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FOR THE DEGREE OF MASTER OF SCIENCE

Effects of Three Phenols on Vitellogenin Synthesis in Chinese minnow, *Rhynchocypris oxycephalus* hepatocytes

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DEPARTMENT OF MARINE BIOLOGY
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This thesis has been examined and approved.

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Contents

국문초록

Vitellogenin (VTG; 난황전구물질) 합성은 estradiol-17b (E_2) 처리에 의해 미성숙한 암컷과 수컷 어류의 간에서 유도되었고, 내분비계장애물질들도 (EDCs) 수컷 어류에서 VTG를 합성시켰다. 수컷 어류에서 VTG 생산은 EDCs estrogenic potential에 대한 sensitive biomarker고 간주된다. bisphenol A (BPA), nonylphenol (NP) 그리고 4-tert-octylphenol (OP)는 내분비계장애물질로 널리 알려졌고 어류에서 estrogenic 활성을 가진다. 이 실험에서, BPA, NP 그리고 OP가 버들치의 초대 간세포 배양에서 *in vitro*에서 VTG합성을 비교하였다.

E_2 처리된 수컷 어류의 plasma에 VTG 분자량은 SDS-PAGE로 분석한 결과 134.0kDa이었다. Western-blot은 제작된 항체 (ab.a-E)가 E_2 처리된 수컷 어류의 plasma와 하나의 band를 형성하였다. 성숙한 암컷과 E_2 처리된 수컷 어류의 VTG는 면역화학적인 방법으로 탐색하였다. 면역확산양상은 암컷 plasma, 난추출물 그리고 E_2 처리된 수컷 어류의 plasma가 항체와 반응하였다. 면역조직화학적 반응은 성숙한 난모세포의 난황과립, 여포세포층 그리고 난막들이 항체와 강하게 반응하였다. 간세포 초대배양을 위해, 간세포는 collagenase의해 분리되었고, 분리된 간세포들은 18℃ 조건의 Leibovize-15 medium에 배양하였다. E_2, 페놀류 (BPA, NP 그리고 OP) 그리고 tamoxifen은 에탄올에 용해시키고 초대 배양이틀 후에 배양액에 첨가하였다. E_2, 페놀류 그리고 tamoxifen은 각각 10^{-6}-10^{-5}M, 10^{-6}-10^{-3}M 그리고 10^{-6}M 농도로 처리하였다. 배양액들은 호르몬 처리 4일 후에 culture plate에서 회수하였다. 배양액에 분비된 VTG는 버들치의 난 추출물에서 개발한 항체를 사용하여 ELISA로 측정하였다. VTG 합성은 E_2와 페놀류 (BPA, NP 그리고 OP) 처리에 의해 유도되었다. E_2 10^{-6}M 이상 농도는 VTG 양의 증가를 초래하였다 ($P<0.05$). BPA 10^{-5}M 그리고 NP, OP 10^{-4}M에서 VTG 합성이 유도되었다($P<0.01$). 그러나, BPA, NP 그리고 OP 10^{-3}M은 독성 효과로 VTG 합성이 유도되지 않았다. 이러한 결과들은 BPA가 버들치 간세포에 있어 estrogenic potential이 높은 것으로 사료된다. E_2와 tamoxifen을 첨가한 처리구는 VTG양이 감소하여 나타났다. tamoxifen은 E_2에 의한 VTG의 생산을 저해하여, anti-estrogen으로 생각되어진다. 앞으로, BPA, NP 그리고 OP가 *in vivo* 하에 VTG 합성을 비교하고, E_2 priming와 anti-estrogen 같은 다양한 호르몬이 VTG 합성효과에 대한 비교검토가 요구된다.

List of Figures

Ⅰ. Introduction

In oviparous female fish, vitellogenin (VTG) is synthesized in liver, exported to the general circulation and incorporated into developing oocytes (Mommsen and Walsh, 1988; Specker and Sullivan, 1994; Wallace, 1985; Wallace and Selman, 1981). Here, VTG is also processed into the yolk proteins that comprise the principal nutrient reserve of developing embyos (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, section, 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 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 such as alkylphenols and BPA that have 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 expoited 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 calibrate 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 (*Rhynchocypris oxycephalus*, Leuciscinae, Cyprinidae), a freshwater native of Jeju in Korea, was used as an experimental model. Ths 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 to their estrogenic potential on VTG synthsis in primary cultures of hepatocytes.

Ⅱ. Material and Methods

1. Experimental fish

The Chinese minnow *R. oxycephalus*, used in the present study were caught in stream of Jeju, Korea. The fish were reared in freshwater tanks with recirculating system (temperature 18℃, 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 during one day before the start of the experiments.

