Assessment of *In Vitro* Oocyte Maturation in Two Gobiid Fish Species, *Chasmichthys dolichognathus* and *Tridentiger trigonocephalus* after Exposure to Benzo[a]pyrene

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ABSTRACT : Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants derived from incomplete combustion of carbons and crude oil. In this study, we investigated the effects of benzo[a]pyrene (B[a]P), a representative PAHs on *in vitro* sex steroid hormone production and germinal vesicle breakdown (GVBD) using isolated oocytes of longchin goby (*Chasmichthys dolichognathus*) and chameleon goby (*Tridentiger trigonocephalus*). Oocytes in diameters of 0.8-0.9 (end vitellogenic stage) and 0.9-1.0 mm (germinal vesicle migratory stage) from longchin goby and 0.5 mm (fully vitellogenic stage) from chameleon goby were used. In GVBD assay, B[a]P at 10 nM stimulated GVBD in the oocytes of 0.8-0.9 mm from longchin goby. B[a]P at 1 nM stimulated GVBD in the oocytes with diameter 0.5 mm from chameleon goby. In steroid production from oocytes of longchin goby, B[a]P at 100 nM decreased testosterone (T) production, B[a]P at 1,000 nM increased estraiol-17β (E_2) production and 10 and 100 nM increased 17,20β-dihydroxy-4-pregnen-3-one (17α 20βP) production in the oocytes with diameter 0.8-0.9 ㎜. B[a]P at 1,000 nM increased E_2 production, 100 and 1,000 nM increased 17α20βP production in the oocytes with diameter 0.9-1.0 mm. In steroid production of oocytes from chameleon goby, B[a]P at 1,000 nM increased E_2 production. B[a]P at 10 nM increased 17α20βP production. In the ratio of E_2 to T (E_2/T), B[a]P at 100 and 1,000 nM increased E_2/T in the oocytes of longchin goby. B[a]P at 100 nM also increased E_2/T in the oocytes of chameleon goby. Taken together, these results suggest that B[a]P have not only weak estrogenic effects but progestogenic effects on oocyte maturation.

Key words : Benzo[a]pyrene, Chameleon goby, Germinal vesicle breakdown, Longchin goby, Oocyte maturation, Steroid hormone

INTRODUCTION

Reproduction of fish is regulated by endocrine factors with hypothalamus-pituitary-gonad axis and controlled by environmental factors such as water temperature and photoperiod (Nagahama et al., 1994). Among endocrine factors, sex steroid hormones produced from gonad play an important role associated with reproductive cycle. In female, estradiol-17β (E_2) acts as a major sex steroid hormone, binds to estrogen receptor (ER) and induces vitellogenesis that uptake vitellogenin, the yolk precursor from liver to oocytes. After vitellogenesis, progestins such as 17α,20β-dihydroxy-4-pregnen-3-one (17α20βP) and/or 17,20β,21-trihydroxy-4-pregnen-3-one (17α20β1P) act as maturation inducing steroid and induce final oocyte maturation including germinal vesicle breakdown (GVBD) and ovulation (Nagahama et al., 1994; Patiño & Sullivan, 2002).

Considerable amount of investigation has been accumulated on various chemicals that can disrupt the endocrine system in vertebrates including teleosts (Colborn et al., 1996). These endocrine disrupting chemicals (EDCs) include sewage effluent, industrial detergents, pesticides and polycyclic aromatic hydrocarbons (PAHs). PAHs are major components of crude oil and could be derived from incomplete combustion of carbons (Van Metre et al., 2000). PAHs have been demonstrated to be mutagenic and carcinogenic che-

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micals (McElroy et al., 1991; Maccubin, 1994), as well as to modulate egg development, growth, maturation, respiration and immune system in various organisms including fish (Amanuma et al., 2008; Kawanishi et al., 2009). Among PAHs, benzo[a]pyrene (B[a]P) is a representative 5-ring PAHs that has strong toxicity. To date, several studies reported that B[a]P may have anti-estrogenic effects such as decreasing of E_2 production and ER expression in some mammals including human (Fertuck et al., 2001; Neal et al., 2007). In fish, B[a]P also decrease plasma E_2 or vitellogenin levels, and inhibit E_2 production (Rocha Monteiro et al., 2000; Patel et al., 2006). In contrast to these studies, B[a]P increase plasma levels of vitellogenin and the expression of vitellogenin mRNA (Smeets et al., 1999; Hoffmann & Oris, 2006). Therefore, the effects of B[a]P on reproductive function of fish are unclear yet and under controversy (Scholz & Mayer, 2008).

