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Induction of *In Vitro* Vitellogenin Synthesis by Bisphenol, Nonylphenol and Octylphenol in Chinese Minnow (*Phoxinus oxycephalus*) Hepatocytes

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Bisphenol A (BPA), nonylphenol (NP), and 4-tert-octylphenol (OP) are known endocrine disrupting chemicals (EDCs) with estrogenic activity in fish. This study compared the effects of BPA, NP and OP on *in vitro* vitellogenin (VTG) synthesis in primary cultures of hepatocytes of the Chinese minnow *Phoxinus oxycephalus*. The VTG secreted into the culture medium was measured using enzyme-linked immunosorbent assay (ELISA), which we developed in this study using an antibody prepared from homogenates of Chinese minnow egg. VTG synthesis was induced by estradiol-17β (E_2) and phenols (BPA, NP and OP) treatment. E_2 at concentrations of 10^{-6} M or higher increased VTG levels significantly ($P<0.05$). Exposure to 10^{-5} M BPA or 10^{-4} M NP and OP induced *in vitro* VTG synthesis ($P<0.01$). However, 10^{-3} M BPA, NP or OP did not induce VTG synthesis. These results suggest that BPA has the highest estrogenic potential in Chinese minnow hepatocytes. Tamoxifen, an anti-estrogen, drastically blocked the production of VTG by phenols (BPA, NP and OP) suggesting that phenols (BPA, NP and OP) may act via binding to estrogen receptor (ER) in Chinese minnow hepatocytes.

In oviparous female fish, vitellogenin (VTG) is synthesized in the liver, exported to the general circulation and incorporated into developing oocytes (Wallace and Selman, 1981; Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994). In oocyte, VTG is processed into the yolk proteins that comprise the principal nutrient reserve of developing embryos (Hara et al., 1984). Administration of estradial-17β (E_2) to immature fish or mature male fish induces VTG accumulation in the blood (Maitre et al., 1986; Takemura and Kim, 2001). Sumpter and Jobling (1995) suggested that the production of VTG in male can be considered a sensitive bioindicator for exposure to exogenous estrogenic compounds (so-called ‘environmental estrogen’).

In the last decade, there has been increasing concern about the impact of synthetic substances in the environment that have the potential to endocrine systems (Colborn, 1995; Colborn and Clement, 1992; Gray et al., 1998; Harries et al., 1996; Matthiessen and Sumpter, 1998; Sumpter and Tyler, 1996; Tyler et al., 1998). Compounds that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, which regulate homeostasis, reproduction, development and behavior, are referred to as endocrine disrupting chemicals (EDCs) (Nishi et al., 2002). Several studies have demonstrated that various widely used chemical compounds can disrupt the sexual development and reproductive competence of fish (Harries et al., 1997; Jobling et al., 1998). Much of the research of the effects of EDCs in the wild has focused on fish (Celius et al., 1999; Jobling et al., 1996; Gray and Metcalfe, 1997), for the following reasons. First, fish are relatively easy to work with; second, the endocrine system of fish is similar to the endocrine systems of higher vertebrates; and finally, wild populations of fish in many locations exhibit disruption of endocrine function (Bond, 1979; Harries et al., 1996; Lye et al., 1997).

At least one important group of EDCs, the alkylphenols (including nonylphenol; NP and 4-tert-octylphenol; OP) and bisphenol A (BPA), is thought to disrupt endocrine

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function by mimicking the actions of endogenous E_2 (Jobling and Sumpter, 1993; White et al., 1994). The estrogenic effects of these compounds include elevation of VTG levels in some species of fish, such as eelpout, *Zoarces viviparus* (Korsgaard and Pedersen, 1998), rainbow trout (Kristine et al., 2003) and tilapia (Kim et al., 2002), and reproductive impairment in carp, *Cyprinus carpio* (Smeets et al., 1999), testicular changes in several species such as platyfish, *Xiphophorus maculatus* (Kinnberg et al., 2000) and eelpout, *Z. viviparus* (Christiansen et al., 1998).

