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A three-dimensional *in vitro* liver spheroid model of HepG2 cells for genotoxicity studies

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## Abstract

Liver's ability to metabolise chemical compounds makes it an appropriate tissue for toxicity testing. Current testing protocols i.e. two-dimensional liver cell systems and animal testing offer limited resemblance to *in vivo* liver cell behaviour in terms of gene expression profiles and metabolic competency; thus, they do not always accurately predict human toxicology. *In vitro* three-dimensional liver cell models offer an attractive alternative for determining the toxicity and genotoxicity of exogenous agents. This study reports on developing a 3D liver model using HepG2 cells by a hanging-drop methodology with particular focus on evaluating spheroid growth characteristics and suitability for genotoxicity testing. The cytokinesis-blocked micronucleus assay protocol was adapted to enable micronucleus detection in the 3D spheroid models. This involved evaluating the difference between hanging vs non-hanging drop positions for dosing of the test agents and comparison of the automated Metafer scoring for micronucleus detection in HepG2 spheroids with manual scoring.

The initial seeding density of 5000 cells/20  $\mu$ l drop hanging spheroids, harvested on day 4 with 75% cell viability was used for all experiments. Albumin secretion (7.8 g/l) and both CYP1A1 and CYP1A2 gene expression was highest in the 3D environment at day 4. An exposure of metabolically activated genotoxicants like B[a]P and PhIP for 24 hours resulted in a 6-fold increase in B[a]P- induced CYP1A1 activity at 3 $\mu$ M dose ( $p < 0.001$ ) and 30-fold increase in PhIP- induced CYP1A2 activity at 5  $\mu$ M dose ( $p < 0.05$ ) in 3D hanging spheroids. Similarly, the MN induction in response to B[a]P and PhIP was 2-fold and 3-fold, respectively, greater in 3D hanging spheroids as compared to 2D format, via manual micronucleus scoring, making hanging spheroids more sensitive to genotoxic agents. In conclusion, HepG2 hanging-drop spheroids are an exciting new alternative system for genotoxicity study than 2D culture due to improved structural and physiological properties.

Keywords: Hanging-drops spheroids, genotoxicity, micronucleus induction, metabolic activation, CYP450 enzymes, genotoxins,

## 1. Introduction

Evaluating the genotoxicity of any chemical substance requires optimization of the model's conditions to mimic or closely reflect human tissues. Traditionally, 2D cell cultures and animal testing have provided important insights into physiologic responses to a variety of test chemicals. However, results from 2D cell culture assays and animal tests offer limited relevance to the complexity of human tissues [1]. For example, 2D assays involve cell monolayers, whereas *in vivo* tissues have 3D geometries leading to different biochemistries. Moreover, 2D cultures possess non-physiological micro-environments, they rapidly proliferate, de-differentiate [2] and may also have abnormal gene expression profiles [3]. On the other hand, due to differences in animal and human physiological, genetic and metabolic systems [4] [5], it is argued that animal test results may not serve as accurate predictors of human toxicological responses [6]. These limitations of 2D culture and animal models necessitate further research and development of 3D human tissue models. Regulations made by the EU Registration, Evaluation, Authorization and restriction of CHemicals (REACH, 1999) also stress the importance of the development of new *in vitro* models that can reveal reliable results at lower cost and a shorter time scale compared to *in vivo* tests [7]. Three-dimensional (3D) cell culture assays involve a cell environment promoting direct cell-cell contact as well as an extracellular matrix (ECM) and are thus considered to be more reflective of *in vivo* cellular responses. The optimization of 3D assays with representative human cell lines can therefore provide optimum conditions for genotoxicity testing.

The liver is an important site in predicting xenobiotic agent-associated toxicity as well as the potential impact of drug usage on liver failure [8] and this makes it a physiologically and clinically relevant tissue for toxicity testing. Primary hepatocytes have liver-specific functions, particularly CYP450 enzymes, and are therefore currently used for the testing of different chemical compounds but are expensive and have short life spans in culture. Hence immortalised, and more cost-effective, cell lines such as the human hepatocellular carcinoma (HepG2) cell line is commonly used [9]. HepG2 cells have low levels of cytochrome P450 (CYP450) and xenobiotic receptors compared to normal liver cells; this is noteworthy as these enzymes are often required to metabolise drugs. The presence of basal enzyme activity and the further induction of CYP450 enzymes are required to assess the toxicity of chemical compounds and therefore low expression of these enzymes make this cell line a poor predictor of the toxic effects of pro-carcinogens in humans [10]. However, the development of 3D assays with higher expression of metabolic enzymes provides an opportunity to develop a cost effective and reliable test. There are several 3D cell culture methods available

for assessment of chemical compound metabolism, e.g., agitation-based approaches, matrices and scaffolds, hanging drop and microfluidic cell culture platforms [11] [12]. Most of them are expensive and require specialized equipment. In this study, we report the development and optimization of a hanging drop 3D spheroid model using the HepG2 cell line as a relatively simple, inexpensive and reliable test model for genotoxicity studies [13]. These spheroids provide tightly packed 3D multicellular aggregates with enhanced cell-to-cell contact and extracellular matrix components. The major advantages of this model are that it does not require any specialist materials, equipment or training and cells are not in direct contact with an extracellular matrix. Earlier studies have demonstrated that HepG2 liver cancer cells and MCF-7 breast cancer cells have been used to produce 3D spheroids which were described as ‘tissue-like’ [13]. These spheroids can survive up to 28 days and have increased Phase I and Phase II enzyme expressions, albumin and others liver-specific markers when compared to their monolayer cultures [14]. They also show more physiologically relevant expression of genes involved in xenobiotic metabolism that are higher than typically observed in 2D culture systems, which increases 3D spheroids’ sensitivity to hepatotoxic compounds [15].

