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Effects of Environmental Contaminants and Natural Substances on Vitellogenesis in Tilapia Primary Hepatocytes

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Abstract

The efficacy of estrogenic chemicals and plant materials were assessed to stimulate the vitellogenin (VTG) synthesis in an *in vitro* system based on primary cultures of hepatocytes from female and E_2-primed male Mozambique tilapia, *Oreochromis mossambicus*. Isolated hepatoyctes treated with nonylphenol (NP), bisphenol-A (BP), octylphenol (OP), hexachlorocyclohexane (HCH) and the combination (ALL) as endocrine-disrupting chemicals (EDCs), Solgen 40®, SG40, as a commercial phytoestrogens and plant extracts (fenugreek, FG; aniseed, AS; safflower, SF; flaxseed, FS; fennel, FN; liqurice, LR; pomegranate, PG; soybean, SB) as natural substances, respectively, and their estrogenic activity were compared to control (ethanol treated hepatoyctes). Hepatic VTG levels in medium were determined by ELISA in the primary hepatocytes culture. All tested compounds induced secretion of VTG in hepatocytes of female as well as male tilapia. The induction of the hepatic response was not statistically different when each compounds was compared to control with HCH from EDCs and FG, AS, FN, LQ and PG from plant extracts in female and all cases of EDCs and plant extracts (except SG40 and SB) in male hepatoyctes. The EDCs and plant materials tested were considerably less potent than estradiol. Sensitive tilapia hepatoycte assay for measuring VTG are valuable tools for identifying the presence of environmental estrogens.

Keywords: Hepatocytes, phytoestrogens, tilapia, xenoestrogens, vitellogenin

Çevresel Kirleticilerin ve Doğal Maddelerin Tilapya Birincil Hepatositlerinde Vitellojenesize Etkileri

Özet

Bazı kimyasalların ve bitki materyallerinin estrojenik etkinliği *in vitro* sistemi içinde vitellojenin (VTG) sentezi uyarımı dişi ve estradiol uygulanmış erkek Mozambik tilapyalardan, *Oreochromis mossambicus*, elde edilmiş birincil hepatosit kültüründe değerlendirilmiştir. Sırası ile endokrin bozucu kimyasallar (EDCs) olarak; nonylphenol (NP), bisphenol-A (BP), octylphenol (OP), hexachlorocyclohexane (HCH) ve hepsinin kombinasyonu (ALL) ile ticari fitoestrojen olarak; Solgen 40®, SG40 ve doğal materyaller olarak bitki tohumlarının özütleri (çemen, FG; anason, AS; aspir, SF; keten, FS; rezene, FN; meyan kökü, LR; nar, PG; soya, SB) kullanılmıştır. İzole edilmiş hepatositler bu bileşimlere maruz bırakılmış ve onların estrojenik aktivitesi kontrol (etanol uygulanmış hepatosit) ile karşılaştırılmıştır. Hepatik VTG seviyeleri birincil hepatosit kültüründeki medyum içinde ELISA ile tespit edilmiştir. Test edilen bütün bileşimler dişi ve erkek tilapyaların hepatositlerinde VTG salgılanmasına neden olmuştur. Hepatik tepkinin indüklenmesi dişilerde EDCs'lerden HCH' de ve bitki özütlerinden FG, AS, FN, LQ ve PG' de ve erkeklerde de bütün EDCs'lerde ve bitkilerde de SG40 ve SB dışında kontrolden farklı bulunmamıştır. Bu denemede kullanılan EDCs'ler ve bitki materyallerinin estrojenik etkisi estradiole göre oldukça azdır. Çevresel estrojenin varlığını tespit etmede VTG ölçümü için kullanılan tilapya hepatosit tahlili değerli bir araç özelliğini taşımaktadır.

Anahtar Kelimeler: Hepatositler, fitoestrojenler, tilapya, ksenoestojenler, vitellojenin.

