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A 3D fish liver model for aquatic toxicology: Morphological changes and Cyp1a induction in PLHC-1 microtissues after repeated benzo(a)pyrene exposures

April L. Rodd*, Norma J. Messier, Charles A. Vaslet, and Agnes B. Kane

Department of Pathology & Laboratory Medicine, Brown University, Providence, RI 02912, USA

Abstract

To identify the potential environmental impacts of aquatic pollutants, rapid and sensitive screening tools are needed to assess adaptive and toxic responses. This study characterizes a novel fish liver microtissue model, produced with the cell line PLHC-1, as an *in vitro* aquatic toxicity testing platform. These 3D micro-tissues remain viable and stable throughout the 8-day testing period and relative to 2D monolayers, show increased basal expression of the xenobiotic metabolizing enzyme cytochrome P450 1A (Cyp1a). To evaluate pulsed, low-dose exposures at environmentally relevant concentrations, microtissue responsiveness to the model toxicant benzo(a)pyrene was assessed after single and repeated exposures for determination of both immediate and persistent effects. Significant induction of Cyp1a gene and protein expression was detected after a single 24 h exposure to as little as 1 nM benzo(a)pyrene, and after a 24 h recovery period, Cyp1a expression declined in a dose-dependent manner. However, cell death continued to increase during the recovery period and alterations in microtissue architecture occurred at higher concentrations. To evaluate a pulsed or repeated exposure scenario, microtissues were exposed to benzo(a)pyrene, allowed to recover, then exposed a second time for 24 h. Following pre-exposure to benzo(a)pyrene, *cyp1a* expression remained equally inducible and the pattern and level of Cyp1a protein response to a second exposure were comparable. However, pre-exposure to 1 μ M or 5 μ M of benzo(a)pyrene resulted in increased cell death, greater disruption of microtissue architecture, and alterations in cell morphology. Together, this study demonstrates the capabilities of this PLHC-1 microtissue model for sensitive assessment of liver toxicants over time and following single and repeated exposures.

Keywords

Animal alternatives; Fish; Morphology; Hepatocytes; Cytochrome P450 1A (Cyp1a); Polycyclic aromatic hydrocarbons (PAHs)

*Corresponding author. april_rodd@brown.edu (A.L. Rodd).

Author contributions

Experimental design: ALR and ABK. RNA isolation and RT-qPCR: NJM and CAV. All data analysis and remaining experiments: ALR. Drafting manuscript: ALR and ABK.

1. Introduction

Modern aquatic environments contain a wide range of complex contaminant mixtures, placing both local species and nearby communities at risk of exposure. In addition to persistent historical contaminants present in many waterways, new materials of concern are being identified for toxicological assessment (Hutchinson et al., 2013; Schwarzenbach et al., 2006; Selck et al., 2016). To understand the risks that these contaminants pose to aquatic habitats, toxicity testing has been conducted using whole animal *in vivo* assays, monolayer cell culture assays *in vitro*, and more advanced *in vitro* cell assays. In environmental toxicology, there is increasing interest in models that predict realistic exposure scenarios using repeated or chronic exposures at lower doses to provide more environmentally relevant results and to limit animal use in toxicity testing (Baron et al., 2012; Bhattacharya et al., 2011; Klaper et al., 2014; Pampaloni et al., 2007).

In this study, the well-characterized polycyclic aromatic hydrocarbon (PAH) benzo(a)pyrene is used as a model organic pollutant (IARC, 2012). PAHs like benzo(a)pyrene are found in aerosols, soils and sediments across the globe, and are known to accumulate in marine organisms (Juhasz and Naidu, 2000). Benzo(a)pyrene is a Group 1 carcinogen which undergoes complex metabolism that generates reactive intermediates that can catalyze the formation of reactive oxygen species and DNA adducts (IARC, 2012; Verma et al., 2012). This study focuses on induction of the xenobiotic metabolizing enzyme cytochrome P450 1A (Cyp1a) as a biomarker of exposure and an adaptive cellular response. This is an important pathway in the biotransformation of toxicants leading to their metabolic activation and excretion of metabolites. Cyp1a induction is a downstream event following aryl hydrocarbon receptor (AhR) activation, which occurs through binding of a number of endogenous and exogenous ligands (Denison and Nagy, 2003). Even at low levels, benzo(a)pyrene is a potent inducer of Cyp1a activity, with naïve killifish demonstrating significantly increased Cyp1a enzymatic activity after exposure to concentrations as low as 10 µg/L (39.6 nM) (Lee and Anderson, 2005; Verma et al., 2012; Wills et al., 2009). Increased basal metabolic capability has been detected in fish adapted to highly contaminated sites and is associated with differences in DNA damage and overall liver pathology (Wills et al., 2010). Both enzymatic activity and gene expression levels have been used as biomarkers in field and experimental studies to provide a quantitative measure of exposure to PAHs and other pollutants (Costa et al., 2013; Kopecka-Pilarczyk and Schirmer, 2016; Stagg et al., 2000).