Among the various fish species, gobiid fish are appropriate species for investigating the effects of EDCs with their small size, easy to handle, and strong tolerance (Robinson et al., 2007). The longchin goby, *Chasmichthys dolichognathus* inhabits in coastal waters and tidal pools of Korea and Japan (Chung, 1977). The chameleon goby, *Tridentiger obscurus* inhabits in coastal waters, brackish waters and mud flat of Korea, Japan and China (Chung, 1977). Both of two gobiid species have similar reproductive characteristics that spawn adhesive eggs and attach them to rocks (Kim & Han, 1990; Baek et al., 1985). The purpose of this study was to investigate the effects of B[a]P on *in vitro* sex steroid hormone production and GVBD rate using isolated mature oocytes of two gobiid fish species, longchin goby, *Chasmichthys dolichognathus* and chameleon goby, *Tridentiger trigonocephalus* for verifying and comparison of B[a]P-effects on oocyte maturation.

MATERIALS AND METHODS

1. Chemicals

B[a]P (Sigma-Aldrich Chemical, St. Louis, Missouri, USA) was prepared as stock solutions (1 mM) by dilution in ethanol. These were diluted further in incubation media. The ethanol concentration in the incubation medium was kept at less than 0.1%. Standard testosterone (T), E_2 and 17α20βP were purchased from Sigma-Aldrich Chemical. Antiserum for T was purchased from Sigma-Aldrich Chemical, and those for E_2 and 17α20βP were a kind gift from Dr. Alexis Fostier (INRA, Rennes, France). Radioactive [2,4,6,7-^{3}H]-T and [2,4,6,7-^{3}H]- E_2 were obtained from Amersham Life Science (England). Radioactive [1,2,6,7-^{3}H]-17α20βP was obtained by enzymatic conversion from [1,2,6,7-^{3}H]-17α-hydroxyprogesterone followed by Scott's method (Scott et al., 1982).

2. Fish and *In Vitro* Maturation of Oocytes

The longchin gobies (5.8-6.8 ㎝ in body length) were captured in tide pools at Chongsapo, Busan, Korea, during the spawning season (April-May). The chameleon gobies (4.7-5.5 ㎝ in body length) were captured in eelgrass bed on Dongdae bay, Namhae, Korea. The ovaries were taken from several mature females to obtain oocytes of 0.8-0.9 and 0.9-1.0 ㎜ in diameter from longchin goby (end-vitellogenic and germinal vesicle migratory stage, respectively, Baek et al., 2007, 2008) and oocytes of 0.5 ㎜ in diameter from chameleon goby (fully vitellogenic stage, Hwang, 2011). Some pieces of ovary from each individual were fixed in Bouin's solution for 24 hrs. The fixed samples were washed, dehydrated and embedded with paraffin. Serial sections of 4-6 ㎛ thickness were prepared and slides were stained in Mayer's hematoxylin and 0.5% eosin and mounted with malinol. Histological samples were observed under a light microscope (BX50, Olympus, Japan).

After dissecting the ovaries into small pieces in ice-cold balanced salt solution (132.96 mM NaCl, 3.09 mM KCl, 0.28 mM $MgSO_4 \cdot H_2O$, 0.98 mM $MgCl_2 \cdot H_2O$, 3.40 mM $CaCl_2 \cdot H_2O$, 3.65 mM HEPES), approximately 20 fully grown follicle-enclosed oocytes were incubated in each well of 24-well culture plates containing 1 ㎖ of Leibovitz L15 medium (Gibco, Invitrogen, Paisley, UK). The pH and

osmolarity of the media were adjusted to 7.7 and 300 mOsm, respectively. At the beginning of incubation, B[a]P were added to the media at concentrations of 1-1,000 nM. The plates were incubated for 24 h at 18℃ with constant gentle shaking. After incubation, the incubation media were collected and stored at −80℃ until measurement of the sex steroid levels. The oocytes were fixed with clearing solution (ethanol : formalin : glacial acetic acid = 6 : 3 : 1). The location of the germinal vesicle (GV = nucleus) was observed under low-power magnification using a dissecting microscope. The number of oocytes completing GVBD, i.e., dissolution of the nuclear membrane, was counted in each well and calculated as a percentage.

