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Induction of hepatic enzymes and oxidative stress in Chinese rare minnow (*Gobiocypris rarus*) exposed to waterborne hexabromocyclododecane (HBCDD)

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Abstract

The objective of this study was to evaluate the sub-lethal toxicity of hexabromocyclododecane (HBCDD) in fish. Adult Chinese rare minnows as in vivo models were exposed to waterborne HBCDD from 1 to 500 μg/l for 14, 28 and 42 days. Hepatic CYP1A1 (ethoxyresorufin-*O*-deethylase, EROD) and CYP2B1 (pentaoxyresorufin-*O*-dependentylase, PROD) activities were measured. At the same time, molecular biomarkers of oxidative stress were also assayed in the brain, including reactive oxygen species (ROS), lipid peroxidation products (thiobarbituric acid-reactive substances, TBARS), DNA damage and protein carbonyl, as well as superoxide dismutase (SOD) activity and glutathione (GSH) content. DNA damage was evaluated using the Comet assay on erythrocytes. Besides, the content of HBCDD in whole fish was determined after 42 days exposure. The results show that HBCDD could induce EROD and PROD at 500 μg/l after 28 days exposure, and at 100 to 500 μg/l after 42 days exposure ($P < 0.05$), respectively. ROS formation in fish brain was observed to be increased in both time- and dose-dependent manner due to HBCDD exposure. The significant increases in TBARS and protein carbonyl contents occurred in fish brain after 28 and 42 days exposure ($P < 0.05$). Significant DNA damage in erythrocytes by Comet assay was also found in the 100–500 μg/l exposure groups ($P < 0.05$) after 42 days exposure. Moreover, significant depletion in brain GSH content occurred in all treated groups ($P < 0.05$) and apparent inhibition in SOD activity in brain was observed in the groups of 10–500 μg/l concentrations during 42 days exposure. The results demonstrate that increasing duration of HBCDD exposure induced EROD and PROD activities, caused excess ROS formation, finally resulted in oxidative damage to lipids, proteins and DNA and decreased antioxidant capacities in fish. Chemical analysis of HBCDD in whole fish showed accumulation up to 654 μg/g wet weight.

Keywords: Hexabromocyclododecane; Oxidative stress; Chinese rare minnow; Ethoxyresorufin-*O*-deethylase (EROD); Pentaoxyresorufin-*O*-dependentylase (PROD); Antioxidant

1. Introduction

Hexabromocyclododecane (HBCDD) is a brominated aliphatic cyclic hydrocarbon used as an additive flame-retardant in thermal insulation building materials, upholstery textiles, electronics and even in isolation put beneath road surfaces, although it does not fulfill any purpose there (Bernes, 1998; de Wit, 2002). HBCDD is now considered to be ubiquitous contaminant and has been detected in practically all environmental media, even in human blood and mother's milk (Noren and Meironyte, 2000; Robin et al., 2005). HBCDD has been found in both urban and rural air across Sweden and in polar bears from very remote sites in Greenland and Svalbard in the Arctic Ocean (Lindberg et al., 2004).

The direct acute toxicity of HBCDD appears to be low, but its sub-lethal effects cannot be ruled out (Robin et al., 2005). It has been reported that HBCDD could affect detoxification enzyme activities (cytochrome P450 activities; ethoxy-resorufin-*O*-deethylase (EROD) and pentaoxyresorufin-*O*-deethylase (PROD)) in rat and juvenile rainbow trout (Silke et al., 2006; Ronisz et al., 2004). Neonatal exposure of mice to HBCDD could induce developmental neurotoxic effects including aberrations in spontaneous behavior, learning, and memory function (Eriksson et al., 2006). Moreover, HBCDD has been reported

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to alter the normal uptake of neurotransmitters in rat brains (Mariussen and Fonnum, 2003). However, these studies focused on high doses (>1 mg/kg) of HBCDD and short-term exposure of mammals. Few studies have addressed sub-lethal effects of HBCDD in aquatic vertebrates in vivo. Although the concentrations of HBCDD in water have been detected to range from 0.01 to 65.6 μg/l (Hunziker et al., 2004), food-chain studies have shown that HBCDD can be bioaccumulated and biomagnified (Leonards, 2004). Therefore, it is possible for HBCDD to reach a critical level in aquatic organisms and cause irreversible damage after long-term and low-dose exposure in aquatic environment.