Three dimensional (3D) cell culture provides a bridging technology between conventional monolayer assays and *in vivo* toxicology (Astashkina and Grainger, 2014; Pampaloni et al., 2007). Micro-tissues develop 3D cell-cell interactions, increased cell density, and a microenvironment that better models tissues *in vivo* (Achilli et al., 2012b; Fukuda and Nakazawa, 2005; Shamir and Ewald, 2014). However, they also present challenges associated with imaging through thicker tissues, maintaining long-term viability, and adapting monolayer toxicity assays to 3D systems (Astashkina and Grainger, 2014; Pampaloni et al., 2007). A number of 3D hepatocyte models developed with a range of cell culture techniques have been published using mammalian primary cells and cell lines for toxicity testing (Godoy et al., 2013; LeCluyse et al., 2012). Many of these models require

the use of scaffolding materials made of either an inorganic substrate or a biological material to simulate extracellular matrix (Shamir and Ewald, 2014). This engineered extracellular matrix guides microtissue formation in scaffold-based systems, while models that use non-adherent wells or the hanging drop method rely on cell-cell adhesion, the cellular cytoskeleton, and autocrine and paracrine signaling for microtissue self-assembly and organization (Achilli et al., 2012a; Godoy et al., 2013; Youssef et al., 2011). These 3D mammalian microtissue models show increased maintenance of differentiation and viability of hepatocytes longer than *in vitro* monolayer cultures, increasing their relevance and utility for toxicity testing (Gunnness et al., 2013; Ramaiahgari et al., 2014; van Zijl and Mikulits, 2010).

As an alternative to fish toxicity testing, more advanced *in vitro* models in aquatic ecotoxicology are needed to assess the impacts of chronic or repeated exposures experienced in the environment (Handy, 1994). While fish cell lines are increasing in availability, compared to mammalian cells few are available from validated depositories for *in vitro* toxicity testing (Hahn, 2011; Lee et al., 2009; Schirmer, 2006). New *in vitro* fish liver models have been developed including spheroids grown from primary rainbow trout hepatocytes in low adherence plates (Baron et al., 2012; Uchea et al., 2015) and an *ex vivo* model using cod liver slices (Eide et al., 2014). However, the rainbow trout model showed loss of *cyp1a* expression by day 5 when compared to the original primary cells, indicating the importance of establishing baseline responsiveness throughout the duration of cell culture (Uchea et al., 2015). The model presented here uses the fish liver cell line PLHC-1 to form a 3D spheroidal microtissue through self-assembly in a scaffold-free agarose hydro-gel (Napolitano et al., 2007). The PLHC-1 cell line, originally derived from an hepatocellular carcinoma induced in the teleost fish *Poeciliopsis lucida*, is known to have functional AhR-dependent pathways that are activated by polycyclic aromatic hydrocarbons (PAHs) and dioxin-like compounds resulting in induced Cyp1a expression and activity (Della Torre et al., 2011; Hestermann et al., 2002; Hightower and Renfro, 1988; Huuskonen et al., 1998; Kienzler et al., 2012; Thibaut et al., 2009). Using induction of Cyp1a, apoptosis, and alterations in morphology as endpoints, this study demonstrates the responsiveness of PLHC-1 microtissues to pulsed PAH exposures and the ability to identify both acute and persistent effects of toxicant exposure.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of reagent grade or higher and purchased from Sigma unless otherwise specified. To limit adsorptive loss of benzo(a)pyrene to plastic surfaces, all benzo(a)pyrene stock solutions and exposure media were prepared in glass, and all spheroid exposures were conducted in glass containers (Chlebowski et al., 2016; Madureira et al., 2014).

2.2. Cell culture and microtissue formation

PLHC-1 cells (species *Poeciliopsis lucida*, ATCC #CRL-2406) in 2D monolayer and in 3D microtissues were cultured at 30 °C with EMEM medium (ATCC #30-2003) supplemented with 5% fetal calf serum, penicillin/streptomycin, and 5% CO₂. Microtissues were

assembled using a non-adherent agarose hydrogel formed with a negative mold for small spheroid formation (Microtissues, Inc, #12-256). The resulting gel contains a series of round wells 400 μm in diameter that are then seeded with cells as previously described (Napolitano et al., 2007). Briefly, monodispersed PLHC-1 cells were added to the upper chamber of an equilibrated hydrogel at 500 cells/microtissue and allowed to settle into the micro wells for 20 min. Media was added to the rest of the well and the micro-tissues were cultured with normal media changes every 2–3 days until exposure, fixation, or harvest.

2.3. Exposure to benzo(a)pyrene

For the first benzo(a)pyrene exposure, microtissues were transferred to glass bottles (with vented caps) after 4 days of culture and exposed to 1 nM to 5 μM benzo(a)pyrene for 24 h. Each set of samples used to assemble a concentration-response curve was generated from a single population of cells to minimize the effect of cell heterogeneity. Monolayer PLHC-1 cells were also evaluated for *cyp1a* induction in response to a single benzo(a)pyrene exposure, with cells cultured in glass petri dishes for 24 h, then exposed to 1 nM to 5 μM benzo(a)pyrene for 24 h. For evaluation of microtissue recovery, the medium was completely removed by rinsing the samples, then equilibrating microtissues and hydrogels for 30 min in serum-free medium. The wash medium was then replaced with normal culture medium and microtissues were allowed to recover for an additional 24 h. Microtissues were then either collected or received a second 24 h exposure to benzo(a)pyrene in the same glass bottles. Monolayer samples were not used for repeated exposure because of overgrowth of cells cultured for extended periods of time (Fig. S2) and the loss of damaged cells observed during the wash period after treatments. Samples were collected for analysis as described in the sections below.

