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Oxidative stress and apoptosis of *Carassius auratus* lymphocytes induced by nonplanar (PCB153) and coplanar (PCB169) polychlorinated biphenyl congeners *in vitro*

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Abstract

Among all the 209 kinds of polychlorinated biphenyls (PCBs) congeners, nonplanar and coplanar PCB congeners have different levels of toxicity on mammal cells such as neuronal cells, but little is known about their toxicity on fish cells although PCB congeners usually have high bioaccumulation abilities in the detected fish bodies. This study showed that 2,2',4,4',5,5'-hexacholorbiphenyl (PCB153, nonplanar congener) and 3,3',4,4',5,5'-hexacholorbiphenyl (PCB169, coplanar congener) caused apoptosis on the isolated crucian carp (*Carassius auratus*) lymphocytes and the induced cytotoxicity was structure-dependent. According to the laser confocal microscope observations, apoptosis was clearly distinguished by condensation of nucleus, shrinkage and formation of apoptotic bodies. DNA fragmentation was detected by agarose gel electrophoresis. These typical morphological and biochemical characteristics indicate the occurrence of apoptosis on fish lymphocytes. According to the flow cytometry analysis, after the cells were exposed to 10 μmol/L PCBs for 3 h, the apoptotic percentage induced by PCB153 was 23.41%, while that induced by PCB169 was even higher (31.03%). Furthermore, incubating PCBs with fish lymphocytes enhanced levels of reactive oxygen species (ROS) and malondialdehyde (MDA), clearly indicating the presence of oxidative stress and lipid peroxidation. Our data also demonstrate that the different cytotoxic effects induced by coplanar and nonplanar PCBs were correlated with their structural characteristics and the coplanar congener was more cytotoxic than nonplanar congener. This study suggests that cytotoxicity mechanisms of the PCB congeners on fish lymphocytes depend on their planarity and chemical structures.

Key words: apoptosis; lymphocytes; malondialdehyde (MDA); reactive oxygen species (ROS)
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Introduction

Polychlorinated biphenyls (PCBs) are a class of widely dispensed and persistent environmental contaminants with halogenated aromatic hydrocarbons (Kodavanti *et al*., 1995). They consisted of 209 congeners with varying degrees of chlorination and substitution and usually can be divided into coplanar congeners with one or no chlorine substituent and nonplanar congeners with more chlorine substitution in the *ortho* positions. The toxicities of nonplanar and coplanar PCBs congeners are found to be structure-dependent (Chu *et al*., 1996; Safe, 1994; Sánchez-Alonso *et al*., 2003). The coplanar congeners 3,3',4,4' (PCB77), 3,3',4,4',5 (PCB126) and 3,3',4,4',5,5' (PCB169) are the most toxic PCBs on mammal cells (Storelli *et al*., 2004).

For the lipophilic chemical properties, PCBs are easily bioaccumulated in fatty tissues (Safe, 1994). The bioaccumulation of PCBs in fish has been well documented by Hites *et al*. (2004) (range 10–95 ng/g wet weight), Carline *et al*. (2004) (69–126 ng/g) and others (Maule *et al*., 2007; Stachel *et al*., 2007). Thus, for fish, long-term exposure to PCBs at low doses is possible, and immunotoxicity from PCBs is important.

Earlier studies on mammals have shown that PCBs can cause apoptosis in various cells (Oskam *et al*., 2004; Gregoraszczuk *et al*., 2003; Lee *et al*., 2003). However, little is known about the apoptosis effects on fish lymphocytes. Fish lymphocytes are important immune cells in the immune system, and play a crucial role in both humoral and cellular immunity of fish. Usually, reduction of lymphocyte apoptosis may lead to autoimmunity, whereas excessive apoptosis will cause immune deficiency. Thereby, the apoptosis of fish lymphocytes has a great influence on the homeostasis of the immune system (Xiang *et al*., 2008).

