AN IN VITRO TEST FOR ESTROGENICITY COMBINING CULTURED HEPATOCYTES AND AN ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

by
George H. Monteverdi
Richard T. Di Giulio

Nicholas School of the Environment
Duke University

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George H. Monteverdi and Richard T. Di Giulio
Ecotoxicology Laboratory
Nicholas School of the Environment
Duke University
Durham, North Carolina 27708-0328

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Abstract

An in vitro assay has been developed to screen for estrogenic activity of single chemicals or complex mixtures. This method combines primary hepatocyte cultures from the channel catfish (Ictalurus punctatus) with an enzyme-linked immunosorbant assay (ELISA) to detect and quantify the production of vitellogenin (VTG), a liver-derived, estrogen-induced lipoprotein. A variety of environmentally relevant chemicals and chemical mixtures were tested including the polyaromatic hydrocarbon benzo[a]pyrene (BaP), the alkylphenolic surfactants 4-tert-octylphenol (OP) and p-nonylphenol (NP), the chlorinated insecticide o,p'-DDT, the plant derivative stigmastanol, and a number of waste waters from pulp and paper mills. In addition, the effects of estradiol (E2), the synthetic estrogen diethylstilbestrol (DES) and the antiestrogens trans-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene (tamoxifen) and 7α-[9-(4,4,5,5,5-pentafluoropentylsulfanyl)nonyl]estra-3,17β-diol (ICI-182,780) were also examined. The following compounds were found to be estrogenic: DES > E2 >> OP > o,p'-DDT > NP. Tests with BaP, stigmastanol, tamoxifen, ICI-182,780 and four paper mill effluents exhibited no, or undetectable, estrogenic activity. Further, stigmastanol and the mill effluents were also tested for anti-estrogenic activity in cells incubated in media containing both DES and stigmastanol or effluent. Compared to DES alone, none of these treatments caused a significant reduction in the media concentrations of VTG. The detection limit for this assay was typically 15-25 ng VTG/mL medium. Both the results of the screening and the experimental limit-of-detection were in agreement with data published by other researchers for work with other species.
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Summary and Conclusions

The potential threat of endocrine disrupting compounds in aquatic systems has garnered a great deal of attention from both the scientific community and the public at large. The ubiquitous nature of many of these compounds, and the fact that many of them enter our waterways as components of complex industrial- or municipal-waste streams, has fostered the desire for effective methods of screening for endocrine effects. One such effect, that of xenobiotic-associated estrogenicity (xenoestrogenicity) has received particular attention, primarily because of the important role estrogen plays in early life-stage development and the reproduction. The goal of our work was to develop a reliable screening assay that would afford the opportunity to examine the estrogenicity of a wide range of environmentally-relevant chemicals and chemical mixtures.

The major findings of our studies were as follows:

1) Estradiol (E2), diethylstilbestrol (DES), the alkylphenolic surfactants 4-tert-octylphenol (OP) and p-nonylphenol (NP), and the chlorinated insecticide o,p'-DDT all induced detectable levels of vitellogenin synthesis in cultured channel catfish hepatocytes. The relative potencies of the compounds were:

   DES > E2 >> OP > o,p'-DDT > NP

   The VTG-induction associated with each of these treatments was significantly (≥ 60%) inhibited by the presence of the antiestrogen tamoxifen. These data indicate that these compounds elicit their effect through the estrogen receptor.

2) Stigmastanol (a plant derivative commonly found in pulp mill effluents), the polynuclear aromatic hydrocarbon benzo(a)pyrene, a number of waste waters from pulp and paper mills, and the antiestrogens tamoxifen and ICI-182,780 exhibited no, or undetectable, estrogenic activity.

3) Stigmastanol and the pulp mill effluents were also tested for anti-estrogenic activity in cells incubated in media containing both DES and stigmastanol or effluent. Compared to DES alone, none of these treatments caused a significant reduction in the media concentrations of VTG.

4) The detection limit for this assay was typically 15-25 ng VTG/mL medium. Both the results of the screening and the experimental limit-of-detection were in agreement with data published by other researchers for work with other species.

This WRRI-funded work has developed an effective, in vitro xenoestrogen screen that utilizes primary hepatocyte cultures from the channel catfish (Ictalurus punctatus), an environmentally and commercially important species in North Carolina and the Southeastern United States. In addition to its utility for screening individual chemicals, the experimental design developed here allows for examination of a wide variety of
aquatic pollutants, including complex mixtures of chemicals such as those often associated with industrial and municipal waste treatment processes.
Recommendations

The screening protocol described here was intended to allow for the screening of both individual chemicals and complex mixtures. Because our primary hepatocyte cultures require the use of aqueous media, however, the screening of strongly hydrophobic compounds is difficult. The presence of low concentrations of solvent in the culture medium greatly improves assay reproducibility. As designed, this methodology should continue to prove useful for the screening and identification of aquatic xenoestrogens.
Introduction

Use of VTG as an Indicator of Estrogenicity

A recent U.S. EPA-sponsored workshop resolved that humans and wildlife have suffered adverse health effects after exposure to endocrine disrupting chemicals (Kavlock et. al., 1996). One area of specific concern is the potential for commonly occurring, environmentally persistent chemicals to elicit an estrogen-like response in numerous animals, including fishes. The presence of so-called “xenoestrogens” in aquatic systems has created the need for simple, cost-effective screening methods for estrogenic compounds. Over the past two years, we have developed an in vitro screening method utilizing primary hepatocyte cultures from the channel catfish (Ictalurus punctatus), with vitellogenin (VTG) production serving as the measured indicator of estrogenic activity.

