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Short communication

Detection of water toxicity using cytochrome P450 transgenic zebrafish as live biosensor: For polychlorinated biphenyls toxicity

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ABSTRACT

Cytochrome P450 (CYPs) is significant in degradation of endogenous substrates and detoxification of carcinogens, therefore it is a biomarker for assessment of polycyclic aromatic hydrocarbons (PAHs) level in aquatic environment. In the present study, a transgenic line of zebrafish had been generated using a CYP-green fluorescence protein (CYP-GFP) construct, driven by CYP1A1 promoter. Polychlorinated biphenyls (PCBs) were used as toxicant, in concentrations of 0.02 μg/ml, 0.04 μg/ml, 0.08 μg/ml, 0.4 μg/ml, and 0.8 μg/ml. The transgenic control fish showed low intensity of fluorescence in the liver. After exposed to PCBs, zebrafish had morphological changes such as expansion of yolk, contortion of tails and inflation of pericardial area. Green fluorescence signals were found to express according to concentrations and time. The green fluorescence signal was most intense after treatment with 0.08 μg/ml PCBs. However, the maximum area of green fluorescent signal was found at 0.04 μg/ml PCBs. GFP started to express at 3 h exposure to PCBs, increasing its intensity until 6 h exposure, and then level off. Since the GFP expression is fast responding and is sensitive to low PAHs concentrations, transgenic fish is a good tool for live imaging and monitoring of aquatic contamination.

1. Introduction

Polychlorinated biphenyls (PCBs) are one of the polycyclic aromatic hydrocarbons (PAHs). They are industrial wastes from heat transfer fluids, hydraulic lubricants, dielectric fluids for transformers and capacitors, plasticizers, wax extenders, adhesives, organic diluents, dedusting agents, pesticide extenders, cutting oils, carbonless reproducing paper and flame retardants, which may cause toxicity to human by bioaccumulation (Safe, 1993). Coplanar PCBs are aromatic hydrocarbon receptor (AHR) ligands, which exhibit similar toxic effects as 2,3,7,8-tetrachlorodibenzodioxin (TCDD). The major exposure route of human is consumption of contaminated aquatic organisms.

CYP1A1 is also an important enzyme for detoxification of exogenous substrates. It also metabolizes many compounds that are important regulators of development (Henriksen et al., 2000; Navas and Segner, 2000; Wu et al., 2001). CYP1A1, CYP1A2, and CYP1B1 genes are regulated by the aromatic hydrocarbon receptor.

Normally CYP1A1 expression is negligible. However, when PAHs are present to induce the expression, high levels of mRNA, protein and enzyme activities could be detected. Many of the PAHs are being metabolized (Nebert et al., 2004). The suggested mechanism of CYP1A1 responses to PCBs is that PCBs bind as ligands to AHR. AHR then translocates to nucleus to dimerize with aryl-hydrocarbon receptor nuclear translocator (ARNT) (Hoffman et al., 1991; Reyes et al., 1992). The AHR/ARNT complex binds to a DNA enhancer sequences known as xenobiotic response elements (Gonzalez and Nebert, 1985; Fujisawa-Sehara et al., 1986) and propagate the induction signal to the promoter region, hence the CYP1A1 gene expression.

Zebrafishes are widely used in the transgenic technique with GFP (Higashijima et al., 2000; Motoike et al., 2000; Kobayashi et al., 2001; Mattingly et al., 2001). The advantages of generating transgenic zebrafishes are that the genome project is nearly finished and the rather simple systems has been successful in transgenic technique as shown by many studies. The transgenic zebrafishes do not only allow the real time monitor on the water condition, but also the dynamics of organs and systems in responses to toxins, as well as studies on zebrafish development (Teraoka et al., 2003a).

Using zebrafish for PAHs screening is a widespread technique. Since zebrafishes are easy to be manipulated, it is used for studies in the aspects of binding to AHR (Abnet et al., 1999; Mattingly et al., 2001), induction of CYP (Henry et al., 1997), reproduction (Orn et al., 1998,) and bioaccumulation (Andersson et al., 2001).

