

Comparative Toxicity of PCBs and PBDEs Using Human Cancer Cell Lines and Zebrafish Embryos

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Abstract: Six polychlorinated biphenyl (PCB) congeners, one technical PCB mixture, and two polybrominated diphenyl ether (PBDE) congeners were studied for their cytotoxicity on four human cancer cell lines and developmental toxicity on zebrafish embryos with or without chorions. All chemicals were tested at four concentrations for exposure time periods ranging from 1 to 6 days. The findings showed no adverse effects on cell growth for any chemicals except that high concentrations of PBDE 209 caused significant inhibition of proliferation in two Human Colon Cancer cell lines, RKO and HCT116, after 3~6 day exposure. PBDE 209 had no effect on Breast Cancer Cells ZR75-1 and MDA-MB-231. In contrast, continuous exposure of zebrafish embryos beginning at 5~6 hour post-fertilization to PCBs and PBDEs for 6 days produced concentration-dependent increases in malformation and mortality. The degree of sensitivity varied with congener and the presence of a chorion. Specifically, PBDE 209 showed no adverse effect on zebrafish embryos with intact chorions while this chemical led to malformation in 43.33% of larvae without chorions at the highest concentration and exposure time period, suggesting chorions act as barriers for high molecular weight chemicals such as PBDE 209. The PCB structure also influenced the embryonic response. For example, PCB 126 induced severe developmental toxicity at $1\mu\text{mol}\cdot\text{L}^{-1}$ in embryos with or without chorions, while PCB 105 produced no observable effects even at $10\mu\text{mol}\cdot\text{L}^{-1}$. The developmental toxicity of these chemicals in descending order is PCB 126 \approx PCB 156 > PCB 1254 (Aroclor1254) > PBDE 47 > PCB 77 > PCB 105 \approx PCB 118 \approx PBDE 209. The findings indicate that the toxic response varies between individual cells and in developing embryos where cellular complexity is very different.

Keywords: PCB; PBDE; human cancer cells; zebrafish embryos; toxicity

1 Introduction

Polychlorinated biphenyls (PCBs) are a class of polychlorinated aromatic hydrocarbons that have been found as ubiquitous contaminants in the environment. Because of their low degree of reactivity, non-flammable, high electrical resistance, good insulating properties, and stability when exposed to heat and pressure, PCBs have been widely used for many different industrial purposes such as dielectric and

heat transfer fluids, plasticizers, wax extenders and flame retardants. Although their production has been ceased since the late 1970s, they are still found throughout the environment as complex mixtures, and in fact they are among the most extensively investigated persistent environmental pollutants (Kimbrough, 1995; Naert *et al.*, 2006). Polybrominated diphenyl ethers (PBDE) are a class of brominated hydrocarbons used as flame retardant additives and have only recently emerged as a major

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environmental pollutant (Siddiqi *et al.*, 2003). Because of their resistance to degradation, their pervasiveness in the environment and human tissues resembles that of PCBs.

PBDEs and PCBs are very similar in their chemical structures, and both can theoretically have 209 congeners. Although the toxicity of PBDEs is not as well understood as that of PCBs, recent studies have shown that these two classes of chemicals are similar in many toxicological aspects such as thyroid dysfunctions (Zhou *et al.*, 2001; Madia *et al.*, 2004; Zoeller, 2005; Eriksson *et al.*, 2006), cytochrome P450 activities (Grinwis *et al.*, 2001; Quabius *et al.*, 2002; Cantón *et al.*, 2006), reproductive harm (Kuriyama *et al.*, 2005; Talsness *et al.*, 2005; Beckett *et al.*, 2008), and Ca signaling pathways (Coburn *et al.*, 2008). PBDEs are still currently in use, and have thus been called “the PCBs of the future” (Siddiqi *et al.*, 2003). There are also concerns that incomplete incineration and fire accidents produce brominated dioxins and furans from PBDEs which could be lethal in extremely low doses (Sakai *et al.*, 2002).

