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Responses of Various Biomarkers in Common Carp (*Cyprinus carpio*) Exposed to Benzo[*k*]fluoranthene

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Polycyclic aromatic hydrocarbons (PAHs) derived from leakage of fossil fuels and incomplete combustion of organic materials have been considered as harmful contaminants in environments. This study evaluated the effect of benzo[*k*]fluoranthene (BkF), one of the PAHs, using the multiple biomarkers and applied the integration model with those biomarker responses. After 10 days of the exposure at the measured concentrations of BkF (6, 25, and 45 μg L^{-1}), the changes of the four biomarkers, that is, 7-ethoxyresorufin-O-deethylase (EROD), DNA single-strand breaks (Comet), acetylcholinesterase (AChE) and vitellogenin (VTG) in the common carp (*Cyprinus carpio*) were observed. The standardized values of four biomarker responses were computed and integrated as star plots, representing Integrated Biomarker Respnse (IBR) values. DNA damage was induced in a dose-dependent manner, and increased significantly compared with that in the control. EROD and VTG levels were significantly elevated at low concentrations of BkF. On the other hand, AChE activities were not altered by BkF. IBR values increased as the exposure concentrations increased. Thus, the metabolic, endocrine and genetic changes of the biomarker responses in the common carp exposed to BkF should be considered in the case of the ecological risk assessment of the BkF in fish and it can be used as a biomonitoring tool in aquatic ecosystems. In addition, star plots can be used as a useful analysis tool in multibiomarker integration approach.

Key words : Benzo[*k*]fluoranthene, carp, biomarker, star plot

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants, mainly formed during the incomplete combustion of wood and fossil fuels (Ciecierska and Obiedziński, 2007). Many PAHs are potentially carcinogenic and ubiquitous contaminants of much concern in aqueous environments (Evanson and Van Der Kraak, 2001). It induces the formation of mixed function oxygenase (MFO), especially cytochrome P4501A1 (CYP1A1) in fish liver which has been employed as a biomarker of the exposure to the hazardous organic pollutants from other monitoring studies (Stegeman and Lech, 1991; Bucheli and Fent, 1995; Goksøyr *et al.*, 1996). While the MFO system is essential for the biotransformation of PAHs, its induction could produce damaging side effects through the formation of intermediates that are highly reactive, mutagenic, and carcinogenic (Stegeman and Lech, 1991). For instance, a strong linkage has been shown between the prevalence of vitellogenesis and CYP1A induction in flounder (Kirby *et al.*, 2007).

Various biomarkers in fish species have been used as a tool for the ecotoxicological assessments in many countries (Peakall and Walker, 1994; Str-

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mac and Braunbeck, 2000; Kirby *et al.*, 2007). However, lacks of the information often limit the complete assessments. For instance, data on biomarker responses to PAHs in freshwater fish have not been enough, though such information is essential for the PAH monitoring and its environmental assessment. The full potential of the biomarker-based monitoring approach as a tool for ecological risk assessment is needed to be quantified and Beliaeff and Burgeot (2002) proposed the integrated biomarker response (IBR) computed as the star plot area to summarize biomarker responses and to simplify their interpretation in biomonitoring programs.

Benzo[*k*]fluoranthene (BkF) is the most carcinogenic among 400 PAHs and its exposure in aquatic ecosystems is increasing (Duan and Wei, 2000). BkF is one of the most abundantly found pollutants among the sixteen kinds of the PAHs detected in aquatic systems (Oh *et al.*, 2003). The common carp (*Cyprinus carpio*) is one of the extensively used species for monitoring freshwater contamination. In particular, this model fish has been successfully used for evaluating the acute toxicity and studying metabolism of contaminants (Bongers *et al.*, 1998).

The objective of this study was to investigate the responses of the four biomarkers in common carp (*Cyprinus carpio*) exposed to a pure PAH compound such as benzo[*k*]fluoranthene. The four biomarker assays included the 7-ethoxyresorufin-O-deethylase (EROD), DNA single-strand breaks (Comet), acetylcholinesterase (AChE), and vitellogenin (VTG). Integrated biomarker response (IBR) used as the integration model for the interpretation of those biomarker responses.

