Multi-Cellular Organotypic Liver Models for the Investigation of Chemical Toxicity and Liver Fibrosis

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Sophia M. Orbach

Abstract (Academic)

The liver is responsible for lipid and glucose metabolism, protein and bile synthesis and the biotransformation of xenobiotics. These functions, performed by hepatocytes, are dependent on heterotypic interactions with other liver cell types and the stratified microarchitecture of the organ. *In vitro* liver models provide insights into the role of each cell type and perturbations upon external stimuli. Despite the dissimilarities to *in vivo* and rapid dedifferentiation, most liver studies utilize hepatocyte monocultures. These models lack heterotypic interactions causing inaccurate assessments of toxicity and disease. Only a limited number of 3D hepatic models incorporate the major liver cell types, and these cultures primarily focus on the hepatocyte response. We have developed 3D liver models that include all major hepatic cell types and recapitulate the layered architecture of the organ. These models maintain hepatic functions for up to four weeks and can be used to isolate the role and response of each cell type. We used these models to study two critical aspects of the organ – acute hepatotoxicity and liver fibrosis.

There are tens of thousands of chemicals with undetermined effects on the human body. High concentrations of xenobiotics can cause acute liver damage and failure. Liver impairment can result in multiple organ failure, hepatic encephalopathy and death. Therefore, it becomes critically important to investigate hepatotoxicity in a time, cost and resource effective manner. Our 3D liver models were validated for hepatotoxicity testing with acetaminophen, a prototypic drug. We then adapted and optimized the models for high-throughput hepatotoxicity testing with automated procedures and primary human hepatic cells.
Liver fibrosis and cirrhosis are well-established consequences of chronic chemical exposure, infection and alcoholism. The initiating factors, end stages and resolution of fibrosis have been extensively studied. However, there is minimal information on the role of the local microenvironment in the progression of the disease from diseased to healthy tissue. We designed 3D liver cultures with a mechanical gradient to gradually model this transition through spatial and temporal perspectives. These findings demonstrate the versatility and accuracy of these 3D hepatic models in the investigation of liver toxicity and fibrosis.
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Sophia M. Orbach

Abstract (General Audience)

The liver is responsible for lipid and glucose metabolism, protein and bile synthesis and the biotransformation of chemicals. These functions, performed by hepatocytes, are dependent on interactions with other liver cell types and the layered organization of the organ. In vitro liver models provide insights into the role of each cell type and functional changes upon external stimuli. Despite the dissimilarities to animal models, most liver studies utilize hepatocyte-only cultures. These models lack interactions with other cell types causing inaccurate assessments of toxicity and disease. Only a limited number of 3D hepatic models incorporate the major liver cell types, and these cultures primarily focus on the hepatocyte response. We have developed 3D liver models that include all major hepatic cell types and model the layered architecture of the organ. These models maintain hepatic functions for up to four weeks and can be used to isolate the role and response of each cell type. We used these models to study two critical aspects of the organ – acute hepatotoxicity and liver fibrosis.

There are tens of thousands of chemicals with undetermined effects on the human body. High concentrations of chemicals can cause acute liver damage and failure. Liver impairment can result in multiple organ failure, coma and death. Therefore, it becomes critically important to investigate hepatotoxicity in a time, cost and resource effective manner. Our 3D liver models were validated for hepatotoxicity testing with acetaminophen, the active ingredient with Tylenol®. We then adapted and optimized the models for high-throughput hepatotoxicity testing with automated procedures and primary human hepatic cells.
Liver fibrosis and cirrhosis are well-established consequences of chronic chemical exposure, infection and alcoholism. The initiating factors, end stages and resolution of fibrosis have been extensively studied. However, there is minimal information on the role of the local microenvironment in the progression of the disease from diseased to healthy tissue. We designed 3D liver cultures with a stiffness gradient to gradually model this transition through spatial and temporal perspectives. These findings demonstrate the versatility and accuracy of these 3D hepatic models in the investigation of liver toxicity and fibrosis.
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Chapter 1: Background and Introduction

1.1 Liver Anatomy and Function

1.1.1 Gross Anatomy

The liver is located in the abdomen and is the largest and heaviest solid organ in the body, accounting for 2-5% of the mass of a human adult (Figure 1.1) [1, 2]. The human liver is divided into four lobes. The two larger lobes are called the right and the left and the two smaller lobes are the quadrate and the caudate. The liver receives blood from the heart through the hepatic artery (~30% volume) and from the intestine through the portal vein (~70% volume) providing oxygen and nutrients, respectively [1-3]. Blood flows from the liver to the rest of the body through the inferior vena cava.

![Liver Location](image)

Figure 1.1: Location of the liver in the human body. Adapted with permissions from [4].

1.1.2 Liver Microarchitecture

The liver is divided into hexagonal lobules, 1-2 mm in diameter. There are approximately one million lobules in a human liver [3, 5]. These lobules are oriented around a central vein with tributaries of the portal vein, hepatic artery, and bile duct along the periphery grouped into portal
triads [5, 6]. Hepatocytes are the parenchymal cells of the liver and are organized into cords separated by sinusoids. Sinusoids are small blood vessels with a fenestrated and discontinuous endothelium [1, 5]. The non-parenchymal liver cells (NPCs) are liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs) [1]. Figure 1.2 depicts the sinusoidal region of the liver and the relative location of each cell type in a healthy organ.

![Figure 1.2](image)

**Figure 1.2**: The sinusoidal region of the liver illustrating hepatocytes, LSECs, KCs, and HSCs and their locations. The interfacial region between the hepatocytes and LSECs is the Space of Disse. Adapted with permissions from [4].

### 1.1.3 Cell Types and Functions

Hepatocytes are the parenchymal cells of the liver and account for ~70% of the organ by volume [2, 5]. Opposing sides of the hepatocytes are in contact with the Space of Disse, resulting in two basal poles (unlike most epithelial cells that only have one) [2]. The Space of Disse is a protein-rich interface of extracellular matrix (ECM) components that separates hepatocytes from the bloodstream and LSECs [2, 7, 8]. This interface promotes the transfer of nutrients and signaling molecules between the sinusoid and the hepatocytes. The basal poles of hepatocytes are covered with microvilli that extend into the Space of Disse and increase the surface area and transfer efficacy of proteins and nutrients.

Hepatocytes are responsible for the majority of liver functions [2, 3, 5, 6]. This includes the uptake, storage, metabolism, and release of amino acids, carbohydrates, lipids, and vitamins.
Hepatocytes also maintain glucose homeostasis and synthesize serum proteins, particularly albumin [5]. These cells are responsible for the majority of biotransformation of xenobiotics to water-soluble products that can be excreted from the body. Additionally, hepatocytes produce and secrete bile into the bile canaliculi to improve digestion and eliminate cholesterol.

LSECs contain fenestrae, or pores, that are approximately 100-200 nm in diameter [1-3, 5]. These pores lay in clusters called "sieve plates". Fenestrae control the exchange of compounds between blood and hepatocytes [2]. The fenestrae diameter is easily altered by xenobiotics and disease. Fenestrae of healthy LSECs promote the transport of molecules smaller than 250 kDa including chemicals, nutrients, hormones, proteins, and other plasma components [5, 6]. LSEC functions include scavenging of waste products, angiogenesis and regulation of blood pressure [1-3, 5]. LSECs also endocytose ECM components including glycoproteins, hyaluronic acid (HA), collagen, and fibronectin [9]. The fenestrae and lack of basement membrane differentiate LSECs from other endothelial cells [2, 5].

KCs are resident stellate-shaped macrophages located in the liver sinusoid [1, 2, 6]. They account for approximately 80% of macrophages in the body [6, 10]. KCs process old, damaged, altered, and foreign material through endosomal and lysosomal pathways [9]. These cells secrete pro- and anti-inflammatory cytokines in a healthy liver to maintain homeostasis.

HSCs are spindle-shaped cells found in the Space of Disse [2, 5]. Major functions of HSCs include the production and regulation of ECM components and the storage of lipid droplets and retinoids (vitamin A). The liver stores between 50% and 80% of retinoid in the body, with 80-90% of the organ supply in the HSCs. HSCs promote organ regeneration through differentiation and proliferation.
1.2 Liver Toxicity

1.2.1 Liver Response to Chemical Exposure

The liver is the first major organ subjected to many ingested chemicals once they exit the gastrointestinal tract (Figure 1.1) [1]. The biotransformation of these substances in the liver protects other organs. However, byproducts of biotransformation can result in hepatic injury. Extensive and repeated hepatic damage or disease can cause liver failure [3]. Severe complications of liver failure include multiple organ failure, elevated blood ammonia concentrations, abnormal brain function, and loss of consciousness. Once liver failure occurs, there is a 70-95% risk of mortality within weeks to months of onset.

Biotransformation of xenobiotics can result in toxic metabolites [1]. Phase I reactions include oxidation, reduction and hydrolysis. Phase I biotransformation most often occurs through cytochrome P450 (CYP) oxidation. These enzymatic reactions commonly form toxic intermediates [6]. Phase II reactions include the conjugation of xenobiotics with glucose, sulfate, glutathione (GSH), amino acids, and acetyl or methyl groups. Phase II reactions generally eliminate the toxic substances through excretion [1]. The relationship between the production of toxic intermediates from phase I biotransformation and detoxification through phase II reactions determines the extent of liver damage [6].

1.2.2 Activation of NPCs

When LSECs are exposed to xenobiotics, their fenestrae increase in diameter, thereby enabling red blood cells to cross the endothelium (Figure 1.3A) [5, 6]. This migration alters the structure of LSECs and leads to their contraction forming gaps between the individual cells and a breakdown of the endothelial barrier. The combination of these factors results in the collapse of the sinusoidal wall and hemorrhage into the hepatocytes.
KCs become activated through phagocytosis of cellular debris (Figure 1.3B) [6]. Activated KCs release reactive oxygen species (ROS), nitric oxide and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukin 1 (IL-1), and interleukin 6 (IL-6) [5, 6, 9]. These cells also secrete anti-inflammatory cytokines such as IL-10 and interferon gamma (IFNγ). These cytokines serve as chemotactic signals that recruit inflammatory cells. Thus, activated KCs promote the migration of neutrophils, lymphocytes, and monocytes to damaged sites [6]. These inflammatory cells remove dead and damaged cells from the liver but can result in further inflammation, injury and oxidative stress.

Activated HSCs proliferate and migrate to injured sites resulting in an increased number of these cells in a damaged liver tissue (Figure 1.3C) [5, 6]. The cells produce excess ECM proteins and proteoglycans that stiffen the liver matrix causing formation of scar tissue and fibrosis. Once HSCs are activated they lose their ability for retinoid and lipid storage.

Figure 1.3: General toxicity pathways for (A) LSECs, (B) KCs and (C) HSCs.
1.2.3 Common Hepatotoxicants

1.2.3.1 Acetaminophen (APAP)

APAP is the active ingredient in Tylenol® and the most frequently used drug in the United States [11-15]. APAP acts as a fever reducer and pain reliever. While safe at therapeutic doses, APAP can cause hepatotoxicity at higher doses. APAP is the number one cause of acute liver failure in the United States responsible for approximately 50,000-80,000 emergency room visits, 26,000 hospitalizations, and 500 deaths annually. The well-established pathway of APAP hepatotoxicity makes this drug an ideal prototypic toxicant [5, 9].

APAP is metabolized in the liver through three pathways (Figure 1.4) [11, 16-19]. Glucuronidation is responsible for the metabolism of about 55% of a therapeutic dose of APAP. Sulfation metabolizes an additional 30% of the dose. These processes form nontoxic conjugates that are safely excreted from the body. CYP enzymes oxidize the remaining APAP to a toxic metabolite, \(N\)-acetetyl-\(p\)-benzoquinoneimine (NAPQI). At therapeutic concentrations, NAPQI is reduced by GSH to a nontoxic conjugate. When excess APAP is ingested, it is this third pathway that leads to hepatotoxicity. CYP2E1 is primarily responsible for the oxidation of APAP to NAPQI. CYP1A2 and 3A4 have been implicated to a lesser extent.

Excess NAPQI formation depletes cytoplasmic and mitochondrial GSH [16, 17, 19]. Unbound NAPQI binds to thiol groups in proteins altering their functions, activating nuclear factor erythroid 2-related factor 2 (Nrf-2) (Figure 1.4). In a healthy liver, Nrf-2 is bound to Kelch-like ECH-associated protein 1 (Keap1) that retains Nrf-2 in the cytoplasm. NAPQI oxidation releases Nrf-2 allowing it to translocate to the nucleus. This transcription factor binds to the antioxidant response element (ARE) in the promoter region producing antioxidant enzymes that partially counter oxidative disturbances.
Significant liver injury occurs when more than 90% mitochondrial GSH is depleted [11, 17, 19]. The depletion of GSH results in an influx of reactive nitrogen species (RNS) and ROS. This activates the c-Jun N-terminal protein kinase (JNK) pathway (Figure 1.4). The reactive species oxidize apoptosis signaling-regulating kinase 1 (ASK-1) and disociates thioredoxin. This, in turn, phosphorylates mitogen-activated protein kinase kinase (MAP2K) that activates JNK through phosphorylation.

**Figure 1.4:** Hepatotoxicity pathway in the hepatocytes as a result of APAP overdose. APAP = acetaminophen, UGT = UDP-glucuronosyltransferases, SULT = sulfotransferases, CYP = cytochrome P450, NAPQI = N-acetyl-p-benzoquinoneimine, GST = glutathione S transferases, GSH = glutathione, RNS = reactive nitrogen species, ROS = reactive oxygen species, ASK-1 = apoptosis signaling-regulating kinase 1, MAP2K = mitogen-activated protein kinase kinase, JNK = c-Jun N-terminal protein kinase, MPT = mitochondrial permeability transition, ATP = adenosine triphosphate, iNOS = inducible nitric oxide synthase, NO = nitric oxide, Keap1 = Kelch-like ECH-associated protein 1, Nrf-2 = nuclear factor erythroid 2-related factor 2, ARE = antioxidant response element.
JNK is a death kinase typically associated with apoptosis [11, 17-19]. The transport of JNK to the mitochondria induces mitochondrial permeability transition (MPT). MPT is defined as a sudden increase in the permeability of the inner membrane of the mitochondria to ions and solutes with molecular weights under 1,500 Da [20, 21]. This process inhibits mitochondrial respiration and reduces adenosine triphosphate (ATP) in the cell. The decrease in ATP prevents apoptosis, as the cells become energy limited. Therefore, APAP-induced cell death primarily results in hepatic necrosis.

1.2.3.2 Ethanol (EtOH)

Excessive alcohol consumption is the number one cause of liver disease in industrialized countries [3]. Over 90% of ingested EtOH is processed in the liver [6]. Alcohol-induced liver disease is often detected through elevated resting blood ethanol, aspartate and alanine aminotransferase and high-density lipoprotein levels [22]. There are three stages of alcoholic liver disease: steatosis, alcoholic hepatitis, and cirrhosis. Hepatitis progresses to cirrhosis in one-third of all patients [3, 23]. Alcoholic liver disease causes death through hepatic encephalopathy, gastrointestinal hemorrhage, hepatic and renal failure, and hepatocellular carcinoma [3].

Three enzymes metabolize EtOH – alcohol dehydrogenase (ADH), CYP2E1 and catalase [24, 25]. ADH and CYP2E1 biotransformation are the two primary pathways, whereas catalase is only active at high concentrations of EtOH. Each of these mechanisms forms acetaldehyde, a toxic metabolite. Aldehyde dehydrogenase (ALDH) metabolizes acetaldehyde to nontoxic acetic acid. Acetic acid is converted to ketone bodies in the liver, resulting in the formation of free fatty acids and triglycerides [26, 27]. The triglycerides are stored in nearby adipocytes that continue fatty liver development even upon cessation of the chemical [28]. Hepatic steatosis results from
this EtOH-induced lipogenesis. Ketone bodies can also increase acute ethanol toxicity by increasing CYP2E1 and the corresponding conversion of EtOH to acetaldehyde [29].

Acute and chronic alcoholic liver disease occurs through inflammation and oxidative stress, particularly through the secretion of TNFα by KCs [30, 31]. TNFα alters lipid signaling in the hepatocytes, which interferes with the mitochondrial electron transport chain and induces oxidative stress [31, 32]. Inflammation and oxidative stress can also be induced independently of KCs through CYP2E1 [33, 34]. In vivo, hepatic inflammation is exacerbated through KC activation by lipopolysaccharide released from increased gut permeability [35]. EtOH-induced oxidative stress increases as hepatocyte GSH decreases, sensitizing the cells to further TNF-α injury [36-38].

1.2.3.3 Isoniazid (INH)

INH is an effective anti-tuberculosis drug [39-42]. However, it causes liver toxicity in up to 20% of patients and hepatitis in 1-2%. Of all drugs currently on the market, INH has one of the highest potentials for hepatotoxicity [41]. Liver toxicity from this drug is primarily detected through increases in serum alanine aminotransferase [42]. In vivo, INH toxicity generally occurs between one week and six months from the onset of treatment [43]. INH can induce oxidative stress, mitochondrial damage and apoptosis [40]. However, the primary mode of cell death is necrosis [40]. INH can activate KCs and other macrophages, inducing an immune response [43]. The role of the adaptive immune system has been further validated by the delayed onset of toxicity in some patients [41, 43].

INH biotransformation occurs through two pathways – metabolism by n-acetyl transferase 2 (NAT2) to a nontoxic metabolite and hydrolysis/CYP2E1 metabolism to toxic hydrazine [40, 42, 44]. Toxicity is more likely in patients that have low basal NAT2 and high basal CYP2E1
expression [40]. INH undergoes biotransformation through CYP2E1, but the drug inhibits the expression of CYP1A2, 2A6, 2C19, and 3A4 [45]. Once INH is converted to hydrazine, GSH drastically decreases within several hours [46]. Oxidative stress results from the depletion in GSH and the inhibition of ARE by INH [40]. Hydrazine induces mitochondrial damage, which is the largest contributing factor to INH-induced toxicity [41, 43]. Hydrazine has also been associated with the development of fatty liver.

1.2.3.4 *Perfluorooctanoic Acid (PFOA)*

PFOA is a fluoropolymer and surfactant with commercial and industrial purposes, primarily Teflon manufacturing [47]. Human exposure primarily occurs through ingestion from contaminated water and food [48]. PFOA is stable in aqueous environments for hundreds of years with a half-life of 2.3-years in the human body [49]. Once ingested, PFOA is easily detectable in serum [48]. Due to the environmental persistence, the United States Environmental Protection Agency (EPA) has placed stringent limitations on the allowable water levels [49]. In the last ten years, the health advisory for PFOA has decreased from 0.4 to 0.07 µg/l.

PFOA is a peroxisome proliferator that induces metabolic diseases [48, 50-53]. High levels have been associated with cancer, organ disease, infertility, obesity, high cholesterol, and developmental defects [48-54]. Unlike many drugs, PFOA does not undergo phase I and II biotransformation and the parent compound is responsible for cellular damage [47, 55]. The toxicant primarily targets the liver and can result in organ enlargement, peroxisome proliferation, hepatitis, and inhibition of fatty acid metabolism [51, 56-59]. PFOA interferes with mitochondrial bioenergetics, resulting in the formation of ROS and apoptosis [60-62]. PFOA has also been associated with hepatic steatosis, necrosis, inflammation, and fibrosis [63].
1.3  *In Vitro* and *In Vivo* Models to Investigate Hepatotoxicity


1.3.1  *In Vivo* Models

*In vivo* systems can provide holistic information on how an organism responds to a xenobiotic [64]. These models measure the absorption, distribution, metabolism and elimination of the toxic component from each organ [1, 64]. However, the use of the whole animal leads to high complexity of the system and the role of a single organ is difficult to isolate. Typical *in vivo* models include rats, mice, rabbits, guinea pigs, pigs, cattle, sheep, and monkeys [64]. None of these models mimic the human response due to species-dependent differences in drug metabolism [64, 65]. Additionally, *in vivo* models are expensive, require significant loss of animal life and are often considered unethical. These reasons underscore the need for quality *in vitro* hepatotoxicity studies.

1.3.2  Cell Types Used for Liver Studies

1.3.2.1  Primary Cells

Primary human or animal hepatocytes are the preferred cells to predict drug biotransformation *in vitro* [9, 66-70]. These cells are isolated through collagenase digestion and purification through density-gradient centrifugation [9, 71, 72]. These cells can be cryopreserved, but there is a loss in function upon thawing [9]. Maintaining the sensitivity of primary cells to chemical treatment over an extended period of time has become a significant challenge in liver tissue engineering [73].
Acquiring functional and reliable primary human hepatocytes is the most significant obstacle to their use [9, 74-77]. High quality primary human hepatic cells are often difficult and expensive to acquire, therefore rodent cells are most commonly used [5, 9]. A primary benefit to rodent models is their ease of use [78]. This includes their size, low sentience, ability for gene manipulation, and reduced costs. Additionally, biotransformation of xenobiotics in rodents is widely reported for numerous drugs and pharmaceuticals [79-85]. For these reasons, studies on rodents or cells derived from their livers serve as good initial models for hepatotoxicity.

1.3.2.2 Immortalized Human-Derived Cell Lines

HepG2 cells are derived from liver tissues with well-differentiated hepatocellular carcinoma and are the most commonly used and best characterized cell line [9]. These cells are adherent, hepatocyte-like and form small aggregates in a monolayer. HepG2 cells are easy to maintain relative to primary human hepatocytes and are often used for toxicology studies. However, there are significant differences between the metabolic processes of HepG2 cells and primary hepatocytes on a transcriptomic level. Messenger RNA (mRNA) levels of several CYP enzymes are significantly reduced in HepG2 cells including CYP1A1, CYP1A2, CYP2E1, and CYP3A4 [86]. mRNA levels of phase II enzymes such as SULTs, UGTs, and GSTs are also reduced in HepG2 cells. These differences lead to unreliable and erroneous results when HepG2 cells are exposed to xenobiotics.

HepaRG cells are also derived from hepatocellular carcinomas [9, 87]. Confluent monolayers consist of two distinct cell types. One resembles primary human hepatocytes while the other resembles cholangiocytes, epithelial bile duct cells. Treatment with dimethyl sulfoxide (DMSO) can preferentially differentiate HepaRG cells into hepatocyte-like cells. The metabolic profile of differentiated HepaRG cells is closer to primary human hepatocytes than other immortalized cell lines [9, 88]. Despite these similarities, mRNA levels of CYP2E1 and CYP3A4 are also reduced
in HepaRG cells compared to primary human hepatocytes. The decrease in mRNA of these two CYP enzymes is approximately 10-fold and 50-fold in HepaRG and HepG2 cells, respectively [86]. Phase II enzymes in HepaRG cells are similar to primary cells.

1.3.2.3 Induced Pluripotent Stem Cells

More recently, induced pluripotent stem cells (iPSCs) have been utilized as a self renewing source of human hepatocytes [9]. Somatic cells can be obtained from specific individuals, which could enable patient-specific studies of idiosyncratic hepatotoxicity [5, 89]. iPSCs can be differentiated into hepatocytes by the addition of medium supplements such as activin A, fibroblast growth factor, Wnt3a, insulin, dexamethasone, hepatocyte growth factor, and oncostatin M [9, 90, 91]. Hepatocyte-like iPSCs have been utilized in 2D [9, 92-94] and 3D [9, 95-97] liver models for toxicity testing. There are two major disadvantages with the use of hepatocyte-like iPSCs. The first is that the expression of biotransformation enzymes resembles that of fetal hepatocyte, rather than mature hepatocytes [9]. However, a recent study has demonstrated that a 3D configuration and the addition of cyclic adenosine monophosphate can address these issues [98]. The second major disadvantage is that there is substantial variability between cell lines as a result of the source and differentiation protocols [90].

1.3.3 Liver Models

1.3.3.1 Hepatotoxicity Testing in 2D Models

Hepatocyte monolayers are assembled by seeding hepatocytes on protein-based substrates (Figure 1.5A) [99, 100]. In such cultures, hepatocytes rapidly lose hepatocyte-specific functions and expression of biotransformation enzymes within 24-72 h of seeding [99, 101]. For these reasons, hepatocyte monolayers are less sensitive to chemicals compared to the native organ resulting in a significant underestimation of toxicity [102-105]. Despite such inaccuracies, hepatocyte monolayers are still commonly used for toxicity studies [73, 102-105].
Figure 1.5: 2D models of liver toxicity. (A) Hepatocyte monolayer (B) collagen sandwich (C) co-culture of hepatocytes with other cell types including NPCs, endothelial cells, fibroblasts, and other epithelial cells.