3. Radioimmunoassay

After incubation, steroids in aliquots of media were extracted twice using five volumes of ethylacetate : cyclohexane (1 : 1). Then, the T, E_2 and 17α20βP levels were measured by RIA following Kobayashi's method (Kobayashi et al., 1987). The intra-assay coefficients of variance were 2.3% (n = 3), 3.4 % (n = 3) and 3.2% (n = 3) for the T, E_2 and 17α20βP assays, respectively, and the respective inter-assay coefficients of variance were 12.5% (n = 5), 11.5% (n = 5) and 9.5% (n = 5). The minimum detectable limits were 10.0, 11.6 and 12.5 pg/㎖ for T, E_2 and 17α20βP, respectively.

4. Statistics

All data were expressed as means with the standard error of the means (SEM) and tested for normality using the Kolmogorov-Smirnov test using SPSS software (version 17.0) for Windows (SPSS, Chicago, IL, USA). Non-parametric Kruskal-Wallis test followed by the Bonferroni adjustment was tested due to the assumptions of normality and equal variance were failed. A value of $p<0.05$ was considered statistically significant.

RESULTS

1. Histological Observation of Oocytes

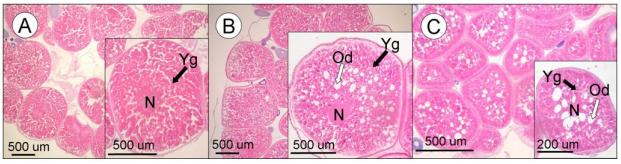

Fig. 1. Histological observations of oocytes for *in vitro* incubation. A, Oocytes of 0.8-0.9 ㎜ in diameter from longchin goby; B, Oocytes of 0.9-1.0 ㎜ in diameter from longchin goby; C, Oocytes of 0.5 ㎜ in diameter from chameleon goby. N, nucleus; Od, oil droplet; Yg, yolk globule.

In 0.8-0.9 ㎜-diameter oocytes of longchin goby, the yolk granules (Yg) were spread throughout the ooplasm and nucleus (N) was located near the center of oocytes and just prior to migrate (Fig. 1A). In 0.9-1.0 ㎜-diameter oocytes of longchin goby, migration of N was observed and several oil droplets (Od) were distributed in the ooplasm (Fig. 1B). In 0.5 ㎜-diameter oocytes of chameleon goby, ooplasm was filled with Yg and Ods about 50 ㎛ were observed around the nucleus. The nucleus was still in the middle of ooplasm (Fig. 1C).

2. Effects of B[a]P on GVBD

In longchin goby, there was no significant effect on GVBD in the oocytes of 0.9-1.0 ㎜ diameter although B[a]P treatment with 1 nM increased GVBD slightly (45.24 ±7.14%) compared with controls (32.50±2.50%, Fig. 2A). B[a]P treatment with 10 nM resulted in a significant increase in GVBD (48.41±3.54%) compared with controls (32.50± 2.50%, $p<0.05$) in the oocytes of 0.8-0.9 ㎜ diameter (Fig. 2B). In chameleon goby (Fig. 2C), B[a]P treatment with 1 nM resulted in a significant increase in GVBD (33.36± 1.22%) compared with controls (19.45±1.78%, $p<0.05$).

3. Effects of B[a]P on Sex Steroid Production

In the oocytes of 0.9-1.0 ㎜ in diameter from longchin goby (Fig. 3), exposure to B[a]P at 100 nM resulted in a significant decrease of T production (89.85±6.70 pg/㎖) compared with controls (159.79±6.06 pg/㎖, $p<0.05$), whereas B[a]P treatment with 1,000 nM resulted in a significant increase in the production of E_2 (3,219.19±150.51 pg/㎖)

