Effects of hexachlorobenzene on antioxidant status of liver and brain of common carp (Cyprinus carpio)

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Abstract

Hexachlorobenzene (HCB)-induced oxidative damages have been published in rats while the effects have not yet been reported in fishes. Juvenile common carps (Cyprinus carpio) were exposed to waterborne HCB from 2 to 200 μg/L for 5, 10 or 20 days. Liver and brain were analyzed for various parameters of oxidative stress. There were no significant changes of glutathione (GSH) content and superoxide dismutase (SOD) activity in liver after 5 or 10 days exposure, whereas obvious drops were observed at higher concentrations after 20 days exposure. Significant decreases of GSH content and SOD activity in brain were found during all the exposure days. In brain, HCB also significantly elevated the contents of reactive oxygen species (ROS), thiobarbituric acid-reactive substances (TBARS, as an indicator of lipid peroxidation products), glutathione disulfide (GSSG), and activities of nitric oxide synthase (NOS), glutathione peroxidase (GPx), and glutathione reductase (GR), and inhibited activities of acetylcholinesterase (AchE) and glutathione S-transferase (GST). The results clearly demonstrated that environmentally possible level of HCB could result in oxidative stress in fish and brain was a sensitive target organ of HCB toxicity.

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Keywords: Acetylcholinesterase; Antioxidant enzymes; Glutathione; Lipid peroxidation (LPO); Oxidative stress; Reactive oxygen species

1. Introduction

Hexachlorobenzene (HCB) is one of the most widespread persistent organic pollutants. Although the use of HCB was banned in most countries in the 1970s, it is still released into the environment as a by-product in several industrial processes (Courtney, 1979). HCB has a broad range of toxic effects on experimental animals, including hepatotoxicity, immune suppression, neurotoxicity, disturbance of thyroid function, reproductive toxicity and carcinogenicity (ATSDR, 2002). Because of its chemical stability, persistence, and long-range transport, HCB can be found throughout the environment (IPCS, 1997; Barber et al., 2005).

Many waterborne organic contaminants can stimulate production of ROS and result in oxidative damage to aquatic organisms (Livingstone, 2001). Under normal conditions, ROS and other pro-oxidants are continually detoxified and removed in cells by antioxidant defense systems comprising both antioxidant enzymes and small molecular free radical scavengers. If the production of ROS overwhelms antioxidant system, an imbalance between the generation and removal of ROS can produce oxidative stress (Tocher et al., 2002). Such processes may in turn provoke alterations in molecular and membrane structures and functions, leading to cell and tissue damage. Many oxidant-mediated biomarkers, including both enzymatic and molecular parameters, are used in environmental risk assessment of xenobiotics (van der Oost et al., 2003).

In mammals, porphyria caused by HCB exposure had been studied extensively. Though the mechanism of
HCB-induced hepatic porphyrins accumulation is not yet fully elucidated, reactive species was considered to contribute to the impairment of uroporphyrinogen decarboxylase (URO-D) or the URO-D inhibitor generation (Billi de Catabbi et al., 1997). Another study also found a close correlation between the oxidant-mediated parameters in HCB-exposed rat liver and levels of urinary coproporphyrin (Almeida et al., 1997). It suggested that oxidative stress could play an important role in HCB-induced hepatotoxicity. As regards HCB-induced biochemical effects on the central nervous system, previous studies reported alterations in regional brain concentration of serotonin, dopamine and norepinephrine (Bleavins et al., 1984) and alteration phospholipid metabolism (Cochon et al., 2001). To our knowledge, oxidative stress in brain has not been reported following HCB intoxication. Although there are many studies focused on the bioaccumulation and acute toxicity of HCB in fish, oxidative damage intoxication are seldom seen. Liver has been known as the most important target organ of metabolism and detoxification for many toxicants, whereas the brain may be particularly susceptible to oxidative damage because it contains a large amount of polyunsaturated fatty acids and consumes 20% of the body’s oxygen (Travacio et al., 2000). So it is necessary to verify HCB-induced oxidative damage in fish, especially in brain tissue.