Several studies have shown that oxidative stress and lipid peroxidation are involved in PCBs-induced apoptosis. Treatment with PCB132 can induce formation of reactive oxygen species (ROS) in rat sperm (Hsu *et al*., 2007). PCB52 was also found to induce ROS generation and

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lipid peroxidation in neuronal SK-N-MC cells (Lee *et al*., 2004). In response to PCBs waterborne exposure, the hepatic lipid peroxidation levels in males of *Girardinichthys viviparus* were increased in a gradual way (Vega-López *et al*., 2007). Thomé *et al*. (1995) found Aroclor 1254 (PCBs mixtures) at concentrations exceeding 25 μmol/L can cause irreversible damage to cultured hepatocytes and the enrichment of malondialdehyde (MDA). So oxidative stress and lipid peroxidation may be involved in PCBs-induced apoptosis in fish lymphocytes.

Therefore, this study was designed to investigate the apoptosis effects on isolated lymphocytes of *Carassius auratus* of two structural different PCB congeners, non coplanar 2,2'4,4',5,5'- hexachlorobiphenyl (PCB153), and coplanar 3,3',4,4',5,5'-hexacholorbiphenyl (PCB169). It was found that both PCB153 and PCB169 caused apoptosis of *C. auratus* lymphocytes with intracellular ROS generation and an increased MDA. Our results strongly suggest that the cytotoxicity of PCBs congeners on fish lymphocytes is also structure-dependent.

1 Materials and methods

1.1 Chemicals

PCB153 (CAS No. 35065-27-1) and PCB169 (CAS No. 32774-16-6) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest grade available from commercial sources. For each experiment, both PCB153 and PCB169 were dissolved in dimethyl sulfoxide (DMSO) and were diluted with cell culture medium to the concentration indicated with a final DMSO concentration less than 0.1%. The control experiments were performed with the same concentration of DMSO diluted by phosphate buffered saline (PBS) without PCBs.

1.2 Experimental fish

Carassius auratus (6–12 months old) of both sexes, weighing approximately 400–500 g, were obtained from the hatchery of the Freshwater Fisheries Institute of Zhejiang (China). All experimental fish were reared and maintained in aerated water at temperature of (25 ± 1)°C. They were fed with pellet food at a daily ration of 0.7% of their body weight. All fish were held in the laboratory for at least 10 days prior to use in experiments, which allows for acclimatization and evaluation of overall fish health. Only healthy fish, as determined by general appearance and level of activity, were used in the studies.

1.3 Isolation of lymphocytes and cell culture

Lymphocytes of *C. auratus* were prepared as previously described by Kemenade *et al*. (1995) but with slight modification. Briefly, *C. auratus* was killed by decapitation, and then the head, kidney and spleen were removed. Single cell suspensions were obtained by teasing the tissue (in serum-free RPMI-1640 culture medium) through a nylon sieve. The cells were washed twice in ice-cold culture medium without serum, collected and layered on 1.5 volumes lymphoprep (Sigma, USA, density adjusted to 1.077 g/mL). Following 30 min centrifugation at 3000 r/min, the lymphocyte layer was collected, and washed three times with PBS. The number of cells was determined by a haemacytometer, and the cells were cultured in antibiotic-free RPMI-1640 medium (Gibco, USA) with 5% fetal calf serum (FCS) in a CO_2 atmosphere at 27°C for 5 h to remove the adherent cells. Non-adherent lymphocytes were then carefully collected and recultured in RPMI-1640 medium.

1.4 Assessment of apoptosis by laser confocal microscope

Lymphocytes (2×10^6) were seeded into 24-well plates and cultured as described above. The cells were exposed to PCB153 and PCB169, respectively, at a concentration of 10 μmol/L for 3 h. Controls were treated with PBS. After induction, PCBs-treated cells and controls were harvested, washed twice with PBS, stained with 0.01% Acridine Orange (Sigma-Aldrich Chemical Co., USA) for 5 min at room temperature. After being blot-dried, the stained cells were observed under a ZEISS laser confocal microscope (LSM510META, ZEISS Co., Ltd., Germany) at 510 nm wavelength.