Introduction

Aquatic environment appears to be at risk by man-made and natural chemicals with suspected endocrine activity. Those environmental substances, called xenoestrogens or endocrine-disrupting chemicals (EDCs), such as ethinylestradiol-17α (an active component of contraceptive pills, Larsson *et al*., 1999), dichlorodiphenyl trichloroethane, DDT, (an organochlorine pesticide, Leanos-Castaneda *et al*.,

2002), nonylphenol (a degradation product of alkylpehnol polyethoxylate, Arukwe *et al*., 2000) and polychlorinated biphenyls, PCBs, (a persistent organic pollutant, Vega-Lopez *et al*., 2006) take action as an endogenous estrogen and adversely affect the hormone systems of the teleosts. Beside synthetic chemicals, a large number of plant-derived substances, phytoestrogens, also present naturally either in dietary ingredient or in the environment. Phytoestrogens are structurally and functionally similar to estrogen, include certain isoflavones, flavonoids, lignans and coumestans and show biological activity in man and animals (Dixon, 2004; Waring *et al*., 2008). Phytoestrogens are able to stimulate vitellogenin (VTG) synthesis *in vivo* in Siberian sturgeon (Pelissero *et al*., 1991a) and common carp, *Cyprinus carpio*, (Turker and Bozcaarmutlu 2009), *in vitro* in rainbow trout, *Oncorhynchus mykiss*, hepatocyte (Pelissero *et al*., 1993) and *in vivo / in vitro* in Siberian sturgeon, *Acipenser baeri*, and rainbow trout (Latonnelle *et al*., 2002).

Hepatocytes in the liver of oviparous vertebrates synthesize VTG, a glycolipophosphoprotein, which serves as the precursor for yolk proteins following estrogen interaction with the estrogen receptor (Lazier and MacKay, 1993; Ng and Idler, 1983). Estrogens stimulate VTG synthesis in the mature female genital tract and play a critical role in establishing and maintaining the reproductive system (Arukwe and Goksoyr, 1998). Although VTG synthesis is not detectable in juvenile and mature male fish due to low endogenous estrogen, upon exposure to estrogen and estrogen-mimicking compounds can increase the induction of VTG in both sexes. Therefore, VTG is considered as a biomarker of estrogenic activity *in vivo* and *in vitro* conditions (Sumpter and Jobling, 1995; Arukwe and Goksoyr, 1998; Jobling and Tyler, 2003).

The induction of this biomarker in teleosts has been used for EDCs screening and environmental monitoring (Kime *et al*., 1999; Nakada *et al*., 2004; Hutchinson *et al*., 2006). Since the VTG response can also be measured in isolated fish hepatocytes, the use of this bioassay has been suggested as an *in vitro* screen for identifying estrogen-active substances (Smeets *et al*., 1999b; Segner and Braunbeck 2003; Navas and Segner, 2006a). Assay for VTG may represent sensitive and rapid methods for screening potency of environmentally relevant compounds (Monteverdi and Di Giulio, 1999).

The purpose of the present study was to determine the estrogenic activity of some EDCs (nonylphenol, bisphenol-A and octylphenol as alkylphenol derivates and hexachlorocyclohexane as a pesticide) and plant materials (fenugreek, *Trigonella foenum-graecum*; aniseed, *Pimpinella anisum*; safflower, *Carthamus tinctorius*; flaxseed, *Linum usitatissimum*; fennel, *Foeniculum vulgare*; liqurice, *Glycyrrhiza glabra*; pomegranate, *Punica granatum*; soybean, *Glycine max,* and Solgen 40®, commercial soybean extract, as a phytoestrogen) based on Mozambique tilapia, *Oreochromis mossambicus*, hepatocyte bioassay in female and estrogen-primed male tilapia by immunological methods.