In inland waters, HCB was frequently detected, with concentration generally below 1 ng l^{-1} (IPCS, 1997). However, higher values have been reported in aquatic systems that receive industrial discharges and surface runoff. For instance, 2–90.3 μg l^{-1} HCB were measured in some areas of Mississippi River (EPA, 1976). As high as 300 μg l^{-1} HCB was measured in industrial wastewater samples (Schmitt et al., 1990). Wu et al. (1997) reported that concentrations of HCB ranged up to 57 mg kg^{-1} in sediments of Lake Ya-Er near a chemical plant, in Hubei province, China. In this study, common carp, one of the widely used fish models in aquatic toxicology, was chosen as the test organism, and exposed to 2–200 μg l^{-1} of HCB to examine whether these environmentally realistic and sub-lethal levels of HCB can cause oxidative damage in fish. To compare the sensitivity of the liver and brain to HCB toxicity, time-courses of SOD and GSH changes under HCB exposure were followed in both tissues. As a more sensitive target organ than liver, brain ROS, NOS, TBARS, Gpx, GR, GST, GSSG and AChE were selected for indicating the oxidative and antioxidant defense status.

2. Materials and methods

2.1. Chemicals

HCB was obtained from Merck (Darmstadt, Germany), with purity 99% or greater. 2,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 1,1,3,3-tetraethoxy-propane (TEP), 1-chloro-2,4-dinitrobenzene (CDNB), GSH, GSSG and Folin’s reagent were purchased from Sigma (USA). N-Echylmaleimide (NEM) was purchased from Alfa Aesar (USA). All other reagents were of analytical grade.

2.2. Animals, treatment and sampling

Juvenile common carp of both sexes, with body weight 10.29 ± 1.38 g (mean ± SD), were obtained from hatchery of Institute of Hydrobiology (far from contamination area). Fish were randomly distributed into several equal aquariums with a water/fish ratio of 2.91 g^{-1}. During the experiment, pH value of the water was 7.7 ± 0.1, and the water temperature was controlled at 20 ± 2 °C. The light cycle was 16:8 h (light/dark) and the dissolved oxygen level was 7.0 mg l^{-1}. The fish were fed daily with commercial fish pellets at a quantity equivalent to 1% of the total body weight on fixed time and the extra food was removed. After two-weeks acclimation, fish were divided into six groups of ten individuals each (n = 10). The nominal concentrations of HCB used were 2, 10, 50, 100 and 200 μg l^{-1}. HCB was dissolved in acetone with a final concentration less than 0.2%oo, and the control groups received 0.2%oo acetone only. The exposure solution was statically renewed each day. The juveniles were exposed to HCB for 5, 10 and 20 days and the alterations of SOD and GSH were measured in brain and liver. ROS, NOS, TBARS, Gpx, GR, GST, GSSG and AChE were detected in brain of the fish exposed to HCB for another 10 days under the same conditions. No mortality was observed during whole experiment.

At the end of each exposure, fish were killed by decapitation. Brain and liver were dissected, rinsed with ice-cold physiological saline and stored at −80 °C for analysis. The tissues were homogenized with glass-glass homogenizer for 1 min in 0.8 ml ice-cold Tris buffered saline (10 mM Tris-HCl, 0.1 mM EDTA-2Na, 10 mM sucrose, 0.8% NaCl, pH 7.4). After addition of 2 μl perchloric acid, 180 μl homogenate was centrifuged at 4000 × g for 20 min at 4 °C and the supernatant was stored at −80 °C for GSH and GSSG analysis. Another portion of homogenate was removed for TBARS and protein measurements and others were centrifuged at 2500 × g for 20 min at 4 °C and the supernatant was used for various biochemical analysis. Most measurements were made using an Agilent 8453 UV–Visible spectro-photometer.

