Ecotoxicology (2012) 21:2419–2429
DOI 10.1007/s10646-012-0965-5

TECHNICAL NOTE

Towards a more representative in vitro method for fish ecotoxicology: morphological and biochemical characterisation of three-dimensional spheroidal hepatocytes

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Accepted: 8 June 2012 / Published online: 26 June 2012

Abstract The use of fish primary cells and cell lines offer an in vitro alternative for assessment of chemical toxicity and the evaluation of environmental samples in ecotoxicology. However, their uses are not without limitations such as short culture periods and loss of functionality, particularly with primary tissue. While three-dimensional (spheroid) technology is now established for in vitro mammalian toxicity studies, to date it has not been considered for environmental applications in a model aquatic species. In this study we report development of a reproducible six-well plate, gyratory-mediated method for rainbow trout (*Oncorhynchus mykiss*) hepatocyte spheroid culture and compare their functional and biochemical status with two-dimensional (2D) monolayer hepatocytes. Primary liver spheroid formation was divided into two stages, immature (1–5 days) and mature (≥6 days) according to size, shape and changes in functional and biochemical parameters (protein, glucose, albumin and lactate dehydrogenase). Mature spheroids retained the morphological characteristics (smooth outer surface, tight cell–cell contacts) previously described for mammalian spheroids as demonstrated by light and scanning electron microscopy. Glucose production and albumin synthesis were significantly higher in mature spheroids when compared to conventional 2D monolayer cultures ($P < 0.01$) and increased as spheroids matured ($P < 0.01$). Basal lactate dehydrogenase (LDH) leakage significantly decreased during spheroid formation and was significantly lower than 2D cultures ($P < 0.01$). It is therefore suggested that mature spheroids can maintain a high degree of functional, biochemical and morphological status over-time in culture that is superior to conventional 2D models and can provide realistic organotypic responses in vitro. Trout spheroids that take ~6–8 days to reach maturity would be suitable for use in acute toxicological tests and since it is possible to culture individual spheroids for over a month, there is potential for this work to lead towards in vitro bioaccumulation alternatives and to conduct high throughput screens of chronic exposure. This is an important step forward for developing alternative in vitro tools in future fish ecotoxicological studies.

Keywords Spheroid · Monolayer · Rainbow trout · Hepatocyte · Functionality · Morphology · In vitro toxicology

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Introduction

The development and validation of reliable in vitro methods that offer an alternative to conventional in vivo studies in experimental animals is rapidly becoming an important criterion, particularly in toxicological studies (Mazzoleni et al. 2009). Current, or moreover, conventional 2D in vitro culture methods such as primary tissue culture, immortalised and transformed cell lines have been used extensively in a wide-range of toxicological studies to identify diverse end-points to elucidate specific toxic effects in target or surrogate organs (Raisuddin and Jha 2004; Zucco et al. 2004; Xiao et al. 2007; Bornschein et al. 2008; Reeves et al. 2008; Vevers and Jha 2008; Papis et al. 2011).

The use of fish primary cells and cell lines are of importance in ecotoxicological assessment with research

suggesting their suitability in replacing or supplementing in vivo animal tests (Babich and Borenfreund 1986, 1987; Castano et al. 1994; Jha 2004). In addition, there are significant calls for the development of robust in vitro technologies to support regulatory ecotoxicology. For example in Europe there are demands from the regulatory directives and guidance documents (controlling the use of chemical and biological products) for the development of alternative methods (e.g. Registration Evaluation and Restriction of Chemicals (REACH 2009); European Medicines Agency (EMA 2006); plant protection products directive (PPPR 2009). Similar regulatory developments have also taken place in the US (Voluntary EPA-High Production Volume Challenge Programme) and in Japan (TTREC-Toxicity Testing Reports for Environmental Chemicals (Schrattenholz and Klemm 2006). It is therefore not surprising that an important vision for toxicological studies in 21st century is to overcome the currently used animal tests with in vitro systems to gain insights of the mechanisms of actions of toxicants to protect human and environmental health.

Compared to mammalian toxicology however, in vitro technologies for fish seem to be less well developed, perhaps because of the perceived difficulty of fish cell culture, or perhaps because traditionally it has been relatively simple to expose fish directly to the compound of interest. For ethical and financial reasons the principles of the 3Rs (reduction, refinement and replacement) have been actively pursued for vertebrate based studies, however with the large numbers of compounds which now face the potential of regulatory testing (>100,000) alternatives to in vivo testing are being aggressively pursued across the globe for ecotoxicological testing. Primary cells of fish origin in particular express many of the differentiated cellular structures and functions of their source tissues (Dowling and Mothersill 2001; Segner and Cravedi 2001), however short culture periods and loss of organotypic functionality limit their use in chronic toxicity studies and conventional culture methods do not fully represent the growth conditions or 3D architecture of in vivo tissues.

In mammalian studies, the use of 3D cell culture systems address some of the problems associated with conventional 2D culture techniques (Pampaloni et al. 2007). Cells grown in 3D systems develop into tissue-like structures, more similar to those formed in living organisms. Their shape and environment are important in determining their growth behaviour, gene expression (Kim 2005) and specific biochemical organ-functions (Ma et al. 2003; Xu et al. 2003a, 2003b). In addition, important enzymes involved in bio-transformation systems are maintained over-time in culture, highlighting their stability in functionality and biochemical performance (Liu et al. 2007).

