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Optimization of a precision-cut trout liver tissue slice assay as a screen for vitellogenin induction: comparison of slice incubation techniques

P. Schmieder *, M. Tapper, A. Linnum, J. Denny, R. Kolanczyk, R. Johnson

US Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804, USA

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Abstract

An in vitro male rainbow trout liver slice assay has been developed for long-term incubation of precision-cut slices for the detection of vitellogenin (VTG) protein induction in response to xenobiotic chemicals. The assay was optimized to allow 72 h of incubation of slices to maximize detection of VTG, while maintaining slice viability. Two methods of incubation frequently used with rat liver slices were compared: (1) slices were submerged in media (11°C) and cultured in 12-well plates (PL) with continuous shaking; or (2) slices were floated onto titanium screens, placed into glass vials, and held under dynamic organ culture (DOC) conditions (11°C). Slices (200 μm) in modified L-15 media were exposed to 1.0 μM 17β-estradiol (E2) or diethylstilbestrol (DES). Protein from media and slice was sampled for Western blot analysis, using a polyclonal antibody to detect appearance of VTG protein. Maximum VTG was seen at 72 h, with detectable protein at 24 and 48 h in slices and media following PL incubation. In contrast, slices incubated in DOC showed little detectable VTG above background levels after 72 h. This difference was not attributable to protein loss to vial or plate surfaces. Standard viability assays did not reveal any differences between slices incubated in PL or DOC. However, histopathological examination revealed earlier and more severe vacuolization in slices incubated in DOC. Significantly more E2 uptake and conversion to water-soluble metabolites was noted in PL, compared with DOC, as well as more production of VTG in response to DES and E2, correlated with less histologic change. The in vitro assay described allows tissue-level assessment of estrogenicity in aquatic organisms, and will be useful for assessing not only comparative species receptor binding and transactivation, but also the role of tissue-specific activation factors in the estrogenic response of fish. Published by Elsevier Science B.V.

Keywords: Environmental estrogens; *Oncorhynchus mykiss*; Fish metabolism; In vitro liver slice assay

1. Introduction

The US Environmental Protection Agency is developing tools appropriate for evaluating chemicals for their potential to disrupt the endocrine systems of humans, other mammals, and wildlife

* Corresponding author. Tel.: +1-218-529-5161; fax: +1-218-529-5003.

E-mail address: schmieder.patricia@epa.gov (P. Schmieder)

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species including fish. As part of this process, there is continued interest in the development of in vitro models for screening and prioritization of chemicals for further in vivo testing. In oviporous species, the binding of 17β-estradiol to the estrogen receptor (ER), and subsequent interaction of the receptor–ligand complex with estrogen-responsive elements (ERE) on the DNA, results in transcription of the vitellogenin (VTG) gene and, ultimately, production of the egg-yolk precursor, protein vitellogenin. Vitellogenin is normally produced in the liver of female fish and transported to the ovaries, where it is incorporated into the egg. Male fish, which possess functional estrogen receptors but normally produce very small quantities of VTG (Copeland et al., 1986; Sheahan et al. 1994), have been shown to produce large quantities of VTG when exposed to xenobiotics capable of interacting with the ER (Pelissero et al., 1993; Jobling et al., 1996; Lech et al. 1996). This endpoint is thus evolving as an indicator of alterations in hormone synthesis, metabolism, or ER activation in aquatic species.

A variety of in vitro models used to assess chemically induced estrogenicity were reviewed by Zacharewski (1997). These include competitive ER binding, cell proliferation, protein expression/enzyme activity, and recombinant systems, including ERE-regulated reporter gene systems; chimeric receptors; and yeast-based assays. All of these systems have various advantages and disadvantages, such as inability of binding assays to distinguish agonism and antagonism, dependence of cell proliferative responses on serum factors, variable potencies noted in different cell line clones, and influence of intracellular environments on observed responses, including presence or absence of appropriate transcription factors, etc (refer to Zacharewski, 1997 for a comprehensive review). Additional issues with in vitro models are the lack of retention of metabolic capability in some preparations used, and the potential temperature sensitivity of receptors and other proteins involved in transactivation with models derived from poikilotherms (Sheahan et al., 1994; Petit et al., 1995). Thus, a preferred model would retain tissue-specific transcription factors (Katzenellenbogen et al., 1996), maintain metabolic capability for xenobiotic bioactivation, as well as detoxification, and allow ligand–receptor interactions, transcription, and translation to occur optimally at the physiological temperature of the organism under study. While there have been some in vitro systems utilizing transfected cell lines with fish ER and VTG reporters, or cell aggregates derived from aquatic species, there has not been an in vitro aquatic model run at physiological temperature of the organism that has incorporated uninterrupted cell-to-cell communication with multiple cell types, endogenous metabolic capability, tissue specific co-activator and co-repressors, and that has been used as a screen for estrogenicity. This, however, may be possible with the use of precision-cut fish liver slices.

Over the past decade, tissue slices have gained popularity as an in vitro model for studies of metabolism as well as chemical effects research with mammalian organisms due to their representiveness of in vivo processes (reviews include Fisher et al., 1995a,b; Ekins, 1996; Olinga et al., 1997a). There have also been some studies in which tissue slices from aquatic species were used, specifically rainbow trout and catfish (Gilroy et al., 1996; Kleinow et al. 1996; Oganesian et al., 1996; Singh et al., 1996). Precision-cut liver tissue slices are generally prepared by removing the liver from the organism, using a coring tool to extract an 8–10 mm core, and then slicing pieces of tissue from the core that are of a consistent thickness, e.g. 200–500 μm (Krumdiek et al., 1980). The slice of liver tissue therefore contains many intact cell layers (number dependent on slice thickness), maintains tissue architecture and intercellular communication, and contains all liver cell types normally present in the organism in addition to hepatocytes.

As the use of tissue slices has increased, researchers have attempted to optimize slice incubation methods, especially important in long-term culture of slices (Olinga et al., 1997b). One method for slice incubation uses a roller incubation system, often referred to as dynamic organ culture (DOC) (Fisher et al., 1995a,b, 1996; Drahushuk et al., 1996; Gokhale et al., 1997; Price et al., 1998). In this method, tissue slices are loaded onto screens and placed in scintillation

vials, which are horizontally mounted on a device that allows the vials to be rotated vertically in a circular orbit. The slices on the screen continually move between incubation medium and the gas phase. Another method for tissue slice incubation places the tissue slice into media in multi-well plates (PL) which are rotated on a shaking platform (Dogterom and Rothuizen, 1993; Olinga et al., 1997a,b). Both systems allow for control of temperature, gas mixture, and humidity. Various arguments were made as to the advantages or disadvantages of certain slice incubation systems used with rat liver tissue slices (Fisher et al., 1995a,b; Olinga et al., 1997b). However, to our knowledge, a comparison of such methods has not been done with fish liver tissue slices.

The present study was undertaken to develop a method for long-term (72 h) culture of rainbow trout liver slices, optimizing for production of VTG protein in response to estrogenic stimulation, for eventual testing of the model as a possible screening tool for estrogenicity. In addition, the study compared protein production and metabolism of 17β-estradiol in two incubation systems, DOC and shaken PL.

2. Methods

2.1. Chemicals

Leibowitz-15 (L-15) media with glutamine without phenol red was purchased from Gibco BRL (Grand Island, NY). 17β-Estradiol (1,3,5-[10]-estratriene-3,17β-diol) (E2), diethylstilbestrol (DES), fetal bovine serum (FBS), and penicillin/streptomycin (P/S) solution were procured from Sigma Chemical Co. (St. Louis, MO). Ethanol was purchased from Quantum Chemical Co. (Tuscola, IL). All other chemicals were purchased from Sigma.