CYP1A1 induction is a common biomarker for exposure to contaminants particularly PAHs. Many studies have employed bioassay

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to measure the ethoxyresorufin O-deethylase (EROD) activity. However, EROD has the limitations that it cannot locate the induction of CYP1A1 in animals as well as evaluate the development of toxic response (Collodi et al., 1994; Köhler et al., 1992). The use of reporter construct is an alternative to overcome these limitations.

In present studies, CYP1A1 promoter region was fused to reporter gene, GFP, to evaluate the PCB toxic response. The expressions of GFP start with PCB binding as a ligand to the AHR and propagate the induction signal to the promoter region. Subsequently, GFP expresses. In this study, we found that the GFP expressions are fast responding and are sensitive to low PAHs concentrations, which make the transgenic fish a good tool for live imaging and monitoring of aquatic contamination.

2. Materials and methods

2.1. Zebrafish embryos collection

Adult zebrafish was purchased at local aqua shop and maintained according to the zebrafish book (Westerfield, 2000). In brief, zebrafish was kept at 28 °C aquarium water. Male and female fish was allowed to breed after 14:10 night/dark cycle. Equal numbers of male and female were inhabited in tank. An egg collecting box with artificial water plant was placed in tank when the light is on. Eggs were collected within 1 h later for microinjection. Then the embryos were raised in distilled water in petri dish.

2.2. CYP-GFP plasmid construction and microinjection

A 333 bp zebrafishes CYP 1A promoter synthesized (Techdragon Limited) according to CYP 1A promoter sequence (accession EF419300) was cloned into the commercial pZsGreen1-1 Vector (Clonetech #632473). CYP promoter and pZsGreen1-1 Vector were cut by XhoI and BamHI (New England Biolabs Inc.) and ligated by T4 DNA ligase (Promega). The CYP-GFP plasmid was injected into one-cell stage embryos using microinjector (Harvard Apparatus) under stereomicroscope (Olympus).

2.3. Immunohistochemistry

Whole-mount staining was performed according to previous studies (Westerfield, 2000). The primary antibody is Mouse monoclonal anti-Cytochrome P450 1A1 + 1A2 (Abcam, 1:1000) for CYP1A1 activity and the secondary antibody is Alexa 488 anti-mouse. The fish were mounted on glass slide for observation under microscope.

2.4. PCB treatment

PCB Congener Mix (Perkin Elmer) was used. The concentration of investigation of CYP1A1 activities is 0.02 μg/ml, 0.04 μg/ml, 0.08 μg/ml, 0.4 μg/ml, 0.8 μg/ml PCB. The experimental fish were treated after 7 days of microinjection. The control group of fish was maintained in double distilled water and the treatment group was maintained in PCB solution for 1 day, and then images were captured.

Fish in treatment were placed in confocal culture dish. Images were taken by Olympus FV 1000 Confocal Scanning Microscope with 488 nm excitation and stereomicroscope. Images were analyzed by image analysis software MetaMorph.

3. Results

3.1. Development of zebrafish embryos after transfection

At 1 day post fertilization (dpf), the microinjected zebrafishes was found to be elongated. Head and tail could be distinguished. No organ could be identified yet at this stage. At 2 dpf, pigment started to develop. Eyes could be identified. Head–trunk angle was increasing. Yolk began to shrink. Yolk extension could be observed. Liver could not be distinguished yet. At 3 dpf, zebrafishes were ready to hatch in general. Pigments were observed at the ventral side and on the yolk ball. In the transfected embryos, green fluorescence was seen to scatter on the anterior end of yolk ball. At 4 dpf, yolk kept on shrinking. Green fluorescence was becoming concentrated within the fish bodies (data shown in supplementary information).

At 5 and 6 dpf, the green fluorescence was seen to concentrate in the anterior part of yolk ball and along the intestinal tract. The triangular shape of liver which contained higher levels of fluorescence could be recognized (data shown in supplementary information).

