPLC-grade purity (Chromservis, s.r.o., CZ). Detection limit for acetylsalicylic acid was 50 $ng.mL^{-1}$. Limit of quantification for acetylsalicylic acid was 64 $ng.mL^{-1}$. The coefficient of variation is 4.5%.

Water quality parameters

The basic physical and chemical parameters of diluting water used in toxicity test were: acid neutralization capacity ($ANC_{4.5}$) 2.2–2.8 $mmol.L^{-1}$, chemical oxygen demand (COD_{Mn}) 1.8–2.3 $mg.L^{-1}$, total ammonia below the limit of determination (<0.04 $mg.L^{-1}$), NO_3^- 19.6–25.2 $mg.L^{-1}$, NO_2^- below the limit of determination (<0.02 $mg.L^{-1}$), Cl^- 12.1–16.2 $mg.L^{-1}$, Σ Ca^{2+}+Mg^{2+} 3.1 $mmol.L^{-1}$.

Histopathological examination

The fish were prepared for histological examination, fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned on microtome at a thickness of 4 μm, and stained with haematoxylin and eosin. Histological changes of kidney, liver, gills and skin were examined by light microscopy.

RESULTS

Behaviour, mortality and histopathological changes

No changes in fish behaviour were observed in ASA-treated groups in comparison with the control group during the test. In experimental group exposed to the highest concentration of ASA (250 $mg.L^{-1}$) the mortality was 12.2%, whereas in the group with the lowest concentration (0.004 $mg.L^{-1}$) was 1.4%. In the control group the mortality was 0%. No histopathological

changes were found on skin, gills, and liver in any tested concentration.

Growth rate

At the beginning of the experiment body weight of fish did not differ significantly between the groups, while at the end of the experiment body weights of fish in tanks with the concentrations of ASA 120 and 250 mg.L^{-1} were significantly higher (p<0.05) compared to the control group. Final body weight of each group is shown in Figure 1.

The results of specific growth rate r for the tested groups in comparison with the control group are shown in Figure 2. Specific growth rate was found significantly higher (p<0.05) in fish with ASA concentrations of 120 and 250 mg.L^{-1}.

Effect of ASA on biotransformation and antioxidant enzymes

A gradual increase of GST activity was found in fish exposed to ASA concentrations 0.004, 40, 120 and 250 mg.L^{-1} compared to the control group (Figure 3). But only in the groups exposed to ASA concentrations 40, 120 and 250 mg.L^{-1} the GST activity was statistically significantly higher (p<0.01, p<0.05) compared to the control group.

The activity of GR was increased in all tested groups exposed to ASA (0.004, 0.4, 40, 120 and 250 mg.L^{-1}) compared to the control group (Figure 4). The highest values and significantly different from control values (p<0.01) of GR activity were found in the groups exposed to ASA concentrations 0.4, 40 and 120 mg.L^{-1}.

An increase in CAT activity was found in fish exposed to ASA concentrations 0.004, 0.4, 40 and 120 mg.L^{-1} (Figure 5) compared to the control group. But only in the group exposed to ASA concentrations 40 mg.L^{-1} CAT activity was significantly higher (p<0.01) than control while other experimental concentrations (0.004, 0.4, and 120 mg.L^{-1}) had no significant effect (p>0.05). A decrease in CAT activity was found in fish exposed to ASA at 250 mg.L^{-1}, but this result was not significant (p>0.05) compared to the control.

Higher GPx activity was detected in fish exposed to ASA in all tested groups (0.004, 0.4, 40, 120 and 250 mg.L^{-1}) compared to the control group. However, significantly higher (p<0.05) GPx activity compared to

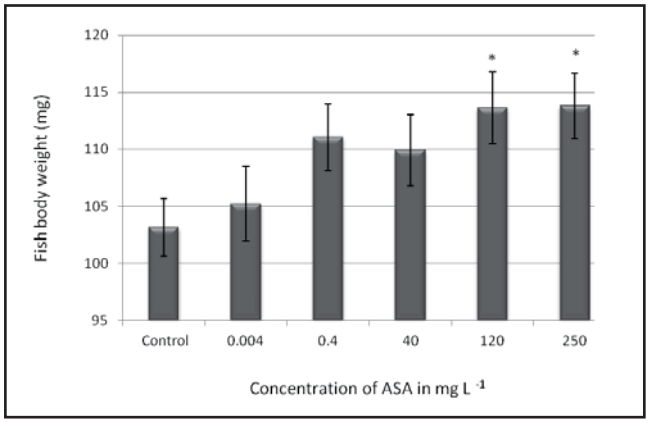

Fig. 1. Comparison of body weight for control group and tested concentrations at the end of the test (*p<0.05, values in mean ± SEM).