The main objective of this study was the development of a 3D hanging drop procedure using HepG2 cells to evaluate the genotoxic effect of carcinogenic chemicals for the first time. The compounds benzo[*a*]pyrene (B[*a*]P) and 2-amino-1-methyl-6-phenylimidazo(4,5-*b*) pyridine (PhIP) were selected on the basis of their ability to form DNA adducts as a result of metabolic activation to a DNA-reactive form [16]. A currently recommended Organization for Economic Co-operation and Development (OECD) 487 [17] guideline for *in vitro* micronucleus tests, was selected as a basis for evaluating the reliability of 3D models for genotoxicity investigations and to study the dose-responses of B[*a*]P and PhIP in both 2D and 3D models. CYP450 expression, activity and albumin production were used as indicators of the enhanced metabolic capability of the 3D models.

## **2. Material and Methods**

### *2.1 Chemicals*

Benzo[*a*]pyrene (B[*a*]P) (Sigma, UK) and 2-amino-1-methyl-6-phenylimidazo(4,5-*b*) pyridine (PhIP) (Calbiochem, EMD Chemicals Inc. Germany) were used in this study and stored according to the manufacturer’s instructions. B[*a*]P and PhIP were both diluted in DMSO (Fisher Scientific). Dilutions from the master stocks were made freshly for each replicate.

## 2.2 *Cell culture*

The human hepatocellular carcinoma cell line, HepG2, obtained from ECACC, was cultured in DMEM (GIBCO<sup>®</sup>, Paisley, UK) supplemented with 10% foetal bovine serum (FBS, GIBCO<sup>®</sup>, Paisley, UK) and penicillium/Streptomycin 100X (GIBCO<sup>®</sup>, Paisley, UK). Sub-culturing or processing of HepG2 cells was performed by trypsinisation with trypsin/EDTA (0.05%) solution (GIBCO<sup>®</sup>, Paisley, UK). The cells were maintained in culture at a density between 1- 3 x 10<sup>5</sup> cells/ml or cm<sup>2</sup> and sub-cultured every 5 days, until they reached confluency. Cultures were examined using x40 objective on a Zeiss Axiovert 25 light microscope.

## 2.3 *Hanging spheroid preparation from different cell density of HepG2 monolayers*

HepG2 monolayers (Fig 1 A) were used to form the hanging spheroids using a hanging drop method. Initially, cell density 5000 and 10000 per 20 µl drop of growth medium were made. The drops were placed on the inner side a 9.4 cm Petri dish (Greiner bio-one, UK) lid as shown in (Fig 1 B). Lids contained either 50 or 100 drops depending on the analysis. The cellular suspension was gently mixed via pipetting to ensure the cells were thoroughly suspended with the media. To prevent the drops from drying, the 9 cm Petri dish was filled with 20 ml PBS (inner compartment). The whole set up was placed very gently in the incubator at 37°C and 5% CO<sub>2</sub> atmosphere.

The cell viability in the hanging drop was maintained by adding 6 µl of growth media to each drop on day 3 and day 6. The light colour of the hanging spheroid (indicator of the live and dead cells colonization) and size of hanging spheroid (indicator of cell growth) were observed and measured on day 4 and day 7 using Nikon Eclipse 50i (Nikon) attached to a computer (Windows 7) using Cellsense software (OLYMPUS). The experiments were done three times, and area/ diameter are shown as the mean of three replicates.

## 2.4 *Cell viability of hanging spheroids with 0.4% Trypan blue solution*

To determine the viability of hanging spheroids within the hanging drops, trypan blue was used. Five hanging spheroids were harvested on day 4 and day 7, transferred into a centrifuge tube and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 1 ml PBS (GIBCO<sup>®</sup>, Paisley, UK), centrifuged and again re-suspended into 200 µl of the trypsin/ EDTA. This was incubated for 8 minutes at 37°C, followed by mixing with a pipette to ensure that spheroids were completely dissociated. The action of trypsin was inhibited by adding 1 ml of complete growth medium prior to centrifugation at 1200 rpm for 5 min, and the supernatant was then discarded. The tube was left inverted for a couple of minutes on medical wipes (Kimcare<sup>®</sup>, Kimberly-Clark<sup>®</sup>) to remove all traces of any

remaining liquid. Again 100  $\mu$ l of growth medium was added and mixed well. Live and dead cell counts were taken using a standard trypan blue methodology.

### 2.5 *Effect on albumin production*

Levels of albumin were determined using a commercially available kit (BCG (Bromocresol green) Albumin Assay Kit, Sigma-Aldrich)). The experiment was performed according to the manufacturer's instructions. The medium along with 3 hanging spheroids were collected on day 4, in 1.5 ml centrifuge tube, briefly centrifuged to settle the spheroids at the bottom of tube and 15  $\mu$ l of the supernatant was used to measure the albumin secretion in the medium. The known quantities of albumin were used to establish the standard curve.

