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ORIGINAL ARTICLE

Cytotoxic effects of 4-octylphenol on fish hepatocytes

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Abstract The present study was conducted to determine cytotoxic effects of 4-octylphenol (4-OP) on primary cultured hepatocytes of pearl mullet (*Alburnus tarichi*). Lactate dehydrogenase (LDH) release, malondialdehyde (MDA) level, antioxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST)] and glutathione (GSH) content were measured after 24-h exposure to 4-OP. 4-OP caused dose- and time-dependent increases in LDH release. Significant induction of MDA level and decrease in GSH content were found. SOD and GPx activities were decreased while GST activity was increased. These findings suggest that 4-OP leads to cytotoxicity by depressing antioxidant defenses in fish hepatocytes.

Keywords 4-Octylphenol · Cytotoxicity · Antioxidant defenses · Isolated fish hepatocytes · *Alburnus tarichi*

Introduction

Alkylphenol polyethoxylates (APEOs) are nonionic surfactants widely used in the detergent, paint and plastics industry, as well as in pesticides, herbicides and cosmetics and many formulated products (Nimrod and Benson 1996). The APEOs enter the aquatic environment through landfill leachates and sewage treatment effluents. In this environment APEOs degrade to alkylphenolic compounds such as 4-nonylphenol, 4-octylphenol (4-OP), 4-*tert*-octylphenol (*t*-OP) and bisphenol A (Du et al. 2008; Sharma et al. 2009). Concentrations of these products in the aquatic environment were reported to be at µg/L levels (Ahel et al. 1994; Snyder et al. 1999). These compounds mimic natural hormones and cause disruption in endocrine systems (El-Dakdoky and Helal 2007; Tanaka and Grizzle 2002). Apart from endocrine disrupting effects, they can accumulate within the organs of aquatic species (Ahel et al. 1993) and cause toxic effects such as developmental deformities and organ toxicity (Cakal Arslan and Parlak 2007; Hernandez-Rodriguez et al. 2007). Recent studies showed that alkylphenolic compounds have the potential to cause toxic effects in mammalian tissues through free radicals and reactive oxygen species (ROS), and oxidative damage by attacking biomolecules including lipid, DNA and protein (Aydoğan et al. 2008; Korkmaz et al. 2010).

Pearl mullet (*Alburnus tarichi* Güldenstädt 1814) is an endemic fish species, a member of the *Cyprinid* family, inhabiting the Lake Van Basin, in the Eastern Anatolia Region of Turkey. It is the only vertebrate species in the lake, and female and male individuals display the anadromous character of the species by

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migrating to freshwater inlets for spawning at the reproduction period (April–June). They return to the lake after spawning (Danulat and Selcuk 1992). The fish has economic importance, the annual catch being about 11,000 tons. Recent studies showed that pearl mullet might be exposed to endocrine disrupting chemicals in its ecological niche (Unal et al. 2014). Although pearl mullet has been chosen as an indicator species in some studies, little information has been available on its sensitivity to environmental contaminants, including alkylphenols.

As far as we know, an integrated investigation of cellular toxicology of 4-OP in fish has not been conducted. This study was conducted to investigate the effect of 4-OP on cellular damage and antioxidant systems of pearl mullet, using freshly isolated hepatocytes.

Materials and methods

Fish

Adult pearl mullet were sampled at the end of the reproductive season by electrofishing from the river Karasu, which drains into Lake Van. Fish were transported to the laboratory in aerated coolers filled with river water, and acclimated for 2 months in 60-L glass aquaria with dechlorinated tap water aerated by air pumps. During this time fish were maintained under natural photoperiod (12 h day–night cycle) at an average temperature of 22 °C, and were fed commercial trout granule food. Ethical regulations were followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments.

Cell culture medium and chemicals

The cell culture medium, Leibovitz's L15 (L-15) with L-glutamine (cat. no: L1518), was purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibiotic/antimycotic solution and collagenase (type IV) were also purchased from Sigma. The 4-OP was purchased from Sigma-Aldrich and dissolved in absolute ethanol (Merck, Darmstadt, Germany). The kit for the LDH analysis was purchased from Abcam (Cambridge, UK) and the kits for the antioxidant enzyme activity measurements were purchased from Randox Laboratories (Crumlin, UK). The thiobarbituric acid (TBA); butylated hydroxytoluene; trichloroacetic acid; ethylene diamine tetraacetic acid; reduced GSH; metaphosphoric acid; 5,5′dithiobis-(2-nitrobenzoic acid); 1-chloro-2,4-dinitrobenzene (CDNB); potassium dihydrogenphosphate (KH_2PO_4); ethylene glycol tetraacetic acid (EGTA) and sodium chloride used in this study were technical grade and supplied by Sigma-Aldrich. All other chemicals were analytical grade, and were obtained from the same source.