VTG is a phospholipoglycoprotein synthesized by the liver (or equivalent organ) of virtually all oviparous (i.e., egg laying) animals including most fish, birds, reptiles, amphibians and invertebrates. Production of VTG (vitellogenesis) in oviparous fish species occurs in response to increased blood concentrations of circulating estrogens, normally triggered by environmental stimuli such as the increased water temperature and photoperiod (i.e., increased daylight hours) associated with the reproductive season. Estradiol (E2), primarily produced by the ovary, enters the circulation and leads to the production of VTG through interaction/bindning with hepatic estrogen receptors. Following synthesis, VTG is secreted by the hepatocytes, enters the circulation and is sequestered by growing oocytes. Following incorporation into the oocyte, VTG is enzymatically processed into a variety of cleavage products, including lipovitellins and phosvitins. These stores of proteolyzed vitellogenin form the nutrient basis for embryonic and early life-stage development.

Although livers of male fish are capable of producing VTG, endogenous production of VTG is female-specific, as male fish normally lack plasma estrogen levels sufficient to elicit an appreciable vitellogenic response. Xenoestrogenic compounds, however, can trigger vitellogenesis in both male and female animals by entering the circulation and interacting with hepatic estrogen receptors. In our work, it is the phenomenon of VTG production by hepatocytes of male fish that serves as a measure of estrogenicity.

Studies Conducted by Other Researchers

Previous workers have suggested that VTG synthesis may serve as a biological indicator of estrogenic activity (Jobling and Sumpter, 1993; Purdom et al., 1994; White et al., 1994), and enzyme-linked immunosorbant assay (ELISA) techniques have been developed for a number of fish species including: the Atlantic salmon (Salmo salar) (Olin and Von Der Decken, 1987), Siberian sturgeon (Acipenser baeri) (Pelissero et al., 1991), tilapia (Oreochromis mossambicus; Kishida and Specker, 1993), and channel catfish (Goodwin et al., 1992). Most of these assays, including that for channel catfish, were developed to determine levels of VTG in blood plasma. Subsequently, other groups have developed in vitro VTG assays that have combined hepatocyte...
culturing and an ELISA. Our experimental design was initially based on a protocol developed in rainbow trout (*Oryzchus mykiss*) by Pelissero et al. (1993), and uses primary (i.e., non cell-line) cultures of hepatocytes isolated from male channel catfish by *in situ* perfusion with collagenase D (see Materials and Methods, Section II. A). Following determination of cell count and viability, the hepatocytes are cultured in medium containing various concentrations of the compound of interest. Synthesized VTG is secreted from the hepatocytes into the culture medium and is measured in media aliquots by ELISA (see Section II. C). Initially, our ELISA protocol followed the design of Goodwin et al., (1992), but was subsequently modified in response to the difficulties discussed below.

A number of groups are now combining cell culturing with an ELISA to evaluate the potential estrogenicity of aquatic pollutants. Jobling and Sumpter (1993) demonstrated the ability of 4-tert-octylphenol (OP) and p-nonylphenol (NP) to induce VTG synthesis in cultured fish (rainbow trout) hepatocytes. Subsequently, White et al., (1994) demonstrated the estrogenicity of OP and NP as well as other alkylphenolic compounds, including 4-nonylphenoxycarboxylic acid (NP1EC) and 4-nonylphenoldiethoxylate (NP2EO), in the same system. Our method utilizes cultured hepatocytes from the channel catfish, a species with great relevance to the waterways of North Carolina and the Southeast.

**Goals and Accomplishments of our Work**

The work supported by WRRI was intended to provide a simple, cost-effective means of assessing the estrogenicity of selected aquatic pollutants. While the compounds initially examined were selected based on their common occurrence and environmental persistence, the method we have developed can be used to evaluate a wide range of chemicals, including complex chemical mixtures such as effluents from municipal and industrial waste treatment facilities.

We have successfully completed studies evaluating the estrogenicity of suspected xenoestrogens OP, NP and *o,p'*-DDT, the common hydrocarbon pollutant benzo(a)pyrene (BaP), the plant extract stigmastanol, and effluents from a number of pulp mills that utilize differing waste water treatment processes.
Materials and Methods

The method we have developed combines primary hepatocyte cultures from the channel catfish with an enzyme-linked immunosorbent assay (ELISA) for quantification of VTG. Our methods are described below, and additional details are provided in Appendices A, B and C.

Hepatocyte Isolation Procedures

Channel catfish hepatocytes were isolated following *in situ* perfusion through the atrium and sinus venosus which lie directly caudal to the liver through the transverse septum. This method is based on the retrograde perfusion procedure previously described by Meredith (1988) in the rat, and adapted successfully for channel catfish by Koban (1986). Our technique also included some modifications from a method used for the isolation of trout hepatocytes (Blair et al., 1990).

The fish (usually 400-500 g b.w.) was anesthetized in buffered deionized water containing the anesthetic 3-aminobenzoic acid ethyl ester (MS-222; see Appendix A). Anesthesia was maintained throughout the procedure by a recirculating system that passed MS-222 continuously over the gills. The fish was placed ventral side up on a surgical table and the following incisions made: 1) a longitudinal incision from the anus to the transverse septum; 2) two lateral incisions along the caudal edge of the transverse septum exposing the liver, with the skin lifted clear of the liver and held with hemostats; and 3) one shallow lateral cut of the tissue in front of and below the approximate position of the heart to expose the heart. While holding the bony plate clear of the heart, two longitudinal cuts were made towards the transverse septum. The plate was then removed from the fish using bone snips, leaving the heart exposed.