Despite the fact that PCBs and PBDEs have been studied extensively for their toxic effects on various cell lines, tissue and animal models, few studies explored their effects at environmentally relevant concentrations. A recent study with PBDEs on cynomolgus monkey hepatocytes showed no significant induction of CYP1A after 48h of exposure to PBDE 47, 99, 100, 153, 154, 183, and 77 at a low concentration range of 0.01 to 10 $\mu\text{mol} \cdot \text{L}^{-1}$ (Peters *et al.*, 2006). The present study is thus designed to evaluate the comparative toxicity of PCBs and PBDEs at environmentally relevant concentrations on human cancer cell lines and zebrafish embryos. Zebrafish have recently emerged as a popular model vertebrate for investigating chemical toxicity (Hill *et al.*, 2005). Comparative studies between cell lines and living organisms may help elucidate how chemicals exhibit toxicity in these two different systems.

2 Materials and methods

2.1 Tested chemicals

The PCBs selected for this study were 77, 105, 118, 126, 156, and 1254. Among which, PCB126 and 1254 are studied frequently. PCB 105, 118, and 156 are suspected to cause breast cancers (Demersl *et al.*, 2002; Li *et al.*, 2005). PCB 77 exposures produce development toxicity in mice (Wang and Kelow, 2001). PBDEs tested in this study include PBDE 209 and 47, as PBDE 47 is the most abundant PBDE congener detected in environmental monitoring studies (Darnerud *et al.*, 2001; Stapleton *et al.*, 2003) and PBDE 209 is one of the most frequently studied PBDE congener. The eight PCB and PBDE congeners (>99.0% pure) were all purchased from AccuStandard. The technical mixture of Aroclor1254 (hereafter referred to as “PCB 1254”) dissolved in dimethyl sulfoxide (DMSO, Sigma) was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). It consists of 53 PCB congeners and has an average molecular weight of 327. Primary stock solutions of 2 $\text{mmol} \cdot \text{L}^{-1}$ were prepared by dissolving 2 μmoles (e.g., 583.98 μg of PCB 77) solute in 1 mL of DMSO in a glass tube, and stored at -20°C . Secondary stock solutions (200 $\mu\text{mol} \cdot \text{L}^{-1}$) were prepared by dissolving 100 μL primary stock solution in 900 μL DMSO. The third (20 $\mu\text{mol} \cdot \text{L}^{-1}$) and fourth stock solutions (2 $\mu\text{mol} \cdot \text{L}^{-1}$) were prepared in the same way.

2.2 Cell culture

Human colon carcinoma HCT116 and RKO cells, breast carcinoma ZR75-1 and MDA-MB-231 cells were used in this study. The HCT116 cells were cultured in McCoy's 5A medium, MDA-MB-231 cells were cultured in Leibovitz-15 medium, and the other two cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) with high sugar content (4500 $\text{mg} \cdot \text{L}^{-1}$ glucose). All culture media (pH 7.2, Gibco Brl, Life Technologies, Breda, The Netherlands) were supplemented with 10% fetal calf serum (Hangzhou Sijiqin, Hangzhou, China). Cells

were incubated at 37°C in 5% CO₂.

2.3 Exposure of cells

The above stock solutions were diluted 100-fold in culture media to create a series of working solutions at 0.02, 0.2, 2, and 20 μmol · L⁻¹. Cells in logarithmic growth phase were seeded in 96-well plates at a density of 2000 cells per well in 50 μL. Cells were allowed to attach in the well for 2h before adding an equal volume (50 μL) of working solution, yielding final chemical exposure concentrations of 0.01, 0.1, 1, and 10 μmol · L⁻¹ and a solvent concentration of 0.5% v/v. Control wells include medium controls without cells and vehicle controls with 0.5% DMSO. Cells were then cultured at 37°C in 5% CO₂ for 1 to 6 days with replacement of 50 μL fresh medium containing the same chemical concentration at day 3. One plate was used for MTT assay each day.