MATERIALS AND METHODS

1. Test chemical and fish

Test chemical, benzo[*k*]fluorathene (BkF, 98.7% pure), was obtained from Sigma-Aldrich. Because of its low solubility in water, stock solution of the BkF was prepared by dissolving it in N,N-dimethylformamide (<100 mg L^{-1}), and then it was diluted with carbon-filtered and dechlorinated tap water to make nominal concentrations of 10, 30, and 50 μg L^{-1} for 10 days. Only dechlorinated tap water was used in the control.

Common carp (*Cyprinus carpio*) were obtained from a Chungcheongnam-do Experimental Station for Inland Waters Development (Nonsan City, S. Korea) and held in 2000 L tanks with flowing water at 23 ± 2°C. The fish in the culturing tanks were fed once a day with commercial fish food (Fishtop feed No. 2®, Woosung Feed, S. Korea). The fish were starved at least for 24 h in order to ensure the gut clearance before the experiments.

2. Exposure design

The dosing apparatus consisted of flow-through systems with four 100 L aquaria (10, 30, 50 μg L^{-1}, and control), receiving carbon-filtered and dechlorinated tap water (pH, 7.0; alkalinity, 25.1 mg L^{-1} as $CaCO_3$; total hardness, 42.5 mg L^{-1} as $CaCO_3$). In each aquarium, water flow was set at a rate to achieve at least two complete turnovers per day. To avoid any effects from chemicals other than the tested compound, all the exposure systems were made with glass, Teflon®, and stainless steel components. BkF was delivered to the aquaria from the concentrated stock solutions using syringe pumps (Kloen Co. Ltd., USA). Flow of the BkF into the test vessels was regulated in order to maintain the nominal concentrations. Ten fish were held in each tank tested. The photoperiod was 16 h : 8 h light : dark, and water temperature was maintained at 23 ± 1°C. Fish were not fed during the tests to minimize the loss of chemical concentrations in the water via adsorption to organic particulates.

After 10-day exposure, all fish were taken out from the tanks, and their sex was determined by observing the gonad. The whole fish were blotted on filter papers, weighed (total weight), and measured (total length). Liver, brain, and blood from each fish were taken and stored in eppendorf tubes at −80°C.

3. Analysis of benzo[*k*]fluorathene concentrations in the test solutions

The concentrations of BkF in each test solution were measured on day 0, 3, 7, and 10. Forty mL of the test solutions were taken from each treatment group. Then, 20 mL of ethyl acetate (Burdick and Jackson, USA) was added to the sample and then shaken for 20 min. Eighteen mL of the mixture was collected and evaporated under N_2 in a nitrogen evaporator (N-EVAP, Organomaion Associates, JNC, USA). The residue was dissolved in 1 mL of acetonitrile (Burdick and Jackson, USA). A 20 μL aliquot of each sample was

analyzed by high-performance liquid chromatography (HPLC) with a Hewlett-Packard HP 1200 series (Palo Alto, CA, USA) equipped with a diode-array detector at the wavelength of 296 nm. The HPLC separation was conducted using Phenomenex C_{18} column (150×4.6 mm, 5 μm). The mobile phase was eluted with acetonitrile : water (80 : 20, v/v) solution. The limit of detection for BkF under these conditions was 1 μg L^{-1}. The concentrations of the test substance were expressed in term of geometric mean of the measured concentrations.

4. Enzyme activities

1) Ethoxyresorufin-*O*-deethylase activities

Liver samples were homogenized in ice with 10 volumes of phosphate buffer (50 mM, pH 7.8) and centrifuged at 73,000g for 30 min. at 4℃. The supernatant was centrifuged again at 16,000 g for 60 min at 4℃. The pellet (microsomes) was suspended in phosphate buffer. Ethoxy resorufin-*O*-deethylase (EROD) activities in the microsomes were determined using reaction product (resorufin) by a fluorescence plate reader (Fluoroskan Ascent, Thermo Labsystems, Finland) with excitation and emission filters set at 530 nm and 590 nm, respectively. Protein concentrations in the samples were measured by the fluorescamine assay (Kennedy and Jones, 1994).