2.4. Morphological characterization

Live microtissues were imaged in the hydrogel array with phase contrast brightfield microscopy over 8 days of culture to visualize microtissue formation. To image cells throughout the microtissues, they were stained with Hoechst 33342 (Molecular Probes #H3570), optically cleared using Clear^{T2} and visualized as an intact microtissue using confocal fluorescent microscopy (Kuwajima et al., 2013). For histological evaluation, microtissues were embedded in Tech-novit 7100 (Heraeus Kulzer) *in situ* in the hydrogel array using the two-step embedding protocol previously described (Kabadi et al., 2015), then sectioned at $3 \pm 0.5 \mu\text{m}$. For comparison, PLHC-1 cells were plated on collagen I-coated coverslips (Corning #354089) in monolayer at 1 million cells/well in a 6-well plate. Microtissue sections and monolayer coverslips were stained with hematoxylin and eosin (H&E) to evaluate cell morphology in untreated cultures and after benzo(a)pyrene exposure. Spheroid radius was calculated using a minimum of 15 spheroids from each H&E-stained slide and measuring compact spheroid radius using Spot software. The 10 largest spheroids were used from each slide as representative of the center of the spheroid and used for subsequent analysis. As a measure of hepatocyte differentiation, glycogen stores were assessed in untreated microtissues using periodic acid Schiff's reagent counter-stained with hematoxylin (PAS/H) (Segner, 1998). All images were taken at approximately the center of microtissues for consistency. To examine ultrastructure, individual microtissues were

processed for transmission electron microscopy and embedded in Durcupan with osmium staining (Deerinck et al., 2010).

2.5. Biochemical characterization

For measurement of protein and DNA content, samples were cultured for 2–8 days and then collected as a cell lysate. The 2D samples were plated at 1 million cells/well in a 6-well plate, and a total of 2048 microtissues (plated at an initial density of 1.024 million cells) were pooled for each microtissue sample. For collection, hydrogels were transferred to a new plate then flushed with phosphate buffered saline (PBS) to wash out the microtissues. After collecting the loose microtissues, additional PBS was added, the gels flipped upside down and the plate centrifuged at 1000 rpm for 5 min at 4 °C. The pooled microtissue suspension was then pelleted by centrifugation and the supernatant removed. The pellet was washed with additional PBS to remove any residual media and the microtissues were lysed with 0.25% Triton X-100 in PBS with cOmplete protease inhibitor (Roche). After vortexing, samples were cleared by centrifugation at 15,000 rpm for 15 min at 4 °C and stored at –80 °C until further analysis. For protein quantification, samples were processed using a BioRad DC Protein Assay (BioRad #5000112). To quantify DNA, the lysate used for total protein quantification was digested overnight with 200 µg/mL proteinase K and analyzed with a PicoGreen Quant-iT kit (Invitrogen #P11496). Protein and DNA concentrations were measured using a SpectraMax M2 and analyzed with SoftMax Pro software.

2.6. RNA collection and analysis

To examine the effect of 3D cell culture on basal expression of *cyp1a*, a single pool of cells was plated in monolayer (1 million/well in a 6-well plate) or as 3D microtissues (1024 spheroids pooled per sample). Both samples were cultured for 2 or 4 days, then collected for RNA isolation. Monolayer cells were lysed directly into TRI Reagent (MRC #TR118) and microtissues were collected as described for protein, with the elimination of the final pellet wash. The microtissue pellet was then lysed in TRI Reagent and processed for RNA isolation (Chomczynski, 1993).

Expression of *cyp1a* in PLHC-1 cells was determined after a single benzo(a)pyrene exposure, following a 24 h recovery period, or after repeated benzo(a)pyrene exposures. Microtissues and monolayer cells were collected and lysed with TRI Reagent. In all exposures to benzo(a)pyrene, dimethyl sulfoxide (DMSO) was used for stock solutions at a final concentration no greater than 1%, with the vehicle-treated group exposed to the highest amount of solvent used in a given experiment. No additional cell death or significantly increased *cyp1a* gene expression was observed across the range of DMSO used throughout this study (data not shown).

In all samples, isolated RNA was used for RT-qPCR determination of *cyp1a* expression with modifications to previously described protocols (Nolan et al., 2006). Briefly, total RNA was DNase I treated using RNase-free DNase I recombinant (Roche) and reverse transcribed with SuperScript III (Life Technologies) using random primers. The cDNA was precipitated overnight at 4 °C with glycogen (Ambion #9510) as carrier and recovered as outlined by the supplier. The dried cDNA pellet was suspended in nuclease-free water for amplification. The

Applied Biosystems® ViiA™7 and GoTaq qPCR Master Mix (Promega) supplemented with additional MgCl₂ were used for the qPCR following manufacturer's recommendations. The primers and conditions used for RT-qPCR are shown in Supplemental Table S1. Samples were analyzed and normalized to 18S RNA, and results expressed as delta Ct values or fold change relative to vehicle-treated samples (Livak and Schmittgen, 2001). Delta Ct is calculating by taking the cycle threshold value (Ct) for the target gene (*cyp1a*) and subtracting the Ct for the reference gene (18S). Fold change, also known as the comparative Ct method, normalizes the delta Ct values of experimental groups to the vehicle control and uses this delta delta Ct value to calculate $2^{-(\text{delta delta Ct})}$ (Schmittgen and Livak, 2008). All statistical analyses were completed using GraphPad Prism 6. For the single exposure samples (both monolayer and microtissues) and recovery samples (microtissue only), delta Ct values were used to compare vehicle-treated samples to all others using a one-way ANOVA and Dunnett's multiple comparisons test to generate multiplicity adjusted *p*-values. To analyze basal expression and expression after repeated exposures, a two-way ANOVA was used with Tukey's multiple comparisons test for comparison to the vehicle-treated group with multiplicity adjusted *p*-values. To analyze differences in compact spheroid radii, a two-way ANOVA with Dunnett's multiple comparisons test was used to determine the effect of pre-exposure to benzo(a)pyrene, resulting in multiplicity adjusted *p*-values. To compare *cyp1a* expression in treated 2D or 3D samples, multiple *t*-tests were performed to compare delta Ct values at each concentration. Data generated from different pools of cells were not statistically compared because of the heterogeneity observed within this cell line and resultant variability between experiments.

