Investigating Acetaminophen Hepatotoxicity in Multi-Cellular Organotypic Liver Models

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ABSTRACT

In vivo studies clearly demonstrate the participation and subsequent death of non-parenchymal liver cells (NPCs) with corresponding hepatocyte effects. This results in a critical need to investigate how major liver cell types function cohesively during hepatotoxicity. However, virtually no studies replicate these phenomena *in vitro*. We report the design of multi-cellular three-dimensional (3D) organotypic liver models of primary rat hepatocytes, liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs). LSECs and KCs were separated from hepatocytes by a detachable membrane that emulates the physical and chemical properties of the Space of Disse. Acetaminophen (APAP)-induced changes to cellular function and phenotype were investigated. LSECs exhibited approximately 40% cell death at 20 mM APAP. KCs exhibited decreased interleukin-10 and increased tumor necrosis factor-alpha and interferon-gamma secretion. The secretion of these proteins altered hepatocyte function and signaling. Both LSECs and KCs maintained phenotypic markers. At 20 mM APAP, the 3D models exhibited aspartate aminotransferase to alanine aminotransferase ratios from 2.1-2.5 and 45% glutathione depletion, corresponding to what is seen *in vivo*. At 10 and 20 mM APAP, the 3D models exhibited cell death, primarily through necrosis. Therefore, the 3D cultures described in this report demonstrate significant potential as realistic models for hepatotoxicity studies.

KEYWORDS

Acetaminophen; liver; organotypic culture models; multi-cellular

ABBREVIATIONS

2DHL = 2D co-culture of hepatocytes and liver sinusoidal endothelial cells, 3DHL = 3D culture of hepatocytes, collagen/hyaluronic acid multilayer and liver sinusoidal endothelial cells, 3DHLK = 3D culture of hepatocytes, collagen/hyaluronic acid multilayer, liver sinusoidal endothelial cells and Kupffer cells, ALT = alanine aminotransferase, APAP = acetaminophen, AST = aspartate aminotransferase, CS = collagen sandwich, CYP = cytochrome P450, DMEM = Dulbecco's modified Eagle medium, ECM = extracellular matrix, GSH = glutathione, HM = hepatocyte monolayer, HRP = horseradish peroxidase, HSCs = hepatic stellate cells, IFN- γ = interferon gamma, IL-10 = interleukin 10, KCs = Kupffer cells, LSECs = liver sinusoidal endothelial cells, MTT = thiazolyl blue tetrazolium bromide, NAPQI = *N*-acetyl-*p*-benzoquinoneimine, NPCs = non-parenchymal liver cells, PBS = phosphate buffered saline, PVDF = polyvinylidene fluoride, TNF- α = tumor necrosis factor alpha

INTRODUCTION

The liver is primarily responsible for the biotransformation of many toxicants and xenobiotics (Arias et al., 2001). Such biotransformation can induce hepatotoxicity, a phenomenon investigated *in vivo* and *in vitro* (Godoy et al., 2013; Kaplowitz, 2005; Klassen, 2013). 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) (Arias et al., 2001). 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 (Godoy et al., 2013; LeCluyse et al., 2012). LSECs serve as the first line of defense when a toxicant enters the hepatic microcirculation (Ito et al., 2003; Sorensen et al., 2015). 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 (Bataller and Brenner, 2005; Godoy et al., 2013; Ito et al., 2003). 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 (Godoy et al., 2013; Ito et al., 2003; Ju et al., 2002). 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) (Godoy et al., 2013; LeCluyse et al., 2012). 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 (Bhatia et al., 1999; Godoy et al., 2013; LeCluyse et al., 2012). Some 2D co-culture studies have reported hepatocyte-NPC interactions in the presence of toxicants (DeLeve et al., 1997; Milosevic et al., 1999; Nelson et al., 2015). 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 (Godoy et al., 2013). Common 3D liver models include bioreactors (Allen et al., 2005; Mueller et al., 2013), microfluidic devices (Toh et al., 2009; Trietsch et al., 2013), gel entrapped hepatocytes (Lan et al., 2010; Shen et al., 2007), spheroids (Gunnness et al., 2013; Messner et al., 2013), and scaffolds (Kostadinova et al., 2013; Toyoda et al., 2012). 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.* (2013) assembled a 3D hepatic model for toxicity with a NPC fraction (seeded on a Nylon scaffold) above hepatocytes. 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 (Kim et al., 2010; Kim and Rajagopalan, 2010; Larkin et al., 2013). 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 (Larkin et al., 2013). Additionally, cytochrome P450 (CYP) 1A1/2 and CYP3A enzyme activities were maintained (Kim and Rajagopalan, 2010; Larkin et al., 2013). 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 (McGill and Jaeschke, 2013). *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 (Hinson et al., 2010; McGill and Jaeschke, 2013). 2D