Toxicity of many contaminants to aquatic organisms is mediated through oxidative damage, where reactive oxygen species (ROS) are formed. The formation of ROS may be enhanced by xenobiotics through induction of cytochrome P450 system, Fenton reaction involving free metal ions, or uptake of lipophilic xenobiotics into membranes resulting in disturbance of electron flow between components of the cytochrome P450 system (Lemaire and Livingstone, 1993). Under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or glutathione (GSH) peroxidase (Tocher et al., 2002). If the production of ROS overwhelms the antioxidant system, an imbalance between the formation and removal of ROS can produce oxidative stress. Oxidative stress is imposed on organism as a result of three factors: first, an increase in oxidant generation, second, a decrease in antioxidant protection, and third, a failure to repair oxidative damage. Oxidative stress can lead to damages in DNA, proteins, lipids and decrease in antioxidant protection. The DNA damage includes DNA base modifications, single- and double-strand breaks and the formation of apurinic/apyrimidinic sites, which may lead to tumorigenicity (Girard and Boiteux, 1997). Ronisz et al. (2004) has reported that HBCDD could induce the activities of antioxidant enzyme catalase in juvenile rainbow trout after 28 days exposure at 50 mg/kg (bw) by injection. However, no more data is available to prove ROS formation and oxidative damage in aquatic organisms exposed to HBCDD. Therefore, more studies are required to confirm the putative oxidative stress-inducing activity of HBCDD, e.g., detection of ROS production and measurement of oxidative damage.

In this study, the sub-lethal toxicities of long-term HBCDD exposure were evaluated in Chinese rare minnow as an in vivo model by measurement of CYP450 activities and oxidative damages. CYP450 activities measured in terms of EROD and PROD activities have been used as biomarkers of environmental pollution (Van der Oost et al., 2003). The oxidative damage was assayed by measurement of lipid, protein and DNA damages, and correlative antioxidant enzyme activities.

2. Material and methods

2.1. Chemical

HBCDD (99% purity) was obtained from Aldrich (Milwaukee, WI, USA). Thirteen mass ($^{13}C_{12}$-α, β and γ) labeled HBCDD isomers (each >98% purity) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Stock solutions of HBCDD were prepared in dimethyl sulfoxide (DMSO). Other reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

2.2. Animals and treatment

2.2.1. Experimental animals

Adult, 4–6-month-old Chinese rare minnow were used in the study. They were 5.3–8.3 cm in length and 4.0–8.0 g in weight. These fish were cultivated in our lab. The fish were maintained in a 14: 10 h light/dark cycle at 23–26 °C and fed three times a day with commercial pelleted food (Caihong, Guangzhou, China). Enough food was added to each stock aquarium, so that every fish was able to eat and no food remained after approximately 5 min.

2.2.2. Exposure to HBCDD

A laboratory-constructed 120 L semi-static system was employed. Nominal concentrations of HBCDD at 1, 10, 100 and 500 μg/L were selected for this study. HBCDD was dissolved in DMSO with a final concentration less than 0.06‰ (v/v). The control fish were exposed to the nominal concentration of 0.06‰ DMSO, corresponding to the highest concentration of DMSO used in the HBCDD treatments. The number of fish exposed in each group was 45. The photoperiod was a 14:10 h light/dark cycle. Water temperature ranged from 23 to 26 °C. During the experiment, residual food in the test chambers was removed every day and 80% of the volume was renewed once every 2 days. The test equipment and chambers were cleaned once a week. Chinese rare minnows were exposed to HBCDD for 14, 28 and 42 days. No mortality was observed during whole experiment.

2.2.3. Sampling

At the end of each exposure, blood was drawn from fish tail vessel into heparinized capillaries (Eppendorf, Hamburg, Germany) and transferred into centrifuge micro-tubes containing 500 μl Hank's Balanced Salt Solution (HBSS) buffer. Blood cells were used in comet assay.

EROD and PROD were measured in the liver. ROS, TBARS, protein carbonyl, SOD and GSH were detected in the brain of the fish. The brain and liver were dissected, maintained on ice and stored at −80 °C for analysis. The livers and brains were homogenized with glass-homogenizer for 1 min in 0.25 ml and 0.4 ml ice-cold Tris buffered saline (10 mmol/l Tris–HCl, 0.1 mmol/l EDTA-2Na, 10 mmol/l sucrose, 0.8% NaCl, pH 7.4), respectively. After the addition of 2 μl perchloric acid, 40 μl of the brain homogenate was centrifuged at 4000 × *g* for 20 min at 4 °C and the supernatant was stored at −80 °C for GSH analysis. Another portion of the brain homogenate was taken out for TBARS and protein measurements. Other portions were centrifuged at 2500 × *g* for 20 min at 4 °C and the supernatant was used for various biochemical analyses. All homogenates were kept in −80 °C until analyses which were carried out within 1 week.

At end of 42 days, some of the exposed fish were frozen at −20 °C for future chemical analysis.