An MTT assay was used to assess the cell survival and proliferation. In brief, 50 μL MTT reagent (2g · L⁻¹) was added to each well, and cells were incubated for 2~3h at 37°C. The culture medium was then removed, and DMSO (100 μL) was added to each well. After 10min on a shaker at room temperature, the optical density at 550nmol · L⁻¹ of each well was obtained in a plate reader (DIALAB, ELX800G). Cell viability was recorded as the mean optical density (Coburn *et al.*, 2008) for treatment groups or vehicle controls minus the mean OD for medium controls without cells.

2.4 Fish husbandry, embryo collection and dechoriation

Specific pathogen free AB line zebrafish were obtained from Oregon State University, and raised and kept at standard laboratory conditions of 28°C on a 14:10 light/dark photoperiod (Westerfield, 1995) in a recirculating system. The fish were fed three times daily with either the zebrafish diet (Zeigler, Aquatic Habitats, Apopka Florida) or live artemia (Jiahong Feed Co., Tianjin, China). Embryos were collected within half hour post-fertilization

(hpf) from group spawns (male/female ratio of 1:1), and were rinsed with fish water and kept in embryo media (EM) (Westerfield, 1995) at 28°C prior to experiments. For dechorionated embryos, pronase E was used to remove chorions. In detail, 200 embryos at 4.5hpf were exposed to 5mL pronase E solution (0.5g · L⁻¹) for 15min at 28°C. EM was added to stop the treatment, and embryos were then rinsed thoroughly at least 3 or 4 times to remove the pronate. Embryos fall out of their chorions with gentle swishing during the rinse.

2.5 Exposure of zebrafish embryos

Chemical exposure was initiated at 50%-epiboly stage (5~6hpf). Stock solutions were diluted 200-fold in EM to create a series of working solutions at 0.01, 0.1, 1, and 10 μmol · L⁻¹. Experiments were conducted in 6-well plates with 10 embryos per well in 3mL working solution. The control exposures consisted of EM alone or EM containing 0.5% DMSO. The experiment lasted for 6d without EM changes during exposure. Plates were covered with lids to avoid evaporation.

Embryos were examined daily for developmental progress, hatch, mortality, and malformation, and dead embryos were removed to avoid possible adverse effects (e.g., bacterial growth or deterioration of water quality in the EM). Percent hatch was calculated as the number of embryos hatched during the course of the experiment divided by the total number of embryos. The percent mortality was calculated as the number of embryos dead during the course of experiment divided by the total number of embryos. Cessation of heartbeat and circulation were used as end points for mortality. The percent malformation was calculated as the number of embryos having any deformities (including dead animals resulted from malformation) after hatch divided by the total number of embryos. For each experimental run, all treatments were replicated three times (3 wells per treatment), and all experiments were replicated a minimum of two times, thus yielding a minimum of 60 embryos per treatment

group.

2.6 Data analysis

Data were analyzed using one-way analysis of variance (ANOVA) (SAS 9.0, SAS Institute Inc., Cary, NC). When a significant difference ($\alpha = 0.05$) was observed among treatments, Tukey's Honestly Significant Difference Procedure was used for pair-wise comparisons. Results were presented as means \pm SD, and probability values of $p < 0.05$ were considered to be significant. Percentage data for hatch, mortality, and malformation were arcsine-square root transformed prior to analysis.

3 Results

3.1 Exposure of cells

Except for PBDE 209, all tested PCBs and PBDE 47 (up to $10 \mu\text{mol} \cdot \text{L}^{-1}$ for 6d exposure) showed no adverse effects on cell proliferation for all four human cancer cell lines (Fig.1). Though not statistically significant, PCB 126 and PCB 156 at $10 \mu\text{mol} \cdot \text{L}^{-1}$ had lower OD values than control wells (Fig.1). PBDE 209 at $10 \mu\text{mol} \cdot \text{L}^{-1}$ caused significant inhibition of cell proliferation in RKO after 5d exposure and in HCT116 after 3d exposure (Fig.2). PBDE 209 at the concentration of $1 \mu\text{mol} \cdot \text{L}^{-1}$ was

