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Impact of cell types and culture methods on the functionality of in vitro liver systems – a review of cell systems for hepatotoxicity assessment

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Abstract

Xenobiotic safety assessment is an area that impacts a multitude of different industry sectors such as medicinal drugs, agrochemicals, industrial chemicals, cosmetics and environmental contaminants. As such there are a number of well-developed in vitro, in vivo and in silico approaches to evaluate their properties and potential impact on the environment and to humans. Additionally, there is the continual investment in multidisciplinary scientists to explore non-animal surrogate technologies to predict specific toxicological outcomes and to improve our understanding of the biological processes regarding the toxic potential of xenobiotics. Here we provide a concise, critical evaluation of a number of in vitro systems utilised to assess the hepatotoxic potential of xenobiotics.

Keywords; in vitro toxicology, xenobiotic safety, hepatotoxicity, 3D cell culture models, liver spheroids.

Introduction

The major constituent cell type of the liver is the hepatocyte, a parenchymal cell which makes up to 80% of the entire liver mass and performs the majority of the liver functions [1]. The remaining liver mass of ~20% is made up of a number of non-parenchymal cells (NPCs) such as; stellate cells (SCs), liver sinusoidal endothelial cells (LSECs), biliary epithelial cells (BECs), Kupffer cells (KCs) acting as in situ macrophages, and other immune cells, including lymphocytes and neutrophils [2]. Characterised by its anatomical position and intrinsic biochemistry, the liver is involved in the metabolism and clearance of numerous xenobiotics. While the metabolic transformation of xenobiotics is usually considered as a detoxification process, some compounds which are not toxic may subsequently be converted into toxic substrates in the liver. For example, a notable compound that has been intensively investigated in this regard is acetaminophen (APAP) [3]. The pathophysiology, disease course and management of acute liver failure caused by APAP toxicity still needs to be fully elucidated, however, APAP hepatotoxicity has been shown via the use of in vitro models, to follow a predictable timeline of hepatic failure [4].

The scientific basis of xenobiotic action and activity is complicated due to the variance in predictability of primary and secondary metabolites, as well as variability in individual susceptibility within the population [5]. This is true not only for humans but for other species utilised as experimental models. For example, our understanding of the mechanisms involved in the occurrence of adverse drug reactions (ADRs) and drug-induced liver injury (DILI) in humans is also an area that remains limited [6]. ADRs currently represent a major encumbrance to the development of new therapeutics with ~21% of drug attrition attributed to toxicity during the development process [7]. Despite a wealth of research utilising a variety of model systems in the field of xenobiotic safety, our comprehensive understanding of the mechanisms underpinning the impact of xenobiotics either on human health or on the environment is not fully established partly owing to the complexity of understanding exposure scenarios [8]. As such, the rigorous testing requirements and challenges in the global regulatory arena remain, and are apparent in all industries.

Current in vitro model systems developed to assess hepatotoxicity have a number of limitations including:

- Current mainstream 2D models fail to capture the complexities of multicellularity as well as the lack of the intricate 3D microenvironment, such as direct cell-cell and cell-tissue interactions.
- Primary human liver cell isolation is a complicated procedure that requires well-trained staff and established cooperation with the surgical department performing liver resections. However, cryopreserved human hepatocytes are available commercially.
- In vitro models provide limited viability for the study of long-term effects, such as responses to low-level chronic exposure.
- Limited availability of certain in vitro platforms to all researchers.

The use of animals in science is a global practice and the main purposes of animal experiments, both in vivo and in vitro, are to gain basic biological knowledge for fundamental medical research, to test the toxicity of xenobiotics and ultimately contribute towards the discovery and development of novel drugs, and the development of vaccines and medical devices [9]. However, due to species-species differences in mechanistic responses, it is often difficult to assess results in animal experiments and translate these findings to predict the in vivo response in humans [10]. In addition to the ethical considerations, there is an increased desire to implement the 3R's (Replacement, Reduction and Refinement) of animal experimentation in research [11-13], which is shifting the emphasis on producing more relevant and representative in vitro (human cell and cell line) models [14-17].

This review discusses the development of in vitro platforms and expands on the focus of 3D spheroid and co-culture models and their increasingly integral role in xenobiotic hepatic safety assessment.

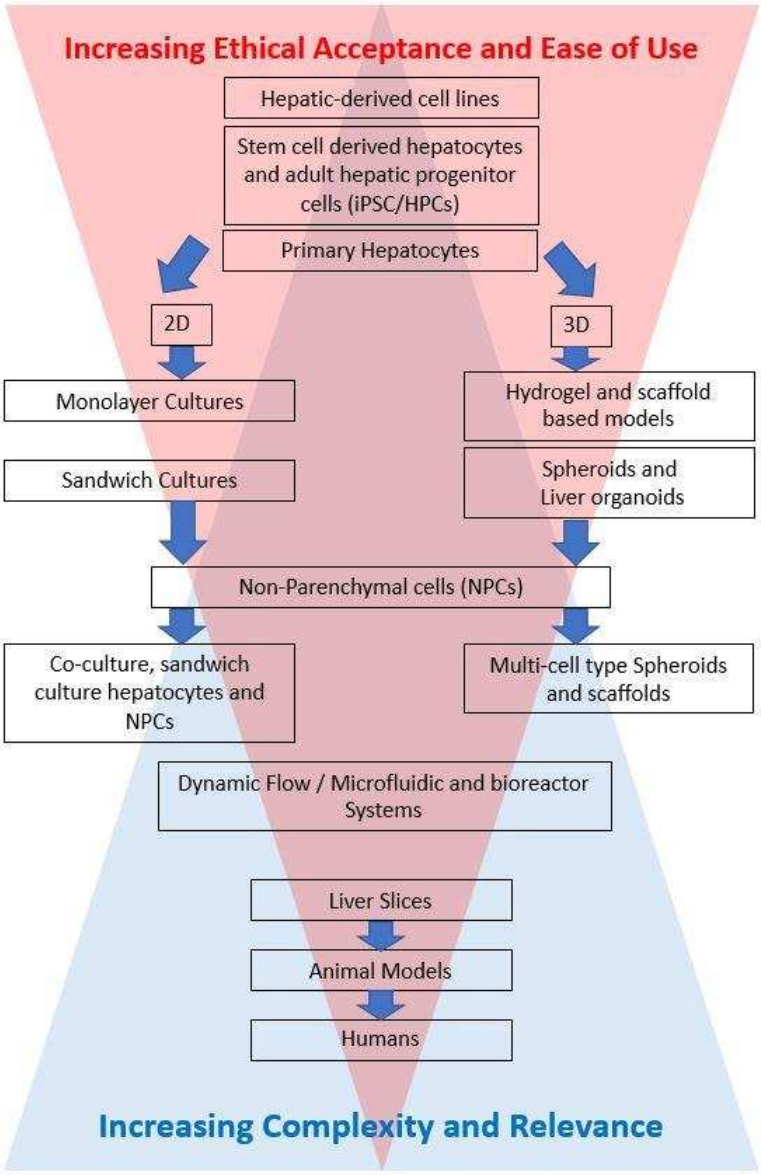
In Vitro Liver Models Utilised for Human Hepatotoxicity Prediction

The main aim of an in vitro liver model is to be able to capture relevant and useful end points, such as assessing the toxicity potential of novel xenobiotics, ADRs and modifications in transporter functionality. For example, simple vesicle models can be used to investigate the uptake and efflux properties of specific transporters [18], demonstrating that in vitro models do not necessarily have to recapitulate the natural in vivo microenvironment in order to be utilised successfully. Further to that, there are a number of in vitro liver models that differ depending on their culture conditions and conformations, cell types used and other additional culture

parameters. These platforms include isolated primary hepatocytes, hepatic-derived cell lines and liver slices. More conventional cellular model systems such as simple monolayer cultures are easier to manipulate in the laboratory and are much more widely accepted ethically than the use of animal models, but immortalised cell lines and 2D hepatocyte cultures maybe less representative of the in vivo liver.

For certain compounds and other endpoint analyses, a more complex model that recapitulates the in vivo microenvironment more closely is required. As such, approaches including 3D platforms, co-cultures and/or those that incorporate flow parameters such as bioreactor technologies may prove to be better suited to capture these end points. Continuing development in the area of 3D cell culture technology has meant several technologies have been established to culture cells in these more complex environments. These include matrix-free systems for some cells but also include the addition of hydrogels and scaffold technologies, and also the more recently established stem cell-derived hepatocyte-like cells and liver organoids [19-22]. Another degree of complexity in these systems has been introduced with the inclusion of fluid-flow to emulate sheer stress and nutrient exchange seen in vivo as a way to improve functionality and relevance [23, 24] (Fig 1).