1.5 DNA extration and agarose gel electrophoresis

Lymphocytes (3×10^6) were exposed to PCB153 and PCB169 at a concentration of 10 μmol/L for 3 h. Controls were treated with PBS for 2 h. After induction, lymphocytes were collected and washed in ice-cold PBS. The cells were lysed in 0.5 mL of lysis buffer (100 nmol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% SDS, 200 mg/mL proteinase K, pH 7.5) for 16 h at 37°C. Thereafter, cell lysates were first extracted with phenol-chloroform-isoamyl alcohol (25:24:1, *V/V/V*), then with chloroform-isoamyl alcohol (24:1, *V/V*), precipitated overnight at –20°C in 2 vol. ethanol in the presence of 0.3 mol/L acetate, and recovered by centrifugation. Pellets were air dried, resuspended in TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA), and digested with 1 mg/mL RNase A for 2 h at 37°C. Electrophoresis was performed with a 1% agarose gel, which was stained with ethidium bromide.

1.6 Intracellular reactive oxygen species (ROS) assay

Assays of intracellular ROS of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) were performed as described by Enane *et al*. (1993). For $O_2^{\bullet-}$ assay, cells that had been incubated by PCB153 and PCB169 at the exposure level of 1, 10, and 100 μmol/L for 3 h, respectively, were plated in a 96-well plate at a density of 1×10^5 cells /well. 100 μL nitroblue tetrazolium (NBT, Serva Chemical Co., dissolved in PBS at 1 mg/mL) was added into the cell cultures for 1 h at 27°C, and then the cells were fixed with methanol before washed twice in PBS. After the cells were centrifuged at 3000 r/min for 5 min, 20 μL of 2 mol/L KOH and 140 μL DMSO were added to the cells. The cells were read at 630 nm in a microplate reader.

For H_2O_2 assay, 1×10^5 control and PCBs treated cells/well were mixed with 100 μL PRS buffer (140

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mmol/L NaCl, 10 mmol/L K_2HPO_4-KH_2PO_4 buffer, pH 7.0, 5.5 mmol/L glucose, 0.56 mmol/L phenol red and 100 μg/mL horseradish peroxidase), and maintained for 1 h at 27°C, then added 10 μL of 1 mol/L NaOH to stop reaction, the cells were read at 630 nm in a microplate reader.

1.7 Malondialdehyde (MDA) assay

MDA levels were evaluated to estimate the extent of lipid peroxidation in the cells. The assay was carried out using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The MDA content was colorimetrically measured following the manufacturer's instructions.

1.8 Statistical analysis

Experimental data were presented as means ± standard deviations (SD) of six independent experiments performed in triplicate. Where appropriate, samples were analyzed by using the Student's two-tailed *t*-test, and statistical significance was defined as $P < 0.05$ and $P < 0.001$.

2 Results

2.1 Apoptotic morphological observation by laser confocal microscope

Morphological changes of PCB153- and PCB169-induced lymphocytes were determined by Acridine Orange staining and observed by laser confocal microscopy (Fig. 1). The results showed that, after induction at the exposure level of 10 μmol/L for 3 h, the induced lymphocytes displayed a series of morphological changes such as condensation of nucleus, shrinkage and formation of apoptotic bodies, which were designated as typical evidence of apoptotic features. In contrast, control cells exhibited a normal appearance.

2.2 DNA fragmentation of PCBs-induced lymphocytes

Biochemically, the typical characteristics of apoptosis is the cleavage of nuclear DNA at the internucleosomal regions, resulting in 3'-OH termini and multimers of 180–200 bp. DNA fragmentation of PCBs-induced cells was also evaluated by the agarose gel electrophoresis. As the results predicted, characteristics of internucleosomal DNA ladder patterns with the size of 180–200 bp were detected in DNA extracted from cells treated with PCB153 and PCB169, but not in DNA from those of controls (Fig. 2). The results from agarose gel electrophoresis assessment made it clear that PCBs congeners could trigger apoptosis in *C. auratus* lymphocytes *in vitro*.