2) COMET assay

The alkaline comet assay with fish blood cells was conducted following the published methods (Kim *et al.*, 2003). Fish blood cells were dispersed and immobilized onto the agarose gel coated on microscope slides. The slides were placed in a solution to lyse and disperse the cell components, leaving the DNA immobilized in the agarose. Following electrophoresis, the slides were rinsed in a neutral buffer and the gel and its contents were fixed using ethanol. The DNAs in the fixed slides were stained with ethidium bromide. A computerized image analysis system (Komet version 4.01, Kinetic Imaging Ltd., UK) was used to determine the tail moment which is the product of the percentage of DNA in the tail and the tail length.

3) AChE activities

Brain samples were thawed, homogenized in ice with 5～10 volumes of phosphate buffer (0.1 M, pH 7.6), and centrifuged at 10,000 g for 20 min. The supernatant (postmitochondrial supernatant, PMS) was used to assay AChE activities. AChE activities were expressed in terms of PMS protein contents determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, USA) using bovine serum albumin as a standard. Activities of the PMS towards the diagnostic substrates acetylthiocholine was assayed by the modified Ellman method (Jung *et al.*, 2007). A microplate reader method was used based on the absorbance measurements using a filter with a transmission (max. 415 nm).

4) VTG assay

Blood sample taken from each fish was centrifuged at 3,000 g for 60 min. at 4°C. The supernatant (plasma) was collected and frozen at −80°C for the later ELISA analysis. VTG concentrations were measured with a carp VTG enzyme-linked immumosorbent assay kit (Biosense Lab., Norway).

5) Integrated biomarker response

The procedures for the integrated biomarker response by Beliaff and Burgeot (2002) were shown as follows, with some modifications. Briefly, data were standardized to allow direct visual comparison of the biomarker responses at the test concentrations. Standardized data (Y) was calculated as below:

$$Y=(X-m)/s,$$

where X=the value of each biomarker responses
m=mean value of the biomarker
s=standard deviation of the biomarker

The minimum value (*min*) for each biomarker was obtained in the standardized data (Y). Finally, the score (S) was computed as $S=Y+|min|$, where $S\geq 0$ and $|min|$ is the absolute value.

Star plots were then used to visualize the biomarker results. A star plot radius coordinate represents the score of a given biomarker. When the S_i and the S_{i+1} are assigned as two consecutive clockwise scores of a given star plot, the area of the star plot (IBR value) obtained by the sum of the four triangle areas can be calculated as

$$\text{IBR value}=[(S_i\times S_{i+1})+(S_{i+1}\times S_{i+2})+(S_{i+2}\times S_{i+3})+(S_{i+3}\times S_i)]/2.$$

5. Statistical analysis

Statistical analysis was done using SPSS statistical package programs (ver. 10.0). One-way

ANOVA was used to compare the variables between the control and the treatments. The significance level was set at $P < 0.05$. Duncan' program was performed to determine whether there was any significance detected or not.

RESULTS

The concentrations of benzo[*k*]fluorathene (BkF) were measured at 0, 3, 7, and 10 days and were expressed in term of the means of the measured concentrations. The concentrations of BkF in test solutions are presented in Table 1. Nominal concentrations of 10, 30, and 50 μg L^{-1} were measured as 6, 25, and 45 μg L^{-1}, respectively. Compared to nominal concentrations, the measured concentrations were maintained in the range of 60~90%. The level of DNA single-strand breaks was determined in the erythrocytes of the carp blood using comet assay (Fig. 1). DNA damage was induced in the presence of the BkF and it was significantly increased compared with the control ($P < 0.05$). Hepatic EROD activity of the carp was determined by measuring the dealkylation of ethoxyresorufin. As shown in Fig. 2, the activities were similar to the induction pattern of DNA damage, and it was significantly increased at the lowest concentration of the BkF ($P < 0.05$). Acetyl-

Table 1. Concentrations of the test solutions during the exposure.