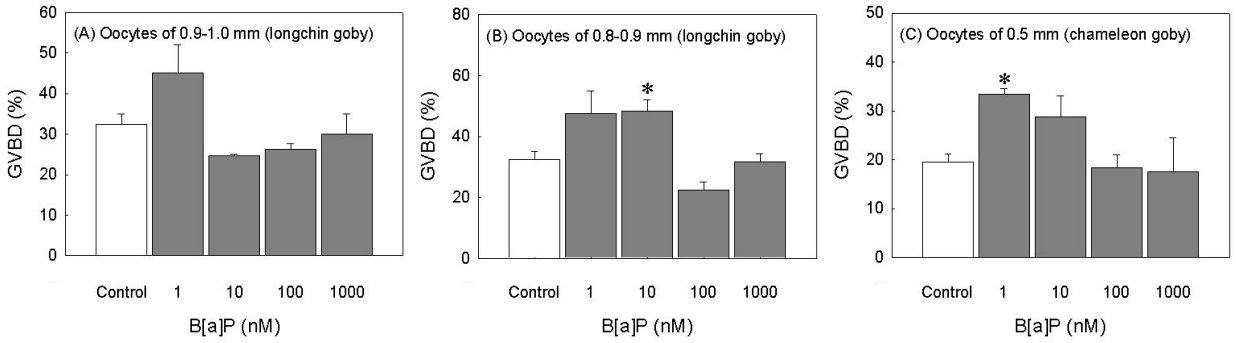

Fig. 2. Effects of B[a]P on *in vitro* GVBD of oocytes from longchin goby and chameleon goby. Values are the mean±SE of triplicates. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks indicate significant differences from controls (p<0.05). A, Oocytes of 0.9-1.0 ㎜ in diameter from longchin goby; B, Oocytes of 0.8-0.9 ㎜ in diameter from longchin goby; C, Oocytes of 0.5 ㎜ in diameter from chameleon goby.

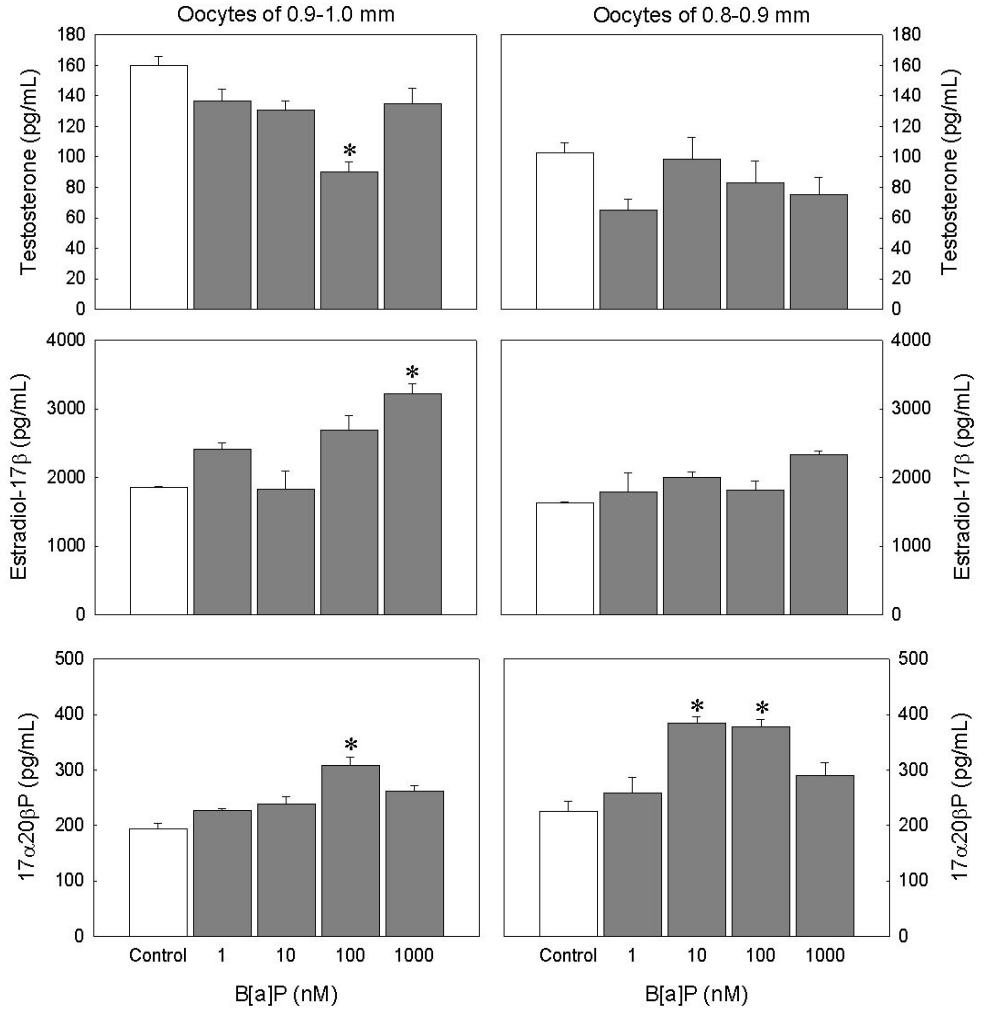

Fig. 3. Effects of B[a]P on *in vitro* steroid production of oocytes from longchin goby. Values are the mean±SE of triplicates. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks indicate significant differences from controls (p<0.05). A, Oocytes of 0.9-1.0 ㎜ in diameter; B, Oocytes of 0.8-0.9 ㎜ in diameter.

compared with controls (1856.10±17.32 pg/㎖, $p<0.05$). B[a]P at 100 nM resulted in a significant increase in the production of 17α20βP (308.52±14.58 pg/㎖) compared with controls (193.78±10.41 pg/㎖, $p<0.05$).