Following severance of the bulbous arteriosus, a glass hemostat (cannula) was inserted through the rostral portion of the bulbous arteriosus and through the heart until it reached the liver sinus. The cannula was secured at this point by suture and a small steel clamp. The perfusion buffers were pumped into the liver by a peristaltic pump. Initially, a calcium-free buffer containing EDTA (Buffer A; recipes for all buffers used in this procedure are provided in Appendix A) was used to flush the liver of blood and prevent coagulation. After 10 minutes, a collagenase-D- and calcium-containing buffer (Buffer B) was introduced and perfusion continued for another 20 minutes. When the liver was thoroughly blanched and softened it was excised, placed in a glass petri dish containing a calcium wash buffer (Buffer B without collagenase), and diced with sterile razor blades. Next, the diced tissue was passed through a sterile sieve to remove all connective tissue and the cell solution retained. Following removal of cell debris by centrifugation, the hepatocytes were resuspended in Leibovitz L-15 (L-15; Gibco BRL) nutrient medium, counted and evaluated for viability by the trypan blue exclusion technique described below.

Plating and Maintenance of Hepatocytes

Following isolation, 2 ml/well aliquots of cell solution (1 x 10^6 cells/ml) were placed in 24-well polystyrene tissue culture plates (Corning). Cells were allowed to form a monolayer on the bottom of the wells in the absence of any experimental treatments. After 24-hours, media of all wells were changed with appropriate treatments being introduced. As an experimental positive-
control, the synthetic estrogen diethylstilbestrol (DES) was included in all studies. The anti-
estrogen tamoxifen was also used in these studies to verify that the response observed (i.e.,
vitellogenesis) was associated with the estrogen receptor (ER). That is, tamoxifen acted to
dampen VTG production induced by chemicals acting via the ER. Subsequent media changes
occurred at either 24- or 48-hour intervals over the following 4-6 days. Media removed at these
time points were analyzed for both VTG content (ELISA) and cell viability (LDH assay) by the
methods discussed below. Initial experiments also examined the potent and specific ER
antagonist ICI-182,780 (Zeneca) as a modulator of VTG. Results from these studies, however,
showed ICI-182,780 to be a potent but inconsistent inhibitor of VTG induction in our system. As
a result, tamoxifen was selected as the antiestrogen to be used in all studies reported here.

Assessment of Cell Viability

Trypan blue exclusion (TBE) assay. Trypan blue is large molecular weight, biological dye that
penetrates and stains cells displaying significant plasma membrane damage. In these experiments,
trypan blue exclusion (TBE) was interpreted as a measure of the viability of isolated hepatocytes.
Although TBE was primarily used immediately prior to cell plating, it was also used for quick-
and-simple assessments of cell viability during experimental runs.

The assay was conducted as follows. An aliquot (300 µL) of the cell suspension was diluted 1:2
with L-15. Five-hundred microliters of the diluted suspension were then transferred to a vial
containing 100 µL 0.4% trypan blue using a pipette tip from which the end has been snipped off
(to prevent cell damage). The mixture was incubated at room temperature for 2 minutes. After
gentle mixing, aliquots were transferred by Pasteur pipette to a 0.1 mm-deep Bright-Line
hemocytometer (Hausser Scientific) and examined microscopically at 100X magnification. Viable
and non-viable (blue stained) cells were then counted with large clumps of cells counted as a
single cell. Viability was calculated as:

\[
\text{Viability} = \frac{\text{total cells not containing dye}}{\text{total cells}}
\]

The hemocytometer has 9 grids, each grid with a volume of \(10^{-4}\) ml. Typically, 5 grids were
counted and the viable cell density was calculated as:

\[
\text{Cells/ml} = \frac{(\text{total viable cells/ # grids counted}) \times \text{dilution factor} \times (1.2 \times 10^4)}{9}
\]

For example, if 5 grids were counted with a total of 194 viable and 15 non-viable cells in a 1:5
dilution:

\[
\text{Viability} = \frac{194}{194 + 15} = 93\%
\]

\[
\text{Viable cells/ml} = \frac{(194/5) \times 1.2 \times 10^4}{5} = 2.33 \times 10^6 \text{ cells/ml}
\]

Following calculation of viable cell density, the cell suspension was diluted to yield a density of 1
x \(10^6\) cells/mL and the cells were plated as described above.
Lactate dehydrogenase (LDH) assay. LDH is a cytoplasmic enzyme found in hepatocytes that catalyzes the NADH-dependent reduction of pyruvate to lactate. Damaged and dying cells will leak LDH into culture media. This LDH can be measured indirectly by monitoring the decrease in spectrophotometric absorbance due to the LDH-catalyzed oxidation of NADH. Cell viability was assessed by comparing the rate of NADH oxidation in culture media samples with the rate observed in lysed-cell preparations (Bergmeyer, 1983). The chemical composition of the solutions used in the LDH assay are provided in Appendix B. The LDH assay served as our primary method of assessing cell viability once cell cultures were established.

Sample preparation

For each treatment and control the following protocol was followed for LDH analysis. Cultured cells were resuspended, and an aliquot of this cell suspension (200 μL / sample) was centrifuged for 20 seconds at 12,000 x g. A portion of the supernatant (100 μL) was collected and stored at 0-4°C until LDH analysis. A second 200 μL aliquot of the cell suspension was transferred to a microfuge tube containing an equal volume of Triton X-100 to lyse the cells. The tubes were kept on ice and mixed with a vortex mixer several times during the next 10 minutes. The mixtures were then centrifuged at 12,000 x g for 20 seconds and a 100 μL portion transferred to a clean tube. If it was necessary to postpone analysis, stored samples were kept at -10°C and assayed within 3-5 days. Because activity may drop after only 24 hours (Bergmeyer, 1983), postponement of analysis occurred infrequently. The second set of samples represented the total potential LDH activity of the hepatocyte extract, and were analyzed along with the first set of collected samples by the protocol discussed below.