Figure 1. Schematic of commonly used liver models. There are a multitude of liver models that differ in their translational relevance to humans. Systems vary from complex animal models that present significant ethical challenges as well as species variation issues, to primary human hepatocytes that, although deemed more relevant, suffer from inter-donor variability, rapid dedifferentiation in vitro along with sparse availability. On the other end of the spectrum are the more conventional cellular models that are easier to manipulate in the laboratory and are much more widely accepted ethically but these immortalised cell line models are less representative of the in vivo liver. Sandwich cultured hepatocytes retain more in vivo-like properties, including polarised excretory function, enhanced morphology and viability of hepatocytes compared to monolayer cultures, however these models still lack complex cellular interactions and the 3D microenvironment. Cells can



be grown in a 2D monolayer setting or the more complex 3D conformation with 3D set-ups considered to be more representative of the native liver. The complexity of both 2D and 3D models can be increased with the addition of non-parenchymal cells, again producing a more representative model via the adoption of a multicellular system, and the addition of flow with some systems incorporating highly complex microfluidic devices.

Primary Human Hepatocytes (PHH)

Primary human hepatocytes (PHH) in monolayer cultures are generally still considered the gold standard in vitro model for metabolism studies and toxicity investigations [25, 26]. When isolated effectively, PHH demonstrate a number of favourable characteristics such as phase I and II metabolic enzyme activity, expression of liver-specific transporters, glucose metabolism, ammonia detoxification, as well as urea secretion and albumin production [27]. However, there are a number of problematic issues with this system; (i) loss of liver-specific function/dedifferentiation (PHH lose their specific-liver function rapidly in vitro, including Cytochrome P450 (CYP) expression, and therefore are unsuitable for long-term and repeat-dose studies) [28, 29]; (ii) the isolation procedure of hepatocytes is itself difficult (there is scarce availability of tissue and considerable inter-donor variability that impacts on the reproducibility of end point measurements) [30]; (iii) classical 2D/monolayer cell culture does not recapitulate the complex 3D in vivo microenvironment. PHH in vitro are still widely used despite the difficulties associated with culturing, isolating, cost, inter-donor variation, acquisition etc. Much research has therefore been directed towards using cryopreserved hepatocytes, hepatic-derived cell lines and other alternatives.

Research has demonstrated that one way to improve and retain hepatocyte phenotype is to culture cells in a 3D conformation [31-33]. Mammalian cells in vivo grow in a 3D setting; therefore 2D cell cultures are ineffectual at recreating a microenvironment that is representative of this native in vivo configuration [33]. 2D cultures also fail to maintain phenotypic characteristics over the duration of the culture period [34]. Other strategies to improve PHH function and survival in vitro include the use of growth factors, cytokines and other supplementation within the growth media [35]. However, research has shown that one of the most successful

techniques in retaining hepatocyte function has been their co-culture with other cell types including NPCs [14-16].

Since many toxic responses in vivo are mediated by complex interplay amongst a multitude of cell types, the predictive capabilities of isolated hepatocytes are limited and therefore, there is a need to establish models that integrate NPCs within the culture platform [36]. Research has shown that intricate hepatocyte-NPC interactions affect the response after exposure to specific compounds. An example of this is vinyl chloride monomer (VCM) which is metabolically activated within hepatocytes, causing hepatocellular cancer [37]. However, a long-term effect of VCM is that it gives rise to haemangiosarcoma, a rare tumour that arises from the LSECs [38]. Furthermore, toxic responses are not only mediated by the association of the cells within these multiplexes, but also by the complex 3D interaction involving NPCs and the extracellular matrix (ECM) which is believed to be crucial in regulating and maintaining hepatic function in vivo [39].

The differences between cells grown on flat culture surfaces versus novel 3D formats such as extracted ECM attachment surfaces, has been documented since the early 1970's [40]. With decades of research being conducted since then, the compelling similarities of in vivo morphologies and behaviours of cells grown in 3D environments have been well demonstrated [41]. Consequently, it is widely agreed that culturing cells in 3D provides a much more in vivo-like platform and this format is extensively used in an array of disciplines within scientific research such as: cancer medicine/tumour-immune system interactions [42, 43] regenerative medicine and tissue fabrication technologies [44], and in the field of toxicology [28].

There are certainly a number of in vitro pharmacological models that have been developed to assess uptake, metabolism and detection of undesired effects, along with a vast number of publications that have addressed a number of desirable endpoints. However, only a small fraction of these models will inevitably become standardised industrial tools [45]. In part this is due to the specific internal requirements of industries and their capacity to incorporate these emerging technologies into their existing experimental framework. Industry screening comprises a battery of models that address single end points and in combination make up the tools for xenobiotic safety evaluations. Even though it is widely accepted that 3D cell culture provides a more in vivo-like model, with large sets of historical data at their disposal and potential difficulties in comprehensive

characterisation and automation of novel 3D models, the widespread adoption of these 3D platforms into the already well-established battery of screening tools remains a challenge [46].

Liver Slices

There are a number of desirable characteristics attributed to liver slices when compared with other in vitro liver models. Unlike primary cell isolations, liver slices do not require incubation with proteolytic enzymes and therefore cell-cell interactions and other cellular components remain largely undisrupted. The maintenance of this microarchitecture provides a more in vivo-like model. Additionally, with many in vitro systems, the conditions of isolation vary from species to species; counter to this, a reproducible and repetitive procedure is used to prepare and incubate liver slices from different species making this model particularly suitable to perform inter-species studies [47].

Liver slices have been utilised extensively in the field of hepatotoxicity and DILI investigations with the main advantage of this system being that the liver microarchitecture remains intact with all liver cell types being present, along with zone specific CYP450 activity [47]. Human liver tissue can either be obtained from excised tissue removed during surgical procedures such as a partial hepatectomy or from the non-transplanted donor tissue [48]. Such liver slices have been utilised as an in vitro method for the prediction of human specific toxicity by toxicogenomics investigations. However, human liver slices used from different donors, many of whom have underlying conditions, result in the introduction of inter-individual variability. This in turn means reproducibility of the investigations can be difficult to achieve [28]. Animal tissue on the other hand, is more readily available and can be controlled via perfusion methods using preservation solutions or simple buffers [28]. It has been shown that albumin production and phase II enzyme expression remain relatively stable for a period of up to 96 hours of culture, with the studies typically lasting between 30 minutes and 5 days using rat liver slices [49-51]. The main limitation with using freshly cut liver slices is their longevity, meaning that repeat-dose studies cannot be achieved with this model beyond 3 days. Inter-individual variability has also been seen in liver slices taken from different rats within a strain [52, 53].

It is well known that the long-term conservation of metabolic competence for in vitro models is difficult to achieve but it has been reported that metabolic capacity is better preserved in human liver slices when compared

to PHH [48, 54]. However, conflicting reports have demonstrated that xenobiotic metabolism in liver slices is impaired after 24 hours of culture [55]. Research has demonstrated good in vitro to in vivo correlations for the qualitative metabolism of xenobiotics in liver slices obtained from multiple species, however, the use of liver slices may be limited to identifying low- and high-clearance compounds [47].

Despite their short-term viability, liver slices have been used extensively over the years to investigate metabolism and toxicity of a number of xenobiotics. Olinga et al. showed that in human liver slices, all hepatocytes within the slice had an equal rate of metabolism of lidocaine [54]. Elferink et al. further evaluated the utility of human liver slices as an in vitro platform for the prediction of human-specific toxicity by toxicogenomics. They found that human liver slices retained a relatively stable expression of transporters and enzymes that are involved in drug metabolism during a 24 hour culture period [56].

Liver slices have also been used in conjunction with bioreactor platforms such as the multiwell plate platform engineered by CN BIO Innovations [23]. This combined approach has been utilised as a means of increasing the complexity and representativeness of the liver slice platform as fluid shear stress has been shown to improve liver-specific functional output [57]. Liver slices are placed into multiwell chambers of the plate and media flow controlled by a pneumatic underlay. The bioreactor is produced from polystyrene and has two connected chambers, one for the media reservoir, and the second is the reactor chamber. This reactor chamber can be used for culturing liver slices (and for the culture of isolated hepatocytes) with polycarbonate scaffolds [58]. This engineered platform enables the cells or liver slices to be cultured in an environment close to that of the in vivo liver. The system incorporates media flow, oxygen gradients and shear stresses. The experimental set up is able to recapitulate oxygen gradients similar to that seen within the liver sinusoid (145 µM to 50 µM at a flow rate of 0.25 mL/minute) [58]. Hepatocytes cultured using this system have improved longevity when compared with conventional monolayer cultures. However, liver slices utilised in this platform are still not able to provide a model for repeat-dose toxicity studies due to their short term culture longevity and viability.

Hepatic-derived Cell Lines

To overcome some of the previously mentioned limitations with PHH, immortalized hematoma-derived/hepatocellular carcinoma-derived cell lines have been utilized extensively. Cell lines previously used

in toxicological investigations include; HepG2, C3A, HepaRG and Huh7 [59-62]. Use of these cell lines inherently overcome the issue associated with inter-individual variability of primary hepatocytes [63], and are characterised by having a relatively stable phenotype, ease of manipulation in the laboratory along with unlimited life-span [59]. The main limitation with utilising these cell lines is that they generally possess reduced metabolic competence due to lack of expression of key metabolising enzymes [64].