Materials and Methods

Experimental Fish

The average weight of 380±10 g female and 570±20 g male adult Mozambique tilapia used in this study were obtained from rivers in the Okinawa Prefecture, Japan. Fish were kept in a tank to acclimate with aeration and running water and fed daily with a commercial tilapia feed at the Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan.

Induction of VTG in Male Fish

Estradiol-17β (E_2, Sigma Chemical Company, MO, USA) was dissolved in ethanol and injected into males intraperitoneally at a concentration of 10 µg/g body weight a week before hepatocyte culture. The effect of E_2 injection on the induction of VTG in male was assessed by untreated male fish which injected with ethanol (the data was not shown).

Isolation and Culture of Tilapia Hepatocytes

Hepatocyte isolation and primary culture was performed according to the tilapia hepatocyte (TLP-HEP) assay by Takemura and Kim (2001). The liver was removed from the anesthetized fish and transferred into a watch glass. A two-step perfusion technique was used, in which the liver was perfused with first a Ca^{2+}-free buffer (136.9 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5.0 mM $NaHCO_3$, pH 7.6) for 15 min and subsequently with buffer containing collagenase (Wako Pure Chemicals, Osaka, Japan) at a concentration of 0.3 mg/ml at room temperature. The softened liver was then minced with a razor blade and strained through 200 and 50 µm nylon meshes. The cell suspension was washed four times by centrifugation at 50 g for 90 s at 10°C with a buffer containing 1.5 mM $CaCl_2$. Isolated hepatocytes were seeded in 6-multiwell plates (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) with Leibovitz-15 culture medium (Sigma Chemical Co.) containing 5 mM $NaHCO_3$, penicillin (70 mg/ml), polymixin B (50 mg/ml) and amphotericin (2.5 mg/ml) and incubated at 25°C under atmospheric air with saturated humidity. The media were replaced every 48 h until the monolayer formation is maintained for 6 days. Cells were daily checked by visual inspection under microscope.

EDCs and Plant Materials Exposure

The estrogenic potency of a number of chemicals including nonylphenol, (NP, Wako Pure Chemicals, Lot No: HCN9822), octylphenol, (OP, Sigma Chemical Company, Lot No: 06315CE), bisphenol-A, (BP, Aldrich Chemical Company, Lot No: AU115118BS) and hexachlorocyclohexane, (HCH, Sigma Chemical Company, Lot No: 07325DD) were dissolved in ethanol added to the culture media at 10^{-6} M with a single treatment. In addition to these individual compounds, commercial soy bean extract, Solgen 40® extract (SG40, Solbar Plant Extracts Ltd. Ashdod, Israel) was tested in EDC group due to reported genistein amount. The percentage of genistein was 63.5% based on dry weight in the Solgen 40® extract (certificate of analysis by HPLC, Solbar Plant Extracts Ltd. Ashdod, Israel). The SG40 was reconstituted with ethanol and adjusted to desired concentration (0.32 mg genistein/ml) for the medium according to genistein amount.

All plant materials were seeds, except liqurice (root), and purchased from health-food stores in Bolu, Turkey. Thirty grams powdered plant samples were soxhlet extracted with 350 ml ethyl acetate for 8 hours. Ethyl acetate portion of the extract was evaporated under vacuum. The resulting extracts after the removal of solvent mixture on a rotary evaporator were obtained in the following yields (w/w, %): fenugreek, FG, 1.97; aniseed, AN, 5.0; safflower, SF, 8.18; flaxseed, FS, 13.0; fennel, FN, 4.2; liqurice, LR, 1.3; pomegranate, PM, 6.0 and soybean, SB, 6.1. Plant samples contained the oil obtained from the extraction of 30 g of plant material and were reconstituted with ethanol at 50% (v/v) prior to addition to cultures. The total concentration of each reconstituted extract was 0.01% in the medium.

