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Effect Evaluation of Benzo[a]pyrene on Multiple Biomarkers in Common Carp (*Cyprinus carpio*)

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잉어 (*Cyprinus carpio*)의 다중바이오마커를 이용한 Benzo[a]pyrene의 영향평가

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요 약

수백여 종의 개별물질이 불완전 연소 혹은 유기물의 열분해로 인해 발생되는 다환방향족 탄화수소(PAHs)는 환경에서 중요한 오염원이 되고 있다. 본 연구는 다양한 바이오마커를 이용하여 수서생태계에 벤조피렌(benzo[a]pyrene)과 같은 다환방향족 탄화수소의 영향을 분석하였고, 이에 대한 통합적 결과 모델을 도출하였다. 즉, 잉어(*Cyprinus carpio*)를 이용하여 여러 농도의 벤조피렌(3, 12, 34 μg/L, 측정농도 기준)에 10일간 노출시킨 다음, DNA single-strand break, ethoxyresorufin-*O*-deethylase (EROD), acetylcholine esterase (AChE)와 vitellogenin (VTG)의 농도를 측정하였다. 벤조피렌은 잉어의 DNA 손상을 유도하였고, 낮은 농도에서 EROD와 VTG의 유의적인 활성을 보였으나, 신경전달물질과 관련이 깊은 AChE 효소활성에는 영향을 미치지 않았다. 이 결과를 star plot를 이용하여 통합 및 분석하였으며, 노출농도에 따른 통합반응지수(integrated biomarker response value: IBR)로 나타내었다. 이런 다양한 바이오마커의 결과들은 벤조피렌에 대한 어류의 영향과 수생태 모니터링 자료로 이용 가능할 것으로 여겨지며, 통합반응지수는 생태위해성평가에서 유용한 도구로 쓰일 가치가 있는 것으로 평가된다.

Key words : Benzo[a]pyrene, carp, multi-biomarkers, star plot

INTRODUCTION

Aqueous environments receive large inputs of anthropogenic pollutants through industrial and urban discharge, atmospheric deposition, and terrestrial drainage (Shailaja and D'Silva, 2003). Many of polycyclic aromatic hydrocarbons (PAHs) are potentially carcinogenic and ubiquitous contaminants of much concern in aquatic environments (Evanson and Vand 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 stud-

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ies (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 sideeffects through the formation of intermediates that are highly reactive, mutagenic, and carcinogenic (Stegeman and Lech, 1991). Indeed, 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; Strmac and Braunbeck, 2000; Kim *et al.*, 2007; 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 simplify their interpretation in biomonitoring programs.

Benzo[a]pyrene (BaP) was used as a representative PAH compounds in this research because it has been known to exhibit carcinogenic, apoptogenic, mutagenic and endocrine-disrupting activities (Nicolas 1999; Akcha *et al.*, 2000; Yamata *et al.*, 2002; Pan *et al.*, 2006).

The objective of this study was to investigate the responses of the biomarkers in common carp (*Cyprinus carpio*) exposed to a pure PAH compound such as benzo[a]pyrene and to apply the integration model for the interpretation of those biomarker responses. After 10 days of the exposure to the BaP, the changes of the four biomarkers in the common carp (*Cyprinus carpio*) were observed. The four biomarkers (assays) included the 7-ethoxyresorufin-O-deethylase (EROD), DNA single-strand breaks (Comet), acetylcholinesterase (AChE), and vitellogenin (VTG). To quantify the various biomarker responses, integrated biomarker response (IBR) was computed as star plot areas.

MATERIALS AND METHODS

1. Test chemical and fish

Test chemical, benzo[a]pyrene (BaP, 99.8% pure), was obtained from Supelco (Bellefonte, PA, USA). Stock solution of the BaP was prepared by dissolving it in N,N-dimethylformamide (< 100 mg/L), and then it was diluted with carbon-filtered and dechlorinated tap water to make nominal concentrations of 10, 30, and 50 μg/L for 10 days. Only dechlorinated tap water was used in the control.

Common carp were obtained from a Chungcheongnam-do Experimental Station for Inland Waters Development (Nonsan City, S. Korea) and 60 fish held in 2,000 L tanks with flowing water at 23±1℃. The photoperiod was 16h : 8h light : dark. The fish in the culturing tanks were fed once a day with commercial fish food (Fishtop feed No. 2®, Woosung Feed, Daejeon City, S. Korea). The mean length and weight of experimental fish were 12.2±0.9 cm and 20.9±3.7 g, respectively.