2.3 Flow cytometry detection of PCBs-induced lymphocyte apoptosis

Apoptosis of *C. auratus* lymphocytes in response to PCBs was quantified by the assessment of DNA integrity through fluorescence-activated cell sorting after propidium iodide (PI) staining. The histogram of PI staining is a reflection of chromatin content and the amount of apoptotic cells is proportional to the sub-G0/G1-phase. Figure 3 shows that the percentages of apoptotic lymphocytes induced by PCB153 and PCB169 at the exposure level of 10 μmol/L for 3 h were 23.41% and 31.03%, as compared to 3.27% apoptotic cells in the controls.

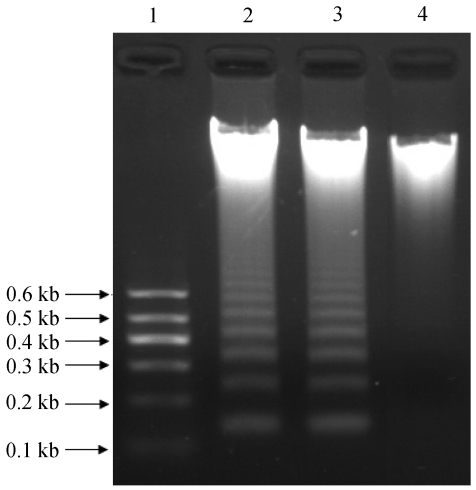

Fig. 2 DNA fragmentation of lymphocytes induced by PCB153 and PCB169 determined by agarose gel electrophoresis. Lane 1: 0.1–0.6 kb molecular size markers of DNA ladder; lane 2: DNA ladder patterns of lymphocytes induced by PCB153 for 3 h at the concentration of 10 μmol/L; lane 3: DNA ladder patterns of lymphocytes induced by PCB169 for 3 h at the concentration of 10 μmol/L; lane 4: control group.

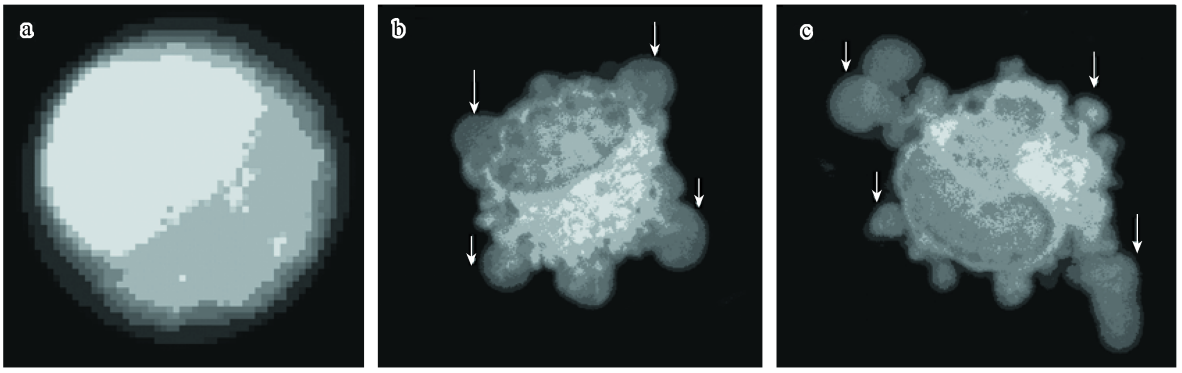

Fig. 1 Morphological changes by PCBs under a laser confocal microscope. Cells were stained with 0.01% Acridine Orange. (a) control cells exhibited normal morphology; (b) cells treated with PCB153 at the concentration of 10 μmol/L for 3 h; (c) cells treated with PCB169 at the concentration of 10 μmol/L for 3 h (original magnification: a, b and c × 1000). The apoptotic bodies were pointed out by the white arrow in b and c.