2.2. Fish

Immature male rainbow trout (*Oncorhynchus mykiss)*, 250–650 g, were used for this study. Fish sex was determined by visual observation of gonads. If sex was not apparent, gonads were removed from fish and examined microscopically. Fish were obtained from the Seven Pines Fish Hatchery, Lewis, WI or McKenzie Farms, Stacy, MN, and allowed to acclimate in flow-through circular tanks in Lake Superior water (11°C, pH 7.7; hardness, 45.3 mg/l) for at least 2 weeks prior to use.

2.3. Slice preparation

Male trout were anesthetized with MS-222 and an incision made to expose the livers. The livers were perfused in situ by insertion of a cannulae in the portal vein followed by perfusion with sterile, ice-cold, clearing buffer (a modified Hanks' balanced salt solution (HBSS) lacking Ca and Mg, pH 7.8) for 5 min to remove red blood cells and prevent clotting. Livers were surgically removed within 10 min on anesthesia, and placed in 4°C sterile slicing buffer (HBSS, pH 7.8 + glucose 900 mg/l) until coring, which was started immediately. Liver cores (8 mm) were prepared using a modified tissue coring press (Model MD2000; Alabama Research and Development, Munford, AL). The cores were immediately placed into a sterilized and chilled (4°C) Krumdiek precision tissue slicer (Alabama Research and Development), which contained sterile slicing buffer. Liver cores were cut into 200 μm slices. To minimize ragged edges, a new blade was used for each core. Slices from one core were loaded into all vials or wells used for control and chemical (E2 or DES) treatments for a given time point (see Section 2.4). Minimal slice preparation time and low buffer temperatures resulted in favorable slice viability. Approximately 75–120 slices were obtained from a 2.5–7.6 g liver. One trout was used per experiment. It took ~10 min for slices to go from core to incubator, and 1–1.5 h from the start of surgery until all the slices were loaded into plates or vials.

2.4. Slice incubation systems

Trout liver slices were incubated by two methods. In the first, DOC (Fisher et al., 1996), 20 ml glass scintillation vials containing media and slice were placed on their side between pegs of a verti-

cally mounted wheel that was rotated at a rate of 2.3 rpm. The unit was housed within a temperature-controlled incubator held at 11°C (Vitron Inc., Tucson, AZ). Slices destined for the DOC incubator were floated onto titanium screens which were inserted into titanium roller inserts and placed in sterile 20 ml vials containing 1.7 ml incubation media (L-15 + 10% FBS + P/S). Vial caps had a 2 mm diameter hole in the center to allow gas exchange. Regulation of humidity was not possible in the roller incubator, but a pan of distilled water was placed in the incubation chamber. The second incubation method allows the agitation of media with slice in a PL. Slices subjected to PL incubation were placed into sterile 12-well polypropylene (Falcon 3043 flat bottom) tissue culture plates, one slice per well, containing 1.7 ml incubation media (L-15 + 10% FBS + P/S). The covered plates were then placed on an orbital shaker (125 rpm) in an 11°C incubator, 80% relative humidity, normal air. Media pH and osmolality were measured daily in both systems throughout the course of the experiment, and there was no media replacement.

2.5. Exposures

E2 (3.7 mM), in 13.6 μl ethanol, was added to 50 ml L-15 incubation media to obtain a final concentration of 1.0 μM E2. Carrier controls consisted of 50 ml L-15 media with 13.6 μl added ethanol. Control, E2, or 1.0 μM DES media (1.7 ml) was added to either wells or vials prior to the addition of liver slices. Slices in each treatment were incubated in DOC or PL for 0, 24, 48, or 72 h. Duplicate slices incubated in individual vials (DOC) or wells (PL) for each treatment, at each time, were sampled for determination of K^+ content, with media removed from the same vial or well for measurement of lactate dehydrogenase (LDH) leakage. Slices in two additional vials and wells were sampled at 0 h (approximately 30 min after start of incubation) for determination of total LDH in slices. Another two vials and wells contained media and slices sampled for determination of VTG protein by Western blotting. Finally, slices were sampled from two vials and wells for histomorphology.

Experiments in which slices were exposed in PL or DOC to 1.0 μM E2 were replicated on three different dates. In two of these experiments, attempts were made to determine the amount of extractable VTG protein that may have been adsorbed onto vial or plate-well surfaces, in addition to routine determination of VTG in media and slice. Two additional experiments were done in which slices were exposed for 72 h to 1.0 μM DES in PL and in DOC incubators.

2.6. Slice viability

The activity of LDH in the media was measured throughout the experiment, and compared with total slice LDH activity at 0 h. Total LDH was determined by removing the slice from the incubation media, blotting it dry and placing it in a microcentrifuge tube with 0.5 ml distilled-deionized water (DIW), and disrupting the tissue by sonication (Virsonic 475; Virtis Co., Gardiner, NY). LDH was measured in incubation media or slice homogenate following the method of Bergmeyer and Bernt (1974). LDH appearance in media was expressed as a percentage of initial measured LDH activity in slices.

Liver slice cellular K^+ content was determined using the technique of Fisher et al., (1996). Briefly, slices were removed from the culture media, blotted dry and placed in pre-weighed microcentrifuge tubes. After determining slice weight, 1 ml DIW was added to each tube and slices were sonicated. Proteins were precipitated from cell lysate by the addition of 20 μl perchloric acid (70%), pelleted by centrifugation, and supernatants were removed and assayed for K^+ using an atomic absorption spectrophotometer (Perkin-Elmer 3100; Perkin-Elmer, Norwalk CN). K^+ concentrations were determined by comparison with a standard curve and were expressed as μmol K^+/g wet weight.

2.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis Western blots

Whole liver slices were removed from the culture media, placed in microcentrifuge tubes, and prepared for polyacrylamide gel electrophoresis

(PAGE)/Western blot analysis by adding 1 ml protein sample buffer (0.0625 M Tris–HCl, 1% sodium dodecyl sulfate (SDS), 0.002 M ethylenediamine tetraacetic acid (EDTA), 2% 2-mercaptoethanol, 5% glycerol, 0.05% phenol red, pH 6.8) and heating at 100°C for 2 min (Laemmli, 1970). The slice was further disrupted by being pulled through a 20 G needle with the aid of a 1 ml syringe. The samples were again heated in a 100°C water bath for 2 min. Incubation media samples were prepared by addition of 40 μl concentrated protein sample buffer (0.3125 M Tris–HCl, 5% SDS, 0.01 M EDTA, 10% 2-mercaptoethanol, 25% glycerol, 0.25% phenol red) to 160 μl sample media, resulting in a protein sample buffer concentration identical to that used with slices. Samples were heated in a 100°C water bath for 2 min prior to loading onto the gel.

An attempt was made to determine possible protein loss through adsorption to plate wells or glass vial surfaces. In one experiment, following removal of both the slice and media at all time points, 1 ml protein sample buffer was added to the vials and wells, and plates and vials were returned to the respective incubators. After being shaken or rotated for 1 h at 11°C, the 1 ml protein sample buffer was placed in microcentrifuge tubes, heated for 2 min in a 100°C water bath, and loaded onto gels. In one experiment, a mass-balance was also done to confirm VTG protein recovery from vial and well surfaces as follows. Media, taken after 72 h of slice incubation with 1.0 μM E2, was sampled for VTG, and then distributed into three vials and three wells, and left in DOC or PL incubator for 72 h. Following incubation, media, vial and plates extracted with protein sample buffer were measured and compared with the initial media concentration.