2. Exposure design

The dosing apparatus consisted of flow-through systems with four 100 L aquaria (10, 30, 50 μg/L, and control), receiving carbon-filtered and dechlorinated tap water (pH, 7.0; alkalinity, 25.1 mg/L as $CaCO_3$; total hardness, 42.5 mg/L 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. BaP was delivered to the aquaria from the concentrated stock solutions using syringe pumps (Kloen Co. Ltd., Summerlin, NV, USA). Flow of the BaP into the test vessels was regulated in order to maintain the nominal concentrations. Ten fish were held in each tank tested. The photoperiod was 16h : 8h light : dark, and water

temperature was maintained at 23±1℃. 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. They 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℃.

3. Analysis of BaP concentrations in the test solutions

The concentrations of BaP 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 & Jackson, Muskegon, MI, 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 & Jackson, Muskegon, MI, 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 DAD detector at the wavelength of 296 nm. The HPLC separation was conducted using Phenomenex C_{18} column (150 × 4.6 mm, 5 μm). An isocratic elution was performed with acetonitrile : water (80 : 20, v/v) solution. The limit of detection for BaP under these conditions was 1 μg/L. The recovery of the BaP at 40 μg/L was 95.6 %. The concentrations of the test substance were expressed in term of arithmetic 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 73,000 × g for 30 min at 4°C. The supernatant was centrifuged again at 16,000 × g for 60 min at 4°C. 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, Helsinki, Finland) with excitation and emission wavelength at 530 nm and 590 nm, respectively. Protein concentrations in the samples were measured by the fluorescamine assay (Kennedy and Jones, 1994). All samples were analyzed in triplicates.

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 in an agarose gel 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. All samples were analyzed in duplicates.

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, Rockford, IL, USA) using bovine serum albumin as a standard. Activities of the PMS towards the diagnostic substrate acetylthiocholine were assayed by the modified Ellman method (Jung *et al.*, 2008). A microplate reader method was used based on the absorbance measurements using a filter

with a transmission (wavelength 415 nm). All samples were analyzed in triplicates.

4) VTG assay

Blood sample taken from caudal vein of male 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., Bergen, Norway). All samples were analyzed in triplicates.

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 for direct visual comparisons 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

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

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 controls and the treatments. The significance level was set at $P<0.05$. Duncan's multiple range test was performed to determine whether there was any significant difference among treatments.

RESULTS

Measured concentrations of BaP in the test solutions are presented in Table 1. Nominal concentrations of BaP-10, 30, and 50 μg/L-were measured as 3, 12, and 34 μg/L (mean concentrations for 10 days), respectively. Compared to the nominal concentra-

Table 1. Concentrations of the test solutions during the exposure

Nominal conc. (μg/L)	Measured concentration (% nominal conc.)				Mean±SD (% nominal conc.)
	Day after exposure				
	0d	3d	7d	10d	
Control	ND*	ND	ND	ND	ND
10	2 (20)	4 (40)	3 (30)	3 (30)	3±1 (30)
30	8 (27)	14 (47)	15 (50)	10 (33)	12±3 (40)
50	30 (60)	34 (68)	38 (76)	35 (70)	34±3 (68)

*ND: Not detected

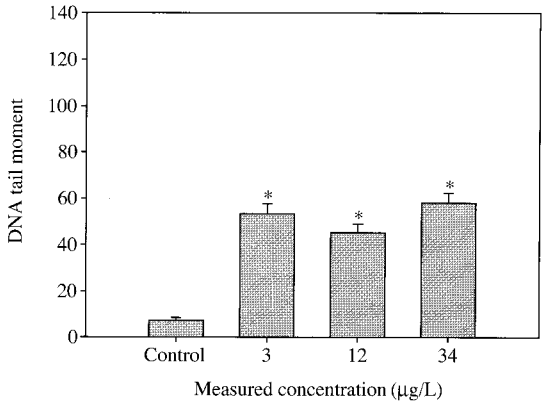

Fig. 1. DNA damage in carp (*Cyprinus carpio*) exposed to 3, 12, and 34 μg/L of measured concentrations of benzo[a]pyrene (n=6). Values are means±SE and marked with an asterisk when significantly different from the control value ($P<0.05$).