3.2. Immunohistochemistry

Immunohistochemistry using CYP1A1 antibody was employed as control to show the localization of CYP1A1 protein with 0.8 μg/ml PCB treatment. The expression is condensed in elongated shape. Control showing triangular CYP1A1 distribution (data shown in supplementary information).

3.3. CYP1A1-GFP expressions in response to PCB

After zebrafishes were microinjected with CYP-GFP plasmids, the green fluorescence signals were assessed after exposures to PCBs. Digital images of 7 dpf fish from confocal microscope (Fig. 1) and stereomicroscope (Fig. 2) were taken after 1 day exposure to PCBs. Most of the GFP signals were found in liver. The control group (Fig. 2A) showed fluorescence signals in liver increased with the concentrations of PCB. The green fluorescence signal was most intense after treatment with 0.08 μg/ml PCB as seen in the images (Fig. 2D and Fig. 2G) and in the graph (Fig. 3A). The expression of GFP was then lowered when the concentration increased to 0.4 μg/ml (Fig. 2E) and 0.8 μg/ml (Fig. 2F). As shown in Fig. 3A, the intensity of the GFP expression was represented by average gray value, which was quantified by Metamorph software. The trend of intensity was found to change according to the concentrations of PCB . This was consistent with the findings by observing the images. Both quantitative analysis and imaging showed that the trend of the CYP-GFP expression increased significantly from control to 0.08 μg/ml of PCB. The average gray value increase 110% (control value: 20.44 ± 1.30, 0.08 μg/ml PCB: 43.02 ± 0.14). However, treatments with higher concentrations of PCBs 0.4 and 0.8 μg/ml resulted in reductions of GFP signals. A dose response relationship is observed between the CYP1A1 expressions with PCB exposures within a limited range of PCBs.

In addition, the area of GFP signals which indicate the subregions of the livers that responded to the PCBs, also changed according to the concentrations. The area expressing CYP-GFP signals was considered to be correlated with the number of liver cells expressing CYP-GFP. The location of liver was found between the heart and yolk sac. The shape of CYP expression of control group (Fig. 2A) is triangular shape, which is the normal shape of liver (Bryson-Richardson et al., 2007). Only part of the liver was expressing CYP-GFP, suggested that CYP1A expressions remained low. After treating with 0.02 μg/ml PCB, the GFP-expressing area in the liver was greatly expanded (Fig. 2B). The shape was still remained triangular. These data suggests that most of the cells in liver are expressing CYP1A in response to PCB. However, when the concentrations of PCB