### 2.6 *CYP Gene expression analysis*

#### 2.6.1 *RNA Extraction*

On day 4, 100 hanging spheroids or HepG2 monolayer from a whole T-25 flask was trypsinized, centrifuged and set the concentration at  $1.5 \times 10^6$  cells/ml in the culture medium. RNA extraction was performed using TRIzol<sup>®</sup> reagent (Invitrogen) (500  $\mu$ l) added to the cell pellets from hanging spheroids or HepG2 monolayer and the contents were transferred to 1.5 ml reaction tubes (Greiner bio-one, UK) and following the procedure as specified by the manufacturer. RNA sample concentration was determined using a NanoDrop spectrometer (ND-1000 Spectrophotometer, Labtech International UK).

#### 2.6.2 *Reverse Transcription-Polymerase Chain Reaction (RT-PCR)*

A QuantiTect<sup>®</sup> Reverse Transcription kit (Qiagen, UK) was used to prepare the complementary DNA (cDNA). GoTaq<sup>®</sup> Flexi DNA polymerase kit (Promega, UK) was used for the RT-PCR reactions of all the gene primers. Forward and reverse primers for  $\beta$ -actin, CYP1A1 and CYP1A2 were purchased from Sigma-Aldrich, UK. The details of primers and RT-PCR are available in a previous paper Shah et al [18].

### 2.7 *Measurement of CYP450 enzyme activity for metabolic competency*

The CYP450 activity was determined using the P450-Glo 1A1 and 1A2 assay kits (Promega, USA), according to the manufacturer's instructions. Fifty hanging spheroids were treated with B[a]P (3  $\mu$ M (LOEL), 4  $\mu$ M and 8  $\mu$ M) and PhIP (5  $\mu$ M, 7.5  $\mu$ M (NOEL), 10  $\mu$ M (LOEL) and 15  $\mu$ M), on day 4. The same concentrations were used to dose the flasks with HepG2 monolayers for 24 h, at 37°C, 5% CO<sub>2</sub>.

Hanging spheroids were collected in the labelled tubes, centrifuged, completely dissociated using trypsin/EDTA, centrifuged again and the supernatant was discarded. Similarly, cells from the flasks were trypsinized, collected, and centrifuged to form a pellet.

All the tubes were left inverted for a couple of minutes to remove all traces of any remaining liquid. For the measurement of CYP1A1, the cells were maintained at  $5 \times 10^5$  cells/ml concentration in culture medium. The assay was performed following the manufacturer's instructions. Results were obtained using a FLOURstar OPTIMA plate reader (BMG Lab technologies Ltd., UK). CYP450 activities were calculated by subtracting the background and normalized to the seeded cell number.

## 2.8 *Development of Cytokinesis-blocked micronucleus (CBMN) assay for hanging spheroids*

The hanging spheroids (200 drops) were prepared in square Petri plates using 5000 cells/ 20  $\mu$ l drop of growth medium and the same concentration was used to obtain the HepG2 monolayers in flasks. The spheroids were left in the hanging drop for 3 days and then on day 4 they were exposed to B[a]P for 24 h in both hanging vs non-hanging positions, to compare the spheroid orientation to the genotoxicity of B[a]P in the 2D system at different concentrations (2  $\mu$ M, 3  $\mu$ M, 4  $\mu$ M and 8  $\mu$ M) based on those used in a previous study Shah et al [18]. The same concentrations were used to dose the flasks with HepG2 monolayers for 24 h, at 37°C, 5% CO<sub>2</sub>. Cytochalasin B (6  $\mu$ g/ml (Merck)) was added for one cell-cycle at the time of dosing of HepG2 spheroids and monolayers. Following comparison of hanging and non-hanging orientations, no difference in genotoxicity outcomes was observed (Fig 1).

After 24 h incubation time, hanging spheroids were collected and centrifuged at 1200 rpm for 5 min. These were washed twice with 1 ml PBS and re-suspended into 300  $\mu$ l trypsin/ EDTA. Tubes were incubated for 8 min at 37°C, followed by further pipetting to ensure that spheroids were completely dissociated. This was then followed by the addition of 1 ml of growth medium, centrifugation and discarding of the supernatant. Again 3 ml of growth media was added into each tube and mixed well. The same procedure was used for monolayer cells.

### 2.8.1 *Cell fixation and slide preparation for the semi-automated Metafer slide scanning system*

The cell suspension in each tube was split into three tubes (1 ml each tube). The cells of the first tube were harvested using a standard 2D method adopted by Seager *et al.* [19] and the second tube harvested using a previous 3D protocol as reported by Chapman *et al.* [20]. The standard 2D method led to insufficient cell numbers due to cell loss via multiple wash steps, whereas the 3D method led to false positive micronucleus results due to excessive debris remaining in the samples (data not shown).