At present, concern about the presence of alkylphenols (including NP and OP) and BPA in the environment has resulted in the development of screening assays to detect substances having hormonal activity. Ashby (2000) reported that *in vitro* screening assays are the most appropriate type of assay for assessing the hazardous effects of alkylphenols and BPA. Several *in vitro* assays for estrogenic potency have been developed. These assays have exploited the hypothesized receptor-mediated mechanism of action of alkylphenols and BPA to identify alleged environmental estrogens, and have helped establish the relative potency of different estrogenic compounds (Zacharewski, 1997). The challenge at present is to shift assays from the culture dish to the whole organism, in order to assess the effects of environmental estrogens *in vivo*. It is not yet known whether *in vitro* assays can be calibrated and validated to assess development and reproductive performance *in vivo* (Zacharewski, 1997; Ashby, 2000). Consequently, studies that compare *in vitro* and *in vivo* effects of potentially hazardous environmental compounds in the whole organism are required to evaluate the usefulness of *in vitro* assays for assessing the whole organism (Zacharewski, 1997).

In the present study, the Chinese minnow (*Phoxinus oxycephalus*, Leuciscinae, Cyprinidae), a freshwater native of Jeju in Korea, was used as an experimental model. This species is an excellent model for studying the effects of EDCs, because it reproduces continuously and is likely exposed to EDCs and environmental pollutant (agrochemicals) in its natural environment. We assessed the ability of three different phenols, namely BPA, nonylphenol (NP) and 4-tert-octylphenol (OP), to induce VTG synthesis in this species, and compared their estrogenic potential on VTG synthesis in primary cultures of hepatocytes.

Material and Methods

Experimental fish

The Chinese minnow *P. oxycephalus*, used in the present study, were caught in streams of Jeju, Korea. The fish were reared in freshwater tanks with recirculating system (temperature 18°C, photoperiod 12L : 12D) at Marine and Environmental Research Institute, Cheju National University, Jeju, Korea. They were fed two times per day with commercial diet. Their body weight range was 1.2 to 2.5 g. They were starved for one day before the start of the experiments.

Estradiol-17β injection

A group of male Chinese minnow (n=10, BW: 1.5~2.0 g) were anesthetized with 2-phenoxyethanol and injected intraperitoneally with 5 μg^{-1} BW of E_2, which was dissolved in ethanol and diluted with peanut oil. 2-phenoxyethanol and peanut oil were purchased from Sigma Aldrich. Three d after the injection, blood was collected from the caudal aorta with heparinized syringe and then centrifuged at 15,000 rpm for 10 min at 4°C to separate plasma. Plasma obtained was used for immunodiffusion, electrophoresis and Western blot. The samples were stored at -70°C until use.

Electrophoresis and Western blot

The plasma samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the method of Laemmli (1970) using a 7.5% separating gel and 4.5% stacking gel. For SDS-PAGE, samples were diluted in sample buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.2% bromphenol blue). Electrophoresis was carried out at 25 mA gel over 4 h. Molecular weight markers (myosin 212.0 kDa, MBP-β-galactosidase 158.0 kDa, β-galactosidase 116.0 kDa, phosphorylase 97.2 kDa, serum albumin 66.4 kDa and glutamic dehydrogenase 55.5 kDa) were used to determine the molecular weights of the proteins of interest in gels. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB R-250; Sigma) for 5~10 min.

Western blotting after SDS-PAGE was performed using a nitrocellulose membrane (Bio-Rad) and transfer buffer (25 mM Tris, 192 mM glycine, and 200 ml methanol per liter). After transfer at 300 mA for 3 h, nonspecific binding of the membranes were blocked in 25 mM Tris-buffered saline containing 0.05% Tween 20 (TBS-T) added 5% skim milk for 1 h at room temperature. The nitrocellulose membranes were incubated with primary antibody at a dilution of 1:10,000 in TBS for 1 h at room temperature. After washing three times with TBS-T, the membranes were treated in TBS-T containing goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:10,000) (Sigma). After washing three times with TBS-T, visualization of immunoreaction was performed by incubating the membrane in a substrate solution of 330 μg/ml nitro blue tetrazolium (NBT; Sigma) and 165 μg/ml of 5-brome-4-chloro-3-indolyl phosphate (BCIP; Sigma) in 0.1 M Tris-HCl buffer containing 100

mM NaCl and 5 mM $MgCl_2$ (pH 9.5). The color reaction was stopped by transferring the membrane into water.

Preparation of antiserum

Preparations for specific antiserum was carried out in accordance with the method of Takemura et al. (1991). Polyclonal antiserum against egg extracts (a-E) of Chinese minnow induced in rabbit. Ovary was removed from sexually mature females and homogenized in 0.01 M phosphate buffered saline (PBS). The homogenate was centrifuged at 15,000 rpm for 10 min at 4°C to obtain the egg extract. An emulsion of antigen and an equal volume of Freund's complete adjuvant was injected intradermally into the back of the rabbit once per week for 4 weeks. One week after the last injection, blood was collected from the ear artery and allowed to clot at room temperature for 1 h. The serum was collected after centrifugation at 10,000 rpm at 4°C for 10 min.