E_2, positive control, were dissolved in ethanol added to the culture media with the concentration of 10^{-5} M. Hepatocyte cultures were exposed to the same amount of ethanol to serve as negative control. Ethanol concentration never exceeded 0.1% in the culture plates. Tamoxifen (TM, Sigma Chemical Company) was added to the medium in female hepatocytes at a concentration of 10^{-5} M as a consistent inhibitor of VTG synthesis in a combination of compounds. The media were collected from the culture plate at 48 h after the treatments and used immediately for measurement of VTG with an enzyme-linked immunosorbent assay (ELISA).

Enyzme-Linked Immunosorbent Assay

VTG levels in the medium of mature female and E2-treated male TLP-HEP culture were determined with ELISA utilizing polyclonal antibodies specific for tilapia VTG. The sandwich ELISA method was similar to that described by Takemura and Kim (2001). A 96-well microtiter plate (Iwaki, Chiba, Japan) was coated with 100 ml of 50 mM carbonate buffer, pH 9.6, containing anti-tilapia VTG antibody at the concentration of 10 mg/ml for 2 h at room temperature or overnight at 4°C. After washing the plate with 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBS-T) using a plate washer type 1575 (Bio-Rad, Hercules, CA, USA), residual protein binding sites on each well were blocked by adding 200 ml PBS-T containing 1% BSA for 1h at room temperature. After washing the plate three times with PBS-T, VTG standard (previously purified from Mozambique tilapia by Takemura and Kim, 2001) and culture media were dispensed in duplicate (100 ml per well) into the plates, which were incubated for 2 h at room temperature. After washing, 100 ml HRP-conjugated anti-tilapia VTG was added to all wells of the plate and incubated for 2 h at room temperature. Following three washes with PBS-T, 100 ml of 0.1 M citrate buffer, pH 4.5, containing 1 mg/ml *o*-phenylenediamine dihydrochloride (Sigma Chemical Company) and 0.04% H_2O_2 was added to the wells. Color development proceeded for 30 min at room temperature and stopped by the addition of 25 ml of 2 N sulfuric acid to the wells. Absorbance of each well was measured at 492 nm using a plate reader model 550 (Bio-Rad).

Statistical Analysis

The absolute values of VTG obtained in each experiment were the means of triplicate and expressed as mean ± standard error of the mean (S.E.M.). The effect of various treatments on VTG levels was analyzed in one way ANOVA using Dunnett's pairwise multiple comparison test against negative control and Duncan's test against tamoxifen treatment at significance levels of 0.05.

Results and Discussion

Induction of VTG was previously observed after treatment of primary hepatocytes culture in tilapia with E_2 and NP *in vitro* (Kim *et al*., 2002). Therefore, use of this culture protocol as an assay for estrogenic activity was conducted with respect to environmental compounds and natural plant materials in the present study.

All EDCs at the concentration of 10^{-6} M, except HCH, significantly induced the secretion of VTG in hepatocytes female tilapia (Figure 1A). In hepatocytes from male tilapia, SG40 (0.32 mg genistein/ml) was the only significant inducer on the VTG (Figure 1B). The level of induction by EDCs was not significantly different from the control in male TLP-HEP (Figure 1B).

The relative potency of estrogenic compounds including OP, BP and NP with others was assessed using *in vitro* VTG induction assays with hepatoyctes