2.3. EROD and PROD analysis

EROD and PROD assay were conducted according to the method of Burke and Mayer (1974). The general reaction mixture was prepared in a 5 ml test-tube. 30 μl homogenate fraction was incubated in a final volume of 1.95 ml containing 96.5 mM potassium phosphate buffer (pH 7.4), 50 μmol/l ethoxyresorufin or pentahoxyresorufin. The fluorescent intensity (blank) was immediately measured at an excitation/emission wavelength of 560/580 nm with a Hitachi F-2500 spectrophotometer (Hitachi, Japan). Then 10 μl of 6 mmol/l NADPH was added to the solution, mixed, and the mixture was allowed to stand for 20 min. The reaction was stopped by adding 0.5 ml of ice-cold methanol. The spectrophotometer was calibrated with 0.01 mmol/l resorufin. The activity was expressed as pmol/min/mg proteins. Total protein concentration was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

2.4. ROS measurement

ROS in fish brain was measured based on the method of Driver et al. (2000) with slight modifications. 20 μl of homogenate was pipetted into each well of a 96-well plate and allowed to warm to room temperature for 5 min. At that time, 100 μl physiological saline, 5 μl of 2,7-dichlorofluorescin diacetate (DCFH-DA, dissolved in DMSO, 10 μmol/l final concentration) was added to every well and the plate was incubated at 37 °C for 30 min. The conversion of DCFH to the fluorescent product DCF was measured using a TECAN spectrofluorometer with excitation/emission at 485/530 nm (Tecan, Switzerland). Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS level was expressed in arbitrary units (DCF mg^{-1} protein).

2.5. Protein oxidation

Protein carbonyls were determined in brain according to the method described by Oberdorster (2004). Nucleic acids in homogenate were removed by adding 10 μl 20% streptomycin to 50 μl homogenate; after incubation at room temperature for 15 min, the DNA was removed by centrifugation at 14,000 × *g* for 5 min. Four hundred microliters of 10 mmol/l 2,4-dinitrophenzylhydrazine (DNPH) in 2 mol/l HCl was added to 40 μl of the DNA-free supernatant, and incubated for 1 h at room temperature vortexing the mixture every 10–15 min. Proteins were precipitated and pelleted by adding 500 μl 20% trichloroactic acid and centrifuging at 14,000 × *g* for 5 min. The pellet was washed at least three times with 50:50 ethanol:ethylacetate mixture to remove unreacted DNPH. The pellet was redissolved at 37 °C in 450 μl guanidine HCl/dithiothreitol, and absorbances at 372 nm read from 200 μl aliquots in duplicate using a plate-reading spectrophotometer. The carbonyl content was calculated from the absorbances using a molar absorption coefficient of 22,000/M cm^{-1}.

2.6. Lipid peroxidation

To determine the degree of lipid peroxidation in the brain, levels of thiobarbituric acid reactive substances (TBARS) were detected according to the method of Ohkawa et al. (1979). The method was calibrated with 1,1,3,3-tetraethoxy-propane (TEP) standard solution. The content of TBARS was expressed as nmol/mg protein.

2.7. Comet assay

The comet assay was performed as described by Singh et al. with some modifications (1988). In brief, 1% low melting point agarose (Ameresco, USA) was dissolved in PBS using microwave heating. 20 μl blood cell solution was added to 300 μl low melting point agarose solution. The polystyrene ponds from the cover of 96-well cell plate (NUNC, Denmark) were used as a slide. Then, 20 μl low melting point agarose containing blood cells was added to every well of a slide. The gel of slide was allowed to solidify in the refrigerator for 5 min. After solidification of the gel, the slide was submersed in the lysing solution (2.5 mol/l NaCl, 100 mmol/l EDTA-2Na, 10 mmol/l Tris–HCl, pH 10; 1% Triton X-100 and 10% DMSO, pH 10) for 1 h. The slide was then placed in unwinding buffer (1 mmol/l EDTA and 300 mmol/l NaOH, pH 13) for 20 min and electrophoresis was carried out using the same solution for 20 min at 12 V. After electrophoresis, the slide was neutralized via three washings (5 min each) with neutralization buffer (400 mmol/l Tris–HCl, pH 7.4). Then the slide was stained with 500 μl of 10 μg/ml ethidium bromide. The slide was examined using Autocomet image analysis system and software (Tritek, USA) after visualization with an Olympus OX71 fluorescence microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. For each treatment group, two wells were prepared from each, 50 randomly chosen cells (total 100 cells), were scored. The parameter scored, Olive tail moment, used for global comet description – i.e. as an indicator of DNA damage – was calculated automatically using the Autocomet image analysis system.

2.8. Antioxidative capacities

The activities of SOD were measured using the Diagnostic Reagent Kit purchased from Nanjing Jiancheng Bioengineering Institute (Jiancheng, China) according to the manufacturer's instructions.

