Evaluation of the toxic effect of peracetic acid on grass carp (Ctenopharyngodon idella) juveniles

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Submitted: 2014-09-23 Accepted: 2014-11-08 Published online: 2014-11-30

Key words: antioxidant system; histopathology; haematological parameters; biochemical parameters; Persteril; micronucleus frequency; fish

Abstract

OBJECTIVES: The aim of present study was to evaluate the effect of peracetic acid (PAA) on haematotological and biochemical indices, antioxidant status, micronucleus induction and histopathological alterations of liver and gill in grass carp.

METHODS: Grass carp (Ctenopharyngodon idella) juveniles were exposed to therapeutic concentrations (1, and 3 mg.l⁻¹) of PAA for a period of 10 days. Selected haematological indices – the erythrocyte count (RBC), haematocrit (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leukocyte count (WBC), and biochemical indices – glucose (Glu), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH) were evaluated in plasma. Activity of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), as well as levels of thiobarbituric acid reactive substances (TBARS) were assessed in gill and liver. Micronucleus frequency in peripheral erythrocytes was counted in control and experimental fish. Histological examinations of gill and liver were performed.

RESULTS: No significant differences were found in haematological parameters measured. Statistically significant (p<0.05) alterations in the activities of AST, CK and LDH were found in treated fish compared to control groups. Fish exposed to 1 mg.l⁻¹ of PAA showed significantly lower (p<0.05) SOD activity in liver and gill while catalase activity indicated a significant decrease (p<0.05) only in gill tissue. Other significant changes were observed in GR activity in gill in both PAA exposed groups, while GR activity in liver remained unchanged. There was no significant difference in the count of micronuclei between control and exposed fish. Haemorrhage, fusion of primary lamellae, degeneration of secondary lamellae, some clubbing on primary and secondary lamellae tips, and lifting of epithelial cells were found in gill tissues in both control and treated fish.

CONCLUSION: The results show that PAA could induce alterations in biochemical parameters in blood plasma, antioxidant enzymes response and histopathological changes in gill; however, it seems that these changes are reversible. Subsequently, lower concentration (1 mg.l⁻¹) is useable as a treatment concentration for grass carp.
**INTRODUCTION**

Peracetic acid (PAA) is a strong oxidant agent which has been known for its germicidal properties for a long time (Duong 2005). It has been widely used as a disinfectant in laboratories, the food, beverage, medical and pharmaceutical industries as well as for the treatment of municipal waste water to inactivate many pathogenic and indicator microbes (Kitis 2004). PAA have been recently approved for the use in aquaculture as a sanitizer (Straus et al. 2012a; Kouba et al. 2012) which has drawn a high attention as an alternative biocide for malachite green and formaldehyde in aquaculture. The use of malachite green for food fish has been forbidden in Europe since 2000 by the reason of its accumulation in exposed fish (Sudova et al. 2007). The application of formaldehyde has been recommended to be limited as regards workers’ safety and possible harmful effects on water body (Pedersen et al. 2013). The most desirable attributes for PAA include its wide spectrum of antimicrobial activity, relative stability in environments containing low organic matter, harmless by-products, neutral residuals, easy-to-use in water bodies (Pedersen et al. 2009). PAA products are commercially available in the form a quaternary equilibrium aqueous solution containing PAA, hydrogen peroxide (HP), acetic acid, and water (Falsanisi et al. 2006). Although there is no comprehensive data about the distinction activity of PAA, two feasible mechanisms are hypothesized. First one is the production of active oxygen (Lefevre et al. 1992; Liberti et al. 1999) and hydroxyl radicals (OH) (Lubello et al. 2002) which would lead to disruption of sulphydril (-SH) and sulphur (-S-S) bonds in the cell membrane proteins (Reichert & Young 1997) and the second one is the disruption of the chemiosmotic function of lipoprotein cytoplasmic membrane, denu- turation of microbial macromolecules and metabolites (Gómez-López 2012).