In the oocytes of 0.8-0.9 ㎜ in diameter from longchin goby, B[a]P treatment with 10 and 100 nM resulted in significant increase in production of 17α20βP (384.29±11.93 and 377.09±13.16 pg/㎖, respectively) compared with controls (225.94±18.46 pg/㎖, $p<0.05$) although there was no significant effect in production of T or E_2.

In the oocytes of 0.5 ㎜ in diameter from chameleon goby (Fig. 4), B[a]P at 1,000 nM resulted in a significant increase in production of E_2 (5,580.82±664.15 pg/㎖) compared with controls (3,698.40±384.51 pg/㎖, $p<0.05$), however there was no significant effect on production of T. B[a]P at 10 nM resulted in a significant increase in production of 17α20βP (237.92±18.94 pg/㎖) compared with controls (150.15±20.02 pg/㎖, $p<0.05$).

For steroid production, the ratio of E_2 to T (E_2/T ratio) was calculated as indices of endocrine disruption by exposure to B[a]P (Fig. 5). The E_2/T ratio has been cited as a sensitive biomarker of sex-steroid concentrations (Bevans et al., 1996; Hwang et al., 2010). In the oocytes of 0.9-1.0 ㎜ diameter from longchin goby, B[a]P at 1 and 10 nM resulted in decrease of E_2/T although there was no significant difference compared with controls. B[a]P treatment with 100 nM resulted in a significant increase in E_2/T ratio (29.84±0.20) compared with controls (19.03±2.01, $p<0.05$).

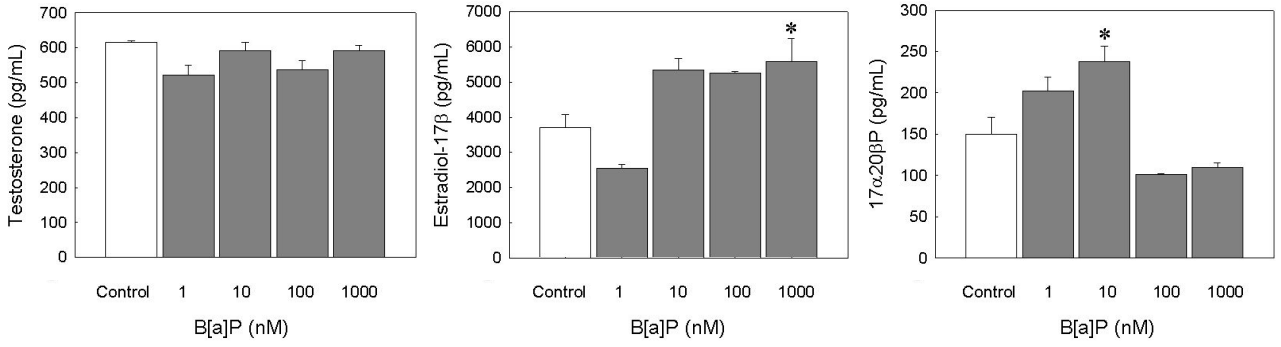

Fig. 4. Effects of B[a]P on *in vitro* steroid production of oocytes from chameleon goby. Values are the mean±SE of triplicates. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks indicate significant differences from controls ($p<0.05$).