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4 and 3D models were investigated to determine the extent and mechanisms of cell death,
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6 changes in GSH and the ratio of aspartate aminotransferase (AST) to alanine
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8 aminotransferase (ALT). Cytokine secretion was investigated to determine if APAP
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10 toxicity resulted in inflammation in these models. A unique feature of these 3D models is
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12 the ability to monitor changes in NPC counts and phenotypes in response to the toxicant
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14 thereby providing a better understanding of APAP hepatotoxicity. Our goal was to
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16 determine how closely the 3D models compared to the biotransformation of APAP
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18 reported in rodents.
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24 MATERIALS AND METHODS

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

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56 Collagen was extracted from rat tails using previously described procedures (Ford et al.,
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58 2015). Briefly, tendons were dissolved in acetic acid and centrifuged at 13,000 x g. A
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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.

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 (Kim et al., 2010; Kim and Rajagopalan, 2010; Larkin et al., 2013). 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 $\geq 97\%$ as determined through trypan blue exclusion. LSEC separation was conducted by differential adhesion and the cells were cultured in fibronectin-coated flasks (Kim and Rajagopalan, 2010; Larkin et al., 2013).

Hepatocytes were seeded as monolayers at 0.5×10^6 cells/well in a 12-well plate on collagen gels (Larkin et al., 2013). 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.

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 **Supplementary Data** 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 (Larkin et al., 2013).

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 1**. For a detailed description of changes in hepatocyte and NPC counts in each culture, refer to the **Supplementary Figure S1**.

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 (Bachmann et al., 1996; Belanger et al., 1987; Lee and Blaufox, 1985). Additionally, these concentrations are consistent with

those used for *in vivo* and *in vitro* studies (**Table 1**). Spent culture medium and cell lysates were extracted after the 24 h APAP incubation.

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 μ L/ml of Hoechst 33342 (cell nuclei), ethidium homodimer III (necrotic) and FITC-annexin V (apoptotic). Imaging was performed on a Nikon TE2000 inverted microscope.

Measuring Overall Cell Viability

The overall cell viability of control and APAP-treated hepatic cultures was assayed using the MTT assay (Mosmann, 1983). 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.

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.

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 (Kim and Rajagopalan, 2010; Larkin et al., 2013). 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).

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 Diagnostics)). 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.

Measuring Changes in GSH in Hepatocytes

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4 In **2DHL** cultures, LSECs were separated from the hepatocytes using a magnetic cell
5 separation process (Larkin et al., 2013). Briefly, the CD32b antibody was conjugated to
6 Dynabeads® (CELLlection™ Kit; Invitrogen Thermo Fisher Scientific) (Tokairin et al.,
7 2002). The LSEC fraction adhered to the beads and was separated using a magnet
8 (DynaMag™, Invitrogen Thermo Fisher Scientific). The supernatant contained the
9 hepatocyte fraction. In the **3DHL** and **3DHLK** cultures, the hepatocyte fraction was
10 obtained upon removal of the multilayer.
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22 Hepatocytes were lysed in PBS (1X) containing 1 mM ethylene diamine tetra-acetic acid
23 maintained at 4 °C. The lysates were centrifuged at 10,000 x *g* for 15 min, and the
24 supernatant was collected and stored at -20 °C. Total GSH was measured using a
25 commercially available colorimetric kinetic assay kit (Glutathione Assay Kit; Cayman
26 Chemicals, Ann Arbor, MI). The absorbance was measured at 405 nm every 5 min for
27 30 min. The total GSH concentration was determined by calibrating to a standard curve.
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38 ***Western Immunoblotting for CYP2E1***

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

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 VivaspinTM 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.

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 $\pm$ standard deviation; n denotes sample size (number of separate experiments). The data presented in this report were collected and analyzed from up to five independent experiments, with three replicates per experiment.

RESULTS

Most *in vitro* studies report changes in viability, hepatocyte function and CYP expression (Allen et al., 2005; Khetani and Bhatia, 2008; Toh et al., 2009). While this information is very helpful, the role of NPCs has largely been ignored in *in vitro* hepatotoxicity investigations. We report how APAP affects i) the model as a whole, ii) each cell type and iii) the secretion of signaling molecules. 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 multi-cellular 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

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**). 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.