**MATERIAL & METHODS**

**Fish and water parameters**

Grass carp (mean weight ± SD, 72.3±14.0 g) were obtained from a local fish hatchery and maintained for 2 weeks in aquaria with dechlorinated tap water. Fish were fed a commercial diet (BioMar, 47%protein, 26% fat) every day, at 2.5% of total body weight. Water quality parameters were: temperature 16.3–18.2 °C; dissolved oxygen >69%; pH 7.2–7.9; ANC4.5 (acid neutralization capacity) 1.2 mmol.L−1; CODMn (chemical oxygen demand) 1.5 mg.L−1; total ammonia 0.04 mg.L−1; sum of Ca2+ + Mg2+ 14 mg.L−1; Cl− 11 mg.L−1; PO43− 0.01 mg.L−1.

**Experimental protocol**

The 10-day test was carried out using duplicate groups of 7 fish held in six 150-l tanks. Grass carp juveniles were subjected to 0 (C, control group), 1 mg.l−1 (E1, experimental group) and 3 mg.l−1 (E2, experimental group) of PAA for ten days. PAA concentrations (1 and 3 mg.l−1) were obtained by addition of Persteril 36 (Eurosarm) two-times a day (at 8:30 a.m. and 4:30
The desired concentration of PAA was first carefully diluted with enough water from the target aquarium and then was applied into the rest of water volume with fish to ensure even distribution of the active ingredient. The test baths were constantly aerated and renewed daily before the first application of PAA. The range of examination consisted of haematological, biochemical and, histopathological analyses, the evaluation of antioxidant status and counting of micronuclei in erythrocytes.

**Fish sampling and haematological examination**

The blood samples were taken by puncturing the caudal vessel with heparinised syringes and the blood was stabilised by 40 IU of sodium heparin (Heparin inj., Leciva, Czech Republic) per 1 ml blood. Immediately after sampling, erythrocyte count (RBC), haematocrit (PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and leucocyte count (WBC) were determined in the blood samples according to Svobodova et al. (1991).

**Biochemical examination**

Blood plasma was separated by centrifugation (10 min at 12,000 × g) at 4°C and then glucose (Glu), total proteins (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK) were determined using VETTEST 8008 Analyser (IDEXX Laboratories, Westbrook, ME, USA). The analyser works on the basis of dry chemistry and colorimetric analysis. The analyses were carried out using testing slides (Multi-layer film slides, Kodak).

**Biomarkers of oxidative stress and antioxidant parameters**

Livers and gill samples were taken, quickly frozen and stored in –80°C until analysis. For the determination of catalase (CAT) and superoxide dismutase (SOD) activities, frozen samples were homogenized in 50 mM potassium phosphate buffer (KH₂PO₄ with 1 mM EDTA, pH7.4), centrifuged at 30,000 × g for 30 minutes, and supernatants were taken. Total SOD activity was assessed according to the method based on the inhibition of nitro blue tetrazolium (NBT) and production of superoxide anions by NADH and phenazine methosulfate (PMS). SOD activity was measured spectrophotometrically at 560 nm and reported as the nmol of NBT per min per mg protein (Ewing & Janero 1995). CAT activity was determined regarding to the decomposition rate of hydrogen peroxide by spectrophotometric method at 240 nm (Abel 1984). Glutathione reductase (GR) activity was evaluated spectrophotometrically by the rate of NADPH oxidation at 340 nm (Carlberg & Mannervik 1975). In order to determine GR and lipid peroxidation rate, the tissues were homogenized in phosphate saline buffer at pH 7.2. The level of TBARS as an index of lipid peroxidation was calculated spectrophotometrically at 532 nm according to the method of TBA (thiobarbituric acid) assay with some slight modifications (Uchiyama & Mihara 1978).

Total protein concentration in various samples was calculated by bicinchoninic acid (BCA) method using bovine serum albumin as a standard (Bradford 1976).

**Micronucleus test**

Peripheral blood samples were taken from caudal vein by heparinized syringe and smeared on microscope glasses. The blood smears were fixed in absolute ethanol for 20 minutes, air-dried and stained by 10% Giemsa solution for 25 minutes. Two slides were prepared for each fish and 1000 erythrocyte cells per slides were analyzed under light microscope (1000×). Micronuclei were identified according to Al-Sabti and Metcalfe (1995) as non-reflective, small ovoid or circular chromatin bodies, displaying the same staining properties and with a diameter 1/3–1/20 of the nucleus.