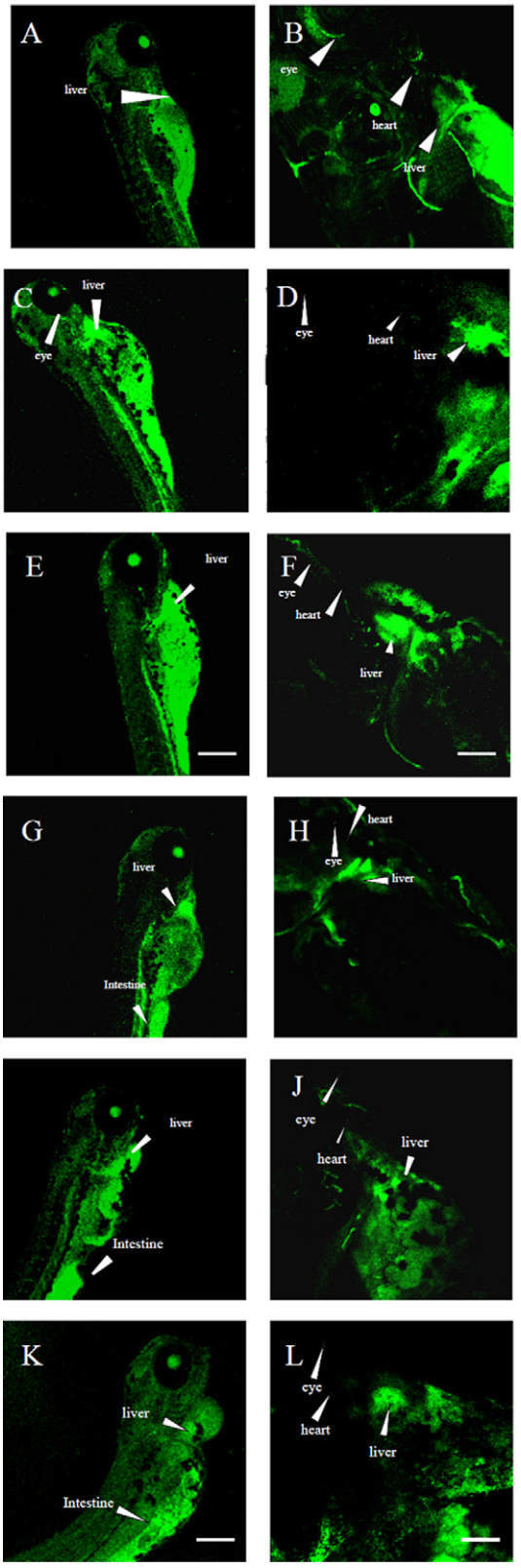

Fig. 1. PCB induced GFP expression under confocal microscope. (A, B) Control fish showing triangular GFP expression, (C–F) 0.02 μg/ml PCB and 0.04 μg/ml PCB showing robust GFP expression in liver, (G, H) 0.08 μg/ml PCB, GFP in liver concentrated as irregular shape. (I, J) 0.4 μg/ml PCB, (K, L) 0.8 μg/ml PCB, showing condensed GFP as dot. GFP showed also in intestines. Scale bar for A, C, E, G, I, K: 25 μm. Scale bar for B, D, F, H, J, L: 50 μm. Arrow showing liver.

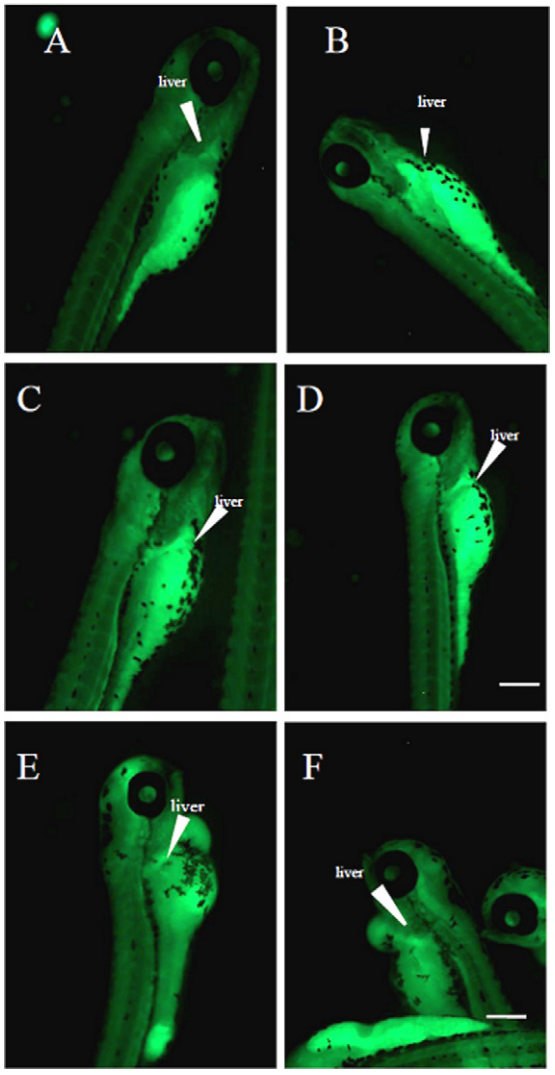

Fig. 2. GFP expression induced by PCB, captured by stereomicroscope. (A) Control fish B, 0.02 μg/ml PCB, showing robust GFP expression in liver. (C, D) 0.04 μg/ml PCB and 0.08 μg/ml PCB, GFP in liver concentrated as irregular shape. (E, F) 0.4 μg/ml PCB, 0.8 μg/ml PCB, showing contortion of tail, pericardial edema, GFP expression in liver further condensed. GFP is also shown in intestines. Scale bar: 300 μm.