Since its isolation in the 1970's, extensive work has been carried out in the fields of toxicological and pharmacological assessment using the HepG2 cell line [49]. These cells possess a number of attractive characteristics such as: (i) nuclear transcription factor (Nrf2) expression, which is essential for drug metabolism and toxicity response [65]; (ii) availability, unlimited growth and the absence of inter-donor variation ensuring reproducible results [66]; and (iii) it is an easy-to handle cell line with uncomplicated culture protocols [67]. Research has targeted the development of classical monolayer formats to more complex 3D models including spheroids, with HepG2 spheroids showing markedly different gene expression when compared to monolayer cultures [68]. Chang and Hughes revealed that significantly more genes related to ECM, cytoskeleton, and cell adhesion were expressed in monolayer cells, whilst genes involved in liver-specific functions of xenobiotic and lipid metabolism were upregulated in HepG2 spheroids [68]. In addition, more genes involved in cell cycle and regulation of growth and proliferation were upregulated in monolayers (Table 1). For example, CYP1A1 and ALB (albumin) expression was ~ 10 and 2-fold higher, respectively in 3D spheroid cultures when compared with monolayers, whilst COL1A1 (alpha 1 type-1 collagen) and GSPG2 (versican) expression was ~ 70 and 11-fold higher, respectively in monolayer cultures when compared with 3D spheroids.

Table 1 – Number of genes upregulated by at least 2-fold in HepG2 monolayers or spheroid as determined by microarray analysis [68].

	Number of Genes	Number of Genes
Category	Monolayer	Spheroids
Total	250	210
Extracellular Matrix	10	0
Cytoskeleton	10	5

Cell Adhesion	21	4
Cell Cycle	13	7
Growth/Proliferation	25	10
Xenobiotic Metabolism	0	6
Lipid Metabolism	4	11
Apoptosis/Cell Death	11	12
Signal Transduction	26	20
Transcription	20	21

It has been demonstrated that with the lack of appropriate levels of CYP expression when compared to PHH, HepG2 cells do not fully represent the phenotype of in vivo hepatocytes and therefore the detection of many hepatotoxic compounds utilising the HepG2 cells line is inaccurate, and for non-liver specific toxins this model may be ineffectual [69]. It is however still the case that 2D cultures of hepatic-derived cell lines are valuable in the early stages of safety assessments and liver cell lines can still provide a convenient and pragmatic tool for early screening and drug safety assessment [28, 70].

C3A cells are a sub-clone of the HepG2 cell line that demonstrate more advantageous characteristics compared with the parent cells. C3A cells are selected for their contact-inhibited growth characteristics, increased albumin production and alpha fetoprotein production alongside their ability to proliferate and thrive in glucose-deficient media [71]. These characteristics have made C3A cells a more representative model for hepatotoxicity studies with a number of researchers utilising this cell type in 3D culture systems [63].

The HepaRG cell line is another hepatocellular carcinoma-derived cell line that has been of interest over the last decade [72]. It is a human cell line that exhibits a number of attractive qualities and unique features when compared to the more commonly used HepG2 cells [61]. HepaRG cells have been shown to express a number of phase II enzymes and membrane transporters comparable to freshly isolated or cultured primary human hepatocytes [64, 67, 73]. HepaRG cells, when seeded at low density, acquire an elongated undifferentiated morphology. They then actively divide and after having reached confluency, form typical hepatocyte-like

colonies surrounded by biliary epithelial-like cells [61]. In addition, much of the literature has reported enhanced CYP450 expression along with improved liver-specific functionality [28, 61, 64, 72].

Guillouzo et al. demonstrated that the HepaRG cell line was more sensitive to metabolism-mediated toxicity when compared with HepG2 cells [61]. They found that HepaRG cells expressed various CYPs (1A2, 2B6, 2C9, 2E1, 3A4) and the nuclear receptors, constitutive androstane receptor (CAR) and pregnane X receptor (PXR) at levels comparable to those found in cultured PHH, and much increased when compared to the expression levels in HepG2 cells. HepaRG cells also expressed phase II enzymes, apical and canalicular ABC transporters and basolateral solute carrier transporters, albumin, haptoglobin as well as aldolase B which is a specific marker of adult hepatocytes. The findings of Guillouzo et al., demonstrate that HepaRG cell models have the potential to replace PHH models for xenobiotic metabolism and toxicity studies [61]. McGill et al. concluded that HepaRG cells are a useful model to study mechanisms of APAP hepatotoxicity in humans [74]. They found that HepaRG cells that were exposed to varying concentrations of APAP resulted in glutathione depletion, APAP-protein adduct formation, mitochondrial oxidative stress, peroxynitrite formation, mitochondrial dysfunction, and lactate dehydrogenase (LDH) release. This analysis indicated that these key mechanistic propagators of APAP-induced cell death were the same as in the in vitro rodent models and cultured primary mouse hepatocytes [74].

Gerets et al. carried out a comprehensive assessment of the HepaRG cell line, investigating mRNA levels and CYP activity in response to a number of inducers [64]. This study characterised PHH, HepG2 and the novel HepaRG cell lines in direct comparison with each other. All of the cells in this investigation were cultured in a monolayer multiwell format and were compared with regard to their metabolism and potential to detect hepatotoxicity. Gerets et al. concluded that HepG2 cells in this 2D environment responded weakly to the different inducers (β-napthoflavone, phenobarbital and rifampicin), when compared with PHH and the HepaRG cells at the gene expression and CYP activity levels, whilst HepaRG cells appeared to be most suitable for these induction studies. However, HepaRG cells were not as predictive for hepatotoxicity as PHH and were more comparable to HepG2 cells [64].

One of the main limitations with the HepaRG cell line as a model for hepatotoxicity investigations is the long culture procedure that is required. Cells are seeded at low densities and after a period of 14 days, cells are able to differentiate into hepatocyte-like and biliary epithelial-like cells. This pre-differentiation culture phase incurs cost and also time when compared to more commonly used cell lines such as HepG2/C3A cells. Specialist culture media and supplements are required for the entirety of the culture procedure and licensing is required to culture the cells meaning the cost of culturing the HepaRG cells can be as much as 100 times more expensive than the more commonly used cell lines. As a research tool this means that availability to all researchers is limited. However, terminally differentiated, commercially available cryopreserved HepaRG cells can be obtained [75].

Co-cultures

It has been demonstrated that culturing hepatocytes with other cell types increases their longevity and functionality [76]. The culturing of hepatocytes with NPCs has been investigated since the late 1970's and is still being intensively researched [77]. The predictive capabilities of isolated PHH can be limited [36]. Therefore, in order to represent the multicellularity of the liver, culturing primary hepatocytes with NPCs is an important facet for in vitro cellular models [45]. Much of the research to date has demonstrated that culturing primary hepatocytes, with NPCs not only increases liver-specific functionality, but also improves the longevity of the cultures [15-17]. Whilst there is a wealth of research in co-culture models, the emphasis has shifted to producing 3D co-cultures, where not only multiple cell types can interact but they can grow in a physiologically relevant manner [45]. Figure 2 highlights the various methods for producing co-culture models of hepatocytes that incorporate multiple NPCs within the model.

Research has shown that hepatocyte function and stability is improved regardless of whether the secondary cells used are primary or not. Bhandari et al. showed that when culturing primary rat hepatocytes (PRH) with murine 3T3 fibroblasts, there was a reciprocal relationship whereby the cellular interactions in the co-cultures ensured survival, and increased stability and function of both cell types [14]. Thomas et al. further expanded the work of Bhandari et al. by producing a co-culture model where activated rat SCs were cultured with isolated PRH in a spheroid model. This co-culture spheroid model displayed bile canaliculi-like structures, complex ECM within

the spheroid and, when compared with monoculture spheroids, superior cytochrome P450 functionality [14, 16].