Total GSH content was measured using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)-oxidized GSH (glutathione disulfide, GSSG) recycling assay. The total GSH content was normalized to milligrams of protein. For total GSH determination, GSH was oxidized by DTNB to give GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). Thirty microliters homogenate containing 5% perchloric acid was incubated with 100 μl of 280 μmol NADPH and 15 μl

10 mmol/lol/l DTNB for 10 min at 30 °C to oxidize all GSH to GSSG. GSSG was then reduced by adding 15 μl GSH reductase. The rate of TNB formation was followed at 412 nm and was proportional to the sum of GSH and GSSG present. The rate was compared with a standard curve of GSH in buffer.

2.9. Chemical analysis

Fish after 42 days of exposure were freeze-dried. Then 200 mg of the dried and ground fish was introduced into precleaned thimbles and Soxhlet extracted for 24 h using an n-hexane/dichloromethane (1:1, v/v) solution. For this preparation process, $^{13}C_{12}$-α-HBCDD and $^{13}C_{12}$-γ-HBCDD were added to each sample as surrogate standards. The extract was cleaned up on a multilayer silica gel and alumina column. This column contained 5 g of anhydrous sodium sulfate, 2 g of deactivated silica, 10 g of acidic silica (44% conc. sulphuric acid w/w), 4 g of silica, 5 g alumina (3% organic-free reagent water w/w) and 5 g of anhydrous sodium. The first fraction eluted with *n*-hexane (80 ml) was discarded, the second fraction eluted with *n*-hexane/dichloromethane (1:1, v/v, 150 ml) was for collection of HBCD. After solvent evaporation, the extract was spiked with $^{13}C_{12}$-β-HBCDD to act as a recovery standard and then dissolved in 200 μl acetonitrile.

Analyses of HBCDD in the extract were performed using a Waters Acquity Ultra Performance LC system (Waters, Milford, MA, USA). UPLC separation was achieved using an Acquity UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm particle size, Waters, Milford MA, US), maintained at 45 °C, with a mobile phase flow rate of 0.25 ml/min. The mobile phase contained 50% A (80/20 methanol/acetonitrile, v/v) and 50% B (water with 10 mM ammonium acetate). The program was then ramped to 90% A and 10% B in 9 min and held for 4 min before returning to initial conditions. The sample volume injected was 15 μl. Determination was performed using a Waters Micromass Quattro Premier mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray source in negative ion mode. The ionization source parameters were: capillary voltage 3.0 kV; source temperature 100 °C; dissolving gas temperature 160 °C at a flow rate of 400 l/h (N_2); cone gas flow rate 50 l/h. selected ion recording (SIR) mode was applied for the analysis. MS detection of native HBCDD and $^{13}C_{12}$-HBCDD isomers was based on $[M-H]^-$ (native: *m/z* 640.7 and $^{13}C_{12}$: 652.5, respectively). HBCDD concentrations were calculated by comparing peak areas using the internal-standard technique. Data acquisition was carried out by MassLynx V 4.0 software.

2.10. Statistical analysis

All data were analyzed for significant differences ($P<0.05$) using one-way ANOVA with a Tukey post-test (Origin 7.0; OriginLab, USA). Linear regression analysis was also used to test correlative analysis (used as *R* value) (Origin 7.0; OriginLab, USA).

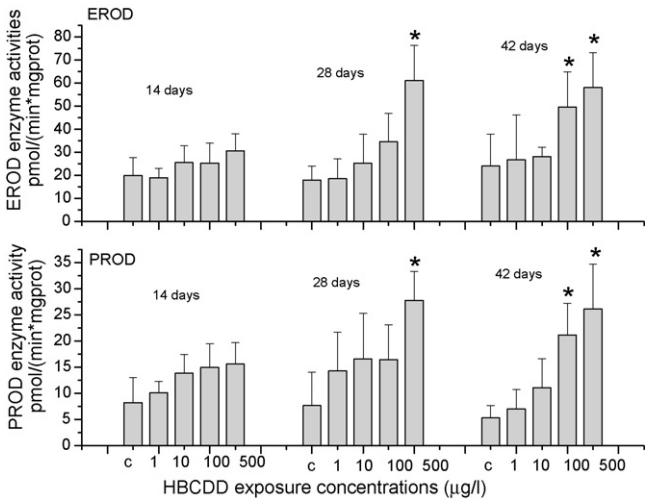

Fig. 1. EROD and PROD activities in fish liver at different exposure times. Values are shown as the mean ± S.D. ($n=10$). Asterisk denotes significant differences from the control at $P<0.05$.