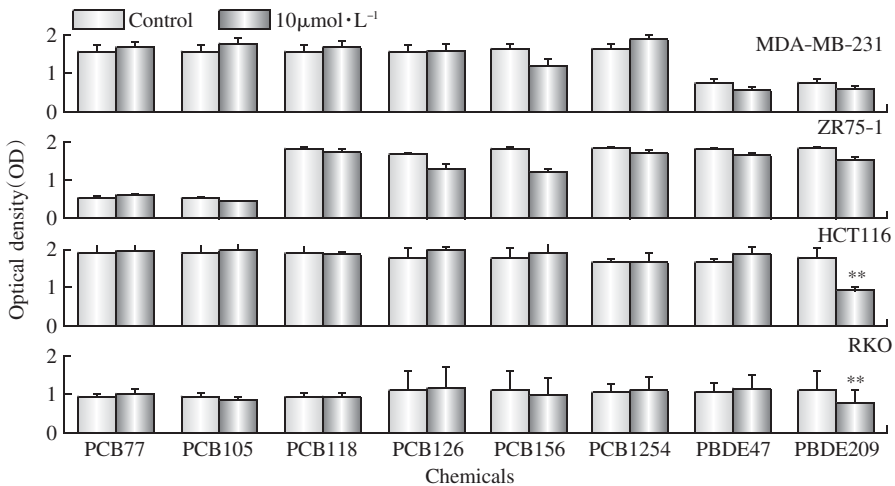


Fig.1 Viability (mean \pm SD) of four human cancer cell lines exposed to PCBs and PBDEs at $10 \mu\text{mol} \cdot \text{L}^{-1}$ for 6 days (Solvent controls contained 0.5% DMSO (v/v) without any chemicals. The mean optical density values were obtained from four replicate wells. *: $p < 0.05$; **: $p < 0.01$)

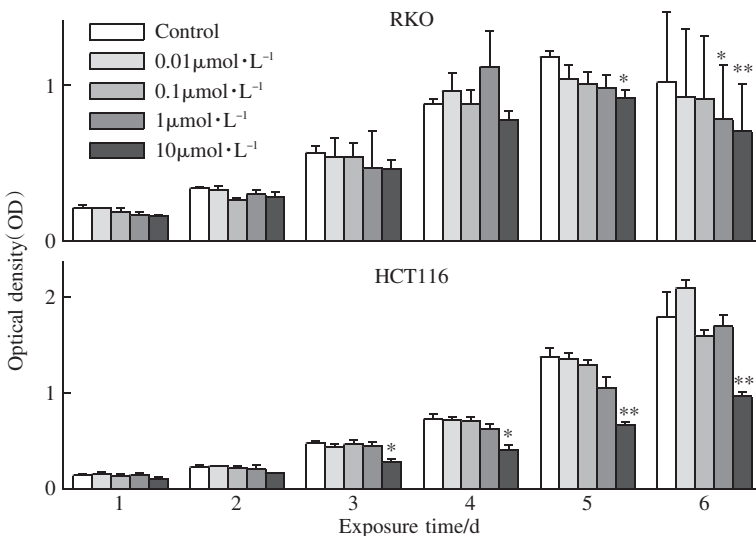


Fig.2 Viability (mean \pm SD) of human cancer cell lines of RKO and HCT 116 after exposed to PBDE 209 at 0.01, 0.1, 1, and $10 \mu\text{mol} \cdot \text{L}^{-1}$ for 1 to 6 days (Solvent controls contained 0.5% DMSO (v/v) without any chemicals. The mean optical density values were obtained from four replicate wells. *: $p < 0.05$; **: $p < 0.01$)

also found to inhibit RKO cell proliferation after 6d exposure (Fig.2). All cell lines cultured in solvent controls with 0.5% DMSO (v/v) did not show any significant difference in OD values from the controls without DMSO for any period of incubation time during the experimental course ($p>0.05$).