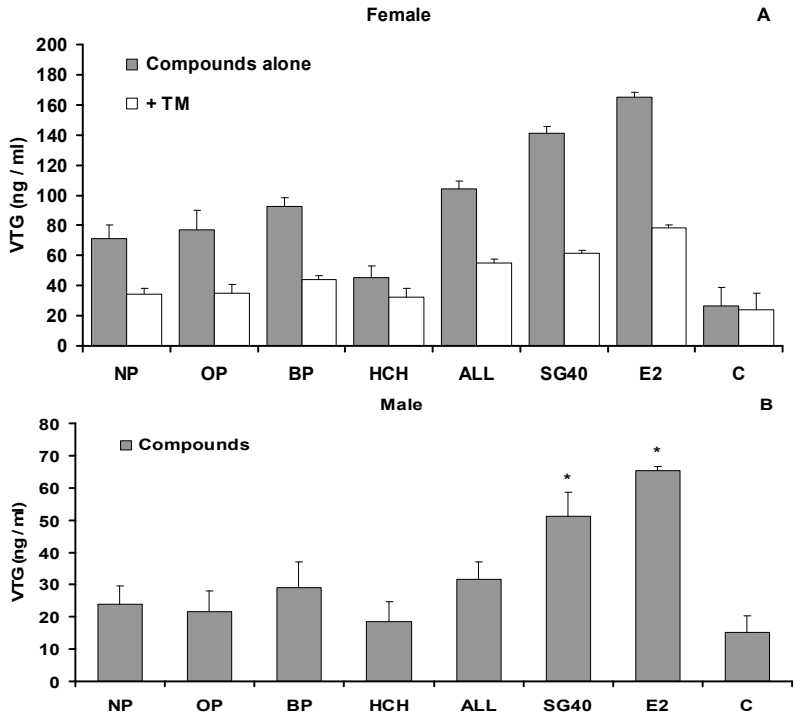

Figure 1. Effect of EDCs (10^{-6} M) and SG40 (0.32 mg / ml) on *in vitro* VTG synthesis in primary cultures of hepatocytes in female (A) with co-treatment of TM (10^{-5} M) and in male (B) tilapias. All values are the mean of a triplicate with ± SEM. In all cases (except HCH), EDCs and SG40 significantly different from control and TM inhibited (except HCH and control), the production of VTG in female ($P<0.05$). * indicates significant difference from the control in male tilapia ($P<0.05$).

from rainbow trout, *O. mykiss,* (Sumpter and Jobling 1995; Christiansen *et al*., 1998; Tremblay and Van Der Kraak 1998), Atlantic salmon, *Salmo salar*, (Celius *et al*., 1999; Arukwe *et al*., 2000; Tollefsen *et al*., 2003), carp, *C. carpio*, (Rankouhi *et al*., 2002; Rankouhi *et al*., 2004)), bream, *Abramis brama*, (Rankouhi *et al*., 2004) and channel catfish, *Ictalurus punctatus*, (Nimrod and Benson 2006; Monteverdi and Di Giulio 1999) and Mozambique tilapia (Kim *et al*., 2002). Ranking of those compounds according to their relative estrogenic potencies was similar among the various studies and found as BP>OP>NP. The previously demonstrated estrogenicity of those EDCs in different fish led to their inclusion in our studies. For instance, Monteverdi and Di Giulio (1999) investigated VTG induction in channel catfish hepatocytes by OP and NP at 10^{-8}, 10^{-6}, 10^{-5} M. While OP was able to induce VTG already at the lowest concentration tested, 10^{-8} M, NP treatment resulted in detectable VTG synthesis only for the highest test concentration, 10^{-5} M. Celius *et al.* (1999) tested BP, NP and HCH in Atlantic salmon hepatocytes at 10^{-6} M, and found that it was sufficient to induced synthesis of VTG protein. Kim *et al*. (2002) reported that in cultured tilapia hepatoyctes, NP induced VTG at concentration of 10^{-4} M. They also suggested that differences in the induction level of VTG among the different NP products.

Hepatoyctes treated with mixtures of all chemicals (10^{-6} M) had elevated levels of VTG compared to control, however response to the combination of chemicals were slightly greater from those treated with a single compounds in female and male tilapia (Figure 1A and 1B). In such experiments, it has been demonstrated that NP, OP, BP and HCH with some others induced VTG and that a mixture of all compounds elevated the level of VTG compared to treatment with the compounds used under single exposure in Atlantic salmon (Celius *et al*., 1999; Arukwe *et al*., 2000) and rainbow trout (Sumpter and Jobling, 1995).