2.7. Immunofluorescence assays

Visualization of benzo(a)pyrene uptake was performed using live day 4 microtissues, which were pre-stained with the nuclear dye DRAQ5 (Thermo Scientific #62254) then exposed to 5 μM benzo(a)pyrene and examined for benzo(a)pyrene autofluorescence at an excitation wavelength of 405 nm (Barhoumi et al., 2000). To assess changes in Cyp1a protein expression and apoptosis in microtissues, samples were exposed to benzo(a)pyrene (described in Section 2.3) then fixed in 10% neutral buffered formalin. Microtissues were embedded for frozen sectioning as previously described (Kabadi et al., 2015). For monolayer samples, cells were plated in 8-chamber LabTekII Chamber slides (100,000 cells per chamber) or 96-well glass-bottomed plates (50,000 cells per well) and treated with benzo(a)pyrene for a single exposure or a single exposure followed by a 24 h recovery period. These samples were fixed with 10% neutral buffered formalin and processed for immunofluorescent detection of Cyp1a. Microtissue frozen sections were used for immunofluorescent detection of Cyp1a, filamentous actin and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL-labeling) of apoptotic cells. Briefly, frozen sections and monolayer samples were permeabilized with 0.25% Triton X-100 then blocked with 3% goat serum + 5% bovine serum albumin + 0.3 M glycine in PBS. Samples were incubated overnight with primary Cyp1a antibody (Cayman Chemical #17315, diluted 1:100), briefly washed with 0.25% Triton X-100, then incubated with a fluorophore-conjugated secondary antibody (Life Technologies A11035, diluted 1:1000) for 2 h. To visualize the filamentous actin cytoskeleton, sections were permeabilized then stained with rhodamine phalloidin (Molecular Probes R415) for 2 h. For TUNEL-labeling, frozen

sections were processed using the DeadEnd Fluorometric TUNEL system (Promega #G3250) as recommended by the manufacturer. All samples were counterstained with the nuclear dye Hoechst 33342 (Life Technologies/Molecular Probes #H3570) or DRAQ5 (Thermo Fisher) and visualized using laser scanning confocal fluorescence microscopy.

3. Results

3.1. Characterization of PLHC-1 microtissue morphology and function

Morphology and markers of differentiation were characterized in PLHC-1 cells in monolayer or 3D microtissues after 2–8 days. When grown in monolayer, PLHC-1 cells demonstrate the flattened appearance commonly described for cells plated on glass or plastic substrates (Fig. 1A) (Gasiorowski et al., 2013). In contrast, cells incorporated into microtissues take on a three-dimensional (3D) shape and assemble themselves into a more tissue-like structure, forming a compact, homogenous spheroid after 4 days (Fig. 1D and E). Microtissues were formed through self-assembly in scaffold-free, non-adherent agarose molds (Fig. 1C). Microtissue size is a tunable property determined by the number of cells used to seed the microtissue, with too few cells preventing assembly and overly large microtissues experiencing greater loss of viability over long culture times due to their large diameter. When seeded with 500 cells per microtissue, cells aggregate overnight and self-assemble into microtissues over 3 days (Fig. 1D). Beginning as an irregular aggregate, cells become increasingly spheroidal and compact over the next several days of culture.

With both light and electron microscopy, microtissues show intact and healthy cell morphology throughout the spheroid for at least 8 days. Nuclear staining of whole microtissues demonstrates that the dense packing of cells observed in sections is representative of the entire 3D spheroid (Supplemental Fig. S1 and Supplemental Video S1). Microtissues grow slowly over time and while day 2 microtissues contain numerous mitotic figures, by day 4 these have decreased in abundance (Supplemental Fig. S2A). In contrast, 2D monolayer cells continue to proliferate after 4 days, with numerous mitotic figures forming domes of multilayered cells visible in most fields of view (Supplemental Fig. S2B). Additionally, microtissues show little change in size from day 4 to day 8 while cell number in 2D samples continues to increase. Quantification of protein and DNA levels over the same time period reflect these histological changes and confirms the different growth rates in monolayer and in 3D spheroids (Fig. 1I and J). After plating at comparable cell numbers, monolayer cultures show a rapid increase in total protein and DNA from 2 to 8 days. During the same time period, protein and DNA content only slightly increase in 3D microtissues when compared to day 2.

The effect of 3D cell culture on differentiation of the PLHC-1 cells was assessed using glycogen accumulation and basal expression of the xenobiotic metabolizing enzyme cytochrome P450 1A (*cyp1a*). Monolayer samples contain only scattered periodic acid Schiff (PAS)-positive, glycogen-rich cells at low densities (Fig. 1B). In contrast, glycogen-rich cells can be found distributed throughout the microtissue for at least 8 days with discrete, granular regions of intracellular glycogen (Fig. 1F and Supplemental Fig. 3A). Glycogen accumulation in 3D microtissues cultured for 7 days was confirmed using transmission electron microscopy, with well-preserved sub-cellular organelles observed in

cells throughout the microtissue in addition to numerous healthy mitochondria (Fig. 1G and 1H). After 8 days, islands of PAS-positive cells can be seen in overgrown regions of cells in 2D, but the distribution of these cells remains uneven (Supplemental Fig. S3B). Culturing PLHC-1 cells in 3D also had a significant effect on their expression of *cyp1a*. Using a single starting population of cells to control sample variability, cells were used to seed 2D monolayers and 3D microtissues in parallel. After 2 or 4 days, microtissues expressed significantly greater *cyp1a* (4.7 and 2.9 fold, respectively) than cells plated in monolayer ($p < 0.01$) (Fig. 1K). Microtissues also maintain their *cyp1a* expression level, while monolayer PLHC-1 cells have a statistically significant decline after 4 days ($p < 0.05$).