3.2 Exposure of zebrafish embryos

Zebrafish embryos exposed to PCBs and PBDEs beginning at 5~6hpf continuously for 6d revealed adverse effects with an increase in the relative number of fish with malformations and mortality in a concentration-dependent manner, but the degree of their sensitivity varied with chemical and also depended on the presence or absence of chorions (Fig.3). For example, PCB 77, PCB 105, and PCB

118 elicited the lowest responses in mortality for embryos with or without chorions and a low rate of malformation in embryos with chorions. PCB 126 and PCB 156; however, elicited the highest mortality and percent malformation in the groups of fish with and without their chorions. PCB 1254 and PBDE 47 had an intermediate effect on embryonic development. PBDE 209 produced a large increase in the percent malformation, but only in dechorionated embryos. All chemicals at 0.01 and 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ had no significant adverse effect on embryonic development. However, removal of chorions resulted in higher percent mortality for PCB 156 and PBDE 47, and higher percent malformation for PCB 77, PCB 118, PCB 1254, PBDE 47, and PBDE 209 at the highest concentration (all $p<0.05$) (Fig.3).

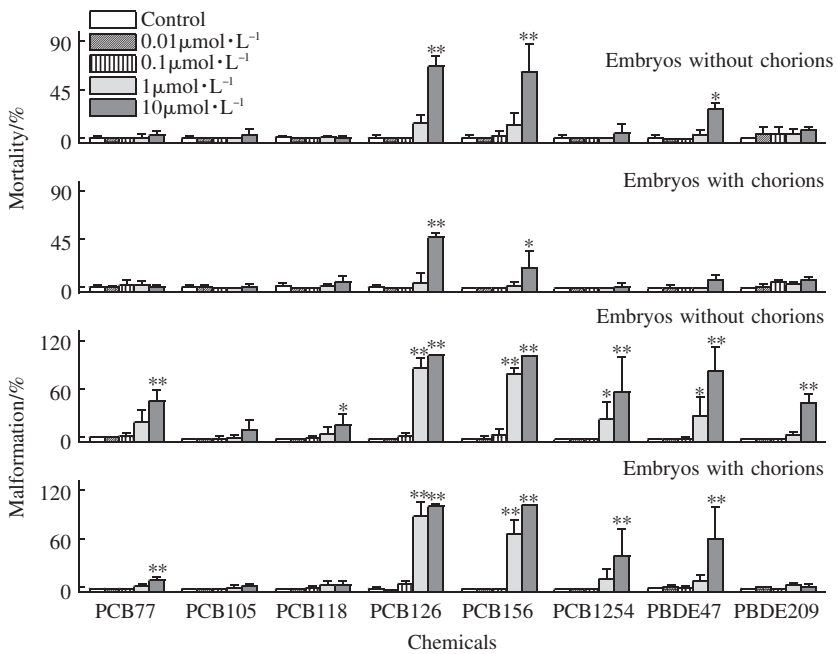


Fig.3 Percent mortality and malformation(mean \pm SD)of zebrafish embryos at 5~6hpf with or without chorions exposed to PCBs and PBDEs at 0.01,0.1,1, and 10 $\mu\text{mol}\cdot\text{L}^{-1}$ for 6 days (The mean values were from three replicate measurements. *: $p<0.05$; **: $p<0.01$)

Exposure time also played a significant role for dechorionated zebrafish embryos exposed to PCB 126, PCB 156, PCB 1254, and PBDE 47 at high concentration of 10 $\mu\text{mol}\cdot\text{L}^{-1}$ (Fig.4). Longer exposure yielded higher percent mortality for PCB 126 and PCB 156, and higher percent malformation for PCB 1254 and PBDE 47.

Percent hatch was not affected by all chemicals within the tested concentration range and exposure

time (data not shown). All embryos exposed to solvent controls with 0.5% DMSO (v/v) developed normally and showed no significant difference in percent hatch, malformation, or mortality as compared to water exposure only controls ($p>0.05$).

