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Microphysiological system for studying fatty liver disease and its impact on drug-induced liver injury

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Introduction

As a result of the increased prevalence of diabetes, obesity and metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) is now the most common chronic liver disease in developed countries (1). NAFLD is a spectrum of pathologies ranging from benign hepatic steatosis through to non-alcoholic steatohepatitis (NASH), which can ultimately lead to cirrhosis and liver cancer. There are currently no FDA approved drugs for the treatment of NAFLD/NASH and there is a clear requirement for better models to understand this disease.

There is additionally a growing awareness of the potential risk factors for drug induced liver injury (DILI) due to the underlying metabolic condition. DILI in NAFLD patients can be exacerbated in two distinct ways; some drugs seem to aggravate pre-existing NAFLD causing more extravagant intracellular lipid accumulation, whereas more frequently, other drugs induce acute hepatitis and liver injury (2). Higher risk of drug-induced acute hepatitis in obesity is proposed to relate to increased activity of several cytochromes P450 (CYPs), which enhance the generation of toxic metabolites (2). When generated in excess, reactive metabolites can induce hepatic oxidative stress, severe mitochondrial dysfunction and cytolysis.

However, the mechanisms by which these processes occur are poorly understood and adverse drug responses due to fatty liver are becoming more common with the ever-increasing obesity epidemic. Therefore, we have developed an advanced in vitro model to explore the relationships and mechanisms that link DILI and NAFLD.

Using a microphysiological system (MPS), we have developed a fully human perfused in vitro NAFLD model, utilising primary human hepatocytes (PHH) cultured in 3D to mimic the liver microarchitecture. Cells are cultured with high concentrations of free fatty acids for up to four weeks to induce intracellular triglyceride (fat) accumulation and mimic hepatic steatosis. Cells in this model were investigated for changes to the CYP activity and affects of known hepatotoxicants were investigated when dosed at or around IC:50 concentrations.

Cryopreserved plateable primary human hepatocytes (PHH) were obtained from ThermoFisher® (USA). 0.6 x 106 hepatocytes were seeded into each well on the PhysioMimix™ OOC LC-12 plate (CN Bio Innovations Ltd) and were cultured in HEP-Lean or HEP-Fat medium which contains physiologically relevant concentrations of glucose, insulin, and high concentrations of saturated and unsaturated free fatty acids. Cells were cultured in the PhysioMimix™ platform for up to 18 days.

Fat accumulation was measured by Oil Red O staining of fixed microtissues, with images acquired on a Nikon Eclipse Ti-E inverse fluorescent microscope. Staining was quantified by absorbance at 515 nm and normalised to total protein content, measured by BCA assay (ThermoFisher). Production of albumin was measured by ELISA (R&D systems), LDH release was quantified using the Cyto-tox96 assay (Promega) and cell viability was assessed using the Cell Titre Glo assay (Promega).

For transcriptomic analysis, total RNA was extracted from scaffolds using TRIzol. cDNA samples were generated and compared using RT2 profiler arrays (Qiagen) specific for genes associated with fatty liver disease. To

assess CYP activity, two methods were utilised; either using the CYP3A-glo assay (Promega) or using LC-MS to quantify specific compound concentrations. Briefly, cells were exposed to a range of compounds specific for different CYP-450 enzymes. The following compounds were used: Phenacetin (CYP1A2), Diclofenac (CYP2C9), S-Mephenytoin (CYP2C19), Bufuralol (CYP2D6), Midazolam (CYP3A4) and Chlorzoxasone (CYP2E1). Each compound was dosed for 48 hours at a starting concentration of 1 μM using a vehicle of 0.1% DMSO. The production of phase I metabolites from each compound was quantified using LC-MS from cell culture medium samples.