While 3D culture technology is now particularly established for mammalian toxicity studies, its potential application as a new tool for aquatic toxicological testing is not known. Limited previous work has demonstrated the use of aggregate culture systems for characterising levels of estrogen receptor and vitellogenin (Vg) mRNAs in rainbow trout (*Oncorhynchus mykiss*) and has been described as a promising in vitro model to investigate the biotransformation pathways in fish and their regulation by endogenous and exogenous compounds (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996). Yet in the last two decades, to our knowledge, no new research into the suitability of 3D cell culture technology in ecotoxicity testing has emerged. Needless to mention, understanding the biochemical and metabolic characteristics of 3D models are important criteria for promoting their application in this field of ecotoxicology. One reason that new work has not emerged may well have been the perceived difficulty of establishing these 3D cultures for fish cells. Longer lived and metabolically more realistic fish cells could allow the development of new predictive screens that would take the potential for regulatory alternatives beyond the current compromise of short-term cultures in the weight of evidence assessments, and provide a platform for further alternatives to in vivo fish studies in ecotoxicology.

Here we report a detailed protocol for the routine formation of 3D rainbow trout primary hepatocyte spheroids. In addition to morphological characterisation using light and scanning electron microscopic (SEM) studies, baseline biochemical organ-functionality were investigated for differences in (a) energy metabolism (b) albumin synthesis and (c) culture viability in 3D spheroid and 2D monolayer hepatocyte culture models from the same individual fish.

Materials and methods

Chemicals and media

Leibovitz's medium (L-15), foetal bovine serum (FBS), trypsin (0.25 %) and versene were obtained from Life Technologies (UK). Collagenase D was obtained from Roche Scientific (UK). Hank's balanced salt solutions (HBSS), trypan blue solution (0.4 %), Poly(2-hydroxyethyl methacrylate) (pHEMA) and other chemicals, reagents and kits were obtained from Sigma-Aldrich (UK) unless otherwise indicated.

Fish husbandry and collection

Female rainbow trout (*Oncorhynchus mykiss*) (wet weight: 168.1 ± 68.1 g) were kept in 180 L holding tanks (de-chlorinated and aerated water; non re-circulating system) at a stocking density of ~40 fish per tank. Water parameters (temperature: 16.0 ± 0.7 °C; pH: 6.7 ± 0.1;

dissolved oxygen: 94.0 ± 2.7 %) were measured daily. A photoperiod of 12 h light and 12 h dark was applied and fish were fed a daily ration of pellets (BioMar, UK) at ~2 % body weight to maintain their weight and health. Fish were starved for 24 h prior to sampling and donor animals were sacrificed by a blow to the head followed by immediate destruction of the brain. Fish were then weighed; surface sterilised with 70 % ethanol and transferred to a downdraft dissection table to control collagenase aerosols.

Cell preparation

The liver was dissociated by a collagenase perfusion method (Nabb et al. 2006) with the following modifications in our laboratory for optimisation of hepatocyte yield and viability. A cannula was inserted into the hepatic portal vein and the liver cleared of blood with 10 ml of calcium and magnesium-free HBSS supplemented with 2.3 mM EDTA and 4.2 mM $NaHCO_3$ (pH 7.4). The liver was then dissociated with 20 ml of calcium and magnesium-supplemented HBSS with additional 0.1 % collagenase D and 4.2 mM $NaHCO_3$ (pH 7.4). Finally, the collagenase was cleared with 3 ml L-15 media (pH 7.4). All stages of the perfusion utilised a peristaltic flow rate of 1 ml min^{-1}. Livers were then mechanically dispersed into a sterile Petri dish and passed through 100 µm gauze. Cells were collected by low-speed centrifugation (50×*g*; 5 min; 18 °C), the supernatant removed and the pellet re-suspended in 20 ml L-15 media. This process was repeated twice more to remove non-viable cells before being re-suspended in a final volume of 20 ml L-15 media supplemented with 10 % FBS. Cell viability was determined by the trypan blue exclusion test (0.2 % final volume) and cell suspensions with a viability of ≥85 % were used for subsequent experiments. Cell suspensions were immediately transferred to micro-plates after isolation to maximise viability.

Cell culture

Spheroid culture

Cell suspensions were diluted (1×10^6 cells ml^{-1}) in L-15 media supplemented with 10 % FBS (pH 7.4) and transferred to non-tissue culture treated six-well micro-plates (Falcon, VWR, UK; 3×10^6 cells $well^{-1}$; 3 ml total volume) that had been pre-coated with 500 µl of 2.5 % p-HEMA solution (dried for 48 h in a sterile culture cabinet) to eliminate hepatocyte attachment. Plates were placed at 18 °C in a refrigerated incubator (MaxQ 4000, Thermo Scientific, UK) on an orbital shaking platform set at a constant rotation speed of 70 RPM. After 24 h, when aggregates had started to form, 1.4 ml of old media was replaced with 1.5 ml of fresh media to compensate for well evaporation as per the protocol followed for mammalian spheroids (Ma et al. 2003). The culture media was then changed every 2 days thereafter (1.3 ml replaced with 1.5 ml of fresh media).

Monolayer culture

Diluted cell suspensions (1×10^6 cells ml^{-1}) in L-15 medium supplemented with 10 % FBS (pH: 7.4), were plated (3 ml $well^{-1}$) in treated-tissue culture six-well micro-plates (Iwaki, Sterilin, UK) and incubated statically at 18 °C. After 24 h, 1.4 ml of old media was replaced with 1.5 ml of fresh media and thereafter every 2 days. Both spheroid and monolayer cultures were prepared from the same donor organ.