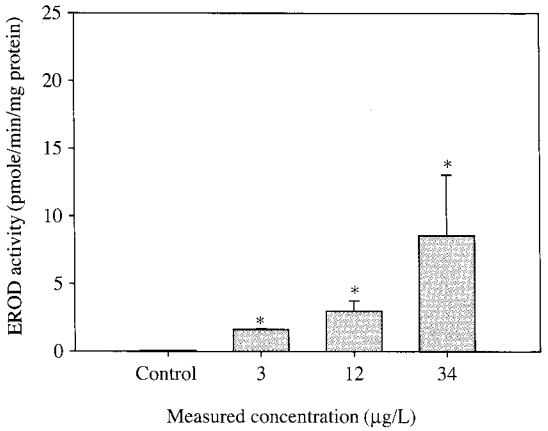

Fig. 2. EROD activities in carp (*Cyprinus carpio*) exposed to 3, 12, and 34 μg/L of the measured concentrations of benzo[a]pyrene (n=4). Values are means± SE and marked with an asterisk when significantly different from the control value ($P<0.05$).

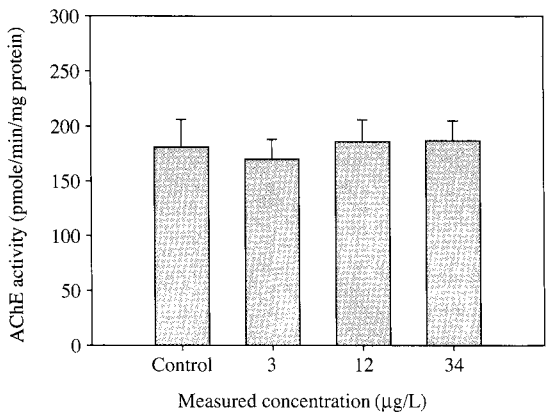

Fig. 3. AChE activities in carp (*Cyprinus carpio*) exposed to 3, 12 and 34 μg/L of measured concentrations of benzo[a]pyrene (n=5).

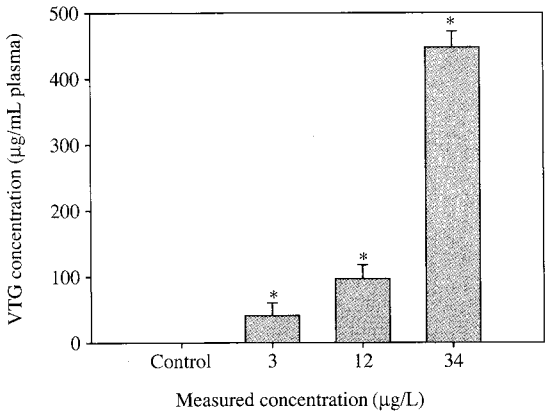

Fig. 4. VTG concentrations in male carp (*Cyprinus carpio*) exposed to 3, 12, and 34 μg/L of the measured concentration of benzo[a]pyrene (n=4). Values are means ±SE and marked with an asterisk when significantly different from the control value ($P<0.05$).

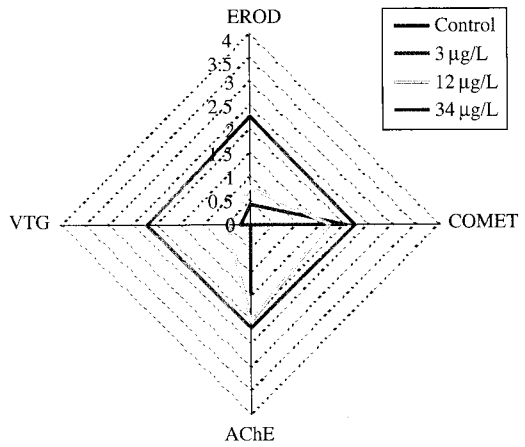

Fig. 5. A biomarker star plot exposed to BaP in the carp (EROD=ethoxyresorufin-O-deethylase; COMET= DNA damage; AChE=acetylcholinesterase; VTG= vitellogenin).

tions, the measured concentrations were maintained in the range of 30~68%. 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 BaP 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. The activities were similar to the induction pattern of the DNA damage (Fig. 2), but was significantly increased even at the lowest concentration of the BaP exposed ($P<0.05$) and in a concentration-dependent manner. Neurotoxic effect was determined by the AChE activities in the brain of the carp. There was almost no change in the level of AChE in the carp after the exposure to BaP for 10 days (Fig.