To remove residual components common to both sexes, four parts of a-E were absorbed with one part of pooled normal male plasma (ab.a-E). The antiserum was obtained after three time with centrifugation at 10,000 rpm at 4°C for 10 min.

Immunodiffusion

Immunodiffusion test was performed according to the method of Ouchterlony (1953). Agarose (Bioneer, USA) was dissolved in 100 ml PBS at a concentration of 1.2%. Gel was reacted with the prepared antibody overnight at 4°C. After washing with PBS for 24 h and drying, the gel was stained to visualize the immune reaction with CBB R-250.

Immunohistochemical staining

Pieces of Chinese minnow ovaries were fixed in Bouin's solution and washed in running water overnight. Pieces were embedded in paraffin and 4~6 μm sections were prepared. After removal of the paraffin, the sections were immunostained using the avidin-biotin complex (ABC kit, Vector Laboratories, Inc.): endogenous peroxidase activity was inhibited with 0.5% periodic acid, non-specific reaction was blocked with normal goat serum in PBS, primary antibody (ab.a-E) was diluted 1:1,000 in PBS and incubated for 12 h at 4°C, secondary antibody (biotinylated goat anti-rabbit antisera) was diluted 1:200 in PBS and incubated for 50 min, ABC kit was diluted with PBS and incubated for 1 h, and 0.05% 3-3 diaminobenzidine (Sigma Co.) in PBS containing 0.003% H_2O_2 was incubated with the sections for 5~10 min. Control sections were obtained by substituting normal goat serum for ab.a-E. Sections were washed three times with PBS between each step. Some sections were stained with haematoxylin-eosin in order to compare with immunohistological observation.

Isolation and culture of Chinese minnow hepatocytes

Isolation and primary cultures of Chinese minnow hepatocytes were carried out in accordance with the methods of Cao et al. (1996). The Chinese minnow was anesthetized with 2-phenoxyethanol (Sigma-Aldrich.) and the liver was carefully removed from the abdominal cavity, transferred onto a glass beaker and perfused with Ca^{2+}-free hepatocyte buffer (HB) (136.90 mM NaCl, 5.40 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, and 5.00 mM $NaHCO_3$, pH 7.6) for 10 min at room temperature. After clearing blood, the liver was digested for 20 min at room temperature with HB containing collagenase (Sigma) at a concentration of 1 mg/ml. The perfused liver section was minced and serially filtered through 100 and 60 μm nylon meshes. The cell suspension was centrifuged three times at 60 g for 1 min at 4°C with HB containing 1.5 mM $CaCl_2$. After final centrifugation, the isolated hepatocytes were seed at a density of 1×10^6 cells/ml in cell culture plates (24-well) (Becton Dickinson, USA) in Leibovitz-15 medium containing 5.00 mM $NaHCO_3$, penicillin (100 μg/ml), polymicin B (10 μg/ml) and streptomycin (70 μg/ml) (Sigma). Viability was >90% as assessed by trypan blue exclusion. The cells were incubated at 18°C under atmospheric air with saturated humidity.

One day after the isolation, the hepatocytes were attached to the wells and appeared as round structures. They conjugated and formed chains at 2 d after culture. At this time, medium was removed and replaced with fresh medium containing test compounds or solvents only.

Hormone and phenols treatment

E_2, three phenols (BPA, NP and OP) and tamoxifen were dissolved in ethanol and added to the culture media after 2 d of preculture. The treatment concentrations of E_2, three phenols (BPA, NP and OP) and tamoxifen were 10^{-6}-10^{-5} M, 10^{-6}-10^{-3} M and 10^{-6} M, respectively. Ethanol concentration in the media never exceeded 0.1%. The media were collected from the culture plate 4 d after hormone treatments (six d culture in total) and used immediately for measurement of VTG levels in the media with an ELISA. E_2, three phenols and tamoxifen were purchased from Sigma.