Typical abnormal morphology of zebrafish larvae associated with PCB or PBDE exposure includes enlarged pericardial sac (Fig.5B), enlarged yolk sac (Fig.5C, E), and minor or severe spinal deformities

(Fig.5D, E) in comparison to the control fish (Fig. 5A). Zebrafish embryos exposed to PCB 1254 at $10\mu\text{mol}\cdot\text{L}^{-1}$ displayed pericardial edema, retarded development, and spinal deformities (Fig.5D).

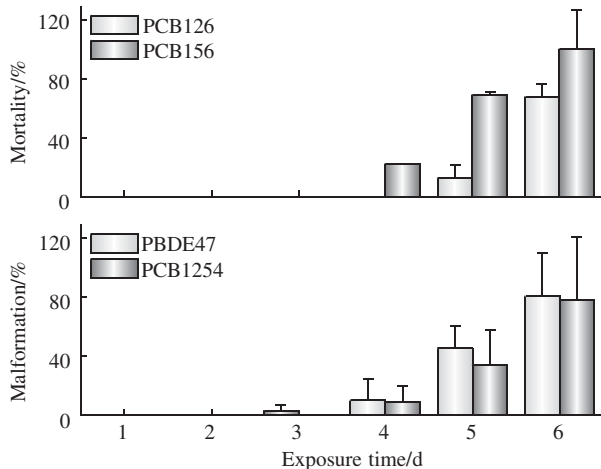


Fig.4 Percent mortality (mean \pm SD) of dechorionated zebrafish embryos at 5~6hpf exposed to PCB 126 and PCB 156 at $10\mu\text{mol}\cdot\text{L}^{-1}$ for 6 days(a), and percent malformation (mean \pm SD) of dechorionated zebrafish embryos at 5~6hpf exposed to PCB 1254 and PBDE 47 at $10\mu\text{mol}\cdot\text{L}^{-1}$ for 6 days(b) (The mean values were from three replicate measurements)

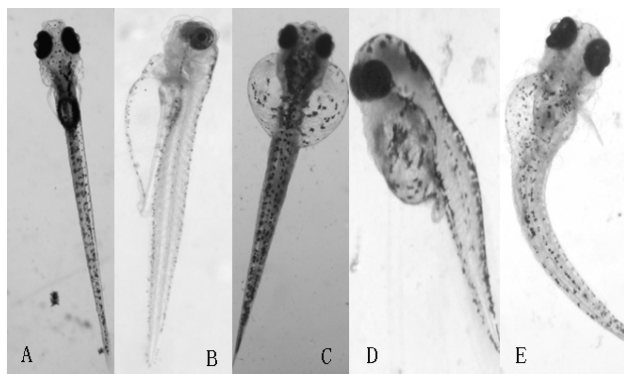


Fig.5 Typical morphology of zebrafish larvae associated with PCB or PBDE exposure: control larva at 144hpf (A), treated larvae with enlarged pericardial sac (B), enlarged yolk sac (C, E), pericardial edema and retarded development (D), and minor or severe spinal deformities (D, E)

4 Discussion

The present study revealed that human cancer cell lines and zebrafish embryos differentially respond to PCBs and PBDEs. Zebrafish embryos are developing organisms with multiple cellular compartments; they are more sensitive to PCB and PBDE toxicity than human cancer cell lines. This sensitivity was further