Size measurements

Diameters of individual spheroids ($n = 20$ individuals, $n = 3$ wells) were calculated from digital images acquired using a microscope mounted digital camera attached to an inverted light microscope (Olympus® CK40-SLP) at a total 100 (10 × 10) magnification. Radius (r) measurements of spheroids and individual hepatocytes were also calculated to determine spheroid/hepatocyte volume ($V = \frac{4}{3}\pi r^3$), which in turn were used to calculate the number of cells per spheroid ($\frac{spheroid\ volume}{hepatocyte\ volume}$). Digital images were analysed using Image J analysis software (Maryland, USA, http://imagej.nih.gov/ij/).

Morphological observations

Morphological changes of developing spheroid and monolayer cultures were observed under light microscopy over 8 days. Surface structure of developing and mature spheroids were observed under Scanning Electron Microscopy (JSM-5600LV, JEOL Ltd, UK) at 1, 4 and 8 days of formation, after fixation in 2.5 % glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h followed by dehydration with 30, 50, 70, 90 and 100 % ethanol, 5 min for each concentration.

Biochemical assays

Cultures and batches

Culture treatments compared in this study were (a) 8 day old spheroids (mature) (b) 2 day old aggregates (immature) and (c) 2 day old monolayers (functional). Each biochemical endpoint was tested in six individual cultures from three individual animals, resulting in a total of eighteen replicates for each culture treatment. The selection of biochemical endpoints was based on previous mammalian

studies investigating liver spheroid functionality (Walker et al. 2000; Ma et al. 2003; Xu et al. 2003a).

Sample collection and preparation

Samples were prepared as described previously (Ma et al. 2003). Old culture medium from each well was completely replaced with 3 ml serum-free L-15 medium 24 h before sampling at each respective time-point. Cell samples were collected 24 h after changing the medium in 2 ml aliquots and transferred to micro-centrifuge tubes. Monolayer cultures were carefully scraped into the surrounding media using a cell scraper. The tubes were centrifuged for 3 min at 900 *g* and the media decanted into separate 500 µl aliquots. The pellet was homogenised for 60 s in 100 µl of homogenisation buffer (2 mM NaH_2PO_4, 2 mM Na_2HPO_4, 0.5 mM EDTA and 145 mM NaCl; pH 7.4) (Xu et al. 2003a) using a micro-tube pestle, followed by 30 s sonication in an ultra-sonic water bath. Homogenates were made-up to a final volume of 1 ml homogenisation buffer and split into 250 µl aliquots. Sample preparation was carried out on ice and media and homogenate samples were stored at −20 °C prior to assay.

Determination of total protein

Total protein was determined using a Pierce BCA protein assay kit (Thermo Scientific, 23227) following manufacturer's instructions. Briefly, a 10 µl aliquot of neat homogenate sample was added to each well of a 96-well micro-plate (Iwaki, Sterilin, UK) in duplicate, followed by 200 µl of BCA reagent. The plate was allowed to stand for 3 min at room temperature (21 °C) then incubated for 30 min at 37 °C. Absorbance was read on a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at 595 nm. For protein per hepatocyte analysis, contents of individual wells were disaggregated in 0.05 % Trypsin (1:5 dilutions with versene) for 10 min at room temperature and 50 µl aliquots used for cell counts. Total protein was determined as described.

Determination of albumin concentration

Albumin concentrations were determined using an Albumin fluorescence assay kit (Fluka, 09753) as described previously (Kessler and Wolfbeis 1992), modified in our laboratory to be suitable for a micro-plate reader in a 96-well assay format. A 50 µl aliquot of neat media or homogenate sample was added to each well of a black 96-well micro-plate (Falcon, VWR, UK) in duplicate, followed by 250 µl of assay reagent. The plate was allowed to stand for 2 min at room temperature and the fluorescence read on a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at em/ex 600/630 nm.

Determination of glucose concentration

Glucose concentrations were determined using an enzymatic glucose kit (Sigma, GAGO20), based on the oxidation of D-glucose to gluconic acid by the enzyme glucose oxidase. This method was modified in our laboratory to be suitable for a micro-plate reader in a 96-well assay format as outlined by (Xu et al. 2003a). A 50 µl aliquot of neat media or homogenate samples was added to each well of a 96-well micro-plate in duplicate, followed by 250 µl of assay reagent. The plate was incubated for 30 min at 37 °C and the absorbance read on a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at 540 nm.

Determination of lactate dehydrogenase (LDH) concentration

LDH concentrations were determined based on the method described by (Scholtz and Segner 1999). A 50 µl aliquot of diluted media (1:5) or homogenate (1:10) sample was added to each well of a 96-well micro-plate in duplicate, on ice, followed by 250 µl of reaction buffer (50 mM Tris/HCL, 0.14 mM NADH; pH 7.5). The plate was allowed to stand at room temperature for 5 min and the reaction started with the addition of 25 µl of 12.1 mM sodium pyruvate dissolved in 50 mM Tris/HCL (pH 7.5). Plates were briefly mixed and the enzyme activities recorded for 20 min at 25 °C, in a micro-plate reader (SpectraMax M5, Molecular Devices, USA) at 340 nm.