further increased to 0.04 μg/ml and 0.08 μg/ml respectively, the GFP expressing areas of livers seems to consolidate into an irregular shape. After treatment with 0.8 μg/ml PCB, the area with the GFP signals was found to minimize to a circular dot (Fig. 1K and L). After treatments with 0.4 and 0.8 μg/ml PCB, GFP expression was also found in the intestinal bulb and other parts of intestine.

The relationship between areas of GFP expression in the liver and concentrations of PCB was analyzed (Fig. 3B). The area of GFP in control group was employed as the baseline level of expression. A significant increase in the area was found after 0.02 μg/ml PCB treatment. The trend then declined steadily from 0.04 μg/ml to 0.4 μg/ml PCB treatments. However, the intensities of GFP were still significantly higher than the control. The area of GFP expression further decreased after treatments with 0.8 μg/ml PCB. The area was found to drop from $3996 \pm 427\ \mu m^2$ (control) to $1852 \pm 324\ \mu m$ (0.8 μg/ml PCB). These results suggested that exposure of high level of PCB might cause damages in the cell or depletion of CYP1A1 expression. At 0.4 and 0.8 μg/ml PCB, GFP expression is also found in the intestinal bulb and other part of intestine.

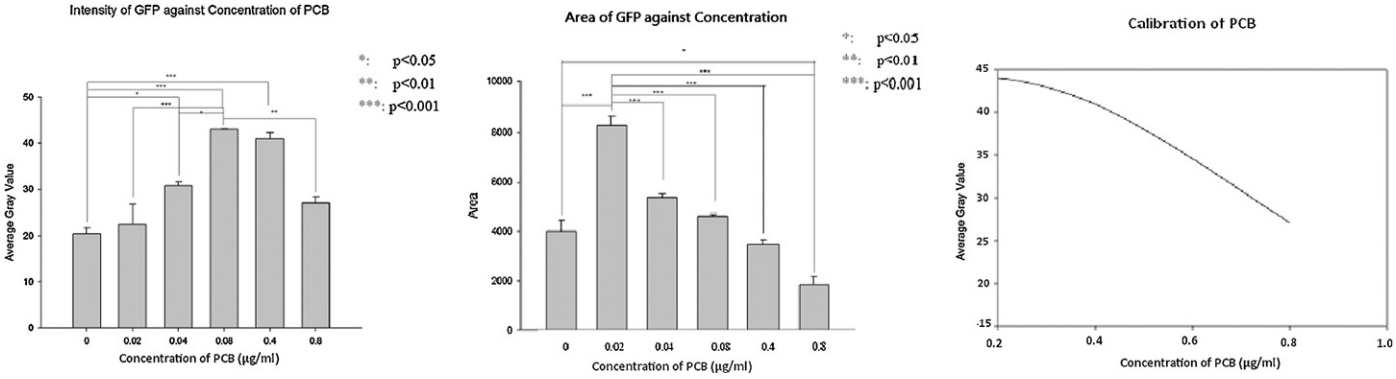

Fig. 3. (A) The graph of GFP intensity against concentration of PCB. GFP intensity is quantified by imaging analysis software Metamorph. Statistical analysis revealed there were significant changes. $N = 5$ each group. Relative standard deviation for 0.00 μg/ml PCB: 11.05%, 0.02 μg/ml PCB: 33.48%, 0.04 μg/ml PCB: 4.62%, 0.08 μg/ml PCB: 0.56%, 0.4 μg/ml PCB: 5.68%, 0.8 μg/ml PCB: 8.40% (B). The graph of area of GFP against concentration of PCB. Area of GFP is quantified by imaging analysis software Metamorph. Statistical analysis revealed there were significant changes. $N = 5$ each group. Relative standard deviation for 0.00 μg/ml PCB: 18.54%, 0.02 μg/ml PCB: 7.93%, 0.04 μg/ml PCB: 4.21%, 0.08 μg/ml PCB: 2.60%, 0.4 μg/ml PCB: 11.33%, 0.8 μg/ml PCB: 30.34%. (C) Calibration curve of PCB.