3.2. Response to a single benzo(a)pyrene exposure

Using benzo(a)pyrene as a model aromatic hydrocarbon, microtissues were initially exposed after 4 days of assembly and growth (Fig. 2A). Passive diffusion of 5 μM benzo(a)pyrene into the spheroid was detected as early as 30 min after exposure (Fig. 2B). Over 6 h, confocal fluorescence imaging of live microtissues indicates uniform penetration and accumulation throughout the microtissue. Between 2 and 6 h of exposure to 5 μM benzo(a)pyrene, increasing levels of Cyp1a protein can be detected using immunofluorescence in monolayer cultures (Supplemental Fig. S4A). Microtissues show a similar time course for response, with increased Cyp1a detected throughout the microtissue after 6 h of exposure to 5 μM benzo(a)pyrene.

Microtissues treated on day 4 are highly responsive to benzo(a)pyrene exposure, with significantly increased *cyp1a* expression measured after 24 h exposure to as little as 1 nM benzo(a)pyrene ($p < 0.01$) (Fig. 2C and E). Concentrations from 1 nM to 5 μM benzo(a)pyrene were tested in microtissues and elicited up to a nearly 17,000-fold increase in *cyp1a*, with all exposure groups statistically different from each other ($p < 0.05$). In comparison, monolayer samples show significant induction of *cyp1a* at doses of 10 nM benzo(a)pyrene and higher. This suggests that the benzo(a)pyrene dose-response is comparable between monolayer and microtissues and that there is no loss of *cyp1a* inducibility in spheroids (Supplemental Fig. S5).

Benzo(a)pyrene exposure also induces Cyp1a protein expression in both monolayer cells and microtissues. Increased levels of Cyp1a and decreased cell number were observed in monolayer samples exposed to either 1 μM or 5 μM benzo(a)pyrene for 24 h (Fig. 3A). Levels of Cyp1a and cell loss are comparable between these groups. Microtissues show greater differences between treatment groups, with changes in Cyp1a protein expression and cell death observed after higher benzo(a)pyrene exposures. Microtissues exposed to 1 μM benzo(a)pyrene have increased expression at the edge of the microtissue with decreasing levels toward the center, which had fluorescence approximately equal to vehicle-treated spheroids (Fig. 3B and Supplemental Fig. S6). In comparison, microtissues exposed to 5 μM benzo(a)pyrene show increased Cyp1a protein throughout most of the microtissue, with highest Cyp1a levels at the outer edge. These microtissues also show an increased number of dead cells detaching from the surface compared to vehicle-treated controls (Fig. 3E). Detached cells were visualized with Hoechst staining in cryosections as brightly stained pyknotic nuclei and labeled as apoptotic using TUNEL staining. Apoptotic cells were

confined to the outer edge of the microtissue and the overall size of the microtissues remained the same as in the vehicle-treated group, indicating survival of the majority of cells within the microtissue.

3.3. Prolonged adverse responses after removal of benzo(a)pyrene

After 24 h of benzo(a)pyrene exposure, treatment media was replaced with normal media for a 24 h recovery period before collection on day 6. In all samples, *cyp1a* gene expression was decreased after the recovery period (Fig. 2C and E). However, 3D microtissues exposed to high concentrations of benzo(a)pyrene (100 nM to 5 μ M) continued to express significantly elevated levels of *cyp1a* compared to vehicle-treated microtissues. In contrast, microtissues that were exposed to low concentrations of benzo(a)pyrene (1 to 10 nM) have relatively lower *cyp1a* expression after the recovery period. This relative reduction is due in part to increasing expression measured in vehicle-treated samples (Supplemental Table S2). When comparing delta Ct values, which are normalized to the reference gene but not to vehicle-treated controls, *cyp1a* levels continue to be elevated relative to the day 5 control after all treatments.

Following the recovery period, additional differences between 2D and 3D cultures can be identified based on morphology and Cyp1a protein expression. In monolayer samples, fluorescent labeling of nuclei and Cyp1a protein immunofluorescence shows increased cell numbers and reduced Cyp1a protein expression during recovery following exposures up to 1 μ M benzo(a)pyrene (Fig. 3C). However, 2D monolayer cultures exposed to the highest dose of 5 μ M benzo(a)pyrene continue to show elevated Cyp1a protein with no increase in cell number. In contrast, 3D microtissues exposed to 5 μ M benzo(a)pyrene show a disruption of the compact microtissue structure and cell loss only on the outer edge of the microtissue (Fig. 3D). During the recovery period, the number of apoptotic cells in microtissues continues to increase following exposure to 1 μ M and 5 μ M benzo(a)pyrene, with a lower level of apoptosis noted in the 100 nM exposure group (Fig. 3F). Samples exposed to 5 μ M benzo(a)pyrene showed significant losses in both RNA yield and quality, limiting the number of replicates analyzed. This decrease is likely associated with the high level of apoptosis detected in these samples, which has been linked to the rapid degradation of mRNA (Thomas et al., 2015). Using both nuclear labeling in cryosections and histological staining, greater toxicity is seen at the periphery of the microtissue with disruption of the compact, even distribution of cells seen in unexposed microtissues (Supplemental Fig. S7). Additionally, Cyp1a protein levels were reduced in all treated 3D microtissues after the recovery period.