Nominal concentration (μg L^{-1})	Measured concentration (% nominal conc.)				Mean ± SD (% nominal concentration)
	Day after exposure				
	0d	3d	7d	10d	
Control	ND*	ND	ND	ND	ND
10	6 (60)	5 (50)	6 (60)	6 (60)	6 ± 1 (60)
30	24 (80)	24 (80)	26 (87)	25 (83)	25 ± 1 (83)
50	33 (66)	47 (94)	50 (100)	49 (98)	45 ± 8 (90)

*ND: Not detected

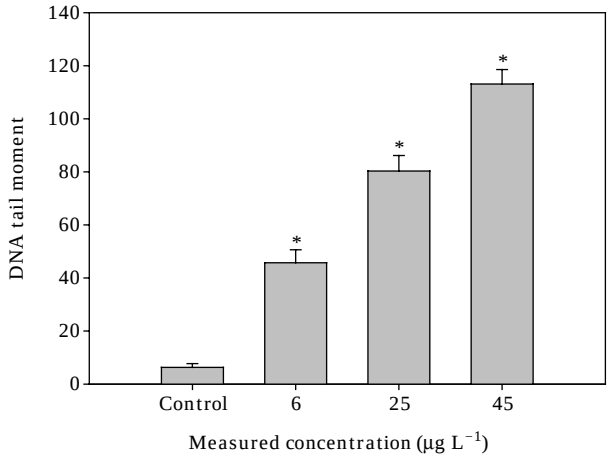

Fig. 1. DNA damage exposed to 6, 25, and 45 μg L^{-1} of measured concentrations of benzo[*k*]fluoranthene (n=10). Values are means ± SE and marked with an asterisk when significantly different from the control value ($P < 0.05$).

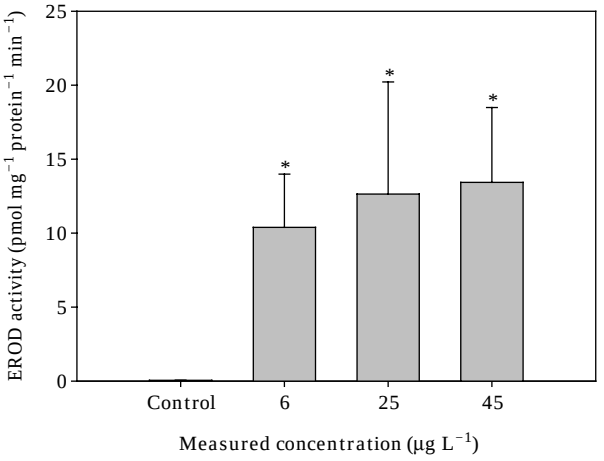

Fig. 2. EROD activities exposed to 6, 25, and 45 μg L^{-1} of the measured concentrations of benzo[*k*]fluoranthene. Values are means ± SE and marked with an asterisk when significantly different from the control value ($P < 0.05$).

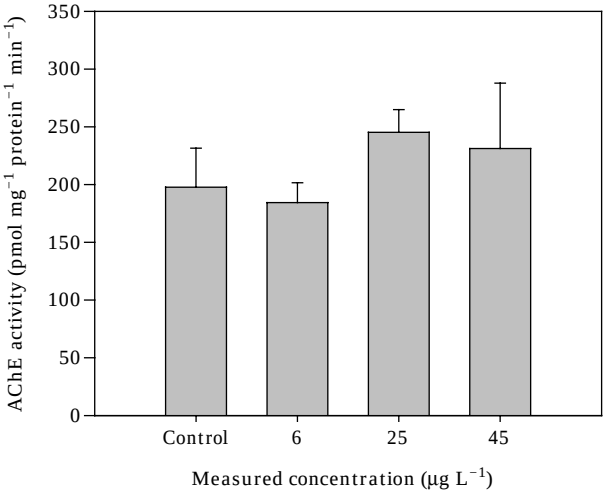

Fig. 3. AChE activities exposed to 6, 25, and 45 μg L^{-1} of measured concentrations of benzo[*k*]fluoranthene (n=4). Values are means ± SE.