### 2.8.2 Slides preparation, cell fixation and staining for Manual Scoring of Slides

The third set of tubes was centrifuged and the cell pellets were re-suspended in 2 ml PBS and mixed gently using a pipette. The slides for the manual scoring were prepared using protocol described by Manshian *et al.* [21]. The Olympus BH2 microscope at  $\times 100$  objective was used to visualise the slides. A minimum of 1000 binucleated cells with non-overlapping nuclei and with intact nuclear membranes within the same cytoplasm, per dose per replicate were scored for the presence of micronuclei (Fenech *et al.* (2003) criteria for scoring [22]). The experiment was repeated three times.

### 2.9 Genotoxic effect of B[a]P and PhIP on 2D monolayers and 3D spheroids using CBMN assay

PhIP and B[a]P were used to compare the difference in chromosomal damage after 24 h exposure in 3D hanging spheroids and 2D HepG2 monocultures. The PhIP concentrations (5  $\mu\text{M}$ , 7.5  $\mu\text{M}$ , 10  $\mu\text{M}$  and 15  $\mu\text{M}$ ) selected were all giving  $<55 \pm 5\%$  cytotoxicity, which is in line with the OECD guideline (487) [17] requirements for use of the *in vitro* micronucleus assay. The B[a]P concentrations used were the same as in CBMN assay developmental studies. The rest of the procedure adopted was similar to that mentioned previously.

### 2.10 Statistical analysis

The area of the hanging spheroid was calculated by taking the mean area of five spheroids. The SPSS software v. 22 was used to perform a one-way ANOVA with a Dunnett's 2-sided post-hoc analysis to identify statistically significant increases or decreases relative to vehicle control levels. The first statistically significant change was representing by  $p \leq 0.05$ . All error bars represent standard error (SE) around the mean of three biological replicates.

## 3. Results

This study aimed to develop the 3D assay conditions to facilitate genotoxicity studies in HepG2 hanging spheroids. We used a hanging drop culture protocol to generate HepG2 hanging spheroids (3D) from trypsinized HepG2 monolayers (2D) (Fig 2A). Cells were placed in a hanging drop culture (Fig 1B) and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  until they formed 3D hanging spheroids (Fig 2C), which took approximately 4 days. The hanging drop culture formed tissue-like cellular aggregates, which provided optimum conditions for the measurement of biomechanical properties, as well as allowing for molecular and biochemical analysis in a physiologically relevant model.

### 3.1 Growth and viability of HepG2 hanging spheroids

The growth of the hanging spheroids was evaluated on days 4 and 7 with seeding cell densities of 5000 and 10000 per 20  $\mu$ l drop of cell culture medium. Both cell densities formed spheroids with reproducible diameters. When an initial seeding density of 5000 / 20  $\mu$ l drop was used, by day 4 the mean diameter of the spheroids was 0.08 cm; this diameter reduced to 0.056 cm by day 7 with a visible change in shape as the spheroids became more compact (Fig 2 D). Similarly, when the initial cell density 10000 / 20 $\mu$ l drop was applied, the spheroid diameter was 0.068 cm and 0.073 cm on day 4 and day 7, respectively, and the shape of the hanging spheroids became less circular (Fig 2D). Light microscopy also indicated darker areas in the centre of the hanging spheroids on day 7 compared to day 4, suggestive of cellular necrosis (Fig 2 D).

Cell viability was subsequently assessed by both trypan blue staining and use of propidium iodide (PI) to establish when necrosis started to increase in the centre of the spheroids. For spheroids with an initial cell density of 5000 cells/ 20  $\mu$ l drop, the cell viability was 100% which gradually decreased to 87% and 75% on day 4 and day 7, respectively. When 10000 cells/ 20  $\mu$ l drop were seeded, the cell viability was lower at 61% on day 4, but recovered slightly on day 7 to 77% compared to initial cell density (Fig 3a). Although there was an increase in cell viability on day 7, the fold change difference was non-significant between both days when compared to the initial cell density.

Propidium iodide staining was used for the identification of apoptotic/necrotic cells in the hanging spheroids (Fig 3 b, c, d, e). The fluorescent images supported the cell viability data. Fewer red dye foci were observed on day 4 in the samples with initial seeding density 5000 cells / 20  $\mu$ l drop (Fig 3 b) as compared to 10000 cells / 20  $\mu$ l drop (Fig 3 d) and they were evenly distributed throughout the hanging spheroid. In contrast to that of day 4, on day 7 stronger and more red dye signals were observed, especially at the centre of the spheroid, representing a higher cell death rate (Fig 3 c, e). Cell density of 5000 cells/ 20  $\mu$ l drop on day 4 and day 7 were chosen to make spheroids for albumin and *CYP1A1* and *CYP1A2* gene expression studies.

### 3.2 Effect of culture time on albumin production in spheroids

Albumin production in HepG2 hanging spheroids was measured on day 4 and day 7, while for 2D monolayers it was measured on day 4 only (Fig 4 A). It was observed that albumin secretion was significantly higher in hanging spheroids on day 4 (7.8 g/l,  $p < 0.01$ ) as compared to day 7 (5.6 g/l,  $p < 0.05$ ). It was also observed that albumin concentration was

significantly ( $p < 0.05$ ) increased in cultures grown as spheroids compared to cells grown in 2D monolayers (3.4 g/l).