2. Estradiol-17β injection

A group of male Chinese minnow (n=10, BW: 1.5~2.0g) were anesthetized with 2-phenoxyethanol and injected intraperitoneally with 5 μg^{-1} BW of E_2, which was dissolved in ehanol and diluted with peanut oil. 2-phenoxyethanol and peanut oil were purchased from Sigma Aldrich Company. After 3 days injection, blood was collected from the caudal aorta with heparinized syringe and then centrifuged at 15,000 rpm for 10 min at 4℃ to seperate their plasma. Plasma obtained was used for immunodiffusion, electrophoresis and Western blot. The samples were stored at −70℃ until use.

3. Electrophoresis and Western bolt

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, 0.2% bromphenol blue). Electrophoresis was carried out at 25 mA gel over 4 h for SDS gels using power supply. Molecular weight markers (myosin 212.0 kDa, MBP-β-galactosidase 158.0 kDa, β-galactosidase 116.0 kDa, phosphorylase 97.0 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 Co.) 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, 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 1h 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 transferred 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.

4. Preparation of antiserum

Preparations for specific antiserum was carried out in accordanece 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 female, and the ovary was homogenized in 0.01 M phosphate buffered saline (PBS). The homogenate was centrifuged at 15,000 rpm for 10 min at 4℃ to obtain the egg extracts. An emulsion of antigen and an equal volume of Freund's complete adjuvant were injected intradermally into the back of the rabbit once per week for 4 weeks. One week after the last injection, blood was collected from an ear artery of rabbit and blood obtained was allowed to clot at room temperature for 1h. The serum was collected after centrifugation at 10,000 rpm at 4℃ 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℃ for 10 min.

5. Imunodiffusion

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 prepared antibody for overnight at 4℃. After washing with PBS for 24 h and dry, gel was stained for represent precipitin reaction with CBB R-250.

6. Immunohistochemical staining

Pieces of Chinese minnow ovaries and livers were fixed in Bouin's solution and washed in running water for overnight. Pieces were embedded in paraffin (56℃) and 4~6 ㎛ 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℃ in chamber, 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% 3-3diaminobenzidine (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 hematoxylin-eosin in order to compare with immunohistological observation.

7. Isolation and culture of Chinese minnow hepatocytes

Isolation and primary cultures of Chinese minnow hepatocytes were done in accordance with the methods of Cao et al. (1996). The Chinese minnow was anesthetized with 2-phenoxyethanol (Sigma-Aldrich, Co.) and the liver was carefully removed from the abdominal cavity of anesthetized fish, 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, 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 filtered through 100 and 60 ㎛ nylon meshes. The cell suspension was centrifuged three times at 60 g for 1 min at 4℃ with HB containing 1.50 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 ㎍/ml), polymicin B (10 ㎍/ml) and streptomycin (70 ㎍/ml) (Sigma Chemical Co.). Viability was >90% as assessed with trypan blue exclusion. The cells were incubated at 18℃ 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 days after culture. At this time, medium was removed and replaced with fresh medium containing test compounds or solvents only.

8. 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 two days 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 days after hormone treatments (six days 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 Chemical Co.

9. 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 μl $well^{-1}$ 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℃, 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 of each well was measured at 450 nM using a precision microplate reader model 650.

10. 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 $P<0.05$.

Ⅲ. Results

1. Electrophoresis and Western blot

VTG was identified by SDS-PAGE as a band with a molecular weight of 134 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 kDa in plasma from E_2-treated males (Fig. 1B), but did not recognize any protein in the plasma of untreated males.

2. 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).