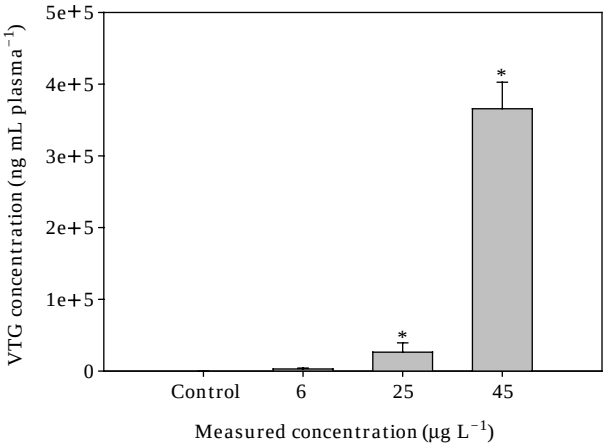

Fig. 4. VTG concentrations exposed to 6, 25, and 45 μg L^{-1} of the measured concentration of benzo[*k*]fluoranthene (n=4). Values are means±SE and marked with an asterisk when significantly different from the control value ($P<0.05$).

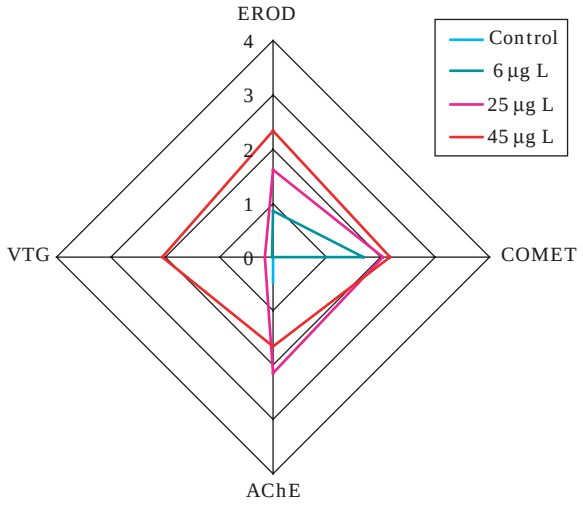

Fig. 5. A biomarker star plot exposed to benzo[*k*]fluoranthene in the carp (EROD=ethoxyresorufin-O-deethylase; COMET=DNA damage; AChE=acetylcholinesterase; VTG=vitellogenin).

cholinesterase (AChE) activity was observed in the brain of the carp. Inhibition of AChE was not changed by BkF for 10 days (Fig. 3). Endocrine effects were measured as the VTG levels in the plasma of the male carp. VTG concentrations were highly elevated at the highest concentration of the BkF compared with that in the control and it was also in a concentration-dependent manner (Fig. 4). Summary of the various biomarker responses in the carp treated with BkF and the computed integrated biomarker response (IBR) are presented in Table 2. And, the computed IBR values were used for the star plot area calculation (Fig. 5).

Table 2. The values of the biomarker responses (EROD=ethoxyresorufin-O-deethylase; COMET=DNA damage; AChE=acetylcholinesterase; VTG=vitellogenin) and integrated biomarker response (IBR) values in the carp exposed to the benzo[*k*]fluoranthene.