In collagen sandwich models (Figure 1.5B) the continued expression of CYP enzymes leads to greater sensitivity to xenobiotics [73, 106-112]. Despite the benefits of the sandwich model, it does not incorporate heterotypic intercellular interactions that occur in the liver. The co-culture of hepatocytes with other cell types enables heterotypic cell-cell interactions (Figure 1.5C) [113]. These cells include NPCs, endothelial cells, fibroblasts, and other epithelial cells. Hepatocyte phenotypes can be maintained up to five weeks in culture when additional cell types are added within a week of seeding [113-115].

Expression of CYP enzymes are up to two-fold higher in co-cultures relative to hepatocyte monolayers [116]. These cultures have been utilized for toxicity studies and exhibit increased function but comparable sensitivity to hepatocyte monolayers [93, 94, 117-120]. This is particularly true for APAP treatment, since CYP2E1 expression is not maintained in 2D co-cultures [116, 117, 119]. However, Nelson et al. found that C3A/human vascular endothelial cell co-cultures exhibited increased ATP in response to acetaminophen relative to monocultures of either cell type [93]. This indicates that hepatocyte-like cell lines do not respond to 2D co-culture similarly to primary cells. Micropatterning of co-cultures has been developed to better replicate in vivo cellular organization in a 2D configuration [5, 9]. These models control the heterotypic
inter-cellular interactions, support the differentiation of iPSCs to hepatocytes and have demonstrated improved sensitivity relative to standard 2D co-cultures [94, 121-125].

1.3.3.2  Hepatotoxicity Testing in 3D Models

3D liver models can maintain hepatocyte phenotype and biotransformation enzyme expression [5, 9]. These models emulate *in vivo* properties of the liver such as heterotypic intercellular interactions, the stratified architecture, maintenance of hepatocyte tight junctions, induction of polarity, kinetic flow, and the presence of ECM components [1-3, 5, 6, 9, 39, 113, 126, 127]. The recapitulation of these properties prevents dedifferentiation and maintains CYP expression and chemical sensitivity over extended periods of time [5, 9, 126]. For these reasons, 3D liver models have become regularly utilized for the investigation of *in vitro* hepatotoxicity [5, 9].

![Figure 1.6: 3D models of liver toxicity. (A) PCLS depicting the maintenance of hexagonal liver lobules and the location of the portal triad and central vein (B) hepatocyte spheroids in a culture well and (C) hepatocytes in a bioreactor system.](image)
Precision-cut liver slices (PCLS) are 100-250 µm thick sections of whole liver that can be cultured in vitro for a maximum of 72 h due to restrictions in oxygen and nutrient diffusion (Figure 1.6A) [5]. However, gene expression of PCLS varies drastically after 24 h in culture [128]. Diffusion is a critical limiting factor in toxicity studies, since chemicals cannot reach all the cells [5]. Administration of PCLS with APAP results in protein profiles similar to in vivo and predicts relevant toxic concentrations [128-130]. Unlike many other in vitro models, the microarchitecture, intercellular interactions and CYP expression are maintained from in vivo [129, 131, 132]. Specifically, CYP expression accurately depicts inter-donor variations from human livers [132].

PCLS models can be used to analyze the role of individual cell types on overall liver toxicity. For example, KCs can be depleted in vivo by gadolinium chloride [133, 134]. PCLS from gadolinium chloride treated rats predict decreased TNFα secretion and APAP toxicity upon KC depletion [133]. Analyzing protein profiles in PCLS are useful for detecting metabolites and protein adducts [131, 133]. However, the proteins upregulated in response to a chemical are not always accurate, as the corresponding predicted mechanisms of cell death do not always match in vivo [130].

Spheroids are small cell aggregates formed through numerous techniques such as the hanging-drop method, seeding on non-adhesive surfaces and rotating cultures (Figure 1.6B) [5, 9]. Spheroids are the most commonly used 3D liver models for hepatotoxicity studies and, unlike PCLS, can maintain biotransformation enzymes for up to five weeks [95, 135-141]. Unlike other liver models, hepatocytes in spheroids become increasingly more sensitive to chemicals after several days in culture due to increased interactions between the cells over time [135, 138, 140, 142]. Sensitivity of the spheroids to chemicals can also be dependent on the surface used to
assemble the model [137]. For instance, spheroids formed on Matrigel™ are more resilient to nanoparticle toxicity than those seeded on collagen. Cells on the outside of spheroids are more sensitive to chemicals than those in the center [96, 143]. Therefore, increases in the diameter of spheroids can be used as a marker of toxicity [96].

Hepatocyte spheroids respond to APAP similarly to in vivo as seen through cell death, mitochondrial damage, GSH depletion, and response to N-acetylcysteine [136, 138, 144]. CYP enzymes are consistently better induced in spheroids than 2D models [95, 140, 141]. Particularly, CYP2E1 is maintained in spheroids for up to three weeks, but not expressed in 2D co-cultures [116, 135, 138]. Therefore, when treated with some compounds, spheroids can be less sensitive than 2D models [96, 138, 141, 145]. However, these responses vary from study to study depending on the type of cell used and method of spheroid formation.

Hepatocytes can be encapsulated in hydrogels to induce polarity and extend hepatocyte function [85, 146-149]. The addition of APAP to hepatocytes in gel entrapped hollow fibers can increase toxicity up to four-fold relative to monolayers [85, 149]. Encapsulation of hepatic cells in alginate hydrogels does not always improve chemical sensitivity relative to 2D models [146, 147]. For example, encapsulated HepG2 cells are more responsive to APAP and diclofenac than monolayers, but are comparably sensitive to quinidine (antiarrhythmic drug) and rifampicin (anti-tuberculosis drug) [147].

1.3.3.3 Investigating Hepatotoxicity Under Dynamic Flow

Bioreactors and liver-on-chip devices incorporate kinetic flow to emulate vascular flow. In vivo, vascular flow replenishes nutrients, provides oxygen and removes cell debris and toxic byproducts (Figure 1.6C) [5, 9]. When culture medium is circulated in a device or a bioreactor, it also enables the ability to investigate the response of multiple chemicals and concentrations[9].
Hepatocytes in devices with flow exhibit increased CYP expression and functional markers, such as albumin and urea, relative to static cultures [124, 142, 150-152].

The incorporation of spheroids and scaffolds within bioreactors improves hepatocyte functions and CYP expression making them suitable for toxicity evaluations [139, 142, 150, 153-159]. Their inclusion in bioreactors results in biotransformation more comparable to in vivo and can maintain liver function up to seven weeks [139, 153, 155, 157, 160-164]. Hepatocyte spheroids in a bioreactor can maintain significant toxicity to chemicals up to three weeks in culture [155, 163]. Such chemicals include APAP, amiodarone (antiarrhythmic drug), diclofenac, metformin (diabetes medication), phenformin (diabetes medication), and valproic acid (anti-epileptic drug) [155]. Additionally, after three weeks in culture, the number of CYP enzymes that maintained in vivo-like expression in dynamic models was four-fold higher than static models [163].

In many bioreactors, hepatocyte toxicity (relative to static cultures) is reduced as a result of the removal of toxic byproducts [154, 155, 157-159]. Depending on the relevance of these metabolites to the mechanism of toxicity, this can lead to results that either converge or diverge from in vivo. For example, hepatocytes in a bioreactor have exhibited a LC50 of 40 mM for APAP in rats, approximately twice that of in vivo [155, 160]. However, the inclusion of J2-3T3 fibroblasts in this culture has been shown to increase the sensitivity to APAP up to three-fold [160]. This demonstrates that in a bioreactor, the inclusion of heterotypic inter-cellular interactions results in a system more representative of in vivo and can overcome the loss of critical metabolites as a result of the flow.

Hepatocytes have been cultured in microfluidic devices to assemble liver-on-a-chip systems [5, 9, 124, 142, 165-167]. Hepatic cells can be cultured in a 2D conformation or encapsulated in ECM proteins as a ‘micro-tissue’ [123]. Toxicity can be measured in these systems via
fluorescence visualization [124, 142, 150-152, 161, 165, 167]. This analysis provides both temporal and spatial variations in toxicity within the culture. Fluorescence visualization can be used to quantify live, dead and apoptotic cells [124, 142, 150, 165, 167], identify cell types, organization and organelles [150, 152, 161], detect changes in protein expression [142, 151, 152, 161], and establish hepatic cell activation [152] in response to chemicals.

Liver-on-a-chip studies have also been conducted using immortalized hepatocyte-like cell lines [142, 150, 151, 165, 167]. Spheroidal cultures of HepG2 cells and NIH-3T3 fibroblasts in a microfluidic device have demonstrated some apoptosis at low concentrations of APAP and extensive necrosis at 20 mM [165]. Necrosis as the primary mechanism of APAP hepatotoxicity is consistent with in vivo studies [19]. Similarly, HepG2/C3A spheroids in microfluidic devices exhibited significant APAP toxicity at 15 mM [142]. When HepG2 cells were cultured on a scaffold in a microfluidic device, they exhibited an approximately 50% decrease in viability at 50 mM EtOH, approximately three-fold lower than the LC50 reported in humans [167, 168]. These results demonstrate that despite the reduced biotransformation capacity in HepG2 cells, their inclusion in microfluidic devices greatly increases the sensitivity of the cells.

There are several liver-on-a-chip models designed to recapitulate the hepatocyte cords and sinusoidal architecture [169-172]. In these models, cylindrical cell chambers are separated from medium perfusion chambers by LSEC-like barriers to emulate fenestrae. Such a design provides a constant influx of nutrients and oxygen while preventing shear stress to the hepatocytes [170, 171]. A variety of chemicals have been tested with these types of models including diclofenac, APAP, INH, rifampicin, quinidine, and ketoconazole (anti-fungal) [169-172]. The majority of these treatments resulted in increased sensitivity in 3D models relative to 2D conformations [170, 171]. The predicted LC50 of APAP, rifampicin and ketoconazole in the 3D models were similar to those reported in vivo [171]. However, the estimated LC50 for diclofenac
and quinidine were approximately three-fold lower than \textit{in vivo}. The increased toxicity of such chemicals \textit{in vitro} is most likely due to the critical role of enterohepatic circulation in the metabolism of the drugs \textit{in vivo} [39].

1.3.3.4 Future Liver Toxicity Models

\textit{In vitro} liver models have recently been developed that more accurately mimic the stratified architecture of the liver [173-175]. These models are the most representative of hepatocyte interactions with the other cell types and the microenvironment. Zhang et al. have utilized 3D printing technique to mimic the vascular interface present in the liver [176]. 3D printing technology enables tuning the mechanical properties of the matrix, the incorporation of features such as micro-channels, and rapid cell deposition [177]. While this methodology has not yet been utilized for toxicity testing, it shows promise for future toxicity testing platforms. Kostadinova et al. published a report wherein a NPC fraction and hepatocytes were seeded on opposing sides of a Nylon scaffold to form a tissue [173]. Hepatocyte function was maintained for 11 weeks, with chemical sensitivity occurring over two weeks. While others have not yet reported toxicity studies using layered models, increases in CYP expression have been shown to occur over a 12-day culture. This model, established by Larkin et al., utilizes a polyelectrolyte multilayer as a Space of Disse mimic to support paracrine heterotypic intercellular interactions and directed signaling [175]. These cultures allow for each type of NPC to be individually visualized and extracted from culture for further analysis. Most multicellular 3D liver models used for hepatotoxicity studies investigate either cumulative toxicity or hepatocyte-only toxicity. The ability to isolate the response of each cell type to a chemical is a unique feature of these 3D liver models. While these cultures have not yet been used to model hepatotoxicity, their stratified architecture, maintenance of CYP expression and ability to isolate each cell type provide a promising platform for studying holistic liver toxicity.
1.4 High-Throughput Toxicity Testing

1.4.1 High-Throughput Screening (HTS)

The EPA has approximately 80,000 chemicals in their registry with unknown or under-studied effects on the human body [178, 179]. The development of toxicity is the number one cause of failure and withdrawal of potential drugs [180]. Preclinical studies are misrepresentative due to a lack of mechanistic or organ-specific information, non-cytotoxic assays, immune response, and *in vitro-in vivo* correlations [180, 181]. HTS was developed to reduce costs, resources and time [180]. Major deficiencies in current HTS models include an overreliance on viability assays and minimal *in vivo* relevance. More recently, 3D HTS models have been assembled to better emulate the *in vivo* organization of the cells [182]. These cultures model lung, kidney, liver, adipose, skin, musculoskeletal, and cardiac tissue [183]. Common 3D models include spheroids, cells on a scaffold and encapsulated cells [182]. Body-on-a-chip models have also been utilized for HTS studies [184].

The fast and standardized methods associated with HTS allow for untargeted toxicity investigations [185]. Big data methods analyze multiple concentrations of tens of thousands of chemicals over thousands of assays. This data is used to prioritize chemicals and drugs for in-depth investigation. The most notable use of big data with HTS is the EPA’s Toxicity Forecaster (ToxCast) initiative developed in 2006 [186, 187]. This program works to establish connections between chemicals and biomolecular targets [186]. These targets are detected with cell-free assays or monolayers of cell lines [188, 189]. Cell-based assays identify changes to secreted factors, proliferation, cell death, and transcription factor activity. Receptor interactions and enzyme activity were primarily detected with the cell-free assays [189]. The findings from the ToxCast initiative have been extensively reported [179, 188-192]. To allow for standardized
methodologies, the ToxCast program only investigates chemicals up to a concentration of 100 µM, which is not physiologically relevant for many ingested drugs [189].

1.4.2 HTS Hepatotoxicity Models

Liver models for HTS studies are assembled with primary hepatocytes or human hepatocyte-like cell lines [5, 193-195]. The vast majority of these models are monolayers of one of these cell types. The standard practice for liver toxicity in drug discovery is the detection of cell death in HepG2 monolayers at EC$_{50}$ [181]. The deficiencies of these cells have been detailed in Section 1.3.2.2. Almost all HTS liver models focus on cell death in response to toxicants [123, 124, 140, 196-199]. High content screening qualitatively investigates the response of chemicals on specific functions in hepatic cells, such as mode of cell death and mitochondrial damage [200]. There are essentially no HTS studies that quantify the interactions of xenobiotics with biochemical targets.

More advanced HTS models include static co-cultures, micropatterned co-cultures and spheroids [193]. Hepatocyte monolayers, co-cultures and sinusoid-like hepatic cultures have been included in microfluidic devices [201]. These systems introduce dynamic flow and are micro-scale, however current technologies allow for a maximum of 12 cultures per device. Commercial microtissues for HTS developed by InSphero and RegeneMed are most representative of the in vivo microenvironment. However, these models are proprietary, limiting potential analyses, and do not recapitulate the native liver organization. Importantly, these cultures do not allow for optimization or cell-specific analyses since the tissue is already formed before other researchers can access the cultures.
1.5 Liver Fibrosis

1.5.1 Fibrosis as a Wound Healing Mechanism

Fibrosis is a wound healing response brought on by chronic inflammation [202, 203]. Before fibrosis occurs, inflammation induces repair mechanisms and cell regeneration. With continued inflammation, damaged cells are replaced with ECM proteins and scar tissue. Myofibroblasts secrete matrix metalloproteinases (MMPs), which degrade ECM proteins, allowing for increased infiltration of inflammatory cells. Myofibroblasts also improve contraction of the wound, promoting both healing and the formation of scar tissue. Fibrosis is defined when the rate of formation of scar tissue surpasses the rate of degradation. Fibrotic diseases account for approximately 45% of all deaths in industrialized countries [202, 204, 205]. Liver fibrosis occurs through chronic or repetitive injury from infection, chemical toxicity, metabolic diseases, and alcoholism [203, 206, 207]. Advanced fibrosis is associated with distorted liver architecture, impedance to blood flow and liver failure [203, 207]. These symptoms primarily occur through excess ECM protein accumulation in the Space of Disse [206]. The most advanced stage of liver fibrosis is cirrhosis, which can lead to organ failure and hepatocellular carcinoma [203, 207].

1.5.2 Components of the Space of Disse

The Space of Disse is primarily composed of ECM components including collagen, glycoproteins, elastic fibers, proteoglycans, and glycoaminoglycans [7, 8]. Collagen is the most abundant protein in the human body [208]. There are ten types of collagen found in the adult liver [8]. Collagens type I, III, IV, and V are most prevalent in the hepatic ECM [8, 209-211]. The amount of collagen in the Space of Disse significantly increases during fibrosis.

Fibronectin is the most prevalent component of the Space of Disse in a healthy liver, connecting the surface of hepatocytes and endothelial cells to collagen [8, 212]. Laminin is an important
architectural protein of basement membranes that regulates development and differentiation [8, 213]. Increased production of laminin during fibrosis contributes to the continuous basement membrane that forms in the Space of Disse [8, 212].

Proteoglycans are proteins with glycosaminoglycan side chains [8, 214]. Proteoglycans found in a healthy liver are heparin, dermatan, and chondroitin sulphates. These proteins maintain structural integrity of the tissue and provide a charged barrier for the passage of molecules through the Space of Disse. There is a five-fold increase in the concentration of proteoglycans during fibrosis. The most prevalent glycosaminoglycan in the liver is HA, a negatively charged compound [8, 215].

1.5.3 Role of Hepatic Cells in Fibrosis

Each of the four hepatic cell types plays a critical and distinct role in the development and progression of fibrosis. HSCs are the cell type primarily associated with the disease [207, 216-218]. Upon repeated liver injury, cytokines such as TGFβ induce the transition from HSC quiescence to an activated state [216]. TGFβ and platelet-derived growth factor (PDGF) are the factors most associated with HSC activation and progression of fibrosis [203, 207, 216]. During fibrosis, cholesterol accumulates in the HSCs, which sensitizes the cells to TGFβ-induced activation [217]. Activated HSCs are marked by the conversion to myofibroblastic cells, increased cell proliferation and migration, increased expression of alpha smooth muscle actin, and secretion of excess ECM proteins [205, 216]. These proteins, particularly collagen type I and proteoglycans, increase the stiffness of the organ [205, 207, 218]. This increased stiffness can further activate the HSCs [203]. HSCs also secrete TGFβ, self-perpetuating the disease [207, 216].
LSECs become defenestrated and form a complete basement in a process known as capillarization [216]. Capillarized LSECs lose their capacity for endocytosis [219]. These cells contract, resulting in collapse of the sinusoids and inhibition of blood flow and oxygen [216, 219, 220]. This process is critical to the development of liver fibrosis, as differentiated LSECs induce the regression of HSCs back to an inactivated state [216]. Culture of activated HSCs with other hepatic cells does not support this reversion [219, 221]. Differentiated LSECs contribute to apoptosis of activated HSCs. Capillarized LSECs secrete an isoform of fibronectin that promotes HSC activation [203].

Activated KCs are highly proliferative during fibrosis [222]. These cells phagocytose dead cells and debris and secrete inflammatory cytokines and chemokines [207, 216]. Specifically, these cells are the primary source of TGFβ. KCs also secrete PDGF and induce expression of its receptors on HSCs, further propagating the disease. These cells also secrete IL-1 and TNFα, which promote the survival of activated HSCs [222].

In fibrosis, hepatocytes become apoptotic and secrete ROS that increase HSC activation [203, 207, 216]. The apoptotic cells are phagocytosed by KCs and HSCs, increasing inflammation and fibrosis [203]. This process also recruits immune cells to the site of injury [207]. It has been suggested that diseased hepatocytes can directly recruit immune cells [223]. Increased infiltration of immune cells can result in hepatocyte necrosis. Diseased hepatocytes are a major source of MMPs and, upon hypoxia, become a source for TGFβ [216]. Hepatocytes also secrete ECM proteins, usually at a scale 120-fold lower than HSCs [223]. However, these cells have been shown to be responsible for up to 10% of abnormal collagen in fibrosis.
1.5.4 Models of Liver Fibrosis

1.5.4.1 In Vivo Models

Fibrosis is induced in rodents through a variety of mechanisms [224]. Chemical-induced fibrosis is a common model due to the ease of use and reproducibility. The most used chemicals are ethanol and carbon tetrachloride [224, 225]. Ethanol induces fibrosis similarly to steatosis, while carbon tetrachloride models fibrosis comparable to chronic toxicity [224]. Steatosis-like fibrosis can also be induced through alterations in food supply, such as the removal of essential amino acids or a high-fat diet. Cholestatic fibrosis can be modeled with surgical methods, such as ligation of the common bile duct [224, 225]. Fibrosis has also been investigated with genetic models that utilize knockdown or knockout mice [224].

1.5.4.2 In Vitro Models

The most common in vitro models of liver fibrosis are monolayers of primary HSCs or HSC-like cell lines [224-227]. The major disadvantage to the use of primary cells is that HSCs spontaneously activate in culture within one week to an extreme myofibroblastic phenotype [224]. In fact, there is only a 12-18% overlap in gene expression of in vitro activated HSCs with their in vivo counterparts [225]. Culture of primary HSCs on ECM proteins, such as collagen, can mitigate the extreme activation [227]. Most HSC-like cell lines exhibit this extreme phenotype in culture [224]. LX-2 cells are the most physiologically relevant cell line, as they can model both the quiescent and activated HSC phenotype [224, 227].

HSCs are also co-cultured with hepatocytes, LSECs or KCs in 2D configurations, Transwell® systems or spheroids [224-226]. Hepatocytes, LSECs and KCs can identify the roles of fatty acid accumulation, angiogenesis and the immune response in fibrosis, respectively [224]. Recent studies with monolayers or combinations of hepatocytes, LSECs and KCs emphasize the role of each cell type in fibrosis and the effects of substrate stiffness [228-230]. Pro-fibrotic
compounds have been tested on advanced tissues of hepatocytes, HSCs and an additional cell type [231, 232]. PCLS are the only fibrotic model to include all four hepatic cell types [224, 225]. PCLS are routinely used as the architecture and cellular organizations found in vivo remain intact [224, 225]. These cultures are primarily used to study the potential of pro- and anti-fibrotic chemicals and drugs [225, 227]. However, cells in PCLS begin to rapidly lose viability after 48 h, making them unsuitable for long-term investigations [224]. In culture, PCLS spontaneously exhibit increased expression of fibrotic markers [227]. Importantly, this increase in fibrosis is due to increased cell death and damage in the center of the liver slices from decreased oxygen and nutrient diffusion. Despite these limitations, PCLS cultures have been used for fibrosis studies up to one week [226].

1.6 Space of Disse Mimic

The Space of Disse is critical to the maintenance of polarity in hepatocytes. It enables the transport of signaling molecules between the endothelium and the underlying layer of hepatocytes [2]. The inclusion of a Space of Disse mimic in the design of a liver model is essential to accurately model the interactions between the cells and their microenvironment [233]. Since the Space of Disse regulates hepatic cell function [2, 5, 9], there is a critical need to incorporate such an interfacial region in any liver-mimetic organotypic model.

The inclusion of ECM components in a Space of Disse mimic promotes cellular interactions comparable to the native microenvironment [233]. However, there is a need to control the concentration and composition of components in such a mimic. Layer-by-layer (LbL) deposition provides a platform that incorporates such components into a thin film [233, 234]. The physical
and chemical properties of these thin films can be modulated by input parameters resulting in a Space of Disse mimic that closely emulates its biological counterpart.

1.6.1 LbL Deposition of Polyelectrolytes

Polyelectrolyte multilayers (PEMs) are formed through LbL deposition of polyelectrolytes [234, 235]. The underlying premise of these PEMs is the sequential deposition of alternately charged polyelectrolytes to obtain a self-assembled PEM. Typically, LbL deposition is conducted by dipping a substrate in each polyelectrolyte solution for a desired period of time [235]. Traditionally this assembly is completed on a charged substrate, but can be conducted on uncharged, hydrophobic substrates (Figure 1.7) [175, 235]. The substrate is exposed to water between polyelectrolyte solutions to remove excess ions. In addition to dip-coating, spraying and spin coating of polyelectrolytes can be used for PEM assembly [235].

![Figure 1.7](image)

**Figure 1.7:** Schematic of solution dipping for LbL assembly on an uncharged substrate. The substrate is first dipped in an anionic PE solution and then washed so only adhered components remain. The substrate is then dipped into a cationic PE solution and washed. This process forms one bilayer.