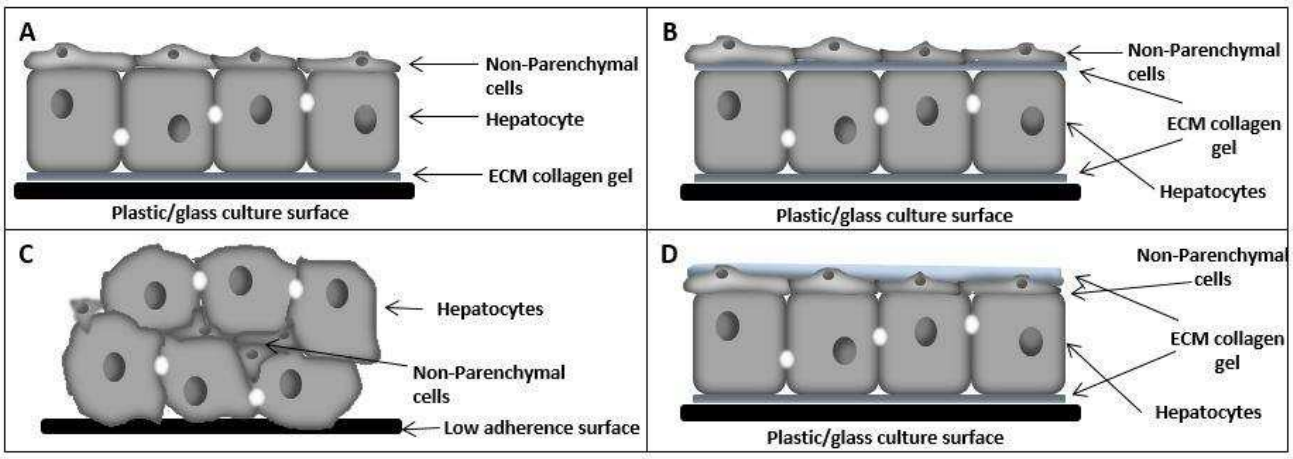
Peters et al. were able to demonstrate that PRH co-cultured with rat liver epithelial cells displayed higher levels of albumin secretion and the longevity of CYP enzyme activity was enhanced when compared to conventional PRH monolayer cultures. It was concluded that this co-culture model was the most applicable method for investigating cytokine-mediated induction of acute-phase proteins, due to there being a 3-fold increase in fibrinogen secretion in comparison with monolayer cultures [78].

Kang et al. produced a model system whereby PRH and LSECs were cultured on the opposite sides of a transwell membrane, allowing prolonged viability for a period of up to 39 days, as well as the stable presence of hepatocyte-specific differentiation markers [79]. Dedifferentiation of primary hepatocytes is a commonly discussed limitation of classical in vitro liver platforms. However, the model system developed by Kang et al. showed that PRH maintain this differentiated status for an extended period as verified by mRNA expression of albumin, transferrin, and hepatocyte nuclear factor 4 [79].

KCs have been the focus of much research and it is accepted that this NPC plays a role in the development of DILI. Jemnitz et al. produced a 2D co-culture model of PRH and KCs and concluded that the hepatocyte-KC co-culture model provided a good platform for the prediction of chemical hepatotoxic potential [80]. KCs have also been shown to detect hepatocyte stress and damage from model hepatotoxins in vitro, leading to the release of cytokines [81]. Hepatocytes cultured in isolation are not able to capture this release of inflammatory response, further strengthening the view that co-culture and, in particular, co-culture with KCs may increase the sensitivity of in vitro liver models to DILI and specific hepatotoxins [81].

BECs line the biliary tracts and are often targets of liver pathologies such as cholestatic liver disease and because of this, BECs have been the subject of much NPC research [28]. Auth et al. developed a model where hepatocytes were co-cultured with BECs and demonstrated substantially increased protein synthesis and urea production. Hepatocytes in isolation exhibited low levels of CYP450 activity; however, in co-culture with BECs, CYP450 activity remained stable for up to 3 weeks [82]. Auth et al. concluded that co-culture of human hepatocytes with BECs restored the synthetic and metabolic liver function in vitro [82].

Figure 2. Schematic of a selection of in vitro co-culture liver models. (**A**) shows hepatocyte cultures that have been grown on a collagen coated surface and then overlaid with NPCs. (**B**) demonstrates the much-utilised sandwich culture method whereby hepatocytes are cultured between two layers of collagen and then subsequently overlaid with NPCs. (**C**) demonstrates the structural formation of hepatocyte spheroids including NPCs. In this conformation there are multiple and direct cell-cell contacts between the parenchymal cells and the NPCs. There are a number of methods for culturing hepatocyte spheroids, however it is becoming more common to utilise low-attachment surfaces. (**D**) sandwich culture whereby NPCs are in direct contact with the hepatocytes and then subsequently sandwiched between two layers of collagen matrix.



3D Liver Microtissues

Spheroids

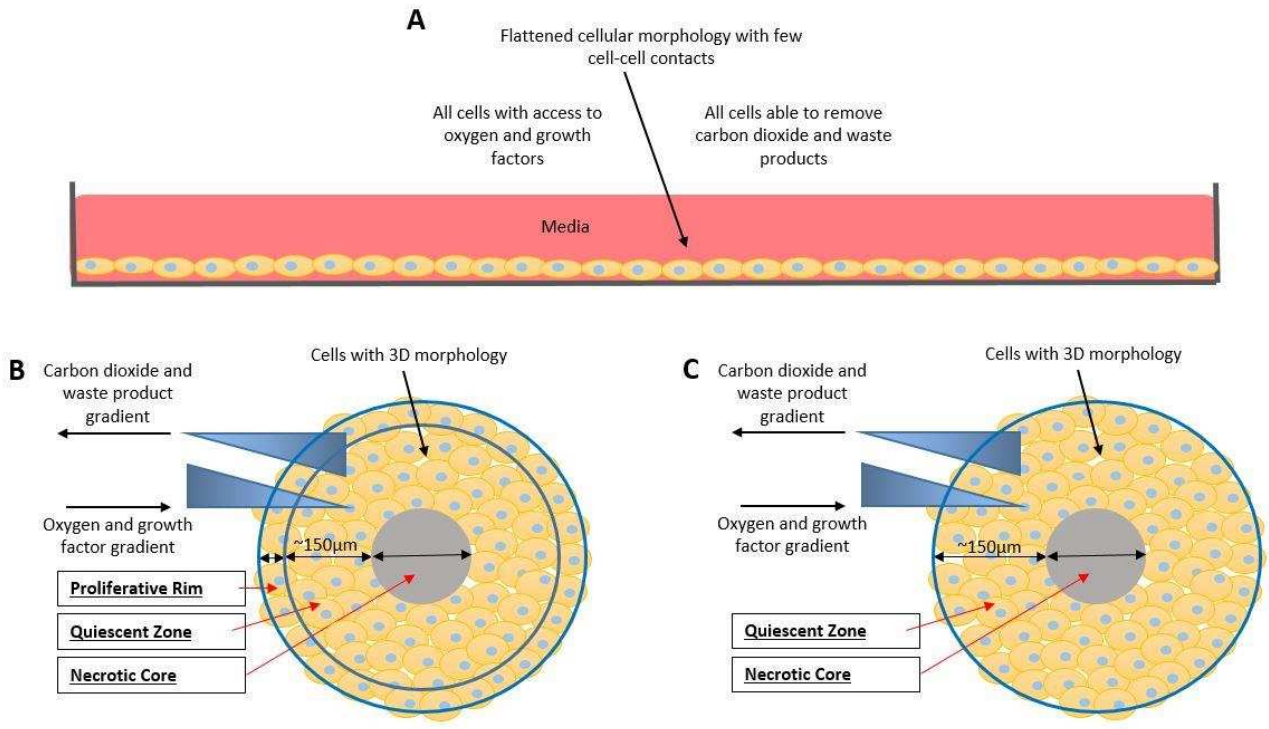
3D cultures of hepatocytes and hepatic-derived cell lines is a rapidly developing field, whereby researchers and bioengineers endeavour to capture the complexity of the microenvironment with a view to improving the liver-specific functionality, longevity and relevance of the cultured cells [49]. The recent progress in 3D in vitro liver spheroid models may improve the ability to predict hepatotoxicity of novel compounds, in part owing to the better recapitulation of the native physiology of the liver [83]. It has been shown that the re-establishment of cellular polarisation is critical in maintaining gene expression and hepatocyte-specific function [84]. With 2D cultures of hepatocytes unable to model the multiple apical and basolateral membranes of the in vivo hepatocytes, it is crucial that liver models are capable of restoring this highly-complex microenvironment. There are now a number of 3D liver approaches which help restore this highly-complex microenvironment including

hydrogel [19, 44, 85, 86] and scaffold based technologies [87], as well as the production of "hepatospheres" or liver spheroids [83, 88]. For the latter, techniques to produce spheroids have become progressively more refined and accessible and they are being increasingly utilised to assess areas such as xenobiotic penetration, metabolism and hepatotoxicity [89].

The basic underlying principle for the production of spheroids is that monodispersed cells (isolated cells from fresh tissue or cell lines) are capable of reforming a 3D configuration via self-reaggregation if adhesion to the substrate they are being cultured in is prevented [90]. According to the prevalent theory of self-assembly, in the absence of external influences, cells will self-organize into a spherical conformation as a result of specific local interactions amongst the cells themselves [85]. In conjunction with this, the differential adhesion hypothesis (DAH) states that tissues are treated as liquids composed of mobile cells whose varying degrees of surface adhesion causes them to reorganize spontaneously in order to minimize their free energy [91]. Thus, cells will migrate to be near other cells of comparable adhesive capacity in order to maximize the strength of the bonds between them. This in turn produces a more thermodynamically stable structure [85].