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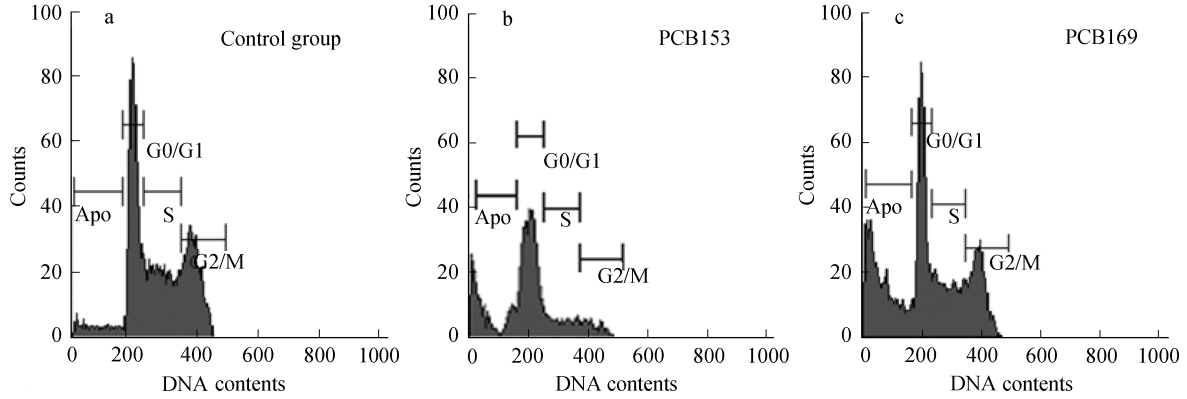

Fig. 3 Lymphocytes apoptosis induced by PCB153 and PCB169 were analyzed by flow cytometry. (a) PBS-treated control cells in which the apoptotic cells percentage is 3.27%; (b) lymphocytes induced by PCB153 at the concentration of 10 μmol/L for 3 h in which the apoptotic percentage is 23.41%; (c) lymphocytes induced by PCB169 at the concentration of 10 μmol/L for 3 h in which the apoptotic percentage is 31.03%.

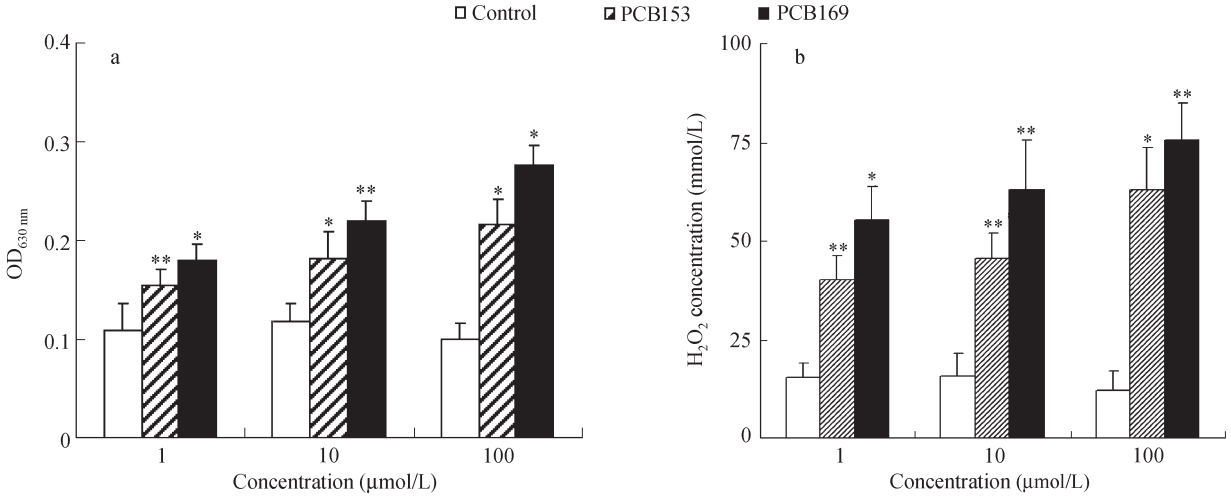

Fig. 4 Changes of intracellular $O_2^{\cdot -}$ (Fig. 4a) and H_2O_2 (Fig. 4b) after *Carassius auratus* lymphocytes were incubated with 1, 10 and 100 μmol/L PCB153 and PCB169 for 3 h, respectively. Data represent the means±S.D. of nine independent experiments each performed in triplicate. Significant difference from the control is designated by $^*P < 0.05$ and $^{**}P < 0.001$.