Polyelectrolytes used for the development of PEMs consist of polymers, colloids, biomacromolecules (DNA, RNA), and small molecules (such as peptides) [234-238]. PEMs are self-assembled as each layer binds to adjacent polyelectrolytes through a combination of
electrostatic attraction, donor/acceptor interactions, hydrogen binding, and covalent binding [235]. PEM thickness, stiffness and porosity are dependent on the concentration of the polyelectrolytes, adsorption time, temperature, rinse time, pH, and choice of substrate [234, 235]. Modifying each of these parameters permits control over the physical properties of a PEM.

The thickness of a PEM is directly linked to the pH and ionic strength of the polyelectrolytes [234, 235]. Polyelectrolytes at low ionic strength form thicker, looped structures [234, 239]. High ionic strength polyelectrolytes exhibit a rod-like, thinner conformation. Increasing the number of bilayers increases PEM thickness in a linear or exponential fashion [234]. Linear growth occurs in PEMs formed from synthetic, highly charged polyelectrolytes. Exponential growth occurs with weakly charged, biological polyelectrolytes due to diffusion of charge throughout the PEM. Increasing the number of bilayers can have a direct effect on stiffness and cellular adhesion [234, 240]. In the next section, we outline the advances made in Space of Disse mimics in the Rajagopalan group.

1.6.2 Previous Work on 3D Liver Models in the Rajagopalan Research Group

There is currently no long-term in vitro hepatic model that accurately mimics the cellular interactions and complex functions of the liver [5, 9]. The Rajagopalan research group developed a multicellular organotypic liver model that uses a PEM to emulate the Space of Disse [174, 175, 241]. This model recapitulates the layered architecture of the liver while promoting the interactions between the hepatocytes and NPCs.

The first Space of Disse mimic was a PEM made of chitosan (cationic) and HA (anionic) assembled directly on the hepatocytes [174, 241]. These PEMs were assembled with 5-15 bilayers. The thickness and Young’s modulus (YM) ranged from 30-105 kPa. The second mimic was a detachable and free-standing PEM assembled on hydrophobic poly-tetrafluoroethylene
These PEMs were 12.5 bilayers and cross-linked with 8% (w/v) glutaraldehyde to prevent degradation in aqueous environments. The PEMs had a hydrated thickness of approximately 650 nm and YM of approximately 35 kPa. The thickness of the PEMs was identical to in vivo values and stiffness values were very close to those reported for healthy liver tissues [242-244].

The optimal seeding ratio of primary rat LSECs to hepatocytes was first determined in a two-cell model [241]. LSECs were seeded on top of the hydrated PEM at a ratio of 1:20 to the hepatocytes. The cultures were further extended to a three-cell model through the addition of LSECs and primary rat KCs onto the PEM [175]. These cultures were used to optimize the initial ratio of KCs to hepatocytes to 1:10.

The two-cell and three-cell 3D liver models were compared to HM, CS and 2D co-culture counterparts over 16 days [175]. Albumin levels increased over 3-fold in the 3D models, but only 1- to 2-fold in the 2D models. There was a two-fold increase in CYP1A1 in the two-cell 3D model relative to the next best performing systems. The 3D models promoted proliferation in the hepatocytes while the 2D comparisons decreased in number throughout the culture. Remarkably, as each cell type proliferated throughout the culture, the ratios of NPCs to hepatocytes remained comparable to in vivo [245, 246]. These three-cell 3D models are the foundation for several of the current projects in the Rajagopalan group.

1.7 Research Objectives

The motivation behind this work is the need for a multi-cellular organotypic liver model that can be used to recapitulate in vivo-like hepatotoxicity and liver disease. The design of such a model would allow for in vitro investigations to replace early in vivo studies and reduce the cost, time and resources of current testing. An accurate in vitro model can be used to identify cell-cell
interactions and cell-matrix interactions that become muddled *in vivo*. The models described in this thesis utilize ECM components found in the Space of Disse to mimic the native stratified architecture of the organ. We aim to demonstrate the versatility of these models in the investigation of chemical-induced hepatotoxicity and liver fibrosis. Our specific research objectives are

1. Investigate the ability of the multi-cellular organotypic liver models to identify *in vivo*-like hepatotoxicity through the use of a prototypic toxicant, APAP
2. Adapt the liver models for high-throughput hepatotoxicity testing of human and rodent cells
3. Utilize the liver models to study the role of the microenvironment in the progression of fibrosis from diseased to healthy tissue
Chapter 2: Investigating Acetaminophen Hepatotoxicity in Multi-Cellular Organotypic Liver Models


2.1 Introduction

The liver is primarily responsible for the biotransformation of many toxicants and xenobiotics [2]. Such biotransformation can induce hepatotoxicity, a phenomenon investigated in vivo and in vitro [5, 6, 247]. The metabolism of drugs and chemicals is accomplished through continuous interactions between parenchymal (hepatocytes) and non-parenchymal liver cells (NPCs). The NPCs are primarily liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs) and hepatic stellate cells (HSCs) [2]. Hepatocytes are separated from LSECs and KCs by a protein-rich interface called the Space of Disse. This interface is composed of extracellular matrix (ECM) components and plays a critical role in the transfer of nutrients and exchange of signaling molecules between the different cell types.

In vivo toxicity evaluations have clearly illustrated protective and inflammatory roles of NPCs when the liver is exposed to toxicants [5, 9]. LSECs serve as the first line of defense when a toxicant enters the hepatic microcirculation [248, 249]. These cells die upon exposure to toxicants, thereby protecting hepatocytes. Alterations to NPC functions and phenotypes occur upon liver injury, in a process known as activation [5, 207, 248]. For example, during liver injury, KCs secrete pro- and anti-inflammatory cytokines that recruit neutrophils to the site of injury and regulate oxidative stress and inflammation [5, 134, 248]. However, the mechanisms by which NPCs simultaneously ameliorate and exacerbate hepatotoxicity are not understood.
Despite the important contributions of NPCs towards hepatotoxicity, in vitro studies have focused on monocultures, such as the hepatocyte monolayer (HM) or collagen sandwich (CS) [5, 9]. These models do not capture the wide range of heterotypic inter-cellular interactions in the liver. Two-dimensional (2D) co-cultures have primarily focused on the effects of fibroblasts, epithelial cells, endothelial cells, or NPCs on hepatocyte function and phenotype [5, 9, 113]. Some 2D co-culture studies have reported hepatocyte-NPC interactions in the presence of toxicants [84, 93, 250]. These 2D systems are easy to assemble but are not representative of the native three-dimensional (3D) microenvironment.

To date, 3D models have been utilized to investigate the maintenance of hepatic phenotypes and hepatocyte toxicity [5]. Common 3D liver models include bioreactors [160, 251], microfluidic devices [171, 172], gel entrapped hepatocytes [85, 146], spheroids [138, 252], and scaffolds [173, 253]. To the best of our knowledge, these models have not been used to elucidate specific changes to NPCs upon toxicant exposure. In one study, Kostadinova et al. assembled a 3D hepatic model for toxicity with a NPC fraction (seeded on a Nylon scaffold) above hepatocytes [173]. The NPCs proliferated and secreted ECM proteins forming a Space of Disse mimic. However, detailed investigations into NPC-specific death and phenotypic changes were not reported. In summary, toxicity investigations using 3D liver models have not demonstrated how specific NPCs respond to a toxicant and how they alter signaling mechanisms. These gaps in knowledge can be addressed by the design of in vitro models that mimic the hepatic 3D microenvironment, where each cell type can be easily isolated for further analyses. Such models may enable accelerated investigations on efficacy and toxicity for drugs and chemicals.

We previously reported the design and assembly of 3D organotypic hepatic models comprised of primary rat hepatocytes, LSECs and KCs [174, 175, 241]. These liver-mimetic models contained a detachable membrane that mimicked the physical and chemical properties of the
Space of Disse. These models maintained hepatic functions and phenotypes over 16 days. A unique feature of these 3D models was that the ratio of hepatocytes to NPCs was identical to in vivo, demonstrating similarity to the native hepatic microenvironment [175]. Additionally, cytochrome P450 (CYP) 1A1/2 and CYP3A enzyme activities were maintained [174, 175]. Since CYP expression is critical for biotransformation, such 3D models are promising for conducting toxicity evaluations.

We report a detailed toxicity investigation on 3D organotypic hepatic models comprised of primary rat hepatocytes (H), LSECs (L), KCs (K), and a multilayer composed of alternating layers of type I collagen and hyaluronic acid. The 3D liver models of hepatocytes and LSECs (3DHL) or hepatocytes, LSECs and KCs (3DHLK) were compared to 2D co-cultures (2DHL) and hepatocyte monocultures (HM and CS). We tested the liver models by administering a prototypic hepatotoxicant, acetaminophen (APAP). Although APAP is nontoxic at therapeutic doses, acute drug induced liver injury occurs upon overdose [11]. In vivo, APAP is oxidized by CYP2E1 to the active metabolite N-acetyl-p-benzoquinoneimine (NAPQI). Excess NAPQI results in the depletion of glutathione (GSH), generation of reactive oxygen species and eventually necrosis [11, 19]. 2D and 3D models were investigated to determine the extent and mechanisms of cell death, changes in GSH and the ratio of aspartate aminotransferase (AST) to alanine aminotransferase (ALT). Cytokine secretion was investigated to determine if APAP toxicity resulted in inflammation in these models. A unique feature of these 3D models is the ability to monitor changes in NPC counts and phenotypes in response to the toxicant thereby providing a better understanding of APAP hepatotoxicity. Our goal was to determine how closely the 3D models compared to the biotransformation of APAP reported in rodents.
2.2 Materials and Methods

Dulbecco’s modified Eagle medium (DMEM), phosphate buffered saline (PBS, 10X), penicillin-streptomycin, human plasma fibronectin, primary KCs, 10% tris-glycine gels, and polyvinylidene fluoride (PVDF) membranes were purchased from Thermo Fisher Scientific (Carlsbad, CA). 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, glucagon, calcium chloride, hydrocortisone, sodium dodecyl sulfate, hydrogen peroxide, glutaraldehyde, calf thymus DNA, hyaluronic acid, sulfuric acid, collagenase type IV, protease inhibitor cocktail, and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were ordered from Fisher Scientific (Pittsburgh, PA) unless otherwise stated. All experimental procedures were approved by and conducted in accordance with Virginia Tech’s Institutional Biosafety Committee.

2.2.1 Extraction of Type I Collagen

Collagen was extracted from rat tails using previously described procedures [228]. Briefly, tendons were dissolved in acetic acid and centrifuged at 13,000 x g. A 30% (w/v) sodium chloride solution was slowly added to the supernatant and centrifuged at 8,500 x g. The resulting solution was dialyzed in 1 mN hydrochloric acid. The final collagen suspension was sterilized with chloroform and maintained at a pH of 3.1.

2.2.2 Isolation and Culture of Hepatocytes and LSECs

Primary hepatocytes and LSECs were isolated from female Lewis rats (175-199 g; Harlan Laboratories, Indianapolis, IN) using a two-step in situ collagenase perfusion method [174, 175, 241]. Animal care and the procedure for liver excision from rats were approved by and conducted in accordance with the Virginia Tech Institutional Animal Care and Use Committee. A typical surgical and isolation procedure resulted in 100-150 million hepatocytes with viability ≥
97% as determined through trypan blue exclusion. LSEC separation was conducted by differential adhesion and the cells were cultured in fibronectin-coated flasks [175, 241].

Hepatocytes were seeded as monolayers at 0.5 x 10^6 cells/well in a 12-well plate on collagen gels [175]. Wells were coated with 0.25 ml collagen and gelation was induced by increasing the temperature to 37 °C. In CS models, a second layer of collagen was added 24 h post-hepatocyte seeding.

All cultures were maintained in hepatocyte medium consisting of DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin-streptomycin, 0.5 IU/ml insulin (MP Biomedicals, Solon, OH), 14.3 ng/ml glucagon, and 7.6 μg/ml hydrocortisone. Spent culture medium was collected at the end of each 24 h period and maintained at -80 °C until analyzed. All cultures were maintained in a humidified gas environment at 37 °C containing 10% carbon dioxide.

2.2.3 Assembly of Multi-cellular Models

Primary rat hepatocytes were seeded on collagen gels. Collagen/hyaluronic acid multilayers were placed directly above the hepatocytes and hydrated with hepatocyte medium for approximately 1 h (refer to Section 2.5 for assembly of the multilayers). The assembly of these models occurred 72 h post hepatocyte seeding to allow the formation of a confluent monolayer. LSECs and KCs were seeded and cultured on hydrated multilayers. Initial seeding ratios were 1:20 (LSECs:hepatocytes) and 1:10 (KCs:hepatocytes). For 2D co-cultures, LSECs were seeded directly on the hepatocytes at a ratio of 1:20 (LSECs:hepatocytes) 72 h post-hepatocyte seeding [175].
When referring to a specific multi-cellular culture the following notation is used. The absence or presence of the collagen/hyaluronic acid multilayer is denoted by a “2D” or “3D” prefix, respectively. Rat hepatocytes, LSECs and KCs are abbreviated as “H”, “L” and “K”, respectively. A visual description of the types of multi-cellular models is presented in Figure 2.1. For a detailed description of changes in hepatocyte and NPC counts in each culture, refer to Figure 2.8.

Figure 2.1 Experimental set-up and design. Schematics of each of the models investigated for APAP toxicity (a) HM (b) CS (c) 2DHL (d) 3DHL (e) 3DHLK. (f) Timeline of model assembly, APAP addition and culture analysis where hepatocyte isolation is denoted as day 0. (g) Abbreviations used for the models investigated for APAP toxicity.
2.2.4 Administering APAP to Hepatic Cultures

APAP (CAS Number: 103-90-2) was dissolved in hepatocyte medium and administered on day 4 of culture for 24 h. The drug was administered at concentrations of 0 mM (untreated; control), 10 mM, 20 mM, and 40 mM. These concentrations were derived from in vivo rodent pharmacokinetic properties [254-256]. Additionally, these concentrations are consistent with those used for in vivo and in vitro studies (Table 2.1). Spent culture medium and cell lysates were extracted after the 24 h APAP incubation.

Table 2.1: Commonly used doses and concentrations of APAP in rodent models. The corresponding concentrations to each in vivo dose were calculated through the use of pharmacokinetic parameters [85, 120, 122, 160, 171, 252, 257, 258].

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<th>Dose (mg/kg)</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>~10</td>
</tr>
<tr>
<td>800</td>
<td>~20</td>
</tr>
<tr>
<td>1,500</td>
<td>~40</td>
</tr>
</tbody>
</table>

2.2.5 Determining the Mechanism of Hepatocyte Death in Response to APAP

The numbers of live, necrotic and apoptotic cells were determined using a commercially available kit (Apoptotic, Necrotic, and Healthy Cells Quantification Kit; Biotium, Hayward, CA). 24 h after APAP treatment, cells were incubated with 50 µL/ml of Hoechst 33342 (cell nuclei), ethidium homodimer III (necrotic) and FITC-annexin V (apoptotic). Imaging was performed on a Nikon TE2000 inverted microscope.

2.2.6 Measuring Overall Cell Viability

The overall cell viability of control and APAP-treated hepatic cultures was assayed using the MTT assay [259]. Cultures were incubated for 4 h with 0.5 mg/ml MTT in phenol-red free DMEM. The resulting formazan crystals formed in live cells were dissolved in 0.04 N hydrochloric acid (diluted in isopropanol). The absorbance was measured at 570 nm.
2.2.7 Changes in Hepatic Cell Counts Upon APAP Administration

To obtain information on changes in the number of LSECs and KCs in the multi-cellular models, the cells were labeled with nontoxic, fluorescent, membrane permeable dyes prior to seeding. LSECs and KCs were incubated with red (LSECs; PKH 26 Red Fluorescence Cell Linker Kit; Sigma-Aldrich) and green (KCs; PKH 2 Green Fluorescence Cell Linker Kit; Sigma-Aldrich) dye, respectively. Cytoplasmic labeling was performed as per the manufacturer’s protocols and imaged using a Nikon TE2000 inverted microscope. LSEC and KC viability in monocultures was determined by trypan blue exclusion. Hepatocyte viability in multi-cellular models was determined by measuring protein content.

2.2.8 Imaging LSECs and KCs by Immunostaining

2DHL, 3DHL and 3DHLK cultures were fixed in 2% (w/v) glutaraldehyde in PBS (1X) and sequentially exposed to 0.1% (v/v) Triton X-100 and a 1% (w/v) bovine serum albumin in PBS blocking solution with rabbit serum [175, 241]. The cultures were then incubated with a mouse anti-rat sinusoidal endothelial-1 antibody (CD32b; ImmunoBiological Laboratories, Minneapolis, MN) to identify LSECs. Thereafter, either a FITC-conjugated monoclonal ED-2 antibody (CD163; AbD Serotec, Bio-Rad, Hercules, CA) was added to identify KCs or a FITC-conjugated monoclonal CD31 antibody (Abcam, Cambridge, MA) was added to identify dedifferentiated LSECs. This was followed by incubation with a TRITC-conjugated secondary rabbit anti-mouse IgG antibody (Abcam) to visualize LSECs. Imaging was conducted on a Zeiss LSM confocal microscope (Oberkochen, Germany).

2.2.9 Measuring the Concentrations of ALT and AST

The concentrations of ALT and AST in spent hepatocyte culture medium were determined using commercially available kits (ALT (SGPT) Reagent (Colorimetric, Endpoint Method; Teco Diagnostics, Anaheim, CA) and AST (SGOT) Reagent (Colorimetric, Endpoint Method; Teco
Manufacturer recommended protocols were followed. The absorbance was measured at either 505 nm (ALT) or 530 nm (AST). A calibrator (Teco Diagnostics) was used to convert absorbance values to enzyme concentrations.

2.2.10 Measuring Changes in GSH in Hepatocytes

In 2DHL cultures, LSECs were separated from the hepatocytes using a magnetic cell separation process [175]. Briefly, the CD32b antibody was conjugated to Dynabeads® (CELLection™ Kit; Invitrogen Thermo Fisher Scientific) [260]. The LSEC fraction adhered to the beads and was separated using a magnet (DynaMag™, Invitrogen Thermo Fisher Scientific). The supernatant contained the hepatocyte fraction. In the 3DHL and 3DHLK cultures, the hepatocyte fraction was obtained upon removal of the multilayer.

Hepatocytes were lysed in PBS (1X) containing 1 mM ethylene diamine tetra-acetic acid maintained at 4 °C. The lysates were centrifuged at 10,000 x g for 15 min, and the supernatant was collected and stored at -20 °C. Total GSH was measured using a commercially available colorimetric kinetic assay kit (Glutathione Assay Kit; Cayman Chemicals, Ann Arbor, MI). The absorbance was measured at 405 nm every 5 min for 30 min. The total GSH concentration was determined by calibrating to a standard curve.

2.2.11 Western Immunoblotting for CYP2E1

The hepatocyte fraction from each culture was lysed and incubated with a protease inhibitor cocktail of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, E-64, leupeptin, and pepstatin A. The total protein concentration was measured using a commercially available kit (Coomassie (Bradford) Protein Assay Kit; Thermo Scientific). Gel electrophoresis was performed with a 10% tris-glycine gel followed by protein transfer to a PVDF membrane. CYP2E1 and β-actin (housekeeping protein) were identified through exposure to primary
antibodies for CYP2E1 (rabbit polyclonal anti-cytochrome P450 enzyme CYP2E1; EMD Millipore, Billerica, MA) and β-actin (rabbit monoclonal anti-β-actin; Cell Signaling Technology, Danvers, MA), respectively. The membrane was exposed to a secondary horseradish peroxidase (HRP)-conjugated antibody (anti-rabbit IgG; Bio-Rad). The membrane was developed with a chemiluminescent HRP substrate (Clarity Western ECL Substrate; Bio-Rad) and imaged on a Chemidoc™ XRS+ system (Bio-Rad). Image analysis was conducted using Image Lab™ software (Bio-Rad).

2.2.12 Assaying the Levels of Pro- and Anti-Inflammatory Cytokines

Interleukin 10 (IL-10) concentration was measured using a commercially available kit (Rat IL-10 ELISA Kit; Thermo Scientific). To measure the levels of tumor necrosis factor alpha (TNF-α), spent culture medium samples were concentrated through the use of Vivaspin™ 2 Centrifugal Concentrators (Sartorius, Goettingen, Germany). The concentration of TNF-α was measured using a commercially available kit (Rat TNF-alpha Quantikine ELISA Kit; R&D Systems, Minneapolis, MN). The interferon gamma (IFN-γ) concentration was measured using a commercially available kit (IFN-γ ELISA Kit, Rat; Thermo Scientific). The concentrations of IL-10, TNF-α and IFN-γ were individually determined by calibrating to standard curves.

2.2.13 Statistical Analysis

Statistical significance and $p$-values were calculated by a Student’s t-test, assuming unequal variance while applying the Bonferroni (multiple hypothesis testing) correction. Statistical significance, unless specifically noted, compared untreated (0 mM APAP) to each treatment condition (10, 20 and 40 mM APAP). Reported $p$-values are the products of individual $p$-values and the number of comparisons. For all significance testing $\alpha = 0.05$. All data are reported as mean ± standard deviation; $n$ denotes sample size. The data presented in this report were
collected and analyzed from up to five independent experiments, with three replicates per experiment.

2.3 Results

Most *in vitro* studies report changes in viability, hepatocyte function and CYP expression [122, 160, 171]. While this information is very helpful, the role of NPCs has largely been ignored in *in vitro* hepatotoxicity investigations. We report how i) APAP affects the model as a whole, ii) it affects each cell type, iii) it affects the secretion of signaling molecules, and iv) the incorporation of NPCs can begin to address the gap between *in vivo* studies and *in vitro* models. We have broadly classified our results with hepatocyte monocultures (HM and CS) and 2D and 3D multicellular liver models (2DHL, 3DHL, and 3DHLK) into three sections that provide information at different levels of investigation:

A. Changes in viability of each cell type
B. Maintenance of NPC phenotypes
C. Changes in liver toxicity markers

2.3.1 Changes in Viability of Each Cell Type in Response to APAP

Changes in overall viabilities of each culture model upon APAP administration were investigated using the MTT assay. HM, CS and 2DHL cultures only exhibited significant cell death at 40 mM APAP (approximately 30%; \( p < 0.05 \)) (Figure 2.2). In contrast, the 3DHL and 3DHLK models exhibited approximately 10% cell death at 10 mM and approximately 20% cell death at 20 mM (\( p < 0.05 \)). Viability decreased by approximately 45% in the 3D models at 40 mM APAP (\( p < 0.05 \)). These results demonstrate that the 3D models exhibited greater sensitivity to APAP.
Figure 2.2: Cell viability of APAP-treated models relative to untreated conditions measured with the MTT assay. Changes in viability of all cell types were determined 24 h after APAP administration. Results are expressed as percent of control, mean ± SD, n ≥ 3, *p < 0.05 relative to untreated samples. Statistically significant cell death occurred at 20 mM APAP in only the 3D models.

Since the MTT assay measures the viability of the entire culture, detailed studies were undertaken to determine effects of APAP exposure on each cell type. APAP exposure of hepatocytes decreased protein content in the 3D models by 20% whereas hepatocyte protein levels in the 2DHL cultures decreased by 32% (Table 2.2). The effects of APAP on LSECs and KCs were greater than on hepatocytes.

Apoptosis/necrosis staining was conducted to investigate the mechanism of hepatocyte death (Figure 2.3). In the CS and 3D models, the primary mode of cell death was necrosis, consistent with prior studies on APAP [11, 19]. At 20 mM APAP, apoptotic cells in CS (3.2 ± 0.8%), 3DHL (7.4 ± 1.0%) and 3DHLK cultures (11.5 ± 3.2%) were a small fraction of total cell death. In contrast, HM and 2DHL models exhibited a high percentage of apoptotic cells. Even untreated cultures exhibited 23.3 ± 6.6% (HM) and 15.0 ± 4.8% (2DHL) apoptotic cells.
Figure 2.3: Analyzing the type of hepatocyte cell death in response to APAP. Representative images of live (Hoechst dye, blue), necrotic (ethidium homodimer III, red) and apoptotic (annexin V, green) cells in the liver models treated with 0 mM (a-e) and 40 mM (f-j) APAP. The graphs depict the percentages of live (blue), necrotic (pink) and apoptotic (green) cells calculated in each culture after treatment with APAP for 24 h for (k) HM, (l) CS, (m) 2DHL, (n) 3DHL, and (o) 3DHLK cultures, mean ± SD, n = 3, *p < 0.05 relative to untreated samples. There are an increased number of apoptotic cells in the HM and 2DHL models in both untreated and treated models relative to the other cultures. In the CS, 3DHL and 3DHLK models there is a significant increase in the number of necrotic cells in response to APAP treatment.
Table 2.2: Changes in cellular responses to APAP in 2D and 3D multi-cellular liver models and NPC monolayers. Effect of APAP on hepatocytes was determined through changes in protein content. Effects on LSECs and KCs were determined by cell counts, comparing numbers prior and 24 h post APAP-treatment. Results expressed as percent of control (0 mM APAP), mean ± SD, n = 3.