### 3.3 *HepG2 hanging spheroids were metabolically more competent than HepG2 monolayers*

The CYP450 gene expression was assessed using *CYP1A1* and *CYP1A2* mRNA in 2D format (day 4 only) and 3D hanging spheroids at day 4 and day 7 (Fig 4 B). In a similar manner to albumin, the fold change in *CYP1A1* and *CYP1A2* mRNA expressions were higher in 3D hanging spheroids in comparison to 2D HepG2 monolayers. It was noted that the fold change gene expression for *CYP1A1* was higher at day 4 whilst *CYP1A2* was higher on day 7 in 3D hanging spheroids.

A comparison of their respective CYP1A1 and CYP1A2 enzyme activities both in 2D monolayers and 3D hanging spheroids after 24 h exposure of B[a]P and PhIP were in Fig 5. The basal enzyme activity of CYP1A1 and 1A2 was higher in 3D hanging spheroids as compared to cells within the 2D format. B[a]P- induced CYP1A1 and PhIP-induced CYP1A2 enzyme activities were higher in 3D hanging spheroids after 24 h exposure to B[a]P and PhIP respectively as compared to HepG2 cells grown in 2D monolayers. The significant increase in B[a]P-induced CYP1A1 activity was observed at 3 $\mu$ M dose of B[a]P in both the 2D monolayer and 3D hanging spheroids ( $p < 0.05$  and  $p < 0.001$ , respectively) (Fig 5 A) but the CYP1A1 activity was 6-fold more in 3D hanging spheroids compared to 2D monolayers. In 2D monolayers, the significant increase in PhIP- induced CYP1A2 activity was observed at 10  $\mu$ M dose of PhIP, while in 3D spheroids it was noticed at a lower (5 $\mu$ M) dose of PhIP ( $p < 0.05$ ) (Fig 5 B) with 30-fold increased activity. These results indicated an overall increase in the activity of CYP1A1 and CYP1A2 enzymes in 3D hanging spheroids as compared to 2D monolayers.

### 3.4 *Comparison of genotoxicity in 2D monolayers and 3D hanging spheroids with different chemical exposures*

Due to the aforementioned challenges of using the Metafer system for spheroid analysis, the slides were prepared by cytopsin for manual scoring and micronucleus frequencies were assessed by visualisation under a light microscope. The first significant increase in micronucleus frequency in binucleated cells was observed at a 3 $\mu$ M dose of B[a]P both in the hanging and non-hanging positions of the spheroids. The difference in genotoxicity between 2D monolayers and 3D hanging spheroids was compared after 24 h exposure of both B[a]P and PhIP (Fig 6). The BN frequency in 3D hanging spheroid cultures was less than the 2D monolayers due to the slow proliferation rate of the cells in 3D environment (Fig 6 A and B).

When micronucleus frequency generated as a result of exposure to B[a]P was compared in 2D monolayers versus 3D HepG2 hanging spheroids exposures, it was shown in both test systems that a 3µM dose of B[a]P induced the first significant increase ( $p<0.05$ ) in micronucleus frequency in binucleated cells, above the vehicle control (Fig 6A). However, it was noted that the micronucleus frequency induced in the 3D hanging spheroids was 2-fold higher than that in 2D monolayers at their first significant dose.

In 2D monolayers treated with PhIP, the first significant dose leading to MN induction was 10µM ( $p<0.001$ ), at which the micronucleus frequency in binucleated cells was 1.9%, relative to its vehicle control. While in 3D hanging spheroids the first significant ( $p<0.05$ ) micronucleus frequency in binucleated cells was 3.66% at 5µM, from its vehicle control (Fig 6 B). The micronucleus frequency for 3D hanging spheroids compared to 2D monolayer for PhIP was 1.1 fold higher at the first significant dose (5 µM). The results therefore indicate that the 3D hanging spheroids were more sensitive to exposure to genotoxic agents.

#### **4. Discussion**

The development and application of 3D human tissue models in (geno)toxicity testing might bridge the gap between 2D assays and animal testing. As the current regulatory guidelines for genotoxicity testing are mainly focused on 2D models, further optimisation of *in vitro* 3D tissue culture models is required. In relation to this, the objective of this study was to assess the feasibility of a 3D HepG2 *in vitro* model specifically for genotoxicity studies, evaluating its performance using B[a]P and PhIP. The hanging drop method was specifically chosen here due to its versatility and low cost. The hanging drop method also offers advantages over other 3D culture approaches, as cells are not in contact with an artificial matrix, supporting the maintenance of *in vivo*-like cell morphology and behaviour [23].

##### *4.1 Effect of contact inhibition on area and cell viability in 3D spheroids*

The visual inspection of hanging spheroids via light microscope images and their average area measurements indicated a decrease in spheroid areas as the culture time progressed (on day 7) (Fig 2 D). Such a decrease may be due to contact inhibition, which is greater in 3D structures as it is induced from both lateral and vertical directions in comparison to monolayers where it occurs only laterally. A similar study conducted by Mueller *et al.* [24] reported that due to contact inhibition, the diameter of HepG2 spheroids increased up to day 6

and then decreased up to day 10 when the initial cell density was 2000 HepG2 cells which is possibly due to lateral and vertical contact inhibition (33).