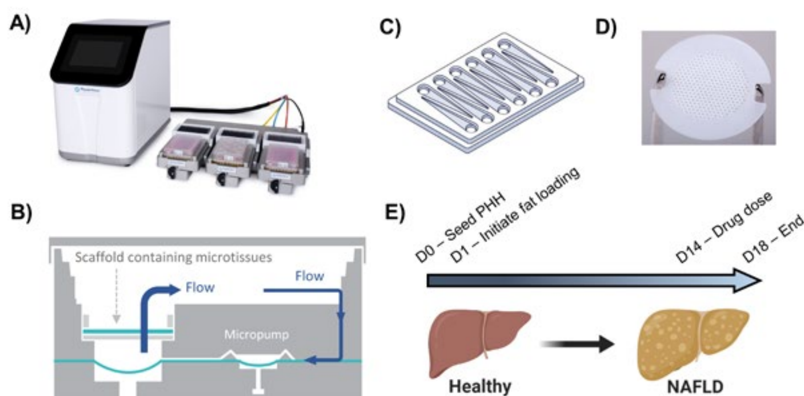


Figure 1 – Human in vitro MPS NAFLD model

A) The in vitro model utilises the PhysioMimix™ OOC cell culture system, which uses open well plates designed for the culture of primary liver cells in 3D in an engineered scaffold. B) Schematic representation of individual culture well on LC-12 liver-on-chip plate. C) Schematic of LC-12 multi-well plate. D) The scaffolds held within each well on the plate are continually perfused with cell culture medium throughout the experiment to provide biomechanical stimuli and oxygen provision (3). E) The NAFLD model is generated by culturing PHH in high fat medium for up to 28 days. For studying DILI, compounds were dosed after 14 days of fat loading.

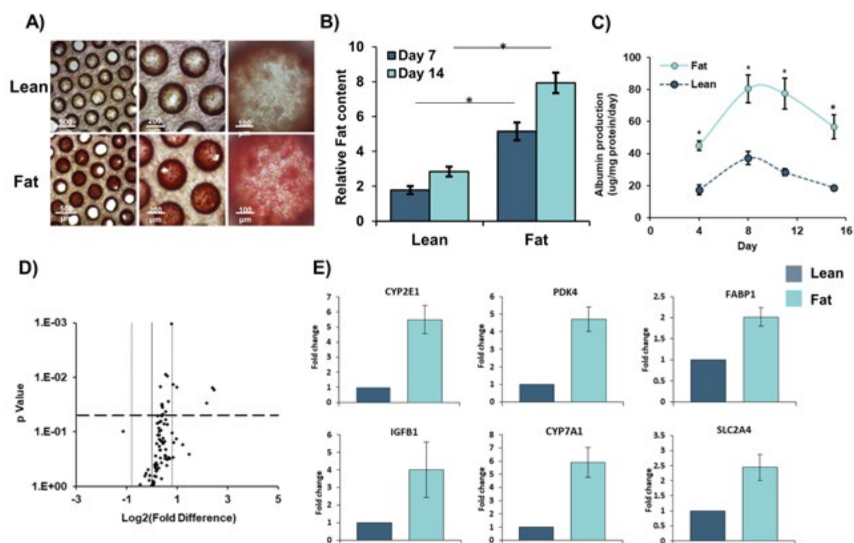


Figure 2 – Primary Human Hepatocytes accumulate intracellular fat over time. Those cultured in fat media show altered gene expression profiles.

PHH were cultured for 14 days under fat and lean conditions. Fat loading was measured by Oil Red O staining of microtissues and gene expression was compared using Liver RT2 Profiler PCR Arrays. A) Cells were imaged for intracellular fat accumulation, and B) quantified by absorbance at 510 nm and normalised to total protein content. C) Albumin production was quantified by ELISA and gene expression changes between lean and fat cultures D) were defined by a fold change >1.8 and P = <0.05. E) Fold change in expression in fat vs lean condition of key genes. Data are mean ± SEM from 9 independent cultures (three donors per condition and n=3 per donor); * = P < 0.05. Data taken from previous publication Kostrzewski et al. 2017 (3).