3. Results

3.1. EROD and PROD

Fig. 1 presents the responses of EROD and PROD in fish liver after exposure to HBCDD. EROD and PROD activities were not significantly induced in any group after 14 days exposure. However, HBCDD caused a significant increase of both EROD and PROD activities at the concentration of 500 μg/l after 28 days exposure ($P<0.05$). After 42 days exposure, EROD and PROD were both significantly induced by HBCDD at concentrations 100 μg/l and above ($P<0.05$).

3.2. ROS, protein carbonyl, lipid peroxidation and DNA damage

Levels of ROS, lipid peroxidation and protein carbonyl in brain and DNA damage in erythrocyte are summarized in Fig. 2. Although a slight hint for an increase of ROS was observed in all exposed groups, there was no significant induction in ROS formation in any group after 14 days of exposure. A significant increase in ROS formation was evident at the high concentration (500 μg/l) treatment after 28 days exposure ($P<0.05$) when compared with the control. The generation of ROS was induced by 22.0% at 1 μg/l, 28.1% at 10 μg/l, 60.1% at 100 μg/l and 108.4% at 500 μg/l HBCDD after 42 days exposure.

The levels of protein carbonyls were not significantly increased compared to the control group in any group at 14 days of exposure. But, a significant increase was observed in the groups of 100 and 500 μg/l after 28 days exposure ($P<0.05$). After 42 days exposure, the increased values observed were 76%, 84%, and 89% over control values at concentration of 10, 100 and 500 μg/l, respectively ($P<0.05$).

There was no significant change in TBARS in fish brain after 14 and 28 days exposure when compared with the control group.