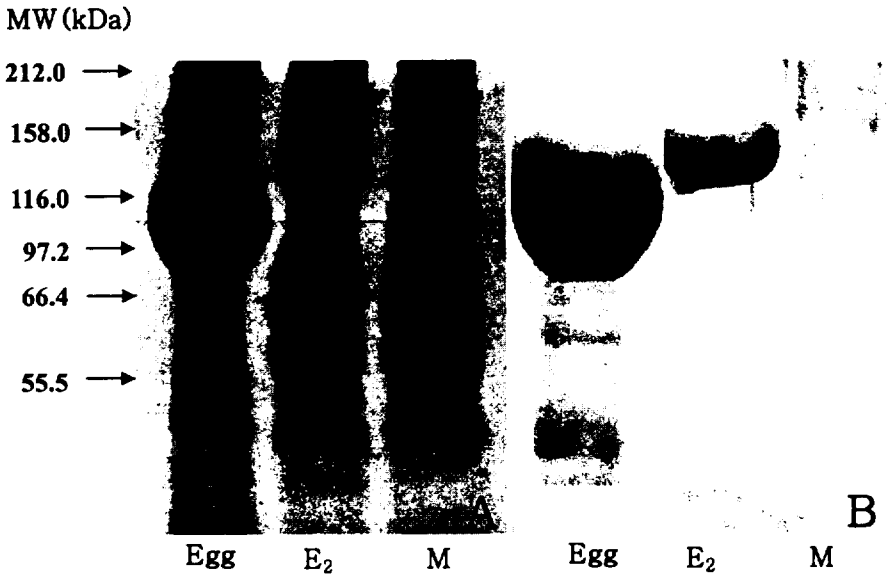

Fig. 1. (A) SDS-PAGE (7.5%) pattern of the plasma proteins from the E_2 treated male (E_2), untreated male (M) and egg extracts (Egg) of Chinese minnow. Plasma samples were stained with CBB R-250. Molecula weight (MW) markers were myosin (212.0 kDa), MBP-β-galactosidase (158.0 kDa), β-galactosidase (116.0 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 run with SDS-PAGE (7.5%) and blotted onto the nitrocellulose membrane. The antibody (ab.a-E) was used at 10,000.

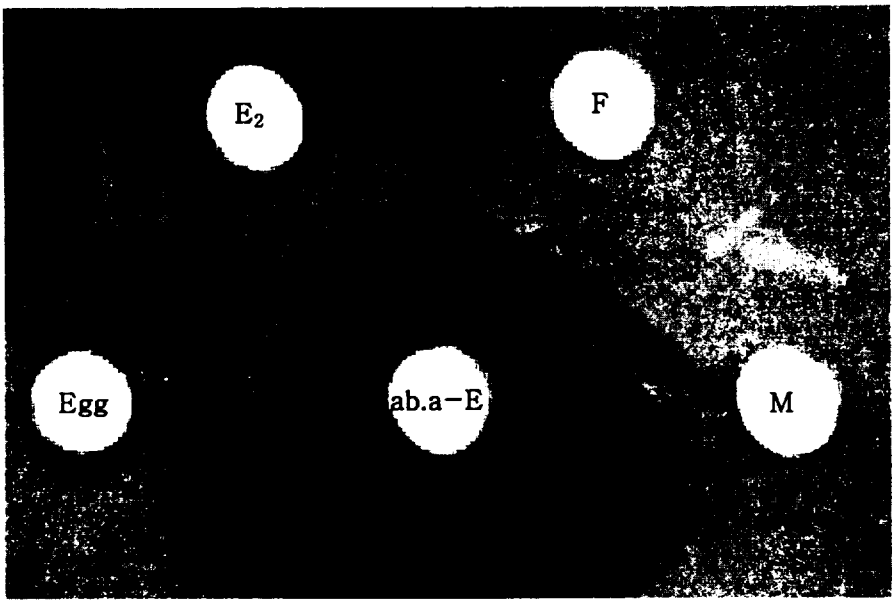

Fig. 2. Immunodiffusion patterns of untreated male plasma (M), vitellogenic female plasma (F), plasma from E_2 treated male fish (E_2) and egg extracts (Egg) against ab.a-E.

3. 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 liver and ovaries of mature and immature females were stained with hematoxylin-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 an immunopositive reaction (Fig. 3D). In addition, in liver stained with hematoxylin-eosin (Fig. 3E and F), the cytoplasm of hepatocytes in liver from mature females reacted positively to the ab.a-E antibody (Fig. 3G), but similar samples from immature females did not exhibit any immunoreactivity (Fig. 3H). There was no immunopositive reaction in control ovary and liver samples (ab.a-E antibody omitted, data not shown).