In addition to these individual compounds, we evaluated the estrogenicity of some suspected plant materials used in food and health industry. A large number of plants such as soy bean, flaxseed and pomegranate contain phytoestrogens (Dixon 2004) and presents in dietary ingredients of plant origin. Incubation of female hepatoyctes with plant materials (SB, SF and FS) elevated VTG level significantly as compared with control (Figure 2A). VTG induction by SB exposure in male was only significant from the control among the tested crude plan extracts (Figure 2B). No significant difference in VTG levels was observed with other plant materials in male tilapia hepatoyctes (Figure 2B). Although the present study demonstrates for the first time the relative estrogenic effect of some crude plant extracts with regards to vitellogenesis in tilapia, the active metabolites of the tested phytoestrogens must be known to determine the maximum estrogenicity in *in vitro* conditions. If the

metabolism of phytoestrogens is taken into account, *in vitro* assays provide a good system to estimate estrogenic potencies of natural substances (Mueller, 2002). Previously, phytoestrogens such as formononetin, daidzein, genistein and equol were synthesized from the various plants and the estrogenic activity of these compounds was tested by the induction of vitellogenin secretion in the plasma of yearling sturgeon *in vivo* and in the hepatoyctes of rainbow trout *in vitro* conditions at the concentration of 10^{-5} M (Pelissero *et al*., 1991b; Pelissero *et al*., 1993). They found that all compounds were weakly estrogenic with possessing about one thousandth the potency of E_2. The estrogenic potency of the isoflavones ranged differently between Siberian sturgeon and rainbow trout and was highest with equol in both species when effects of dietary phytoestrogens were tested *in vivo,* at 0.2, 2 and 20 mg kg^{-1} feed, and *in vitro*, at 10^{-5} M, conditions (Latonnelle *et al*., 2002).

E_2 was found to be the most potent estrogen for the induction of VTG in hepatocytes from females and males tilapia among the all tested compounds and plant materials in this study. Similarly, E2 was shown to be most effective in inducing VTG synthesis by hepatoyctes of fish (Kim *et al*., 2002; Rankouhi *et al*., 2002; Tollefsen *et al*., 2003; Rankouhi *et al*., 2004).

In order to observe the reduction of VTG secretion by anti-estrogenic compounds, a co-treatment experiment was performed with TM, inhibitor for the estrogen receptor. The hepatocyte VTG response to the estrogenic compounds is specific, as evident from the fact that co-treatment with TM is able to inhibit the VTG induction (Lazier *et al*., 1996; Peyon *et al*., 1996; Smeets *et al*., 1999a). Hepatocytes from females were simultaneously exposed to EDCs at the concentration of 10^{-6} M and plant crude extracts of 0.01% with 10^{-5} M TM. TM was found to be a potent inhibitor of EDC (except in HCH) and plant extract-induced VTG synthesis at all the tested compounds in female hepatocytes (Figure 1A and 2A). The significant decrease in VTG induction by the presence of TM may imply that compounds bring out their effect through the estrogen receptor. An anti-estrogenic effect of TM on vitellogenesis has also been observed in rainbow trout (Pelissero *et al*., 1993; Celius *et al*., 1999), channel catfish (Monteverdi and Di Giulio, 1999), common carp (Smeets *et al*., 1999a), Nile tilapia, *O. niloticus*, (Leanos-Castaneda *et al*., 2002) and Mozambique tilapia (Kim *et al*., 2003). TM was tested in female hepatocytes only, since greater VTG levels in females facilitate the detection of a decrease in VTG production.