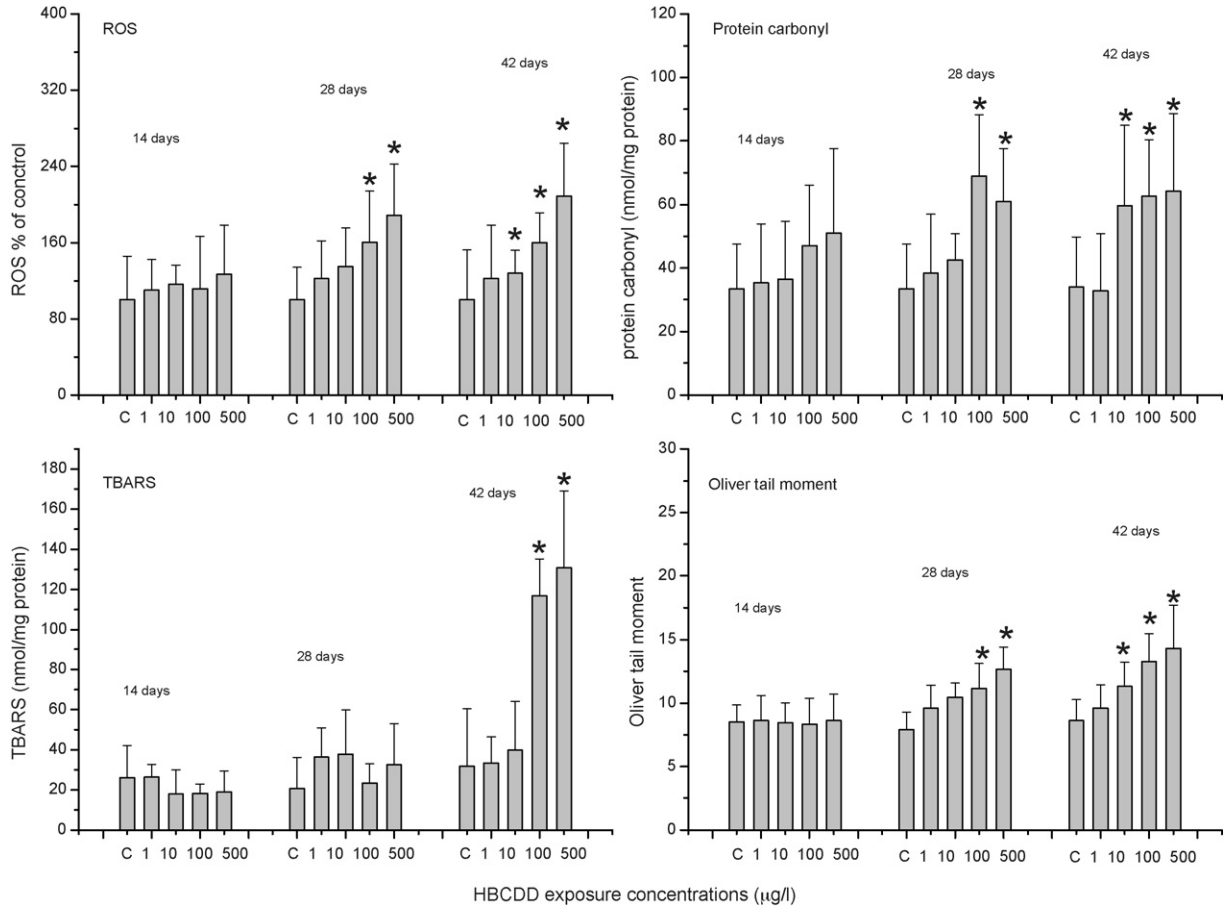

Fig. 2. Effects of different concentrations of HBCDD on ROS formation, the content of TBARS, protein carbonyl in the brain and the Olive tail moment in erythrocytes at different exposure times. Values are shown as the mean ± S.D. ($n = 10$). Asterisk denotes significant differences from the control at $P < 0.05$.

However, after a longer-term exposure (42 days) this biomarker was significantly elevated at HBCDD concentrations of 100 and 500 μg/l ($P < 0.05$).

The level of DNA damage was expressed as the mean Olive tail moment. Olive tail moment increased significantly only in the fish exposed to 500 μg/l of HBCDD for 28 days as comparison with the control ($P < 0.05$). However, Olive tail moment increased also at 100 μg/l HBCDD concentration after 42 days exposure ($P < 0.05$). A good dose–response relationship was observed between Olive tail moment and the concentrations of HBCDD after 42 days exposure ($R = 0.9916$).

3.3. SOD and GSH

GSH level and SOD activity in fish liver exposed to HBCDD for 14, 28 and 42 days are shown in Fig. 3. GSH level was depressed in both time- and dose-dependent manner. Significant depletion in GSH level occurred in all treatment groups after 42 days of exposure ($P < 0.05$). SOD activity did not change significantly in any group exposed for 14 days. A longer exposure resulted in a significant decrease in the SOD activities ($P < 0.05$).

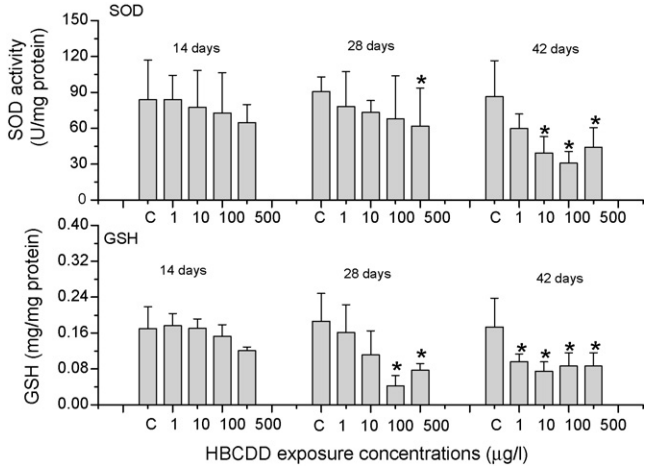

Fig. 3. Effects of different concentrations of HBCDD on the contents of GSH and on the activities of SOD in fish brain after 14, 28 and 42 days of exposure. Values are shown as the mean ± S.D. ($n = 10$). Asterisk denotes significant differences from the control at $P < 0.05$.

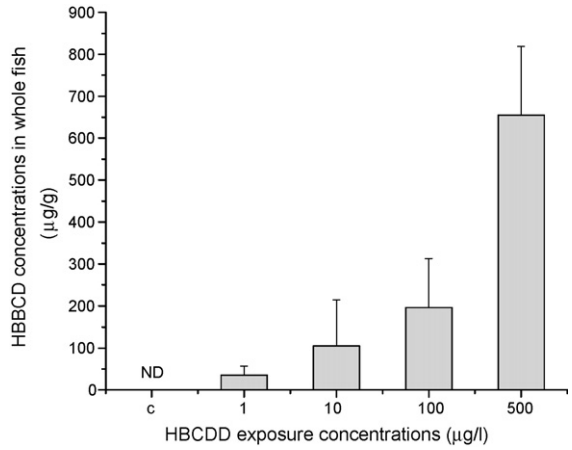

Fig. 4. Chemical analysis of HBCDD in whole fish after 42 days exposure.

3.4. Chemical analysis

Internal HBCDD levels are presented in relation to wet weight for comparison with exposure levels (Fig. 4). After 42 days of exposure, HBCDD in whole fish was detected in all exposure groups. As expected, the HBCDD levels in whole fish increased with increasing exposure concentration. Internal HBCDD levels were 34 μg/g (wet weight) when nominal concentration in water was 1 μg/l. A maximum level of 654 μg HBCDD/g (wet weight) was reached in whole fish from an animal exposed to waterborne 500 μg/l.