One of the main advantages of culturing cells in a spheroid is the increased cell-cell interactions and cell-ECM interactions when compared to 2D monolayer culture [33]. The majority of cells are in close contact with each other and are able to communicate and produce their own ECM. Cells within a spheroid have virtually 100% of their surfaces in contact with neighbouring cells unlike a 2D monolayer. On this basis, cells in a spheroid conformation mimic much more closely the cells natural in vivo-like state. Figure 3 illustrates the differences between monolayer cells and cells grown in a 3D spheroid model.

Figure 3. Comparison of monolayer cells and cell cultured in a spheroid. (**A**) Monolayer cells become flattened, have very few cell-cell contacts, unlimited access to the media as well as ease of waste product expulsion into the media. (**B**) Proliferative cell lines grown within the spheroid have numerous cell-cell contacts, do not become flattened and retain an in vivo-like morphology. Cells on the periphery of the spheroid proliferate and have greater access to media and can remove waste products easier than those cells situated in the centre of the spheroid. These cells have less access to the nutrients within the media and less access to oxygen due to an oxygen diffusion gradient. Waste products may also accumulate in this central area, and potentially this may cause necrotic regions. Over the duration of the culture period, the size of the spheroid can increase dramatically. (**C**) Non-proliferative cells such as PHH/PRH grown within a spheroid again have numerous cell-cell contacts and retain an in vivo-like morphology. Similar nutrient and solute gradients form within the spheroids. However, as there is no proliferative rim, the overall size of the spheroid remains relatively constant over-time, reducing the formation of necrotic areas due to hypoxia.



A number of hepatic-derived cell lines have been utilised extensively in research including; C3A, HepG2, HepaRG and Huh7 [33, 63, 92, 93]. These cell lines are capable of forming 3D liver spheroids and the resultant models are most commonly being used in the early stages of assessing xenobiotic safety [28]. HepG2 cells cultured as a spheroid model show the morphological characteristics of hepatocyte-like cells as well as the

formation of bile canalicular-like structures. HepG2 spheroids also exhibit a highly compact structure with tight cell-cell interactions [94]. Studies by Li et al. and Ramaiahgari et al. have assessed a number of key functional outputs including; (i) cellular interactions as shown by E-cadherin, electron microscopy, β1-integrin and β-catenin that are indicative of polarity; (ii) epithelial characteristics (CK7/8); and (iii) proliferative capabilities (Ki-67) [33, 86]. Wrzesinski et al. along with others have also investigated end points such as albumin and urea production, and metabolic competence via CYP activity (CYP1A1, CYP1A2, CYP3A4, & CYP7A1) [60, 63, 86]. These studies have conclusively elucidated that spheroids, and perhaps in general, 3D cultures of the HepG2 cell line show enhanced liver-like functionality when compared to the more traditional 2D cultures. However, it is widely accepted that with their low metabolic competence [28], HepG2 spheroids may be limited in their use as a model for toxicological investigations and may underestimate the toxicity potential of compounds [67].

The formation of bile canalicular-like structures within HepG2 spheroids has been increasingly investigated in recent years [86, 95]. Much of the work has shown the formation of these structures but further investigation into whether or not they are functional in producing bile salts and their subsequent transport is required [28]. It has been shown in work previously undertaken with the HepG2 and C3A cell lines [33, 94, 95], that there are several quantifiably useful end points such as albumin, urea secretion and ATP content that can be used to confirm in vitro 3D liver model phenotype. Recently Gaskell et al. demonstrated secondary structure functionality in C3A spheroids via the transport of CMFDA by the canalicular transporter MRP2 [63]. This line of investigation has yet to be fully characterised in primary hepatocyte spheroids and would help strengthen the case that 3D spheroid cultures may be better placed to assess hepatobiliary transporter-based compounds. Nevertheless, HepG2 or C3A cells have poor metabolic competencies when compared with PHH in 2D and this is one of the main limitations with these commonly used cell lines [66].

There are a limited number of publications using HepaRG cells in a 3D liver microtissue model [28]. However, with the accumulation of studies detailing more comparable functionality to that of PHH [61, 64, 72, 96] and improved functionality when compared with the more commonly used cell lines such as HepG2 and C3A cells in 2D culture, it is anticipated that a 3D HepaRG model may bridge the gap between conventional monolayer cultures and in vivo physiology.

Gunness et al. reported the production of 3D organotypic cultures using HepaRG cells via the high-throughput hanging drop method [97]. They were able to maintain the cultures for 3 weeks and showed conservation of high liver-specific function for the duration of culture via phase I enzyme (CYP3A4, CYP2E1) and transporter activity (MRP2), expression of liver-specific proteins (albumin, urea) and response to a number of drugs (APAP, troglitazone and rosiglitazone). In order to assess whether the 3D HepaRG cultures were a more appropriate model to study drug toxicity, 2D HepaRG cultures were set up in parallel with the 3D cultures over 3 weeks. 3D HepaRG cultures showed higher sensitivity for APAP and troglitazone toxicity, and the 3D cultures maintained high levels of liver-specific functionality, including phase I enzyme and transporter activity, and also production of liver-specific proteins including albumin and urea. These investigators therefore suggested that these 3D organotypic HepaRG cultures provide a suitable in vitro tool for assessment of drug-induced hepatotoxicity [97].

HepaRG cells when cultured differentiate into hepatocyte-like cells and biliary-like cells and it has been demonstrated that bile canalicular-like structures form throughout 3D models [98]. The fact that the HepaRG cell line differentiates into two distinguishable cell types means that the resultant cultures are intrinsically co-cultures in nature. Compared with the more commonly used hepatic-derived cell lines, HepaRG cells possess many more advantages with regards to specific functional output, formation of secondary structures, upregulated metabolic capacity and this makes them much more comparable to PHH [64, 73, 99].

The main advantage of 3D models, and in particular the spheroid model, is that very few cells are required to produce a functional spheroid [28]. For example, we have been able to demonstrate that a functional PRH spheroid can be produced from as little as 2000 cells/well on a 96-well, liquid-overlay plate.

PRH spheroid models are well characterised and have been used since the 1980's [32]. These spheroids have been shown to have a smooth outer surface with numerous pore-like openings leading to secondary structures shown to be similar to bile canaliculi [31]. As well as the formation of these bile canalicular-like structures, cells within the spheroid have shown polarisation as assessed by staining of apical HA4 and basolateral HA321 membrane bounds proteins [31], and dipeptidyl peptidase 4 (DPP IV) by immunohistochemical staining as an apical membrane marker [100]. Much of the initial work was carried out on characterising the cellular

morphology and polarity in conjunction with the formation of bile canaliculi [31, 101]. However, more recent work has involved examining intra-cellular interactions and communication [95] along with oxygen concentration and gradients throughout the spheroids [102, 103].

Due to metabolism and uptake of numerous solutes by hepatocytes, the composition of blood changes as it flows along the sinusoids from the periportal zone to perivenous zone. Concentration gradients of substrates, products, and hormones are formed and subsequently drive liver zonation [104]. This sinusoidal zonation is extremely important to discuss when looking at hepatotoxic potential of xenobiotics. If we look at APAP toxicity for example, glucuronidation, the dominant pathway of conjugation at high APAP concentrations (>5 mM), has been shown to be more rapid in perivenous cells than in periportal cells. Prolonged exposure to high concentrations of APAP damages perivenous cells expressing higher levels of CYP2E1 than periportal cells [105]. This demonstrates that perivenous hepatocytes exhibit increased APAP vulnerability and extensive glutathione depletion when compared with periportal cells, and emphasises the importance of being able to recapitulate liver-specific zonation and solute gradients in vitro

As one of the circulating signals, oxygen plays an important role in modulating zonation along the liver sinusoid. Its partial pressure is about 60 to 65 mm Hg (84-91 μmol/L) in the periportal blood and falls to about 30 to 35 mm Hg (42-49 μmol/L) in the perivenous blood [106, 107]. Research utilising liver spheroids has become progressively more interested in the physiological oxygen tension along the sinusoid, with increasing focus on trying to experimentally recapitulate oxygen profiles within 3D liver models.

Oxygen demand and concentration throughout the in vitro spheroid models remains an interesting point of research because it is desirable that all the cells are viable and free from necrosis. Much of the literature describes that spheroids with a diameter >150 μm form a necrotic core due to hypoxia and lack of nutrients (see figure 3) [21]. For an in vitro model used in cancer medicine for example, necrosis is a desirable characteristic because; larger tumour spheroids are characterised by an external proliferating rim, an internal quiescent zone, and a necrotic core resembling the cellular heterogeneity of solid in vivo tumours [108]. However, for a model that attempts to recapitulate the in vivo-like liver microenvironment, this is an undesirable characteristic. Being able to determine the oxygen diffusion and consumption within spheroids, and using this information to mimic the oxygen profile seen within the liver sinusoid, would provide a more accomplished model than classic monolayer

culture, and a more comparable one to that of the liver in vivo. In the field of 3D tumour cell culture, much research has been dedicated to the quantitative description of tumour vascular networks whilst the consideration of oxygen consumption is largely neglected. Whilst oxidative respiration in standard 2D cell culture has been widely studied, this aspect of characterisation has been lacking with 3D in vitro liver models [109].