LDH analysis

As noted above, LDH catalyzes the oxidation of NADH to NAD⁺. This assay used a spectrophotometer (Shimadzu UV-260) to monitor the decrease in sample absorbance (at 339 nm) due to oxidation of NADH. Samples were prepared immediately prior to spectrophotometric analysis as follows: A 50 μL sample aliquot was added to 2.5 mL of a Tris-HCl/NaCl/NADH solution, mixed and warmed to 30°C (see Appendix B for all solution recipes for the LDH assay). Five-hundred microliters of a Tris-HCl/NaCl/pyruvate solution were then added, the samples mixed briefly on a vortex mixer and placed in the spectrophotometer. Exactly 30 seconds after being placed in the spectrophotometer absorbance at 339 nm was recorded and a stopwatch started. The absorbence was recorded every 30 seconds for the next 2 minutes.

Calculation of LDH activity

LDH activity was calculated using the equation:

\[
\text{Activity (U/L)} = \frac{(\delta A)(V)(1 0 0 0)}{(c)(d)(\delta t)(v)} = 9682 \left(\frac{\delta A}{\delta t}\right),
\]

Where: \( V = 3.05 \text{ ml} \)
\[ \varepsilon = 6.298 \times 10^{-1} \text{ L/mmol-min} \]
\[ d = 10 \text{ mm} \]
\[ v = 0.05 \text{ ml} = 5 \times 10^{-3} \text{ L} \]
\[ t = \text{ time in minutes} \]
\[ A = \text{ activity (U/L)} \]

Units of activity can be converted to specific activity by dividing by protein concentration (g/L) to yield activity as U/g total protein. For these conversions, total protein was determined using the standard BioRad protein assay. Relative LDH leakage (i.e., cell viability) was determined by comparing the LDH activity measured in the first set of samples (media supernatant) with the activity measured in the cell extract (second sample set):

\[
\text{Percent LDH leakage} = \frac{\text{activity of supernatant}}{\text{activity of cell extract}} \times 100
\]

Viability was considered good when percent leakage was less than 10%. The LDH assay was run for each treatment group at the time of sample collection for VTG determination.

**Selection of Treatment Chemicals and Media Collection**

The hepatocyte culturing and VTG ELISA methods were used to evaluate the estrogenic potency of a number of chemicals including estradiol (E2), the synthetic estrogen diethylstilbestrol (DES), the polyaromatic hydrocarbon benzo(a)pyrene (BaP), the surfactants 4-tert-octylphenol (OP) and p-nonylphenol (NP), the chlorinated insecticide \( o,p' \)-DDT, and the antiestrogens \( \text{trans-1-(4-\beta-\text{dimethylaminooethoxyphenyl)-1,2-diphenylbut-1-ene (tamoxifen) and 7a-[9-(4,4,5,5,5-}\text{pentafluoropentylsulfinyl)nonyl]estra-3,17\beta\text{-diol (ICI-182,780).} \)

In addition to these individual compounds, we evaluated the estrogenicity of reconstituted pulp mill effluents, and stigmastanol, a plant extract commonly found in pulp mill effluents at concentrations <10 ppb.

E2 and DES were both used during method development. Based on its more potent and reproducible induction of vitellogenesis, DES was chosen to serve as the experimental positive control. Throughout these studies, DES was used to assess the inducibility of VTG in cultured hepatocytes and to test the efficacy of the antiestrogens. The previously demonstrated estrogenicity of OP, NP (Soto et al., 1991; Jobling and Sumpter, 1993; White et al., 1994) and \( o,p' \)-DDT (Palmer and Palmer, 1995) in different animal systems lead to their inclusion in our studies. Stigmastanol and lyophilized mill effluents from the aeration stabilization lagoons of two bleached kraft mills, an unbleached kraft mill and a deinking recycled fiber mill in the southeastern US were provided by the National Council for Air and Stream Improvement (NCASI).

Most chemicals we examined display limited water solubility and, therefore, were first dissolved in dimethylsulfoxide (DMSO) or ethanol (EtOH) to produce stock solutions. Treatment media were prepared by careful addition of the stock solutions to volumes of L-15 media. In media containing either a single treatment (e.g., OP) or a combination of compounds (e.g., OP and tamoxifen), the total concentration of DMSO or EtOH was 0.3%. Media for control groups were made by addition of the appropriate volumes of DMSO or EtOH. Paper mill effluents were

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reconstituted to desired concentrations by direct addition of L-15. Control groups for these experiments did not contain DMSO or EtOH.

Treatment media were introduced 24-hours after the cells were plated. Subsequently, 1.9 mL aliquots of media were removed and an equal volume of similarly-prepared, new media were added every 48-hours for 2-6 days. Media samples were analyzed for VTG using the ELISA discussed below and LDH as described above.

Principles of ELISA and Methods of Detection

Over the past 25 years, ELISAs have become a preferred system for assaying soluble antigens and antibodies. Factors contributing to the popularity of ELISAs include their sensitivity, the long shelf-life and ease of preparation of the reagents, and the lack of radiolabeled compounds. Common protocols include: indirect-, direct competitive-, antibody-sandwich-, and double antibody-sandwich methods. The ELISA we have developed as part of this work is a direct competitive ELISA. This format was chosen because of our ability to purify sufficient quantities of VTG and, moreover, because it requires smaller quantities of primary antibody than other methods (e.g., antibody sandwich methods). We chose to use a secondary antibody conjugated with the enzyme alkaline phosphatase (AP) because of this enzyme’s rapid catalytic rate, availability and resistance to inactivation by reagents in the assay. A cartoon depicting a direct competitive ELISA is provided as Figure 1.