4. Discussion

In the present study, although EROD and PROD activities had no significant induction after 14 days exposure, HBCDD was found to induce of EROD and PROD in fish at the highest concentration after 28 and 42 days exposure. EROD and PROD activities in fish liver were elevated by HBCDD three-fold at 500 μg/l after 28 days exposure comparison with the control, while EROD and PROD were induced three-fold at 100 μg/l after 42 days exposure. This result is not in accordance with other reports. For example, Ronisz et al. (2004) reported that HBCDD had an inhibitory effect on EROD in juvenile rainbow trout after 28 exposure days to 500 mg/kg (body weight) dose by injection. However, Silke et al. (2006) reported that HBCDD had no effect on EROD in rat after 28 days of treatment to 200 mg/kg (body weight) by gavage. The reason for different results is still not known and needs further study. At the same time, Silke et al. (2006) also reported that HBCDD led to a significant induction of PROD activity in male rats at 10 and 100 mg/kg (bw) after 28 days exposure, while it caused no significant induction in female. However, in the present study, we found PROD activity was significantly induced at 500 μg/l after 28 days exposure. But we did not distinguish a difference of PROD activity between sexes.

It has recently been recognized that under certain circumstances, P450s can produce reactive oxygen species (ROS) that result in oxidative stress to organism (Schlezinger and Stegeman, 2001). In the present study, the long-term exposure to HBCDD could cause the increased capacity of microsomes to produce ROS in fish brain (Fig. 2). According to the results of the present study, HBCDD was identified as an inductor of cytochrome P450 in Chinese rare minnow hepatocytes. Therefore, HBCDD-induced ROS production could involve HBCDD metabolized by P450 through redox cycle.

Brain has a high mitochondrial oxidative metabolism to meet the high ATP demand for neural processing in fish (Soengas and Aldegunde, 2002). Furthermore, brain contains a large amount of easily oxidized polyunsaturated fatty acids. And moreover, brain has a relatively low antioxidant defense system (Mates, 2000). On the contrary, antioxidant enzymes in liver show a higher activity than other organs in fish (Lemaire et al., 1994) indicating a high capacity to tolerate oxidative stress. Furthermore, in previous study, it was found that brain was a more sensitive target organ to oxidative damage than liver (Song et al., 2006). So, in this study, we selected the brain as an object of study. The induction of ROS could enhance the oxidation of polyunsaturated fatty acids and lead to peroxidation. Lipid peroxidation has often been used as an effective biomarker of toxic pollutants in fish (Livingstone, 2001). Lipid peroxidation has usually been indicated by TBARS in fish (Wilhelm et al., 2001; Oakes and Van Der Kraak, 2003). In the present study, we observed that HBCDD-induced TBARS increased in dose- and time-response manner. A significant increase in TBARS levels was seen only in the fish exposed to ≥100 μg/l of HBCDD for 42 days exposure. There was a good correlation between TBARS levels and ROS formation after 42-day exposure ($R = 0.782$, Fig. 5). The results show that ROS produced during a long exposure of HBCDD could cause the damage to lipid in the brain of Chinese rare minnow. The lipid peroxidation

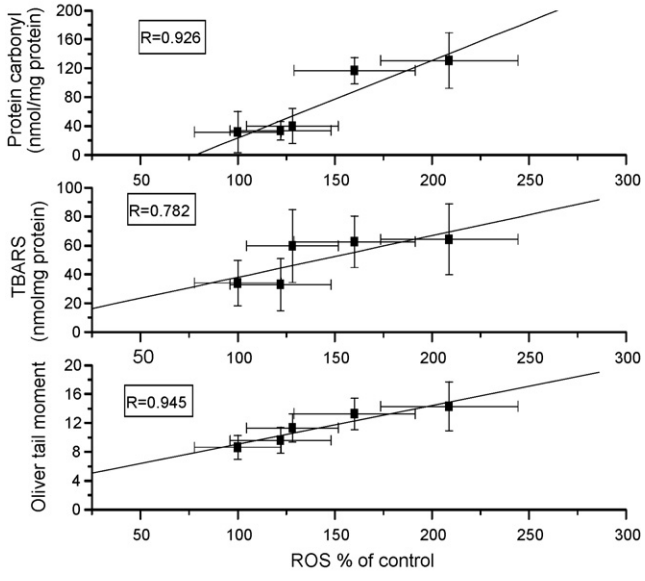

Fig. 5. Correlation analysis between protein carbonyl and TBARS content in the brain, Olive tail moment in erythrocytes and ROS content in the brain after a 42-day exposure. R represents correlation coefficient. Each value represents the mean ± S.D. ($n = 10$).

process influences membrane fluidity as well as the integrity of biomolecules associated with the membrane (membrane bound proteins or cholesterol). Since these lipids in fish are in close proximity to electron transport chains and heme iron proteins, which can act as sources of reactive oxygen species under normal conditions, the lipids may sustain high degrees of damage (Dix and Aikens, 1993). These highly oxidizable lipids may then, in turn, attack nearby proteins causing the formation of an excess of protein carbonyls (Almroth et al., 2005).

