Evaluating Liver Toxicity in Bioprinted Mini Livers

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Abstract

Drug-induced liver injury (DILI) affects the liver's ability to metabolize and detoxify substances, but its underlying mechanisms are largely unknown. To accurately and reproducibly predict DILI in humans, there is a significant need for *in vitro* liver models that replace costly and low-throughput 2D cell culture systems, animal studies and lab-on-a-chip models. Here, we present a new method of "droplet in droplet" (DID) bioprinting to produce physiologically relevant liver models for hepatotoxicity studies. These models, or mini livers, were produced using a BIO X to droplet-print hepatic (HepG2 and LX2) and nonhepatic (HUVEC) cells encapsulated in type I collagen. After 7 days of culture, mini livers were exposed to acute and high doses of acetaminophen or flutamide, then evaluated for changes in cell viability, albumin secretion, alanine aminotransferase (ALT) activity and lipid accumulation. Increased ALT activity and low albumin and lipid production in mini livers suggested a cytotoxic response to both drugs. The results of this study further validate 3D bioprinting as a viable and medium- to high-throughput solution for modeling hepatic tissue and screening idiosyncratic drug reactions.

Introduction

Drug-induced liver injury (DILI) is a leading cause of liver disease and acute liver failure (ALF). The risk factors for DILI are elusive, but drug properties and disposition can affect DILI development and play a major role in drug attrition and withdrawal from market. In fact, preclinical studies in 2015 showed that 50% of drug candidates failed due to liver toxicity, justifying the need for a model that can predict drug toxicity and mirror abnormalities associated with DILI (Chen, 2015). Recently, abnormalities like upregulated alanine aminotransferase (ALT) and reduced albumin production have been studied in 3D models like hanging droplets and spheroids (Shah, 2018). These models, however, are unrepresentative and low-throughput. As an alternative, bioprinted tissue models can be used for medium- to high-throughput drug screenings, to reduce drug attrition and fast-track preclinical phases of drug development (Ramaiahgari, 2014).

This study evaluates the effects of two drugs on mini livers that were produced using a new method of "droplet in droplet" (DID) bioprinting. DID bioprinting allowed for controlled cellular arrangements and cell-matrix interactions, and provided unique multi-layered models to study drug penetration and response. The compounds used in this study, acetaminophen (APAP) and flutamide (FLU), are frequently used to evaluate liver toxicity. These compounds are categorized by severity, for which Category 1 is considered "Severe Clinical DILI," Category 2 is "High Clinical DILI," and Category 3 is "Low Clinical DILI Concern" (Proctor, 2017). FLU and APAP fit into Categories 1 and 3, respectively. FLU is an antiandrogen used to treat prostate cancer; and APAP is a widely used analgesic and COX-3 inhibitor. At high doses, both drugs have been linked to hepatic toxicity, oxidative stress, ALF and DILI, which makes them ideal for evaluating the efficacy and functionality of bioprinted liver models (Behrends, 2019; Zhang, 2018).

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Materials and Methods

Cell Preparation

Two hepatic (HepG2 and LX2) and one nonhepatic (HUVEC) cell line were cultured according to suggested protocols and passaged every 3–4 days. HepG2 were grown in MEM α with GlutaMax and supplemented with 1% Sodium Pyruvate (Gibco, Cat #11360070) and 1% MEM Non-Essential Amino Acids Solution (Gibco, Cat #11140050). LX2 cells were grown in IMDM (Gibco, Cat #12440053), and HUVECs were cultured in EGM-2 Growth Medium (Lonza, Cat #CC-3156) with SingleQuots Supplements (Lonza, Cat #CC-4176). All media were supplemented with 10% FBS (Gibco, Cat #16000044) and 1% penicillin streptomycin (Gibco, Ref #1509-70-063).

Bioink Preparation and Droplet-in-Droplet Bioprinting

A 3 mg/mL concentration of Coll I bioink (CELLINK, SKU #IK4000002001) was neutralized and prepared for bioprinting. An ice-cold cartridge was loaded with $5x10^6$ cells/mL at a ratio of 1:1:2 (LX2:HUVEC:HepG2). Mini livers were bioprinted in an untreated 96-well plate (Thermo Fisher Scientific) using the Droplet Print function on a BIO X (CELLINK, SKU #000000022222). A temperature-controlled printhead (TCPH, SKU #000000020346) set to 8°C was used to dispense collagen droplets onto a cooled printbed set to 8°C–10°C. After the first round of droplet printing, samples were incubated for 3 minutes at 37°C and returned to the BIO X for a second round of droplet printing using the same parameters. The resulting encapsulated droplets were thermally crosslinked for 20 minutes at 37°C and provided with 200 µL cocktail media (25% IMDM + 25% DMEM + 50% MEM) per well. Media was refreshed every 2–3 days.