Enzyme-linked immunosorbent assay

An indirect ELISA method was carried out according to Arukwe et al. (1997). A 96-well microplate was coated with 100 l $well^{-1}$ of standards (serial dilution of VTG) and culture media diluted 1:1,000 in 50 mM carbonate buffer (pH 9.6) for 2 h at room temperature or overnight at 4°C, then washed three times with PBS-Tween 20 (pH 7.4,

containing 0.05% Tween 20). The residual protein binding sites on each well were blocked by adding 200 ml PBS-Tween 20 containing 1% BSA for 1 h at room temperature. After washing the microplate three times with PBS-Tween 20, 100 µl primary antibody (ab.a-E), which was diluted 1:10,000 with PBS-Tween 20, was added to all wells of the plate. Incubation was done for 2 h at room temperature. After washing, each well received 100 µl of HRP-conjugated antibody (secondary antibody) diluted 1:10,000 in the same buffer. The plate was then incubated for 2 h at room temperature. Following three washes with PBS-Tween 20, 100 µl of 0.1 M citrate buffer (pH 4.5), containing 1 mg/ml o-phenylenediamine dihydrochloride (Sigma) and 0.04% H_2O_2 was added to the wells. Color development proceeded for 30 min at room temperature and 25 µl of H_2SO_4 was added to each well to stop the reaction. Absorbance was measured at 450 nM using a precision microplate reader model 650.

Statistical analysis

VTG levels in the culture media were expressed as mean±standard error of the mean (SEM) for three wells. Data were analyzed by one-way ANOVA followed by Scheffe's F-test. The criterion used for statistical significance was $P<0.01$ and $P<0.05$.

Results

Electrophoresis and Western blot

VTG was identified by SDS-PAGE as a band with a molecular weight of 134.0 kDa in the plasma of male fish treated with exogenous E_2, but was not observed in the plasma of untreated males (Fig. 1A). The specificity of antibody, which was raised against Chinese minnow VTG, was confirmed by Western blot. Specifically, the antibody revealed a single band with an estimated molecular weight of 134.0 kDa in plasma from E_2-treated males (Fig. 1B), but did not recognize any protein in the plasma of untreated males.

Immunodiffusion

Vitellogenic female plasma, egg extracts and plasma from E_2-treated males all reacted with antiserum containing ab.a-E antibody, forming a single precipitation product. In contrast, untreated male plasma exhibited no reaction to the antibody (Fig. 2).

Immunohistochemical localization of VTG

Ovaries contained two types of oocyte: mature oocytes in which yolk granules were visible, and immature oocytes at the perinucleolus stage. The ovaries of mature and immature females were stained with haematoxylin-eosin and incubated with ab.a-E antibody (Fig. 3A and B). The yolk granules, follicle layer and thin egg envelope in the oocytes of vitellogenic female fish reacted positively to the ab.a-E antibody (Fig. 3C), whereas immature oocytes failed to exhibit immunopositive reaction (Fig. 3D). There was no immunopositive reaction in control ovary samples (ab.a-E antibody omitted, data not shown).

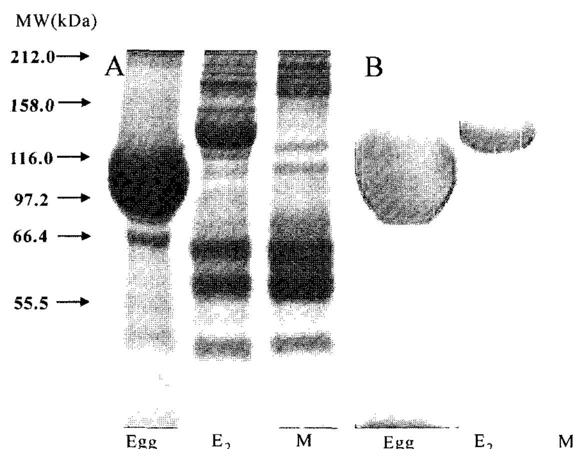

Fig. 1. A, SDS-PAGE (7.5%) pattern of the plasma proteins from the E_2 treated male (E_2), untreated male (M) and egg extracts (Egg) of Chinese minnow. Plasma samples were stained with CBB R-250. Molecular weight (MW) markers were myosin (212.0 kDa), MBP-β-galactosidase (158.0 kDa), β-galactosidase (116.0 kDa), phosphorylase (97.2 kDa), serum albumin (66.4 kDa) and glutamic dehydrogenase (55.5 kDa). B, Western blot of plasma samples. Plasma samples were resolved in SDS-PAGE (7.5%) and blotted onto the nitrocellulose membrane. The antibody (ab.a-E) was used at 10,000.