Protein carbonylation is one result of protein oxidation. Beside highly oxidizable lipids, ROS could directly attack protein and lead to the formation of carbonyl (Stern, 1985; Bainy et al., 1996). The formation of carbonyl proteins is non-reversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting, owing to increased susceptibility to protease action, in breakdown of proteins by proteases. Oxidative modification leads to proteolytic degradation, which may affect the structure, function and integrity of proteins (Carney et al., 1991). We observed the increase in protein carbonylation in time and dose-dependent manner as a result of HBCDD exposure. There was a good relationship between protein carbonyl level and ROS formation after 42 days of exposure ($R = 0.926$, Fig. 5). This result indicates that normal protein metabolism was disrupted, which resulted in the accumulation of damaged molecules. This result also suggested that protein carbonyl was more sensitive than TBARS as a biomarker of oxidative damage. This result is consistent with the suggestion of Almroth et al. (2005).

DNA damage resulting from attack by ROS includes base oxidation, deoxyribose damage, DNA single strand breaks, abasic sites, deamination, nitration, and DNA-protein crosslinks (Nishimoto et al., 1991). ROS induce deoxyribose damage by removing hydrogen to generate single strand breaks as well as oxidized abasic sites in the DNA (Nigro et al., 2002). This study shows that HBCDD resulted in the damage to erythrocyte of Chinese rare minnow. There was a clear time- and dose-dependent response to HBCDD which was associated with the elevation of ROS production. Interestingly, there was a good correlation between ROS production and DNA damage after 42 days exposure ($R = 0.945$, Fig. 5). This shows that ROS attacked DNA and led to DNA strand break.

Cellular antioxidant defense systems in biological systems are depleted when exposed to environmental pollutants (Winston and Di Giulio, 1991). The nonenzymatic antioxidant systems mainly consists of substances of low molecular weight, such as vitamins C and E, β-carotene, GSH, etc. (Kohen and Nysks, 2002). SOD catalyses the dismutation of superoxide anion radical to H_2O and H_2O_2. SOD provides the first defense against reactive oxygen toxicity (Pandey et al., 2003) and is usually used as a biomarker to indicate oxidative stress. In this study, SOD activity was significantly inhibited after 28 days exposure to 500 μg/l of HBCDD. However, for longer-term exposure, SOD activities were inhibited at concentrations of 10 to 500 μg/l HBCDD. In the present study, the inhibition of SOD activities observed may possibly reflect the damage to the enzyme due to ROS production in accordance with protein carbonyl level increase.

As an antioxidant, GSH plays a major role in cellular metabolism and free radical scavenging (Athanasios et al., 2006). On one hand, GSH serves as a cofactor for glutathione transferase, which facilitates the removal of certain chemicals and other reactive molecules from the cells. On the other hand, GSH can also interact directly with certain ROS (e.g., hydroxyl radical) for their detoxification as well as perform other critical activities in the cell. GSH is important to development process of brain. Its depletion can result in cell degeneration due to oxidative stress caused by pollutants. In the present study, the GSH contents in the brain of fish exposed to the highest concentration HBCDD were only 41.0%, 24.2% and 22.4% of the control (14, 28, 42 days exposure, respectively). These values approached a depletion stage. Therefore, our results suggest that depletion of GSH in brain indicates that the balance between the oxidative and antioxidant systems was broken. The intracellular imbalance may render the cells more susceptible to oxidative damage because of abnormal levels of ROS and simultaneous decline in the antioxidant defense mechanism, leading to damage to cellular organelles and changes of certain enzymatic activities.

Besides, the present study also confirms that HBCDD is bioavailable to a lower aquatic vertebrate species, Chinese rare minnow, when exposed in an environmentally relevant concentration. HBCDD induced P450 enzymes and resulted in oxidative damage after 42 days exposure, which were related to HBCDD accumulated in fish.

5. Conclusion

Our results show that HBCDD induces the detoxification enzymes CYP1A1 and 2B1 activities and increases ROS formation in the Chinese rare minnow after an exposure of 4–6 weeks to concentrations of 100–500 μg/l. Besides the overproduction of ROS resulted in oxidative damage to lipids, proteins and DNA and also in a concomitant decrease in antioxidative capacity measured through decreased in brain SOD activity and GSH content after such exposure. The long-term or chronic effects of HBCDD exposure on aquatic organisms should receive great attention and further investigations.

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