**Histopathology**

Liver and gill from each fish were fixed in 10% neutral buffered formalin. The fixed tissue samples were dehydrated through a series of graded ethanol, cleared in xylene, and embedded in paraffin. Sections were cut using a rotary microtome at 3–4 µm, stained with haematoxylin and eosin (H&E) and observed under light microscope.

**Statistical analysis**

The statistical analysis was performed using the STATISTICA (version 8.1 for Windows, StatSoft). Data was expressed as means ± SD and p-value less than 0.05 was considered as statistically significant. Kolmogorov-Smirnov and Bartlett’s tests were conducted to evaluate normality and homoscedasticity of variance respectively. One-way ANOVA was applied in the case of data normal distribution. If the data showed a “non-normal” distribution, a non-parametric test (Kruskal-Wallis) was used.

**RESULTS**

**Mortality**

No mortality occurred in control and E1 groups. In contrast, high mortality rate (71.5%) was observed in fish exposed to 3 mg l⁻¹ PAA (E2).

**Haematological and biochemical indices**

Haematological and biochemical values are shown in Table 1 and 2. There are no significant differences in haematological parameters between PAA exposed groups (E1 and E2) and control. On the other hand, the results of biochemical values show a significant increase in AST, CK and LDH activities in E1 group compared with control.
Biomarkers of oxidative stress and antioxidant parameters

No significant differences were observed in the levels of TBARS in liver and gill tissues between control and exposed groups. SOD activity in liver and gill tissue was significantly lower \((p<0.05)\) in treated fish (E1, E2).

There was a significant decrease in CAT activity of gill in both groups of E1 and E2 compared with control, whereas in liver significant decrease was just observed in E group. On the other side, GR activity significantly increased in gill tissues of E1 and E2 groups compared with control (Tables 3 and 4).

Micronucleus frequency

Micronucleus assay revealed no significant differences in E1 and E2 groups compared with control.

Histopathology

The liver of exposed fish (E1 and E2) showed normal physiological structure, while gill tissue revealed some pathological alterations in secondary and primary lamellae including haemorrhagiae, fusions, focal degeneration of secondary lamellae, clubbing of primary lamellae tips and lifting of epithelial cells (Figure 1). Furthermore, as side findings, there were observed undifferenced protozoan parasites in histological section of gill of control group.

DISCUSSION

Peracetic acid is a disinfectant, which was relatively recently introduced into aquaculture for fish treatment (Elia et al. 2006). Regarding the growing interest in the application of PAA as therapeutic agent, toxicity data on different fish species is needed for the safe application security.

Haematological and biochemical indices in peripheral blood provide a reliable index of health status in various organisms including fish (De Pedro et al. 2005). The activity of enzymes can be affected as a result of environmental stress, diseases and tissue damage (Kor-Siakpere et al. 2011). The present study indicated higher

Tab. 1. Haematological parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C (n=14; mean±SD)</th>
<th>E1 (n=14; mean±SD)</th>
<th>E2 (n=4; mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (T.l⁻¹)</td>
<td>2.20±0.13</td>
<td>2.32±0.51</td>
<td>1.95±0.08</td>
</tr>
<tr>
<td>WBC (G.l⁻¹)</td>
<td>42.56±7.45</td>
<td>37.94±7.15</td>
<td>29.25±7.75</td>
</tr>
<tr>
<td>PCV (l.l⁻¹)</td>
<td>0.31±0.03</td>
<td>0.32±0.02</td>
<td>0.30±0.01</td>
</tr>
<tr>
<td>Hb (g.l⁻¹)</td>
<td>66.99±9.18</td>
<td>65.56±7.22</td>
<td>63.02±8.24</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>142.02±13.41</td>
<td>142.10±30.32</td>
<td>155.34±5.09</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>29.63±4.34</td>
<td>28.84±7.55</td>
<td>32.20±2.90</td>
</tr>
<tr>
<td>MCHC (l.l⁻¹)</td>
<td>0.22±0.03</td>
<td>0.21±0.02</td>
<td>0.21±0.03</td>
</tr>
</tbody>
</table>

* all samples missed for this group
Values with the different alphabets within each row are significantly different \((p<0.05)\)