Measured concentration (μg L^{-1})	Score of biomarkers				IBR value
	EROD	Comet	AChE	VTG	
Control	0	0	0.47	0	0
6	0.86	1.67	0	0.02	0.72
25	1.61	2.03	2.15	0.15	4.10
45	2.33	2.16	1.65	2.05	8.38

DISCUSSION

The aim of this study was to examine the responses of the various biomarkers in the carp treated with PAH such as benzo[*k*]fluorathene (BkF). DNA damages in the carp exposed to BkF at the measured concentrations of 6, 25, and 45 μg L^{-1} increased significantly (Fig. 1). No data on the DNA damage by BkF using comet assay are currently available for fish, but Ericson and Lennart (2008) reported BkF induced DNA damage in the intestine of the juvenile northern pike (*Esox lucius*) which was measured by DNA adduct formation. In the study with the benzo[*a*]pyrene consisting of five benzene rings such as BkF, DNA damage increased in the mussels exposed to BaP-contaminated sediment (Akcha *et al.*, 2000). Genotoxicity of BaP has been demonstrated in mussel haemocytes and digestive gland cell (Mitchelmore *et al.*, 1998).

The MFO system concerned with BkF biotransformation was highly active in the carp, showing the induced EROD activities after the exposure to BkF in a concentration-dependent manner (Fig. 2). Even at the lowest concentration (nominal concentration of 10 μg L^{-1}) of BkF, the enzyme activity was significantly induced. This result was consistent with the previous report that EROD activities after the BkF exposure increased at the nominal concentration of 10 μg L^{-1} in

the flounder (Kirby *et al.*, 2007) and scallop (Pan *et al.*, 2005). In a study with ten polycyclic aromatic hydrocarbons (PAHs) by Bosveld *et al.* (2002), BkF induced the highest EROD activity in the H4IIE cell (rat hepatoma cell line) among the PAHs tested.

Little information is available on the PAH effects on the neurotransmission activities, though Akcha *et al.* (2000) reported that aceylcholinesterase (AChE) activities decreased in the mussels exposed to the BaP-contaminated sediment. However, in this study, there were little changes in the AChE activities of the brain in the carp exposed to the tested BkF concentrations compared with that of the control (Fig. 3). Shin *et al.* (2003) reported that AChE activities in the head portion of the medaka exposed to PAH such as fluoranthene was not changed, but those in the body portion was inhibited significantly. The results indicated that BkF might not inhibit the AChE activities in the head portion of carp. It is suggested to investigate the AChE activities in the whole fish not just in the head portion of the fish.

VTG is normally synthesized in sexually maturing females, and male fish do not produce it. However, estrogenic compounds can induce male fish to synthesize VTG (Seo *et al.*, 2007). There are several lines of evidence that vitellogenesis was affected by environmental polycyclic aromatic hydrocarbons (Anderson *et al.*, 1996; Tintos *et al.*, 2006; Kirby *et al.*, 2007). Nicolas (1999) reported that PAHs induced the vitellogenesis in fish. In this study (Fig. 4), VTG concentrations in the plasma of the male carp treated with BkF increased in a concentration-dependent manner.

Star plots were used for visualization and quantification of the biomarker responses (Fig. 5). Integrated biomarker responses (IBR) for the DNA damage, EROD, AChE, and VTG activities were computed (Table 2). According to increasing exposure concentrations, IBR scores and graphic panel tended to increase. The IBR scores can be used for the statistical comparison of various biomarker data affected by BkF and integration can be processed by substituting each biomarker value with a numerical value. Though the IBR scores can not provide any critical criteria, it can be used as an exploratory tool for the biological responses. The application of the IBR is in a beginning stage and the use of this will be expanded when more data is accumulated. Beliaeff and Burgeot (2002) reported that star plot could readily be compared across survey stations and organic contaminants and it was useful graphic aid for exploratory analysis of data in multibiomarker approach. Fränzle (2006) represented that various indicators depicted integral graph as amoeba diagram. This approach leads to models for improving both the technical practicability and the data quality of biomonitoring approaches. By establishing the analysis of various biomarkers in fish and the method to analyze its pattern, it might be able to increase the probability to determine the presence of the exposure to PAHs in the environments and to extrapolate the amount of its exposure.

In conclusion, BkF caused DNA damage, cytochrome P450 induction, and vitellogenesis in the common carp, indicating that those effects should be considered in the case of the ecological risk assessment of the PAHs especially in fish. In addition, those biomarkers can be used for the monitoring of PAHs contamination in aquatic ecosystems.

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