3.4. Response to repeated benzo(a)pyrene exposures

To assess repeated exposures, 3D microtissues were exposed to benzo(a)pyrene on day 4 and on day 6 for 24 h each, with a 24 h recovery period between exposures (Fig. 2A). Microtissues were initially exposed to either 10 nM or 1 μ M benzo(a)pyrene, then to 100 nM to 5 μ M benzo(a)pyrene for a second exposure. Based on gene expression, previous benzo(a)pyrene exposure does not alter the inducibility of *cyp1a* expression in 3D microtissues (Fig. 2D). Additionally, through comparison of delta Ct values, the upregulation of *cyp1a* after 1 and 5 μ M benzo(a)pyrene is equivalent to that seen in

microtissues exposed on day 4 despite higher basal expression in the vehicle-treated group of the repeated exposure study (Fig. 2F).

In 3D cultures that received no second exposure to benzo(a)pyrene, significant differences in *cyp1a* expression persisted for 48 h after the initial benzo(a)pyrene exposure ($p < 0.05$). Compared to microtissues that received only the vehicle, samples exposed to 10 nM benzo(a)pyrene on day 4 showed reduced *cyp1a* expression 48 h after the benzo(a)pyrene was removed. In contrast, microtissues that were exposed to 1 μ M benzo(a)pyrene continued to show elevated expression, indicating a prolonged induction following the single exposure and extended recovery period.

To assess alterations in morphology and Cyp1a protein localization after repeated exposures, microtissues were first exposed to either 1 μ M or 5 μ M benzo(a)pyrene. Following the second exposure, Cyp1a protein expression is comparable between exposure groups (Fig. 4B). As observed with gene expression, induction of Cyp1a protein was not altered by previous exposure to benzo(a)pyrene. In comparison, after the second benzo(a)pyrene exposure microtissues show increased cell death at the periphery and greater disruption of microtissue architecture (Fig. 4A). Microtissues receiving a single benzo(a)pyrene exposure on day 6 of culture show similar adverse effects to those exposed on day 4, with PAH exposure associated with cell death and morphological changes at the microtissue periphery (Fig. 4C).

Pre-exposure to 1 μ M or 5 μ M benzo(a)pyrene increases cell death, decreases microtissue size, and disrupts microtissue architecture. Histology of the 3D microtissues shows dead and dying cells detached from the spheroid periphery and empty regions within the microtissues indicative of cell loss. The fragmented nuclei in cells near these regions suggest that they are clustered foci of cell death (Fig. 4C). The radius of the compact spheroid area decreases with increasing doses of benzo(a)pyrene and is significantly altered by pre-exposure to benzo(a)pyrene. While initial exposure to 5 μ M benzo(a)pyrene decreases the size of the spheroid after second exposure, those pre-exposed to 1 μ M benzo(a)pyrene have increased spheroid radius (Fig. 5A). Visualization of the filamentous actin cytoskeleton shows that the regular architecture and uniform cell density through the microtissue is disrupted, with scattered foci of cells with cytoskeletal collapse (asterisks in Fig. 5C) in addition to detachment of apoptotic cells at the periphery. This disrupted organization is not observed in spheroids exposed only once to benzo(a)pyrene (Fig. 5B).

4. Discussion

As a fish-specific model for aquatic toxicology, these microtissues are presented as an alternative to animal use that has greater utility than traditional, monolayer *in vitro* assays. While other microtissue studies show problems with necrosis at the center, our microtissues maintain viability and differentiation for at least 8 days, creating the opportunity for extended low-dose exposures (Mueller-Klieser, 1997). The microtissues also show signs of better differentiation, with increased *cyp1a* gene expression and more even distribution of glycogen in our microtissues as compared to monolayer cells. To assess the toxicity of metabolically activated contaminants like aromatic hydrocarbons, maintenance of liver

differentiation and *cyp1a* expression is critical. The increased *cyp1a* expression seen in our microtissues also indicates that their cellular physiology is closer to primary fish hepatocytes, increasing the biological relevance of this system (Thibaut et al., 2009). The apparent increase in *cyp1a* basal expression observed in the fish liver microtissues cultured between 4 and 7 days appears to be correlated with time in culture, suggesting that cell function may improve over time as the spheroids mature.

While there are challenges associated with *in situ* visualization of biomarkers, these microtissues can be assessed using techniques optimized from both monolayer *in vitro* assays and *in vivo* tissue histology to characterize adverse effects at sublethal and toxic concentrations (Kabadi et al., 2015). Using both gene and protein expression of Cyp1a, we were able to demonstrate the sensitivity of our model to the exposure to aromatic hydrocarbons. Aromatic hydrocarbons like benzo(a)pyrene enter cells *via* passive diffusion and can be observed by visualization of their autofluorescence, allowing us to observe uptake into the 3D microtissue (Barhoumi et al., 2000; Madureira et al., 2014). Unlike cells in monolayer, which are exposed through direct contact with the medium, the multilayered structure of a 3D microtissue is a barrier to toxicant diffusion or transport similar to *in vivo* liver (Godoy et al., 2013; Olinga et al., 2001). However, we observed fairly even distribution of the benzo(a)pyrene within the microtissue, suggesting that at this size uptake of freely diffusible chemicals is not a limitation for the microtissue response. Additionally, technical differences between the monolayer and microtissue experiments could have contributed to the differing response at 5 μ M benzo(a)pyrene. While the gene expression studies were conducted entirely in glass, monolayers used for the immunofluorescent detection of Cyp1a protein were plated in chamber slides. The plastic of the chambers could have adsorbed small amounts of benzo(a)pyrene, leading to continued benzo(a)pyrene exposure and Cyp1a upregulation during the recovery period (Chlebowski et al., 2016).