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**). 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 3**). In the **CS** and 3D models, the primary mode of cell death was necrosis, consistent with prior studies on APAP (Hinson et al., 2010; McGill and Jaeschke, 2013). At 20 mM APAP, apoptotic cells in **CS** ($3.2 \pm 0.8\%$), **3DHL** ($7.4 \pm 1.0\%$) and **3DHLK** cultures ($11.5 \pm 3.2\%$) 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 $23.3 \pm 6.6\%$ (**HM**) and $15.0 \pm 4.8\%$ (**2DHL**) apoptotic cells.

At all concentrations of APAP, LSEC and KC monolayers exhibited at most 10% cell death (**Table 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 (Hinson et al., 2010; McGill and Jaeschke, 2013; Sellaro et al., 2007; Zinchenko et al., 2006). 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**). This is consistent with *in vivo* studies that reported significant LSEC death upon APAP administration (DeLeve et al., 1997; Ito et al., 2003). 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 (Antoniades et al., 2012; Zigmond et al., 2014).

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** (**Supplementary Table S1**). In contrast, untreated **2DHL** cultures exhibited fewer CD32b-expressing cells compared to **3DHL** or **3DHLK** models (**Figure 4**). The number of CD32b-expressing LSECs was statistically lower in the **2DHL** models than those counted using cytoplasmic staining (**Supplementary Table S1**), 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 (**Supplementary Table S1**).

We further investigated the decrease in CD32b-expressing cells associated with an increase in LSECs expressing CD31 (a marker for dedifferentiated LSECs) (**Figure 5**; further information in **Supplementary Table S2**) (Elvevold et al., 2008). 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.

Changes in Liver Toxicity Markers in Response to APAP

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 (Dufour et al., 2000). This ratio ranged from 0.64-1.22 in the 2D models upon exposure to 10 and 20 mM APAP (**Figure 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.

Measuring Changes in Hepatocyte GSH and CYP2E1

The concentration of total GSH was measured in hepatocyte lysates (**Figure 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 7a**).

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 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 7b**), whereas IFN- γ and TNF- α increased by approximately 50% and 11%, respectively (**Figures 7c and 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 7e** (Ishida et al., 2002), 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 (**Supplementary Table S3**). 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.

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 demonstrate differences in 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 (Bataller and Brenner, 2005; Godoy et al., 2013; Hinson et al., 2010; Ito et al., 2003). For example, LSECs contract and lose their fenestrae and scavenging abilities (Godoy et al., 2013; Hinson et al., 2010; Ito et al., 2003). Activated KCs proliferate and secrete pro- and anti-inflammatory cytokines (Godoy et al., 2013; Hinson et al., 2010). 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 (**Supplementary Figure S2**). These data suggest that the inclusion of NPCs was critical to the maintenance of sensitivity to APAP that occurred within the 3D models.