Figure 1: Depiction of a competitive, enzyme-linked immunosorbant assay (ELISA) for vitellogenin (VTG)
ELISAs using various techniques have been developed to detect and quantitate specific antigens including fish VTG (Olin and Von Der Decken, 1987; Nunez Rodriguez et al., 1989; Goodwin et al., 1992; Kishida and Specker, 1993; and Perez and Callard, 1993). Assay procedures of our ELISA were as follows. All but one well of a 96-well Maxisorb microtiter plate (Nalge/Nunc International) were coated with 200 µL (1 µg VTG/well) purified channel catfish VTG in 0.017 M borate-buffered saline (BBS; pH 9.6). The remaining well (reagent blank) was coated with 200 µL (4 µg/mL) bovine serum albumin-BBS solution (BBS-BSA; Sigma) and the plate was stored overnight at 4°C.

Next day, the wells were rinsed 3X with BBS and blocked for 1 hour at room temperature with 1% BSA (200 µL) in BBS. Following blocking, wells were washed 3X with 200 µL BBS containing 0.1% Tween-20 (BBS-T) to remove unbound protein. All standards and samples were preincubated for 1 hour with a monoclonal anti-channel catfish VTG antibody (Goodwin et al., 1992). The first (standard curve) row was then loaded with doubling dilutions (100 µL/well) of a known concentration of VTG in BBS. The second row was loaded with 100 µL doubling dilutions of a VTG control. The next five rows of the plate were loaded with doubling dilutions (100 µL/well) of media from experimental cultures, and the final row was loaded with fresh L-15 that had not been used for culturing. The plate was covered and placed on an orbital shaker at room temperature. After 2-hours, the plate was washed with BBS-T (200 µL/well) to remove ligand-antibody complexes not bound to the wells. Aliquots (100 µL/well) of an alkaline phosphatase-conjugated goat-anti-mouse IgG (Sigma) diluted 1:1000 were then added to each well and allowed to incubate for 2-hours at room temperature. The wells were washed again with 200 µL/well BBS-T and 100 µL/well aliquots of a p-nitrophenyl phosphate (pNPP) solution (1 mg pNPP / mL 0.2 M Tris buffer) were added to each well and left at room temperature. Alkaline phosphatase catalyzes a reaction converting pNPP to p-nitrophenol, a yellow compound absorbing light at λ=405 nm.

Following proper color development (typically 10-20 minutes), the reaction was stopped by addition of 50 µL of 3M NaOH and measurement was made at λ = 405 nm by an automated colorimetric plate reader. A standard curve was generated from absorbencies measured in wells of the first row, using the well coated with BSA instead of VTG as the reagent blank. The VTG concentrations for the control (second row) and culture media samples were read with respect to the standard curve.

Additional information regarding the ELISA and its requisite reagents, materials, and equipment is provided in Appendix C.

VTG Purification and Antibody Acquisition

Purified VTG for these experiments was obtained through isoelectric focusing (IEF) of serum from DES-induced channel catfish. IEF allowed for the purification of the large quantities of highly-purified VTG necessary for the ELISA. The IEF technique was as follows. A BioRad Rotofor® isoelectric focusing (IEF) chamber was pre-run using tissue culture-grade distilled water until a reading of 2.0 mA was obtained. The chamber was then prefocused for 30 minutes using
2% v/v ampholytes (BioLyte; BioRad), pH range 3-10. Focusing was conducted at 12 watts constant power. Serum (0.5-1.0 mL), collected from a DES-induced channel catfish, was used to determine the isoelectric point of VTG. Initial focusing of channel catfish serum was conducted using pH gradient of 3 to 10. Each of the 20 chambers was drained into separate tubes and their respective pH values recorded. Aliquots from each chamber were then subjected to sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blotting to detect the presence of VTG. Chambers containing VTG, based on Western blot, were pooled and refocused at pH 4.0 to 6.5, 12 watts constant power. A minimum of 5 chambers from the initial focusing were combined to provide sufficient ampholytes for reestablishing the pH gradient. After 4 hours the chamber contents were drained under vacuum, their pH measured, and 200 μL aliquots were taken for Western blot.

IEF chambers containing VTG were identified by SDS-PAGE and Western blotting. An average pH and associated standard error were then determined over the pH range containing VTG. Rotofor chambers containing VTG were then lyophilized. As needed, lyophilized samples were reconstituted in buffered deionized water for use in ELISA, SDS-PAGE, and Western blots.

The monoclonal antibody (primary antibody) to channel catfish VTG used in our experiments did not require further purification by us as it failed to cross-react appreciably with other proteins in preliminary screening assays. Moreover, the titer and experimental volumes of primary antibody solution used in previous work (Goodwin et al., 1992) proved efficacious in our assay. The secondary antibody used in our work was an AP-conjugated, goat-anti-mouse IgG antibody obtained from Sigma Chemical Company.

Additional Analyses

In addition to ELISA analysis, SDS-PAGE/Western blot analyses were conducted to qualitatively identify VTG and determine if other media components or culture products might interfere with the proper functioning of the ELISA. For instance, if either of the antibodies used cross-reacted with a non-VTG media component, then the ELISA would produce spurious results. Experiments conducted during our early methods development identified no such confounding factors (data not shown).