Statistical analysis

Univariate analysis of variance (ANOVA) with post hoc Tukey HSD was used for comparisons between culture treatments. $P < 0.05$ was accepted as significant.

Results

Liver biometry

Liver hepatocytes were readily isolated by our collagenase perfusion method. Livers (1.3 ± 0.3 g) from wet weighed rainbow trout (105.7 ± 18.5 g) produced an average hepatocyte yield per g liver of 8×10^7 ($n = 10$). Average viability was 92 ± 5 % as determined by trypan blue dye exclusion. Hepatocyte yields from larger rainbow trout (wet weight: 230.4 ± 29.1 g; liver weight: 2.4 ± 0.4 g) were approximately two-fold higher per g liver ($14.8 \pm 3.0 \times 10^7$; $n = 10$), however prolonged perfusion periods and

higher collagenase concentrations were required to achieve this and on occasions, produced cultures with poor viability.

Cell suspensions were purified by low-speed centrifugation prior to seeding to separate out hepatocytes, as whole liver cell suspensions did not re-aggregate into spheroids under gyratory-mediated conditions. Un-purified cell suspensions contained a mixture of hepatic cell types of which four (viz. sinusoidal endothelial cells, stellate cells, Kuppfer cells and hepatocytes) were recognisable under light microscopy. Hepatocytes contributed to ~80 % of the cell suspension (Segner 1998). Following low-speed centrifugation, hepatocytes represented approximately 95 % of the total cell population.

Morphological maturation

Single hepatocytes maintained under constant gyratory-mediated culture conditions re-aggregated to form small, irregular aggregates after 24 h in culture (Fig. 1a). Over a period of 3–4 days these smaller aggregates joined together to form larger aggregates, exhibiting a more spherical-like shape, with an average diameter of 53 ± 12 μm ($n = 180$) at 4-days old (Fig. 1c). After 6–8 days in culture the diameter of the aggregates had increased (67 ± 13 and 83 ± 14 μm respectively, $n = 180$) and the shape became more stable forming neat, regular liver spheroids (Fig. 1e). Hepatocytes isolated from smaller rainbow trout (105.7 ± 18.5 g) tended to form neater and more spherical spheroids compared to those formed from larger trout (230.4 ± 29.1 g). The number of hepatocytes per individual aggregate or spheroid also increased with maturity with an ~50 % doubling of cell number in the transition from aggregate to spheroid (Table 1). Spheroids maintained their neat, spherical structure and routinely survived up to 16 days in culture and in on-going studies using this protocol, large primary hepatocyte spheroids have survived in excess of 30 days in our laboratory.

The SEM analysis (Fig. 1b,d,f) further illustrates the morphological maturation of aggregates to spheroids, with 8 day old spheroids exhibiting a smooth outer surface and tight cell–cell contacts (Fig. 1f) compared to 4-day old spheroids, where the boundaries between individual hepatocyte contacts are more defined (Fig. 1d). Aggregates in the early stages of spheroid formation exhibit weak cell–cell contacts where well-defined single cells are still visible (Fig. 1b). Therefore, aggregating hepatocytes can be morphologically classified as 'mature' spheroids after 6–8 days in culture and those during 1–5 days of aggregation classified as 'immature spheroids'.

In monolayer cultures, single hepatocytes attached to the well-surface within 1–2 h and after 24 h had begun to regroup into rows of cell networks. After 48 h in culture tightly-packed rows of hepatocytes were visible, however this tight, neat structure soon began to disaggregate and after 8 days in culture, hepatocytes had begun to detach from the surface. Preliminary experiments demonstrated a rapid decrease in liver functionality of monolayer hepatocytes particularly between 4–8 days, which is well documented in mammalian studies (Walker et al. 2000). They were therefore classified as 'functional' between 2–4 days in culture.

Maintenance in culture

The addition of FBS greatly improved the re-aggregation and degree of organisation of hepatocytes in both monolayer and suspension cultures, however attachments of monolayers to the micro-plate surface were weak and could be easily removed by gentle pipetting. Choice of culture media did not seem to have a significant effect on longevity of cultures. Preliminary optimisation studies of culture conditions found L-15 media supplemented with additional 10 % FBS a simple and easily reproducible media recipe for maintaining hepatocytes in culture (Lipsky et al. 1986). A range of rotation speeds (60–90 RPM) were tested based on previous studies (Flouriot et al. 1993; Walker et al. 2000; Xu et al. 2003a). Rotation speeds above 70 RPM resulted in irregular spheroids with weak cell–cell contacts. Slower rotation speeds (60–65 RPM) caused a degree of clumping between developing spheroids. A subsequent routine rotation speed of 70 RPM combined with a constant well volume (3 ml) was deemed optimum for maintaining developing spheroids.

Changes in protein profile

Each of the values of the biochemical parameters was normalised against total protein per well. Protein per hepatocyte was constant between monolayer and immature spheroid cultures, however in mature spheroid cultures an approximate two-fold decrease in protein per hepatocyte was observed when compared with monolayer and immature spheroid cultures ($P < 0.01$) (Fig. 2).

Changes in glucose profile

The change in intracellular and extracellular glucose in monolayers and during spheroid formation is shown in Fig. 3a. Glucose secretion by mature spheroids was significantly higher than that in monolayers ($P < 0.01$) and immature spheroids ($P < 0.01$). No difference was observed between monolayer and immature spheroid cultures. Total intracellular glucose was constant during spheroid formation (Fig. 3b) but was significantly lower than that observed in monolayer cultures ($P < 0.05$).