<table>
<thead>
<tr>
<th>APAP Concentration (mM)</th>
<th>Hepatocytes (%)</th>
<th>LSECs (%)</th>
<th>KCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2DHL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 5.1</td>
<td>100.0 ± 10.7</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>97.8 ± 11.3</td>
<td>84.6 ± 11.1</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>86.0 ± 8.4</td>
<td>81.8 ± 10.7</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>66.4 ± 9.4</td>
<td>56.0 ± 14.2</td>
<td>-</td>
</tr>
<tr>
<td><strong>3DHL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 7.3</td>
<td>100.0 ± 10.6</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>112.6 ± 17.3</td>
<td>74.2 ± 5.1</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>106.7 ± 1.9</td>
<td>57.6 ± 8.4</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>81.2 ± 4.0</td>
<td>45.7 ± 7.0</td>
<td>-</td>
</tr>
<tr>
<td><strong>3DHLK</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100.0 ± 5.1</td>
<td>100.0 ± 11.6</td>
<td>100.0 ± 12.8</td>
</tr>
<tr>
<td>10</td>
<td>110.7 ± 1.1</td>
<td>68.7 ± 6.0</td>
<td>114.7 ± 9.0</td>
</tr>
<tr>
<td>20</td>
<td>96.3 ± 0.7</td>
<td>60.1 ± 10.8</td>
<td>134.3 ± 18.3</td>
</tr>
<tr>
<td>40</td>
<td>78.6 ± 1.0</td>
<td>38.5 ± 8.4</td>
<td>52.5 ± 9.5</td>
</tr>
<tr>
<td><strong>LSEC Monolayer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>100.0 ± 13.7</td>
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<td>10</td>
<td>-</td>
<td>102.1 ± 8.4</td>
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<tr>
<td>20</td>
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<td>94.9 ± 1.6</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>107.3 ± 8.7</td>
<td>-</td>
</tr>
<tr>
<td><strong>KC Monolayer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>100.0 ± 3.1</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>96.3 ± 13.3</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>-</td>
<td>100.5 ± 18.5</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>-</td>
<td>89.5 ± 8.5</td>
</tr>
</tbody>
</table>

At all concentrations of APAP, LSEC and KC monolayers exhibited at most 10% cell death (Table 2.2). Hepatocytes in monocultures easily change phenotype, so the absence of APAP sensitivity in monolayers may be attributed to their de-differentiation, inability to metabolize APAP and the lack of exposure to subsequent NAPQI-related effects [11, 19, 261, 262]. In the multi-cellular liver models, LSEC death was significantly higher in the 3D cultures compared to the 2DHL cultures in response to APAP and exceeded that of hepatocytes (Table 2.2). This is consistent with in vivo studies that reported significant LSEC death upon APAP administration [248, 250]. LSEC death at 10 and 20 mM APAP was 33% and 40% in the 3DHLK cultures,
respectively, and 14% and 18% in the 2DHL model, respectively. In the 3DHLK cultures, KCs appeared to proliferate upon exposure to 10 and 20 mM APAP (approximately 15% and 34%, respectively) relative to untreated samples ($p < 0.05$). Similar proliferation of KCs has been observed upon liver injury [263, 264].

### 2.3.2 Maintenance of NPC Phenotypes in Response to APAP

As no previous studies have reported APAP-induced changes to NPC phenotypes, phenotypic markers of NPCs were investigated for multi-cellular models using immunofluorescence staining for CD32b (LSECs) and CD163 (KCs). LSEC counts were measured using CD32b staining (phenotypic marker) and a non-specific cytoplasmic dye. The difference in number of LSECs measured with the two dyes was statistically insignificant in the 3D models (Table 2.4). In contrast, untreated 2DHL cultures exhibited fewer CD32b-expressing cells compared to 3DHL or 3DHLK models (Figure 2.4). The number of CD32b-expressing LSECs was statistically lower in the 2DHL models than those counted using cytoplasmic staining (Table 2.4), suggesting the possibility of dedifferentiation. In the 3DHLK cultures, KCs stained for CD163 after exposure to all APAP concentrations, with some decrease observed upon 40 mM APAP, a similar feature was exhibited when the cytoplasmic stain was used. The similarity of staining characteristics of NPCs in the 3D models suggests that they maintain phenotypic markers (Table 2.4).

We further investigated the decrease in CD32b-expressing cells associated with an increase in LSECs expressing CD31 (a marker for dedifferentiated LSECs) (Figure 2.5; further information in Table 2.5) [265]. Cells exhibiting both CD31 and CD32b were identified as dedifferentiated. A similar observation was made with the 2D co-cultures, as they exhibited the largest number of dedifferentiated LSECs. CD31-expressing cells in 2DHL cultures were up to 7-fold higher than corresponding 3DHLK models. Similarly, CD31-expressing cells in 3DHL cultures were up to
2.6-fold greater than in 3DHLK models. Based on these comparisons, it was evident that KCs improved the maintenance of the LSEC phenotype.

**Figure 2.4:** Immunostaining of NPCs with CD32b (red) and CD163 (green) in multi-cellular 2D and 3D models. APAP was administered 24 h prior to obtaining images. CD32b stains for LSECs and is more highly expressed in the 3D models than the 2D models. CD163 stains for KCs and is expressed at all concentrations of APAP, but visibly decreased only at 40 mM APAP.

**Figure 2.5:** Immunostaining of NPCs with CD32b (red) and CD31 (green) in multi-cellular 2D and 3D models. APAP was administered 24 h prior to obtaining images. Yellow arrows denote co-localization and cells that are dedifferentiating. CD32b is expressed in LSECs that have maintained phenotype, whereas CD31 is expressed in LSECs that have dedifferentiated. Increased dedifferentiation of LSECs occurred in the 2DHL models relative to the 3D models.
2.3.3 Changes in Liver Toxicity Markers in Response to APAP

2.3.3.1 Investigating Changes in ALT and AST Concentrations

The concentrations of ALT and AST in spent culture medium were measured to assess hepatic injury. In serum, an AST/ALT ratio greater than 2.0 is a clinical marker for liver damage [266]. This ratio ranged from 0.64-1.22 in the 2D models upon exposure to 10 and 20 mM APAP (Figure 2.6a). In the 3DHL models, the AST/ALT ratio was 1.33 ± 0.25 and 2.12 ± 0.08 at 10 and 20 mM APAP, respectively. In the 3DHLK cultures, this ratio was 1.73 ± 0.46 and 2.46 ± 0.17 at these concentrations. While all models exhibited AST/ALT ratios greater than 2.0 at 40 mM APAP, this ratio was 4.28 ± 0.69 and 4.67 ± 0.48 in the 3DHL and 3DHLK cultures, respectively.

2.3.3.2 Measuring Changes in Hepatocyte GSH and CYP2E1

The concentration of total GSH was measured in hepatocyte lysates (Figure 2.6b). GSH decreased 22-40% in all cultures upon administration of 10 mM APAP ($p < 0.05$). At 20 mM APAP, GSH decreased by approximately 70% ($p < 0.05$) and 45% ($p < 0.05$) in 2D and 3D models, respectively. The presence of NPCs in the 3D models may have contributed to the maintenance of GSH. Western immunoblotting of CYP2E1 was conducted on hepatocyte lysates and was detected in the 3D cultures treated with APAP. HM, CS and 2DHL cultures did not exhibit well-defined bands for CYP2E1 at any APAP concentration (Figure 2.7a).
Figure 2.6: Changes in liver enzymes and hepatocyte GSH. (a) Ratio of AST to ALT in culture medium following treatment with APAP for 24 h. The ratio of AST to ALT exceeds 2.0 in vivo during liver damage [267]. This was observed in all models upon exposure to 40 mM APAP. However, AST/ALT ratios only exceeded 2.0 after 20 mM APAP in the 3D liver models. (b) GSH content in the hepatocyte fraction of all cultures following treatment with APAP for 24 h. At 20 mM APAP, GSH is depleted approximately 45% in the 3D liver models and approximately 70% in the 2D models, suggesting possible hepatoprotection in the 3D models, mean ± SD, $n \geq 3$, *$p < 0.05$ relative to untreated samples.
2.3.3.3 Measuring Changes in Pro- and Anti-inflammatory Cytokines

IL-10 (anti-inflammatory), TNF-α (pro-inflammatory) and IFN-γ (pro-inflammatory) are cytokines secreted primarily by KCs, although there is evidence of secretion by other liver cells (Hinson et al., 2010). Cytokine levels were measured to determine association of APAP toxicity with inflammation. In untreated HM, CS, 2DHL, and 3DHL cultures, the concentrations of these cytokines were statistically insignificant from fresh culture medium (Table 2.3). In untreated 3DHLK cultures, cytokine concentrations were at least 35-fold (IL-10), 2.5-fold (TNF-α) and 1.6-fold (IFN-γ) higher than the other cultures. These data suggest that the three cytokines were primarily secreted by KCs in vitro, which was subsequently verified by determination of cytokine secretion in spent culture medium from 3DHLK cultures. IL-10 decreased approximately 4-fold after exposure to 20 mM APAP (Figure 2.7b), whereas IFN-γ and TNF-α increased by approximately 50% and 11%, respectively (Figures 2.7c and 2.7d). At 40 mM APAP, due to excessive KC death, all cytokine concentrations decreased. Furthermore, AST secretion in the 3DHLK models was approximately 1.5-fold higher than the 3DHL cultures. The proposed interaction of these observations is presented in (Figure 2.7e) [268], which suggests KC proliferation at this concentration could have resulted in increased pro-inflammatory cytokine secretion. This increase in IFN-γ upon APAP exposure may relate to the increase in hepatocyte apoptosis and higher AST secretion.

To determine if the proximity of hepatocytes and NPCs in the 3DHLK models contributed to changes in cytokine secretion, values obtained from 3DHLK cultures were compared to those from hepatic cell monocultures (Table 2.6). Cytokine levels of fresh medium and hepatocyte or LSEC monocultures were found to be statistically insignificant. IFN-γ was significantly higher in the 3DHLK models than KC monocultures from 0-20 mM APAP. At 40 mM APAP, there was significantly higher TNF-α in the 3DHLK cultures than in KC monocultures.
Figure 2.7: Markers of oxidative stress associated with APAP-induced toxicity. (a) Western immunoblotting of hepatocyte lysates for cytochrome P450 2E1 (CYP2E1) and β-actin (housekeeping protein). Cytokine secretion in the 3DHLK models measured (b) IL-10, (c) TNF-α and (d) IFN-γ mean ± SD, n = 3, *p < 0.05 relative to untreated samples. All cultures were treated with APAP for 24 h prior to obtaining hepatocyte lysates or cytokine concentrations. (e) Pathway depicting how cytokines secreted by KCs impact hepatocyte viability and functions. The connection between increased IFN-γ secretion, hepatocyte apoptosis and AST/ALT is shown. Boxes shaded in blue correspond to measurements reported in this study. Boxes shaded in green correspond to signaling pathways reported in the literature.
Table 2.3: Cytokine concentration in unused hepatocyte medium (denoted as “medium”) and untreated (0 mM APAP) liver models, mean ± SD, n = 3. Cytokine is listed as “undetectable” when the concentration was below the detection limit provided by the manufacturer.

<table>
<thead>
<tr>
<th>Model</th>
<th>IL-10 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>3.06 ± 0.08</td>
<td>Undetectable</td>
<td>3.38 ± 0.31</td>
</tr>
<tr>
<td>HM</td>
<td>3.34 ± 0.72</td>
<td>Undetectable</td>
<td>3.90 ± 0.26</td>
</tr>
<tr>
<td>CS</td>
<td>3.42 ± 1.03</td>
<td>Undetectable</td>
<td>3.42 ± 0.07</td>
</tr>
<tr>
<td>2DHL</td>
<td>3.50 ± 0.24</td>
<td>Undetectable</td>
<td>3.97 ± 0.52</td>
</tr>
<tr>
<td>3DHL</td>
<td>3.73 ± 0.67</td>
<td>Undetectable</td>
<td>2.98 ± 0.15</td>
</tr>
<tr>
<td>3DHLK</td>
<td>132.50 ± 32.32</td>
<td>8.61 ± 0.86</td>
<td>6.21 ± 0.39</td>
</tr>
</tbody>
</table>

2.4 Discussion

The gap between in vivo and in vitro toxicity studies can lead to differing results. In this study, responses of a 3D multi-cellular liver model were examined in the presence of a prototypic hepatotoxicant, APAP. We used this model to obtain a more comprehensive understanding of changes to parenchymal and non-parenchymal cells when exposed to this toxicant.

When the liver is exposed to toxicants, NPCs become activated, resulting in altered function and phenotype [5, 19, 207, 248]. For example, LSECs contract and lose their fenestrae and scavenging abilities [5, 19, 248]. Activated KCs proliferate and secrete pro- and anti-inflammatory cytokines [5, 19]. The varying profiles and altered phenotypes of NPCs during liver toxicity are important factors to consider when testing the efficacy or toxicity of drugs and chemicals.

In order to isolate the effect of NPCs, 3D models were also assembled that contained only a collagen/hyaluronic acid multilayer above the hepatocyte layer (no NPCs; denoted as 3DH). The response to APAP in the 3DH cultures was similar to 2D cultures (Figure 2.9). These data suggest that the inclusion of NPCs was critical to the maintenance of sensitivity to APAP that occurred within the 3D models.
Closer examination of each cell type in the 3DHL and 3DHLK models revealed additional information. For example, hepatocyte death in these models occurred primarily via necrosis, identical to studies conducted in vivo [11, 19]. However, increased secretion of IFN-γ by KCs resulted in approximately 11% apoptosis in the 3DHLK models [268]. In contrast, HM and 2DHL cultures exhibited significant apoptosis even in the absence of APAP suggesting that they are less suitable for studies on mechanisms associated with cell death. Although, hepatocytes in the 3D models exhibited increased expression of CYP2E1 and other markers of toxicity, the improved albumin secretion suggests that the NPCs acted to support the maintenance of hepatocyte-specific functions. The loss of CYP2E1 protein in the 2D models is consistent with reports in the literature that demonstrate 2D co-cultures do not maintain hepatocyte CYP2E1 expression [269]. The lack of expression of this enzyme could explain the insensitivity to APAP in the 2D cultures (Figure 2.7A; Figure 2.10).

In vivo studies have reported that the sinusoidal endothelium suffers the greatest initial impact upon exposure to hepatotoxins [248, 250, 270]. In the present study, this was only observed in the 3D cultures. Additionally, only the 3D models consistently expressed CD32b, a marker of LSEC phenotype [271]. Interestingly, the addition of KCs resulted in the simultaneous maintenance of LSEC phenotype and increased LSEC sensitivity to APAP. Although the exact mechanisms for LSEC death are not fully understood, we hypothesize that the decrease in IL-10 may have played a role. There is evidence that LSECs undergo classical and alternative activation due to exposure to cytokines [270]. Specifically, removal of IL-10 from culture medium resulted in the transition of LSEC phenotype from M2 (anti-inflammatory) to M1 (pro-inflammatory). Such LSEC activation is also associated with the production of inducible nitric oxide synthase, which is directly correlated to cell death in response to APAP [272]. This could
explain the increased LSEC death exhibited in the **3DHLK** models and merits further investigation.

The proliferation and activation of KCs results in increased inflammation [263, 264]. In the present study, cell death in KC monocultures was minimal and did not correlate to APAP concentration, suggesting loss of sensitivity. In direct contrast, KC proliferation and death in the **3DHLK** models was dependent on APAP concentration. Furthermore, APAP treatment resulted in up to a 9-fold decrease in IL-10 with a concomitant increase in TNF-α and IFN-γ. Taken together, these results indicate that in response to APAP, KC activation results in inflammation in the **3DHLK** models.

*In vivo* hepatotoxicity studies have demonstrated that increased liver toxicity occurs through the production of pro-inflammatory cytokines [273] or decrease of anti-inflammatory cytokines [272]. Changes in cytokine concentrations in the **3DHLK** models indicate a shift in KC phenotype from M2 to M1 upon exposure to APAP. Paracrine signaling appears to have modulated cytokine secretion. For example, at 40 mM APAP, the TNF-α secretion was higher in the **3DHLK** models than the KC monocultures. This can be attributed to increased TNF-α secretion from hepatocytes in the presence of KCs [5, 120, 274]. The secretion of IFN-γ was also significantly higher in the **3DHLK** models. These results suggest that the increases in TNF-α and IFN-γ were facilitated through heterotypic inter-cellular interactions and merit further investigation.

It is critically important to note that at 20 mM APAP (800 mg/kg APAP, Table 2.1), only the 3D models exhibited AST/ALT ratios from 2.1-2.5, identical to values obtained *in vivo* [257, 258]. At 10 and 20 mM APAP, the increased AST/ALT ratio in the **3DHLK** models parallels that reported previously [268]. *In vivo*, rats treated with 800 mg/kg APAP exhibited a 40% reduction in GSH
At the corresponding \textit{in vitro} concentration (20 mM) only the 3D models exhibited a 45% decrease in GSH. At longer time-points only the 3D models exhibited sensitivity to APAP. This property makes the 3D models relevant for investigations that require longer testing periods (further information can be found in the Figure 2.11).

The strong relationships between the 3D models and data obtained \textit{in vivo} demonstrate the potential use of these multi-cellular models for drug testing. These appear to be excellent 3D \textit{in vitro} hepatic models for emulating changes observed in hepatic cell types, closely mimicking events that occur in livers of APAP-treated rodents. The ability to include and isolate NPCs in organotypic liver models described here provides capability to conduct systematic measurements of toxicant effects on each cell type. The future inclusion of HSCs in the 3D models will further increase insights into HSC-hepatocyte interactions that modulate response to toxicants. Experiments designed to delineate intricate signaling pathways between NPCs and hepatocytes will provide additional insights. Our current experiments demonstrate that the 3D organotypic models offer potential for a new avenue to bridge the gap between \textit{in vitro} and \textit{in vivo} toxicity investigations.

\section*{2.5 Supplementary Material}

\subsection*{2.5.1 Supplementary Data}

\subsubsection*{2.5.1.1 Assembly of a Detachable Polymeric Space of Disse}

Type I collagen (cationic) and hyaluronic acid (anionic) were used to assemble detachable multilayers as previously described [275]. Collagen was dissolved in 1\% (v/v) glacial acetic acid in 18 MΩ•cm deionized water and hyaluronic acid was dissolved in 18 MΩ•cm deionized water. Both solutions were maintained at a concentration of 1.5 mg/ml at a pH of 4.0. Hydrophobic poly-tetrafluoroethylene (McMaster-Carr, Elmhurst, IL) substrates were prepared as previously
reported [175, 275]. Collagen/hyaluronic acid multilayers were assembled using a robotic deposition process (Nano-Strata, Tallahassee, FL). The absorption time for each polyelectrolyte layer was 30 min with a deionized water rinse step (10 min) between depositions of successive layers. A bilayer is defined as the deposition of one cationic (collagen) and one anionic (hyaluronic acid) polyelectrolyte. Fifteen bilayer multilayers were cross-linked with 8% (w/v) glutaraldehyde for 30 s, rinsed and vacuum dried and have been previously characterized [275]. Collagen/hyaluronic acid multilayers were sterilized under germicidal UV for 1 h before cell culture.

2.5.1.2 Investigating Untreated (0 mM APAP) 2D and 3D cultures

The trends in proliferation of hepatocytes, LSECs and KCs were investigated in untreated cultures from day 4 to 7 (Figure 2.8). These measurements were conducted to ascertain the stability of the models over the culture period. The number of hepatocytes in CS, 3DHL and 3DHLK cultures was consistently 70-85% higher than corresponding HM or 2DHL models. There was no significant change in the number of hepatocytes over time for any model. LSEC proliferation was observed only in the 3D cultures. In vivo, the hepatocyte to LSEC ratio is reported to be approximately 6:1 [175]. This ratio increased from 5.8:1 to 11.3:1 in 2DHL cultures from day 4 to day 7 due to extensive LSEC death. In contrast, this ratio was approximately 8.0:1 and 7.0:1 on day 7 in the 3DHL and 3DHLK models, respectively. These data demonstrated that the 3D models promoted optimal cellular ratios and were therefore appropriate for further investigations on hepatotoxicity.

2.5.1.3 Investigating APAP Toxicity on Day 7

To determine if the 3D liver models would exhibit APAP sensitivity at a later time point, the drug was administered on day 6. Cell death (determined by MTT) was not observed in HM cultures even at 40 mM APAP suggesting a complete loss of sensitivity at this time point (Figure 2.10a).
The CS and 2DHL models exhibited only an approximately 20% decrease in viability at 40 mM APAP ($p < 0.05$). In the 3D models, overall decreases in viability were approximately 5% and 15% at 10 mM and 20 mM, respectively. These values were similar to those obtained on day 5 (Figure 2.2) indicating hepatic cells in the 3D models maintained their sensitivity to APAP. The response of the NPCs to APAP was also monitored on day 7 (Figure 2.10b). Trends in LSEC death were consistent with observations from day 5 (Table 2.2). Once again, KCs exhibited proliferation at 20 mM APAP and significant death at 40 mM APAP.

On day 7, the AST/ALT ratios in the 3D models were within 15% of the values on day 5 at 10 mM and 20 mM APAP (Figure 2.6 and Figure 2.10c) indicating continued toxicity. The concentration of ALT on day 7 was within 20% of the value on day 5 in the 3D models when treated with 40 mM APAP (Figure 2.10d). In contrast, on day 7, the ALT concentration in the 2D models decreased up to 80%. Since ALT is a liver specific enzyme, these trends demonstrate that the 3D models maintain hepatic phenotypes better than the 2D models [266, 267]. These results suggest that even at later time points, the organotypic 3D liver models are sensitive to APAP and hold promise for hepatotoxicity testing.

2.5.1.4 Measuring Changes in Albumin Secretion

Hepatocyte function was determined by measuring albumin secretion in spent culture medium. The albumin concentration was established by an enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody to rat albumin [276]. The albumin concentration was measured at 490 nm. Standard curves were generated using diluted rat albumin in hepatocyte medium.

Changes in albumin secretion by hepatocytes were investigated in all models (Figure 2.11a). In general, the 3D models maintained higher levels of albumin than the hepatocyte-only models in response to APAP. These data indicate that despite the increased reduction in the number of
NPCs in the 3D models, damage to hepatocyte-specific functions was reduced. This can be attributed to the collagen/hyaluronic acid multilayer and NPCs protecting the hepatocytes from APAP toxicity. The trends in albumin secretion on day 7 (Figure 2.11b) were similar to those obtained on day 5 wherein HM, CS and 2DHL cultures exhibited greater decreases in albumin secretion than the 3D cultures.

Figure 2.8: Time-dependent changes in (a) hepatocytes and (b) NPCs in untreated models from day 4 to day 7 in culture, mean ± SD, n = 3 *p < 0.05 relative to day 4 cell numbers.
**Table 2.4:** Comparison of NPC counts as determined through cytoplasmic staining and antibody staining on day 5 of culture after APAP treatment for 24 h treatment. The cytoplasmic stain was conducted by incubating the cells with membrane permeable dyes prior to their addition to the multi-cellular cultures. The antibody stain was conducted by immunofluorescence staining for phenotypic markers (CD32b for LSECs and CD163 for KCs), mean ± SD, n ≥ 9 images per condition.