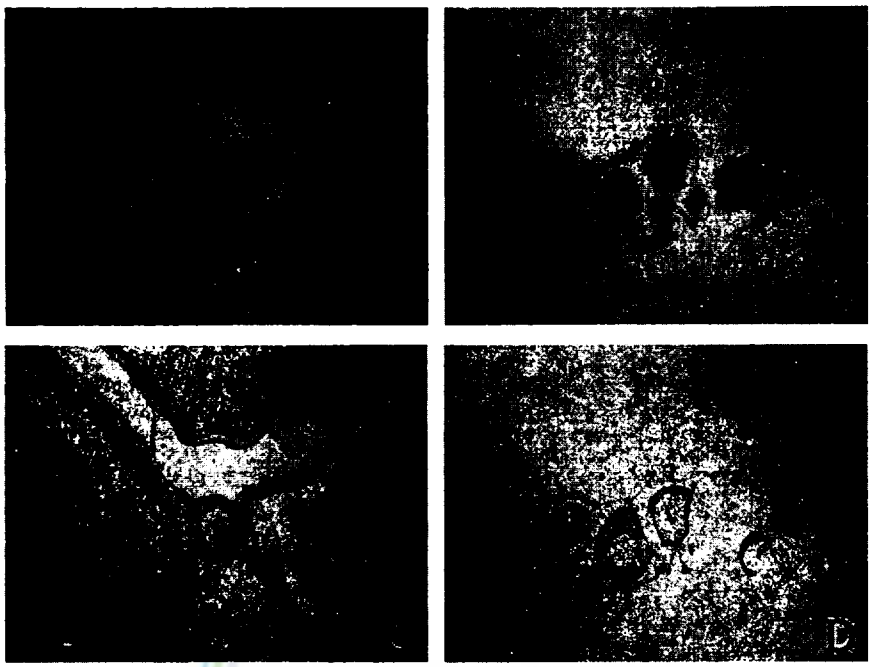

Fig. 3. Immunohistochemical observation of oocyte with ab.a-E. Haematoxylin-eosin staining (Mature oocyte; A and Immature oocyte; B); immunological staining with ab.a-E (Mature oocyte; C and Immature; B). EE; egg envelope, FL; follicle layer, PO; perinucleolus oocyte, YG; yolk granules. Scale bars =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

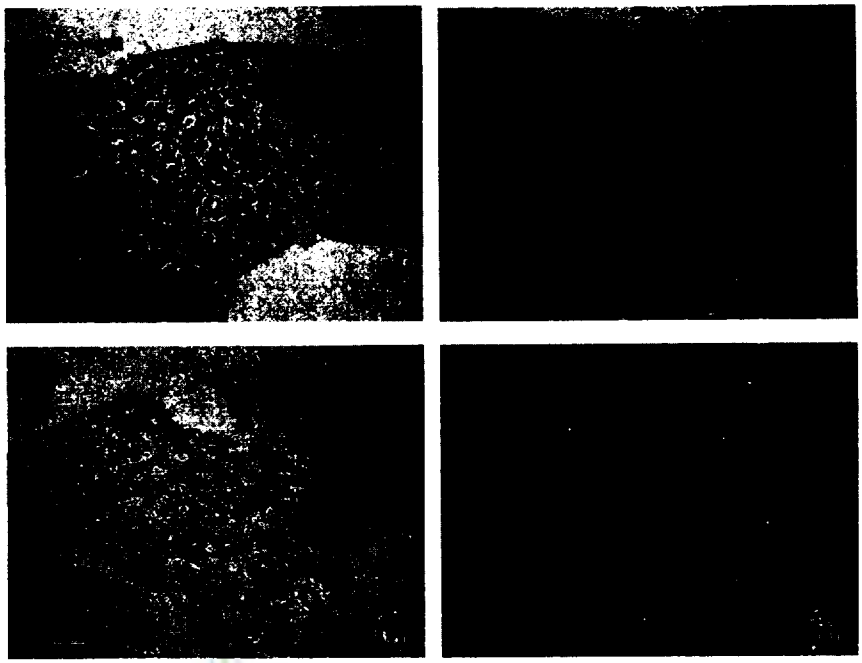

Fig. 3. Immunohistochemical observation of liver with ab.a-E. Haematoxylin-eosin staining (Mature liver; E and Immature liver; F); immunological staining with ab.a-E (Mature; G and Immature liver; H). Scale bars = 100 ㎛.

4. Enzyme-linked immunosorbent assay

Indirect ELISA was carried out using ab.a-E antibody. The precision of the assay was determined by repeated measurement of control samples. The intra-and inter-assay coefficients of variation were 7.2 and 8.6%, respectively. Figure 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).