2.4 Detection of reactive oxygen species (ROS)

To define the role of ROS in PCBs-treated cells, changes in intracellular $O_2^{\bullet -}$ and H_2O_2 levels in response to treatment with different PCBs concentrations are shown in Fig. 4. Dose-dependent increases in $O_2^{\cdot -}$ (Fig. 4a) and H_2O_2 (Fig. 4b) were observed in PCBs-incubated lymphocytes. Compared to the control cells, significant ($P < 0.05$) increases in both $O_2^{\bullet -}$ and H_2O_2 were typically noticed in PCBs-treated cells at the concentration of 1, 10 and 100 μmol/L. Levels of ROS induced by PCB153 groups were lower than those induced by PCB169 groups. Generation of intracellular ROS induced by both PCB153 and PCB169 were dose-dependent.

2.5 Analysis of MDA

According to Fig. 5, both PCB153 and PCB169 can significantly increase MDA formation ($P < 0.05$), depending on the incubation levels. Dose-dependent increases in MDA contents were observed in PCBs-incubated lymphocytes. Exposure to PCB153 and PCB169 at 10 and 100 μmol/L increased MDA as compared to control and 1 μmol/L treatments. Clearly, the MDA elevation induced by PCB153 and PCB169 was also dose-dependent. Furthermore, the increases in MDA contents were greater when cells were exposed to PCB169 than when exposed to PCB153 at all the exposure levels.

3 Discussion

Recent studies have demonstrated many PCBs congeners can induce apoptosis and exhibit immunotoxicity on mammals cells. Yoo *et al*. (1997) reported apoptosis-mediated immunotoxicity of PCBs in murine splenocytes. Shin *et al*. (2000) found that 2,2',4,6,6'-pentachlorobiphenyl induced apoptosis in human monocytic cells. However, apoptosis-inducing activity has not been studied in fish cells. As the toxicity of individual PCBs is structure-dependent (Chu *et al*., 1996), little is known about the different toxicities between coplanar and nonpolanar PCBs. So the present study was designed to investigate the apoptosis induced by nonplanar PCB153 and coplanar PCB169 and to outline the possible mechanism relating to structural characteristics. Our study strongly suggests that both PCB153 and PCB169 can induce *C.*

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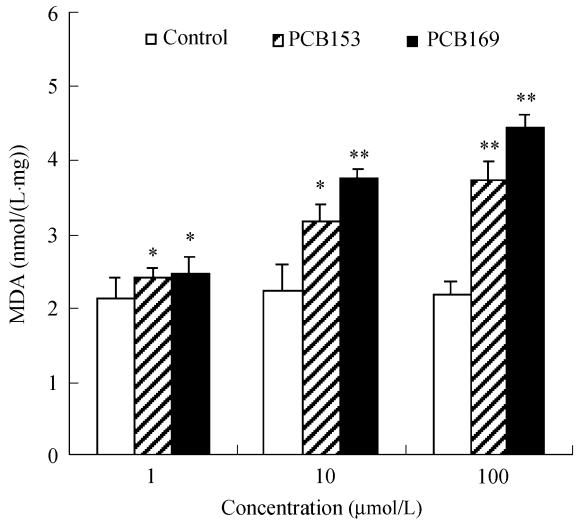

Fig. 5 Changes of MDA contents after *Carassius auratus* lymphocytes were incubated with 1, 10 and 100 μmol/L PCB153 and PCB169 for 3 h, respectively. Data represent the means±S.D. of nine independent experiments each performed in triplicate. Significant difference from the control is designated by $^{*}P < 0.05$ and $^{**}P < 0.001$.

auratus lymphocytes apoptosis *in vitro*, and there was difference in the apoptosis toxicity between coplanar PCB169 and nonplanar PCB153. The induction of the characteristic morphological features of apoptosis, such as condensation of nucleus, shrinkage and formation of apoptotic bodies and the biochemical features of DNA fragmentation confirm the occurrence of apoptosis in fish lymphocytes caused by PCBs. We also found that oxidative stress was the major event in PCB-induced apoptosis, which involved the increase of ROS and MDA.