3.4. Morphological changes of transgenic zebrafish after exposure to PCBs

When the transgenic zebrafishes were exposed to different concentrations of PCB, morphological changes were observed as an important indicator of toxic responses. In the control group of zebrafishes which were treated with double distilled water, most of the fishes could keep their positions horizontally and in balance when at rest (Data shown in supplementary information). When transgenic zebrafishes were exposed to 0.02 μg/ml (Data shown in supplementary information), 0.04 μg/ml (Data shown in supplementary information) and 0.08 μg/ml (Data shown in supplementary information) for 1 day, there were an increasing number of fishes that were lying on their lateral side. When compared to the control fishes (Data shown in supplementary information), the PCB-treated fishes had their morphology unchanged which could still stay straight from head to tails (Data shown in supplementary information). When the concentrations of PCBs increased ten fold to 0.4 μg/ml (Data shown in supplementary information), all of the exposed fishes were seen lying on their lateral side. These fishes lying on either lateral side could not flip over. The yolk sac expanded and their tails were contorted. At concentration of 0.8 μg/ml PCB (Data shown in supplementary information), the situation was becoming severe. The yolk sac swelled and the tails were heavily contorted.

These morphological changes highly affected the locomotion of the fishes. The control zebrafishes exhibited normal swimming patterns: swimming straight forward with smooth movements. When the concentrations of PCBs increased to 0.02 μg/ml, 0.04 μg/ml and 0.08 μg/ml respectively, the zebrafishes were seen to lose their balance and they had increasing difficulties in swimming. They could not maintain the horizontal and balance position during swimming, which made them hard to swim straight forward. At 0.4 μg/ml PCB, the motility of the fish greatly decreased. The tails were totally contorted so that the fishes could only swim in circular patterns. At 0.8 μg/ml PCB, the fish lose their motility and could hardly swim. The morphological changes and behaviors in the PCB exposed zebrafishes displayed a dose-response patterns, namely in the contortion of tails and motility. The contortion of tails increased as the concentration of PCB increased. The decreased motility of fish was believed to be caused by the increase of contortion of tails. It is also observed that in Fig. 2, the area between heart and trunk known as pericardial area increased the volumes after treatments with higher concentrations of PCB (0.4 and 0.8 μg/ml). The area became inflated.

3.5. Time course of toxic responses of PCBs in transgenic zebrafish

One advantage of using GFP is the live and real time monitoring of the toxic responses. 7 dpf zebrafishes were captured at every hour during the exposure of 0.8 μg/ml PCB (Data shown in supplementary information). The zebrafishes did not show any sign of GFP expression until 3 h of exposure (Data shown in supplementary information). It was thought that the CYP1A system took 3 h for PCB toxic response to occur.

The GFP fluorescence signals grew stronger at 4 h and the signals occurred in a circular dot in the liver (Data shown in supplementary information). These indicated that CYP1A expression was upregulated while PCB induced damage to liver and CYP1A1 system. After 5–6 h of exposure (Data shown in supplementary information), the circular dot further reduced in size with expression of robust green fluorescence. After 20 h of exposure, no further change in the levels and patterns of expression of the green fluorescence signals was detected when compared with those of 5–6 h exposure. These data indicated that the toxic responses of PCBs in the fish livers were at maximum after 5–6 h exposure. Similarly, changes in fluorescence intensity of the GFP expression with time was represented by average gray value, which was quantified by Metamorph software. The trend of change according to time is displayed in Fig. 6. Quantitative analyses showed that the trend that the GFP signals started to occur after 3–4 h of exposure to PCB. At 5 h of exposure, the intensity of GFP expression started to level off and reached the maximum. At 20 h of exposure of PCBs, the intensity remained at the same level as that of 5 h exposure, showing that the toxic responses of PCBs in fish livers was developed between 0 and 5 h after exposure.