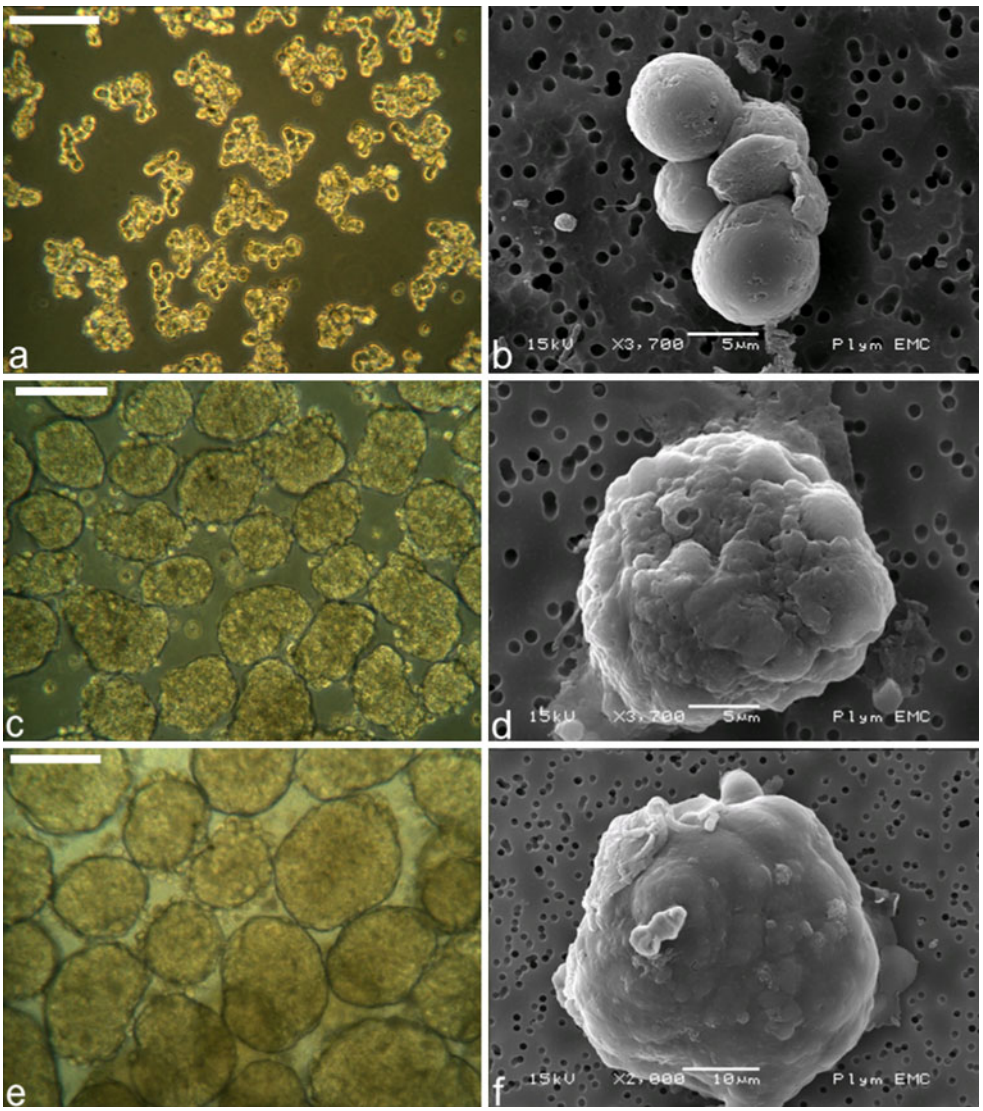

Fig. 1 Light and scanning electron microscope images taken during hepatocyte aggregation and spheroid formation: **a-b** Small aggregates, 24 h after plating; **c-d** Immature spheroids, 4 days after plating; **e–f** Mature spheroids, 8 days after plating; the *scale bar* represents 100 µm (**a**, **c**, **e**). *Scale bars* in **b**, **d**, **f** are defined

Table 1 Size measurements of spheroids

Days	Diameter (µm)	Volume ($\times 10^4$ µm^3)	Cells spheroid^{-1}
4	53 ± 12	9 ± 6	127 ± 90
6	67 ± 13	18 ± 10	252 ± 147
8	83 ± 14	32 ± 17	457 ± 247

Values are expressed as mean ± SD, $n = 20$ individuals/$n = 9$ wells. Typically, hepatocytes collected from 105.70 ± 18.50 g trout are 10.90 ± 0.90 µm in diameter. Mammalian hepatocytes are typically much larger, for example those of a similar sized rat are 24.10 ± 0.10 µm in diameter and spheroids produced from this measure 200 ± 25 µm in diameter upon reaching morphological maturity (Ma et al. 2003)

Changes in albumin profile

Rate of albumin secretion of both monolayers and liver spheroids is shown in Fig. 3c. A significant increase in albumin secretion was observed during liver spheroid formation ($P < 0.01$) and both immature and mature spheroids had a significantly higher secretion rate than that in monolayer cultures ($P < 0.01$), with an approximate five-fold increase observed between monolayers and mature spheroids. No difference in total intracellular albumin was observed between mature and immature spheroids, the highest of which was observed in monolayer cultures which was significantly higher than in spheroids ($P < 0.01$) (Fig. 3d).