High contact inhibition can lead to lower cellular proliferation in 3D hanging spheroids [25] which we observed as reduced hanging spheroids diameter (Fig 2 D), cell viability (Fig 3) and increasing PI fluorescence (Fig 3 b and d). The presence of high red fluorescence indicated larger areas of necrosis possibly as a result of hypoxia (Fig 3 c, e) as compared to the areas with less/no red fluorescence representing viable cells. Earlier studies have shown an inverse relationship between the spheroid diameter and cell viability with a survival threshold of 200  $\mu\text{m}$  [26] [27]. Cell death was observed in our study after PI staining at both day 4 and day 7 (Fig 3) and associated with hanging spheroid cell diameters of greater than 200  $\mu\text{m}$ .

The effect of contact inhibition in term of cellular proliferation prevailed from day 4 to day 7 and caused the decrease or levelling off of other metabolic activities such as albumin secretion and CYP1A1 expression levels (Fig 4 A and B). Similarly, Muller *et al.* [24] observed an early increase (day 3) and later decrease of albumin secretion in their study.

#### 4.3 Effect on CYP1A1 and CYP1A2 expression

Gene expression analysis indicated significant variations between 2D monolayers and 3D HepG2 hanging spheroids for basal and induced CYP genes. *CYP1A1* and *CYP1A2* expression was significantly higher in 3D hanging spheroids (Fig 5 A and B). Nakamura *et al.* [28] also reported considerably higher basal expression of these genes in HepG2 spheroids as compared to 2D HepG2 monolayers, where expression was found to be very low. Up-regulation of *CYP1A1* and *CYP1A2* expression is linked with the activation of factors such as the nuclear receptors, aryl hydrocarbon receptors (AhR), constitutive androstane and pregnane X receptors [24] [15] [29]. These findings indicate that spheroids provide a cell environment closer to that *in vivo*, compared to that of 2D systems, for interaction with chemicals and their expression of enzymes.

An important aspect of toxicity testing is the assessment and prediction of CYP1A enzyme induction by certain chemicals, such as B[a]P and PhIP. Our study shows that 3D hanging spheroids have significantly higher induction of CYP1A1 and CYP1A2 following a 24 h exposure to B[a]P and PhIP when compared with 2D HepG2 monolayers across all the doses evaluated in this study (Fig 5 A and B). For example, a 5  $\mu\text{M}$  PhIP dose resulted in significantly higher activity of CYP1A2 and MN induction in 3D hanging spheroids as compared to 2D monolayers (Fig 5 B and 6 B). It is important to note that CYP enzyme

induction is chemical-specific, with different expression profiles resulting from treatment with different chemical compounds.

#### 4.4 Genotoxicity of B[a]P and PhIP in 3D

The MN assay results with B[a]P (Fig 6) indicate that in HepG2 hanging spheroids, this chemical induces a statistically significant increase in MN frequency at 3  $\mu$ M which is in line with the previous study with 2D monolayers [18]. In contrast to B[a]P, PhIP significantly increased MN frequency at 5  $\mu$ M in 3D hanging spheroids but only at the higher dose of 10  $\mu$ M in 2D monolayers following 24 h treatment.

It is worth mentioning that the cytokinesis-block proliferation index (CBPI) observed in our 3D hanging spheroids were low compared to the 2D monolayers (Fig 6). This could be attributed to limitation of cell division in the spheroids where it is mainly cells in the outer layers that divide, while cells towards the core of the spheroids are less likely to divide and hence show a lower proliferation index. Apoptosis / necrosis may also contribute, but we have shown through the cell viability studies that conditions were maintained to minimise this. The effect of treatments and the presence of contact inhibition on day 5 influences the proliferation index, as we dosed the spheroids seeded with 5000 cells/ 20  $\mu$ l drop on day 4 but processed them on day 5. On day 5, the size of spheroid was large enough that this would not only affect the distribution of oxygen, CO<sub>2</sub> and nutrients but also cytochalasin B in 3D hanging spheroids [30]. In 3D cell cultures all of these functions were performed by diffusion [31]. The diffusion rate is less effective in larger hanging spheroids as compared to 2D monolayers, and results in heterogeneous distribution of nutrients, accompanied by effects on cell cycle stage distribution and reduced cell proliferation rate. It is acknowledged that the significant rates of apoptosis and necrosis within the core of these spheroids is not representative of *in vivo* liver and this therefore could be considered a limitation of these particular spheroid models.

### Conclusion

Our findings suggest that the culture of HepG2 cells as hanging spheroids is a superior model to the traditional 2D monolayer culture due to the greater structural and physiological resemblance to *in vivo* conditions. Our studies have also highlighted the importance of optimizing initial seeding density, as this affects spheroid size, viability and physiological properties. Similarly, our optimized assays improved sensitivity of the test as we were able to evaluate the effect of B[a]P and PhIP at lower concentrations than those previously used for 2D cell assays. We have also demonstrated the successful application of the CBMN assay to

HepG2 spheroids to detect DNA damage induced by genotoxicants that require metabolic activation.