Protein extracts (50 μl) from slices, incubation media, or vials/well eluates were loaded onto SDS polyacrylamide gels (4% stacking, 9.5% resolving) 1.5 mm thick. Gels were loaded into an electrophoresis chamber (Protean IIxi Cell; Biorad, Hercules, CA) and an electrical force was applied; 70 mA through stacking gel and 40 mA through resolving gel (Laemmli 1970). Following running of the gel, proteins were electrophoretically transferred for 16 h (Trans-Blot Cell; Biorad) onto nitrocellulose as described by Towbin et al. (1979).

Nitrocellulose membranes were probed with a VTG polyclonal antibody (Ab) generously provided by A. Hara, Hokkaido, Japan. Membranes were washed in PBST (phosphate buffered saline (PBS) pH 7.4 + 0.05% Tween 20) for 30 min, followed by 1 h in blotto buffer (5% carnation dry milk in PBS) (Damato et al., 1988). The membrane was then incubated in VTG Ab 1:2000 in blotto buffer 4% normal goat serum (Chemicon Inc.; Temecula, CA) for 4 h at 37°C. Following a wash in PBST, the membranes were incubated in a goat anti-rabbit immunoglobulin G horseradish peroxidase labeled Ab 1:500 (Chemicon Inc.), blotto buffer, 4% normal goat serum (NGS) solution for 1.5 h at 37°C. Membranes were developed with 3,3′-diaminobenzidine (DAB)/NiCl and 3% H_2O_2.

Protein migration distances and band density were determined using broad range protein standards 202 kDa myosin, 109 kDa β-galactosidase, 78 kDa bovine serum albumin, 46.7 kDa ovalbumin, 34.5 kDa carbonic anhydrase, 28.8 kDa soybean trypsin inhibitor, 20.5 kDa lysozyme, 7.4 kDa aprotinin. (Sigma Chemical Co.) ImagePro Plus software (Media Cybernetics, Silverspring, MD).

2.8. Estradiol metabolism

To determine the relative uptake and disposition of E2 in media and trout liver slices incubated in PL compared with those incubated in DOC, liver slices were placed in 1.7 ml L-15 media containing ^{3}H-E2 prepared in the following manner. A solution containing 0.58 nmol in 90 μl (9:1 toluene:EtOH) of ^{3}H-E2 ([2,4,6,7,16,17-^{3}H]Oestradiol, code TRK587, batch 188; Amersham, UK) (1 μCi/μl) was added to 89.2 nmol unlabeled E2 in 90 ml L-15, resulting in a final ^{3}H-E2 exposure concentration of 1 μM and 1 μCi/ml. Incubations were carried out as previously described, and four replicate samples were taken from both PL and DOC systems at 0, 2, 4 and 24 h. Controls consisted of ^{3}H-E2 media added to wells and vials which contained no

slices. Slice samples were removed from the media, washed (three times) in L-15 media to remove residual labeled media from the slice, then placed in a centrifuge tube containing 1 ml DIW and sonicated for 10 s to disrupt the slice. Upon addition of 1.5 ml ethyl acetate (EtOAc), tubes were vortexed, centrifuged, and the EtOAc fraction removed. The procedure was repeated, EtOAc fractions combined, mixed, and 0.5 ml removed for scintillation counting. In addition, 0.2 ml of the remaining aqueous phase was counted to determine concentration of unextractable label. Media samples (1 ml) from wells or vials were transferred to centrifuge tubes, then extracted with EtOAc and subsampled as already described. The subsampled EtOAc and aqueous extracts were added to 15 ml scintillation cocktail (Packard UltimaGold) and counted on a liquid scintillation analyzer (Packard Instrument Co.; Downers Grove, IL). Concentration of ^{3}H-E2 in stock media prior to placement in wells or vials was determined in duplicate, and used as a basis for mass-balance determinations. Differences in disposition and metabolism of ^{3}H-E2 in slices in the two incubation systems was assessed by inspection of means and standard deviations, and additionally assessed using analysis of variance Balanced Design (Minitab Inc.; State College, PA) to detect effects of the two incubation techniques and time.

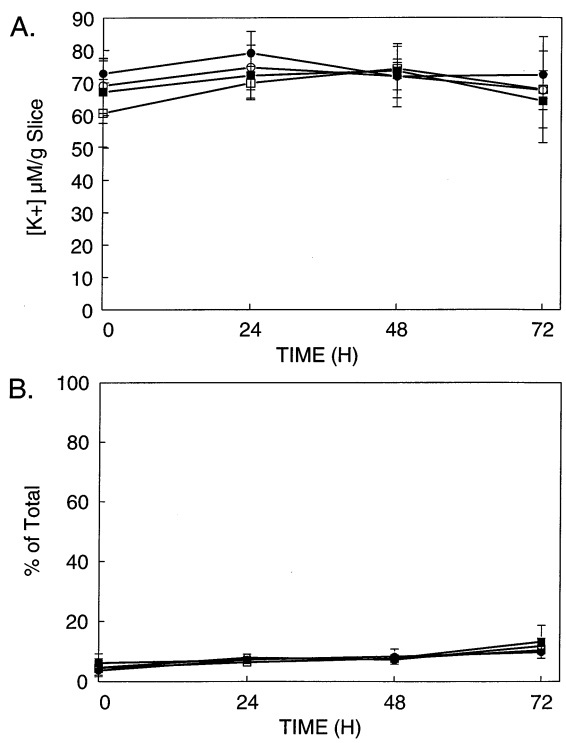

Fig. 1. Slice viability assessed by (A) potassium content per gram wet slice weight and (B) LDH leakage into media as a percentage of total LDH per slice. Symbols: CTRL-PL (□), CTRL-DOC (○), E2-PL (■), E2-DOC (●). Values represent the mean ± S.D. of three separate experiments.

2.9. Histology

Duplicate liver slices (control or E2 exposed) were obtained for histological analysis at 0, 24, 48, and 72 h. Fixation began by placing each tissue slice in a vial with ice-cold fixative (0.1 M PBS (pH 7.4), 4% formaldehyde 1% glutaraldehyde (w/v)). Fixation continued overnight at room temperature by placing the vials in a rotator. After 24 h in fixative each slice was quartered into four pie-shaped pieces and placed in 0.1 M phosphate buffer. The slices were post-fixed for 10 min in 1% osmium tetroxide prior to flat embedding in epoxy resin (Araldite 504). Each tissue slice was sectioned normal to its flat axis (800 nm thick) and sections were stained with borate-buffered (pH 8.3) azure B. All observations were done by light microscopy with transmitted brightfield illumination.

3. Results

Trout liver slices (200 μm) cultured in L-15 + 10% FBS + P/S media at 11°C were incubated in either shaken 12-well plates (PL), where they were submerged in media throughout the incubation period, or roller DOC culture, where they were placed on titanium screens allowing movement of slice in and out of culture media. The L-15 media was maintained at constant pH (within 0.2 U) and osmolality over 72 h, and both incubation systems resulted in healthy slices, as determined by no significant change in K^{+} content in slices (Fig. 1A) and no increase in LDH leakage into the media (Fig. 1B).