2.3. ROS measurement

ROS was measured based on the methods of Driver et al. (2000) with slight modifications. Homogenates (20 μl) were pipetted into 96-well plates and allowed to warm to room temperature for 5 min. At that time, 100 μl physiological saline, 5 μl of DCFH-DA (dissolved in DMSO, 10 μM final concentration) was added to each well and the plates were incubated at 37 °C for 30 min. The conversion of DCFH to the fluorescent product DCF was measured using a TECAN spectrophotometer with excitation at 485 nm and emission at 530 nm. 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).

2.4. Enzymatic activities

The activities of SOD, NOS, GPx, AChE were measured using the Diagnostic Reagent Kit purchased from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacturer’s instructions. Activities of SOD, NOS, and GPx were all referred to their total enzyme forms. GST assay was carried out by the modified method of Habig et al. (1974) using CDNB as substrate. GST was determined kinetically by monitoring changes in absorbance at the 340 nm for 3 min at 28 °C in 100 mM sodium phosphate buffer (pH 6.5) containing 1 mM CDNB and 1 mM GSH. The CDNB was dissolved in ethanol. In all cases, the final concentration of ethanol in the assay mixture did not exceed 5% (v/v). Blanks had the same conditions replacing the sample with phosphate buffer. The extinction coefficient used for GST was 9.6 mM⁻¹ cm⁻¹ at 340 nm. One unit (U) of GST activity was defined as the amount of enzyme producing 1 μmol CDNB conjugate formed in 1 min. The brain GST activities were expressed as U mg⁻¹ protein.

2.5. Lipid peroxidation

To determine the degree of LPO in brain, levels of TBARS were assayed according to the method of Ohkawa et al. (1979). Tissue homogenate (200 μl) was added to 50 μl of 8.1% SDS and 20 μl of butylated hydroxytoluene (0.02% in 95% ethanol, w/v). The samples were vortexed and incubated for 10 min at room temperature. Subsequently, 375 μl of 20% acetic acid (pH 3.5) and 375 μl of thiobarbituric acid (0.8% in 0.05 M NaOH) was added to the mixture, vortexed and placed into a 95 °C water bath for 60 min. The samples were then allowed to cool immediately under running tap water and centrifuged at 2500 × g for 10 min. The absorbance of the clear supernatant was determined at 532 nm and 600 nm. The absorbance differences were used to calculate TBARS levels. The method was calibrated with TEP standard solution.

2.6. GSH and GSSG measurements

GSH and GSSG levels were measured by method of Hissin and Hilf, 1976). To 10 μl homogenate, 2.8 ml of the phosphated-EDTA buffer (pH 8.0), and 100 μl O-phthalaldehyde solution (2 mg ml⁻¹ in methanol) were added. After thorough mixing and incubation at room temperature for 15 min, fluorescence was determined with a Kontron SFM 25 spectrofluorometer at excitation wavelength 350 nm and emission wavelength 420 nm. The instrument was calibrated against GSH standard. 100 μl tissue homogenate was incubated at room temperature with 40 μl of 40 mM NEM for 30 min to interact with GSH. 0.86 ml of 0.1 M NaOH was added to the mixture. A 100 μl portion of this mixture was taken for measurement of GSSG, using the procedure above for GSH assay, except that 0.1 M NaOH was employed as a diluent rather than phosphated-EDTA buffer. GSSG standard was used to calibrate the results.

2.7. Protein measurement

Protein was assayed by Lowry’s method (1951) using bovine serum albumin as a standard.

2.8. Statistical analysis

All data are presented as mean ± SEM and were analyzed using one-way analysis of variance (ANOVA). When differences were found (P < 0.05), the unpaired two-tailed Student’s t-test was used to compare means between treatment and control groups. Difference was considered to be statistically significant if P < 0.05.