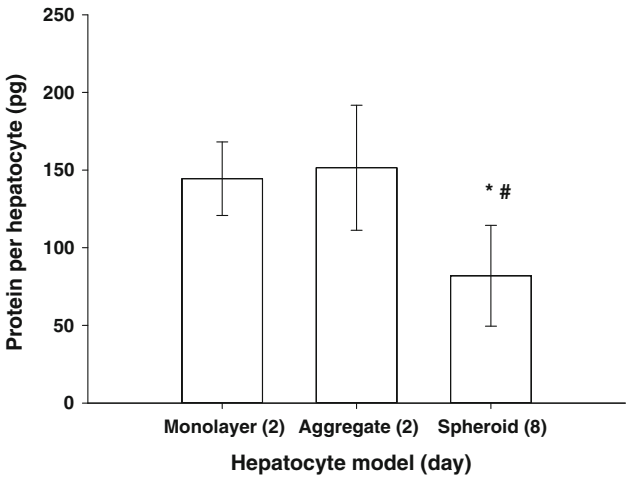

Fig. 2 Protein per hepatocyte values are expressed as mean ± SD, $n = 6$ (wells). * $P < 0.01$, compared with the value of monolayer cultures. $^{\#}P < 0.01$, compared with the value of immature aggregates

Changes in LDH profile

Changes in intracellular and extracellular LDH in monolayers and spheroids are shown in Fig. 3e. As spheroids matured the release of LDH into the surrounding culture media decreased significantly ($P < 0.01$). The highest amount of LDH release was observed in monolayer cultures which were significantly higher than that in spheroids, both immature and mature ($P < 0.01$). The highest amount of intracellular LDH was observed in mature spheroids (Fig. 3f) which was approximately seven-fold higher than that in immature spheroids and monolayers ($P < 0.01$). No difference was observed between monolayers and immature spheroids.

Discussion

Cell isolation procedures and subsequent culture techniques can significantly affect the formation and functionality of fish liver spheroids (Lin et al. 1995; Niwa et al. 1996; Juillerate et al. 1997). In the present study we have demonstrated the importance of optimising isolation and culture parameters for successful spheroid formation such as: fish size, purified hepatocyte suspensions, serum-supplemented culture media and optimal rotation speeds. The use of smaller fish for successfully isolating hepatocytes is apparent in the literature (Lipsky et al. 1986; Pesonen et al. 1992; Flouriot et al. 1993) suggesting that as both fish and liver size increases, so does the physiological and biochemical stress on liver cells due to the requirement of prolonged perfusion and enzymatic digestion periods. Purified suspensions of hepatocytes combined with gyratory-mediated culture techniques have demonstrated successful aggregate formation in fish (Flouriot et al. 1993; Flouriot et al. 1995; Cravedi et al. 1996). In mammalian studies however, un-purified, whole liver cell suspensions can successfully re-aggregate into spheroids (Walker et al. 2000; Ma et al. 2003; Xu et al. 2003a) suggesting that enzymatic cell isolation procedures may damage sensitive non-parenchymal cell types from fish liver.

Serum-supplemented culture media vastly improved the aggregation process of developing spheroids (Flouriot et al. 1993), on which formation and growth of hepatocytes are dependent (Pannevis and Houlihan 1992; Garmanchuk et al. 2010). Gyratory-mediated methods for trout hepatocytes differ significantly to rat hepatocytes, where high rotation speeds are required to induce liver cell aggregation (Walker et al. 2000). This suggests that the difference in overall hepatocyte size between rats (24.1 ± 0.1 μm in diameter) (Katayama et al. 2001) and fish (10.9 ± 0.9 μm in diameter) (present study) dictates the rotational forces required to keep cells in an optimal suspension state to allow aggregation to take place. The difference in size of hepatocytes between species also seems to control the size of mature spheroids as those measured from rat liver were significantly larger (Ma et al. 2003).

Hepatocyte spheroid formation was divided into two stages in this study according to the morphological formation process: immature and mature (Ma et al. 2003). Immature spheroids (1–5 days) under-go the transition from single cells → irregular aggregates → morphologically stable spheroids (see Fig. 1). Mature spheroids (≥6 days) exhibit a relatively stable and regular shape, with an increase in size due to the fusion of smaller spheroids. The classification of fish hepatocyte spheroids based on morphological maturity (Hansen et al. 1998) is important new information, particularly when determining functional status during and after spheroid formation. Between 10–16 days in culture, spheroids continued to increase in size with the fusion of mature spheroids. Beyond 16 days, the fusion process ceased and spheroids were maintained in culture for a period of up to 40 days. However, we are as yet unclear why this is the case for some fish and not others. Further iterative optimisation of culture parameters may prolong the integrity and survival of fish hepatocyte spheroids, which would render them a useful tool for chronic toxicity, bio-accumulation and biotransformation studies.