Hepatocyte isolation

Hepatocytes were isolated in a two-step perfusion method as described by Berry and Friend (1969) and modified by Mortensen et al. (2006). Prior to the cell culture studies, all glassware and instruments were sterilized, and solutions were sterilized by passing through a 0.22-μm filter (Millipore Ireland, Cork, Ireland) before use. Hepatocytes were prepared from five individual fish. Briefly, the liver of the anesthetized fish was carefully excised, transferred onto a glass petri dish, and rinsed with a cold calcium-free solution containing NaCl (7.14 g/L), KCl (0.36 g/L), $MgSO_4$ (0.15 g/L), Na_2HPO_4 (1.6 g/L), NaH_2HPO_4 (0.4 g/L), $NaHCO_3$ (0.31 g/L), and EGTA (20 mg/L) under sterile conditions. The liver was dissected into small pieces in this solution and agitated by pipetting. Next, tissue pieces were rinsed with a clear solution, and the process was repeated until all blood was flushed out. The whitened tissue pieces were then transferred into the same buffer with 0.22 g/L $CaCl_2$, instead of EGTA, and collagenase (0.53 mg/mL). The tissue was digested for 15 min in this solution. The softened tissue was then triturated by pipetting with pipette tips of different size. At the final stage, the cell suspension was gently passed through the needle of a sterile injector for dissociation of the cells. The resulting cell suspension was transferred to sterilized centrifuge tubes and centrifuged at $30\times g$ for 3 min. Cells were washed twice with L-15 medium containing antibiotic–antimycotic 1 % (v/v) and $NaHCO_3$ (0.38 g/L). After the last wash, the cell pellet was resuspended in Leibovitz's L15 (L-15) medium. The cells were counted using a Thoma slide, and cell viability was assessed using a trypan blue exclusion test. The cells were observed to possess more than 90 % viability for cell culture studies.

Hepatocyte culture and exposure

The isolated hepatocytes were seeded at a density of 1×10^6/mL (1000 µL per well) in 48-well culture plates (Greiner Bio-One, Monroe, NC, USA). The cells were allowed to attach for 24 h at 22 °C in a sterile incubator (Binder, Tuttlingen, Germany) without additional O_2/CO_2 prior to chemical exposure. Stock solutions of 4-OP [$CH_3(CH_2)_7C_6H_4OH$; Aldrich] were prepared in absolute ethanol, and the final concentration of ethanol in the medium never exceeded 0.1 % (v/v). After 24 h of preculture, the old medium was removed, and then the attached monolayers of hepatocytes were exposed to 4-OP at concentrations of 10, 25, 50 and 100 µM in fresh L-15 medium (without serum) for 24 h. Control cells received only L-15 medium containing 0.1 % (v/v) absolute ethanol. Four replicates were used in each experiment. The cell cultures were routinely checked for morphological changes and contamination using an inverted microscope (Leica DM 6000, Germany).

Lactate dehydrogenase assay

Cellular damage was monitored by measuring lactate dehydrogenase (LDH) release into the culture medium. After exposures were completed, the culture media were removed from wells by gentle pipetting, transferred to Eppendorf tubes and immediately used for the LDH analysis. Samples were loaded onto a 96-well plate and LDH activity was assayed using a LDH-cytotoxicity assay kit (Abcam, Cambridge, UK), according to the manufacturer's instructions. Finally absorbance values were measured with an ELISA plate reader, model A3 (DAS, Rome, Italy) at 450 nm. All measurements were taken in duplicate, and LDH release was expressed as the percentage of cytotoxicity. Four replicates were used in each experiment.

Biochemical analyses

After gentle pipetting of culture media from the wells, the hepatocytes were rinsed with ice-cold phosphate buffered saline (1 mL, pH 7.4) and cell suspensions (1×10^6/mL) were transferred to Eppendorf tubes. The cells were then lysed using a glass-porcelain ultrasonic homogenizer (Jencons Scientific Co., Leighton Buzzard, Bedfordshire, UK). The homogenate was centrifuged at 20,000×*g* for 15 min. All processes were carried out at 4 °C or on ice. Supernatant fractions were removed and used to determine the lipid peroxidation and antioxidant defenses. Four replicates were used in each experiment.