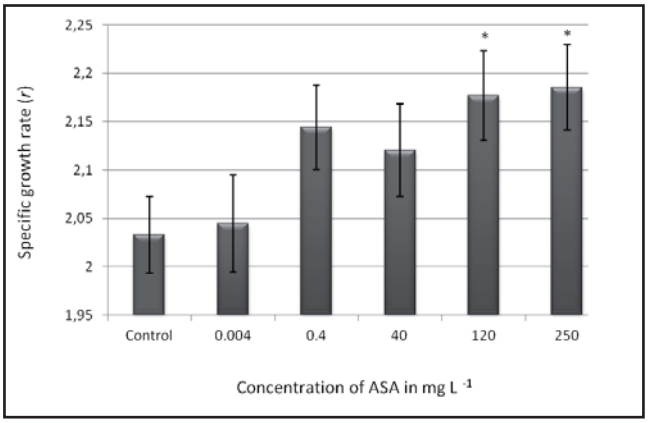

Fig. 2. Comparison of specific growth rate (*r)* for control group and tested concentrations (*p<0.05, values in mean ± SEM).

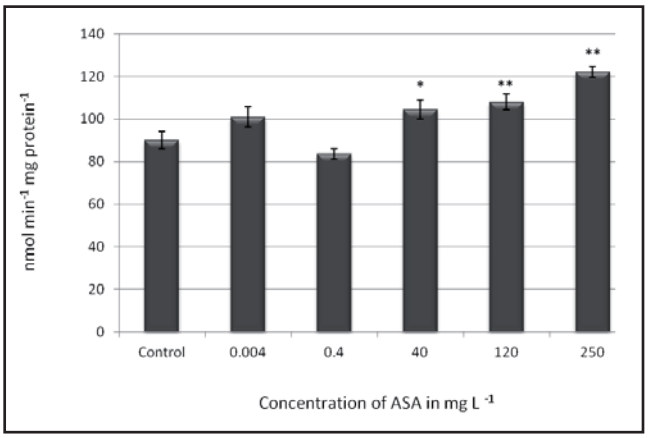

Fig. 3. Activity of GST (**p<0.01, *p<0.05, values in mean ± SEM).

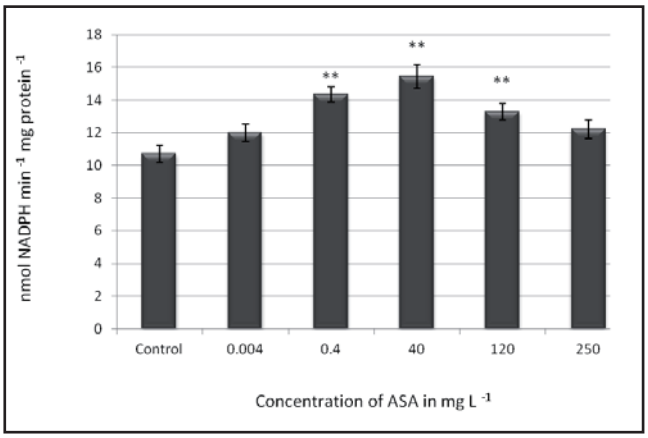

Fig. 4. Activity of GR (**p<0.01, values in mean ± SEM).

control was found only in the groups exposed to 0.004 and 120 mg.L^{-1} of ASA (Figure 6).

Effect of ASA on lipid peroxidation

The concentrations of TBARS were lower in fish exposed to ASA at all tested concentrations (0.004, 0.4, 40, 120 and 250 mg.L^{-1}) compared to the control group. A gradual decrease in TBARS level was found in tested groups exposed to ASA at 0.004, 0.4, 40 and 120 mg.L^{-1}. However, TBARS levels were significantly lower (p<0.01, p<0.05) than control only in fish exposed to 40, 120 and 250 mg.L^{-1} of ASA (Figure 7).