SDS-PAGE gels were prepared with BioRad reagents according to a BioRad-prescribed protocol. Discontinuous (Laemmli) gels were made with an upper, 4.0% acrylamide stacking gel and a lower, 7.5% acrylamide resolving gel, and used in the following protocol:

- samples were diluted 1:4 with SDS reducing buffer, heated to 90°C for 15 minutes, and loaded onto the gel;
- after the samples cooled, the gel was run at 500 volts, 30 milliamps and 250 watts for 0.75 to 1.5 hours; and,
- following the run, proteins were transferred from the gel to a nylon membrane with a BioRad Mini Trans-Blot cell (100 volts, 500 mA, 250 watts for 1.5 hours).
Following protein transfer, the membrane was blocked with nonfat milk, rinsed and incubated for 1.5 hours with a monoclonal antibody to channel catfish VTG diluted 1:1000 in phosphate-buffered saline containing 1% Tween-20 (PBS-Tween). After repeated washings with PBS-Tween, the membrane was incubated for 1.5 hours with a horseradish peroxidase (HRP)-conjugated secondary antibody diluted 1:20,000 in PBS-Tween. Subsequently, the membrane was thoroughly washed with PBS-Tween, drained and incubated with a solution containing luminol and hydrogen peroxide. In the presence of these two compounds, HRP catalyzes a light-producing reaction detectable by exposure to BioMax MR imaging film (Kodak). Proteins that cross-react with the primary antibody appear on the film as black bands.
Results and Discussion

To date we have examined the estrogenicity of E2, DES, tamoxifen, OP, NP, o,p'-DDT, BaP, a number of paper mill effluents and stigmastanol. Of these, tamoxifen, BaP, stigmastanol and the effluents did not induce detectable VTG synthesis in cultured channel catfish hepatocytes. All treatments were analyzed for their ability to induce VTG synthesis, and the results compared with the effects of DES at 10 nM (10^{-8} M). The relative potency of the VTG-inducing compounds was DES > E2 >> OP > o,p'-DDT >> NP. Despite previous reports of estrogenic activity by tamoxifen (Jobling and Sumpter, 1993), we were unable to measure VTG production in cultures exposed only to tamoxifen at the dose used (1 μM).

Experiments with DES, E2, tamoxifen and ICI-182,780

While both DES and E2 were used in preliminary experiments, DES was used as a positive control for all experiments reported here because it displayed greater potency and better inter- and intra-study consistency of VTG induction than E2 (data not shown). These data are in contrast to Pelissero et al. (1993), who observed greater VTG induction potency in rainbow trout hepatocyte cultures treated with E2 versus DES. The reason(s) for this difference is not clear, though possible explanations include a greater rate of E2 metabolism/breakdown, greater binding (and thus “inactivation”) of E2 by VTG in the medium, or simply that catfish hepatocytes possess greater sensitivity toward induction by DES.

Based on previous studies (White et al., 1994; Jobling and Sumpter, 1993), VTG induction by E2 or DES at 10-100 nM was used to determine the relative potency of test compounds. At concentrations greater than 100 nM, both E2 and DES did not remain entirely in solution and these experiments were abandoned. Our tests showed a dose-related increase in VTG synthesis from 10 pM to 100 nM, with little difference in rate of VTG synthesis between 10 and 100 nM for both E2 and DES. This invariance, combined with the greater reproducibility and potency of DES, was the basis for selecting VTG induction by DES at 10 nM as the benchmark against which the estrogenic potencies of other compounds were compared. The dose-related VTG production at the DES concentrations tested are shown in Figure 2. Additional experiments demonstrated the ability of 1 μM tamoxifen to decrease VTG synthesis by ≥ 60% in DES- or E2-treated cells (Figure 2). Despite previous reports of estrogenic activity by tamoxifen (Jobling and Sumpter, 1993), we were unable to measure VTG production in cultures exposed only to tamoxifen at the dose used (1 μM). The estrogen receptor antagonist ICI-182,780 was also examined for its ability to inhibit VTG-synthesis. While often more potent than tamoxifen (≥ 75% inhibition), ICI-182,780 proved inconsistent in its ability to reduce VTG-synthesis by cells cultured with DES or E2 (data not shown).
Experiments with 4-tert-octylphenol (OP), nonylphenol (NP), o,p’-DDT, and benzo(a)pyrene (BaP)

In experiments conducted with the alkylphenolic surfactants OP and NP, both compounds induced detectable VTG synthesis, with OP approximately 6.5-fold more potent than NP (Figure 3). While OP was estrogenic at all concentrations examined, NP induced VTG synthesis only at the highest treatment concentration (10 μM). The general trend of these data are in agreement with previous work (White et al., 1994), but differ in that induction by NP was not observed at 1 μM (10^-6 M). When comparing data from our OP experiments to those collected by workers using other species (White et al., 1994; Jobling and Sumpter, 1993), it should be noted that the use of 1 x 10^6 cells / mL was common among the three studies. On a ng VTG / mL media basis, therefore, OP appears to be approximately 55% (300 ng VTG / mL versus 550 ng VTG / mL at 10μM) as potent in channel catfish hepatocyte cultures as in the rainbow trout cultures of these other workers.

The potency of OP can also be examined in comparison to a positive control. A difference between our studies and those referenced above is that DES served as our positive control while these other workers used E2. In our study, VTG production in response to OP at 10μM was approximately 30% (300 ng VTG / mL versus 940 ng VTG / mL) of the concentration observed in positive control cultures (10 nM DES). In contrast, VTG induction by OP was approximately 85% (600 ng VTG / mL versus 700 ng VTG / mL) of the positive control (10 nM E2) induction.
observed by Jobling and Sumpter (1993). These data support the assertion that OP appears to be less potent in channel catfish hepatocyte cultures than in rainbow trout cultures.