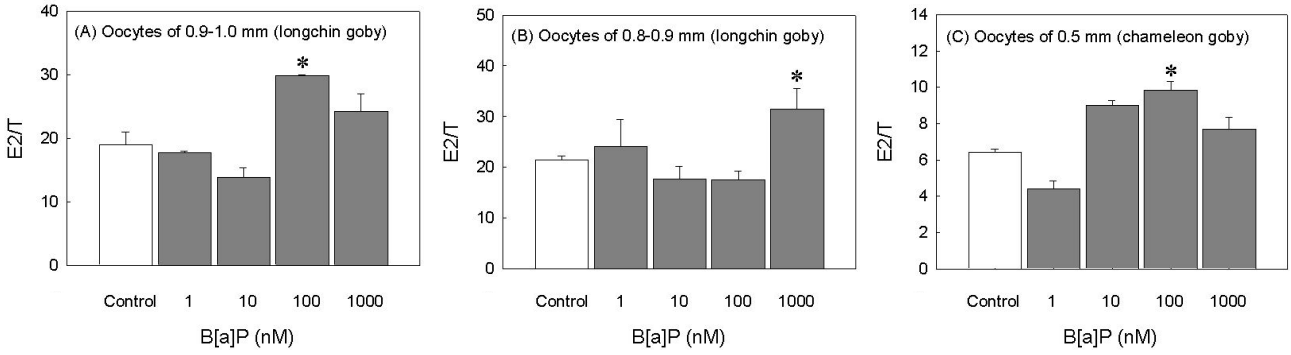

Fig. 5. Effects of B[a]P on the E_2/T ratio in longchin goby and chaemelon goby oocytes. Values are the mean±SE of the ratio of each steroid in triplicates. Data were analyzed using the Kruskal-Wallis test followed by the Bonferroni adjustment. Asterisks show significant differences from controls ($p<0.05$). A, Oocytes of 0.9-1.0 ㎜ in diameter from longchin goby; B, Oocytes of 0.8-0.9 ㎜ in diameter from longchin goby; C, Oocytes of 0.5 ㎜ in diameter from chameleon goby.

In the oocytes of 0.8-0.9 ㎜ diameter from longchin goby, the highest dose of B[a]P resulted in a significant increase on E_2/T (31.52±3.97) compared with controls (21.51± 0.68, $p<0.05$) although there was no significant effect at B[a]P treatments with 1-100 nM. In the oocytes of 0.5 ㎜ diameter from chameleon goby, B[a]P at 1 nM decreased E_2/T slightly, although there was no significant difference. B[a]P treatment with 100 nM resulted in a significant increase on E_2/T (9.82±0.48) compared with controls (6.40±0.19, $p<0.05$).

DISCUSSION

B[a]P is classically associated with its strong toxicity, however recent studies have been demonstrated that B[a]P could act as an EDCs on various wild life including fish (Dong et al., 2008; Schlenk et al., 2008). In fact, B[a]P is lipophilic organic chemical that could be accumulated in lipid-rich tissues such as liver and gonads (Meador et al., 1995). In the present study, we designed the same *in vitro* incubation protocol and analysis condition with two different gobiid fish species for assessment of B[a]P-effects on oocyte maturation. In histological observation, oocytes with average diameters of 0.8-0.9 and 0.9-1.0 ㎜ from longchin goby began to mature after vitellogenesis. Oocytes with average diameters of 0.5 ㎜ from chameleon goby were fully vitellogenic stage, so we thought it is appropriate developmental stage for investigating the effects of B[a]P on consecutive maturation process from vitellogenic stage to maturation stage.

GVBD assay and quantification of steroid production from oocytes had been used as useful index for various contaminants and chemicals (Tokumoto et al., 2004; Hwang et al., 2010). First, we hypothesized that B[a]P may inhibit GVBD and steroid production by its strong toxicity. In the oocytes from longchin goby, B[a]P treatment with lower doses (1 or 10 nM) increased GVBD. Also, production of 17α20βP was increased at B[a]P treatment with 10 or 100 nM. However, the highest dose of B[a]P stimulated E_2 production. Moreover, B[a]P increased E_2/T at 100 or 1,000 nM. In the oocytes from chameleon goby, the results were similar with those of longchin goby that lower doses of B[a]P induced GVBD and production of 17α20βP. However, the highest dose of B[a]P stimulated production of E_2. We supposed that B[a]P could stimulate the production of E_2 as well as final oocyte maturation.

There are very few studies about the effects of B[a]P on GVBD or oocyte maturation. In bovine oocytes, B[a]P has no significant effects on *in vitro* GVBD and cytotoxicity (Luciano et al., 2010). However, B[a]P stimulated and did not inhibit GVBD and production of 17α20βP in the present study. In a recent study, B[a]P increased expression of 20β-hydroxysteroid dehydrogenase (20β-HSD) gene from oocytes of zebrafish (Hoffmann & Oris, 2006). 20β-HSD is a crucial enzyme for conversion of 17α-hydroxyprogesterone to 17α20βP in the ovary (Nagahama et al., 1994). In this regards, B[a]P could increase 20β-HSD, induce production of 17α20βP and GVBD although future study with diverse ranges of B[a]P-dose and expression of 20β-HSD gene should be conducted.