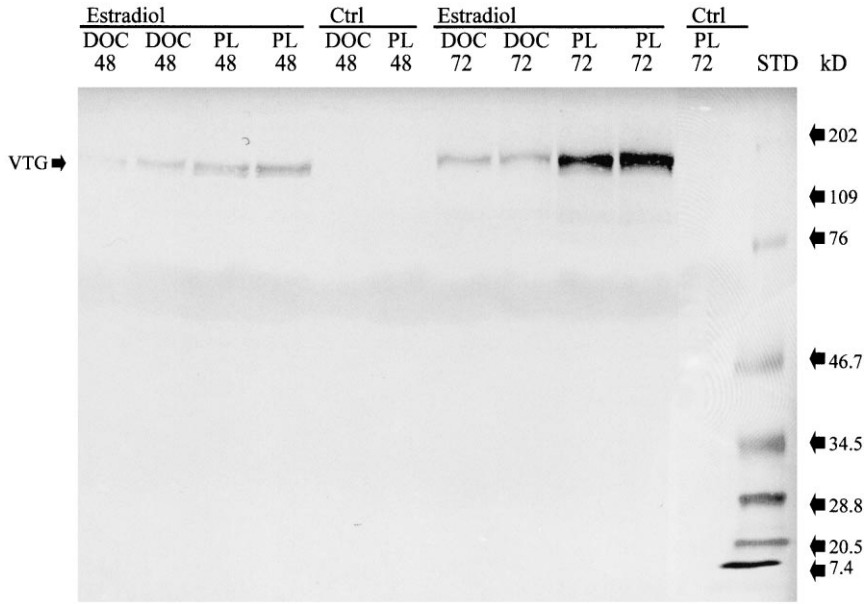

Fig. 2. Western blot of VTG protein secreted into medium from male rainbow trout liver slices exposed to 1 μM 17β-estradiol. Slices which were incubated for 48 or 72 h in shaken PL produced greater amounts of VTG protein than those incubated in glass vials in a roller DOC incubator.

Slices exposed to 1.0 μM E2 in either PL or DOC incubators were tested for their ability to produce VTG protein. Greater amounts of VTG were excreted into the media from slices incubated in PL than DOC. This is illustrated in Fig. 2, with darker bands observable on Western blots from PL media samples at 48 and 72 h when compared with DOC samples taken at the same times. This response was replicated in multiple experiments with E2 (data not shown). Exposure of male trout slices to another estrogenic chemical, DES, also showed the same response, i.e. earlier and greater induction of VTG in PL than DOC, with VTG first detected at 24 h in PL, but not until 48 h in DOC (Fig. 3).

A similar response to that noted in media was seen when VTG was measured within homogenized liver slices. VTG protein appeared in slice and media samples from PL, while none was detected from DOC (Fig. 4). The predominant band in both slice and media samples was determined by SDS-PAGE to be 176.4 ± 12 kDa ($n = 10$) (P176). In the present study, a large VTG band > 250 KDa ($P > 250$) was also identified in media samples. The $P > 250$ band was relatively more intense when β-mercaptoethanol (S-EtOH) was not added to the sample buffer. Concurrently, P176 increased in intensity in samples processed with S-EtOH relative to those without, suggesting

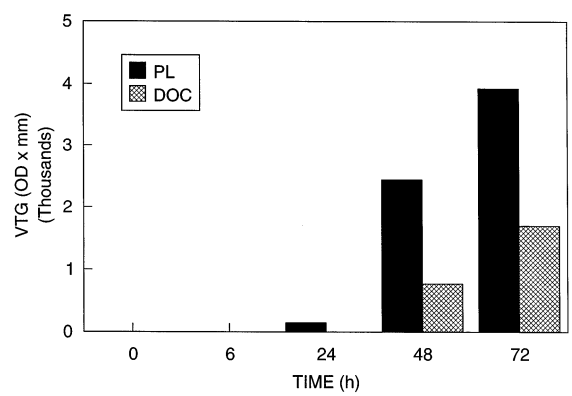

Fig. 3. Secretion of VTG protein into tissue slice incubation media in response to 1 μM DES. Slices incubated in PL produced greater amounts of VTG protein than those incubated in DOC, starting at 24 h and continuing through 72 h without media replacement. Bars indicate optical density × area (mm) of dot blots from media.

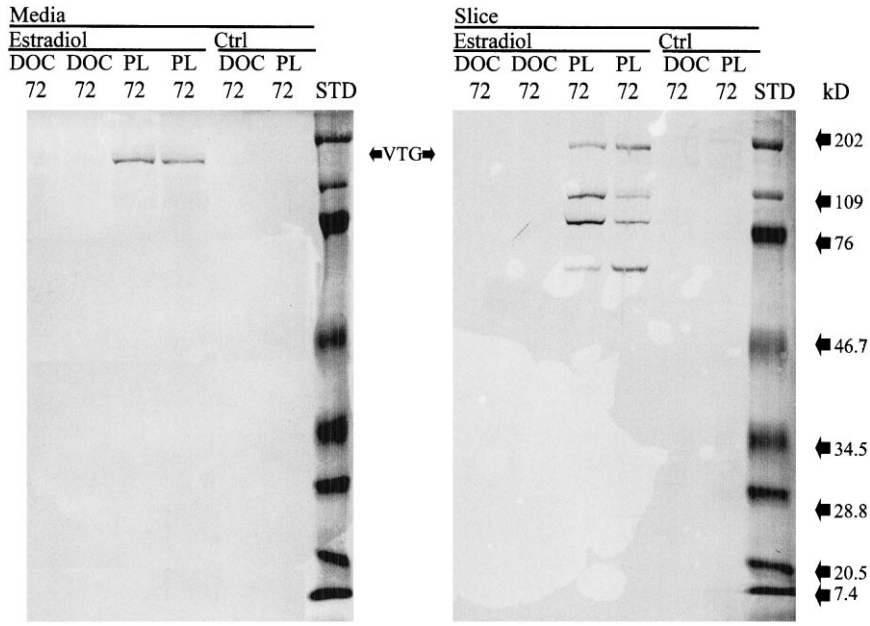

Fig. 4. Western blots of VTG protein secreted into media or measured in homogenized slices incubated for 72 h in shaken PL or roller DOC incubator. Media samples produced one predominant VTG band at 176 kDa, while digested tissue slices produced bands at 176, 106, 70.8, and 61.2 kDa.

$P > 250$ was the dimeric form of Vtg and P176 was the monomer. Interestingly, $P > 250$ was never seen in the slice digests, perhaps because the dimerization that resulted in $P > 250$ did not occur until the VTG had reached the golgi apparatus. Once formed, it was then secreted into the media (Mommsen and Walsh, 1988). Three additional bands, along with P176, typically appeared on Western blots from slices in sample buffer containing S-EtOH; 106.3 ± 12.6, 70.8 ± 10.4 and 61.2 ± 6.2 kDa. These smaller bands may be precursors of the larger forms of VTG. They increased in intensity in the presence of S–EtOH, but were also observed in its absence. However, these lower molecular weight bands were not observed, in the Western blots from media samples.

Due to the large differences observed in VTG protein production from the two incubation systems, attempts were made to determine if VTG was being fully recovered in each system. No detectable VTG was extractable with protein buffer from well or vial surfaces at the completion of the 72 h experiments. Additionally, a media sample spiked with a trout VTG standard was incubated at 11°C for 72 h in PL wells and DOC vials. A small and equivalent amount of VTG was recovered from glass vials and wells, with the majority of the VTG recovered in the media, resulting in a mass balance. Also, titanium screens present normally only in DOC were placed in PL wells during one experiment. Again, greater VTG production was observed in PL than in DOC. Therefore, any differences noted in VTG protein between PL and DOC seems attributable to differences in production and not to differential detection.