The estrogenic activity of these compounds was biologically tested by the induction of vitellogenin

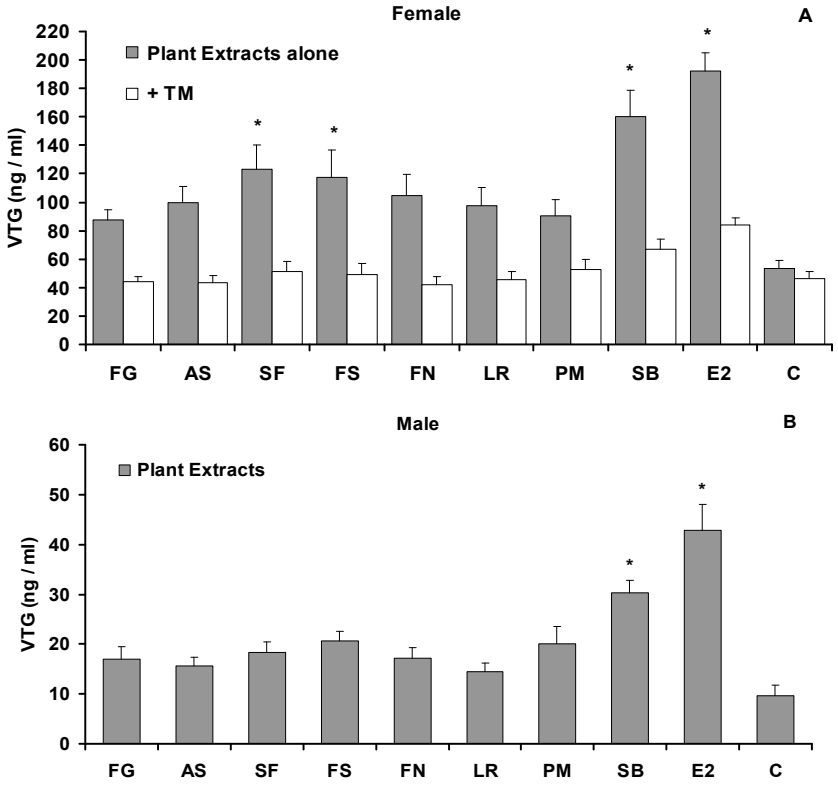

Figure 2. Effect of plant crude extracts on *in vitro* VTG synthesis in primary cultures of hepatocytes in female (A) with co-treatment of TM (10^{-5} M) and in male (B) tilapias. All values are the mean of a triplicate with ± SEM. * indicates significant difference from the control in female and male ($P<0.05$). In all cases (except control), TM inhibited the production of VTG in female tilapia ($P<0.05$).

secretion in female and male tilapia and compared to their respective control groups (ethanol treatment). All EDCs and plant materials tested were considerably higher inducer in female than male tilapia. Induction of VTG in different sex influences the sensitivity of the hepatocytes to estrogen treatment because VTG is not at a detectable level in male hepatoyctes whereas VTG is induced in hepatoyctes from mature females without estrogenic stimulation. Since VTG synthesis is regulated by expression of the estrogen receptors, female hepatoyctes having a higher constitutive expression of estrogen receptor may response faster and greater magnitude to estrogen exposure than male hepatoyctes (Navas and Segner 2006b). However, the estrogen receptor level affects only the absolute magnitude of VTG synthesis but not the relative sensitivity for estrogens in the hepatocytes (Smeets *et al*., 1999a; Kordes *et al*., 2002; Riley *et al*., 2004; Navas and Segner 2006b). Therefore, these authors suggested that the estrogenic potencies of compounds should be assessed with male hepatocytes because the detection of VTG may indicate the sensitivity for estrogens in male hepatocytes which show no or minor basal synthesis of VTG.

Many of these man-made and natural chemicals are widely used in plastic and detergent industry and in alternative medicine and food industry, respectively. Very high volumes of some these estrogenic compounds lead to the appearance of significant amounts in aquatic environment. In conclusion, the induction of VTG in short-term in vivo tests with fish is presented in this study as a screening assay to detect estrogenic activities of chemicals or environmental samples. *In vitro* assays are valuable for reproductive toxicology, inexpensive compared to most *in vivo* experiments and easy procedure for identifying potential estrogenic compounds that could be benefit or risk for humans and wildlife.

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