3. Results

GSH contents and SOD activities in brain and liver of common carp exposed to HCB for different days were summarized in Figs. 1 and 2. In liver, GSH content and SOD

![Fig. 1. GSH contents in brain and liver of common carp exposed to HCB for different days. Each value represents the mean ± SEM. (n = 10). The significant change was shown as (a) P < 0.05 and (b) P < 0.01 when compared with control group values.]
activity showed no significant difference after 5 and 10 days exposure ($P > 0.05$, ANOVA). Twenty days after exposure, GSH content and SOD activity significantly decreased at higher concentrations. In brain, GSH and SOD showed a depressed manner with the dose increase during all the exposure period.

ROS, TBARS and GSSG levels and AChE, NOS, GPx and GR activities in brain of carp exposed to HCB for 10 days were measured and the results were shown in Fig. 3 and Table 1. The generation of ROS was induced by 18.7% at 10 μg l$^{-1}$, 28.4% at 50 μg l$^{-1}$, 90.8% at 100 μg l$^{-1}$ and 64.0% at 200 μg l$^{-1}$ HCB. Similarly, NOS activity and TBARS content were also induced at higher concentration, and moreover good correlations were found between their inductions and ROS formation. In brain, GSH and SOD showed a depressed manner with the dose increase during all the exposure period.

Fig. 2. SOD activities in brain and liver of common carp exposed to HCB for different days. Each value represents the mean ± SEM. (n = 10). The significant change was shown as (a) $P < 0.05$ and (b) $P < 0.01$ when compared with control group values.

4. Discussion

4.1. Effects of HCB exposure on ROS, TBARS and NOS

NADPH-cytochrome P450 reductase acts as an electron donor during the metabolism of xenobiotics by the cytochrome P450-dependent monoxygenase system and can produce superoxide radicals during their redox cycling. Though HCB was not an inducer of the cytochrome P450I A in carp (Cyprinus carpio) hepatocytes (Smeets et al., 1999), the HCB-induced ROS production could due to two causes. Firstly, as a lipid-soluble chemical, HCB can bind to cytochromes and is not readily metabolized, thus uncoupling the electron transport chain from mono-oxygenase activity and consequently favoring the production of reactive species (Ferioli et al., 1984). Secondly, pentachlorophenol, one of HCB major metabolites, is a potent source of ROS during its metabolism (Wang et al., 2001). HCB-induced ROS formation can oxidize most cellular constituents such as DNA, proteins and lipids causing damage to molecules, resulting in reduced enzymatic activity and affecting cellular integrity. Although the ROS formation had not been analyzed in liver, the alteration of antioxidant parameters indicated its generation.

The induction of ROS could enhance the oxidation of polyunsaturated fatty acids, leading to LPO of cell membranes. LPO has often been used as an effective biomarker of toxic pollutants in fish, including Cu, Fe, Cd, PAHs and PCBs (Livingstone, 2001). It was shown that brain TBARS levels were significantly induced by HCB exposure and correlated to the ROS production well ($R = 0.829$, $P = 0.041$). Previous experiments have also shown that HCB exposure induced LPO in rats (Almeida et al., 1997; Billi de Catabbí et al., 1997), and our results were in agreement with their finding.

NOS is the key enzyme in NO synthesis and may be considered as a proxidative enzyme. Once produced NO can interact with the superoxide anion to generate peroxynitrite (Reiter et al., 1997). Elevated NO can cause direct cytotoxicity, but most damage follows formation of peroxynitrite. The mechanism of NOS induction in brain by HCB exposure was not clear in our study, while good correlation was observed between the enzyme activity and ROS formation (Fig. 4). The results suggested reactive nitrogen species could play a role in HCB induced oxidative damage.