In our studies, VTG induction by OP and NP were both decreased significantly by the presence of tamoxifen (Figure 3). These data strongly suggest that OP and NP elicited their effect through the ER.

Experiments conducted with o,p' -DDT reveal that it induces VTG synthesis with a potency similar to OP. In cultures containing 10 μM and 1 μM o,p' -DDT, VTG was measured at 243 ng / mL and 53 ng / mL, respectively, following a 48-hour incubation. Induction at both concentrations was reduced greatly in the presence of 1 μM tamoxifen. Compared to OP, the potency o,p' -DDT appears to be comparable at 10 μM, while at 1 μM, OP is approximately twice as potent. Data from the o,p' -DDT studies and a comparison of the potency of OP and o,p' -DDT are presented in Figures 4 and 5, respectively. As with OP and NP, the VTG induction by o,p' -DDT is significantly decreased in the presence of 1 μM tamoxifen. As with OP and NP, these data suggest that o,p' -DDT binding with the ER was responsible for the VTG induction observed.
We have also investigated the estrogenicity of the aromatic hydrocarbon BaP. In two experiments with BaP, cultured cells were incubated with 10 nM, 10 μM and 100 μM BaP. BaP did not induce VTG synthesis at any of the examined concentrations and appeared to increase cell toxicity dramatically at 100 μM (data not shown).

Paper Mill Effluents and Stigmastanol

Previous workers have found constituents of pulp and paper mill effluents to be estrogenic (Mellanen et al., 1996). Known to us only by coded identification numbers, we examined mill effluents from the aeration stabilization lagoons of two bleached kraft mills, an unbleached kraft mill and a deinking, recycled fiber mill. In studies conducted to date, VTG synthesis was not detected in cultures containing 10-, 10-, and 1-fold dilutions, nor 10-fold increases in effluent concentrations. Cultures containing 10-fold-increased concentrations, however, displayed marked increases in cytotoxicity, with high percentages (60-100%) of cells in all effluents showing signs of degeneration after 48-hours (data not shown). Experiments with the plant extract stigmastanol, a common component of paper mill effluent, showed that this chemical did not induce detectable VTG synthesis.

Stigmastanol and the mill effluents were also tested for anti-estrogenic activity in cells incubated in media containing both DES and stigmastanol or effluent. Compared to DES alone, none of these treatments caused a significant reduction in the media concentrations of VTG at the concentrations tested. Data from the stigmastanol experiments are presented in Figure 6.
Utility of Experimental Design and Protocol

The design, development and implementation of a protocol combining cultured channel catfish hepatocytes and an ELISA have provided us with a powerful tool to examine the potential estrogenicity of selected aquatic pollutants. Currently, we are able to maintain hepatocyte cultures for up to 8 days without loss of VTG inducibility. In addition, the detection limit of the ELISA was approximately 25 ng VTG/mL culture medium. This represents a 600-fold increase in sensitivity compared to the ELISA developed by Goodwin et al. (1992) for VTG in channel catfish plasma. All values reported for experimental treatments were calculated using the regression equation generated from standard curve data. The intra- and inter-assay variations for the data presented here were approximately 10 and 17%, respectively. Specifically, experiments conducted with different batches of hepatocytes showed an average of 17% (range 5-25%) variability in the concentration of VTG measured in similarly treated cultures. The standard curve (i.e., DES only) data presented here are a combination of data from 10 different studies. Standard errors for these data range from 8-19%. These performance characteristics are similar to those reported for VTG assays for other teleost species (Mananos et al., 1994; Goodwin et al., 1992; Nuñez Rodriguez et al., 1989).

Experimental Limitations, Difficulties and Resolutions

A significant limitation inherent in our method is the water solubility of the chemical(s) of interest. Because the hepatocytes are cultured in a water-based medium, the effective concentration of any
chemical in these studies will be determined by its solubility in an aqueous solution containing 0.3% DMSO or EtOH.

The major difficulty encountered during development of these protocols was inadequate binding of purified VTG to the 96-well plates. As part of our methods development we tried a number of different plates including three different Immulon® microtiter plates (Dynex Technologies) and two plates made by Nalge/Nunc (Polysorb® and Maxisorb®). In addition, we made changes to the composition and molarity of the buffers used in the assay. The current combination of Nalge/Nunc Maxisorb® plates and 0.017 M borate-buffered saline (see Methods section) appear to have eliminated this problem yielding data that are both accurate and reproducible.
References


List of Publications

Monteverdi, G.H., and R.T. Di Giulio. In Review. An enzyme-linked immunosorbant assay for estrogenicity using primary hepatocyte cultures from the channel catfish (Ictalurus punctatus). *Archives of Environmental Contamination and Toxicology*

Appendix A - Solutions Used for Isolation of Hepatocytes

1. Buffer A (In HPLC grade Water)

<table>
<thead>
<tr>
<th>Component</th>
<th>MW</th>
<th>500 ml</th>
<th>1000 ml</th>
<th>1500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mM EDTA</td>
<td>372.24</td>
<td>0.0936 grams</td>
<td>0.1872</td>
<td></td>
</tr>
<tr>
<td>110 mM NaCl</td>
<td>58.44</td>
<td>3.214</td>
<td>6.428</td>
<td>9.642</td>
</tr>
<tr>
<td>4 mM KCl</td>
<td>74.55</td>
<td>0.149</td>
<td>0.298</td>
<td>0.447</td>
</tr>
<tr>
<td>25 mM NaHCO₃</td>
<td>84.01</td>
<td>1.0501</td>
<td>2.102</td>
<td>3.1503</td>
</tr>
</tbody>
</table>

pH 7.4
Make 1000 mL.