<table>
<thead>
<tr>
<th>APAP Concentration (mM)</th>
<th>Cytoplasmic Stain</th>
<th>Antibody Stain</th>
<th>Statistical Significance?</th>
</tr>
</thead>
<tbody>
<tr>
<td>2DHL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>38,943 ± 4,172</td>
<td>27,957 ± 3,976</td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>32,958 ± 4,314</td>
<td>25,805 ± 3,142</td>
<td>Yes</td>
</tr>
<tr>
<td>20</td>
<td>31,856 ± 4,172</td>
<td>23,985 ± 3,269</td>
<td>Yes</td>
</tr>
<tr>
<td>40</td>
<td>21,813 ± 5,544</td>
<td>20,856 ± 4,411</td>
<td>No</td>
</tr>
<tr>
<td>3DHL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50,025 ± 5,313</td>
<td>47,534 ± 6,531</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>37,140 ± 2,564</td>
<td>41,622 ± 6,055</td>
<td>No</td>
</tr>
<tr>
<td>20</td>
<td>28,826 ± 4,216</td>
<td>28,486 ± 4,731</td>
<td>No</td>
</tr>
<tr>
<td>40</td>
<td>22,878 ± 3,516</td>
<td>21,162 ± 2,869</td>
<td>No</td>
</tr>
<tr>
<td>3DHLK (LSECs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>60,932 ± 7,053</td>
<td>58,807 ± 5,750</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>41,849 ± 3,642</td>
<td>36,636 ± 4,965</td>
<td>No</td>
</tr>
<tr>
<td>20</td>
<td>36,614 ± 6,566</td>
<td>34,059 ± 4,940</td>
<td>No</td>
</tr>
<tr>
<td>40</td>
<td>23,466 ± 5,095</td>
<td>22,757 ± 4,122</td>
<td>No</td>
</tr>
<tr>
<td>3DHLK (KCs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>16,980 ± 2,180</td>
<td>16,174 ± 1,819</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>19,484 ± 1,524</td>
<td>18,471 ± 4,492</td>
<td>No</td>
</tr>
<tr>
<td>20</td>
<td>22,803 ± 3,100</td>
<td>18,241 ± 4,350</td>
<td>Yes</td>
</tr>
<tr>
<td>40</td>
<td>8,916 ± 1,606</td>
<td>8,447 ± 1,149</td>
<td>No</td>
</tr>
</tbody>
</table>
Table 2.5: Measurement of the fraction of dedifferentiated LSECs in the multi-cellular models on day 5 of culture after APAP treatment for 24 h. The fractions were determined through dual immunofluorescence staining for CD32b (LSECs) and CD31 (dedifferentiated LSECs), mean ± SD, n = 6 images per condition.

<table>
<thead>
<tr>
<th>APAP Concentration (mM)</th>
<th>Fraction Dedifferentiated LSECs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2DHL</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.475 ± 0.062</td>
</tr>
<tr>
<td>10</td>
<td>0.573 ± 0.123</td>
</tr>
<tr>
<td>20</td>
<td>0.390 ± 0.110</td>
</tr>
<tr>
<td>40</td>
<td>0.334 ± 0.062</td>
</tr>
<tr>
<td><strong>3DHL</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.127 ± 0.021</td>
</tr>
<tr>
<td>10</td>
<td>0.129 ± 0.024</td>
</tr>
<tr>
<td>20</td>
<td>0.123 ± 0.058</td>
</tr>
<tr>
<td>40</td>
<td>0.208 ± 0.063</td>
</tr>
<tr>
<td><strong>3DHLK</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.104 ± 0.034</td>
</tr>
<tr>
<td>10</td>
<td>0.083 ± 0.044</td>
</tr>
<tr>
<td>20</td>
<td>0.094 ± 0.053</td>
</tr>
<tr>
<td>40</td>
<td>0.079 ± 0.024</td>
</tr>
</tbody>
</table>

Table 2.6: Comparison of cytokine secretion of KC monolayers against 3DHLK models on day 5 of culture after APAP treatment for 24 h, mean ± SD, n = 3. Results are listed as “undetectable” when the concentration was below the sensitivity limit provided by the manufacturer.

<table>
<thead>
<tr>
<th></th>
<th>APAP Concentration (mM)</th>
<th>KC Monolayer (pg/mL)</th>
<th>3DHLK (pg/mL)</th>
<th>Statistical Significance?</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>84.20 ± 9.37</td>
<td>131.97 ± 32.32</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>16.30 ± 2.21</td>
<td>39.23 ± 27.83</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>9.22 ± 1.09</td>
<td>34.17 ± 17.10</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>10.53 ± 1.42</td>
<td>13.66 ± 2.01</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>11.59 ± 1.56</td>
<td>8.64 ± 0.86</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.74 ± 0.97</td>
<td>9.52 ± 0.69</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>8.73 ± 1.26</td>
<td>9.64 ± 1.96</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Undetectable</td>
<td>9.85 ± 1.80</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.33 ± 0.26</td>
<td>6.21 ± 0.39</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4.82 ± 0.15</td>
<td>9.18 ± 0.94</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>5.75 ± 0.25</td>
<td>9.61 ± 0.57</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>4.18 ± 0.18</td>
<td>6.09 ± 0.46</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.9: The response of 3DH cultures to APAP treatment for 24 h on day 4. (a) Cell viability relative to untreated samples as measured with the MTT assay (b) The percentages of live (blue), necrotic (pink) and apoptotic (green) cells (c) Ratio of AST to ALT in culture medium (d) Changes in albumin secretion and normalized to the change in the untreated samples, mean ± SD, n ≥ 3, p < 0.05 relative to untreated samples.
Figure 2.10: Response of the liver models to APAP treatment on day 6 of culture. (a) Cell viability of the models relative to untreated samples as measured with the MTT assay and (b) changes in NPCs following treatment with APAP for 24 h on day 6 of culture. (c) Ratio of AST to ALT in culture medium following treatment with APAP for 24 h on day 6 of culture. (d) Changes in the secretion of AST and ALT from day 5 to day 7 when treated with 40 mM APAP for 24 h, mean ± SD, n ≥ 3, *p < 0.05 relative to untreated samples.
Figure 2.11: Changes in albumin secretion following treatment with APAP for 24 hours (a) from day 4 to day 5 and (b) from day 6 to day 7. These results have been normalized to the change in the untreated samples, mean ± SD, n ≥ 3, *p < 0.05 relative to untreated samples.
Chapter 3: High-Throughput Toxicity Testing of Chemicals and Mixtures in Organotypic Multi-Cellular Cultures of Primary Human Hepatic Cells


3.1 Introduction

Thousands of chemicals pose risks to human populations and our ecosystem since their potential for toxicity remains untested or unknown [178, 179]. Chemical toxicity has been implicated in birth defects, metabolic diseases, organ failure, cancer, and death [6, 179, 277]. Even chemicals identified as hazardous are often not fully characterized with respect to molecular targets, initiating events and long-term health effects. Moreover, humans are unknowingly exposed to chemical mixtures at unknown concentrations. Investigating toxicity of mixtures is time-consuming and expensive since the number of combinations increases exponentially with the number of chemicals to be tested. The limited understanding of cell and organ responses to chemical mixtures poses a fundamental barrier to the prevention of life-threatening health issues.

High-throughput screening (HTS) in toxicology can begin to address the gaps in identifying adverse effects of exposure to single chemicals or combination of toxicants. HTS studies typically use cultures that are easy to assemble and assays that follow an “add-mix-measure” procedure. The overall goal of HTS in toxicity evaluations is to prioritize chemicals for further analysis, thereby reducing costs and animal use [180, 182]. Currently, the United States Environmental Protection Agency (USEPA) has over 1,000 HTS assays for protein expression,
enzyme activity, phenotypic markers, and secreted factors that are used to prioritize chemicals for in-depth screening through their Toxicity Forecaster initiative [179, 186, 191].

The liver is the primary organ involved in the biotransformation of ingested chemicals [2, 6]. Biotransformation is a critical hepatic function, yet exposure to toxicants and metabolites can cause liver damage [6, 39]. Since the liver is responsible for a wide range of metabolic functions, hepatotoxicity can affect the entire body. While there are numerous studies on the effect of a single toxicant on the liver, there are virtually no HTS reports on how mixtures of chemicals affect hepatic cells [5, 140, 194, 197, 199]. The focus of this study is to develop and use human and rat liver organotypic culture models (OCMs) that can be tested with single or multiple toxicant exposure using high-throughput procedures.

Hepatic biotransformation primarily occurs in the liver sinusoids [2]. Sinusoids are blood vessels comprised of hepatocytes, a protein-rich membrane known as the Space of Disse, liver sinusoidal endothelial cells (LSECs), Kupffer cells (KCs), and hepatic stellate cells. The four liver cell types function cohesively to perform organ functions including chemical biotransformation [2, 39]. Although contributions of all hepatic cells in liver toxicity have been reported, current HTS hepatotoxicity models are primarily comprised of monolayers of primary hepatocytes or hepatic cell lines [5, 194, 195, 198]. A few HTS liver cultures include micro-patterned co-cultures, encapsulated hepatocytes, micro-organoids, and cultures assembled with hepatocytes arranged in a cord-like structure [140, 278-280]. These models do not recapitulate the cellular composition or architecture of the liver and may not match in vivo hepatotoxicity.

Therefore, there is a need for multi-cellular liver OCMs that respond to toxicants in a manner that more closely resembles what occurs in vivo. In this report we describe the design, assembly and toxicant response of multi-cellular hepatic OCMs comprised of either primary
human or rat cells. These OCMs were assembled in 96-well plates (henceforth denoted as µOCMs) using automated procedures, rendering them ideal for large-scale toxicological evaluations. In this study, primary hepatocytes were seeded on collagen-coated wells. Primary LSECs and KCs encapsulated in extracellular matrix proteins were seeded above the hepatocytes forming a stratified three-dimensional (3D) structure that mimicked the liver sinusoid. To the best of our knowledge, this is the first HTS hepatotoxicity model to incorporate three primary hepatic cells.

Effects of the well-studied hepatotoxicants, acetaminophen (APAP), ethanol (EtOH), isoniazid (INH), and perfluorooctanoic acid (PFOA) were investigated [39, 49]. The chemicals were added to hepatic cultures individually or in mixtures. APAP was chosen for testing because overdose of the drug is the leading cause of acute liver failure [39]. Biotransformation of APAP by cytochrome P450 2E1 (CYP2E1) results in the formation of a toxic metabolite, N-acetyl-p- benzoquinone imine (NAPQI). Another test agent was EtOH since it is a well-studied hepatotoxicant and approximately 10% of deaths in those aged 20-64 can be attributed to excessive alcohol consumption [281, 282]. Liver damage associated with EtOH occurs through the formation of toxic acetaldehyde mediated by alcohol dehydrogenase (ADH), CYP2E1 and catalase [6]. Another test chemical, INH, is a commonly prescribed medication for tuberculosis. It induces multiple CYP enzymes such as 2E1, 1A2, 2A6, and 2C19, while inhibiting CYP3A4 [39, 40]. PFOA was included because the USEPA published new advisories for PFOA as an environmental toxicant in 2017. This chemical is very stable and causes hepatotoxicity through mitochondrial damage and decreased ATP production [49].

Since CYP enzymes, particularly CYP2E1, metabolize APAP, EtOH and INH, mixtures of these chemicals provided new insights into potential synergistic and non-synergistic effects. The hepatotoxic response was investigated through a combination of fluorescent and luminescent
HTS measurements of viability, changes in apoptosis, glutathione (GSH) decreases, mitochondrial membrane damage, and CYP2E1 catalytic activity.

3.2 Materials and Methods

Glucagon, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), calcium chloride, hydrocortisone, sodium dodecyl sulfate, collagenase type IV, 7-hydroxytrifluoromethylcoumarin (HFC), APAP, EtOH, INH, and PFOA were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated.

3.2.1 Collagen Extraction

Type I collagen was extracted from rat tails as previously described [228, 275, 283]. Briefly, collagen tendons were dissolved in acetic acid and purified through centrifugation at 13,000 x g. Collagen was precipitated with 30% (w/v) sodium chloride, centrifuged at 8,500 x g and dialyzed in 1 mN hydrochloric acid. The final solution was maintained at a pH of 3.1 and sterilized with chloroform.

3.2.2 Primary Hepatic Cell Cultures

3.2.2.1 Human Cultures

Primary human hepatocytes and KCs were purchased from Thermo Fisher Scientific. Information for the two male hepatocyte donors can be found in Table 3.3. Human hepatocyte cultures were maintained in Williams’ Medium E supplemented with a hepatocyte maintenance supplement of dexamethasone, penicillin-streptomycin, ITS+ (insulin, transferrin, selenium complex, bovine serum albumin, and linoleic acid), GlutaMAX™, and HEPES. Primary human
LSECs were purchased from ScienCell (Carlsbad, CA) and used at a passage number ≤ 5. Cultures were maintained at 37 °C in a humidified environment with 10% carbon dioxide.

3.2.2.2 Rat Cultures

Primary rat hepatocytes and LSECs were isolated from female Lewis rats (175-199 g; Envigo, Indianapolis, IN) as previously described [175, 283]. Animal care and surgical procedures were approved by and conducted in accordance with the Virginia Tech Institutional Animal Care and Use Committee. Hepatocytes were isolated using a two-step in situ collagenase perfusion method. A typical liver excision and isolation resulted in 100-150 million hepatocytes with ≥ 97% viability, as measured by trypan blue exclusion. Hepatocyte cultures were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, 0.5 IU/ml porcine insulin (MP Biomedical, Santa Ana, CA), 14.3 ng/ml glucagon, and 7.6 µg/ml hydrocortisone. The LSEC fraction was obtained by differential adhesion and cultured on fibronectin-coated flasks [175, 283]. Primary rat KCs were purchased from Thermo Fisher Scientific. Cultures were maintained at 37 °C in a humidified environment with 10% carbon dioxide.

3.2.2.3 Non-parenchymal Cells

Rat and human LSECs were maintained in Medium 199 supplemented with 10% FBS, 50 U/ml penicillin-streptomycin, 10 mM L-glutamine, and 12.5 ng/ml endothelial cell growth supplement (Millipore, Billerica, MA). Rat and human KCs were maintained in DMEM supplemented with a mixture of FBS, penicillin-streptomycin, human recombinant insulin, GlutaMAX™, and HEPES provided by the manufacturer. Information for the male KC donors is provided in Table 3.4.
3.2.3 Validating Automated Cell Seeding Procedures

96-well tissue culture plates were coated with 50 µL collagen and incubated at 37 °C for 1 h to induce gel formation. Rat hepatocytes were seeded at a density of 62,500 cells/well. In order to validate automated procedures, collagen, hepatocytes and culture medium were dispensed using a multi-channel pipette (manual) or an EL406 Washer Dispenser (automated; BioTek, Winooski, VT). Hepatocytes were imaged on a Nikon TE2000U inverted microscope (Tokyo, Japan). The differences between manual and automated cell dispensing were evaluated by measuring the number of seeded hepatocytes, cell area, circularity, viability, and function. Circularity was calculated as a marker of cellular morphology using Equation 3.1 [228, 284].

\[ C = \frac{4\pi A}{P^2} \]  
(Equation 3.1)

Where, \( C \) = cell circularity, \( A \) = cell area, \( P \) = cell perimeter

3.2.4 Assembly of Multi-Cellular µOCMs and Collagen Sandwich (CS) Models

Multi-cellular µOCMs and CS cultures were assembled using the BioTek EL406 (Figure 3.1). Primary rat or human hepatocytes were seeded on collagen-coated 96-well plates at 62,500 cells/well and 35,000 cells/well, respectively. Cell counts were selected based on a 1.8-fold increase in human cell areas (889 ± 215 µm²) compared to rat hepatocytes (547 ± 147 µm²) \((n = 50\) cells). After 24 h, LSECs and KCs were mixed in a type I collagen solution containing 1% (v/v) fibronectin and added to the 96-well plates to obtain final cell ratios of 5:1 (hepatocytes:LSECs) and 10:1 (hepatocytes:KCs) in both rat µOCMs (rµOCMs) and human µOCMs (hµOCMs). These ratios are identical to those found in the liver [2, 175, 246, 283]. The solution containing collagen and fibronectin was added to hepatocytes to assemble rat CS (rCS) and human CS (hCS) cultures.
Figure 3.1: Schematic of the assembly of the μOCMs. The BioTek EL406 dispensed cells, medium and collagen into 96-well plates. The hepatocytes were seeded on type I collagen. After 24 h, LSECs and KCs were added. Chemicals were added to the μOCMs 24 h later. Hepatotoxicity was measured with HTS assays over 24 h.

3.2.5 Adding Toxicants to hμOCMs and rμOCMs

24 h after μOCM assembly, chemicals were dissolved in hepatocyte medium and added to the cultures individually or as mixtures. Individual chemicals were added to the cells at either ½ LC₅₀ or LC₅₀, where LC₅₀ is the concentration resulting in 50% cell death (Table 3.1) [6]. Mixtures of APAP + EtOH, APAP + INH, EtOH + INH, and APAP + EtOH + INH were investigated with each chemical at a concentration of ½ LC₅₀.
Table 3.1: Toxicants added to µOCMs. The common use and associated risks for each chemical are listed. The corresponding cytochrome P450 (CYP) isoforms are involved in the biotransformation of the chemical to a toxic metabolite. CYP3A4 is present in humans, but not rats. The symbol “N/A” denotes that the chemical is toxic without the involvement of CYP enzymes [6, 39, 47, 49, 60, 285, 286].

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Use</th>
<th>Risk to Human Health</th>
<th>CYPs</th>
<th>LC_{50} (Rat)</th>
<th>LC_{50} (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen (APAP)</td>
<td>Analgesic and antipyretic</td>
<td>Number one cause of acute drug-induced liver failure</td>
<td>1A2, 2E1, 3A4</td>
<td>20 mM</td>
<td>2 mM</td>
</tr>
<tr>
<td>Ethanol (EtOH)</td>
<td>Alcohol consumption</td>
<td>One in three liver transplants are due to chronic excessive alcohol intake</td>
<td>2E1</td>
<td>200 mM</td>
<td>160 mM</td>
</tr>
<tr>
<td>Isoniazid (INH)</td>
<td>Tuberculosis treatment</td>
<td>Enzymes indicative of liver damage are elevated in ~10% of patients</td>
<td>1A2, 2A6, 2C19, 2E1, 3A4</td>
<td>10 mM</td>
<td>560 µM</td>
</tr>
<tr>
<td>Perfluorooctanoic Acid (PFOA)</td>
<td>Surfactant in Teflon® production</td>
<td>Endocrine disrupter found in drinking water</td>
<td>N/A</td>
<td>500 µM</td>
<td>500 µM</td>
</tr>
</tbody>
</table>

3.2.6 Imaging Live and Dead Cells
Glass-bottomed 96-well plates (Greiner Bio-One, Monroe, NC) were activated with 3-aminopropyl tri-ethoxysilane to promote the adhesion of collagen gels [228]. Live (green) and dead (red) cells were visualized using the LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific) adapted for HTS procedures. Cultures were imaged using a Zeiss LSM confocal microscope (Oberkochen, Germany).

3.2.7 Measuring Changes in Cell Viability Over 24 h
Changes in cell viability over 24 h were determined using the HTS RealTime-Glo™ MT Cell Viability Assay Kit (Promega, Madison, WI) [287]. The MT Cell Viability Substrate and NanoLuc® Enzyme were added to the cultures with the toxicant. Luminescence was measured every 4 h for 24 h using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale,
CA). Luminescence at each time point was compared to the initial value (t = 1 h) and controls to calculate cell death.

Although HTS assays are well established for use with two-dimensional (2D) monolayers [182], there are limited reports on their use in 3D cultures. Therefore, we compared the data obtained with the RealTime-Glo™ MT Cell Viability HTS assay to results obtained from a traditional LIVE/DEAD® Viability/Cytotoxicity Kit. The viability values were 54.4 ± 3.1% (LIVE/DEAD® Viability/Cytotoxicity) and 52.1 ± 10.3% (RealTime-Glo™ MT Cell Viability HTS assay), when PFOA (at LC₅₀) was added to the hµOCMs. The viability values were 63.3 ± 3.2% (LIVE/DEAD® Viability/Cytotoxicity) and 59.2 ± 6.6% (RealTime-Glo™ MT Cell Viability HTS assay), when APAP (at LC₅₀) was added to hµOCMs.

3.2.8 Imaging Phenotypes of Hepatic Cells in the µOCMs

rµOCMs and hµOCMs were fixed in 4% (w/v) glutaraldehyde in phosphate buffered saline (1X) and sequentially exposed to 0.1% (v/v) Triton X-100 and a blocking solution of 20% (v/v) rabbit serum in PHEM buffer [283, 288]. LSECs were identified with a mouse sinusoidal endothelial-1 antibody (CD32b; Novus Biologicals, Littleton, CO) and a TRITC-conjugated secondary antibody (Abcam, Cambridge, MA). KCs were identified with a rabbit CD163 antibody (Abcam) and a FITC-conjugated secondary antibody. Hepatocytes were identified with a sheep serum albumin antibody (Abcam) and a DAPI-conjugated secondary antibody. Imaging was conducted on a Zeiss LSM confocal microscope.

3.2.9 Investigating Changes in Apoptosis

The mode of cell death was investigated with the HTS ApoTox-Glo™ Triplex Assay Kit (Promega) [173, 289]. The Caspase-Glo® 3/7 Reagent was added to the cultures 24 h after
adding chemicals and incubated for 1 h at room temperature. Changes in apoptosis were
determined by comparing the luminescence of treated samples to controls.

3.2.10 Determining Changes in Glutathione (GSH)

GSH concentration was measured using the HTS GSH-Glo™ Glutathione Assay (Promega) [92, 290]. 24 h after chemical administration, cultures were sequentially incubated with the GSH-
Glo™ Reagent and Luciferin Detection Reagent. Luminescence of treated cultures was
compared to the controls to determine changes in GSH.

3.2.11 Measuring Changes in Mitochondrial Membrane Integrity

Mitochondrial membrane integrity was analyzed using the HTS JC-1 Mitochondrial Membrane
Potential Detection Kit (Biotium, Hayward, CA) [291]. After a 24 h treatment, cultures were
incubated for 15 min with 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetra ethyl benzimidazolylcarbocyanine
iodide (a cationic dye) at 37 °C. Ratios of red (healthy; Ex550/Em600) to green (damaged;
Ex485/Em535) fluorescence were used to identify changes in mitochondrial permeability. The ratio
of red to green fluorescence decreased upon mitochondrial damage.

3.2.12 Quantifying Changes in CYP2E1 Activity

CYP2E1 activity was measured using previously established procedures adapted for HTS [292, 293]. Enzymatic activity was estimated by the conversion of 7-methoxy-4-
trifluoromethylcoumarin (MFC) to HFC. Although, CYP2C9 can also result in the formation of
HFC, CYP2E1 is considered to be the primary enzyme that mediates this transformation [292].
10 µM MFC was added to the cultures and incubated for 1 h at 37 °C. The supernatant was
collected and mixed with β-glucuronidase/arylsulfatase and 0.5 M sodium acetate and
incubated for 2 h at 37 °C. The reaction was quenched with 0.25 N Tris in 60% acetonitrile. All
reagents were added using the BioTek EL406. HFC concentrations were determined by
comparing the fluorescence of the treated samples ($Ex_{410}/Em_{510}$) to a standard curve in sterile PBS to measure CYP2E1 activity.

### 3.2.13 Assaying for Secreted Urea

Hepatocyte function was assessed through urea secretion via a commercially available kit (BUN Assay Kit; Stanbio Laboratory, Boerne, TX) [283]. The assay was adapted for HTS by automatically dispensing reagents with the BioTek EL406. Urea concentrations were measured at 520 nm. A standard curve was generated using diluted urea in hepatocyte medium.

### 3.2.14 Statistical Analysis

Statistical significance was calculated by a two-tailed Student's t-test, assuming unequal variance while applying the Bonferroni correction (multiple hypothesis testing) with $\alpha = 0.05$. All data are reported as mean ± standard deviation; $n$ denotes number of independent experiments unless otherwise stated.

When more than one toxicant was added to a culture, their combined synergistic or non-synergistic effects on cell death were calculated using the BLISS independence and Loewe additive models [294-296]. These models are widely used to identify drug-drug interactions in pharmaceutical applications. The BLISS model assumes that the chemicals (toxicants) exert their effect independently while the Loewe model assumes that the interacting chemicals may target the same binding site or use the same mechanism. When cultures were exposed to two toxicants (denoted as “A” and “B”), “$y_A$” or “$y_B$” was the effect (in this case cell death) that occurred upon adding only “A” or only “B”, respectively. In the BLISS model, “$y$” is expressed on a continuous scale between 0 and 1 where $0 \leq y \leq 1$. The combined effect using the BLISS and Loewe models was calculated using **Equations 3.2 and 3.3**, respectively.
\[ y_{BLISS} = y_A + y_B - y_A y_B \]  \hspace{1cm} \text{(Equation 3.2)}

\[ y_{Loewe} = \frac{y_A + y_B - 2 y_A y_B}{1 - y_A y_B} \]  \hspace{1cm} \text{(Equation 3.3)}

Synergistic, antagonistic or additive responses were indicated when \( y_{BLISS} \) or \( y_{Loewe} \) was less than, greater than, or approximately equal to the experimental value, respectively.