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content, a product of lipid peroxidation, in the samples. The MDA concentration was measured spectrophotometrically at 532 nm, using the method described by Jain et al. (1989), based on thiobarbituric acid reactivity. Concentration of MDA was calculated using the molar extinction coefficient (1.56×10^5 mol/L/cm) according to the formula $A = \varepsilon \times C \times L$, where A = absorbance, ε = molar extinction coefficient, and L = path length. The results were expressed as nmol/10^6 cells.

The superoxide dismutase (SOD) activity was measured using a commercial kit at 505 nm and 37 °C, according to the manufacturer's instructions. In this method, a xanthine–xanthine oxidase system was used to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. SOD activity was then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes 50 % inhibition of the rate of reduction of INT under the conditions of the assay. Results were expressed as U/10^6 cells.

The glutathione peroxidase (GPx) activity was measured using a commercial kit at 340 nm and 37 °C, according to the manufacturer's instructions. This assay method is based on Paglia and Valentine (1967). GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The GPx activity was calculated from the decrease in absorbance values. Results were expressed as U/10^6 cells.

Glutathione (GSH) content was measured spectrophotometrically at 412 nm, using the method described by Beutler (1984). The GSH levels were obtained from a standard curve derived from external GSH standards. Results were expressed as nmol/10^6 cells.

Glutathione-*S*-transferase (GST) activity was measured using CDNB as substrate (Habig et al. 1974). The assay was carried out in a quartz cuvette

containing 0.1 M phosphate buffer (pH 6.5), 1 mM CDNB, and 1 mM GSH. The reaction was started by the addition of a 50 µL sample of GST. Absorbance values of the CDNB-glutathione conjugate were assayed spectrophotometrically at 340 nm. Activity was expressed as nmol of CDNB-glutathione conjugate/min/10^6 cells.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences software, version 16.0. The analysis of variance (one-way ANOVA) was used to analyze statistical differences among the treatment groups. Significant differences between control and exposure groups were determined by the post hoc Dunnett's test. Results were expressed as the means ± standard deviation (SD). Values of $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$ were considered to be statistically significant.

Results

LDH is a useful indicator of cellular damage. After 24 h, dose-dependent increases in LDH release were observed following 4-OP exposures with all concentrations ($P < 0.001$). 4-OP-induced cytotoxicity was apparent with concentrations of 100 µM OP (Fig. 1). Results showed that the highest dose of 4-OP (100 µM) is severely toxic for hepatocytes, whereas lower doses could be managed by hepatocytes. Accordingly, antioxidant system parameters were investigated in the lower doses (≤100 µM) of 4-OP.

As shown in Fig. 2, the MDA level significantly increased in hepatocytes treated with a 50 µM dose of 4-OP ($P \leq 0.05$), while increases were not significantly different in the other exposure groups.

Changes in the GSH content and endogenous antioxidant enzymes of isolated hepatocytes exposed to 4-OP concentrations for 24 h are demonstrated in Fig. 3. A statistically significant decrease ($P < 0.01$) in the GSH level was observed with 50 µM 4-OP, but significant changes were not observed at lower doses (Fig. 3A). There was a significant decrease in the SOD activity in hepatocytes exposed to 25 and 50 µM of 4-OP, while there was no effect of 10 µM of 4-OP on SOD activity (Fig. 3B). GPx showed statistically decreased activities ($P \leq 0.001$) in all exposure groups compared with controls (Fig. 3C). GST activity significantly increased in the 25 and 50 µM exposure groups ($P < 0.01$ and $P \leq 0.05$, respectively) while it remained unchanged in the 10 µM 4-OP (Fig. 3D).

Discussion

It has been generally accepted that the release of cytosolic LDH correlates with cellular viability, thus providing an important indicator of plasma membrane damage (El-Shenawy 2010). 4-OP prominently increased LDH release into the medium in a dose-dependent manner, and it was found in this study to be highly cytotoxic at 100 µM concentration. Parallel to

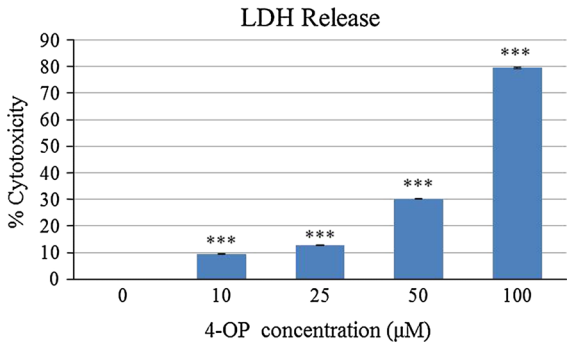

Fig. 1 Effect of 4-OP on LDH release of the isolated hepatocytes of pearl mullet (*A. tarichi*). Values represent the means ± SD of four replicate wells. Significance at ***$P \leq 0.001$ compared with control