4.2. Comparison of the susceptibility to oxidative damage between liver and brain

Previous study revealed that waterborne exposure to 2 μg l$^{-1}$ of HCB did not cause any major changes in hepatic antioxidant enzyme activities in fish (Roy et al., 1995). In our study, hepatic SOD activity and GSH content in 2 μg l$^{-1}$ group were not significantly different from the control group, which consisted with former study and suggested a high antioxidant capacity in liver. Hepatic SOD activity and GSH content were slightly induced after 10
days exposure, and then both were inhibited after 20 days exposure. The results suggested an increase of superoxide radical anion generation of liver and the adaptation to slight oxidative damage during shorter exposure period (i.e., 10 days) and demonstrated that HCB induced oxidative stress in the organ during longer exposure period (i.e., 20 days). 50 lgl/C0 can be suggested as the threshold dose for HCB-induced oxidative damage in liver of common carp, because obvious decreases of GSH content and SOD activity were found upon the concentration. Usually an induction of hepatic SOD activities was observed when exposed to organic pollutant ( Palace et al., 1996 ), however, the excess production of superoxide radicals by themselves or after their transformation to H2O2 cause an oxidation of the cysteine in the enzyme and deactivate SOD ( Dimitrova et al., 1994 ). Decreases of hepatic SOD activity were also found in fish exposed to 2, 4-Dichlorophenol ( Zhang et al., 2004 ).

The responses of brain were clearly different from those of liver in terms of SOD and GSH. Both SOD activity and GSH content in brain were marked depressed after 5, 10 or 20 days treatment, suggesting that they were sensitive biomarkers to HCB exposure and the brain tissue suffered a severe oxidative stress- even after as short as 5 days exposure, and then both were inhibited after 20 days exposure. The results suggested an increase of superoxide radical anion generation of liver and the adaptation to

Fig. 3. Effects of different levels of HCB on the activities of NOS, AchE and on the contents of ROS and TBARS in brain of common carp after 10 days exposure. Each value represents the mean ± SEM (n = 10). The significant change was shown as (a) $P < 0.05$ and (b) $P < 0.01$ when compared with control group values.

Table 1
Effects of HCB on brain GPx, GST, GR and GSSG in common carp 10 days after treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCB (μg l$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>GR (U mg$^{-1}$ protein)</td>
<td>7.03 ± 0.45</td>
<td>6.77 ± 0.33</td>
</tr>
<tr>
<td>GSSG (nmol mg$^{-1}$ protein)</td>
<td>44.23 ± 1.43</td>
<td>41.50 ± 1.69</td>
</tr>
<tr>
<td>GPx (U mg$^{-1}$ protein)</td>
<td>20.47 ± 1.01</td>
<td>18.73 ± 0.75</td>
</tr>
<tr>
<td>GST (U mg$^{-1}$ protein)</td>
<td>150.49 ± 4.00</td>
<td>142.10 ± 3.03</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM (n = 10). The significant change was shown as (a) $P < 0.05$ and (b) $P < 0.01$ when compared with control group values.

Fig. 4. Correlation analysis between NOS activities and ROS contents in common carp brain after 10 days exposure. Each value represents the mean ± SEM (n = 10).
exposure. Brain has a high mitochondria 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 towards oxidative stress. This might explain why the brain was more susceptible to oxidative damage than liver in the present study.

4.3. GSH and GSH-related enzymes in brain

GSH plays a central role in antioxidant system and its depletion is considered as an important biomarker of oxidative stress in fish caused by pollutants (Almar et al., 1998; Pena-Llopis et al., 2001). Brain GSH contents in the highest concentration HCB groups were only 41.0%, 24.2% and 22.4% of controls (5, 10, 20 days exposure, respectively), nearing a depletion stage. Under oxidative stress conditions, ROS are reduced by GSH to GSSG catalyzed by GPx. GSH depletion is considered as an important biomarker of oxidative stress in fish caused by pollutants (Almar et al., 1998; Pena-Llopis et al., 2001). Brain GSH contents in the highest concentration HCB groups were only 41.0%, 24.2% and 22.4% of controls (5, 10, 20 days exposure, respectively), nearing a depletion stage. Under oxidative stress conditions, ROS are reduced by GSH to GSSG catalyzed by GPx. GSH can also consume rapidly and non-enzymatically with hydroxyl radical, NO, N₂O₃ and peroxynitrite (Cnubben et al., 2001). The HCB-induced GSH depletion was also found by former studies. Hepatic GSH content in rats was found to be decreased by 50% within 24 h following oral administration of HCB (200 mg/kg) (Ingebrigtsen et al., 1981). A dose-dependent depletion of GSH in HCB-exposed rats was found in another study (Koss et al., 1987). Our results were in agreement with these works.