The uptake and disposition of ^{3}H-E2 was also examined in slice and media over 24 h to help explain possible differences in incubation systems. First, adsorptive loss of ^{3}H-E2 to vial and well surfaces was determined in the absence of slice, serving as an experimental control (Table 1, control). The initial ^{3}H-E2 concentration of approximately 1.2 nmol ^{3}H-E2/1.7 ml was maintained through 2 h in PL wells, while the Ctrl-DOC dropped from the initial 5 min (0.1 h) value to < 1.0 nmol ^{3}H-E2/vial by 15 min (0.25 h). In an additional experiment (data not shown), media

Table 1
Measured ^{3}H-E2 metabolism in control (1.7 ml media in wells and vials without slices), media (with slices), and in slices incubated in PL wells or DOC vials[a]

Incubator	Time (h)	Total ^{3}H-E2 equivalents					
		Control (no slice) (nmol/1.7 ml)[b]	Media (slice present) (nmol/1.7 ml)[c]	Aqueous fraction (%)	Slice (nmol/slice)[b]	Aqueous fraction (%)	% in slice[d]
PL	0.1	1.24 ± 0.03	1.24 ± 0.03	7	–	–	–
	0.25	1.23 ± 0.01	–	–	0.052 ± 0.004	50	4
	2	1.31 ± 0.03	1.11 ± 0.03	27	0.188 ± 0.009	55	15
	4	0.88 ± 0.15	1.12 ± 0.05	64	0.167 ± 0.018	53	13
	24	1.04 ± 0.11	1.41 ± 0.09	98	0.057 ± 0.010	86	4
DOC	0.1	1.22 ± 0.07	1.22 ± 0.07	6	–	–	–
	0.25	0.99 ± 0.07	–	–	0.017 ± 0.003	51	1
	2	0.99 ± 0.04	0.86 ± 0.07	16	0.057 ± 0.010	61	4
	4	0.85 ± 0.01	0.80 ± 0.05	25	0.056 ± 0.006	58	4
	24	0.93 ± 0.16	0.97 ± 0.04	78	0.037 ± 0.015	77	3

[a] Control, media, and slice ^{3}H-E2 equivalents are total counts, i.e. sum of all ^{3}H-chemical forms present, measured in ethylacetate and aqueous fractions, with percentage of counts found in aqueous fraction reported.

[b] Mean ± S.D. ($n = 2$).

[c] Mean ± S.D. ($n = 4$).

[d] Percentage of total media + slice ^{3}H-E2 counts that are found in the slices at each time point, thus calculation is corrected for lower available ^{3}H-E2 in DOC due to adsorptive losses.

plus ^{3}H-E2 was added to vials and sampled at 2 min, 2, 4, and 24 h. Levels of ^{3}H-E2 dropped from 1.4 nmol/1.7 ml at 2 min to 0.86, 0.77, and 0.78 nmol/1.7 ml in DOC vials after 2, 4, and 24 h, respectively. Thus, a consistent and significant ($P > 0.05$) loss of ^{3}H-E2 is observed in the DOC systems, even in the absence of slice, presumably due to adsorption to glass surfaces as the media is rolled along the vial walls. Virtually all (95%) of the recoverable ^{3}H-E2 in control samples from PL and DOC was found in the organic extractable phase throughout a 24 h exposure.

The amount of ^{3}H-E2 measured in media in the presence of tissue slices was also determined over a 24 h period, and was consistent with previous results in the absence of tissue (Table 1, media). For both PL and DOC systems, in the presence of slices, ~ 1.2 nmol ^{3}H-E2 was initially available in the media, with $> 90\%$ in the organic phase. However, by 2 h, the DOC media had dropped below 1.0 nmol for the duration of the experiment, while ^{3}H-E2 in PL media remained above 1.0 nmol; again, likely due to differences in adsorptive loss. There was a significant ($P < 0.05$) difference in media ^{3}H-E2 due to incubator, as well as time, with a significant interactive effect.

While total counts in media remained fairly constant in PL, a much more rapid conversion of the ^{3}H-E2 equivalents to water-soluble metabolites was observed in PL than with DOC (Table 1, media aqueous fraction). The EtOAc extractable ^{3}H-E2 equivalents rapidly decreased in PL, while the aqueous extractable metabolites increased to 64% of total media counts by 4 h, and 98% at 24 h. An increase in aqueous extractable ^{3}H-E2 equivalents in media was also noted in DOC, but this occurred at a much slower rate with a maximum conversion of 78% reached at 24 h.

There were notable differences in the quantity of ^{3}H-E2 taken up by tissue slices in each system. Slices incubated in PL accumulated 15% of total media + slice counts by 2 h (Table 1, % in slice). The maximum uptake in DOC incubated slices was only 4% at 2 and 4 h. The ^{3}H-E2 equivalents in slices were equally partitioned between aqueous and organic phases through 4 h in PL and DOC systems, but shifted to the majority recoverable in the aqueous fraction by 24 h in both systems. Overall, the amount of total media ^{3}H-E2 equivalents remained more constant in the PL wells, uptake of label into the slices was greater, and there was a faster rate of conversion to aqueous extractable components in slices and media during PL incubation than in DOC.

Freshly prepared (0 h) liver slices demonstrated the standard tubular liver architecture of rainbow trout recently reviewed by Hinton and Couch (1998) (Fig. 5). The sinusoidal space could be easily distinguished. Sections of anastomosing tubules formed primarily of hepatocytes were readily observed. In some regions, the perisinusoidal space of Disse could be identified between the endothelial lining of the sinusoid and the basolateral margin of hepatocytes (Fig. 5). After 12 h in culture, the sinusoidal spaces, although still discernable, were no longer patent regardless of culture method. Also, with time in culture, structural elements of the slice rearrange causing PL slices to get smaller in diameter and thicker. DOC slices did not decrease in diameter, but did appear to get thinner, perhaps due to loss of cells on the outside edge of the slice. The amount of glycogen present in slice hepatocytes was highly variable and did not seem to be affected by either the culture method or E2 treatment (Fig. 5, Fig. 6, panels a–f, and Fig. 7, panels a–f).

Histological examination of slices supported the earlier observation that functional parameters such as protein production and metabolism were maintained to a greater extent in slices cultured in PL than DOC. Based on vacuolar degeneration of hepatocytes, slices from PL were healthier, regardless of sampling time. Within 24 h in DOC, some hepatocytes in both control (Fig. 6, panel a) and E2-treated slices (Fig. 7, panel a) had started to form vacuoles, whereas hepatocytes in slices from PL (Fig. 6, panel d, control and Fig. 7, panel d, E2) had not started to vacuolate. By 48 h in DOC, nearly all the hepatocytes within 25–30 μm of the slice surface showed some vacuolar degeneration (Fig. 6, panel b and Fig. 7, panel b), yet hepatocytes in PL slices, whether unexposed or E2 treated (Fig. 6, panel e and Fig. 7, panel e), did not exhibit this pathology. The cells in 72 h slices from both culture systems were extensively vacuolated. However, PL hepatocytes further

from the slice edge were less vacuolated than those near the surface, while, in DOC, almost all hepatocytes were extensively vacuolated (Fig. 6, panels c and f, and Fig. 7, panels c and f). The morphology of hepatocyte nuclei remained primarily unchanged despite different culturing methods or E2 treatment. Only a few pyknotic or karyolytic nuclei were observed, even by 72 h when vacuolation pathology was extensive; most of these were noted in DOC (Fig. 6, panel c and Fig. 7, panel c). Cell debris was observed throughout the 72 h DOC slices, and macrophages were seen near the central plane. The observation that hepatocyte pathology seemed to originate near the slice surface instead of deep within the slice suggests that the vacuolation degeneration was not attributable to hypoxia or nutrient limitation, which should cause the hepatocellular pathology to originate in the deeper cells first. A comparison of the space of Disse of E2-PL slices (Fig. 7, panels d–f) with control-PL, and control and E2-treated DOC slices (Fig. 6, panels a–f and Fig. 7, panels a–c), indicates that this space stained darkly in E2-PL slices but was lightly stained or unstained in all other cases. The dark staining region in these slices was likely filled with secreted VTG, stimulated by the E2 treatment. This observation is consistent with the measured VTG response in E2-PL being much greater than in E2-DOC. Overall, nearly all cells in DOC slices appear to be irreversibly damaged by 72 h, while many of the deeper cells in the PL slices still appear to be healthy and secreting VTG.