4. Discussion

4.1. CYP1A1 as biomarker for water contamination

It has been suggested that the lethal concentration (LD 50) of zebrafish towards PCB 28, PCB 126 and PCB 153 are 3, 1 and 5 ppm respectively (Sişman and Geyikoğlu, 2007). In our studies, 0.02, 0.04, 0.08, 0.4 and 0.8 μg/ml (ppm) of PCB was used and GFP could be detected at concentration as low as 0.02 μg/ml PCB. It implies that the transgenic zebrafish model is sensitive to PCBs level much lower than LD 50. Although WHO have not suggested international standard for PCBs, several national governments has suggested their own tolerable daily intakes (TDI) (WHO, 2000). In particular, the TDI suggested by Germany is 1–3 μg/kg body weight, that is a maximum of 300 μg PCB for a 100 kg person. The zebrafish model

in our study is capable to detect level much lower than the TDI of Germany. Since consumption of aquatic organisms is the major exposure route of human, monitoring water contamination is necessary. Transgenic zebrafish model is a good way to detect harmful contaminants level in fresh water.

A dose–response relationship from 0.2 to 0.8 μg/ml is obtained in present study (Fig. 3C). This helps in distinguishing the contaminants concentration, such as PCB concentration. The dose-response is mediated by the binding of ligands to AH receptor. Therefore, CYP1A promoter can be used to screen for other AHR binding ligands, especially PAHs in water (Operaña et al., 2007).

4.2. Comparison of live imaging with other assays on liver

In our study, CYP1A1 promoter region fused to reporter gene, GFP. The expression of GFP starts with PCB binding as a ligand to the AHR. CYP-GFP can be used for screening other PAHs present in water as they are also ligands of AHR.

Many studies have employed the bioassay to measure the ethoxyresorufin-O-deethylase (EROD) activity, which is used to estimate the toxicological potency of AHR ligands (Orn et al., 1998; Jones et al., 2010; Troxel et al., 1997). Both EROD activities and GFP expression in liver reflect CYP1A1 activities. Though EROD is a popular assay for CYP1A activities, EROD has the limitations. It involves the quantification of the enzymes fluorimetric determination of 7-ethoxyresorufin-O-deethylase, which is synthesized during CYP1A upregulation (Janosek et al., 2006). CYP-GFP is a more direct method, by which CYP gene expression and protein synthesis can be quantified. While EROD cannot locate the induction of CYP1A1 in animals as well as evaluate the development of toxic response, CYP-GFP expressed in liver as well as in intestinal bulb in our findings (Fig. 1). It has been reported that EROD activity could not be detected until posthatching and in adult zebrafish towards one of the PCBs, Aroclor 1254, which implies that EROD can only be used in larva fishes (Jones et al., 2010; Troxel et al., 1997). However, there are debates that methanol, the carrier of PCBs, can caused inhibitory effects on PCBs (Jones et al., 2010).

Studies also took measurement on liver weight (Orn et al., 1998; Grinwis et al., 2001). By using liver weight assessment, liver enlargement can be assessed, but CYP1A1 activities cannot be evaluated. Both EROD and liver weight assessment involve sacrifices of the experimental animals, while in CYP-GFP monitoring, killing of fish is not necessary. The fish was simply put under microscope for continuous real time monitoring of GFP expression.

Other than cellular methods, gene expression are good approaches to evaluate CYP1A activities, for they can assess interactions with downstream components in pathway (Janosek et al., 2006).

Though few studies worked on the relationship between CYP1A1 and PCBs, many of them concentrated on CYP1A1 and other PAHs, such as TCDD (Tanguay et al., 1999; Mattingly and Toscano, 2001; Teraoka et al., 2003b). *In situ* hybridization were common to evaluate CYP1A1 expression in these studies. The overall trends of CYP1A1 expression in response to PAHs are consistent, although PCBs are less potent than TCDD. However, one of the studies showed that the CYP1A1 mRNA expression is lower in adult fish than in larva fish (Andreasen et al., 2002a).