Induced through activation of the aryl hydrocarbon receptor, Cyp1a expression or activity is a specific biomarker for exposure for benzo(a)pyrene and other organic pollutants (Sarkar et al., 2006). Gene expression was the most sensitive measurement of Cyp1a upregulation, with induction detected after 24 h exposure to only 1 nM benzo(a)pyrene. This low, soluble concentration of benzo(a)pyrene is comparable to the level of PAHs found in the environment, demonstrating the ability of microtissues to detect an environmentally relevant range of organic pollutants. We also observed discretely separated expression values across the concentration-response curve, which allows for quantitative determination of exposure and is important for evaluation of environmental samples.

In parallel with gene expression data, *in situ* detection of Cyp1a protein using immunofluorescence identifies the magnitude of the Cyp1a response in different regions of the 3D structure. While immunofluorescent detection of Cyp1a is less sensitive than gene expression, *in situ* labeling of protein expression allows full utilization of the three-dimensionality of spheroids. Benzo(a)pyrene-treated microtissues show a gradient of Cyp1a protein expression, with the highest levels at the periphery and a lower response at the center. This expression does not correlate with the even uptake of benzo(a)pyrene observed by fluorescence. Instead, we suggest that this is the combined effect of prolonged diffusion of benzo(a)pyrene into the lipophilic compartments of the microtissue and the slower efflux

of metabolites, a process assisted by ABC transporters (Kranz et al., 2014; Luckenbach et al., 2014). Cell death follows this same gradient, with dead cells found primarily at the periphery of treated microtissues. This implies that the cells with the greatest adaptive response, as indicated by induction of Cyp1a, are also the cells experiencing the greatest toxicity. This reinforces the gene expression results, implying that even after exposure to toxic doses of benzo(a)pyrene, surviving cells remain capable of responding. These findings are supported by previous work with primary sea bream hepatocytes in monolayer, which showed induction of apoptosis after 24 h exposure to benzo(a)pyrene (39.6 pM–39.6 μ M), followed by cell death over 72 h and proliferation of surviving cells (Pastore et al., 2014).

After the first exposure, microtissues were allowed to recover for 24 h to assess persistent adverse responses. We found that during the recovery period, Cyp1a expression decreases but microtissues continue to undergo increasing cell death and morphological changes. The persistent upregulation of *cyp1a* gene expression observed after high exposure (>100 nM benzo(a)pyrene) may be the result of benzo(a)pyrene or benzo(a)pyrene metabolites remaining in the microtissue, with continued activation of the aryl hydrocarbon receptor and induced gene expression. Compared to a lower, more readily metabolized dose, the high concentrations of benzo(a)pyrene are likely beyond the cell's ability to be completely metabolized in 24 h, leading to both higher and prolonged intra-cellular exposure (Madureira et al., 2014; Thibaut et al., 2009). The continued upregulation of *cyp1a* during the recovery period may be important for the survival of cells after subsequent or chronic exposures.

Chemical contaminants are associated with chronic, low-dose or repeated exposures and the extended viability of microtissues allows us to incorporate this aspect of environmental exposure by exposing microtissues a second time after the recovery period. Despite the significant changes to spheroid structure and size, the sensitive and concentration-dependent induction of Cyp1a was unaltered by previous exposure to benzo(a)pyrene. Based on the gradient of protein expression observed, we suggest that this maintained responsiveness is due in part to the relative protection of centrally located cells. However, *in situ* detection of Cyp1a protein demonstrates that cells throughout the spheroid are responding to both single and repeated exposures to 5 μ M benzo(a)pyrene, indicating the capability of microtissues to repeatedly upregulate Cyp1a in response to PAHs.

After a single exposure, most of the cell death observed in the spheroids occurs at the periphery, but a number of cellular changes occur throughout the microtissue after repeated exposures. While the differences in monolayer cell number are likely due to cell death or limited proliferation (Madureira et al., 2014), microtissues show a greater complexity in their morphological changes. These studies revealed that repeated benzo(a)pyrene exposure results in altered microtissue architecture and signs of liver pathology including altered cytoskeletal organization, compensatory proliferation, and both necrotic and apoptotic cell death. Both single apoptotic cells and scattered foci of cell necrosis are commonly observed in fish liver following exposure to hepatotoxins (Wolf and Wolfe, 2005). The recapitulation of this pattern of cell death in fish liver microtissues following repeated benzo(a)pyrene exposures demonstrates its ability to reflect the pathology induced by hepatotoxins *in vivo*.

While it has value as an alternative *in vitro* toxicity testing platform, the fish liver microtissue model presented here also has certain limitations. As a spheroid composed of a single cell type, it does not reflect paracrine interactions between the different compartments of the liver, including effects of the endothelium or resident macrophages that could have important implications for toxicity (LeCluyse et al., 2012). Multicellular tissue complexity can be assessed using tissue slices or explant cultures, which maintain the complex, multicellular structure of the liver *in situ* and can be derived from many different species (Eide et al., 2014; Godoy et al., 2013). Another important limitation is static exposure, which does not incorporate the flow of a contaminant as it reaches a tissue from the blood stream. Microfluidic devices that address this dynamic exposure have the additional benefit of incorporating multiple miniaturized systems, building up to an organ or animal on a chip (Huh et al., 2011; Shamir and Ewald, 2014; Sudo, 2014).