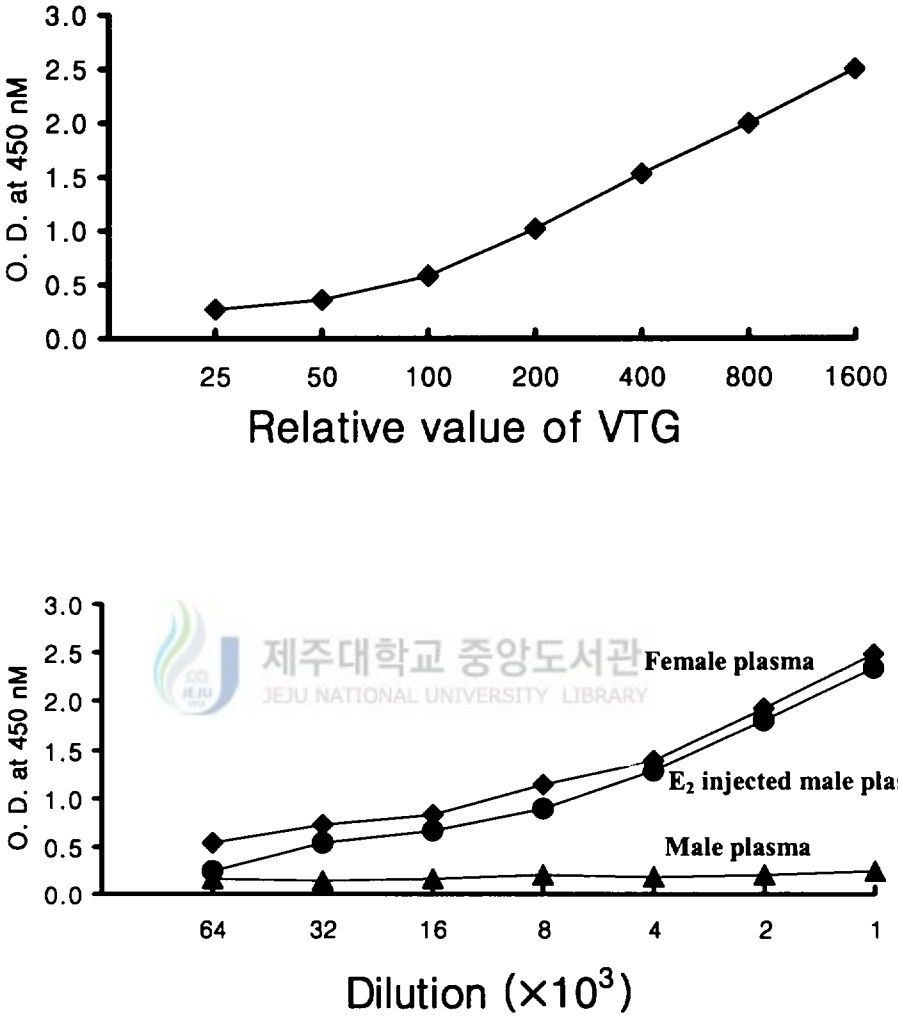

Fig. 4. A typical standard curve of ELISA for ab.a-E. Specificity of ELISA was estimated by parallelism of serial dilutions of vitellogenic female plasma, untreated male plasma and plasma from E_2 treated male fish to standard curve. Each point represents the mean of duplicates.

5. Induction of VTG synthesis by E_2 and phenols

Vitellogenesis was measured in primary cultures of Chinese minnow hepatocytes (Fig. 5). Freshly isolated hepatocytes were round and had lipid granules in the cytoplasm (Fig. 5A). Two days after the onset of culture, the hepatocytes fused together to form chain-like aggregations (Fig. 5B). At this stage, 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 day (4 days after E_2/phenol treatment), E_2 caused a significant increase in VTG synthesis at concentrations $\geq10^{-6}$ M relative to control cultures (Fig. 6). BPA increased VTG synthesis at a concentration of 10^{-5} M, but VTG levels were reduced in response to treatment with 10^{-4} M BPA. NP and OP induced significantly higher VTG levels at a concentration of 10^{-4} M. BPA, NP and OP did not increase VTG synthesis and were toxic at a concentration of 10^{-3} M (Fig. 7-8). Cotreatment of cultures with E_2 and tamoxifen reduced VTG synthesis (Fig. 9).

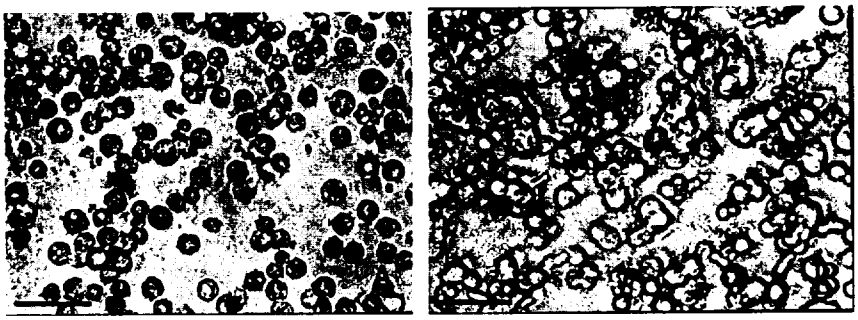

Fig. 5. Phase contrast photomicrographs of cultured Chinese minnow hepatocytes. A; fresh isolated cell, B; colonies of conjugated hepatocytes at 2 days after culture. Scale bars = 25 ㎛.