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4 Closer examination of each cell type in the **3DHL** and **3DHLK** models revealed
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6 additional information. For example, hepatocyte death in these models occurred
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8 primarily via necrosis, identical to studies conducted *in vivo* (Hinson et al., 2010; McGill
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10 and Jaeschke, 2013). However, increased secretion of IFN- γ by KCs resulted in
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12 approximately 11% apoptosis in the **3DHLK** models (Ishida et al., 2002). In contrast, **HM**
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14 and **2DHL** cultures exhibited significant apoptosis even in the absence of APAP
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16 suggesting that they are less suitable for studies on mechanisms associated with cell
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18 death. Although hepatocytes in the 3D models exhibited increased expression of
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20 CYP2E1 and other markers of toxicity, the improved albumin secretion suggests that the
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22 NPCs acted to support the maintenance of hepatocyte-specific functions. The loss of
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24 CYP2E1 protein in the 2D models is consistent with reports in the literature that
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26 demonstrate 2D co-cultures do not maintain hepatocyte CYP2E1 expression (Donato et
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28 al., 1994). The lack of expression of this enzyme could explain the insensitivity to APAP
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30 in the 2D cultures (**Figure 7A**; **Supplementary Figure S3**).
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38 *In vivo* studies have reported that the sinusoidal endothelium suffers the greatest initial
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40 impact upon exposure to hepatotoxicants (DeLeve et al., 1997; Ito et al., 2003; Liu et al.,
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42 2013). In the present study, this was only observed in the 3D cultures. Additionally, only
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44 the 3D models consistently expressed CD32b, a marker of LSEC phenotype (Geraud et
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46 al., 2010). Interestingly, the addition of KCs resulted in the simultaneous maintenance of
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48 LSEC phenotype and increased LSEC sensitivity to APAP. Although the exact
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50 mechanisms for LSEC death are not fully understood, we hypothesize that the decrease
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52 in IL-10 may have played a role. There is evidence that LSECs undergo classical and
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54 alternative activation due to exposure to cytokines (Liu et al., 2013). Specifically,
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56 removal of IL-10 from culture medium resulted in the transition of LSEC phenotype from
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58 M2 (anti-inflammatory) to M1 (pro-inflammatory). Such LSEC activation is also
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4 associated with the production of inducible nitric oxide synthase, which is directly
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6 correlated to cell death in response to APAP (Bourdi et al., 2002). This could explain the
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8 increased LSEC death exhibited in the **3DHLK** models and merits further investigation.
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13 The proliferation and activation of KCs results in increased inflammation (Antoniades et
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15 al., 2012; Zigmond et al., 2014). In the present study, cell death in KC monocultures was
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17 minimal and did not correlate to APAP concentration, suggesting loss of sensitivity. In
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19 direct contrast, KC proliferation and death in the **3DHLK** models was dependent on
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21 APAP concentration. Furthermore, APAP treatment resulted in up to a 9-fold decrease in
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23 IL-10 with a concomitant increase in TNF- α and IFN- γ . Taken together, these results
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25 indicate that in response to APAP, KC activation results in inflammation in the **3DHLK**
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27 models.
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33 *In vivo* hepatotoxicity studies have demonstrated that increased liver toxicity occurs
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35 through the production of pro-inflammatory cytokines (Gardner et al., 2003) or decrease
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37 of anti-inflammatory cytokines (Bourdi et al., 2002). Changes in cytokine concentrations
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39 in the **3DHLK** models indicate a shift in KC phenotype from M2 to M1 upon exposure to
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41 APAP. Paracrine signaling appears to have modulated cytokine secretion. For example,
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43 at 40 mM APAP, the TNF- α secretion was higher in the **3DHLK** models than the KC
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45 monocultures. This can be attributed to increased TNF- α secretion from hepatocytes in
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47 the presence of KCs (Cao et al., 2005; Godoy et al., 2013; Nastevska et al., 1999). The
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49 secretion of IFN- γ was also significantly higher in the **3DHLK** models. These results
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51 suggest that the increases in TNF- α and IFN- γ were facilitated through heterotypic inter-
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53 cellular interactions and merit further investigation.
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4 It is critically important to note that at 20 mM APAP (800 mg/kg APAP, **Table 1**), only the
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6 3D models exhibited AST/ALT ratios from 2.1-2.5, identical to values obtained *in vivo*
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8 (Ahmed et al., 2011; Kikkawa et al., 2006). At 10 and 20 mM APAP, the increased
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10 AST/ALT ratio in the 3DHLK models parallels that reported previously (Ishida et al.,
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12 2002). *In vivo*, rats treated with 800 mg/kg APAP exhibited a 40% reduction in GSH
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14 (Ahmed et al., 2011). At the corresponding *in vitro* concentration (20 mM) only the 3D
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16 models exhibited a 45% decrease in GSH. At longer time-points only the 3D models
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18 exhibited sensitivity to APAP. This property makes the 3D models relevant for
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20 investigations that require longer testing periods (further information can be found in the
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22 **Supplementary Figure S4**).
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29 The strong relationships between the 3D models and data obtained *in vivo* demonstrate
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31 the potential use of these multi-cellular models for drug testing. These appear to be
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33 excellent 3D *in vitro* hepatic models for emulating changes observed in hepatic cell
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35 types, closely mimicking events that occur in livers of APAP-treated rodents. The ability
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37 to include and isolate NPCs in organotypic liver models described here provides
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39 capability to conduct systematic measurements of toxicant effects on each cell type. The
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41 future inclusion of HSCs in the 3D models will further increase insights into HSC-
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43 hepatocyte interactions that modulate response to toxicants. Experiments designed to
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45 delineate intricate signaling pathways between NPCs and hepatocytes will provide
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47 additional insights. Our current experiments demonstrate that the 3D organotypic models
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49 offer potential for a new avenue to bridge the gap between *in vitro* and *in vivo* toxicity
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51 investigations.
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58 **ACKNOWLEDGEMENTS**