With the estrogenic potency of B[a]P, B[a]P increased expression of brain P_{450} aromatase (CYP19A2) gene, ERβ and vitellogenin genes from liver although there was no significant effect on the expression of ovarian P_{450} aromatase (CYP19A1) gene in adult zebrafish (Hoffmann & Oris, 2006). The authors imply that B[a]P-induced endocrine disruption in zebrafish is binding of B[a]P-metabolites to the ER and up-regulation of aromatase and vitellogenin genes expression. Fertuck and colleagues reported that several kinds of B[a]P metabolites have the ability to bind to the ER and induce ER-mediated gene expression *in vitro* (Fertuck et al., 2001). In contrast to these study, Neal and colleagues reported that PAHs including B[a]P may have a significant antiestrogenic effect through an aryl hydrocarbon receptor (Ah-R) mediated mechanism (Neal et al., 2007). Moreover, in one study, B[a]P inhibited the *in vitro* production of E_2 from ovarian tissue of European flounder, *Platichthys flesus* (Rocha Monteiro et al., 2000). However, the differences in the results would be explained

by different experimental conditions. In that study, the tested concentration (15 μM) was extremely higher than that of the present study (~ 1 μM). In addition, the ovarian tissue from European flounder were frozen, stored and then thawed for *in vitro* incubation. In a recent study by Dong et al., (2008), B[a]P decreased expression of CYP19A2 in the brain of embryo and adult killifish, *Fundulus heteroclitus*. Moreover, B[a]P decreased CYP19A1 expression in the immature ovary. However, we consider the increased E_2 levels and E_2/T at higher B[a]P-doses may come from that B[a]P has estrogenic potency *in vitro*. For understanding more detailed mechanism for B[a]P-exposure on process of oocyte maturation, expression of CYP19A1 gene or activity of CYP19A1 should be investigated with identification of B[a]P metabolites and their estrogenic activity. Taken together, these results suggest that B[a]P have not only weak progestogenic potency at 10-100 nM treatment but estrogenic potency at 100-1,000 nM treatment.

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REFERENCES

Amanuma K, Tone S, Nagaya M, Matsumoto M, Watanabe T, Totsuka Y, Wakabayashi K, Aoki Y (2008) Mutagenicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benz otriazole (PBTA-6) and benzo[a]pyrene (BaP) in the gill and hepatopancreas of rpsL transgenic zebrafish. Mutat Res 656:36-43.

Baek HJ, Kim HB, Lee TY, Lee BD (1985) On the maturity and spawning of the longchin goby, *Chasmichthys dolichognathus* (Hilgendorf). Bull Kor Fish Soc 18:447-483.

Baek HJ, Hwang IJ, Kim KS, Lee YD, Kim HB, Yoo MS (2007) Effects of BPA and DES on longchin goby (*Chasmichthys dolichognathus*) *in vitro* during the oocyte maturation. Mar Environ Res 64:79-86.

Baek HJ (2008) Production of C21 steroids in longchin goby, *Chasmichthys dolichognathus* during oocyte maturation. Dev Reprod 12:107-112.

Bevans HE, Goodbred SL, Miesner JF, Watkins SA, Gross TS, Denslow ND, Schoeb T (1996) Synthetic organic compounds and carp endocrinology and histology in Las Vegas Wash and Las Vegas and Callville Bays of Lake Mead, Nevada, 1992 and 1995. Water Resources Investigations Report 96-4266, US Department of the Interior-US Geological Survey, Carson City, NV, USA 12.

Chung MK (1977) The fishes of Korea. Ilji-sa, Seoul, pp 1-727.

Colborn T, Dunmanski D, Myers JP (1996) Our stolen future. Penguin Books, New York, pp 1-306.

Dong W, Wang L, Thornton C, Scheffler BE, Willett KL (2008) Benzo(a)pyrene decreases brain and ovarian aromatase mRNA expression in *Fundulus heteroclitus*. Aquat Toxicol 88:289-300.

Fertuck KC, Kumar S, Sikka HC, Matthews JB, Zacharewski TR (2001) Interaction of PAH-related compounds with the and isoforms of the estrogen receptor. Toxicol Lett 121:167-177.

Hoffman JL, Oris JT (2006) Altered gene expression: a mechanism for reproductive toxicity in zebrafish exposed to benzo[a]pyrene. Aquat Toxicol 78:332-340.

Hwang IJ, Kim HW, Kim JK, Lee YD, Baek HJ (2010) Estrogenicity of 4-nonylphenol and diethylstilbestrol on *in vitro* oocyte maturation of the dusky tripletooth goby, *Tridentiger obscurus*. Anim Cells Syst 14:161-167.

Hwang IJ (2011) The effect of benzo[a]pyrene on reproductive characteristics of chameleon goby, *Tridentiger trigonocephalus*. Ph.D. Thesis, Pukyong National University, Busan, Korea.