enhanced when chorions were removed from embryos, suggesting chorions serves as barriers for chemicals such as PBDE 209 which has a high molecular weight. Our findings suggest that the response to chemical exposure is different between individual cells and developing embryos in part because the complexity of embryonic development offers multiple potential targets for these compounds during filtration, differentiation and organogenesis. A recent study evaluating the immune function of juvenile chickens after *in ovo* exposure to PCB 126 showed that PCB 126 decreased the thymus and bursa mass but not their cellularity (number of live thymocytes or bursal cells) at 1.79ng TEQ per egg (Lavoie *et al.*, 2007). Adverse effects of PCBs and PBDEs on immune, nervous, circulatory and reproductive systems have been documented previously (e.g., Darnerud *et al.*, 2001; Van den Heuvel *et al.*, 2001; Naert *et al.*, 2006). Exposure experiments produced a large number of malformations and then outcome in death. Different PCB exhibits different patterns of abnormalities. When the seriousness of abnormalities was high enough, it always turned into death. The experiments extended the exposure time to two weeks would cause all the deformity of zebrafish even under low concentrations outcomed into death. It illustrated that the malformation was caused by chemicals exposure but not naturally occurring. Our observations of adverse effects of PCBs and PBDEs on developing zebrafish embryos further support previous findings on the adverse effects of these chemicals on the development of major organ systems. However, it is possible that the difference in responses between human cancer cell lines and zebrafish embryos to PCBs and PBDEs may also be due to specie differences at the molecular level and future studies involving the use of zebrafish cell lines would help explore such possibilities.

PBDE 209 was the only chemical that exhibited an inhibitory effect on cell proliferation, and this adverse effect was only found for the RKO and HCT116 cells both of which are Colon Cancer Cells, suggesting that sensitivity varies with cell

lines. The public health concern of PBDEs has been mainly focused on less brominated PBDEs such as tetra- or penta- because of their high affinity for lipids, and thus high bioaccumulation in animals and humans (Covaci *et al.*, 2003). Because of this, penta- and octa- brominated congener mixtures have been banned in Europe and production has stopped voluntarily in the United States. In comparison, the deca- brominated congener (PBDE 209) is of less concern because it has low bioaccumulative and biological activities, and is still currently in use in the United States. Nevertheless, the main public health concern of the deca- brominated congener is possible degradation to less brominated and more toxic congeners in the environment after their release (Watanabe and Sakai, 2003). However, the present study showed direct evidence for the adverse effect of PBDE 209 on human cancer cell lines and dechorionated zebrafish embryos. Our findings also agree with recent study on human hepatoma cells Hep G2 where PBDE 209 at $10 \sim 100 \mu\text{mol} \cdot \text{L}^{-1}$ caused growth inhibitory effects through ROS- induced cellular apoptosis (Hu *et al.*, 2007). As demonstrated in the present study, PBDE 209 produced no adverse effects on zebrafish embryos with chorions while exposure to PBDE 49 at the highest concentration and longest exposure time led to malformations in 80% of larvae. This suggests that zebrafish chorions can be readily penetrated by PBDE 49, but not PBDE 209, while both chemicals can exert adverse effects on developing embryos if such barrier were removed. As noted above, the high molecular weight of PBDE 209 may prevent its entry through the chorion.

The toxicity of PCBs depends on the congener structures. In general, a large number of chlorines present on the molecule together with the absence of ortho carbons (2 or 2' and/or 6 or 6') renders a congener to be particularly toxic and bioaccumulative. In the present study, the developmental toxicity of PCBs on zebrafish embryos confirmed the trend of PCBs toxicity in descending order as $126 \approx 156 > 1254 > 77 > 105 \approx 118$. Notably, PCB 105 has the

same number of chlorines as that of PCB 126, however, PCB 105 contains ortho carbons while PCB 126 does not. PCB 126 caused a significant increase in the percent mortality and malformation at concentrations as low as $1 \mu\text{mol} \cdot \text{L}^{-1}$ for embryos with and without chorions, while PCB 105 showed no observable effect even at $10 \mu\text{mol} \cdot \text{L}^{-1}$. These congener structure specific effects have been previously identified by Messeri *et al.* (1997) where PCB 3,3', 4,4' caused a decrease in K^+ -stimulated catecholamine content and a rapid increase of self-generated catecholamine content in chromaffin cells, and PCB 2,2', 4,4' had no effect on catecholamine content and release. It is worth noting that PCB 126 and 156 inhibited ZR75-1 growth though not statistically significant. This implies that these two PCBs may have anti-estrogen activity as ZR75-1 is estrogen-dependent. The lack of observable or significant effect on cell proliferation for all tested PCBs on all four human cancer cell lines may indicate that concentrations of $0.01 \sim 10 \mu\text{mol} \cdot \text{L}^{-1}$ PCBs are well below the cytotoxicity threshold for individual cultured cells. This observation also suggests that these types of cell lines may not be appropriate models for the assessment of the impact of environmental chemicals on the vitality of multicellular organisms.