DISCUSSION

The literature unfortunately does not provide us with specific data on the effects of ASA on fish, therefore we have to compare our results with the effects of other pharmaceuticals, which belong to the NSAIDs (diclofenac) or have similar effect as ASA (dipyrone).

We found increased body weight and specific growth rate almost in all experimental groups exposed to ASA compared to the control group. These results are contrary to findings reported by Stepanova *et al.* (2013), who evaluated subchronic effects of diclofenac on early stages of common carp. Their data demonstrate that diclofenac, which belongs to NSAIDs, did not have any effect on body weight and growth. We cannot exactly explain the reason of increased body weight and growth rate, but some studies described the effect of salicylates on the release of several important hormones influencing homeostasis in fish (van Anholt *et al.* 2003; Gravel and Vijayan 2007a, b; Gravel *et al.* 2009). Many studies have demonstrated that salicylic acid and other salicylates affect various physiological and biochemical activities of plants and may regulate their growth and productivity (Arberg 1981; Hayat *et al.* 2010).

In our study the mortality at the highest tested concentration of ASA (250 mg.L^{-1}) was 12.2%. No histopathological changes were found. Similar results were observed during the testing of diclofenac on early stages of common carp *(Cyprinus carpio)* (Stepanova *et al.* 2013). But a number of studies which have examined subchronic effects of other NSAIDs are in contrast to our histopathological findings. E.g. Pamplona *et al.* (2011) studied subchronic effects of dipyrone on silver catfish *(Rhamdia quelen)*. They found renal lesions including necrosis areas and the changes in the renal parenchyma. Another example is Triebskorn *et al.* (2004), who demonstrated cytological alterations caused by diclofenac in liver, kidney and gills of rainbow trout *(Oncorhynchus mykiss)*.

Very low increase of mortality in the highest concentration of ASA supports the hypothesis that this chemical substance has low acute toxicity. Praskova *et al.* (2012) investigated the acute toxicity of NSAIDs and she found mean LC50 value for ASA to be 567.7 mg.L^{-1} for embryos and 274.6 mg.L^{-1} for juvenile zebrafish.

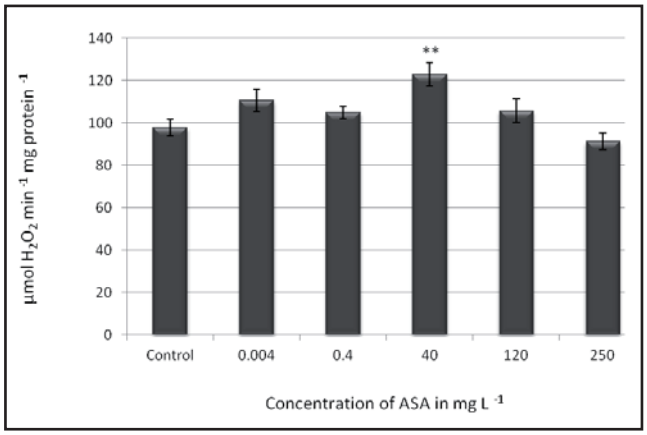

Fig. 5. Activity of CAT (**p<0.01, values in mean ± SEM).

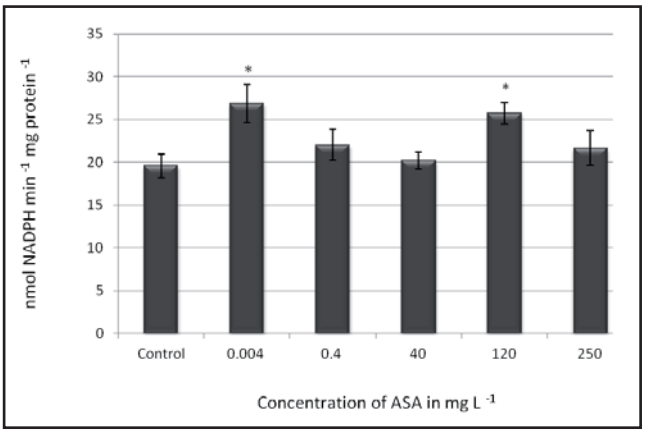

Fig. 6. Activity of GPx (*p<0.05, values in mean ± SEM).