Results

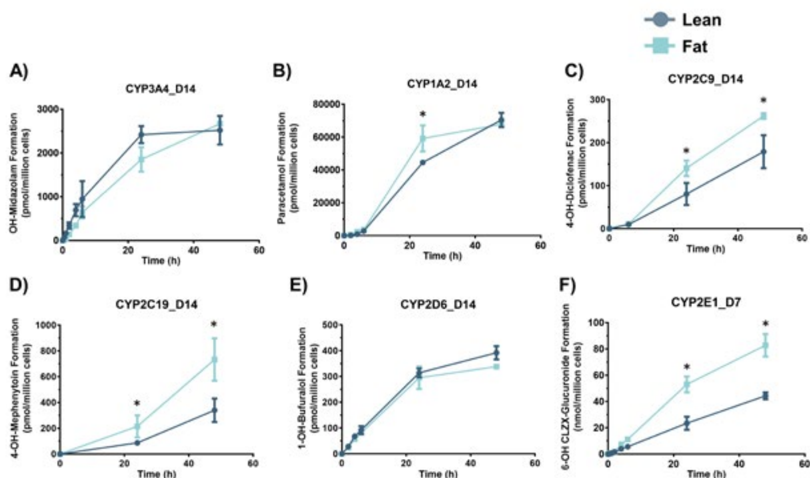
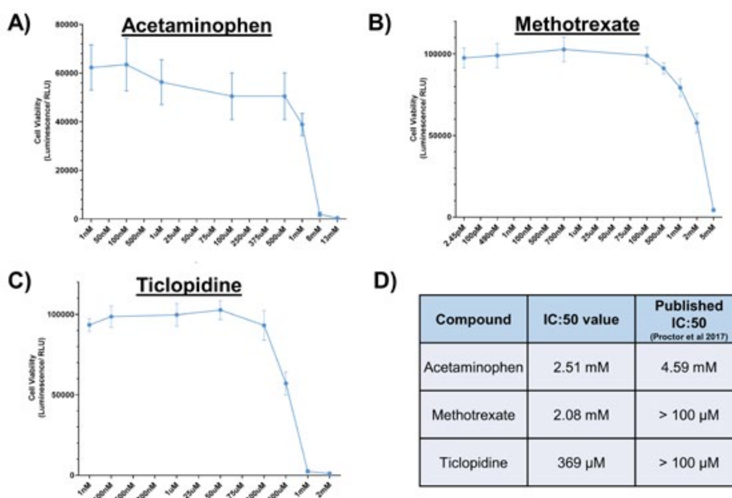


Figure 3 – Hepatocytes in NAFLD model have altered metabolic activity

PHH were cultured for 14 days under fat conditions and then dosed with probe compounds to assess metabolic activity of key CYP-450 enzymes. Activity of each enzyme was determined by the production of phase I metabolites over 48 hours. The presence of the metabolites was quantified using LC-MS and a standard curve of the metabolite. All compounds were dosed at day 14 expect for CLZX (CYP2E1 target) which was dosed at day 7. All data are a minimum of n = 3. * = $P < 0.05$.



Compound	IC:50 value	Published IC:50 (Proctor et al 2017)
Acetaminophen	2.51 mM	4.59 mM
Methotrexate	2.08 mM	> 100 µM
Ticlopidine	369 µM	> 100 µM

Figure 4 – Determining IC:50 concentrations for compounds known to cause DILI

PHH were cultured for 72 hours on collagen-coated 96-well plates, under lean media conditions. They were exposed to varying concentrations of A) Acetaminophen, B) Methotrexate and C) Ticlopidine for 48 hours before cell viability was assessed using Cell Titre Glo. D) IC:50 values for each compound were generated and compared to published data (4). Data shown are a mean ± SD and a minimum of N=4.