5. Conclusion

The 3D fish liver microtissue platform presented here offers a novel screening tool for aquatic toxicity testing. Using a combination of histological and molecular techniques, this study demonstrates the utility of this 3D microtissue model for aquatic toxicology testing of organic pollutants under environmentally relevant conditions. Throughout repeated benzo(a)pyrene exposures, the fish liver microtissues showed changes in adaptive response, microtissue architecture, and cell death (Fig. 6). Focusing on Cyp1a induction, this model provides a highly sensitive, quantitative screening tool for induction of xenobiotic metabolism over a wide range of doses associated with adaptive or adverse outcomes. In addition to cellular alterations, this model can be used to identify disrupted microtissue architecture that is relevant for potential changes in liver morphology *in situ*. This opens the possibility of identifying sublethal adaptive responses using this fish-specific toxicity testing platform as an alternative to expensive, time-consuming *in vivo* assays. This aquatic toxicity testing platform is suitable for screening of potential adverse impacts of the ever-evolving mixture of historical pollutants and newly identified contaminants in modern aquatic environments (Hutchinson et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2017.02.018>.

Fig. 1.

Characterization of microtissues. (A and B) Cells in 2D monolayers cultured on glass coverslips for two days, then stained with either hematoxylin and eosin (H&E) (A) or periodic acid Schiff (PAS) (B), which stains glycogen magenta. Scale bar 50 μ m. (C) To form 3D microtissues, a monodispersed cell suspension is added to an agarose hydrogel mold that contains an upper chamber and an array of 256 microwells. The cell suspension is added to this upper chamber and settles into the microwells with gravity. Over time, the cells self-assemble into a spheroidal microtissue. (D) Microtissue assembly occurs over the first 3 days, beginning as an irregular aggregate of cells that becomes more compact and spheroidal by day 4. (E and F) In plastic sections, day 4 microtissues show intact cell morphology by H&E (E) and uniform distribution of glycogen by PAS (F). Scale bar 50 μ m. (G and H) Transmission electron microscopy reveals intact ultrastructural morphology [N – nucleus; Nu – nucleolus; G – glycogen; Mi – mitochondria; M – microvilli]. Scale bar 2 μ m. (I and J) Changes in protein (I) and DNA content (J) over time for 2D and 3D cultures (relative to day 2). $N = 3$. (K) Basal *cyp1a* gene expression in 3D microtissues and monolayer samples, relative to day 2 monolayer cultures. $N = 3$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 2.

Benzo(a)pyrene exposure induces *cyp1a* expression. (A) Microtissues are cultured 4 days before the first 24 h exposure to benzo(a)pyrene. They are then washed for a 24 h recovery period, followed by a second 24 h exposure to of benzo(a)pyrene. (B) Benzo(a)pyrene uptake was detected after 30 and 60 min (insets) and increases continuously for 6 h (panel). (C) *Cyp1a* gene expression (normalized to 18S) after a single benzo(a)pyrene exposure and after the 24 h recovery period or (D) following a second benzo(a)pyrene exposure. All fold changes are relative to their respective vehicle-treated control group. (E and F) Gene expression data are also shown as delta Ct (normalized to 18S expression) for comparison between groups and to indicate statistically significant differences. Dotted lines at 10 and 20 allow for easier comparison of values between graphs. $N=3$ (errors bars show standard deviation) for all data shown except 5 μ M benzo(a)pyrene with recovery, which was $N=1$ (error bars show standard deviation among technical replicates; data point was therefore excluded from statistical analysis). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 3.

Cyp1a protein expression and TUNEL assay after single exposure followed by recovery. (A and B) Immunofluorescent detection of Cyp1a protein in monolayer cultures (A) and 3D microtissue cryosections (B) after a single 24 h exposure to benzo(a)pyrene. (C and D) Immunofluorescent detection of Cyp1a protein expression in monolayer (C) and 3D microtissue cryosections (D) after recovery period. (E and F) TUNEL labeling of apoptotic cells in 3D microtissue cryosections after 24 h exposure (E) and following recovery (F). All scale bars 50 μm.

Fig. 4. Histology and Cyp1a protein expression after repeated exposure to benzo(a)pyrene. (A) Microtissue histology (A) and Cyp1a immunofluorescence (B) after single or repeated exposures to benzo(a)pyrene. Scale bars 50 μ m. (C) High magnification of vehicle treated microtissues (i) and the cellular changes after two exposures to 5 μ M benzo(a)pyrene (ii–vi). After repeated exposure to a high dose of benzo(a)pyrene (ii–vi), microtissues show clusters of apoptotic (ii) and necrotic cells (iii), focal cell loss (iv), mitotic figures indicating compensatory proliferation (v), and detachment of cells from the spheroid periphery (vi). Cellular changes indicated with arrowheads. Scale bar 25 μ m.

Fig. 5.

Microtissue architecture and size after repeated exposure. (A) The compact spheroid region of each microtissue was circled and measured for analysis of radii after single and repeated exposure to benzo(a)pyrene. $N=3$. Microtissue architecture visualized using rhodamine phalloidin to detect filamentous actin cytoskeleton after a single (B) or repeated (C) exposure to benzo(a)pyrene. Red asterisks indicate areas of cytoskeletal collapse. Scale bars 50 μm . * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 6.

Summary. Over the first 24 h following exposure to a high dose of benzo(a)pyrene (yellow star), microtissues show an adaptive response as measured by Cyp1a expression (green line) and initiate the process of cell death (red line). After benzo(a)pyrene has been removed, induction of Cyp1a protein and gene expression declines, but cell death continues to increase. During this recovery period, morphological changes (blue line) develop and alterations in the spheroid architecture can be detected. After the second exposure, adaptive responses are again induced, but cell death and morphological changes continue to increase.