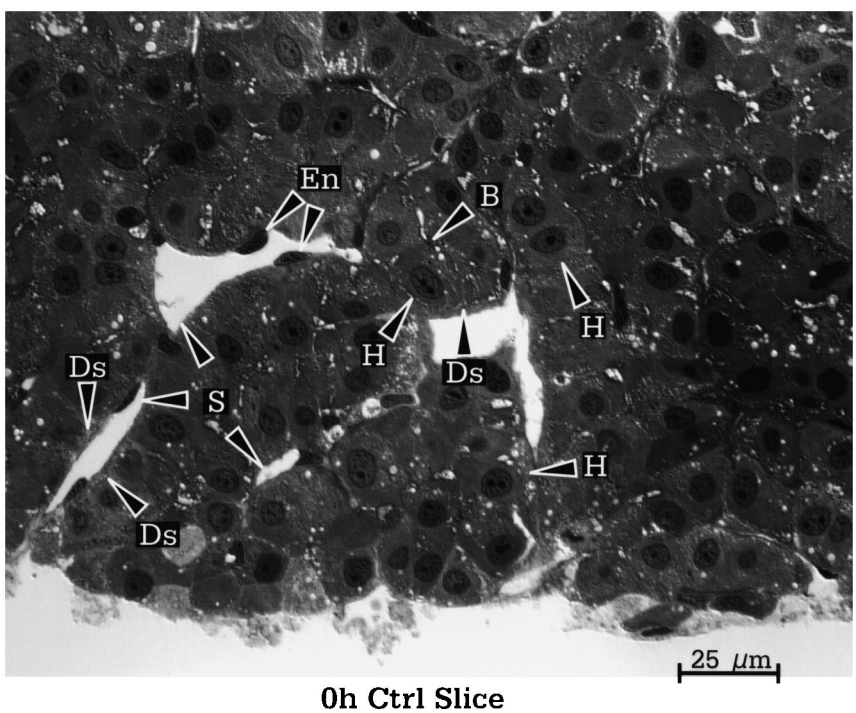

Fig. 5. Histology of freshly cut trout liver slice with one edge of the slice forming the lower border of the image. The tissue (800 nm thick, cut normal to flat axis, epoxy embedded, azure B stained, imaged by light microscopy with transmitted illumination) is comprised primarily of hepatocytes (H) arranged in one-cell-thick tubules that are circular in cross-section with a biliary space (B) in the central region of the tube. The white regions along the outer margins of the tubules are the sinusoidal spaces (S). Note the nuclei of endothelial cells (En) which line the sinusoidal space and the very thin white line between the sinusoidal space and the basolateral margin of heptocytes, the space of Disse (Ds). B, biliary region; H, hepatocyte; En, endothelial cell nucleus; Ds,space of Disse; S, sinusoidal space. Scale, 25 μm.

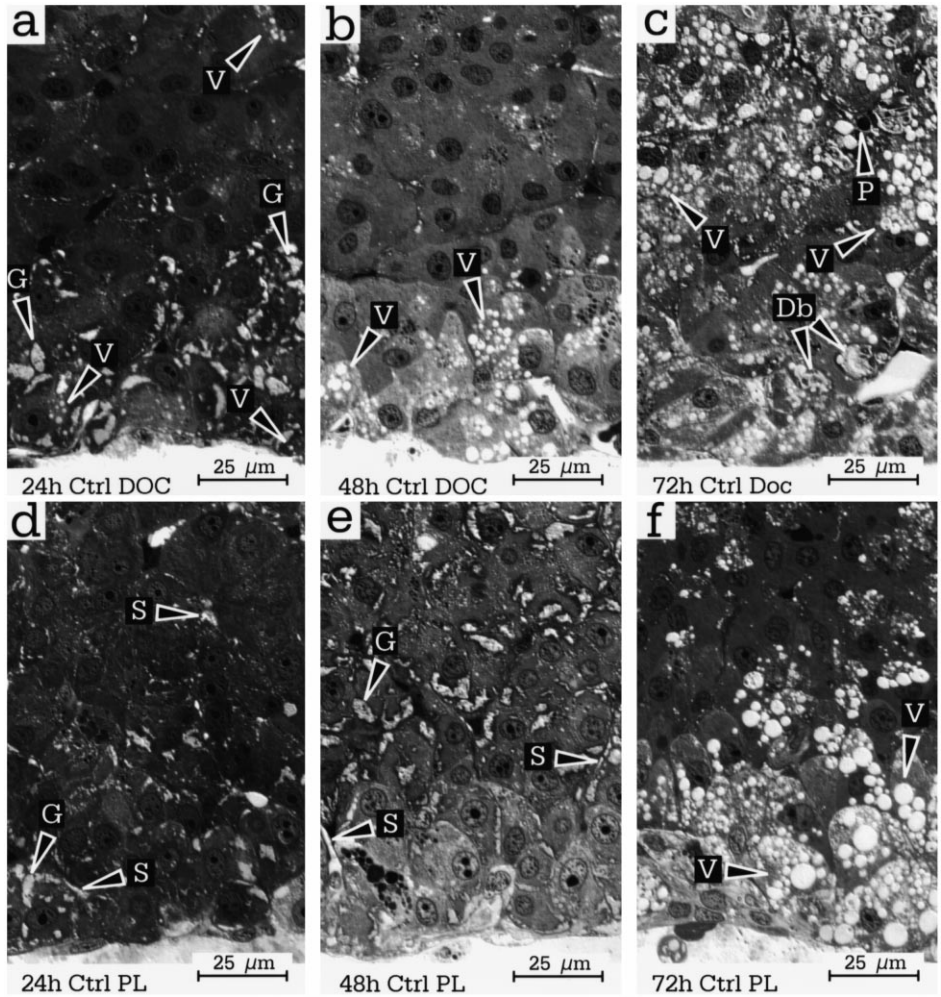

Fig. 6. Control trout liver slices in DOC and PL culture after 24, 48, and 72 h. Histological and imaging methods are the same as described in Fig. 5. In DOC (panels a–c) within 24 h, small vacuoles (V) can be seen in hepatocytes near the edge of the slice. By 48 h, the vacuoles have increased in number and size. The 72 h DOC slice shows extensive vacuolar degeneration throughout the slice. Pyknotic nuclei (P) and cellular debris (Db) can also be observed within the slice. The presence of glycogen (G), the light PAS-positive granular material present in some hepatocytes was highly variable across time and culture methods. The PL slices (panels d–f) are not vacuolated in 24 or 48 h, but show some vacuolar degeneration (V) by 72 h. However, compared with 72 h DOC, 72 h PL only rarely had pyknotic nuclei. Hepatocytes in control PL slices, in contrast to E2-treated slices (see Fig. 7), do not stain darkly in regions adjacent to sinusoid (S). G, glycogen; V, vacuole; Db, cellular debris; P, pyknotic nucleus; S, sinusoidal space.