As shown in Fig. 3, two types of PCB congeners exhibited different apoptosis abilities. At the exposure concentration of 10 μmol/L, the apoptosis percentage induced by PCB169 (coplanar PCB) was much higher than that induced by PCB153 (non coplanar PCB), indicating that PCBs molecule structural characteristic is an important factor for their effects on apoptosis. As we all know for both PCB153 and PCB169, they are hexacholorbiphenyls with the same number of Cl atoms in the molecules, while the main structural difference between them should be the Cl atoms substituted positions. Obviously, PCB153 has more chlorine substitution in the *ortho* positions than PCB169, which may result in the lower apoptosis cytotoxicity. For neuronal cells, Sánchez-Alonso *et al*. (2003) also found that the extent of apoptosis generated was greater for the non-*ortho*-substituted planar congener (PCB77) than for the di-*ortho*-substituted nonplanar congener (PCB153). Our results further proved that the toxicological response of PCBs should be intimately associated with congeners' structures.

Reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), the targets of which include DNA, lipids and proteins, were thought to cause damage (Nordberg and Arner, 2001). $O_2^{\bullet -}$ and H_2O_2, derived from oxidative respiration, can react directly with cellular components and are the most frequent ROS (Fleury *et al*., 2002). Recently, intracellular ROS generation was found to be an intimate mechanism for apoptosis (Cook *et al*., 2004). Using NBT assay, we showed that both PCB153 and PCB169 could induce a dose-dependent increase of ROS formation in *C. auratus* lymphocytes *in vitro* (Fig. 4). PCB153 increased intracellular $O_2^{\bullet -}$ by 1.4- to 2.2-fold and H_2O_2 by 2.1 to 3.8-fold, as compared with untreated lymphocytes. In comparison, PCB169 increased 1.7 to 2.8-fold for intracellular $O_2^{\bullet -}$ and 2.7 to 4.7-fold for H_2O_2. Our study demonstrated that ROS burst occurred after PCBs stimulation. Our results were in accordance with the findings of Hsu *et al*. (2007), where another PCB congener (PCB132) induced the increase of ROS formation in rat sperm. Lin and Lin (2006) also reported that PCB126 and PCB153 can induce ROS formation in human T47D and MDA-MB-231 breast cancer cells.

The oxidative stress level of lymphocytes was also assessed by the MDA assay. MDA is a byproduct of free radical attack on lipids and it is also a marker of membrane lipid peroxidation resulting from the interaction of ROS and the cellular membrane (Aslan *et al*., 1997). As shown in Fig. 5, there was no significant difference among MDA contents induced by 1 μmol/L PCB153, 1 μmol/L PCB169 and control groups. However, in 10 μmol/L PCB153 and PCB169 groups, MDA contents were 1.4- and 1.6-fold higher than the basal level. 100 μmol/L PCB153 and PCB169 further increased the MDA contents which were 1.7- and 2.0-fold higher than the control groups. MDA formation was obviously increased with PCBs doses and the enhancement of MDA production may be modulated by a possible increase in ROS induced by PCBs. Gultekin *et al*. (2000) found that erythrocyte lipid peroxidation induced by organophosphate insecticide chlorpyrifos-ethyl was also mediated by increased ROS.

In conclusion, we were able to ascertain that PCBs congers exhibited cytotoxic effects on isolated *C. auratus* lymphocytes due to the induction of apoptosis, and that intracellular ROS and MDA played an important role in PCBs-induced apoptosis. All the above evidence also supported the idea that the apoptosis cytotoxic effects induced by PCBs are correlated with their structural characteristics and the coplanar congener was more cytotoxic than non-coplanar congener. This finding provides a new understanding of PCBs effects on fish lymphocytes. Further studies should be conducted to elucidate the precise mechanism.

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