Table 2. Scores (EROD=ethoxyresorufin-O-deethylase; COMET=DNA damage; AChE=acetylcholinesterase; VTG=vitellogenin) and integrated biomarker response (IBR) values in the carp exposed to the benzo[a]pyrene

Measured concentration	Scores of the biomarkers				IBR value
	EROD	Comet	AChE	VTG	
Control	0	0	1.41	0	0
3	0.43	2.00	0	0.20	0.47
12	0.80	1.65	2.05	0.47	3.01
34	2.30	2.20	2.18	2.19	9.82

3). Endocrine effects were measured as VTG levels in the plasma of the male carp. VTG concentrations were highly elevated at the highest concentration of the BaP exposed (34 μg/L) compared with the control and it was also in a concentration-dependent manner (Fig. 4). The results of the various biomarker responses in the carp treated with BaP were integrated in Fig. 5. Integrated biomarker response (IBR) was then computed as the star plot area (Table 2). As the exposure concentrations increase, IBR scores tended to increase. The IBR values in control, 3, 12, and 34 μg/L were 0, 0.47, 3.01, and 9.82, respectively.

DISCUSSION

The aim of this study was to examine various biomarkers in the carp treated with PAH such as BaP. DNA damages in the carp exposed to BaP at the measured concentrations of 3, 12, and 34 μg/L was increased significantly (Fig. 1). DNA damage in the flatfish dab exposed to 50 mg/kg BaP has been significantly induced (Van Schancke *et al.*, 2000) and the genotoxicity of BaP has been demonstrated in mussel hemocytes and digestive gland cell (Mitchelmore *et al.*, 1998). For instance, Akcha *et al.* (2000) reported that DNA damage increased in the mussels exposed to BaP-contaminated sediment. The effect of chronic exposure to the benzo(a)pyrene (BaP) on the DNA strand breaks was investigated in bluegill sunfish and fathead minnow and it declined in the initial phase and then, increased at the later phase during 30 days of the exposure (Shugart, 1988).

The MFO system concerned with BaP biotransformation was highly active in the carp, showing the induced EROD activities after the exposure to BaP in a concentration-dependent manner (Fig. 2). Even at the lowest concentration (nominal concentration of 10 μg/L) of BaP, the enzyme activity was significantly induced. This result was consistent with the previous reports that EROD activities were strongly increased after the BaP exposure in fish (Lemaire-Gony and Lemaire, 1992; Wolkers *et al.*, 1996; Rotchell *et al.*, 1999; Padros *et al.*, 2000). In addition, a study by Bols *et al.* (1999) using a rainbow trout liver cell line found the induction of the EROD activities by BaP.

Little information has been available on the PAH effects on the neurotransmission activities. Akcha *et al.* (2000) reported that AChE activities decreased in the mussels exposed to BaP-contaminated sediment. In contrast, there was little change in the AChE activities of the brain from the carp exposed to the tested BaP concentrations compared with that of the control in this study (Fig. 3).

VTG concentrations in the plasma of the male carp treated with BaP increased in a concentration-dependent manner (Fig. 4). As VTG is normally synthesized in sexually maturing females, male fish do not produce it. However, male fish can be induced to synthesize VTG when exposed to estrogenic compounds (Seo *et al.*, 2007). Vitellogenesis may also be affected by pollutants with known affinity for the estrogenic receptor, such as PAHs. Nicolas (1998) reported PAHs induced the vitellogenesis in various fish. There have been 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).

Star plots were used in this study 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). As the exposure concentrations incre-

ase, IBR scores and graphic panel tended to increase. Beliaeff and Burgeot (2002) reported that star plot could readily be compared across survey stations and across organic contaminants. Star plot could also be useful graphic aid for exploratory analysis of data in multi-biomarker approach. Fränzle (2006) represented that various indicators depicted integral graph as amoeba diagram. These approaches can lead the models by improving both the technical practicability and the data quality in biomonitoring approaches. The IBR values calculated in this study present the effect of the BaP and it has a limitation to identify the health conditions of the fish. By analyzing various biomarkers in fish and by establishing the method to analyze its patterns and scores, it is expected 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, BaP caused DNA damage, cytochrome P450 induction, and vitellogenesis in the common carp, showing that those can be used as the potential biomarkers in fish to monitor B(a)P contamination in aquatic ecosystems. In addition, star plots can be used as a useful analysis tool in multi-biomarker integration approach. As a future study, more PAHs and biomarkers will be investigated and it is expected to generalize and classify the fish health conditions with IBR value

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