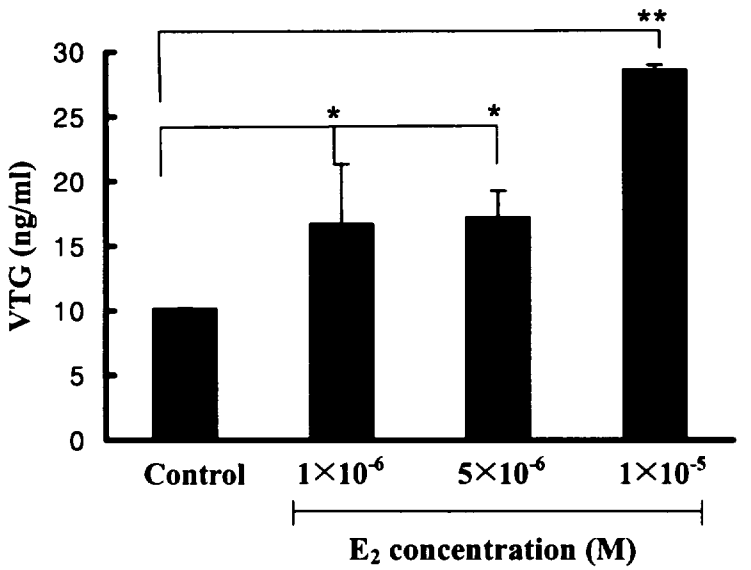

Fig. 6. Effects of E_2 treatment on *in vitro* VTG synthesis in Chinese minnow hepatocytes cultured in L-15 medium. Various concentrations of E_2 were added into the medium 2 days after the onset of culture and the hepatocytes were cultured with E_2 for 2 day. Asterisks * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively.

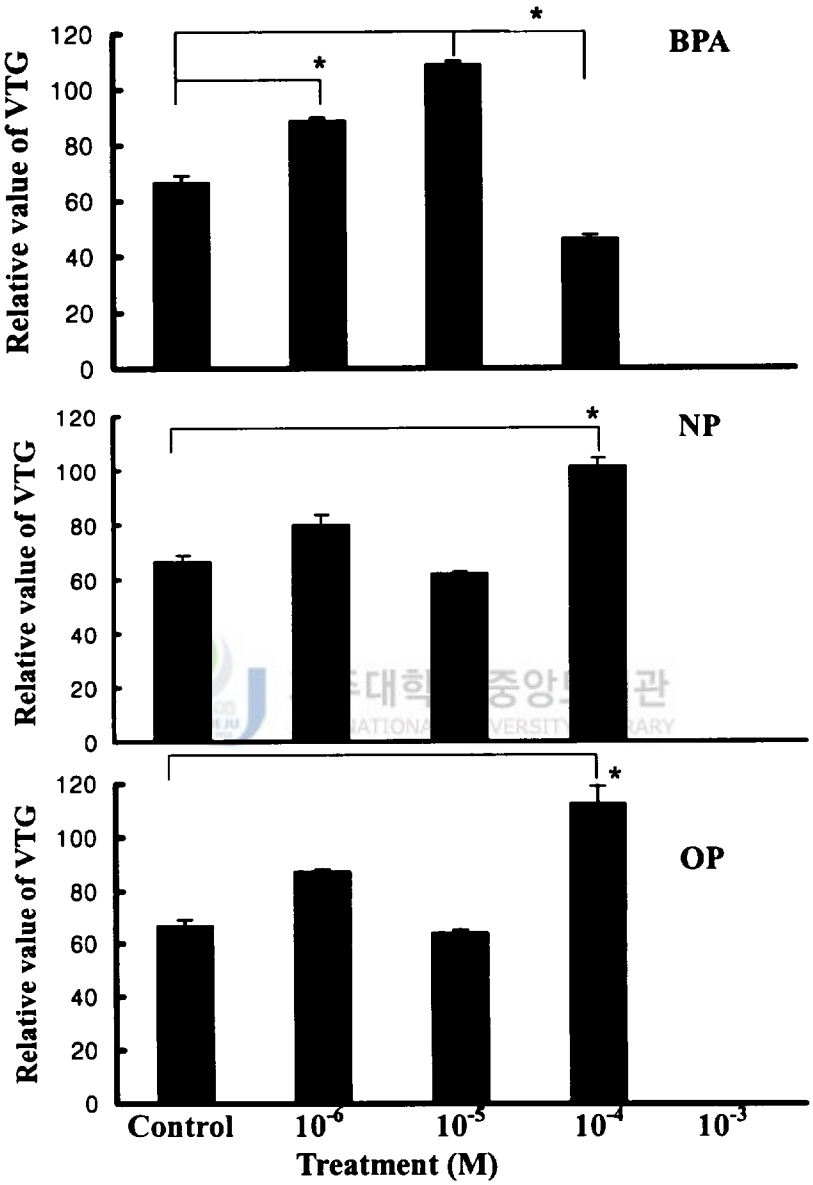

Fig. 7. Induction of *in vitro* VTG synthesis by BPA, OP and NP (from 10^{-6} M to 10^{-3} M) treatment in the primary cultures of Chinese minnow hepatocytes. Asterisks* indicates significantly difference at $P<0.01$, respectively.