Although production of PCBs has been discontinued for nearly four decades and efforts have also ceased or at least decreased the usage of various PBDEs, these persistent organic pollutants will remain in the environment for years to come without any significant degradation. Their presence in various environmental matrices at relatively low concentrations for a long duration could result in adverse consequences for various ecosystems as well as human health. Sensitive molecular biomarkers that facilitate high throughput screening of these organic pollutants at environmentally relevant concentrations (generally very low) will help us better regulate these chemicals and also help prevent new chemicals from being widely used before they pose adverse health effects to the public. Future research efforts

may not only use zebrafish to identify endpoints of toxicity and elucidate the mechanisms, but also to develop sensitive molecular biomarkers for large-scale and high throughput chemical screening.

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PCBs 和 PBDEs 对人类癌细胞和斑马鱼胚胎的毒性对比

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摘要: 研究了 6 种多氯联苯 (PCBs) 3,3',4,4'-四氯联苯 (PCB 77)、2,3,3',4,4'-五氯联苯 (PCB 105)、2,3',4,4', 5-五氯联苯 (PCB 118)、3,3',4,4',5-五氯联苯 (PCB 126)、2,3,3',4,4',5-六氯联苯 (PCB 156) 和商业型混合多氯联苯 Aroclor 1254, 两种多溴联苯醚 (PBDEs) 2,2',4,4'-四溴二苯醚 (PBDE 47)、十溴二苯醚 (PBDE 209) 对人类癌细胞生长和斑马鱼脱膜与不脱膜胚胎发育的影响。8 种化合物均使用 0.01、0.1、1.0、10 $\mu\text{mol}\cdot\text{L}^{-1}$ 4 个浓度进行 1~6d 的暴露实验。结果表明, PBDE 209 在最高浓度 10 $\mu\text{mol}\cdot\text{L}^{-1}$ 下对结肠癌细胞 HCT116 (暴露 3d 后) 和 RKO (暴露 5d 后) 具有显著的生长抑制作用, 所有化合物均对乳腺癌细胞没有显著影响。相比之下, 化合物对受精后 5~6h (5~6hpf) 的斑马鱼胚胎的毒性效应显得比较明显, 而各化合物对胚胎的致畸和致死效应又不相同, 其毒性强弱依次为 PCB 126 \approx PCB 156 > PCB 1254 (Aroclor 1254) > PBDE 47 > PCB 77 > PCB 105 \approx PCB 118 \approx PBDE 209。其中 PBDE 209 在未脱膜暴毒后均无致畸与致死现象, 脱膜暴毒后最高浓度才表现出显著意义的致畸作用, 而 PBDE 47 在最高浓度下可产生高达 80% 的致畸率, 这说明胚胎绒毛膜具有有效阻挡大分子物质如 PBDE 209 进入的作用。PCBs 的毒性效应与其空间结构密切相关。如 PCB 126 和 PCB 105 具有相同的分子式, 前者在 1 $\mu\text{mol}\cdot\text{L}^{-1}$ 下就引起了显著的致死和致畸效应, 而后者即使在 10 $\mu\text{mol}\cdot\text{L}^{-1}$ 下也没有显著的效应。实验结果也说明不同类型的实验对象所展示的毒性效应并不相同, 化合物对体外培养的细胞和发育中的胚胎具有不同的影响。

关键词: 多氯联苯; 多溴二苯醚; 人类癌细胞; 斑马鱼胚胎; 毒性