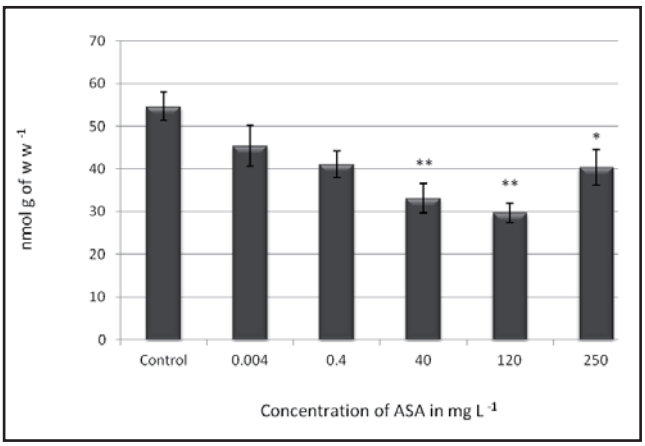

Fig. 7. Content of TBARS (**p<0.01, *p<0.05, values in mean ± SEM).

In this study, significantly higher GST activity compared to control was found in concentrations of ASA 40, 120 and 250 mg.L^{-1}. GST is a biotransformation enzyme, which provides cellular protection against the toxic effects of a variety of environmental and endogenous chemicals. Its main function in the endogenous metabolism is the detoxification of xenobiotics and

products of oxidative stress (Di Giulio & Hinton 2008). In our study, higher GST activity indicates that high concentrations of ASA caused increased activity in the detoxifying system. The effect of ASA on GST activity in *Danio rerio* after 96 hours exposure was investigated by Praskova *et al.* (2012). They found higher GST activity in concentrations 340, 380 and 420 $mg.L^{-1}$. Stepanova *et al.* (2013), who studied effects of diclofenac on early stages of common carp, also found significant increase in the GST activity at the highest tested concentration of diclofenac (3 $mg.L^{-1}$). By contrast, Pamplona *et al.* (2011) documented decreased GST activity in liver of silver catfish exposed to 50 $mg.L^{-1}$ of dipyrone.

The CAT and GPx are enzymes which metabolize reactive oxidative species (ROS). Whereas CAT is restricted to H_2O_2 elimination only, GPx can detoxify H_2O_2 and also reduces fatty acid peroxides. During the reactions catalyzed by GPx which involve reduction of a peroxide substrate to its corresponding alcohol reduced glutathione is oxidized to glutathione disulfide. GR is an enzyme, which catalyzes the conversion of glutathione disulfide to reduced glutathione. The main function of reduced glutathione is the protection of cells from chemical insult. The balance of glutathione is provided by glutathione synthesis, export of oxidized and conjugated glutathione, and activity of GR (Chance *et al.* 1979; Di Giulio & Hinton 2008).

Many environmental contaminants stimulate ROS production and result in oxidative damage (Livingstone 2001; Di Giulio & Hinton 2008). We found an increase in the activity of CAT, GPx and GR. Increased activity of these enzymes leads to the elimination of ROS, which may occur after exposure to ASA. Different results were reported by Stepanova *et al.* (2013), who studied effects of diclofenac on early stages of common carp. They documented decreased activity of GR at the highest tested concentration of diclofenac (3 $mg.L^{-1}$) and no effect of diclofenac on the activity of GPx. No changes in CAT activity in *Rhamdia quelen* liver after dipyrone exposure were observed by Pamplona *et al.* (2011).

The level of TBARS is used to measure the extent of lipid peroxidation. Malondialdehyde is one of the end products of lipid peroxidation (Hodgson 2004; Lushchak 2011). In our study, ASA caused decrease in lipid peroxidation in all tested concentrations. Stepanova *et al.* (2013) reached the same results in their study with diclofenac. By contrast, Pamplona *et al.* (2011), who investigated effects of dipyrone on *Rhamdia quelen*, found no changes in lipid peroxidation products in silver catfish liver.

CONCLUSION

In this study it was found that the ASA has positive effects on body weight and specific growth rate in zebrafish. As well our results indicate that ASA has an effect on some biomarkers of oxidative stress (GST, GPx, GR, CAT and TBARS). The subchronic exposure of zebrafish to ASA causes the increase in activities of antioxidant and biotransformation enzymes and decreases lipid peroxidation. Based on these results, we can conclude that ASA may have a negative impact on some biochemical processes connected with the production of ROS in aquatic organisms. On the other hand low concentrations of ASA have a positive influence on lipid peroxidation, which is reduced.

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