4. Discussion

This study extends earlier work by presenting optimized culture parameters and incubation conditions for the 72 h culture of rainbow trout liver tissue slices for the determination of VTG protein synthesis in response to estrogenic compounds, specifically E2 and DES. To our knowledge, this study is the first to describe optimized conditions for detection of VTG induction in the trout model and the first to compare the impact of slice incubation methods on viability, functional parameters, and metabolism in rainbow trout liver tissue slices. There have been a few studies utilizing precision cut trout liver slices as an in vitro model in recent years. Gilroy et al. (1996) used liver

slices from control and dieldrin pre-treated trout to investigate the subsequent uptake and disposition of several exogenous and endogenous compounds, including estradiol. Singh et al. (1996) held slices in culture for 5 h, and measured oxidative and conjugative metabolism of model substrates biphenyl and naphthol, and in addition provided histomorphological assessment of trout liver slice response to the hepatotoxicants allyl formate and allyl alcohol over 24 h. A study conducted by Fisher et al. (1996) utilized trout liver slices in long-term culture (10 days) for assessment of heavy metal effects on K^+ retention, alanine transferase (ALT) leakage and [^{3}H]-leucine incorporation.

The optimization of incubation parameters as well as other culture conditions and the ability to assess the health and viability of an in vitro tissue preparation are especially important in long-term culture, i.e. days instead of hours (Olinga et al. 1997b). Fisher et al. (1995a) proposed a rank order for use of viability parameters to assess

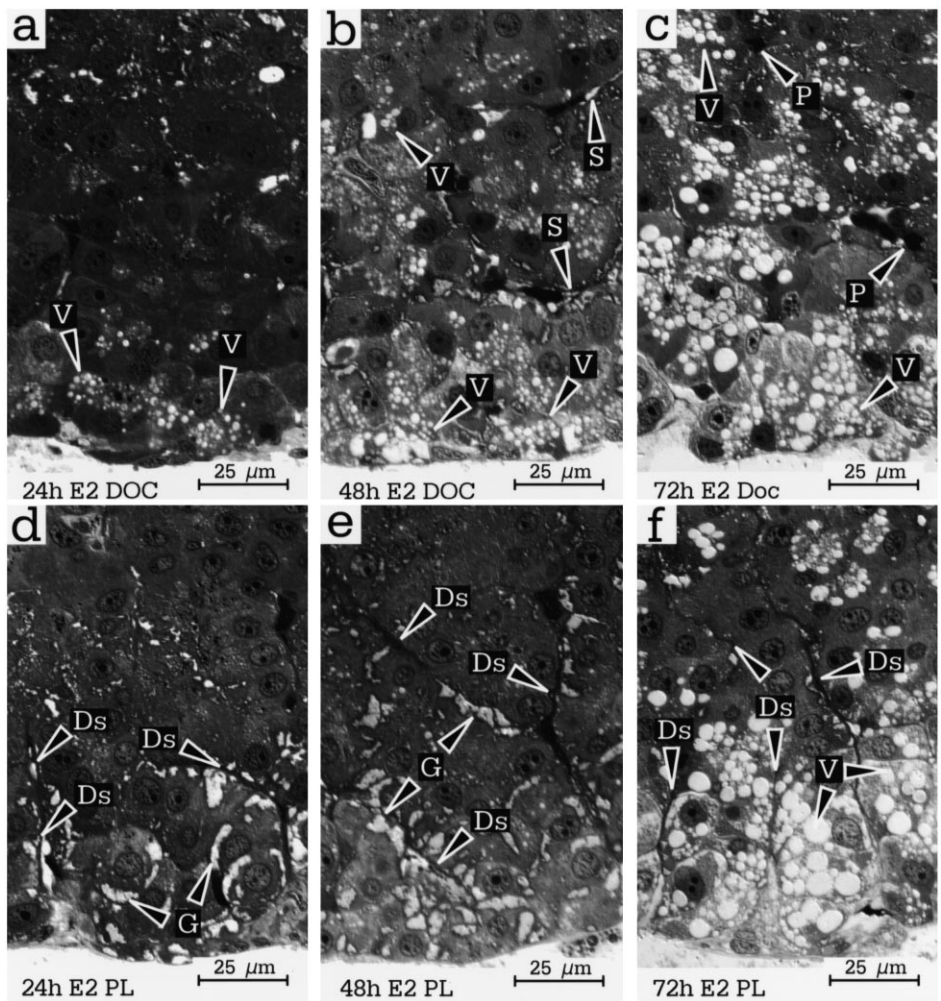

Fig. 7. E2-treated trout liver slices in DOC (panels a–c) and PL (panels d–f) culture after 24, 48, and 72 h. Histological and imaging methods are the same as described in Fig. 5. The progression of vacuolar degeneration is similar to that observed in control slices (Fig. 6). Notice the spaces of Disse (Ds) next to the sinusoids (S) in the estrogen treated PL slices are darkly stained. Ds, space of Disse; G, glycogen; V, vacuoles; P, pyknotic nucleus; S, sinusoidal space.

health of rat liver slices, as follows: adenosine triphosphate content > K^+ retention > protein synthesis > enzyme leakage > 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide reduction. The two incubation systems used in the present study, multi-well PL and roller DOC, performed identically when evaluating the commonly used viability endpoints of slice K^+ retention and LDH leakage. Initial K^+ levels in trout slices measured in this study were identical to those measured by Fisher et al. (1996). LDH leakage also remained low in this study, similar to ALT leakage measured previously in trout slices (Fisher et al., 1996). Protein synthesis, also listed by Fisher et al. (1995a) as an important viability parameter, was the primary endpoint of concern in the present study. The measurement of VTG induction in the liver of trout serves as a indication of estrogenic potential of xenobiotic chemicals (Jobling and Sumpter, 1993; Pelissero et al., 1993; Sheahan et al., 1994; White et al., 1994; Petit et al., 1997). While production of VTG was noted in trout liver slices and detected in incubation media in both systems tested in response to E2 and DES, the incubation of slices in multi-well PL with slices submerged in media throughout the exposure seemed far more favorable for the endpoint of interest than did incubation of trout slices in DOC, where they are alternately exposed to liquid and gas phases.

One of the proposed advantages of slice incubations in roller systems like DOC was that slices would receive more adequate oxygenation (Fisher et al., 1995a,b; Olinga et al., 1997b). Trout liver slices 200 μm thick used in the present study seemed to be adequately oxygenated in either system, based on tissue pathology. Oxygenation may not be limiting due to the low but physiologically relevant incubation temperature used (11°C) and increased oxygen solubility at low temperatures, although several experiments with rat slices incubated at higher temperatures have not shown oxygenation problems, especially when relatively thin slices (200–300 μm) are used (Olinga et al., 1997b; Price et al., 1998).