### 3.3 Results

#### 3.3.1 Comparing Manual and Automated Seeding Procedures

Automated procedures decreased the assembly time of \( \mu \text{OCMs} \) by approximately four-fold (Table 3.2). This is a significant improvement that increases the applicability of the liver \( \mu \text{OCMs} \) in large scale toxicity testing. Hepatocyte number, viability, morphology, and function were investigated to determine whether the automation process affected cells. When primary rat hepatocytes were seeded, there were 43,869 ± 1,544 (manual) and 43,189 ± 2,942 (automated) cells/well \((n = 6)\), indicating less than 2\% variation between the two methods. Hepatocyte viabilities were 85.7 ± 4.4\% and 86.1 ± 3.4\% for manual and automated cell seeding, respectively \((n = 6)\) (Figure 3.2a,b). Hepatocyte morphology was assessed by quantifying cell area and circularity (Figure 3.2c,d). Manually seeded hepatocytes exhibited area and circularity values of 585 ± 153 \(\mu\text{m}^2\) and 0.708 ± 0.079, respectively \((n = 50 \text{ cells})\). Automated seeding resulted in hepatocyte area and circularity values of 574 ± 160 \(\mu\text{m}^2\) and 0.731 ± 0.081, respectively \((n = 50 \text{ cells})\). Hepatocyte function was assessed via urea concentration, which was 22.1 ± 2.1 \(\mu\text{g/ml}\) (manual) and 23.3 ± 2.0 \(\mu\text{g/ml}\) (automated) \((n = 8)\). These results clearly indicate that automated seeding did not impact hepatocyte number, viability, morphology or function.
Table 3.2: Time for assembly of one 96-well plate of μOCMs assembled with manual (pipette) or automated (BioTek EL 406) dispensing.

<table>
<thead>
<tr>
<th>μOCM Assembly Step</th>
<th>Manual Time (min)</th>
<th>Automated Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coat plates with collagen</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Seed hepatocytes</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>Medium change</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Assembly of μOCMs</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>Hydration of μOCMs</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>

Figure 3.2: Representative images of (a, b) live (green) and dead (red) hepatocytes (scale bar = 100 μm) and (c, d) hepatocyte morphologies of untreated cells (scale bar = 20 μm) seeded in 96-well plates. Cells were seeded with (a, c) a multi-channel pipette (manual) or (b, d) the BioTek EL406 (automated). Images were taken 4 h after hepatocyte seeding.

3.3.2 Investigating Cellular Phenotype in μOCMs

In order to ensure that hepatic cells maintained phenotype in the μOCMs throughout the length of the culture period, immunostaining was conducted with cell-specific antibodies (Figure 3.3). Phenotypes were assessed after 72 h in culture, a time point associated with the dedifferentiation of parenchymal and non-parenchymal hepatic cells [4, 5, 241]. Hepatocytes in
**hμOCMs (Figure 3.3a)** and **rμOCMs (Figure 3.3b)** expressed albumin (blue). LSECs and KCs were identified by the expression of CD32b (LSECs, red) and CD163 (KCs, green). The distance in the z-direction between hepatocytes and the first layer of non-parenchymal cells was 6.7 ± 2.6 µm and 8.9 ± 3.0 µm for **hμOCMs** (n = 24 images) and **rμOCMs** (n = 23 images), respectively.

![Figure 3.3: Immunofluorescence staining of the (a) hμOCMs and (b) rμOCMs after 72 h in culture.](image)

Hepatocytes, LSECs and KCs were stained for albumin (blue), CD32b (red) and CD163 (green), respectively. Images are the projection of z-stacks spanning the μOCMs, scale bar = 50 µm.

In the **hμOCMs**, the hepatocyte:LSEC and hepatocyte:KC ratios were 5.5 ± 0.9 and 10.3 ± 1.7, respectively (n = 24 images). In the **rμOCMs**, the hepatocyte:LSEC and hepatocyte:KC ratios were 5.4 ± 0.9 and 11.9 ± 1.6, respectively (n = 23 images). These ratios are virtually identical to those found in vivo [175, 246].

### 3.3.3 Measuring Cell Death Over 24 h

Viability was assessed every 4 h for 24 h in response to APAP, EtOH, INH, and PFOA to obtain information on the rate of cell death upon exposure to each toxicant. Final viability measurements were obtained 24 h after exposure to a toxicant. In response to APAP at LC$_{50}$, the percentage of dead cells in the **hμOCM** and **hCS** cultures was 41% and 11%, respectively (Figure 3.4a). In the **hμOCMs**, cell death primarily occurred 20-24 h after the addition of APAP, which was identical to trends observed in vivo [83, 297, 298]. At 24 h after exposure to EtOH (at LC$_{50}$), the **hμOCMs** and **hCS** cultures exhibited 44% and 22% cell death, respectively (Figure
INH (at LC₅₀) induced 41% (hµOCMs) and 33% (hCS) cell death at 24 h (Figure 3.4c). Exposure to PFOA (at LC₅₀) for 24 h resulted in 48% and 27% cell death in the hµOCMs and hCS cultures, respectively (Figure 3.4d). Cell death occurred throughout the entire 24 h treatment in response to EtOH, INH and PFOA in the hµOCMs. Representative images of live and dead cells at 24 h are shown in Figure 3.4e-j.

![Figure 3.4](image)

**Figure 3.4:** Changes in viability measured by the RealTime-Glo™ MT Cell Viability Assay over 24 h when hµOCMs were exposed to (a) APAP, (b) EtOH, (c) INH, and (d) PFOA. The horizontal gray line indicates 50% cell death, n = 3. Representative images of live (green) and dead (cells) in the (e-g) hCS and (h-j) hµOCM models, 24 h after adding chemicals, scale bar = 100 µm.
In the rµOCMs, APAP, EtOH and INH (all at LC$_{50}$), induced 45%, 39% and 40% cell death at 24 h, respectively (Figure 3.9). In contrast, cell death was only 24% (APAP), 10% (EtOH) and 16% (INH) in the hCS models at the same time point. PFOA induced approximately 45% cell death in both the rCS cultures and rµOCMs at LC$_{50}$. Higher cell death in the µOCMs was attributed to the presence of LSECs and KCs (see Discussion).

### 3.3.4 Determining Mode of Cell Death

The mode of cell death was examined for each chemical by investigating changes in apoptosis 24 h after adding a toxicant. In hµOCMs, apoptosis increased by 67% and 51% (in comparison to controls) in response to APAP and INH at LC$_{50}$ (Figure 3.5a). There was no change in the number of apoptotic cells in the corresponding hCS models. In the hµOCMs, in response to EtOH, apoptosis increased 66% at $\frac{1}{2}$ LC$_{50}$ but only 45% at LC$_{50}$. This trend is similar to prior studies that have reported a shift from apoptosis to necrosis upon increasing EtOH from a sub-lethal to lethal concentration [6, 30]. 24 h after addition of PFOA (at LC$_{50}$), apoptosis increased 74% and 33% in the hµOCM and hCS models, respectively.

When APAP, INH and PFOA were added at LC$_{50}$ to the rµOCMs, apoptosis increased 120%, 35% and 126%, respectively (relative to controls) (Figure 3.10a). EtOH increased apoptosis 71% at $\frac{1}{2}$ LC$_{50}$ but only 21% at LC$_{50}$. Apoptosis did not occur in the rCS models upon treatment with any chemical. The increased apoptosis in the µOCMs can be correlated to the presence of KCs that are known to secrete pro-inflammatory molecules during toxicant exposure [299].
Figure 3.5: (a) Percent increase in apoptosis and (b) percent decrease of GSH in the *hµOCM* 24 h after exposure to chemicals. Results are compared to untreated samples (0%, x-axis), *p < 0.05 relative to the hCS control, # p < 0.05 relative to the *hµOCM* control, n = 3.

### 3.3.5 Measuring Changes in GSH

GSH concentrations were measured to evaluate toxicant-induced oxidative stress [6, 39]. In the *hµOCM* s, GSH was depleted 52%, 56% and 74% after 24 h treatment with APAP, EtOH and INH (all at LC<sub>50</sub>), respectively (Figure 3.5b). The corresponding decreases in GSH in the hCS models were only 36% (APAP), 28% (EtOH) and 17% (INH). The *hµOCM* s exhibited 1.4 – 4.4-fold greater reduction in GSH compared to the corresponding hCS cultures. The same trend prevailed when comparing *rµOCM* s (1.6 – 3.7-fold greater GSH decrease) to rCS cultures (Figure 3.10b). Interestingly, PFOA reduced GSH by < 10% in both models for humans and rats. Since CYP enzymes do not oxidize PFOA, GSH depletion due to binding or oxidation did not occur [47, 55].

### 3.3.6 Measuring Damage to Mitochondrial Membranes

The JC-1 assay was used to detect changes in mitochondrial membrane integrity 24 h after chemical treatment. In the *hµOCM* s, mitochondrial integrity decreased 36% (APAP), 22% (EtOH), 34% (INH), and 37% (PFOA) at LC<sub>50</sub> (Figure 3.6a). In the hCS models, mitochondrial
integrity decreased 26%, 9%, 38%, and 39% in response to APAP, EtOH, INH, and PFOA (all at LC$_{50}$), respectively (see Discussion). In $\mu$OCMs, the mitochondrial membrane integrity decreased 40%, 20%, 28%, and 47% in response to APAP, EtOH, INH, and PFOA (all at LC$_{50}$), respectively (Figure 3.11a). In the rCS models, the corresponding decreases were 26% (APAP), 3% (EtOH), 7% (INH), and 45% (PFOA).

![Figure 3.6](image)

**Figure 3.6:** (a) Red/green JC-1 ratio and (b) HFC formation rate in the $\mu$OCMs 24 h after chemical treatment. The red/green fluorescence ratio decreased in cells with damaged mitochondrial membranes. Increased conversion of MFC to HFC corresponds to increased CYP2E1 activity, *p < 0.05 relative to the hCS control, # p < 0.05 relative to the $\mu$OCM control, n = 3.

### 3.3.7 Evaluating CYP2E1 Activity

The catalytic activity of CYP2E1 was measured through the conversion of MFC to its fluorescent product, HFC. In this study, the catalytic activity of CYP2E1 was measured for three reasons. First, it is more relevant to the biotransformation of the toxicants investigated in this study. Second, CYP2E1 activity has been shown to differ in traditional *in vitro* cultures comprised of monolayers from studies conducted *in vivo* [5, 300]. Since the $\mu$OCMs are multi-cellular 3D structures, measuring enzymatic activity was appropriate. Third, the catalytic activity of CYP2E1
can be measured using HTS procedures that follow the “add-mix-read” guidelines. Interestingly, the \(h\mu OC\)Ms exhibited lower HFC formation (Figure 3.6b) compared to \(h\)CS models 24 h after exposure to all toxicants, especially at the \(LC_{50}\) concentration.

In the \(h\mu OC\)Ms (at \(LC_{50}\)), the rate of HFC formation decreased 1.8-fold and 1.6-fold relative to the controls upon APAP and INH administration, respectively. In contrast, HFC formation was unchanged in the \(h\)CS models in response to these two chemicals. HFC formation did not change upon EtOH exposure in the \(h\mu OC\)Ms but the rate of formation of HFC increased up to 2.6-fold in the \(h\)CS models. Exposure to PFOA did not impact HFC formation in the \(h\mu OC\)Ms due to the lack of CYP2E1-mediated biotransformation of the chemical [47, 55].

In response to APAP and INH at \(LC_{50}\), HFC formation decreased 1.5-fold in the \(r\mu OC\)Ms but only by 20% in the \(r\)CS models (Figure 3.11b). There was about a 1.5-fold increase in HFC formation upon EtOH treatment (at \(LC_{50}\)) in both rodent models. Similar to the human models, there was no change in HFC formation in response to PFOA in the \(r\mu OC\)Ms.

### 3.3.8 Determining Toxicity in Response to Chemical Mixtures

Exposure to toxicants in vivo usually occurs in mixtures. However, toxicity studies are primarily conducted with individual chemicals. In order to address this gap, cell death was measured 24 h after the addition of mixtures of either two or three toxicants. The concentration of each toxicant in the mixture was \(\frac{1}{2} LC_{50}\). Specifically, the mixtures were APAP + EtOH, APAP + INH, EtOH + INH, and APAP + EtOH + INH (Figure 3.7a-d). The measured cell death in cultures exposed to mixtures was analyzed using the BLISS and Loewe models to determine synergistic and non-synergistic interactions. In the \(h\mu OC\)Ms, and \(h\)CS cultures, mixtures of APAP + EtOH, EtOH + INH and APAP + INH + EtOH resulted in synergistic cell death (Figure 3.7a, c, d and Figure 3.12). The APAP + INH mixture did not follow this trend either in human or rat cultures (Figure
3.7b and Figure 3.12). The APAP + EtOH + INH mixture was synergistic for rCS cultures but not for rµOCMs. Interestingly, the BLISS or Loewe models resulted in virtually identical results.

Figure 3.7: Comparison of experimental cell death values for (a) APAP + EtOH, (b) APAP + INH, (c) EtOH + INH, and (d) APAP + EtOH + INH at 24 h to values obtained from the BLISS independence and Loewe additive models for hCS cultures and hµOCMs, n = 3. The bar graphs represent the experimental results for individual chemicals or mixtures. The dashed lines indicate the BLISS and Loewe values for the hCS cultures (red) and hµOCMs (blue).
3.4 Discussion

The primary goals of this study were to assemble primary human multi-cellular hµOCMs using automated procedures and to test the toxicity upon the application of single or multiple toxicants. The studies were designed to understand how a chemical may impact human livers and to identify how mixtures of toxicants lead to hepatotoxicity through synergistic and non-synergistic interactions. The µOCMs described in this study were assembled using automated procedures that markedly reduced assembly time rendering such cultures ideal for large-scale high throughput toxicity evaluations. The hµOCMs were assembled using primary male human hepatic cells. Overall, the multi-cellular hµOCMs exhibited more sensitivity to the four toxicants compared to mono-cellular hCS cultures.

Reports on HTS for hepatotoxicity testing usually focus only on cell death [140, 196-198, 301]. Some studies have reported an additional marker of toxicity, such as hepatocyte function or GSH levels [124, 199]. In this study, phenotypic markers, cell death, mode of cell death, GSH depletion, mitochondrial membrane damage, urea secretion (Figure 3.13) and CYP2E1 catalytic activity were measured on human and rat cultures for single and mixtures of chemicals. These measurements were conducted to obtain a comprehensive understanding of human hepatic responses to toxicant exposure.

The higher cell death in the OCMs was attributed to the presence of non-parenchymal hepatic cells. For example, KCs are known to secrete reactive oxygen species (ROS) and inflammatory cytokines that can cause hepatocyte and LSEC death [283] (Figure 3.8). Since LSECs line the sinusoids, they have been reported to die early in response to toxicant exposure [250, 283].
Figure 3.8: Interactions and cellular effects of high concentrations of APAP, INH and EtOH with CYP2E1. Each of these chemicals is oxidized by CYP2E1 to the toxic metabolites N-acetyl-p-benzoquinone imine (NAPQI by APAP), hydrazine (HYD by INH) and acetaldehyde (MeCHO by EtOH). EtOH is also metabolized by alcohol dehydrogenase (ADH) to MeCHO, which undergoes biotransformation by aldehyde dehydrogenase (ALDH) to non-toxic acetate, which is further metabolized by acetyl coenzyme A (acetyl-CoA) to acetoacetate (ACA), a major ketone body (KB) (Teresinski et al., 2002). ACA increases the expression of CYP2E1 through activation of the protein kinase B (Akt), extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (p38MAPK) pathways (Abdelmegeed et al., 2005). NAPQI, HYD and MeCHO induce the formation of ROS and the secretion of inflammatory cytokines. This leads to an increase in apoptosis, decrease in GSH and mitochondrial membrane integrity (JC-1). Hepatocytes express approximately 10-fold higher CYP2E1 compared to KCs (Koivisto et al., 1996). ROS and cytokines exacerbate inflammation in all cell types (purple). These molecules induce the formation of prostaglandins, eicosanoids and growth factors (GF) in the LSECs that can induce necrosis. LSEC byproducts are transported back to the hepatocytes and KCs, resulting in continued expression of ROS and cytokines. Blue lines correspond to APAP-specific pathways. Green lines correspond to INH-specific pathways. Pink lines correspond to EtOH-specific pathways. Dashed lines indicate cell signaling.
The RealTime-Glo™ MT Cell Viability Assay provided valuable information on the rate of cell death, which has not been widely reported in hepatotoxicity evaluations using HTS approaches. The periodic luminescence measurements over 24 h provided unique insights on how quickly cells were affected by the chemicals. For example, APAP administration resulted in significant cell death approximately 18-20 h post-administration. This time-point correlates to reports on liver dysfunction occurring 24 h after APAP overdose [19]. Although the rate of conversion for EtOH was not measured in this study, a uniform cell death rate was observed in the $h\mu$OCMs over the 24 h culture. In the human body, EtOH undergoes clearance at a constant rate of approximately 160 mg/l/h through CYP2E1, ADH, catalase, and aldehyde dehydrogenase biotransformation [302]. The present study also examined cell death after exposure to INH. Hydrolysis and CYP-mediated metabolism of INH result in hepatotoxic metabolites [40]. Hydrolysis occurs immediately upon INH administration, whereas CYP2E1 oxidation begins 10-12 h later [303, 304]. The dual action of these mechanisms likely contributed to cell death observed over the entire treatment.

The difference in GSH depletion between the $h\mu$OCMs and hCS cultures points to previously noted differences in the expression of CYP2E1. We previously reported increased CYP2E1 expression in response to APAP upon the inclusion of LSECs and KCs in OCMs [283]. The biotransformation of APAP, EtOH and INH through CYP2E1 results in toxic metabolites that bind to GSH thereby causing its depletion [6, 39]. This biotransformation can also activate KCs that secrete ROS that oxidize GSH to GSSG, further decreasing GSH (Figure 3.8) [305].

The mitochondrial membrane integrity was significantly lower in the $\mu$OCMs compared to CS cultures across species for APAP, EtOH and INH treatment. This observation can be attributed to the CYP2E1-mediated metabolism of these toxicants that causes mitochondrial injury. The $\mu$OCMs contain KCs that express CYP2E1 upon toxicant exposure [33, 306, 307], leading to
higher mitochondrial injury. Since PFOA was not metabolized by CYP2E1, the values for mitochondrial membrane integrity were virtually identical between \( \mu \text{OCMs} \) and \( \text{CS} \) cultures.

In the specific case of CYP2E1, the lower catalytic activity (conversion of MFC to HFC) can be attributed to elevated levels of inducible nitric oxide synthase (iNOS) in hepatocytes and KCs upon exposure to high concentrations of a chemical [33, 306, 307]. iNOS leads to the formation of nitric oxide (NO) that binds to the active site on the CYP2E1 protein, reducing its activity as a protective mechanism against ROS [308, 309]. Another mechanism that decreases CYP2E1 activity \textit{in vivo} is protein stabilization, which prevents its ubiquitination but renders the enzyme inactive, as is the case for APAP and INH toxicity [310-312].

A unique aspect of EtOH metabolism is the formation of ketone bodies as a result of biotransformation (\textbf{Figure 3.8}) [27, 29, 303]. In the \( \mu \text{OCMs} \), the inclusion of KCs could have lead to the phagocytosis of ketone bodies (Meng et al., 1992), preventing further expression of CYP2E1. In the \( \text{hCS} \) cultures the lack of KCs and phagocytosis could have resulted in increased CYP2E1 expression and activity.

\( \mu \text{OCMs} \) were assembled with rodent and human cells to investigate species-dependent responses. The expression of biotransformation enzymes is known to differ between rats and humans as shown by the different LC\(_{50}\) values (\textbf{Table 3.1}). For example, CYP3A4 is involved in the biotransformation of approximately 50% of drugs in humans, but is not found in rat livers [6, 313]. In humans, this enzyme participates in the biotransformation of both APAP and INH. However, APAP induces CYP3A4 while INH inhibits enzymatic activity. In response to APAP, the increase in apoptosis (2-fold) and the decrease in GSH (1.3-fold) were higher in \( \mu \text{OCMs} \) than the \( \text{hOCMs} \). In contrast, INH induced 1.3-fold greater apoptosis and 2-fold more GSH depletion in the \( \text{hOCMs} \) relative to the \( \mu \text{OCMs} \). The chemical mixture of APAP + INH resulted
in lower cell death in the human models compared to the rodent cultures. The simultaneous inductive and inhibitory effects on CYP3A4 may have resulted in lower death in the human models. Such species-dependent results highlight the importance of multi-cellular human liver models to evaluate hepatotoxicity.

Evaluating the toxicity of mixtures is time-consuming and expensive since the number of combinations increases exponentially with the number of chemicals to be tested. In this study, the effect of mixtures demonstrated that EtOH had a synergistic effect when combined with APAP or INH. This did not occur with the dual mixture of APAP + INH.

The increased sensitivity to chemicals observed in the OCMs underscores the need to assemble multi-cellular hepatic models and to move away from monolayers or co-cultures. The human and rat μOCMs used in this study demonstrate that multi-cellular 3D liver models can be rapidly assembled using automated procedures rendering them highly relevant as platforms for large-scale toxicity evaluations.
3.5 Supplementary Material

Table 3.3: Information for the two male human hepatocyte donors. Changes in viability in response to APAP and PFOA, each at LC$_{50}$, for these two donors in the hCS models after a 24 h treatment, $n \geq 3$. There were no significant differences between donor 1 and donor 2 in response to either toxicant.

<table>
<thead>
<tr>
<th></th>
<th>Gender</th>
<th>Race</th>
<th>Age</th>
<th>BMI</th>
<th>APAP Treatment</th>
<th>PFOA Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>Male</td>
<td>Caucasian</td>
<td>60</td>
<td>25</td>
<td>89.3 ± 10.5%</td>
<td>73.6 ± 10.9%</td>
</tr>
<tr>
<td>Donor 2</td>
<td>Male</td>
<td>Caucasian</td>
<td>55</td>
<td>22</td>
<td>88.3 ± 4.0%</td>
<td>71.0 ± 8.0%</td>
</tr>
</tbody>
</table>

Table 3.4: Information for the three male human KC donors. Changes in viability in response to APAP and PFOA, each at LC$_{50}$, for donors 1 and 2 in the hµOCMs (hepatocytes, LSECs, and KCs) after a 24 h treatment, $n \geq 3$. The KCs obtained from donor 3 were only used to determine KC phenotype of untreated samples (Figure 3.2). There were no significant differences between donor 1 and donor 2 in response to either toxicant.

<table>
<thead>
<tr>
<th></th>
<th>Gender</th>
<th>Race</th>
<th>Age</th>
<th>BMI</th>
<th>APAP Treatment</th>
<th>PFOA Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>Male</td>
<td>Caucasian</td>
<td>51</td>
<td>27.8</td>
<td>59.2 ± 6.6%</td>
<td>52.1 ± 10.3%</td>
</tr>
<tr>
<td>Donor 2</td>
<td>Male</td>
<td>Caucasian</td>
<td>41</td>
<td>28</td>
<td>63.3 ± 3.2%</td>
<td>54.4 ± 3.1%</td>
</tr>
<tr>
<td>Donor 3</td>
<td>Male</td>
<td>Caucasian</td>
<td>43</td>
<td>25.9</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 3.9: Changes in viability over time of rµOCMs exposure to (a) APAP, (b) EtOH, (c) INH, and (d) PFOA as determined by the RealTime-Glo™ MT Cell Viability Assay. Luminescence was measured every 4 h for 24 h. The gray line indicates 50% cell death, n = 3. Representative images of live (green) and dead (cells) in the (e-g) rCS and (h-j) rµOCM models 24 h after adding chemicals, scale bar = 100 µm.
Figure 3.10: (a) Percent increase in apoptosis and (b) percent decrease of GSH in the $r\mu$OCMs 24 h after chemical treatment compared to untreated samples (0%, x-axis), *$p < 0.05$ relative to the $rCS$ control, #$p < 0.05$ relative to the $r\mu$OCM control, $n = 3$.