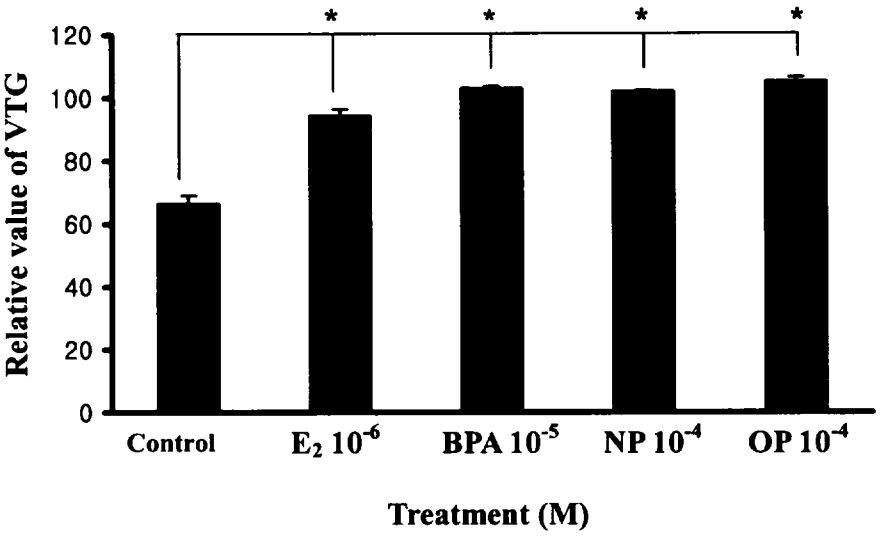

Fig. 8. Comparison of E_2 (10^{-6}) and three different phenols (BPA, NP and OP) on *in vitro* VTG systhesis in the primary culture of Chinese hepatocytes. Asterisks indicate significant difference at $P < 0.01$.

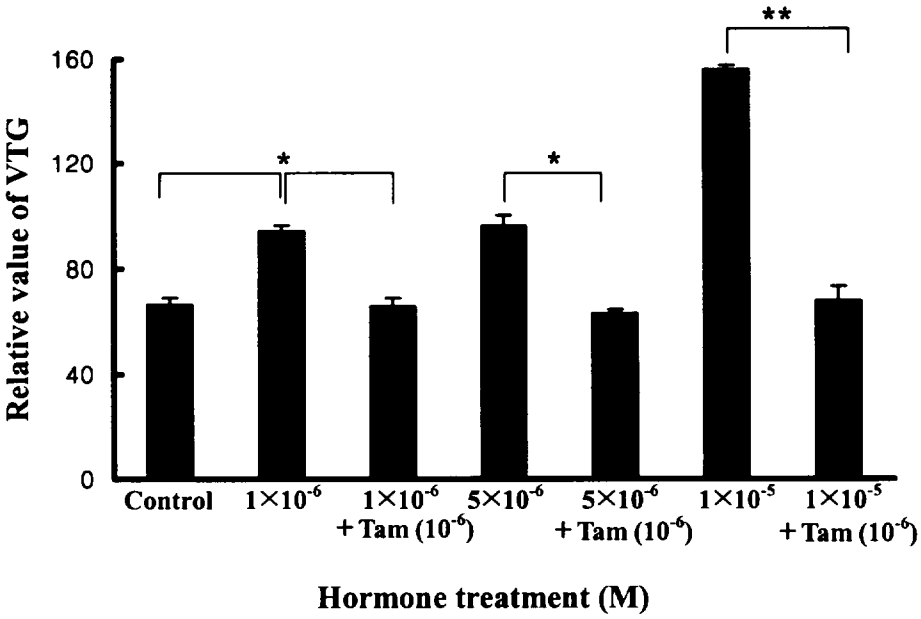

Fig. 9. Effects of co-treatment of E_2 and tamoxifen on *in vitro* VTG synthesis in primary cultures of Chinese minnow hepatocytes. Two days after the onset of culture, E_2 and tamoxifen were added into the medium. Asterisks * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively.

Ⅳ. 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 precursory form 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 134 kDa, which is similar to VTG characterizeds 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 Westen blot in the present study was VTG.

Imunodiffusion 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 containig 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 and liver of mature females. 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 anti-VTG antibody from the plasma 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 species of fish (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 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 present study, hepatocytes from female and male fish produced VTG in response to 1×10^{-6} M or higher concentrations of E_2, even though E_2 priming was not used. 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 madiate 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 the death of hepatocytes. These results reflect differences in the ability of different phenols to induce VTG synthesis. In addition, they suggest 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 and tamoxifen reduced VTG synthesis. Therefore, tamoxifen can block the increased production of VTG results from treatment with E_2, and, therefore, can be considered to be antiestrogenic.