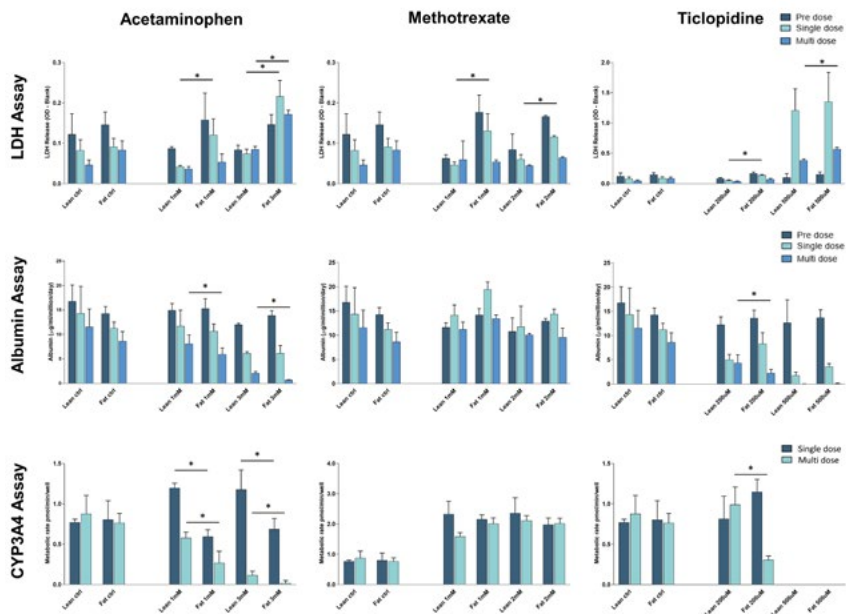


Figure 5 – Fat loading of hepatocytes increases susceptibility to DILI by acetaminophen and ticlopidine

PHH were cultured for 14 days under fat conditions and then dosed with concentrations of compound around the IC:50 value previously determined. PHH microtissues were dosed with a single 48 hour dose or two 48 hour doses (multi dose) of each compound. All compounds were dosed in lean media with a vehicle of 0.1% DMSO. Control lean and fat microtissues were dosed with vehicle alone. Cell health was assessed by measuring LDH release in cell culture medium, albumin production in cell culture medium and determining CYP3A4 activity of hepatocytes following dosing with the different compounds. Data are mean ± SD, n = 4. * = $P < 0.05$.

Conclusions

Utilising the MPS platform PhysioMimix™ we have generated a human in vitro model of NAFLD. PHH were cultured in fat containing medium which induced key features of the early stages of the clinical disease including intracellular fat loading, an increase in albumin production and changes to the expression of key genes (including those involved with metabolism and insulin resistance). We further investigated the metabolic activities of PHH following fat loading to ascertain how the activities of CYP enzymes are modulated by the intracellular lipid accumulation. Mimicking in vivo (clinical) observations (2) we saw increases in a wide range of enzymes including: CYP1A2, CYP2C9 (1.5 fold), CYP2C19 (2-fold) and CYP2E1 (2.5 fold) activity. In addition, we observed a minor reduction in CYP3A4 activity following fat loading. The impact of these metabolic changes on the liver cells in regards to their susceptibility to DILI was explored by assessing the toxicity of Acetaminophen, Ticlopidine and Methotrexate in the in vitro NAFLD model.

Both Acetaminophen and Ticlopidine caused more extravagant DILI responses in fat loaded cells, causing increased release of LDH, reducing the production of albumin and reducing CYP3A4 activity (as a measure of cell viability). These changes were often significantly more prominent when the compounds were dosed multiple times onto the hepatic tissues. Dosing with Methotrexate did not appear to cause DILI in the presence of the fat loaded hepatocytes. Together this data demonstrate how the MPS platform and associated NAFLD model capture the key features of the early stages of human NAFLD and demonstrate a varied response to DILI as has been observed in clinical studies.

This approach will be a highly useful tool for analysing the toxicity profiles of novel compounds and how they behave (cause DILI) in diverse patient subsets.

References

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