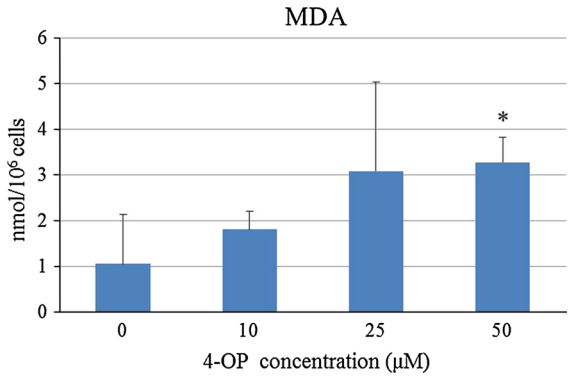

Fig. 2 Effect of 4-OP on MDA level of the isolated hepatocytes of pearl mullet (*A. tarichi*). Values represent the means ± SD of four replicate wells. Significance at *$P \leq 0.05$ compared with control

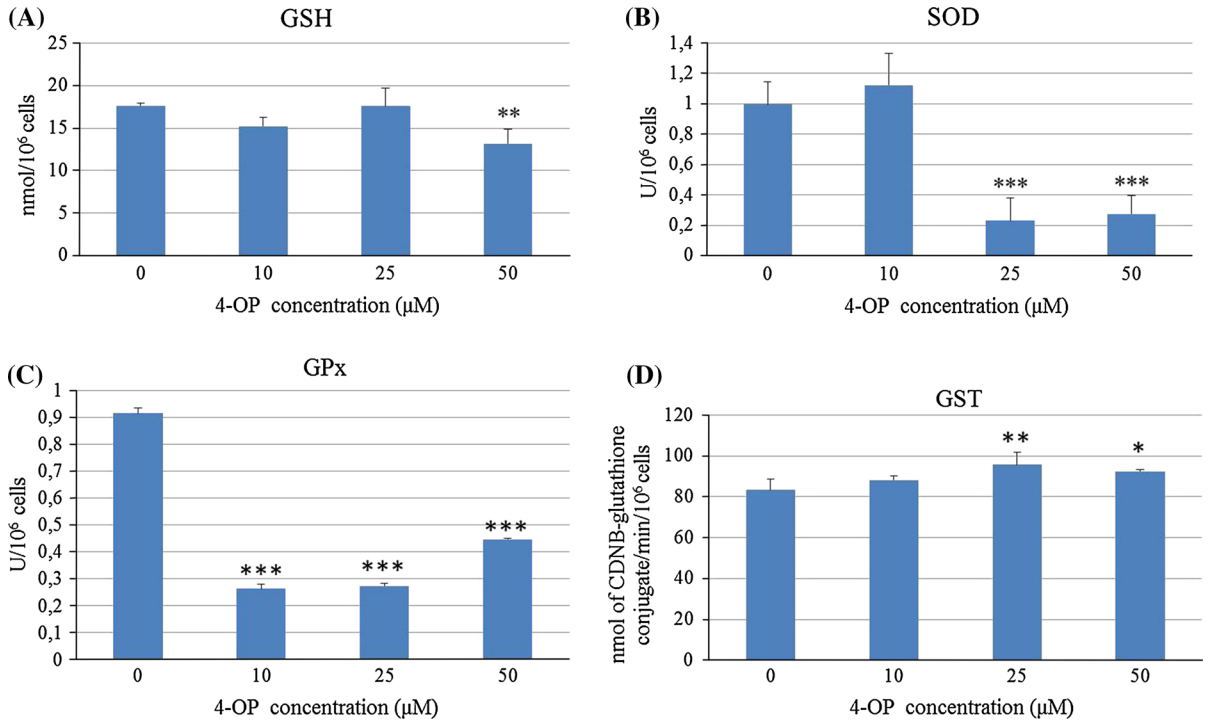

Fig. 3 Effect of 4-OP on GSH content (**A**), SOD (**B**), GPx (**C**) and GST (**D**) activities of the isolated hepatocytes of pearl mullet (*A. tarichi*). Values represent the means ± SD of four replicate wells. Significance at $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared with control

our findings, Toomey et al. (1999) reported that *t*-OP caused cell death in a time- and dose-dependent manner, and most hepatocytes of bullhead catfish (*Ameriurus nebulosus*) died through apoptotic cell death at a higher concentration (100 μM) of *t*-OP. In contrast, it was reported that 4-OP significantly reduced germ cell number in cultured human fetal gonads at a concentration of 10 μM (Bendsen et al. 2001). The results of the present study provide the first indication of hazardous potential effects of 4-OP on pearl mullet hepatocytes.