Enzyme-linked immunosorbent assay

Indirect ELISA was carried out using ab.a-E antibody. The precision of the assay was determined by repeated

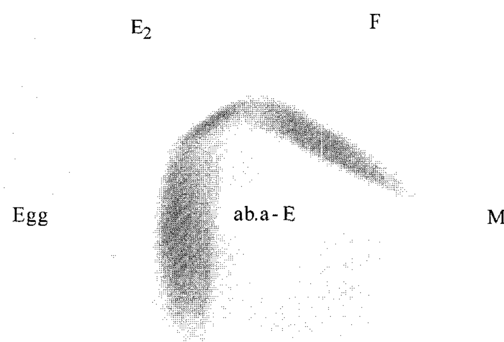

Fig. 2. Immunodiffusion patterns of untreated male plasma (M), vitellogenic female plasma (F), plasma from E_2 treated male fish (E_2) and egg extracts (Egg) against ab.a-E.

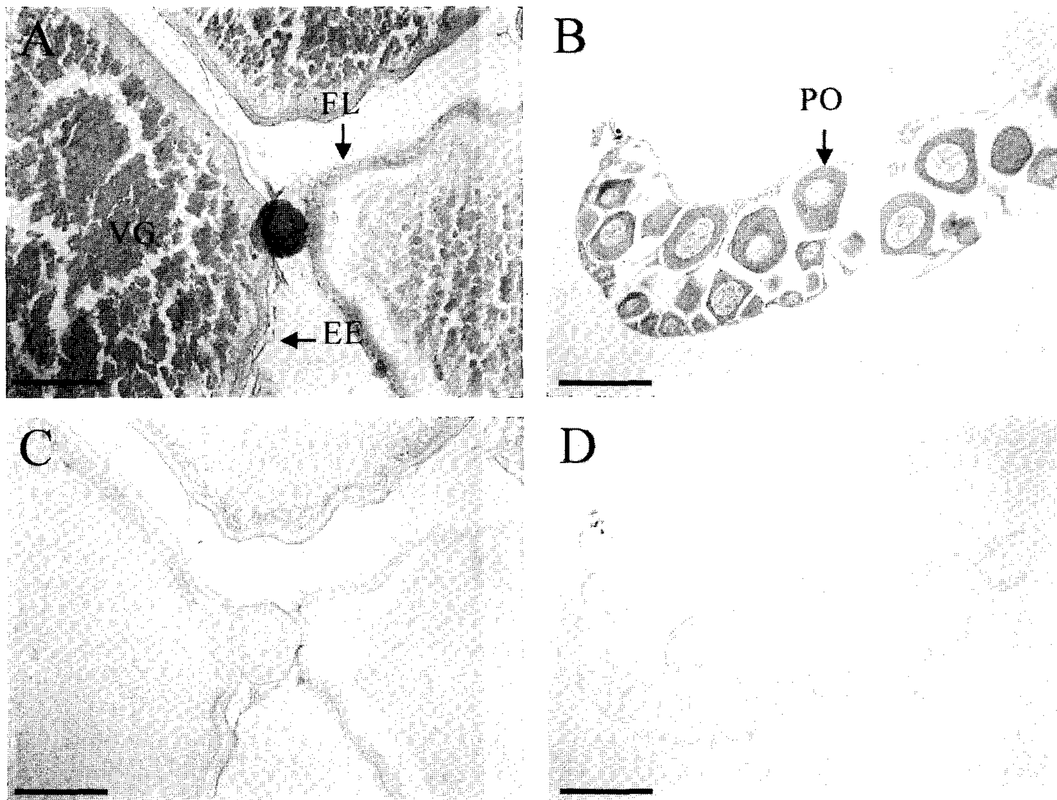

Fig. 3. Immunohistochemical observation of oocyte with ab.a-E. Haematoxylin-eosin staining (mature oocyte; A and Immature oocyte; B); immunological staining with ab.a-E (mature oocyte; C and Immature; D). EE, egg envelope; FL, follicle layer; PO, perinucleolus oocyte; YG, yolk granules. Scale bars=100 μm.

measurement of control samples. The intra-and inter-assay coefficients of variation were 7.2 and 8.6%, respectively. Fig. 4 shows typical assay curves for vitellogenic female plasma, untreated male plasma, and plasma from E_2-treated males. A series of standards and several antigens were compared to determine the ability of antiserum to recognize VTG. Vitellogenic female plasma and plasma from E_2-treated males incubated with ab.a-E antibody paralleled the standard curve. By contrast, untreated male plasma did not react against ab.a-E antibody (Fig. 4).