GPx catalyzes the reduction of both hydrogen peroxide and lipid peroxides and is considered as an efficient protective enzyme against lipid peroxidation. In our study, GPx activity in brain decreased in 10 µg l⁻¹ group, as an adaptive response to maintain the homeostasis of reduced GSH. However, with the concentration of TBARS increasing, GPx activity was then induced to reduce the lipid peroxide; meanwhile, there was an enhanced GSSG formation in 100 µg l⁻¹ treatment group. GSSG content returned to normal in 200 µg l⁻¹ group, and the result suggested that GSSG was exported from the cell to maintain the cellular redox by ATP-dependent transport protein, implicating that severe oxidative stress depletes cellular GSH (Leier et al., 1996). The role of GR is to maintain the cytosolic concentration of GSH. The mechanism of regulation of GR activity in fish is still unknown; however, the content of GSSG was supposed to be a critical factor in the induction of GR activity in rainbow trout liver (Stephensen et al., 2002). In the present study, GR activity was induced, in response to the enhanced GSSG content at 100 µg l⁻¹, which confirmed the former suggestion.

GST is a multicomponent enzyme involved in the detoxification of many toxicants and plays an important role in protecting tissues from oxidative stress (Fournier et al., 1992). In the present study, brain GST activity was inhibited, suggesting the failure of adaptive response and indicating an severe oxidative stress status in the organ. The depressed GST level result from the oxidative damage in aquatic organisms was also observed in T. tubifex exposed to fenhexamid (Mosleh et al., 2005) and in catfish exposed to dimethoate (Hamed et al., 1999).

4.4. Neurotoxic effect

AChE is involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings, which is vital for normal functioning of sensory and neuromuscular systems, and is a common biomarker used in neurotoxic effect. AChE inactivation may have resulted from either amino acid residue and/or membrane lipid oxidation (Ballinger et al., 2005). For instance, the AChE deactivation in rat erythrocytes by pyrethroid was correlated with the LPO status (Kale et al., 1999). Moreover, AChE inhibition in the arginine-treated rats could be prevented by the pre-treatment with vitamins E and C (Wyse et al., 2004). In the present study, dose-dependent inhibition of AChE activity (R = -0.817, P = 0.047) was found and the parameter had good correlations to GSH (R = 0.903, P = 0.013) and SOD (R = 0.891, P = 0.017). So the AChE deactivation in carp brain was supposed to be the result of concomitant oxidative damage and the parameter was also a sensitive biomarker to this pollutant. The present study showed that HCB could cause neurotoxic effect on fish. Therefore, further investigations are needed in the near future.

5. Conclusions

The results demonstrated that waterborne HCB at environmental realistic concentrations could result in oxidative stress in common carp. Compared to liver, brain was more sensitive to the oxidative damage and supposed to be one of the most important target organs of HCB intoxication, preventing continuous nerve firings, which is vital for normal functioning of sensory and neuromuscular systems, and is a common biomarker used in neurotoxic effect. AChE inactivation may have resulted from either amino acid residue and/or membrane lipid oxidation (Ballinger et al., 2005). For instance, the AChE deactivation in rat erythrocytes by pyrethroid was correlated with the LPO status (Kale et al., 1999). Moreover, AChE inhibition in the arginine-treated rats could be prevented by the pre-treatment with vitamins E and C (Wyse et al., 2004). In the present study, dose-dependent inhibition of AChE activity (R = -0.817, P = 0.047) was found and the parameter had good correlations to GSH (R = 0.903, P = 0.013) and SOD (R = 0.891, P = 0.017). So the AChE deactivation in carp brain was supposed to be the result of concomitant oxidative damage and the parameter was also a sensitive biomarker to this pollutant. The present study showed that HCB could cause neurotoxic effect on fish. Therefore, further investigations are needed in the near future.

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