This study further demonstrated that 4-OP treatment caused significant decrease in the GSH level of hepatocytes, indicating oxidative stress. In accordance with our finding, GSH depletion was also reported in other organs such as liver (Aydoğan et al. 2008), testis (Kumar et al. 2002) and kidney (Koriem et al. 2014) of *t*-OP-exposed animals. GSH, a tripeptide consisting of L-glutamine, cysteine and glycine, can react directly with and detoxify ROS (Stephensen et al. 2002). In addition, under slight oxidative stress, GSH levels can increase as an adaptive mechanism through an increase in its synthesis. However, severe oxidative stress may suppress GSH levels due to the failure of adaptive mechanisms (Zhang et al. 2004). GSH is used as a conjugating molecule by GST to facilitate the excretion of xenobiotics. A decline in GSH content may arise from increased utilization of GSH, directly or as a result of converting GSSG by GST, and insufficient GSH production by glutathione reductase (Monterio et al. 2006).

Many environmental pollutants were reported to disrupt the balance between oxidants and antioxidants in the cells resulting in excessive ROS production or oxidative stress. MDA, a main oxidative degradation product of lipid peroxidation, functions as a marker of oxidative injury to biological membranes (Strmac and Braunbeck 2002). Studies have demonstrated that OP exposure is also capable of promoting increased levels of lipid peroxidation in the liver in vivo, and lipid peroxidation induced by *t*-OP exposure arises from ROS production (Korkmaz et al. 2010; Koriem et al. 2014). The results of the present study showed that the MDA content significantly increased in hepatocytes

after 4-OP treatment. The increase in MDA content in the present study could be attributed to the production of ROS as a result of oxidative stress.

SOD is an enzyme catalyzing the dismutation of superoxide anion to hydrogen peroxide and water, and is a first-line defense against oxyradicals. In the present study, SOD activity was significantly reduced after exposure to 4-OP in the hepatocytes. This result agrees with a previous study reporting *t*-OP-induced liver injury and oxidative stress in male rats (Saggu et al. 2014). The decrease in SOD activity is probably due to overproduction of superoxide radicals in the hepatocytes by 4-OP, which cause such an effect that the superoxide radicals, either by themselves or after their transformation to hydrogen peroxide, lead to oxidation of the cysteine in the enzyme and decreases the SOD activity (Dimitrova et al. 1994).

GPx is a selenium-dependent enzyme catalyzing the degradation of organic peroxides and hydroperoxides, using GSH as a substrate, and is considered an efficient protective enzyme against lipid peroxidation (Zhao et al. 2011). Significant decreases in GPx activity compared to controls observed in this study were presumably related to the decreased availability of GSH to reduce free radical impact. Our result is also in accordance with the previously reported decrease in GPx activity after exposure to phenolic compounds due to their direct inhibitory oxidative effects (Wu et al. 2011).

GST is a phase II enzyme involved in the detoxification of xenobiotics by conjugating electrophilic metabolites to glutathione and protecting the cells from oxidative stress. Our results show that 4-OP caused significant increase in GST activity. Increased GST activity has also been reported in various fish species exposed to environmental contaminants such as polychlorinated biphenyls, polyaromatic hydrocarbons and estrogens (Otto and Moon 1996; Lu et al. 2011). Du et al. (2008) also reported elevated levels of hepatic GST activity in rabbitfish (*Siganus oramin*) exposed to *t*-OP, possibly related to increased free radical production.

In conclusion, the present study suggests that 4-OP has toxic effects on isolated hepatocytes of pearl mullet. Toxicity mechanisms of 4-OP are involved in cell membrane damage by increasing LDH release and elevating lipid peroxidation, accompanied by induced oxidative stress as a result of overwhelmed antioxidant system balances. These results may help to elucidate the toxic mechanisms of 4-OP on hepatotoxicity in fish.

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Conflict of interest The author confirms that there are no conflicts of interest.

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