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

environmental contaminates. In addtion, our finding that the estrogenic potential of BPA is greater than NP and OP and that tamoxifen has antiestrogenic effects suggests that it would be useful to investigate the effects of these substances *in vivo*.

V. Summary

Estradiol-17β (E_2) treatment induces that synthesis of VTG (yolk-precursor protein) in the liver of immature female and male fish. Recently, it was demonstrated that endocrine-disrupting chemicals (EDCs) also elevate VTG levels in male fish. Therefore, the production of VTG in male fish is a sensitive biomarker of the estrogenic potential of EDCs. Bisphenol A (BPA), nonylphenol (NP), and 4-tert-octylphenol (OP) are known EDCs with estrogenic activity in fish. This study compared the effects of BPA, NP, and OP on *in vitro* VTG synthesis in primary cultures of hepatocytes of the Chinese minnow, *Rhynchocypris oxycephalus*.

The molecular weight of VTG in plasma from E_2-treated male fish was determined to be 134 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western-blot analysis showed that prepared antibody (ab.a-E) recognized one band in plasma from E_2-treated male. VTG of mature female and E_2-treated male fish was identified using immunochemical methods. Immunodiffusion patterns revealed that the female plasma, egg extracts, and the plasma from E_2-treated male fish reacted against ab.a-E. Immunohistochemical observations showed that ab.a-E stained the yolk granules, follicle layer, and egg envelop in mature oocytes. For the primary hepatocytes culture, hepatocytes were isolated using collagenase, and the isolated hepatocytes were cultured in Leibovitz-15 medium at 18℃. E_2, phenols (BPA, NP and OP), and tamoxifen were dissolved in etanol and added to the culture medium after a two-day preculture. The treatment concentrations of E_2, phenols (BPA, NP and OP), and tamoxifen were 10^{-6}-10^{-5} M, 10^{-6}-10^{-3} M and 10^{-6} M, respectively. Medium was collected from each

culture plate 4 days after hormone treatment. The VTG secreted into the culture medium was measured using enzyme-inked immunosorbent assay (ELISA), which was developed using an antibody prepared from homogenates of Chinese minnow egg. VTG synthesis was induced by E_2 and phenols (BPA, NP and OP) treatment. E_2 at concentration 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 drastically blocked the production of VTG by E_2 in co-treatment with E_2 and tamoxifen. Tamoxifen is considered an anti-estrogen. We should compare the effects of BPA, NP, and OP on VTG synthesis using *in vivo* system, and investigate the effects of various hormones treatment on VTG synthesis, such as E_2 priming and anti-estrogen.

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감사의 글

부족함이 많은 저를 학문의 길로 들어서게 해주시고 논문이 완성되기까지 항상 자상하게 지도해주신 이영돈 교수님께 진심으로 감사드립니다. 그리고, 바쁘신 와중에도 이 논문을 정성스럽게 다듬어주신 최광식 교수님과 여인규 교수님께 감사를 드립니다.

2 년여 동안의 학위과정 중 항상 관심을 가지시고 많은 조언과 충고를 해주신 이정재 선생님, 정상철 선생님, 노 섬 선생님과 이기완 선생님께 감사를 드립니다.

이 연구를 수행하는데 있어서 많은 도움과 격려를 해주신 김세제 교수님과 김기옥 박사님, 그리고 친 형님같이 늘 곁에서 많은 조언을 해주신 김병호 박사님께도 감사를 드립니다.

실험과 자료정리를 위해 많은 시간을 동고동락하며 성심 성의껏 도움을 주신 발생학 실험실의 나오수, 오성립, 송영보, 박성보, 김봉원, 서종표, 이치훈, 최정권, 김성준, 고호진, 김진완, 진숙자 선배님과 고범호, 진영석, 강지웅, 고정남, 한성민, 허상우, 김삼연, 강경미, 허성표, 강승민에게 감사를 드리며, 지금은 멀리 이국땅에서 공부하고 계시고 있지만 항상 격려를 해주신 박용주, 김봉수, 문순주, 김한준 선배님에게도 감사를 드립니다. 학위 과정중 늘 격려해 준 대학원 선, 후배님들에게도 고마움을 전하며, 실험을 하는 동안 실험기기 및 시설을 이용하는데 도움을 준 제주대학교 해양과환경연구소의 변수철 선배님을 비롯한 여러 선생님들께도 감사를 드립니다.

끝으로 오늘이 있기까지 어려운 여건에서도 항상 사랑과 희생으로 뒷바라지에 고생하신 부모님과 누나, 동생들께 감사와 사랑을 전하며 시작에 불과한 이 작은 결실을 바칩니다.