Sakai et al. [95] demonstrated that PRH cultured as spherical multicellular aggregates provided a more useful model than the traditional monolayer culture. Using quantitative polymerase chain reaction it was shown that PRH rapidly lost expression of a number of liver-specific genes when cultured in monolayer from day 1 up to day 5. In contrast, PRH spheroid cultures conferred higher levels of expression of these liver-specific genes when compared to the monolayer cultures for a period of up to 10 days. These results suggested that PRH cultured as spheroids acquire intercellular organisation that may permit maintenance of metabolic competence [88, 95].

As outlined previously, PHH are still considered by many to be the gold standard as an in vitro tool for DILI and toxicity investigations [26]. Despite the number of limitations with primary cells, spheroid systems can be produced from a low cell number, so a large number of spheroids can be produced from a small fraction of a single isolation suspension [28]. It is also important to reiterate that hepatocytes isolated from different donors display marked variations in gene expression levels, and thus may respond differently in hepatotoxicity investigations. 3D spheroid culture, however, enables the production of spheroids utilising cells from a single donor or pooled hepatocytes. The advantage of utilising pooled hepatocytes is that the resultant spheroids may better predict average population drug responses and conversely, spheroids produced from single donors allows for more direct in vivo variability comparisons [110].

Messner et al. characterised a multi-cell type spheroid system incorporating PHH and liver-derived NPCs [111]. This system was shown to be functional for a period of up to 5 weeks, demonstrating that longevity of the cultures is vastly improved compared with the conventional monolayer or sandwich cultures of PHH. Secondary structure formation was confirmed in these spheroids via immunohistochemical staining for the apical transporters MDR1 and BSEP, demonstrating functional polarisation of hepatocytes within the spheroids. In addition, these co-culture spheroids displayed improved longevity, stable albumin production over the duration

of culture period and with KCs showed responsiveness to inflammatory stimuli. In these investigations, Messner et al. were able to incorporate both the 3D microenvironment and multiple cell types within a single model, producing a more representative in vitro tool for the assessment of DILI [111]. These 3D, multicellular models show promise for drug discovery investigations as the much improved longevity and viability of the cells will enable the assessment of long-term effects of compounds over repeat-dose scenarios; an area initially highlighted as a limitation of many of the current commercially available in vitro liver models.

A more recent study carried out by Bell et al. [112] produced PHH spheroids using ultra-low attachment plates. Spheroids in this instance were cultured for a period of up to 5 weeks in serum-free culture medium. Spheroid size decreased over time alongside increasing expression of E-cadherin, suggesting that the cells within the spheroid model are becoming more tightly incorporated via spheroid compaction [113]. MRP2 staining revealed the formation of bile canaliculi-like structures throughout the spheroid body over the 35 day culture period, indicative of stable functional polarisation of hepatocytes [28]. A direct comparison can subsequently be made between the multi-cell spheroids produced by Messner et al. and the monoculture spheroids by Bell et al. Interestingly, both researchers demonstrate improved longevity of up to 5 weeks in culture compared to conventional models via stable albumin production over the duration of the culture period. This demonstrates that co-cultures of NPCs and PHH within this spheroid model may not be essential for improving the longevity. However, the co-culture spheroid models with the inclusion of KC place themselves well to investigate immune-mediated toxicities whereas a monoculture hepatocyte spheroid model may be inadequate for capturing this specific end point analysis. Both models demonstrate preserved hepatic phenotypes and long-term functionality for the investigations into chronic toxicity assays and repeat-dose studies.

There are a number of techniques that have been implemented for the production of PHH and PRH spheroids including, spinner vessels and orbitally shaken flasks [24]. However, limitations of these systems include the inability to control spheroid size, difficulties with manipulation in the lab as well as these systems requiring relatively high cell numbers. Scaffold-free systems that allow the formation of size controllable primary cell spheroids has currently only been performed using a hanging-drop system as described by a Kelm and Fusseneger [114] and the use of ultra-low attachment (ULA) plates described by Bell et al. [112].

Sufficient supply of oxygen to the cells is crucial for a functional 3D in vitro model trying to recapitulate the liver microenvironment. Primary hepatocytes have a relatively high metabolic activity compared with their hepatic cell-line counterparts, and thus, primary hepatocytes have a high oxygen turnover which can be up to ten times greater than other types of non-proliferative cells [115]. Increased levels of albumin and urea production, along with other liver-specific functions have been seen to correlate with higher oxygen uptake rates (OUR) of hepatocytes [115]. The idea that increased functional output increases the oxygen demand on the cells suggests that even the basic set up of in vitro liver models needs to be accurately determined to allow sufficient oxygen to diffuse through the media and into the cells. It also outdates the idea that spheroid diameter is the most crucial factor in determining the formation of central necrosis. It is much more likely that the combination of specific cellular OUR, along with their proliferative characteristics and the experimental set up are equally as important.

Scaffold and Hydrogel Technology

In recent years it has been shown that the cellular microenvironment contributes to the spatially and temporally intricate signalling domain that directs cell phenotype, and thus the idea that cellular scaffolds serve simply as a vehicle with which to assess the expression of specific genes and subsequent functionality has become outdated [44]. Tibbitt et al. concluded that a cell can no longer be thought of as a single entity defined by its genomic material, but must also be regarded in the context of the ECM, soluble growth factors, hormones, and other molecules that regulate organ formation and function [44]. It is better understood that the extracellular microenvironment coordinates intracellular signalling cascades that influences phenotype by altering gene and subsequent protein expression [116, 117].

Spheroids can be produced by embedding hepatocytes into non-adhesive hydrogels [118]. Spheroids form via the process of cellular self-assembly [85], and the cells that self-assemble into spheroids have been shown to achieve increased gene expression and retention of the native cell phenotype when compared to 2D cultures [114]. Even though spheroids have been shown to form without scaffolds and hydrogels, not all cell lines are able to form spheroids via self-aggregation [119] and thus, the 3D microtissue system required is heavily dependent on the cell type being utilised.

Lee et al. [93] were able to produce functional encapsulated spheroids using Huh7 cells. These encapsulated spheroids were functional for a period of up to 3 weeks and the microenvironment in which they were cultured could be adapted depending on the stiffness of the hydrogels. In this case, an in vitro model representative of normal liver could be generated by utilising low stiffness hydrogels, and cirrhotic liver by increasing the stiffness of the gels. Lee et al. also demonstrated that spheroids cultured within the low stiffness hydrogels had the highest rates of proliferation, albumin secretion and CYP450 expression over the culture period.

Another way in which hepatocytes can be cultured to mimic the 3D microenvironment is the use of scaffolds produced from either natural or synthetic material [28]. Natural scaffold systems are thought to allow for biocompatibility with the cells, with the scaffold itself mimicking the native ECM and conferring multiple cell-ECM interactions. However, these naturally liver-derived scaffolds are inherently variable leading to difficulties with experimental reproducibility. Decellularised human livers are considered the ideal ECM alternative because both the 3D microarchitecture and biological features of the native liver are preserved. However, human donor livers are in short supply as decellularised scaffolds, and the intrinsic inter-donor differences means that reproducibility of experiments can be difficult [120]. This limitation can be overcome with the use of synthetic scaffold systems and, similarly to hydrogels, they can be purposely engineered to allow for specific 3D conformations and cell-specific scaffolds [121, 122]. Hepatocytes have been shown to have an affinity for galactose residues such that scaffold systems presenting galactose on their surfaces allows for improved hepatocyte adhesion, leading to an improved functional system [123, 124].

An example of synthetic scaffolds that has been increasingly used within the field of 3D cell culture is the Alvetex® (Reinnervate), which has been produced from cross-linked polystyrene. This system has been shown to be biocompatible and the manufacturing of the scaffold has shown little batch-to-batch variation allowing for more reproducible experimental data [20]. The scaffold is engineered into thin (200 µm) membranes that are able to fit into conventional multiwell plate plasticware. Knight et al. [20] reported that cells seeded on to the scaffold system are able to form close cell-cell interactions and cellular differentiation, allowing the formation of thin tissue-like cultures. Furthermore, the HepG2 cell line has been shown to have improved liver-specific functionality when cultured with the Alvetex® scaffold including higher viability over the culture period and the formation of bile canaliculi within the tissue-like cultures [125]. Rat hepatocytes have also been cultured

using the Alvetex® scaffold system and have been shown to retain their native cuboidal morphology along with much improved viability when compared with conventional monolayer cultures. These 3D cultures display gene expression associated with phase I, II and III drug metabolism under basal conditions along with increased sensitivity to APAP toxicity [126].