Figure 3.11: (a) Red/green ratio determined with a JC-1 kit and (b) HFC formation in the $r\mu$OCMs 24 h after chemical treatment. The red/green fluorescence ratio decreased in samples with mitochondrial membrane damage. Increased HFC formation rate corresponds to increased CYP2E1 activity, *$p < 0.05$ relative to the $rCS$ control, #$p < 0.05$ relative to the $r\mu$OCM control, $n = 3$. 
Figure 3.12: Comparison of experimental cell death values for (a) APAP + EtOH, (b) APAP + INH, (c) EtOH + INH, and (d) APAP + EtOH + INH at 24 h to values obtained from the BLISS independence and Loewe additive models for \( rCS \) cultures and \( r\muOCM \)s, \( n = 3 \). The BLISS and Loewe values were calculated through Equations 3.2 and 3.3, respectively. The bar graphs represent the experimental results for individual chemicals or mixtures. The dashed lines indicate the BLISS and Loewe values for the \( rCS \) cultures (red) and \( r\muOCM \)s (blue).
Figure 3.13: Urea secretion as a marker of hepatocyte function in the (a) human and (b) rat models. Fold change was calculated by dividing the concentration of the treated samples by the value for the controls, *p < 0.05 relative to the CS control, #p < 0.05 relative to the µOCM control, n = 3.
Chapter 4: Investigating the Mechanical Microenvironment on Fibrogenesis in Transitional Multi-Cellular Hepatic Models


4.1 Introduction

Chronic liver disease and fibrosis is the 12th leading cause of death in the United States [314]. Inherently, fibrosis is an uncontrolled wound-healing mechanism resulting in inflammation and accumulation of extracellular matrix (ECM) proteins [202, 203, 206, 207]. Scar tissue from persistent fibrosis disrupts liver architecture, diminishes hepatocyte function and regeneration, decreases vascularization and eventually results in cirrhosis [202, 203, 206, 207]. Without a liver transplant, prolonged cirrhosis can cause hepatocellular carcinoma or complete organ dysfunction [315].

There are four major liver cell types with distinct and critical roles in hepatic fibrosis [203, 206, 207, 216]. Hepatocytes, the parenchymal cells of the liver, become apoptotic and exhibit reduced cell-specific functions, such as biotransformation and protein synthesis during fibrosis [2]. Apoptotic hepatocytes release inflammatory signals and reactive oxygen species (ROS) that activate the non-parenchymal cells (NPCs) [203, 207, 216]. The primary liver NPCs are the hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs) [2].

HSCs are the cells primarily associated with liver fibrosis [2, 203, 206, 207, 216-218]. In a healthy liver, quiescent HSCs store vitamin A and lipids. In a fibrotic liver, activated HSCs
become proliferative, myofibroblastic and the primary source of excess ECM proteins. LSECs in a fibrotic liver become capillarized, inhibiting blood flow and oxygen to the hepatocytes [216, 219, 220]. Activated LSECs have reduced scavenging abilities and secrete signaling molecules that regulate HSC activation [219]. Activated KCs secrete ROS and cytokines that increase inflammation, HSC activation and the progression of fibrosis [206, 207, 216]. KCs further exacerbate inflammation through phagocytosis of apoptotic hepatocytes and the subsequent release of death kinases [216].

Liver fibrosis is initiated by chronic insult through alcoholism, chemical toxicity, infection, nonalcoholic fatty liver disease, or inherited metabolic or autoimmune diseases [205, 207, 216]. These stimuli activate the NPCs, which secrete inflammatory and pro-fibrogenic cytokines and chemokines, perpetuating the disease [206, 207, 216]. Persistent injury and inflammation reduce liver regeneration as ECM proteins replace damaged cells. Excess ECM accumulates in the Space of Disse, an interfacial region that separates hepatocytes from the NPCs [2, 7, 8]. The Space of Disse in a healthy liver is primarily composed of fibronectin and type IV collagen [7, 8, 207]. In a fibrotic liver, collagen types I and III, laminin and hyaluronic acid contribute to an up to 10-fold increase in protein [8, 207]. Increased expression of ECM proteins decreases inter-cellular signaling and induces dedifferentiation of hepatic cells. Increased formation of these proteins further activates HSCs, propagating the fibrotic response [203, 205, 207].

Due to the complex cellular interplay necessary for liver fibrogenesis, the majority of studies are conducted in vivo [224, 225]. Liver fibrosis is induced in rodents through chronic administration of chemicals, such as carbon tetrachloride, changes to diet, bile duct ligation, or genetic modification. In vitro models are utilized for large-scale investigation and to understand specific mechanisms [224-227]. Monocultures of primary HSCs or HSC-like cell lines (primarily LX-2) are the most common in vitro models. However, HSC monocultures adopt an extreme activated
phenotype within 7 days, limiting physiological relevance. HSCs cultured on ECM proteins have been shown to maintain quiescence [224-227, 316]. Only recently, fibrosis has been modeled with monocultures of other hepatic cells, such as hepatocytes and LSECs [228-230]. However, these monocultures lack the requisite inter-cellular interactions.

*In vitro* multi-cellular fibrosis models typically utilize HSCs and hepatocytes [225-227]. These cells have been integrated in two-dimensional (2D) co-cultures, micro-patterned co-cultures and spheroids to promote HSC quiescence and induce hepatocyte fatty acid accumulation [225, 226, 317]. Combinations of HSCs, LSECs and KCs have been used to identify the roles of angiogenesis and inflammation [224, 228, 318]. In the last several years, advanced liver tissues of hepatocytes, HSCs and either LSECs or KCs have been developed as platforms to study compound-induced fibrogenesis [231, 232]. To date, the precision-cut liver slice (PCLS) is the only routine fibrotic model to include the four major liver cell types [224, 227, 319-321]. Key advantages to PCLS models include the exact recapitulation of the native architecture and spontaneous initiation of fibrosis [224, 227]. Major limitations of these models are the precipitous decline in viability after 48 h and that fibrogenesis results from cellular damage within the PCLS.

We developed a three-dimensional (3D) liver fibrosis model containing all four hepatic cell types. A polyelectrolyte multilayer (PEM) with a mechanical gradient was used as a Space of Disse mimic. This transition emulated the spatial and temporal effect of the fibrotic liver on neighboring healthy tissue. To the best of our knowledge, this is the only *in vitro* liver model to study the progression of fibrosis through controlled mechanical cues rather than xenobiotic-induced fibrogenesis [231, 232, 317, 319, 320]. Increased HSC activation, decreased hepatocyte function and increased hepatocyte apoptosis occurred as the stiffness of the PEM increased. Inflammatory markers were identified in the healthy and diseased regions of the models after 8
days. These transitional cultures successfully modeled how fibrosis of the hepatic cells and the matrix are perpetuated through their interactions.

4.2 Materials and Methods

Laminin, high mobility group box protein 1 (HMGB1), hyaluronic acid, glutaraldehyde, N-hydroxysuccimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), oil red O, glucagon, 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), calcium chloride, hydrocortisone, sodium dodecyl sulfate, calf thymus DNA, hydrogen peroxide, sulfuric acid, and collagenase type IV were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all other chemicals were purchased from Thermo Fisher Scientific (Waltham, MA).

4.2.1 Type I Collagen Extraction

Type I collagen was obtained from rat tails through previously established methods [228, 275, 283]. Briefly, tendons were dissolved in 3% (v/v) acetic acid and centrifuged at 13,000 x g. 30% (w/v) sodium chloride was added to precipitate the collagen in the supernatant and centrifuged at 8,500 x g. The collagen was collected and dialyzed in 1 mM hydrocholic acid for a minimum of 48 h. The final collagen solution was maintained at a pH of 3.1 and sterilized with chloroform.

4.2.2 Assembly of PEMs with a Mechanical Gradient

Detachable PEMs were assembled with type I collagen (cationic) and hyaluronic acid (anionic) [275, 283]. Collagen was dissolved in 18 MΩ cm deionized water containing 1% (v/v) acetic acid. Hyaluronic acid was dissolved in 18 MΩ cm deionized water. The polyelectrolyte solutions were maintained at a concentration of 0.75 mg/ml and a pH of 4.0. PEMs were assembled on hydrophobic poly(tetrafluoroethylene) substrates (McMaster-Carr, Elmhust, IL) through robotic deposition (Nano-Strata, Tallahassee, FL). Each polyelectrolyte was absorbed for 30 min with
10 min deionized water rinses between each layer for a total of 30 layers. Homogenous PEMs were cross-linked with 3% (w/v) glutaraldehyde for 1 or 4 min. PEMs with a mechanical gradient were obtained by cross-linking half the PEM for 1 min and the other half for 4 min. PEMs were rinsed with deionized water for 48 h, vacuum dried and sterilized under germicidal UV light for 1 h. The interface of the PEM was visualized by the physical absorption of 0.5 µm red FluoSpheres® [322]. The microspheres were suspended in the 3% (w/v) glutaraldehyde solution used to cross-link the PEM for 4 min. The 1 min side of the PEM was cross-linked with 3% (w/v) glutaraldehyde solution without microspheres.

4.2.3 Characterization of Gradient PEMs

4.2.3.1 Modulus and surface characterization

The Young’s modulus (YM) of hydrated PEMs was measured with a Veeco BioScope II atomic force microscope (AFM; Veeco, Santa Barbara, CA). Measurements were conducted in contact mode using pyramidal SiN cantilever tips (Bruker AFM Probes, Camarillo, CA) with a spring constant of 0.06 N/m. Force-distance curves were determined and the YM was calculated using a modified Hertz cone model (Equations 4.1 and 4.2) [228].

\[
F = k(d - d_0) \quad \text{(Equation 4.1)}
\]

\[
F = \frac{2\tan\alpha}{\pi} \left[ \frac{E}{1 - \nu^2} \right] \delta^2 \quad \text{(Equation 4.2)}
\]

where, \(F\) = applied force, \(\alpha = 18^\circ\), \(E = \text{YM}\), \(k = \text{spring constant (0.06 N/m)}\), \(\nu = \text{Poisson’s ratio (0.4)}\) [275], \(d = \text{deflection of the cantilever}\), \(d_0 = \text{deflection point at contact}\), and \(\delta = \text{indentation}\).

Force-distance curves were fit to 10% of the thickness of hydrated PEMs to avoid substrate effects. Porosity and root mean square (RMS) roughness of PEM surfaces were determined.
with AFM micrographs obtained in contact mode. Pore areas were calculated using ImageJ software (National Institutes of Health).

4.2.3.2 Identification of the gradient region

The distance spanned by the gradient was measured in three stages. First, the initial estimate of the gradient region was identified through differences in transmission of light (Figure 4.1B). This enabled an approximate estimate on the distance spanned as well as the center of the gradient. Second, the fluorescent intensity of rhodamine-conjugated beads embedded in the PEMs was measured using a Nikon TE2000U microscope (Tokyo, Japan). Measurements were conducted on either side of the center (identified in the first step) to obtain information on the 1 and 4 min cross-linked regions (Figure 4.1C). The fluorescent intensity was found to be 7.1-fold higher on the 4 min side of the PEM. The location at which the fluorescent intensity exhibited this change was taken as the center (0,0) of the gradient. Third, AFM measurements to calculate the YM were conducted at ± 250, 500 and 1000 µm from the center (Figure 4.1D). Measurements were conducted on three separate PEMs at three different locations on the vertical axis. The YM at each location on the horizontal axis was compared to the value obtained from homogeneous samples using a Student’s t-test (α = 0.05). The last location at which the YM was statistically insignificant from that of the homogeneous PEMs was taken as the boundary of the gradient.

4.2.3.3 Thickness and optical transparency

The thickness of dry and hydrated PEMs was measured using a DektakXT profiler (Bruker, Billerica, MA) [275]. Hydrated PEMs were incubated in deionized water for 1 h before excess water was removed. Scans that spanned 1000 µm were conducted at five different locations on each PEM. Transmission of light through dry and hydrated PEMs was measured between 400 nm and 900 nm with a SpectraMax M2 UV/vis spectrophotometer (Molecular Devices, Sunnyvale, CA) [275].
Figure 4.1: (A) A detachable PEM assembled with type I collagen and hyaluronic acid. Visualization of the mechanical gradient through (B) decreased light transmission and (C) conjugation of fluorescent microspheres into the side of the PEM cross-linked for 4 min, scale bar = 100 µm. (D) Schematic of the locations on the PEM used to identify the center of the interface with AFM. Measurements were taken over 1000 µm in either direction from the center line at three separate locations on the vertical axis. (E) Schematic of the transitional 3DHLKS models with a gradient PEM. The increase in the number of pink lines on the PEM indicates increased cross-linking and stiffness. This increased stiffness results in NPC activation and hepatocyte apoptosis.

4.2.3.4 Stability in aqueous solution over 7 days

Mass retention of the PEMs in phosphate buffered saline (PBS; 1X) was measured over 7 days. The mass of the dry PEMs was determined before they were submerged in PBS and incubated
at 37 °C for 7 days [275]. The PEMs were removed from the aqueous solution, vacuum dried and weighed to calculate mass change.

4.2.4 Isolation and culture of primary rat hepatocytes, LSECs and HSCs

Primary hepatocytes, LSECs and HSCs were isolated from female Lewis rats (175-199 g; Envigo, Indianapolis, IN) through a two-step in situ collagenase perfusion [175, 283]. Animal care and excision protocols were approved by and conducted in accordance with the Virginia Tech Institutional Animal Care and Use Committee. A typical isolation resulted in 100-150 million hepatocytes with > 97% viability, as measured through trypan blue exclusion. Hepatocytes were seeded at 2.5 x 10^5 cells/well in 24-well plates coated with a collagen gel. LSECs were isolated through differential adhesion and cultured in fibronectin-coated flasks.

Primary HSCs were isolated by the protocol established by Riccalton-Banks et al. [323]. Briefly, the supernatant from the hepatocyte isolation was collected and centrifuged at 50 x g for 5 min. The supernatant was repeatedly centrifuged until no pellet formation was observed. The supernatant obtained after these steps was centrifuged at 200 x g for 10 min. This pellet was suspended in 10 ml hepatocyte medium and centrifuged a final time. The cells were re-suspended in hepatocyte medium and seeded in 6-well tissue culture polystyrene (TCPS) plates. Primary rat HSCs were identified through blue-green autofluorescence of the retinoid-containing lipid droplets [324].

Hepatocytes, HSCs and the multi-cellular models were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, 0.5 IU/ml porcine insulin (MP Biomedical, Santa Ana, CA), 14.3 ng/ml glucagon, and 7.6 µg/ml hydrocortisone. LSECs were maintained in Medium 199 supplemented with 10% FBS, 50 U/ml penicillin-streptomycin, 10 mM L-glutamine, and 12.5
ng/ml endothelial cell growth supplement (Millipore, Billerica, MA). Primary rat KCs (Thermo Fisher Scientific), were cultured in DMEM containing a maintenance supplement provided by the manufacturer. All cultures were maintained in a humidified gas environment (with 10% carbon dioxide) at 37 °C.

4.2.5 Assembly of transitional liver models

Primary rat hepatocytes were seeded on type I collagen in a 24-well plate. After 24 h, the PEMs were placed on the hepatocytes and hydrated with hepatocyte medium for approximately 1 h. LSECs, KCs and HSCs were seeded on top of the PEM. Initial cell ratios were 1:20 (LSECs:hepatocytes), 1:10 (KCs:hepatocytes) and 1:125 (HSCs:hepatocytes) (Figure 4.1E) [175, 283]. The hepatocyte:HSC ratio was determined experimentally. The models in this report are identified through the following notation. Three-dimensional models are denoted by the “3D” prefix. Hepatocytes, LSECs, KCs and HSCs are denoted by “H”, “L”, “K”, and “S”, respectively (Table 4.1).

Table 4.1: Abbreviations and descriptions of the multi-cellular models used in this report.

<table>
<thead>
<tr>
<th>Model</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3DHLKS-21</td>
<td>3D model with hepatocytes, LSECs, KCs, HSCs and a 21 kPa homogenous PEM</td>
</tr>
<tr>
<td>3DHLKS-T</td>
<td>3D model with hepatocytes, LSECs, KCs, HSCs and a gradient PEM</td>
</tr>
<tr>
<td>3DHLKS-43</td>
<td>3D model with hepatocytes, LSECs, KCs, HSCs and a 43 kPa homogenous PEM</td>
</tr>
</tbody>
</table>

4.2.6 Measuring hepatic cell counts

NPCs were stained with non-toxic, fluorescent dyes prior to seeding and counted on days 3 and 8 [283]. LSECs, KCs and HSCs were stained with red (LSECs; PKH 26 Red Fluorescence Cell Linker Kit; Sigma-Aldrich), green (KCs; PKH 2 Green Fluorescence Cell Linker Kit; Sigma-Aldrich) and blue (HSCs; CellTracker™ Blue Fluorescent Probes) dyes, respectively, according to manufacturer protocols. The NPCs were imaged using the Nikon microscope. Hepatocytes
were imaged upon removing the PEM. For each biological replicate of the 3DHLKS-T models, three images were taken at each of the three regions. For each biological replicate of the homogenous 3DHLKS models, six images were taken per well.

4.2.7 Immunostaining of hepatocytes and HSCs

Cultures were fixed with 4% (w/v) glutaraldehyde, permeabilized with 0.1% (v/v) Triton-X and blocked from non-specific binding with 20% (v/v) rabbit serum [283, 288]. To image for intracellular albumin, hepatocytes were incubated with a sheep serum albumin antibody (1:100; Abcam, Cambridge, MA) and a DAPI-conjugated secondary. HSC activation was determined through the expression glial fibrillary acidic protein (GFAP; expressed in quiescent HSCs) and alpha smooth muscle actin (αSMA; expressed in activated HSCs) [217]. The cells were incubated with fluorescently conjugated GFAP Monoclonal Antibody, Alexa Fluor 488 (green; eBioscience, Inc., San Diego, CA) and Alpha-Smooth Muscle Actin Monoclonal Antibody, eFluor 570 (red; eBioscience, Inc.), for GFAP and αSMA, respectively. Cultures were imaged with a Zeiss LSM confocal microscope (Oberkochen, Germany). Hepatocyte area and circularity were calculated using ImageJ software and Equation 4.3 [228, 284].

\[ C = \frac{4\pi A}{P^2} \]  \hspace{1cm} (Equation 4.3)

Where, \( C \) = cell circularity, \( A \) = cell area, \( P \) = cell perimeter

4.2.8 Measuring lipid accumulation in HSCs

Lipid droplets in the HSCs were detected through oil red O staining [175, 325]. Cultures were fixed with 4% (w/v) glutaraldehyde and permeabilized with 0.1% (v/v) Triton-X. The cells were exposed to 1.8% (w/v) oil red O for 15 min. Excess oil red O was washed away with PBS (1X) and the cultures were imaged with the Nikon microscope. For each biological replicate, three images were taken at each of the three regions.
4.2.9 Cytokine secretion

Tumor necrosis factor alpha (TNFα) and transforming growth factor beta (TGFβ) concentrations were measured in spent culture medium with the Rat TNF-alpha Quantikine ELISA Kit (R & D Systems, Minneapolis, MN) and Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (R & D Systems), respectively. Concentrations of TNFα and TGFβ were determined at an absorbance of 450 nm and through calibration with standard curves. The cytokine concentration of fresh medium was subtracted from the values obtained with cellular models.

4.2.10 Mode of hepatocyte death across the gradient

Viable, necrotic and apoptotic cells were stained and counted through the use of a commercially available kit (Apoptotic, Necrotic, and Healthy Cells Quantification Kit; Biotium, Hayward, CA). The NPCs and PEM were removed before staining to isolate the hepatocytes. The hepatocytes were incubated with 50 µl/ml Hoechst 33342 (live), ethidium homodimer III (necrotic) and FITC-annexin V (apoptotic) for 15 min. Cultures were imaged on the Nikon microscope. For each biological replicate, 3-4 images were taken at each of the three regions for a total of 14 images.

4.2.11 Conjugation of laminin and HMGB1 to gradient PEMs

Laminin and HMGB1 were covalently conjugated to the PEMs using carbodiimide chemistry [275, 326]. PEMs were hydrated in PBS (1X) and sterilized under germicidal UV light for 1 h. The concentrations of laminin and HMGB1 solutions were 100 µg/ml and 10 ng/ml [327], respectively. NHS and EDC were added at 10-fold molar excess to the laminin or HMGB1 concentrations. The PEMs were incubated in a protein solution for 2 h at room temperature, rinsed three times to remove unbound protein and maintained in PBS until use.
4.2.12 Statistical analysis
Statistical significance was calculated by a two-tailed Student’s t-test, assuming unequal variance while applying the Bonferroni correction (multiple hypothesis testing) with $\alpha = 0.05$. All data are reported as mean ± standard deviation; $n$ denotes number of independent experiments unless otherwise stated.

4.3 Results
4.3.1 Characterization of PEMs with a mechanical gradient
PEMs with a mechanical gradient were obtained by cross-linking each half of the membrane for 1 or 4 min. This resulted in the two edges of the PEM exhibiting homogeneous YM values and a region in the middle with a gradient in mechanical properties. This gradient was visible through optical and fluorescence microscopy (Figure 4.1). Since fluorescent beads were only added to the glutaraldehyde solution in contact with the PEM for 4 min, only one half of the membrane exhibited fluorescence (Figure 4.1C). Homogeneous PEMs exposed to the cross-linker for only 1 or 4 min were assembled to serve as a reference. The YM of hydrated homogeneous PEMs cross-linked for 1 and 4 min exhibited a YM of 20.6 ± 1.7 kPa (denoted henceforth as 21 kPa; $n = 3$ PEMs, 3 locations/PEM) and 43.3 ± 3.2 kPa (denoted henceforth as 43 kPa; $n = 3$ PEMs, 3 locations/PEM), respectively.

For gradient PEMs, the YM was measured in both directions from the line of separation between the beaded and non-beaded regions of the membrane. AFM measurements were conducted at ± 250, 500 and 1000 µm in either direction (Figure 4.2A). The YM values at 1000 µm from the line were 45.2 ± 7.3 kPa (4 min cross-linking) and 21.7 ± 3.3 kPa (1 min cross-linking). These values were statistically insignificant from those obtained on homogeneous PEMs. Based on the calculated YM values, the mechanical gradient in the membranes spanned...
approximately 2000 \( \mu \text{m} \). The line of separation between beaded and non-beaded regions was taken as the center of the interfacial region and is denoted as such henceforth.

**Figure 4.2:** Characterization of the gradient PEMs. (A) YM of hydrated PEMs showing that values for the homogenous samples were obtained 1000 \( \mu \text{m} \) from the center of the interface, \( n = 3 \) PEMs, 3 locations/region. (B) Thickness of dry and hydrated PEMs across the length of the gradient, \( n = 13-14 \) PEMs, 5 locations/PEM. (C) Transparency of dry and hydrated PEMs as determined through light transmission ranging from 400-900 nm, \( n = 5-6 \) PEMs. (D) Normalized frequency plot of pore size in the PEM across the three regions, \( n = 250-268 \) pores. Representative images of the surface topography for the (E) 21 kPa, (F) interface and (G) 43 kPa regions. AFM micrographs are 25 \( \mu \text{m}^2 \) centered at 0 \( \mu \text{m} \) or \( \pm 1000 \mu \text{m} \).
The thickness of the hydrated gradient PEMs was 1.1 ± 0.3, 1.1 ± 0.2 and 1.2 ± 0.3 µm at the 21 kPa, center of interface and the 43 kPa regions, respectively. These values indicate that the thickness did not vary across hydrated gradient PEMs. The thicknesses at the 1 and 4 min cross-linked regions were statistically insignificant from values obtained with homogeneous PEMs. The thicknesses of 1 and 4 min cross-linked homogeneous PEMs were 1.1 ± 0.2 µm and 1.2 ± 0.2 µm, respectively (Figure 4.2B). The hydrated gradient PEMs exhibited approximately 90% optical transparency (Figure 4.2C) and retained 89.3 ± 3.1% mass over 7 days (n = 3 PEMs).