The amount of total protein of hepatocytes in each well can indirectly reflect the total viable cell number. Total protein decreased during the spheroid maturation process and was lower when compared with functional 2 day old monolayer cultures. In common with mammalian studies (Ma et al. 2003), this is likely to be reflected by loss of cells due to the enzymatic isolation process which was observed during culture maintenance, where only viable cells re-aggregated during spheroid formation. Protein per

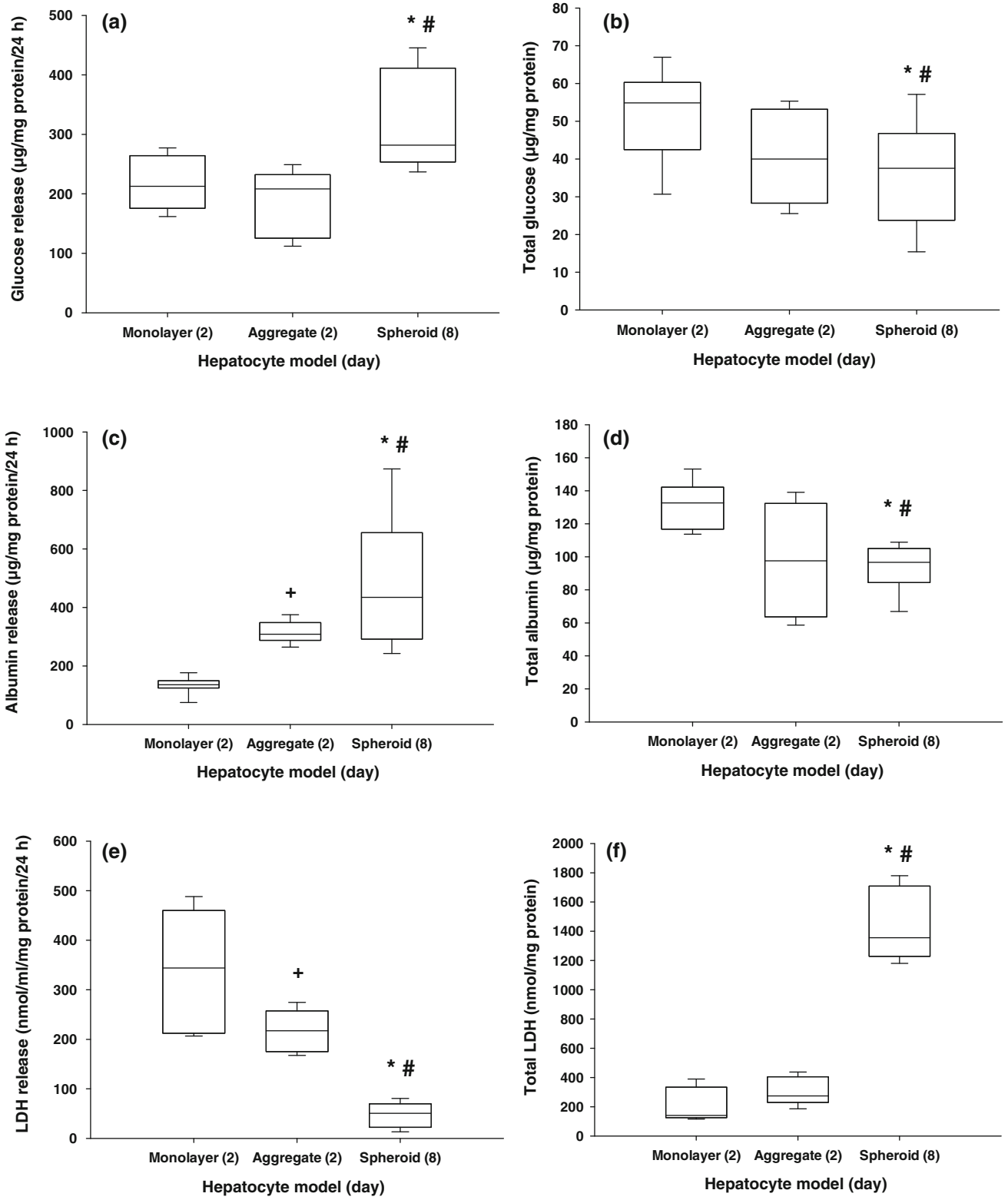

Fig. 3 Changes in glucose, albumin and LDH (intra & extra-cellular) during spheroid maturation compared with monolayer cultures. **a** Glucose release; **b** Total glucose; **c** Albumin release; **d** Total albumin; **e** LDH release; **f** Total LDH. Values are expressed as mean ± SD, $n = 18$ (wells). $^{*}P < 0.01$, compared with the value of monolayer cultures. $^{\#}P < 0.01$, compared with the value of immature aggregates. $^{+}P < 0.01$, compared with the value of monolayer cultures

hepatocyte decreased during the transition from immature to mature spheroids which would suggest a decrease in the viability of spheroids during maturation. However, this contrasts with increased levels of albumin synthesis and significantly lower levels of LDH leakage. This could suggest that aggregated hepatocyte spheroids more closely resembling intact tissue, utilise amino acids as sources of energy such as the conversion to glucose and/or CO_2 during cellular respiration (French et al. 1981).

Gluconeogenesis is the major pathway responsible for glucose production in primary cultures of trout hepatocytes (Mommsen et al. 1999). In mammals, the liver plays an important role in maintaining blood glucose concentration and is also the major organ responsible for converting galactose into glucose and synthesizing glucose from other substances such as pyruvic acid and amino acids (Zubay 1988). The present study demonstrated that fish hepatocyte spheroids may retain the similar function to rat liver spheroids of converting galactose to glucose (Xu et al. 2002; Ma et al. 2003), as the media recipe used in this study contained galactose but no glucose or gluconeogenic controlling hormones. Damage to mitochondria can affect many functions of liver cells, including galactose transformation, gluconeogenesis and glucose secretion, suggesting that glucose secretion may reflect a general state of energy metabolism in spheroids (Xu et al. 2003a).