Induction of VTG synthesis by E_2 and phenols

Vitellogenesis was measured in primary cultures of Chinese minnow hepatocytes. Two after the onset of culture, different concentrations of E_2 (1×10^{-6}-1×10^{-5} M) and BPA, NP and OP (1×10^{-6}-1×10^{-3} M) were added into the culture medium and their effects on VTG synthesis were measured using ELISA.

Control cultures did not receive E_2 or phenols. At 4 d after E_2/phenol treatment, E_2 caused a significant increase in VTG synthesis at concentrations $\geq10^{-6}$ M relative to control cultures (Fig. 5). BPA increased the VTG synthesis at a concentration of 10^{-5} M, but the VTG levels were reduced in response to treatment with 10^{-4} M BPA. NP and OP induced VTG to significantly higher levels at 10^{-4} M. BPA, NP and OP did not increase VTG

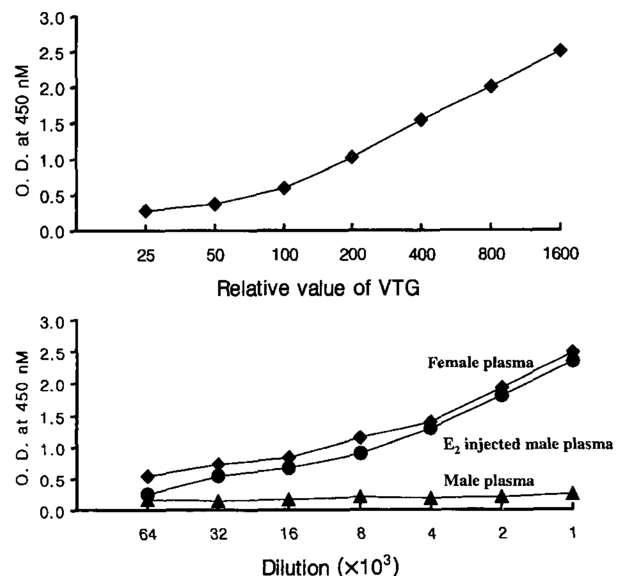

Fig. 4. A typical standard curve of ELISA for ab.a-E. Specificity of ELISA was estimated by parallelism of serial dilutions of vitellogenic female plasma, untreated male plasma and plasma from E_2 treated male fish to standard curve. Each point represents the mean of duplicates.

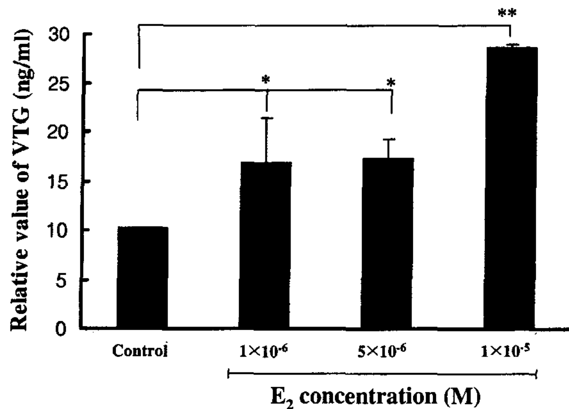

Fig. 5. Effects of E_2 treatment on *in vitro* VTG synthesis in Chinese minnow hepatocytes cultured in L-15 medium. Various concentrations of E_2 were added into the medium 2 days after the onset of culture and the hepatocytes were cultured with E_2 for 2 day. Significant difference at $P < 0.05$(*) and $P < 0.01$(**), respectively.

synthesis and were toxic at 10^{-3} M (Fig. 6-7). Co-treatment of cultures with E_2 or phenols (BPA, NP and OP) and