Kawanishi M, Watanabe T, Hagio S, Ogo S, Shimohara C, Jouchi R, Takayama S, Hasei T, Hirayama T, Oda Y, Yagi T (2009) Genotoxicity of 3,6-dinitro benzo[e]pyrene, a novel mutagen in ambient air and surface soil, in

mammalian cells *in vitro* and *in vivo*. Mutagenesis 24: 279-284.

Kim YU, Han KH. (1990) Early life history and spawning behavior of the gobiid fish, *Tridentiger trigonocephalus* (Gill) reared in the laboratory. Kor J Ichthyol 3:1-10.

Kobayashi M, Aida K, Sakai H, Kaneko T, Asahina K, Hanyu I, Ishii S (1987) Radioimmunoassay for salmon gonadotropin. Nippon Suisan Gakk. 53:995-1003.

Luciano AM, Franciosi F, Lodde V, Corbani D, Lazzari G, Crotti G, Galli C, Pellizzer C, Bremer S, Weimer M, Modina S (2010) Transferability and inter-laboratory variability assessment of the *in vitro* bovine oocyte maturation (IVM) test within ReProTect. Reprod Toxicol 30:81-88.

Maccubin AE (1994) DNA adduct analysis in fish: laboratory and field studies. In: Malins DC, Ostrander GK. (Eds.), Aquatic Toxicology and Cellular Perspectives. CRC Press, Boca Raton, FL, pp 267-294.

McElroy AE, Cahill JM, Sisson JD, Kleinow KM (1991) Relation bioavailability and DNA adduct formation of benzo(a)pyrene and metabolites in the diet of the winter flounder. Comp Biochem Physiol 100C:29-32.

Meador JP, Stein JE, Reichert WL, Varanasi U (1995) Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. Rev Environ Contam Toxicol 143:79-165.

Nagahama Y, Yoshikuni M, Yamashita M, Tanaka M (1994) Regulation of oocyte maturation in fish. In: Sherwood NM, CL Hew (ed.), Fish Physiology. V13. Molecular Endocrinology of Fish. Academic Press, London, pp 393-439.

Neal MS, Zhu J, Holloway AC, Foster WG (2007) Follicle growth is inhibited by benzo-[a]-pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay. Hum Reprod 22:961-967.

Patel MR, Scheffler BE, Wang L, Willett KL (2006) Effects of benzo(a)pyrene exposure on killifish (*Fundulus heteroclitus*) aromatase activities and mRNA. Aquat Toxicol 77:267-278.

Patiño R, Sullivan CV (2002) Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol Biochem 26:57-70.

Robinson CD, Brown E, John A. Craft, Davies IM, Megginson C, Miller C, Moffat CF (2007) Bioindicators and reproductive effects of prolonged 17β-oestradiol exposure in a marine fish, the sand goby (*Pomatoschistus minutus*). Aquat Toxicol 81:397-408.

Rocha Monteiro PR, Reis-Henriques MA, Coimbra J (2000) Polycyclic aromatic hydrocarbons inhibit *in vitro* ovarian steroidogenesis in the flounder (*Platichthys flesus* L.). Aquat Toxicol 48:549-559.

Schlenk D, George S, James M, Hurk PVD, Willet K, Kullman S, Celander M, Gallagher E (2008) Biotransformations. In: Di Giulio RT, Hinton D (ed.), The Toxicology of Fishes. Taylor and Francis Publishers, Washington DC, pp 153-234.

Scholz S, Mayer I (2008) Molecular biomarkers of endocrine disruption in small model fish. Mol Cell Endocinol 293:57-70.

Scott AP, Sheldrick EL, Flint APF (1982) Measurement of 17α,20β-dihydroxy-4-pregnen-3-one in plasma of trout (*Salmo gairdneri* Richardson): Seasonal changes and response to salmon pituitary extract. Gen Comp Endocrinol 46:444-451.

Smeets JS, Holsteijin I, Giesy JP, Seinen W, Van Den Berg M (1999) Estrogenic potencies of several environmental pollutants, as determined by vitellogenin induction in a carp hepatocyte assay. Toxicol Sci 50:206-213.

Tokumoto T, Tokumoto M, Horiguchi R, Ishikawa K, Nagahama Y (2004) Diethylstilbestrol induces fish oocyte maturation. Proc Natl Acad Sci USA 101:3686-3890.

Van Metre PC, Barbara JM, Edward TF (2000) Urban sprawl leaves its PAH signature. Environ Sci Technol 34:4064-4070.

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