It is found that the area of GFP expression decreases to irregular shape as PCB concentration increases. This observation coincides with CYP1A1 expression pattern in immunochemistry. Immunochemistry is used for detecting protein in tissue, while CYP-GFP is the detection of gene expression (Andreasen et al., 2002b). The advantage of GFP over immunochemistry is that immunochemistry is not a live imaging, that continuous monitoring particularly in toxicology on a sample is not allowed.

Studies using CYP1A1 mRNA and protein expression is consistent with imaging method and real time PCR (Mattingly and Toscano, 2001; Tanguay et al., 1999). However, one of the studies reported that using Northern and Western analysis is insensitive. Real time PCR and whole mount immunofluorescence were employed as well to detect unknown increase in CYP1A1 expression (Andreasen et al., 2002a).

4.3. Toxic responses of PCBs

Typical *in vivo* toxic responses of PCB are found to include yolk sac edema, vertebra defect, craniofacial malformation such as double head, triple retina, anaxial body, inhibition of swim bladder inflation and motality (Sişman and Geyikoğlu, 2007). The present results indicate PCBs trigger similar toxic responses including anaxial body shown by contortion of tail, pericardial edema and mortality. These observations are consistent to previous reports. The effects are found to be concentration dependent. Previous studies have confirmed that PCB exerts concentration-dependent CYP1A1 expression, by mortality, EROD activities, liver and ovary somatic indices (Orn et al., 1998; Sişman and Geyikoğlu, 2007).

The area of GFP expressing cell were found to be the highest at 0.02 μg/ml, which believed to be the highest tolerable level of zebrafishes liver towards PCB. The area decreased. It is believed that the GFP expressed in response to the increase of PCB concentration, with the reduction of area that might because of liver degeneration due to metabolic stress and toxic effects (Orn et al., 1998). Liver necrosis and liver degeneration might occur higher than 0.02 μg/ml. The significant increase of area at 0.02 μg/ml, however, might be due to the liver enlargement. Liver enlargement has also been observed in birds (Hoffman et al., 1996) and rats (Van Birgelen et al., 1994). The PCBs effect on zebrafish CYP is also found to be time dependent. The intensity increased from 3 h of exposure to maximum at 5 h. Then the intensity leveled off with slight decrease. It was reported that the CYP1A1 activities reduced after increasing the exposure from 4 weeks to 13 weeks, therefore the GFP expression is expected to drop for longer exposure (Orn et al., 1998).

4.4. GFP expression driven by CYP in zebrafishes and mammals

Apart from zebrafish, there are studies on the relationship of PAHs and CYP1A1 expression in mammals. Some studies employed fluorescence imaging system, flow cytometry, western blot, northern blot and EROD on CYP1A1 expression in mammals and mammalian cells (Chaloupka et al., 1995; Chramostová et al., 2004; Operaña et al., 2007). One of the studies tranfects hepatocytes with GFP driven by human CYP1A1 (CYP1A1GFP) (Operaña et al., 2007) and successfully generate transgenic mouse. CYP1A1 is a good biomarker in monitoring environmental toxicants, either in mammals or in fish. CYP1A1-GFP in zebrafishes are even a more convenient model in quantifying toxicants because their carriage to the field is easy and they can be simply put into water samples instead of oral administration of toxicants. With no complicated instruments, the GFP in zebrafish could be detected.

5. Conclusion

In conclusion, the present data implicated that CYP is a useful target for gene transfection in zebrafish model. The production of CYP-GFP transgenic zebrafish is a useful method in generating a live specimen for detection of PCB pollutants in freshwater and the response time is 7 days. The present findings have strong implications in development of transgenic animals as live biosensors.

The present findings also imply a novel direction in applications of biomaterials.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.10.004.

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