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### **Conflict of interest**

No conflict of interest

### **References**

1. Mazzoleni, G., Di Lorenzo, D., and Steimberg, N., (2009). Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? *Genes Nutr*, 4(1), 13-22.
2. Bhadriraju, K. and Chen, C.S., (2002). Engineering cellular microenvironments to improve cell-based drug testing. *Drug Discov Today*, 7(11), 612-20.
3. Birgersdotter, A., Sandberg, R., and Ernberg, I., (2005). Gene expression perturbation in vitro--a growing case for three-dimensional (3D) culture systems. *Semin Cancer Biol*, 15(5), 405-12.
4. Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., et al., (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regul Toxicol Pharmacol*, 32(1), 56-67.
5. Knight, A., (2007). Systematic reviews of animal experiments demonstrate poor human clinical and toxicological utility. *Altern Lab Anim*, 35(6), 641-59.
6. Long, M.E., (2007). Predicting carcinogenicity in humans: The need to supplement animal-based toxicology. *AATEX*, 14(Special Issue. Proc. 6th World Congress on Alternatives & Animal Use in the Life Sciences.), 553-559.
7. Regulation, E., (1999). No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive. 451-849.
8. Tuschl, G., Lauer, B., and Mueller, S.O., (2008). Primary hepatocytes as a model to analyze species-specific toxicity and drug metabolism. *Expert Opin Drug Metab Toxicol*, 4(7), 855-70.
9. Guo, L., Dial, S., Shi, L., Branham, W., Liu, J., Fang, J.L., Green, B., Deng, H., Kaput, J., and Ning, B., (2011). Similarities and differences in the expression of drug-metabolizing enzymes between human hepatic cell lines and primary human hepatocytes. *Drug Metab Dispos*, 39(3), 528-38.
10. Xu, J.J., Diaz, D., and O'Brien, P.J., (2004). Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. *Chem Biol Interact*, 150(1), 115-28.
11. Breslin, S. and O'Driscoll, L., (2013). Three-dimensional cell culture: the missing link in drug discovery. *Drug Discov Today*, 18(5-6), 240-9.
12. Gomez-Lechon, M.J., Tolosa, L., and Donato, M. T. , (2014). Cell-based models to predict human hepatotoxicity of drugs. *Rev. Toxicology*, 31149-156.
13. Kelm, J.M.e.a., (2003). Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. *Biotechnol. Bioeng.*, 83173-180.
14. Xia, L., Hong, X., Sakban, R.B., Qu, Y., Singh, N.H., McMillian, M., Dallas, S., Silva, J., Sensenhauser, C., Zhao, S., et al., (2016). Cytochrome P450 induction response in tethered

- spheroids as a three-dimensional human hepatocyte in vitro model. *J Appl Toxicol*, 36(2), 320-9.
15. Ramaiahgari, S.C., den Braver, M.W., Herpers, B., Terpstra, V., Commandeur, J.N., van de Water, B., and Price, L.S., (2014). A 3D in vitro model of differentiated HepG2 cell spheroids with improved liver-like properties for repeated dose high-throughput toxicity studies. *Arch Toxicol*, 88(5), 1083-95.
  16. Turesky, R.J., (2007). Formation and biochemistry of carcinogenic heterocyclic aromatic amines in cooked meats. *Toxicol Lett*, 168(3), 219-27.
  17. OECD, (Adopted: 29, July 2016). Organization of economic cooperation and development. Guidelines for the Testing of Chemicals, 487. *In vitro* Mammalian Cell Micronucleus Assay.
  18. Shah, U.-K., Seager, A. L., Fowler, P., Doak, S. H., Johnson, G. E., Scott, S. J., Scott, A. D., and Jenkins, G. J. S. , (2016). A comparison of the genotoxicity of benzo[a] pyrene in four cell lines with differing metabolic capacity. *Mutation Research/ Genet Toxicol Environ Mutagen*, 8088-19.
  19. Seager, A.L., Shah, U.K., Brusehafer, K., Wills, J., Manshian, B., Chapman, K.E., Thomas, A.D., Scott, A.D., Doherty, A.T., Doak, S.H., et al., (2014). Recommendations, evaluation and validation of a semi-automated, fluorescent-based scoring protocol for micronucleus testing in human cells. *Mutagenesis*, 29(3), 155-64.
  20. Chapman, K.E., Thomas, A.D., Wills, J.W., Pfuhrer, S., Doak, S.H., and Jenkins, G.J., (2014). Automation and validation of micronucleus detection in the 3D EpiDerm human reconstructed skin assay and correlation with 2D dose responses. *Mutagenesis*, 29(3), 165-75.
  21. Manshian, B.B., Singh, N., and Doak, S.H., (2013). The in vitro micronucleus assay and kinetochore staining: methodology and criteria for the accurate assessment of genotoxicity and cytotoxicity. *Methods Mol Biol*, 1044269-89.
  22. Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., and project, H.U.M., (2003). HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat Res*, 534(1-2), 65-75.
  23. Foty, R., (2011). A simple hanging drop cell culture protocol for generation of 3D spheroids. *J Vis Exp*, (51).
  24. Mueller D, K.A., Noor F (2011) J Bioengineer & Biomedical Sci and S2:002, (2011). Organotypic Cultures of HepG2 Cells for *In Vitro* Toxicity Studies. *J Bioengineer & Biomedical Sci* S2:002.
  25. Li, C.L., Tian, T., Nan, K.J., Zhao, N., Guo, Y.H., Cui, J., Wang, J., and Zhang, W.G., (2008). Survival advantages of multicellular spheroids vs. monolayers of HepG2 cells in vitro. *Oncol Rep*, 20(6), 1465-71.
  26. Fukuda, J., Okamura, K., Nakazawa, K., Ijima, H., Yamashita, Y., Shimada, M., Shirabe, K., Tsujita, E., Sugimachi, K., and Funatsu, K., (2003). Efficacy of a polyurethane foam/spheroid artificial liver by using human hepatoblastoma cell line (Hep G2). *Cell Transplant*, 12(1), 51-8.
  27. Asthana, A. and Kisaalita, W.S., (2012). Microtissue size and hypoxia in HTS with 3D cultures. *Drug Discov Today*, 17(15-16), 810-7.
  28. Nakamura, K., Kato, N., Aizawa, K., Mizutani, R., Yamauchi, J., and Tanoue, A., (2011). Expression of albumin and cytochrome P450 enzymes in HepG2 cells cultured with a nanotechnology-based culture plate with microfabricated scaffold. *J Toxicol Sci*, 36(5), 625-33.
  29. Nebert, D.W., Dalton, T.P., Okey, A.B., and Gonzalez, F.J., (2004). Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem*, 279(23), 23847-50.
  30. Lin, R.Z. and Chang, H.Y., (2008). Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol J*, 3(9-10), 1172-84.