2. Buffer B

Same as Buffer A with the substitution of 2.5 mM CaCl₂ in place of EDTA.

<table>
<thead>
<tr>
<th>Component</th>
<th>MW</th>
<th>1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM CaCl₂</td>
<td>111</td>
<td>0.2775</td>
</tr>
<tr>
<td>Collagenase D</td>
<td>100 mg / 300 ml</td>
<td></td>
</tr>
</tbody>
</table>

- adjust to 7.4 with 1N HCl.
- Collagenase D from Beringer-Manheim

3. Leibovitz L-15 Medium - Purchased from Gibco.

Buffer and supplement this solution with the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>MW</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM HEPES</td>
<td>238.3</td>
<td>2.383 g</td>
</tr>
<tr>
<td>gentamicin (10 g/ml)</td>
<td></td>
<td>5 mg</td>
</tr>
<tr>
<td>streptomycin (2.5 µg/ml)</td>
<td></td>
<td>50 mg</td>
</tr>
<tr>
<td>tetracycline (10 µg/ml)</td>
<td></td>
<td>5 mg</td>
</tr>
</tbody>
</table>

Raise to pH 7.4 with 2N NaOH.
All solutions should be filtered through 0.22 µm pore size filter.
4. MS-222 Anaesthesia

--Initial: 150 ppm MS-222 (0.15 g/L)

Make 12 L in plastic basin (1.8 g MS-222) and buffer with 3.6 g NaHCO₃.

--Maintenance: 100 ppm MS-222 (0.100 g/L)

Make 16 L in recirculating tub (1.6 g) and buffer with 3.2 g NaHCO₃.

5. Trypan Blue Exclusion Assay:

a. Mix the cell suspension by gently inverting the tube several times.

b. Make a 1:2 dilution of the cell suspension in L-15. Add 0.5 ml of this suspension to a glass culture tube containing 0.1 ml 0.4% trypan blue. Transfer cells using a pipetman tip from which the end has been snipped off.

c. Allow to stain for 2 min. Gently mix and transfer aliquots by pasteur pipet to a hemocytometer. Count viable and non-viable (stained blue) cells. Large clumps of cells should be counted as "1". The hemocytometer has 9 grids, each grid with a volume of 10⁻⁴ ml. It is desirable to count at least 200 cells.

d. Cell viability is calculated as:

\[
\text{Viability} = \frac{\text{total cells not containing dye}}{\text{total cells}}
\]

e. The viable cell density is calculated as:

\[
\text{Cells/ml} = (\text{tot. viable cells} / \# \text{ grids counted}) \times \text{dilution factor} \times (1.2 \times 10^4)
\]

Example: Counted 5 grids with 194 viable, 40 non-viable cells in a 1:5 dilution.

\[
\text{Viability} = \frac{194}{194 + 40} = 83\%
\]

\[
\text{Viable cells/ml} = (194/5) \times 5 \times (1.2 \times 10^4) \\
= 2.33 \times 10^6 \text{ cells/ml}
\]
Appendix B - Lactate Dehydrogenase Assay Solutions:

1. Tris/NaCl solution

<table>
<thead>
<tr>
<th>Component</th>
<th>500ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base (81.3 mM)</td>
<td>4.92 g</td>
</tr>
<tr>
<td>NaCl (203.3 mM)</td>
<td>5.95 g</td>
</tr>
<tr>
<td>Adjust pH to 7.2 with 5 M HCl.</td>
<td></td>
</tr>
</tbody>
</table>

2. Tris/NaCl/NADH solution
   - Dissolve 0.017 g NADH (=0.244 mM), disodium salt, in 100 ml solution.

3. Tris/NaCl/pyruvate solution
   - Dissolve 0.107 g pyruvate (=9.76 mM), dry crystalized monosodium salt, in 100 ml solution.

4. Tris-HCl buffer (0.1 M)
   - Dissolve 3.94 g Tris-HCl in 250 ml final volume water. Adjust to pH 7.4.

5. 1% (v/v) Triton X-100 solution in Tris-HCl buffer.
   - 0.25 ml triton in 25 ml Tris-HCl buffer.

Appendix C - Solutions and Reagents for ELISA:

1. Borate-buffered saline (per 1 L)
   - 6.48 g Na$_2$B$_4$O$_7·$10H$_2$O (0.017 M)
   - 7.01 g NaCl (0.12M)
   - Adjust to pH 9.6 with NaOH

2. Blocking Buffer (BBS-BSA; add to 100 mL BBS)
   - 50 μL Tween 20
   - 0.038 g EDTA (1 mM)
   - 0.250 g bovine serum albumin (BSA)
   - 0.05 g sodium azide (NaN$_3$)

3. Wash Buffer (BBS-T; add to 500 mL BBS)
   - 500 μL Tween 20
   - 0.25 g sodium azide (NaN$_3$)

4. p-nitrophenyl phosphate (pNPP) solution (SIGMA FAST™ Substrate Tablet Set)
   - 50 mL HPLC-grade water
   - 1 SIGMA FASTpNPP tablet (N-2765)
   - 1 Tris Buffer Tablet
   - Mix with magnetic bar stirer

5. Primary Antibody (Gift of Drs. John Grizzle and Tom Steeger)
   - monoclonal mouse-IgG antibody to channel catfish VTG

6. Secondary Antibody
   - alkaline phosphatase-conjugated goat-anti-mouse-IgG from Sigma Chemical Company (St. Louis, MO USA)