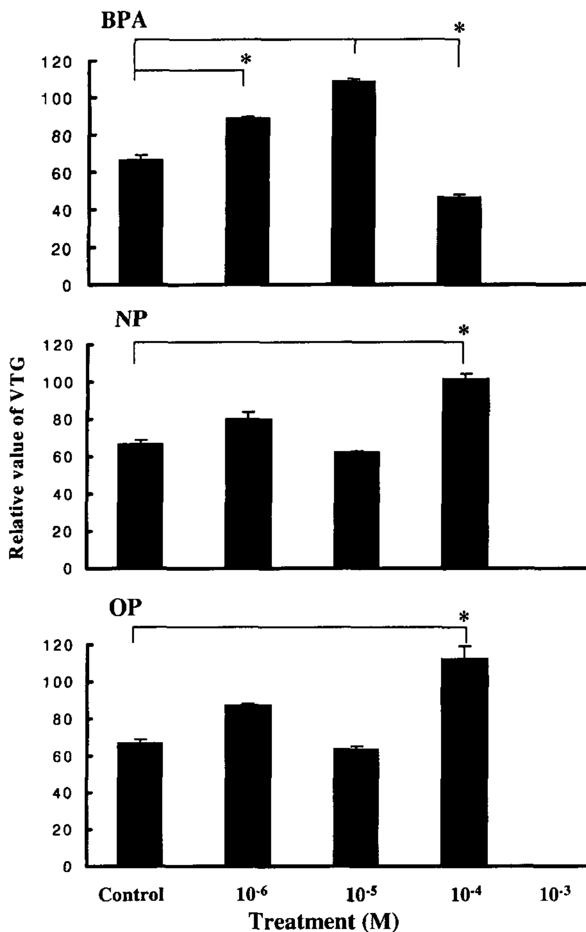

Fig. 6. Induction of *in vitro* VTG synthesis by BPA, OP and NP (from 10^{-6} M to 10^{-3} M) treatment in the primary cultures of Chinese minnow hepatocytes. *Significantly different at $P<0.01$.

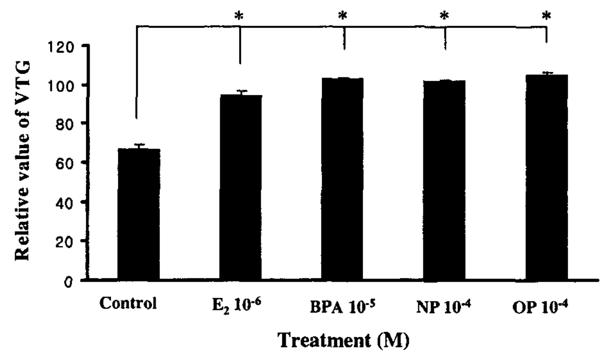

Fig. 7. Comparison of E_2 (10^{-6} M) and three different phenols (BPA, NP and OP) on *in vitro* VTG synthesis in the primary culture of Chinese minnow hepatocytes. *Significant difference at $P<0.01$.

tamoxifen reduced the VTG synthesis (Fig. 8). These results suggest that phenols (BPA, NP and OP) may act via binding to estrogen receptor (ER) in Chinese minnow hepatocytes.

Discussion

VTG in teleost fish has five general characteristics (Hara et al., 1984): it appears in blood of female fish during vitellogenesis, it can be induced in the blood of male and immature female fish by treatment with estrogen, it is a glycolipophosphoprotein complex that binds calcium and iron, it is a precursor of yolk protein, and it reacts with antiserum obtained against egg extract. Several biochemical methods have been used to purify and identify teleost VTG (Wallace and Selman, 1981). In this study, Chinese minnow VTG was immunohistochemically identified using a specific anti-VTG antibody, namely ab.a-E. SDS-PAGE was used to estimate the molecular weight of Chinese minnow VTG. The molecular weight of VTG that was induced in males by treatment with E_2 was

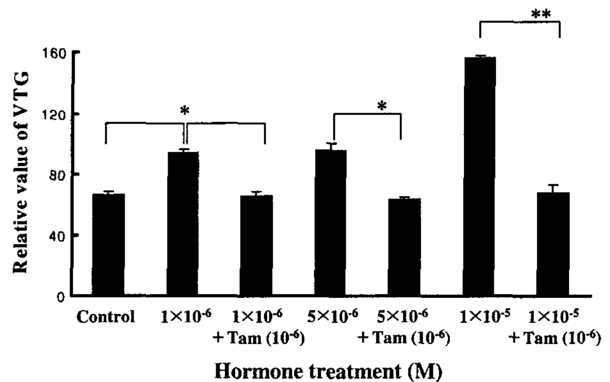

Fig. 8. Effects of co-treatment of E_2 and tamoxifen on *in vitro* VTG synthesis in primary cultures of Chinese minnow hepatocytes. Two days after the onset of culture, E_2 and tamoxifen were added into the medium. Significant difference at $P<0.05$(*) and $P<0.01$(**), respectively.