31. Edmondson, R., Broglie, J.J., Adcock, A.F., and Yang, L., (2014). Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay Drug Dev Technol*, 12(4), 207-18.

## Legends:

Figure 1. Comparison between hanging vs. non-hanging position of drops, exposed to B[a]P for 24 h. No statistically significant difference was observed between the orientations of drop. The first significant increase in micronucleus frequency was observed at 3  $\mu$ M dose of B[a]P relative to control. One thousand binucleated cells were scored manually. Statistically significant results were denoted by \* =  $p < 0.05$ , \*\* =  $p < 0.01$ . Error bars are SE and  $n = 3$ .

Figure 2. Formation of HepG2 spheroids from monolayer cultured cells using hanging-drop method. (A) HepG2 cells cultured in a monolayer (2D). (B) Lid of Petri dish spotted with hanging drops. (C) HepG2 hanging spheroid (3D) in one drop (20  $\mu$ l). (D) Light microscope images of HepG2 spheroids on day 4 and day 7 with initial cell density of 5000 and 10000 cells/ 20  $\mu$ l drop. Images were taken by Nikon Eclipse 50i (scale bar = 100  $\mu$ m/0.01cm).

Figure 3. Quantitative and qualitative visual cell viability progression of hanging spheroids with different initial cell densities. (A) The cell viability in the hanging spheroids on day 4 and day 7. On day 7, the hanging spheroids with an initial cell density of 5000 cells/20  $\mu$ l drop, the cell viability was significantly decreased (t-test) compared to day 4. Bars indicate the  $\pm$  standard error (SE) and  $n=3$ . Fluorescent microscopy images of propidium iodide (PI) stained nuclei of the dead cells in spheroids at different initial cell densities and days (b, c, d, e) (scale bar = 100  $\mu$ m).

Figure 4. Comparison of liver specific metabolic and gene expression levels of 3D hanging spheroids against 2D monolayer cultures. (A) The albumin secretion into the 15  $\mu$ l culture medium is higher on day 4 as compared to day 7 in HepG2 hanging spheroids. Albumin levels were normalized to the total amount of protein per well. (B) Expression of *CYP1A1* and *CYP1A2* mRNA in 3D hanging spheroids at day 4 and day 7 compared to day 4 2D monolayer cells by quantitative real-time PCR. Values were normalized using housekeeping gene  $\beta$ -actin mRNA expression. The y-axis represented the fold change in expression of CYP450 mRNA in 3D hanging spheroids from 2D monolayers. Significant increases compared to the control samples were indicated as follows \* =  $p < 0.05$ , \*\* =  $p < 0.01$ . Error bars are SE and  $n = 3$ .

Figure 5. Basal and induced Phase I cytochrome P450 1A1 and 1A2 enzyme activities. Enzyme activities in 2D monolayers and 3D HepG2 hanging spheroids after 24 h exposure to B[a]P by CYP1A1 (A) and PhIP by CYP1A2 respectively (B). The cell density  $5 \times 10^5$  cells/ml was used for the enzyme activity for 2D monolayers and 3D hanging spheroids. Significant increases compared to the control samples were indicated as follows \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ . Error bars are SE and  $n = 3$ .

Figure 6. The difference in micronucleus frequencies (%MN/BN) and cytokinesis-block proliferation index (CBPI) induced by increasing concentration of B[a]P (A) and PhIP (B) in HepG2 monolayers (2D) and hanging spheroids (3D) after 24 h exposure time. 2D monolayers showed proliferation index than the 3D hanging spheroids. The 2D %MN/BN data comes from Shah *et al.*, 2016. The first significant dose of B[a]P was similar for 2D monolayers and 3D hanging spheroids. After PhIP exposure the first significant dose

appeared earlier in 3D hanging spheroids than the 2D monolayers. Significant increases in chromosomal damage compared to the vehicle control were indicated as follows \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ . Error bars are SE and  $n = 3$ .