One on the main limitations with the hydrogel technologies is that there is poor mass transfer of nutrients, oxygen and xenobiotics and cell retrieval is more difficult [28]. Cell retrieval difficulties have been previously described by Godoy et al., developing the idea that downstream analysis becomes much more challenging with reduced cell numbers [28]. This potentially remains a major caveat of hydrogel systems, as altering the hydrogel stiffness may impact the ability to dissociate cells from the gels themselves. However, with the development of more simplistic methods, the utilisation of non-adhesive hydrogels reduces cell-substrate interactions, thereby increasing the important cell-cell interactions which are vital for retaining functionality as well as the driving process of self-assemble [85]. One of the main advantages of using non-adhesive hydrogels for the production of 3D microtissues is that hundreds of spheroids can be produced with a single pipetting step. This in turn means that the hydrogel method may lend itself to long-term, repeat-dose toxicological investigations [85].

Liver Organoids

Organoids are 3D culture models in which adult stem cells and their progeny grow and are able to recapitulate the natural physiology of the cells in vivo. Organoids have been successfully derived from a number of organ systems for both animals and humans [127]. “Organoid” is a term that, in the past, was used interchangeable for in vitro spheroid models. However, the term organoid refers to “stem cell-derived” self-organising organoids [128]. Organoids can be produced from two types of stem cells which include pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs), and organ-specific adult stem cells (ASCs) such as hepatic progenitor cells (HPCs), which are tissue-specific resident stem cells. Organoid systems have the potential to aid in the development of personalised medicine/treatment strategies and have previously been utilised to investigate a number of disease models [128, 129]. As with hepatic-derived cell lines, 2D culture of PSCs is relatively amenable within the laboratory. However, long-term culture of PSCs with maintenance of stem cell characteristics is a limiting factor [130]. Additionally, 2D cultures fail to produce in vivo cell polarisation and intricate cellular interactions, and cannot recapitulate the complex 3D

microenvironment as seen in vivo [41]. As with hepatic-derived cell lines and primary isolated cells, 3D culture of PSCs has become a rapidly developing field in order to overcome the limitations of monolayer cultures. Huch et al. developed a 3D culture system of HPCs that demonstrated long-term genetically stable expansion (>1 year). The organoid models were derived from both Lgr5+ cells (mouse) and EpCAM+ (normal human liver) ductal cells [22, 131]. It was shown that the original phenotypic epithelial architecture of the cells were maintained and that organoids were differentiated in vitro toward hepatocyte-like and cholangiocyte-like cells [128]. Additionally, upon transplantation of the Lgr5+ organoid into impaired mouse livers, this propagated the formation of functionally mature hepatocytes [131].

Takebe et al. demonstrated the formation of vascularised, functional human liver organoids from human iPSCs via transplantation of liver buds created in vitro (iPSCs-LB) [132]. The researchers were able to show the formation of functional vasculatures that stimulated the maturation of iPSCs-LB into tissue that highly resembled the adult liver. Metabolically competent iPSC-derived tissue demonstrated liver-specific functionality including increased albumin production and human-specific drug metabolism [132]. Commercially available iPSCs have also been used as an in vitro tool for the assessment of hepatotoxic potential with Sirenko et al. demonstrating this with a large number of identified toxic compounds [133]. The researchers used iCell® hepatocytes (Cellular Dynamics International [CDI], Madison WI) which are human iPSC-derived hepatocytes cultured in a 2D multi-well plate platform. The researchers demonstrated that high-content automated screening assays using iPSC-derived hepatocytes were feasible, and additionally this model provided useful information about the potential mechanisms of toxicity. These results suggest that this in vitro liver model may be well placed to assess drug and xenobiotic safety. Although this model does show promise it is clear that monolayer culture of iPSCs is not representative of the liver in vivo and toxic potential of compounds was assessed over a 72-hour period only. Generation of 3D iPSCs may allow for repeat-dose of xenobiotics and it would be interesting to see the potential of this model in assessing chemicals with unknown toxicity and other novel xenobiotics.

3D organoid systems provide an in vitro platform that is highly representative of the in vivo physiology of liver cells, and have developed our understanding of disease development and progression. Liver organoids have also demonstrated accurate recapitulation of disease pathways in vivo. Although much of the research to date concerning liver organoid systems are focused on the developing field of personalised medicine, these 3D in

vitro tools position themselves equally to be utilised within the field of xenobiotic safety and drug toxicity investigations. PSCs have been shown to be a promising model to assess hepatotoxicity in acute treatments, and also in response to chronic drug exposure and repeated-dose investigations, potentially overcoming some of the shortfalls of more commonly used hepatic-derived cell lines [25, 134].

In vitro to in vivo extrapolation (IVIVE)

A number of in vitro cell models have been described within this review. However, it is becoming more apparent that quantitative analyses of the various in vitro liver models is necessary to aid in demonstrating their potential for hepatotoxicity investigations compared to more qualitative measures such as physiological and functional improvements of the cell models. Many reviews have detailed improved physiological and metabolic status of 3D and co-culture in vitro liver models. However, few have combined this with IVIVE as a quantitative classification tool for the different models. IVIVE refers to the transposition of experimental results or data in vitro to predict phenomena in vivo. Extrapolation of intrinsic clearance (CL_{int}) measurements using hepatocytes to give predicted in vivo clearance ($CL_{in\ vivo}$) involves a well-established 'two-step' mechanistic approach. Firstly, the physiological scaling from cell to whole liver and secondly the subsequent modelling of extraction from blood by the liver [28]. There have been a number of investigations that have compared in vitro liver model CL_{int} as a means to develop the predictive capabilities with regards to xenobiotic safety assessments.

Suspensions of PRH have been shown to provide a more accurate estimation of CL_{int} rate when compared to conventional PRH monolayer cultures [135]. Griffin et al. investigated the incubation of seven compounds in both suspensions and monolayer cultures, and the CL_{int} was obtained via metabolite formation or substrate depletion analysis [135]. However, the main limitation with this in vitro system was that cells rapidly dedifferentiated ex vivo in suspension, whereas often the processes of hepatotoxicity manifest themselves over several hours. Therefore, hepatocytes in suspension are unable to maintain viability for the time necessary to capture the development of toxicities for some xenobiotics. As such the assessment of long-term or repeat-dose investigations with this in vitro model will in turn be ineffectual.

Research utilising rat microsomes, hepatocytes and liver slices have indicated adequate accuracy with the aforementioned two-step mechanistic approach [136]. However more recent investigations have demonstrated that rates of drug metabolism and CL_{int} were found to be lower in rat liver slices than in isolated rat hepatocytes

[137]. Other research has indicated that this two-step IVIVE mechanistic approach leads to under-prediction of human in vivo clearance when utilising human hepatocytes and microsomes [138].

Although much of the work to date has particularly focused on suspensions and 2D cultures of cryopreserved and primary isolated hepatocytes, more recent publications have analysed the prospective competence of the more novel HepaRG hepatic-derived cells [139]. Zanelli et al. compared intrinsic clearance of 26 drug compounds in both cryopreserved hepatocytes and the more novel HepaRG cell line [139]. The CL_{int} of the compounds was determined via substrate depletion and the results showed that there was a direct correlation of CL_{int} for both cryopreserved hepatocytes and HepaRG cells (scaled to whole body) for the range of compounds used.

Co-culture bioprinted systems have also been analysed to investigate their potential for hepatotoxicity studies. An example of this is the Hepregen system which is a collagen micropatterned substrate system where hepatocytes are seeded onto a feeder layer of a secondary cell type. When compared to human microsomes, and PHH suspensions, the Hepregen system allowed for longer incubations with 27 known liver-metabolised compounds and was able to generate a greater proportion of the major human metabolites normally found in vivo [140].

Bioreactors and 3D cultures are rapidly becoming incorporated within industry and research as improved predictive platforms for xenobiotic safety assessments. Sivaraman et al. demonstrated this by using a 3D bioreactor system to analyse the functionality of PRH spheroids [141]. This system was developed as it allowed the formation of heterotypic cell interactions, shear stresses via flow, and an in vivo liver-like microarchitecture. Toxicity testing utilising this bioreactor system included studies showing that clearance rates of compounds with known liver metabolism were comparable to those obtained in vivo [36, 49].

Summary

There are a number of advantages that 3D in vitro liver models possess that place them well in the continually developing field of drug discovery and toxicological investigations. These models have been shown to demonstrate improved physiology, longevity and viability over extended culture periods and increasing relevance when compared to classical monolayer cultures. Also, the ability to include multiple cell types within

a single model has been shown to result in improved liver-specific functionality and longevity [45]. The continued development of these in vitro liver models significantly improves their biological relevance and thus increases the chances that xenobiotic-induced toxicities, that may require the complex interplay of a multicellular model, will be identified.