The surface RMS roughness of the hydrated PEMs ranged from 23.0 ± 2.7 to 25.5 ± 4.3 nm across the entire membrane (Table 4.2 and Figures 4.2D-G). The pore density ranged from 16.5 – 17.8 pores/10 µm² at 21 kPa, the interface and 43 kPa. These data demonstrated that substrate rigidity did not result in statistically significant changes to either RMS roughness or pore density (Table 4.2). Approximately 70% of the pores in each region were smaller than 0.05 µm² (Figure 4.2D). At 21 kPa, the interface and 43 kPa, 3.0%, 1.2% and 1.2% of the pores were larger than 1.0 µm² indicating that the number of large pores decreased with increasing stiffness. Representative images of the AFM micrographs are located in Figure 4.2E (21 kPa), Figure 4.2F (interface) and Figure 4.2G (43 kPa).

**Table 4.2**: Characterization of the surface of each region of the hydrated gradient PEMs through surface RMS roughness (n = 4 micrographs) and pore density (n = 6 micrographs).

<table>
<thead>
<tr>
<th>YM</th>
<th>RMS Roughness (nm)</th>
<th>Pores/10 µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 kPa</td>
<td>25.5 ± 4.3</td>
<td>17.8 ± 4.6</td>
</tr>
<tr>
<td>Center of Interface</td>
<td>24.2 ± 2.9</td>
<td>16.5 ± 3.3</td>
</tr>
<tr>
<td>43 kPa</td>
<td>23.0 ± 2.7</td>
<td>16.9 ± 3.4</td>
</tr>
</tbody>
</table>
4.3.2 Ratio of hepatic parenchymal and non-parenchymal cells

The ratio of hepatocytes to HSCs, KCs and LSECs were measured in the 3DHLKS cultures assembled with homogeneous and transitional PEMs. During fibrogenesis, HSCs and KCs are known to exhibit altered proliferation resulting in an inflamed environment [2, 175, 205, 216, 328]. In the 3DHLKS-21 cultures, the hepatocyte:HSC, hepatocyte:KC and hepatocyte:LSEC ratios were $15.4 \pm 1.2$, $10.4 \pm 1.3$ and $6.2 \pm 0.8$, respectively on day 8 (Figure 4.3). These ratios are virtually identical to hepatic ratios found in vivo [2, 175, 246]. In the 3DHLKS-43 cultures, the hepatocyte:HSC, hepatocyte:KC and hepatocyte:LSEC ratios were $12.4 \pm 1.0$, $5.2 \pm 0.5$ and $5.0 \pm 0.5$, respectively on day 8. Although, the hepatocyte:HSC ratio did not change significantly upon increasing PEM stiffness, the hepatocyte:KC ratio was closer to that of an inflamed tissue in the 3DHLKS-43 models either due to significant KC proliferation or hepatocyte death.

Interestingly, in 3DHLKS-T cultures, the hepatocyte:KC ratios on the 21 kPa, interfacial and 43 kPa regions were $5.9 \pm 0.1$, $5.1 \pm 0.3$ and $4.6 \pm 0.7$, respectively. While the hepatocyte:KC ratio in the 3DHLKS-21 models was identical to those found in a healthy liver, the ratio on the 21 kPa region of the 3DHLKS-T cultures was representative of an inflamed environment. Although, the 21 kPa region was only approximately 2000 µm from the 43 kPa side of the PEM, the effect of the stiffer substrate appeared to alter cellular proliferation and death. The hepatocyte:HSC ratio on the 21 kPa, interfacial and 43 kPa regions was $13.2 \pm 0.6$, $11.2 \pm 0.9$ and $9.2 \pm 0.2$, respectively. These values point to increased HSC proliferation or hepatocyte death on the 43 kPa region of the 3DHLKS-T models in contrast to the homogeneous 3DHLKS-43 cultures. Together, these trends clearly point to changes in cell ratios in the multi-cellular 3DHLKS-T. These differences could arise from changes in proliferation, cell death as well as the concentration of soluble signaling molecules.
Figure 4.3: Ratios of hepatocytes to (A) HSCs, (B) KCs and (C) LSECs on day 3 to day 8 in the 3DHLKS-21, 3DHLKS-T and 3DHLKS-43 models, *p < 0.05 relative to 21 kPa, # p < 0.05 relative to day 3, § p < 0.05 relative to homogenous, n = 3.
4.3.3 Activation of HSCs

The activation of HSCs in the 3DHLKS-T cultures models was investigated to determine if these cells exhibited a transition from quiescent to activated cells. This transition in HSC phenotype was investigated through immunostaining for GFAP (quiescent) and αSMA (activated) as well by analyzing the number of lipid droplets per cell as a function of location and time [217, 218]. On day 3 in culture, HSCs cultured on all regions of the 3DHLKS-T cultures expressed GFAP (Figure 4.4A-C) whereas the expression of αSMA was only observed in HSCs at 43 kPa. On day 8, increased αSMA expression was detected in HSCs adhered to the interface and 43 kPa regions but not on the 21 kPa side of the culture (Figure 4.4D-F). The increase in αSMA expression was accompanied with a decrease in GFAP demonstrating that by day 8, HSCs cultured on the gradient and 43 kPa regions were activated and becoming myofibroblastic [203, 217, 218].

The number of lipids per cell as a function of substrate stiffness and time revealed interesting trends. On day 3, HSCs on the 21 kPa region of the 3DHLKS-T cultures exhibited statistically higher lipids/cell (Figure 4.4G). HSCs on the 21 kPa region had a 1.5-fold and 2.7-fold higher number of lipids/HSC compared to cells on the gradient ($p < 0.05$) and 43 kPa ($p < 0.05$) regions, respectively. By day 8, the number of lipids/HSC had decreased on all regions with HSCs on the 21 kPa regions exhibiting 1.2-fold and 2.4-fold higher number of lipids/HSC compared to cells on the gradient ($p < 0.05$) and 43 kPa ($p < 0.05$) regions, respectively. Staining for oil red O [175, 325] showed significant decreases in lipid content in the interface and 43 kPa regions on day 8 (Figure 4.4H-M). These results are consistent with the loss of lipid storage associated with the transition from quiescent to myofibroblastic HSCs.
4.3.4 Changes in cytokine secretion

The secretion of TGFβ and TNFα was measured in all 3DHLKS cultures on day 3 and day 8 to investigate changes in cytokines as a result of activated NPCs. TGF-β is a known profibrogenic agent that simultaneously acts as an anti-inflammatory agent [203, 217, 329, 330]. TGFβ secretion in the 3DHLKS-21 cultures decreased approximately 2.6-fold from day 3 to day 8 in
culture (Figure 4.5A). These trends were similar to other fibrogenic markers identified in cells adherent on the 21 kPa PEMs. In contrast, the concentration of TGFβ increased approximately 2.3-fold and 3.4-fold in the 3DHLKS-T and 3DHLKS-43 cultures, respectively. The increase in a profibrogenic agent indicated that the transitional cultures were undergoing fibrogenesis similar to the cultures assembled using the 43 kPa PEM. The higher value for TGFβ obtained on day 3 with the 3DHLKS-21 cultures could be attributed to the latent form of the protein which has been shown to be secreted by hepatocytes [331].

![Bar chart showing TGFβ secretion](image)

**Figure 4.5:** (A) TGFβ and (B) TNFα secretion in the 3DHLKS models, *p < 0.05 3DHLKS-21, # p < 0.05 relative to day 3, § p < 0.05 relative to 3DHLKS-43, n = 3.

TNFα secretion in the 3DHLKS-21 and 3DHLKS-T cultures decreased by approximately 2.2-fold and 1.5-fold from day 3 to day 8 in culture (Figure 4.5B). Interestingly, TNFα secretion in the 3DHLKS-43 cultures increased approximately 2.3-fold. These trends clearly demonstrate that the KCs were activated in the 3DHLKS-43 cultures compared to the 3DHLKS-T models. Since cytokine concentrations were obtained from spent culture medium for the entire 3DHLKS-T models, relative contributions from each region could not be quantified. TNFα expression is linked to hepatocyte death; therefore changes in cell viability were next investigated.
4.3.5 Investigating hepatocyte viability

Hepatocyte viability and mode of cell death were determined in the 3DHLKS-T models on days 3 and 8 (Figure 4.6). On the 21 kPa side of the culture on day 3, hepatocyte viability was $87.8 \pm 3.0\%$. Viability decreased by approximately 10% and 20% in hepatocytes at the interface and 43 kPa regions, respectively. On day 8, there was an approximately 15.0%, 12.5% and 13.3% loss of viability at the 21 kPa, interface and 43 kPa regions, respectively. A very interesting trend was observed in the percent of apoptotic hepatocytes as a function of time and substrate stiffness. Hepatocytes in contact with the 21 kPa region of the PEM exhibited an approximately 64% increase in apoptosis from day 3 to 8. However, hepatocyte apoptosis increased approximately 30% on the gradient and 43 kPa regions. These trends suggest that hepatocytes in contact with the gradient and 43 kPa regions of the PEM were influencing and altering apoptosis in cells on the softer regions.

Figure 4.6: Changes in (A) viability and (B) mode of cell death in the 3DHLKS-T models in each of the three regions, *$p < 0.05$ relative to 21 kPa, # $p < 0.05$ relative to day 3, $n = 4$. 
4.3.6 Functional changes in the hepatocytes

Albumin expression in the hepatocytes in the 3DHLKS-T models was imaged in each region of the gradient. On day 3, hepatocyte albumin expression was diffuse throughout the entire cell in all three regions (Figure 4.7A-C). By day 8, albumin expression in the hepatocytes decreased as the YM of the PEM increased (Figure 4.7D-F). Additionally, albumin expression became punctate in the gradient and 43 kPa regions.

Figure 4.7: Response of the hepatocytes in the 3DHLKS models. Albumin expression (blue) of the hepatocytes on (A-C) day 3 and (D-E) day 8 in the 3DHLKS-T models, scale bar = 40 µm. Images on day 3 and day 8 are representative of three biological replicates. Hepatocyte (G) area and (H) circularity in the 3DHLKS-T models, *$p < 0.05$ relative to 21 kPa, # $< 0.05$ relative to day 3, $n = 50$ cells.
Hepatocytes across the gradient were further characterized through changes in cell area and circularity. The decrease in hepatocyte differentiation corresponds to a loss of the native polygonal morphology [332, 333]. Specifically, hepatocyte shrinkage is associated with apoptosis of the cell [334, 335]. On day 3, hepatocyte area did not vary with PEM stiffness (Figure 5.7G). However, there were small, but significant increases in the circularity of the cells as the YM increased (Figure 4.7H). From day 3 to day 8, there was no change in hepatocyte area or circularity at the 21 kPa or interfacial regions. At 43 kPa, there was a 35% decrease in hepatocyte area on day 8, which was significantly smaller than the 21 kPa or interfacial regions at this time point. These findings are consistent with the increase in apoptosis and decrease in albumin expression that occurred at 43 kPa.

4.3.7 Changes in elastic moduli of PEMs post-culture

During fibrosis, activated HSCs have been shown to secrete ECM components resulting a 6-10-fold increase in tissue stiffness [203, 206, 207]. Changes in the YM of PEMs post-culture were investigated to determine whether HSCs had indeed caused such changes within an 8-day culture period (Figure 4.8). On day 3, the elastic moduli of the PEM in each of the three regions (21 kPa, gradient and 43 kPa) were statistically insignificant to values obtained from the same regions on hydrated gradient PEMs that were not used for cell-culture. By day 8, the elastic moduli on the 21 kPa and 43 kPa sides of the PEM increased approximately 2-fold and 4-fold, respectively. These trends clearly demonstrate that activated HSCs had indeed secreted ECM proteins and proteoglycans resulting in a statistically significant increase in elastic moduli. Ongoing studies are focused on measuring the concentration of hydroxyproline to estimate the concentration of collagen deposited by cells as well as immunostaining for laminin and heparan sulfate.
4.4 Discussion

Liver fibrosis does not occur uniformly throughout the organ [203, 207]. For example, generation of ROS in the liver has been connected to increased damage in the centrilobular regions of the organ [203]. The initiating factor of fibrosis can also change the distribution of fibrous tissue [207]. Specifically, chronic hepatitis and cholestasis cause fibrosis localized to the periportal region, but alcohol-induced fibrosis occurs in the centrilobular regions. The resulting activation of nearby NPCs corresponds with the release of cytokines, migration of KCs and HSCs and an increase in local stiffness [203, 205, 207, 216, 330]. This causes inflammation and fibrogenesis in the neighboring healthy tissue and allows the disease to spread throughout the organ. There is limited understanding of the role of the local microenvironment in the progression of fibrosis.

In this report, we describe a transitional liver model to investigate the progression of hepatic fibrosis. These models utilize all four major hepatic cell types and recapitulate the in vivo sinusoidal architecture. A PEM assembled with hyaluronic acid and type I collagen, two
components found in the native Space of Disse [8], was used to mimic this peri-sinusoidal space. The PEM was designed with a mechanical gradient to study the progression of liver fibrosis in diseased tissue and to the neighboring healthy tissue. These models are unique, in that, fibrosis was initiated purely through changes to the microenvironment. These models were not exposed to external fibrogenic factors nor were the models assembled with diseased cells from fibrotic rats. Therefore, the progression of liver fibrosis in healthy and diseased tissue could be simultaneously studied through environmental cues and cellular interactions.

The 3DHLKS-T models were compared to the 3DHLKS-21 and 3DHLKS-43 cultures to isolate the impact of the changes in elastic moduli. The hepatocyte:HSC and hepatocyte:LSEC ratios in the 3DHLKS-T cultures were highly comparable to the corresponding homogenous models, indicating a strong dependence on the local microenvironment. However, the hepatocyte:KC ratio at the 21 kPa region of the 3DHLKS-T models was almost 2-fold lower than the ratio in the 3DHLKS-21 models. This decreased ratio at the 21 kPa region the 3DHLKS-T cultures demonstrates the critical role of the transition to the induction of inflammation in healthy tissue. Inflammation was further investigated through the secretion of TGFβ and TNFα. TGFβ increased 2.4-fold and 3.5-fold from day 3 to day 8 in the 3DHLKS-T and 3DHLKS-43 models, respectively, suggesting an increased fibrogenic response in these cultures over time. Interestingly, TNFα decreased 60% and 40% in the 3DHLKS-21 and 3DHLKS-T models, respectively. This cytokine can be found as a transmembrane-bound protein or as a soluble protein [336, 337]. While soluble and bound TNFα induce inflammation, only the soluble protein is correlated to fibrosis. The slight decrease in TNFα in the 3DHLKS-T models could correspond to increased inflammation, but fibrogenesis only throughout the stiffer regions. This demonstrated that diseased tissue in the 3DHLKS-T cultures induced inflammation in the healthy region of the cultures. These data are encouraging since they follow fibrogenic patterns obtained from studies conducted in vivo [203, 205, 207, 216, 330].
There is much variation in the values reported for liver stiffness in healthy and fibrotic tissues [228, 243, 244, 338-343]. This variability is primarily due to differences in sample preparation and techniques used for analysis. Studies report YM for healthy liver tissue ranging from 5 – 20 kPa and a cutoff for fibrosis ranging from 15 – 24 kPa. Therefore, the YM of the PEMs are within physiological range. A YM of 21 kPa is closer to the properties of a healthy tissue, while a YM of 43 kPa is clearly fibrotic. Reports have demonstrated that liver stiffness increases 2-7 fold during fibrosis [340-344]. The 2-fold increase in YM at 21 kPa after culture is consistent with borderline fibrosis, while the 4-fold increase at 43 kPa clearly indicated diseased tissue.

In addition to changing the elastic moduli, PEMs were also modified with two proteins, laminin and HMGB1. These proteins were selected since increased laminin deposition has been found in fibrotic tissue while HMGB1 has been shown to increase the proliferative and migratory behavior of HSCs [327]. The surface of the PEM was chemically modified through protein conjugation. Both laminin (Figure 4.9A) and HMGB1 (Figure 4.9B) were uniformly conjugated across the surface. The fluorescent intensity from laminin was much lower on the 21 kPa regions whereas there was a uniform distribution of fluorescent HMGB1 across all regions. These differences can be attributed to HMGB1 having a lower molecular weight as well as the differences in the input protein concentration. Interestingly, protein modification did not change the proliferation of HSCs, KCs and LSECs (Figure 4.9C-E). Even in the monocultures, the driving force for proliferation was the stiffness of the PEM. On unconjugated PEMs, HSC, KC and LSEC proliferation was 1.1-, 1.5- and 1.8-fold higher at 43 kPa than 21 kPa. Since it is known that the presence of ECM components increases fibrosis in vivo [8, 207], these results suggest that this effect is dependent on inter-cellular interactions.
In vitro fibrosis models typically use cells from diseased animals or initiate fibrosis in culture through administration of fibrogenic compounds or seeding HSCs on TCPS [224-226, 316, 318]. To the best of our knowledge, the only fibrotic liver models assembled with all four liver cell types are PCLS and the 3DHLKS-T models described in this report. PCLS extracted from rat and human livers have exhibited up to 40% cell death after only 72 h, with almost complete loss of viability after 5 days [321, 345-348]. In contrast, the 3DHLKS-T models exhibited a maximum of 40% cell death after 8 days in culture. This finding clearly demonstrates the potential of these models for extended fibrosis studies.

Current in vitro studies do not focus on the role of the local microenvironment on fibrosis in a physiologically relevant manner. In fact, culture of HSCs on TCPS is the only established
method to induce a fibrotic response through mechanical cues [224-226]. However, there are many stages in the process of HSCs transitioning from a quiescent to activated phenotype. This study offers insights into some of these changes where HSCs on the 21 kPa region maintain lipid droplets and GFAP, yet secrete ECM components that result in a 2-fold higher elastic modulus.

In conclusion, we have assembled a multi-cellular transitional liver model that can be used to investigate the progression of fibrosis from diseased to healthy tissue solely through alterations in the microenvironment. This study was completed through the development of a PEM with a mechanical gradient with YM ranging from healthy to fibrotic tissue. These transitional models clearly demonstrate a key dependence of HSC activation and hepatocyte dysfunction on the local stiffness. KC activation was detected throughout the entire culture indicating the inflammation shown to be necessary for perpetuation of the disease. Importantly, significant increases in the stiffness of the PEM through culture were detected, demonstrating the critical ability of the cells to alter their local microenvironment. These results clearly indicate the potential of these models to be used to study liver fibrosis, the need for a transitional model and the importance of mechanical cues in the progression of the disease.
Chapter 5: Conclusions and Future Work

5.1 Conclusions

The research presented in this thesis focuses on the application and versatility of the organotypic liver models developed by the Rajagopalan research group. In this work, we (1) examined the in vivo relevance for acute hepatotoxicity studies, (2) adapted the models for HTS, (3) studied the effects of multiple chemicals and their mixtures with HTS methodologies, (4) designed a PEM with a mechanical gradient, and (5) assembled liver models to study the role of the microenvironment in the progression of fibrosis.

In Chapter 2, APAP was administered to the organotypic liver models as a prototypic toxicant. The models were assembled with primary rat hepatocytes, LSECs, KCs and a Space of Disse mimic. The liver models exhibited significant cell death at LC50, primarily through necrosis. At this concentration, LSEC viability decreased 40% and KCs proliferated 34%. This corresponded to a 74% decrease in IL-10 and 55% increase in IFNγ. Neither LSECs nor KCs dedifferentiated in the liver models over five days, even in response to APAP. At LC50, the AST/ALT ratio was 2.46 and there was a 45% decrease in GSH, identical to values reported in vivo. CYP2E1 expression increased upon all concentrations of APAP. The increase in IFNγ in the liver models at LC50 was connected to increased AST/ALT and hepatocyte apoptosis.

In Chapter 3, the organotypic liver models were adapted for HTS. The stratified architecture was recapitulated by LSECs and KCs encapsulated in collagen containing 1% (v/v) fibronectin. Each component was automatically dispensed into 96-well plates using the BioTek EL406. Automated seeding reduced assembly time 4-fold and did not alter hepatocyte morphology or viability. There was approximately 50% cell death when each chemical was added at LC50. Apoptosis decreased with increasing EtOH indicating a shift to necrosis. APAP, INH and PFOA decreased
mitochondrial membrane integrity. GSH decreased 40-80% with APAP, EtOH and INH at LC50. HFC formation (CYP2E1 activity) decreased in response to APAP and INH, but increased upon EtOH treatment. This increase could be attributed to the formation of ketone bodies in the hepatocytes and KCs. GSH and HFC formation were unchanged upon PFOA treatment. Each of these responses was consistent with the reported mechanisms of action. Synergistic cell death occurred upon treatment with each chemical mixture except APAP + INH.

Chapter 4 describes the assembly of a PEM with a mechanical gradient and its application in a fibrosis model. Fifteen bilayer PEMs were assembled with collagen and HA, each at 0.75 mg/ml and a pH of 4.0. The PEMS were cross-linked for 1 min (21 kPa, healthy liver) or 4 min (43 kPa, fibrotic liver) forming a stiffness gradient spanning 2000 µm. The PEMs were placed on hepatocytes before HSCs, LSECs and KCs were added to assemble the transitional liver models. Proliferation of LSECs and HSCs increased with PEM stiffness. By day 8, the hepatocyte to KC ratio was 4.6-5.9 in each region of the culture, indicating inflammation. HSC activation was detected with increased αSMA expression and decreased lipid content. Increased fibrosis was further observed by increased TGFβ and hepatocyte apoptosis and decreased albumin expression as a function of stiffness. Each of these results corresponded to the development of fibrosis on the 43 kPa side of the model and inflammation, the precursor to fibrosis, on the 21 kPa side. The cells reciprocally affected the PEM, as demonstrated by the 2- to 4-fold increase in stiffness over the culture period.

5.2 Future Work

We designed and utilized organotypic liver models containing all four hepatic cell types and a Space of Disse mimic. The models were investigated for their use in acute hepatotoxicity and fibrogenesis. There is enormous potential to adapt these models for further critical studies.
Future areas of investigation include the (1) usage of the HTS models to study chronic toxicity and the design space of chemical mixtures, (2) combination of gene expression with the models to predict chemically activated pathways and (3) addition of other ECM proteins to the PEM to improve fibrosis studies.

5.2.1 HTS Models for Chronic Toxicity and Chemical Mixture Studies

In Chapter 3, we designed HTS organotypic liver models for acute hepatotoxicity studies. Extensive toxicity occurred when each chemical was administered at LC$_{50}$. However, hepatotoxicity also occurs from chronic administration of low doses of chemicals. The HTS models could be used to investigate the response of low chemical concentrations administered over extended periods. Such a study would require the introduction of HSCs into the µOCMs. HSCs secrete cytokines and ECM proteins, altering inflammation and liver stiffness, factors strongly associated with chronic hepatotoxicity.

We also investigated cell death in response to chemical mixtures, with each chemical added at $\frac{1}{2}$ LC$_{50}$. While this provided an excellent starting point, humans are not exposed to such controlled chemical combinations. This work could be extended by administering chemical mixtures at multiple concentrations to determine the relevant design space. These experiments would dictate the allowable doses of each chemical that do not cause hepatotoxicity when administered in combination.

5.2.2 Determination of Activated Pathways in Response to Toxicants

In Chapters 2 and 3, we investigated changes to biomarkers in the organotypic liver models. However, biomarkers cannot identify specific pathways activated in cells by chemicals. This is particularly true for toxicants with unknown mechanisms of action. RNA-seq can provide information at the transcriptomic level [349]. RNA-seq analysis could be used to determine the
differentially expressed pathways in treated samples. The RNA-seq data can be analyzed with the TopHat software, which aligns RNA readouts to the reference genome and determines differential expression of genes in treated samples [350, 351]. The activated pathways can be predicted through the combination of the RNA-seq data and the PathLinker software developed by the Murali research group [352].

5.2.3 Role of Different ECM Components in the Progression of Fibrosis

In Chapter 4, we investigated the local microenvironment in the progression of fibrosis from diseased to healthy tissue. This was completed with a PEM comprised of type I collagen and HA, two components found in the native Space of Disse. In vivo, the Space of Disse is composed of many ECM proteins (see Section 1.5.2). The inclusion of additional ECM components into the PEM would provide a more accurate Space of Disse mimic. For instance, fibronectin is the prominent component of this interfacial region and type IV collagen is highly prevalent in healthy and diseased livers. These proteins are associated with increased cell binding and promote organ stability. Proteoglycans are highly correlated with increased liver stiffness in fibrosis. The inclusion of such components in the Space of Disse mimic would increase physiological relevance. Additionally, controlling the concentration of each protein in the PEM could isolate the role of the individual components in the progression of fibrosis.
Chapter 6: References


77. Richert, L., et al., Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. Drug Metab Dispos, 2006. 34(5): p. 870-9.