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4 We gratefully acknowledge financial support from the United States Environmental
5
6 Protection Agency (US EPA Grant: R834998, P.R. and M.F.E.) and the National Science
7
8 Foundation (DBI 1062380, P.R.). We also acknowledge financial support from the
9
10 Institute for Critical Technologies and Applied Sciences at Virginia Tech and the
11
12 Computational Tissue Engineering Graduate Education Program at Virginia Tech.
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18 **AUTHOR DISCLOSURE STATEMENT**

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21 No competing financial interests exist.
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Table 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 (Ahmed et al., 2011; Allen et al., 2005; Khetani and Bhatia, 2008; Kikkawa et al., 2006; Messner et al., 2013; Nastevska et al., 1999; Shen et al., 2007; Toh et al., 2009).

Dose (mg/kg)	Concentration (mM)
300	~10
800	~20
1,500	~40

Table 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 $\pm$ SD, $n = 3$.

	APAP Concentration (mM)	Hepatocytes (%)	LSECs (%)	KCs (%)
2DHL	0	100.0 $\pm$ 5.1	100.0 $\pm$ 10.7	-
	10	97.8 $\pm$ 11.3	84.6 $\pm$ 11.1	-
	20	86.0 $\pm$ 8.4	81.8 $\pm$ 10.7	-
	40	68.4 $\pm$ 9.4	56.0 $\pm$ 14.2	-
3DHL	0	100.0 $\pm$ 7.3	100.0 $\pm$ 10.6	-
	10	112.6 $\pm$ 17.3	74.2 $\pm$ 5.1	-
	20	106.7 $\pm$ 1.9	57.6 $\pm$ 8.4	-
	40	81.2 $\pm$ 4.0	45.7 $\pm$ 7.0	-
3DHLK	0	100.0 $\pm$ 5.1	100.0 $\pm$ 11.6	100.0 $\pm$ 12.8
	10	110.7 $\pm$ 1.1	68.7 $\pm$ 6.0	114.7 $\pm$ 9.0
	20	96.3 $\pm$ 0.7	60.1 $\pm$ 10.8	134.3 $\pm$ 18.3
	40	78.6 $\pm$ 1.0	38.5 $\pm$ 8.4	52.5 $\pm$ 9.5
LSEC Monolayer	0	-	100.0 $\pm$ 13.7	-
	10	-	102.1 $\pm$ 8.4	-
	20	-	94.9 $\pm$ 1.6	-
	40	-	107.3 $\pm$ 8.7	-
KC Monolayer	0	-	-	100.0 $\pm$ 3.1
	10	-	-	96.3 $\pm$ 13.3
	20	-	-	100.5 $\pm$ 18.5
	40	-	-	89.5 $\pm$ 8.5

Table 3: Cytokine concentration in unused hepatocyte medium (denoted as “medium”) and untreated (0 mM APAP) liver models, mean $\pm$ SD, $n = 3$. Cytokine is listed as “undetectable” when the concentration was below the **detection** limit provided by the manufacturer.

Model	IL-10 (pg/ml)	TNF- α (pg/ml)	IFN- γ (pg/ml)
Medium	3.06 $\pm$ 0.08	Undetectable	3.38 $\pm$ 0.31
HM	3.34 $\pm$ 0.72	Undetectable	3.90 $\pm$ 0.26
CS	3.42 $\pm$ 1.03	Undetectable	3.42 $\pm$ 0.07
2DHL	3.50 $\pm$ 0.24	Undetectable	3.97 $\pm$ 0.52
3DHL	3.73 $\pm$ 0.67	Undetectable	2.98 $\pm$ 0.15
3DHLK	132.50 $\pm$ 32.32	8.61 $\pm$ 0.86	6.21 $\pm$ 0.39

Figure 1

Hepatocytes
 LSECs
 KCs
 Collagen Gel
 Collagen/Hyaluronic Acid Multilayer

(g)

Model	Abbreviation	Schematic
Hepatocyte monolayer	HM	Fig 1a
Collagen sandwich	CS	Fig 1b
2D co-culture of hepatocytes and LSECs	2DHL	Fig 1c
3D model with hepatocytes, multilayer and LSECs	3DHL	Fig 1d
3D model with hepatocytes, multilayer, LSECs, and KCs	3DHLK	Fig 1e

Figure 2

Figure 3

Figure 4

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Figure 6

Figure 7