In the present study, trout slice culture was extended to 72 h to obtain maximum induction of VTG to optimize the detection of weak estrogenic responses expected upon exposure to some environmental toxicants. Slices in PL culture for up to 72 h secreted large amounts of VTG into the media, while maintaining a healthier appearance than DOC slices. Irreversible changes noted in trout liver slices after 72 h in the current study did not appear to be due to hypoxic damage because initial pathology began at the edge of the slices. Trout liver slices had been previously incubated for 24 h in a shaking water bath by Singh et al. (1996), and were described as generally healthy with no selective damage in the center of the slice, although some glycogen depletion and the presence of mis-shapen nuclei throughout the slice was noted. Trout slice health in long-term culture is somewhat in contrast to observed changes in rat liver slices. Gokhale (1995) saw histomorphological deterioration (coagulation necrosis and hypertrophy), which they believed to be hypoxic injury in rat liver slices maintained on Chee's medium and incubated in multi-well plates on a gyratory shaker maintained in long-term culture (72–96 h). At the same time, they noted no change in functional parameters including inducibility of CYP1A1/1A2, ethoxyresorufin o-deethylase (EROD) activity, albumin mRNA levels and secretion, despite histological change. In a later study, slices incubated in a roller system had morphological change after 3–4 days in culture while maintaining P450 activity (Gokhale et al., 1997). This is similar to the trout slice system in which metabolism, and VTG induction and secretion continued despite noted histological change, although less metabolism and VTG secretion were noted in DOC slices, which exhibited more severe structural damage.

Precision-cut liver slices have gained acceptance as a robust model for the study of metabolic processes in mammalian tissues (Parrish et al., 1995; Drahushuk et al., 1996; Ekins, 1996; Olinga et al., 1997a,b), and also recently with fish (Gilroy et al., 1996; Kleinow et al., 1996; Oganesian et al., 1996; Singh et al., 1996). Advantages in comparison with other in vitro models include ease of preparation, minimal activation of proteolytic enzymes, maintenance of tissue architecture including cell-to-cell communication, as well as ability to incorporate metabolism and biochemical end-

points with histopathological changes. Flouriot et al. (1993) found many of these characteristics to be critical for expression of rainbow trout estrogen receptor (rtER) and VTG genes. Their rainbow trout hepatocyte cell aggregate cultures exhibited kinetics of rtER and VTG mRNAs similar to in vivo kinetics only after reassembly of extracellular matrix components, re-establishment of cell-to-cell contacts and re-creation of three-dimensional cell architecture. Precision-cut liver slices of ~ 200 μm contain multiple undisturbed cell layers with differentiated liver tissue, presence of other liver cell types in association with hepatocytes, intact extracellular matrix, cell-to-cell communication, and metabolic capability. Trout tissue slices cultured in L-15 media in the present study were capable of producing VTG protein detectable after 24 h in PL incubation, with VTG mRNA measurable as early as 4 h (unpublished observations). Detection of VTG in slices incubated in DOC, however, was delayed and did not reach levels measured in PL cultures, presumably due to a combination of decreased uptake of E2 and greater degree of irreversible histological change. The VTG protein excreted into culture medium as determined by Western blotting in the current study was similar to that of Kwon et al. (1993), who identified VTG excreted into media from cultured trout hepatocytes after stimulation with E2 to have a molecular weight of 175 kDa. The VTG found in media and slice are also consistent with a monomeric form of 176 ± 4 kDa, with breakdown products ranging from 120 to 80kDa found by Bon et al., 1997, with a dimeric form of 393 ± 16 kDa. The molecular weight of VTG has been characterized between 342 and 535 kDa, dependent on the estimation method used (Bon et al., 1997). For application as a screening assay, maximization of total VTG detectability, whether in the monomeric or dimeric form is desirable; therefore, characterization of the antibodies used by Western blotting prior to development of more rapid quantitative assays, i.e. enzyme-linked immunosorbent assay, is recommended.

Possible reasons for the dramatic differences in VTG induction observed in the two incubation systems include differences in both E2 uptake and metabolism by the slice. There was rapid uptake of E2 into slices in both systems, with maximum E2 slice concentrations measured at 2 h in this limited kinetic study. However, almost three times more ^{3}H-E2 equivalents accumulated in slices incubated in PL than in DOC. Accumulation of ^{3}H-E2 equivalents in PL slices at 10 min and 2 h was similar to that noted by Gilroy et al. (1996), in which ^{3}H-E2 uptake in trout liver tissue slices was measured in 60 min incubations in glass vials held in an orbital shaker at 14°C. Estradiol was rapidly converted to water-soluble metabolites in the present study, as determined by EtOAc extractions. Ethyl acetate has been shown to separate E2 and its phase I metabolites (estrone, estriol) from sulfate and glucuronide conjugates in the aqueous media (Beleh et al., 1995; Zhu et al. 1996). The apparent conjugation occurred to some extent in each system but at a faster rate in slices from PL, resulting in eventual loss of ^{3}H-E2 equivalents from the slice, especially at 24 h. The conversion of E2 to glucuronide conjugates is known to occur in trout liver. Forlin and Haux (1985) looked at biliary elimination of labeled E2 in rainbow trout and determined the hepatic route to be a major method of elimination with $> 90\%$ of counts in bile determined to be glucuronides. Singh et al. (1996) measured significant rates of conjugative metabolism by trout liver slices incubated in a shaking water bath. Slices exposed to 100 μM 1-naphthol produced glucuronide and sulfate metabolites in nmol/mg protein quantities in 2 h at 15°C. In the present study, significant conjugative metabolism is indicated by the rapid conversion of ^{3}H-E2 from an organic extractable to a water-soluble fraction. Confirmation of sulphation and/or glucuronidation could be achieved by addition of aryl-sulphatase or glucuronidase to aqueous samples with subsequent EtOAC extraction. The present results also indicate that greater E2 uptake and significant metabolic conversion was positively associated with increased production of VTG in slices incubated in the PL system. While a more rapid metabolic conversion of E2 may seem counterproductive to VTG production, in this instance it seems to be an indicator of slice health and maintenance of function. It has also been shown that in trout hepatocytes, estradiol-3-

sulphate was as potent as E2 in stimulating VTG production, and estradiol glucuronide was about 10 times less potent than E2 but still active (Pelissero et al., 1993). Thus, increases in aqueous metabolite concentration may not necessarily mean decrease in activity. The increased E2 uptake into PL slices, which spend the entire incubation period in contact with the medium, in contrast to DOC slices that spend half the time out of the medium, however, may be the most significant reason for the greater VTG induction in this system. Therefore, this optimized PL incubation system is preferred for studies of VTG induction in trout liver slices and may be better suited to long-term (several days) studies using the trout liver slice model.

5. Conclusion

A method is presented for the long-term (72 h) culture of trout liver slices at physiological temperatures in which VTG production can be induced upon stimulation with E2 or DES. Vitellogenin can be monitored in either the slice or media, and significant production is achieved by 48 h, while 72 h may be more ideal for quantitation by Western blot analysis. While slice viability measured by LDH leakage and K^+ retention is similar for slices incubated in either PL or DOC, histological degeneration is noted earlier and to a greater extent in DOC slices. VTG induction is seen earlier and to a greater extent when slices are incubated in a multi-well plate system when compared with a roller system. The male trout tissue slice system presented here will be tested further to determine its utility in screening the estrogenic potential of xenobiotics. This in vitro system has several advantages over other in vitro systems, including retained metabolic capacity, long-term culture, maintenance of intercellular communication and tissue architecture, with an endogenous complement of tissue specific transcription factors, and its ease of preparation and use when incorporating PL incubation. Additionally, the capability provided by this system of assessing rtER activation at physiologically relevant temperatures may be essential for the accurate screening of potentially estrogenic environmental contaminants in aquatic organisms.

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