Tab. 2. Biochemical parameters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C (n=14; mean±SD)</th>
<th>E1 (n=14; mean±SD)</th>
<th>E2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU (mmol.l⁻¹)</td>
<td>3.21±0.83a</td>
<td>2.87±0.42a</td>
<td>–</td>
</tr>
<tr>
<td>TP (g.l⁻¹)</td>
<td>29.00±4.50a</td>
<td>28.30±2.70a</td>
<td>–</td>
</tr>
<tr>
<td>AST (U.l⁻¹)</td>
<td>93.00±24.00a</td>
<td>158.30±30.27b</td>
<td>–</td>
</tr>
<tr>
<td>ALT (U.l⁻¹)</td>
<td>18.80±6.80a</td>
<td>23.50±8.00a</td>
<td>–</td>
</tr>
<tr>
<td>CK (U.l⁻¹)</td>
<td>576.30±100.70a</td>
<td>1 160.00±232.70b</td>
<td>–</td>
</tr>
<tr>
<td>LDH (U.l⁻¹)</td>
<td>1 780.00±821.00a</td>
<td>2 837.50±366.80b</td>
<td>–</td>
</tr>
</tbody>
</table>

Values with the different alphabets within each column are significantly different \((p<0.05)\).

Tab. 3. Lipid peroxidation and antioxidant enzymes activities in liver of grass carp.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (nmol NBT/min/mg protein) (mean±SD)</th>
<th>CAT (H₂O₂/min/mg protein) (mean±SD)</th>
<th>GR (nmol NADH/min/mg protein) (mean±SD)</th>
<th>TBARS (nmol/mg protein) (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n=14)</td>
<td>0.1017±0.0332a</td>
<td>2.0956±0.0683a</td>
<td>0.2274±0.0927a</td>
<td>0.1808±0.0249a</td>
</tr>
<tr>
<td>E1 (n=14)</td>
<td>0.0760±0.0267b</td>
<td>2.1505±0.2668a</td>
<td>0.2292±0.0833a</td>
<td>0.2034±0.0333a</td>
</tr>
<tr>
<td>E2 (n=4)</td>
<td>0.0683±0.0054b</td>
<td>1.3102±0.1425b</td>
<td>0.2708±0.1177a</td>
<td>0.1670±0.0018a</td>
</tr>
</tbody>
</table>

Values with the different alphabets within each column are significantly different \((p<0.05)\).

Tab. 4. Lipid peroxidation and antioxidant enzymes activities in gill of grass carp.

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD (nmol NBT/min/mg protein) (mean±SD)</th>
<th>CAT (H₂O₂/min/mg protein) (mean±SD)</th>
<th>GR (nmol NADH/min/mg protein) (mean±SD)</th>
<th>TBARS (nmol/mg protein) (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n=14)</td>
<td>0.1809±0.0896a</td>
<td>0.0585±0.0119a</td>
<td>0.2399±0.0683a</td>
<td>0.1140±0.0093a</td>
</tr>
<tr>
<td>E1 (n=14)</td>
<td>0.0080±0.0038b</td>
<td>0.0287±0.0072b</td>
<td>0.3535±0.0435b</td>
<td>0.1510±0.0218b</td>
</tr>
<tr>
<td>E2 (n=4)</td>
<td>0.0110±0.0060b</td>
<td>0.0392±0.0124b</td>
<td>0.2889±0.0292b</td>
<td>0.1145±0.0048a</td>
</tr>
</tbody>
</table>