Despite the recent advancements, one of the main limitations of in vitro liver models is the inability to prolong the culture period for repeat-dose and long-term toxicological investigations without extensive necrosis within the in vitro cellular environment. However, PSC models have shown promise in hepatotoxicity investigations including chronic drug exposure and repeated dose scenarios [134]. Most commonly used hepatic cell lines proliferate and as a result, microtissue models such a spheroids will increase in size. With an increase in functionality there seems to be an increase in oxygen consumption by the cells, and this increase in oxygen consumption coupled with excessive growth will inevitably result in the formation of necrotic regions within the model, greatly impacting on the phenotype of the model and the ability of oxygen and key nutrients to diffuse through the 3D culture.

Primary cells have a number of advantages over the hepatic-derived cell lines including the inability to proliferate ex vivo and thus 3D cell models utilising these cells will remain relatively stable in size over time. In fact, it has been shown that primary hepatocytes cultured as spheroids actually contract over the culture period. The up-regulation of key ECM elements and cytoskeletal components causes an initial contraction of the spheroid body. Therefore, over extended culture periods the ability of oxygen and key nutrients to diffuse through the spheroids may not interrupted.

Table 2 shows the multiple cell types and model systems that are used to investigate liver toxicity in vitro, and defines some of the advantages and limitations of these systems. It is clear from the literature that 2D and classic monolayer cultures of hepatic cell lines and primary hepatocytes are rapidly becoming superseded by the continually developing field of 3D, co-culture, bioreactor, and combined approaches. There is a wealth of research to demonstrate that both 3D and co-culture approaches improve liver-specific functionality, sensitivity to xenobiotics, culture longevity, recapitulation of the microenvironment and relevance to that of the in vivo liver, with 3D cell culture becoming the model of choice for many researchers and industrial institutions.

3D cell culture systems that can incorporate flow dynamics for a primary cell type appear to hold the most promise for toxicological studies, due to that fact that many of the liver-specific functions remain stable over time along with the preservation of phase I, II and III genes associated with metabolism. In addition, models that incorporate multiple cell types, not limited to NPCs, have the ability to further enhance the functional and predictive capabilities of the aforementioned 3D systems, through representative cellular morphologies and phenotypes, and intricate cellular-ECM interactions.

Outlook

As the need for more predictive in vitro liver models increases, emerging 3D and bioreactor technologies have started to become increasingly utilised for xenobiotic hepatotoxicity assessments [142]. The incorporation of shear stress and flow has been demonstrated to improve functionality as described previously, and increases the complexity of the model system [23, 57]. These more complex 3D and bioreactor technologies have the potential to capture some more of the intricate physiological aspects of the liver in vivo such as the solute and oxygen gradients of the liver sinusoid, and thus, may be able to better recapitulate the microenvironment of the native liver [7]. It has become clear that collaborative investigations between tissue engineers, toxicologist, applied mathematicians etc. whereby a more detailed assessment of the in vitro liver model set up is analysed, has focused the development of 3D and bioreactor models. While many of these systems show encouraging results, only a small number have provided extensive data that demonstrates the added value for hepatotoxicity investigations for human liver.

Industry, along with academia, is continually developing a multitude of 3D in vitro liver models for toxicological investigations. Prior to these model systems being incorporated and utilised for early compound screening investigations, a pragmatic schedule of detailed evaluation and subsequent validation to show relevant pharmacological and toxicological end points is required. To date, liver organoids and spheroid models show good promise for assessment of hepatotoxicity, however they only partly recapitulate the native liver in vivo and so more complex flow systems, micropatterned plates and bioreactor technologies have started to emerge as other potential candidates. Furthermore, solute gradients, including oxygen, have been identified as key physiological characteristics that play a vital modulating role for liver zonation and subsequent gene expression and metabolism [104]. Recent focus of in vitro liver models has been directed at trying to capture these gradients

in a physiological fashion. In the future, the screening for hepatotoxic potential of novel xenobiotics most likely requires a combined approach whereby multiple in vitro models to cover appropriate end points is needed. This approach combined with the developing field of in silico liver models may better aid in early selection of compounds, and streamline the process by which toxicity investigations are carried out.

In conclusion, multidisciplinary approaches in the development of more complex in vivo-like models will better aid human relevant translational research and will yield potential diagnostic advances that will reduce the risk of hepatotoxic potential at pre-clinical and clinical levels.

Conflicts of interest

There are no conflicts to declare.

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Table 2. Advantages and limitations of currently used in vitro liver models.

Cell Type	**Advantages**	**Limitations**
HepG2	Unlimited source of cells. Repeatability of experiments is more achievable. Easy-to-handle in laboratory with simplistic culturing methods. No inter-donor differences. Some expression of liver-specific enzymes.	Low metabolic competence and rapid loss of expression of liver-specific enzymes/transporters. Loss of cellular polarity. Absence of NPCs.
C3A (HepG2/C3A)	Selected for strong contact inhibited growth characteristics. High albumin production, alpha fetoprotein (AFP) production and ability to thrive in glucose deficient media.	Low metabolic competence and rapid loss of expression of liver-specific enzymes/transporters. Loss of polarity. Absence of NPCs.
HepaRG	Improved liver-specific functionality when compared with the commonly used HepG2 and C3A cells in 2D culture. More comparable to PHH for phase I & II, gene and transporter expression.	More complex culturing methods when compared to more commonly used hepatic cell lines. Expensive consumables required for extended culture periods.

Primary Hepatocytes (Human, Rat)	Improved metabolic competence and more physiologically relevant compared to hepatic-derived cell lines. Availability of cryopreserved hepatocytes. Full expression of liver-specific enzymes. Good transferability of data for in vitro to in vivo models. Historical human data for numerous drugs allows for direct comparison with in vitro models.	Limited availability for researchers and inter-donor variability. Short-term culture time. Rapid loss of expression of liver-specific enzymes. Difficult isolation and subsequent culturing processes. Limitations can be partially overcome by 3D culturing.
Stem cell based approaches	Stem cells proliferate extensively in vitro and can differentiate into hepatocytes. This provides a stable source of hepatocytes for multiple investigations. iPSCs/HPCs have the potential to establish genotype-specific cells, increasing the predictivity of toxicity assays. Potential to develop personalised medicine and hepatotoxicity investigations.	Dedifferentiation concerns after the long-term culture of PSCs. Few thorough investigation in toxicological applications. Complex reprogramming steps (iPSCs.) Variability in phenotype between preparations. Expensive when compared with other hepatic-derived cell lines.

In vitro Approaches	**Advantages**	**Limitations**
Monolayer cultures	Simplistic culture methods and low set-up costs. Good repeatability of experimental data. Can incorporate NPCs improving overall functionality and longevity.	Cannot recapitulate the complex 3D microenvironment. Lack of in vivo-like cellular morphology. Poor gene and subsequent protein expression profiles. Loss of cell polarity.
Sandwich cultures	Sandwich cultured hepatocytes retain more in vivo-like properties, including polarised excretory function and enhanced morphology and viability of hepatocytes compared to monolayer cultures.	Sandwich cultures lack complex cellular interactions and the 3D microenvironment. The expression of genes responsible for many liver-specific functions decreases over time.
Co-Culture	Multi-cellular environment with direct cell-cell interactions mimicking natural environment. Positive reciprocal effect with improved functionality and longevity. Co-culture models can be produced in 2D and sandwich cultures and also within 3D cultures such as spheroids. Recovery of cellular polarity.	Limited availability of NPCs with difficult isolation procedures. Batch to batch variability between NPCs. Differentiation status and viability are varied depending on culture conditions.

Scaffold and Hydrogels	Formation of cellular interactions and representation of native ECM. Improved functionality and sensitivity to APAP. Ability to mimic pathologies via stiffness variation.	Limitations with regards to mass transfer of oxygen and nutrients. Limitations with cell retrieval and subsequent analysis. Poor culture longevity
3D	Recapitulation of 3D microenvironment and ECM properties. Well established cellular interactions leading to improved gene and protein expression. Establishment of cellular polarity. Can incorporate NPCs improving overall functionality and longevity.	More complicated methods of culture. The literature has extensively discussed the formation of necrotic regions within 3D cellular models due to reduced oxygen diffusion to cells within the 3D mass.
Spheroids	Multicellular environments recapitulating native 3D microenvironment. Cell-cell interactions and natural production of ECM. Spheroids can be produced with hepatic cell lines and primary hepatocytes.	Spheroids have a limited size due to formation of necrotic cores (~150 μm). Limitations of oxygen and nutrient diffusion through multicellular aggregates. Comprehensive investigation with regards to optimal spheroid size for specifics cell types has yet to

	Maintain liver-specific functionality over longer periods of time. Enhanced CYP450 and transporter expression. Formation of secondary structures (bile canalicular-like structures). Cellular polarity is recovered. Along with maintenance of native cuboidal morphology.	be done including cell-specific and model-specific OUR. More work needs to be done to improve basis for high-throughput system.
Liver slices	Maintains multicellularity (all NPCs) in appropriate proportions and complex 3D microenvironment. Can be incorporated into flow systems to allow shear stresses.	Short term culture periods meaning liver slices are unsuitable for repeat-dose investigations.

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