A higher rate of glucose secretion and a reduction in total glucose as observed between monolayers and spheroids in this study may reflect significant differences in the metabolic state of 2D and 3D hepatocyte models in fish, although a distinct lack in the scientific literature of glucose studies in 2D cultures for fish makes this difficult to confirm. The secretion of glucose in mammalian and non-mammalian in vitro cultured hepatocytes has been shown to be far lower than that in vivo (Weber and Shanghavi 2000; Xu et al. 2003a), suggesting that in vitro studies have less physiological importance when investigating liver specific function. Mature spheroids however, may reflect similar levels of in vivo glucose production in fish as previously demonstrated in organotypic liver slice models (Morata et al. 1982), suggesting their usefulness as a more in vivo–like model.

Albumin synthesis is a useful marker for the assessment of liver specific function. Culture conditions in particular seem to play a key role in the synthesis of albumin in rat hepatocytes (Hamilton et al. 2001). Albumin synthesis was significantly higher in both developing and mature spheroids compared to monolayer cultures in this study, which could suggest the need for albumin proteins during more complex hepatocyte re-aggregation. Fish hepatocytes cultured in serum-supplemented media exhibit better cell–cell contacts and longevity in culture (Klaunig et al. 1985; Kocal et al. 1988). Hepatocytes cultured in collagen matrix configurations have been shown to maintain a high albumin secretion rate for prolonged periods (Dunn et al. 1989). The spheroid culture proposed in this study utilises a media-based extra-cellular matrix (ECM) which seems to stimulate albumin synthesis, suggesting that rotary-mediated suspension culturing of spheroids could offer an alternative, more physiologically-relevant method to conventional ECM-based spheroid culture methods for fish.

The use of LDH as a marker for chemical induced cytotoxicity is well supported in the literature (Pesonen and Andersson 1992; Bains and Kennedy 2004). It can also be used to assess the basal viability in non-chemical induced hepatocytes as indicators of cell membrane integrity. This is particularly useful when assessing hepatocyte aggregation as previously mentioned, it is possible to elucidate the viable state of spheroids when effects of total protein loss are perhaps not clear. In rat hepatocyte spheroids, the in vivo-like 3D environment permits to prolong hepatocyte viability in vitro (Tong et al. 1992). There was an inverse relationship between LDH leakage and total LDH in the present study. With particular reference to mature spheroids, a low level of basal LDH leakage suggests that membrane integrity within spheroids is maintained whereupon high intracellular levels of LDH could suggest feedback inhibition of enzyme release. This seems unlikely however, as both glucose and albumin release was not inhibited and similar patterns of LDH activity have been observed previously in mammalian liver spheroids (Ma et al. 2003).

Further to our work on primary liver tissue, it is worth considering the use of established fish cell lines in generating spheroids for alternative ecotoxicity assessment. We have successfully formed spheroids from both rainbow trout gonad (RTG-2) and rainbow trout liver (RTL-W1) cell lines in our laboratory (data not shown), with the latter of particular interest in offering a complete alternative to animal-based toxicity studies. Established liver cell lines such as RTL-W1 demonstrate a degree of cytochrome P450-dependent monooxygenase activity (Lee et al. 1993) under 2D conditions, but would require further characterisation to determine morphological, metabolic and biochemical functionality as spheroidal aggregates.

In conclusion, trout hepatocytes can be readily isolated via our proposed enzymatic digestion method with a high viability and hepatocyte yield. Hepatocytes will re-aggregate into 3D spheroids under gyratory-mediated conditions in culture media commonly used in fish in vitro studies. Trout spheroids take $\sim$6–8 days to reach maturity whereupon they would be suitable for use in acute toxicological tests and can survive in culture for a period of up to 40 days, an important criterion for both chronic toxicity and bio-accumulation tests. Trout liver spheroids appear inherently more variable in size than comparable mammalian 3D tissue cultures,

however the biochemical markers suggest that their condition and function can provide realistic organotypic responses in vitro and represent an important step towards future ecotoxicology methods and potentially a better alternative to exposing large numbers of fish to toxicants in order to study the biochemical response (Replacement). Further, since a single fish can provide a large number of spheroids, it is possible to develop assays for large numbers of compounds, or concentrations and time courses, which will reduce the overall number of fish required for testing (Reduction). Finally, as the fish are not exposed to the toxicant before death (Refinement) there is an additional benefit in the reduced requirement for large quantities of the test compound. Further work is currently in progress to determine the biochemical, metabolic and cytotoxic response of 3D fish liver spheroids to reference environmental toxicants, along with the refinement for longer term cultures that may offer potential for chronic in vitro exposures in fish.

Acknowledgments The authors would like to thank Dr. Jinsheng Xu (UWE, Bristol) for his assistance with spheroid culture and Mrs. Lynne Cooper (Plymouth University) for her expertise in primary tissue culture. Mr. Gareth Readman (Brixham Environmental Laboratory) and Mr. Ben Eynon (Plymouth University) are thanked for their help with fish husbandry and Dr. Roy Moate (Plymouth University) for his expertise in electron microscopy. The authors would also like to thank Prof. Lucy Lee (Wilfrid Laurier University, Canada) for her kind donation of RTL-W1 cells. This study was funded by a Biotechnology and Biological Sciences Research Council (BBSRC)/AstraZeneca Case Award to MB.

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