Values with the different alphabets within each column are significantly different \((p<0.05)\).
AST, LDH, and CK activities in fish exposed to PAA. The increase in the activities of the relevant enzymes is well-known as an indicator of physiological disorder due to stress (Velisek et al. 2006) reported under different contamination exposure and stress condition in fish (Banaee et al. 2014a; Agrahari et al. 2007; Almeida et al. 2002). Furthermore, AST is one of important enzymes involved in nitrogen metabolism, amino acid oxidation and liver gluconeogenesis. Increased levels of the aminotransferases can have a vital role in supplying energy under the stress condition (Banaee et al. 2014b). LDH activity is generally associated with the reduction of pyruvate, the final product of glycolytic pathway, which allows keeping glycogenesis. This could be vital in the conditions that additional amount of energy required such as stress situation related to chemical exposure (Diamantino et al. 2001). The elevation of LDH may attribute to a shift from aerobic metabolism to anaerobic pathway in treated fish. CK, engaged in the regeneration of ATP by reversible transfer of phosphate from the phosphoryl group of phosphocreatine to ADP, play an essential role in providing required energy in the process of environmental adaptation (Kori-Siakpere et al. 2011). The reduction in SOD and CAT activities was observed in gill tissues of treated fish, whereas, GR activity was increased. PAA can produce free radicals (Booth & Lester 1995). GR is one of important enzymes engaged in the detoxification of reactive oxygen species through the reduction of oxidized glutathione (GSSG) to reduced form (GSH) (Chang et al. 1978). In general, high activity of GR is proposed as an indices of oxidative stress in living cells (Stageman et al. 1992). Increase in the activity of GR in treated fish could indicate an increment in the consumption of glutathione used by glutathione peroxidase (GPx), one of crucial enzymes involved in antioxidant system, to protect cells against oxidative stress. Weakening SOD and CAT activities observed in our study might be attributed to enhanced ROS production. The ROS overproduction could overwhelm the oxidative detoxification, resulting in decrease of antioxidant enzymes activities or inactivation of enzymes (Kono & Fridovich 1982). Furthermore, PAA causes the inactivation of catalase (Block 1983).

The data obtained for MN assay indicated that micronuclei frequency was not elevated by fish exposure to PAA. Our results are in line with the finding of exposure of common carp with PAA (Sapone et al. 2007) and could be a confirmation for non genotoxic effect of PAA as reported in other studies with other organisms (Maffei et al. 2005; Bolognesi et al. 2004).

The histopathological analysis of liver tissue in both control and treated fish did not show any alterations. Our data from histopathology corresponded to the constant level of ALT activity in plasma as already confirmed that ALT activity is more specific enzyme for the evaluation of liver damage than AST (Singh et al. 2011). Normal structure with slight variations in antioxidant enzyme activities may point out that liver is not a target organ for PAA exposure in fish.

As fish gills have an extensive surface area exposed to the external environment, they could be affected by
multiple factors in water, such as physical and chemical changes (Flores-Lopes & Thomaz 2011). There is rare published data on gill histopathological alterations due to PAA exposure of fish. The histopathological alterations including fusion, club-shape of primary lamellae tips, and lifting of epithelial cells observed in gill tissues during present study have been reported in several studies which have investigated the effects of hydrogen peroxide on fish (Tort et al. 2002; Rach et al. 1997). It is reported that the recovery of minor damage gill can begin relatively fast, but intensive damage caused by hydrogen peroxide can be recovered within weeks (Henriksen et al. 2014). The mechanism of action of PAA is thought to be similar to the mechanism of hydrogen peroxide (Finnegan et al. 2010) and since the main structure of gill revealed unchanged in most cases in our study, histological changes reported in our study may be repairable. The lesions observed in gill tissues in control group could be caused by protozoan parasites. Protozoan parasites constitute the most common parasites group encountered in fish (Omeji et al. 2011). Gill filament fusion, aneurism, filament hyperplasia, epithelial lifting were reported in fish infected by protozoan parasites (Khorramshahr 2012; Baticados et al. 1984; Singh & Kaur 2013). No parasites were found in gill of treated fish which might be due to biocide impact of PAA on protozoan parasites.

Although PAA sub-acute exposure (1 mg.l⁻¹) of grass carp can affect some enzymes of antioxidant system and induces histopathological changes in gill, it seems that these alterations are slight and can be recovered. The present results may propose that the concentration of 1 mg.l⁻¹ of PAA can be applied as a therapeutic concentration in grass carp.

ACKNOWLEDGMENTS

The study was financially supported by the Ministry of Education, Youth and Sports of the Czech Republic – projects „CENAKVA“ (No. CZ.1.05/2.1.00/01.0024), “CENAKVA II“ (No. LO1205 under the NPU I program), and by the Grant Agency of the University of South Bohemia in Ceske Budejovice (No. 018/2014/Z).

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