134 kDa, which is similar to the VTG characterized in medaka (200 kDa; Nishi et al., 2002), coral trout (180 kDa; Takemura and Teruya, 1997), Arctic charr (158 kDa; Johnsen et al., 1999), and zebrafish (134 kDa; Segner et al., 2003). Western blot with ab.a-E antibody revealed a single protein band in the plasma of E_2-treated male fish. These results suggest that the main protein isolated by SDS-PAGE and Western blot in the present study was VTG.

Immunodiffusion patterns revealed a specific reaction with ab.a-E antibody in the plasma of mature female fish and E_2-treated males. The precipitation product in egg extracts containing ab.a-E antibody was the same in female fish and E_2-treated males, but untreated male plasma did not react. In addition, there was a strong immunopositive reaction with the same antibody in the ovary of mature females. Similatly Roubal et al. (1997) identified VTG immunohistochemically in ovaries of English sole (*Pleuronectes vetulus*), rock sole (*Lepidopsetta bilineata*), and starry flounder (*Platichthys stellatus*), using an antibody raised against the plasma VTG of English sole. These results suggest that the antibody used in the present study was specific to VTG.

In the present study, we described the development and validation of indirect ELISA for measuring VTG in Chinese minnow using a polyclonal antibody raised against VTG in egg extracts. The results demonstrated that VTG production could be induced in male Chinese minnow by treatment with E_2, which is consistent with studies on other fishes (Mommsen and Walsh, 1988; Specker and Sullivan, 1994). The parallelism between the standard curve and plasma dilution curve of vitellogenic females and E_2-treated males demonstrated that the antibody used in the present study recognized antigens in these two samples in a similar fashion. The antibody did not show any cross-reactivity with untreated male plasma, indicating that the antibody was specific to VTG.

According to Kwon and Mugiya (1994), VTG synthesis is not induced by the addition E_2 alone. Rather, growth hormone and/or prolactin in addition to E_2 are required to induce the VTG synthesis. Similarly, Peyon et al. (1998) reported that growth hormone was required for the induction of VTG in hepatocytes cultured with E_2. In addition, it has been reported that VTG synthesis is not induced without E_2 priming in hepatocytes cultured with E_2. In the present study, hepatocytes from female and male fish produced VTG in response to 1×10^{-6} M or higher concentrations of E_2, in the absence of E_2 priming. Therefore, E_2 appears to be a strong inducer of VTG synthesis in Chinese minnows, as in other teleosts. However, it is necessary to further investigate how E_2 priming might affect VTG synthesis in this species.

The present study demonstrates that alkylphenols (NP and OP) and BPA can induced VTG synthesis in cultured Chinese minnow hepatocytes. Several authors have reported that VTG synthesis is induced by the same treatment of hepatocytes from channel catfish, *Octalurus punctatus* (Monteverdi and Di Giulio, 1999) and zebrafish, *Danio rerio* (Segner et al., 2003). In primary cultures of rainbow trout hepatocytes, NP induced estrogen receptor and VTG mRNA accumulation (Flouriot et al., 1995). In eelpout (*Zoarces viviparus*), alkylphenols were found to mediate estrogen binding of estrogen receptors (White et al., 1994). Therefore, it is clear that alkylphenols (NP and OP) and BPA have estrogenic effects in fish hepatocytes.

Monteverdi et al. (1999) and Islinger et al. (1999) reported that VTG synthesis was induced by 10^{-6} M NP in hepatocytes from channel catfish and rainbow trout. In the present study, VTG synthesis was induced by NP and OP at a concentration of 10^{-4} M. BPA administered to cultures at 10^{-5} M increased VTG synthesis, but decreased VTG synthesis at 10^{-4} M. Treatment of cultures with phenols at 10^{-3} M caused death of hepatocytes. These results reflect differences in the ability of different phenols to induce VTG synthesis. In addition, It also emphasizas that the estrogenic potential of BPA is greater than that of NP and OP.

By contrast, tamoxifen is a competitive antagonist of estrogen receptor (ER) (Lazier et al., 1996; Mori et al., 1998; Peyon et al., 1997). In the present study, co-treatment with E_2 or phenols (BPA, NP and OP) and tamoxifen reduced VTG synthesis.

In conclusion, the assays used in the present study may be appropriate for field and laboratory investigations of the effects of exposure of Chinese minnows to estrogenic chemicals.

Acknowledgments

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