Drug Treatment and Analysis

After 7 days of culture, mini livers were treated with varying concentrations of APAP [0.1, 0.5, 1, 5, 10, 25, 50 mM] (Abcam) or FLU [10, 25, 50, 75, 100, 150, 200 µM] (Selleckchem) for 72 hours. A colorimetric Bromocresol Green (BCG) Assay (Sigma-Aldrich), ALT Activity Assay (BioVision) and LIVE/DEAD staining kit (Invitrogen) were used to detect albumin production, liver injury and cell viability, respectively. All assays were performed according to their manufacturer's instructions. In parallel, an AdipoRed Assay Reagent (Lonza) was used to detect intracellular lipid accumulation. Samples were digested with Cell Collect G (CELLINK, SKU #LR020000) at 37°C for 30 minutes and resuspended in PBS with 5 µL AdipoRed Reagent (Lonza) for plate reader analysis. Lipid presence was validated using an Oil Red O (ORO) Staining Kit (Abcam). Samples were fixed with 4% paraformaldehyde for 2 hours, incubated in 30% sucrose overnight at 4°C, frozen in OCT and cryosectioned into 8 µm-thick sections. Stained samples were imaged using an EVOS Auto 2 Fluorescent Microscope.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.2.1. All data are expressed as Mean ± SEM of 2 experiments carried out in triplicate. Differences in treated conditions were analyzed using a one-way analysis of variance (ANOVA) and considered significant for p values < 0.05.

Results and Discussion

Cell Growth and Spheroid Formation in Collagen I

In this study, we evaluated cell growth, migration patterns and spheroid formation in our Coll I bioink. By Day 2, HepG2 and LX2 had tightly assembled into small clusters and HUVECs had elongated to form concentric networks (Figure 1). The use of collagen as a scaffold allowed for cellular reorganization, spheroid polarization and cell proliferation throughout culture (data not shown). Furthermore, based on Figure 1, it is apparent that cells permeate the DID model throughout culture and possibly migrate between inner and outer droplet layers.

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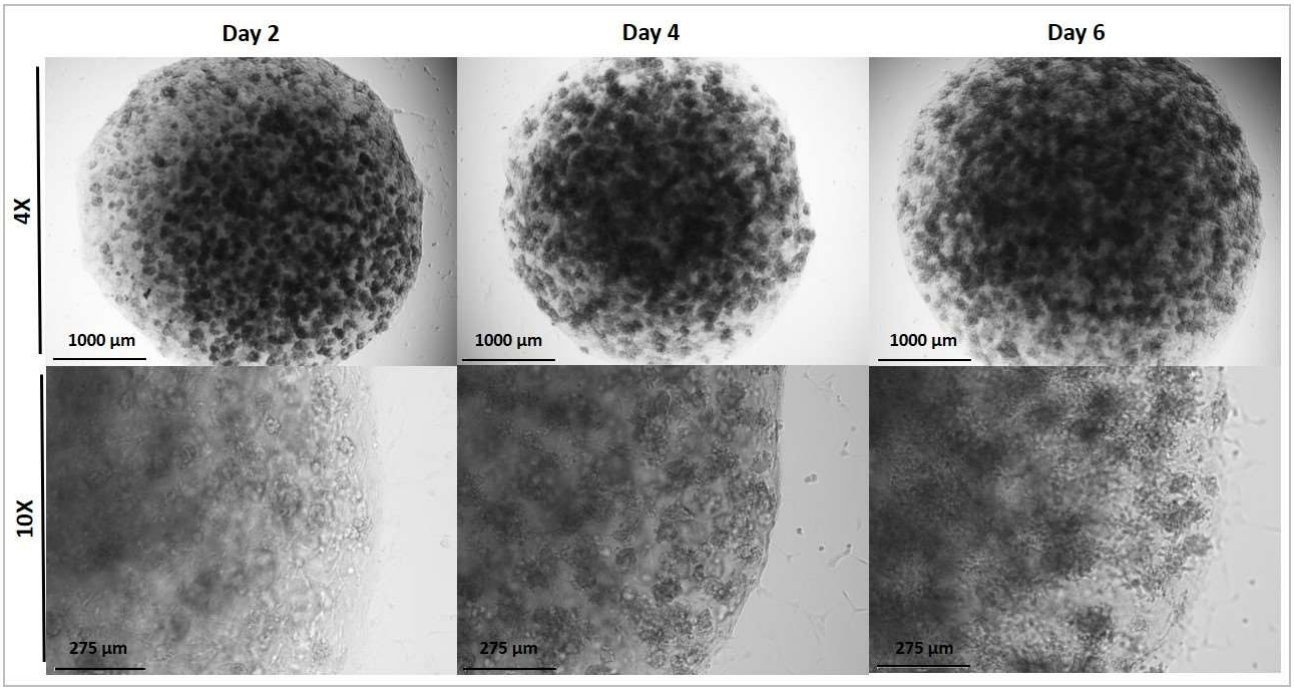

Figure 1. Phase contrast images of mini livers throughout culture display evidence of hepatic spheroid formation, migration and polarization. Scale bar = 1000 µm (4x) and 275 µm (10x).

Drug Treatment and Cytotoxicity in Bioprinted Mini Livers

The results of a toxicity assessment on Day 10 demonstrate a cytotoxic, dose-dependent response to APAP (Figure 2A) and FLU (Figure 2B) in bioprinted mini livers. This decrease in liver function is depicted by reduced albumin secretion and lipid production, and upregulated ALT activity. It is also apparent, based on increased ALT activity, that toxic doses of both drugs have damaging effects on cell viability. The latter is especially evident in Figure 3, in which LIVE/DEAD images indicate a significant decrease in cell viability at higher concentrations of APAP or FLU.

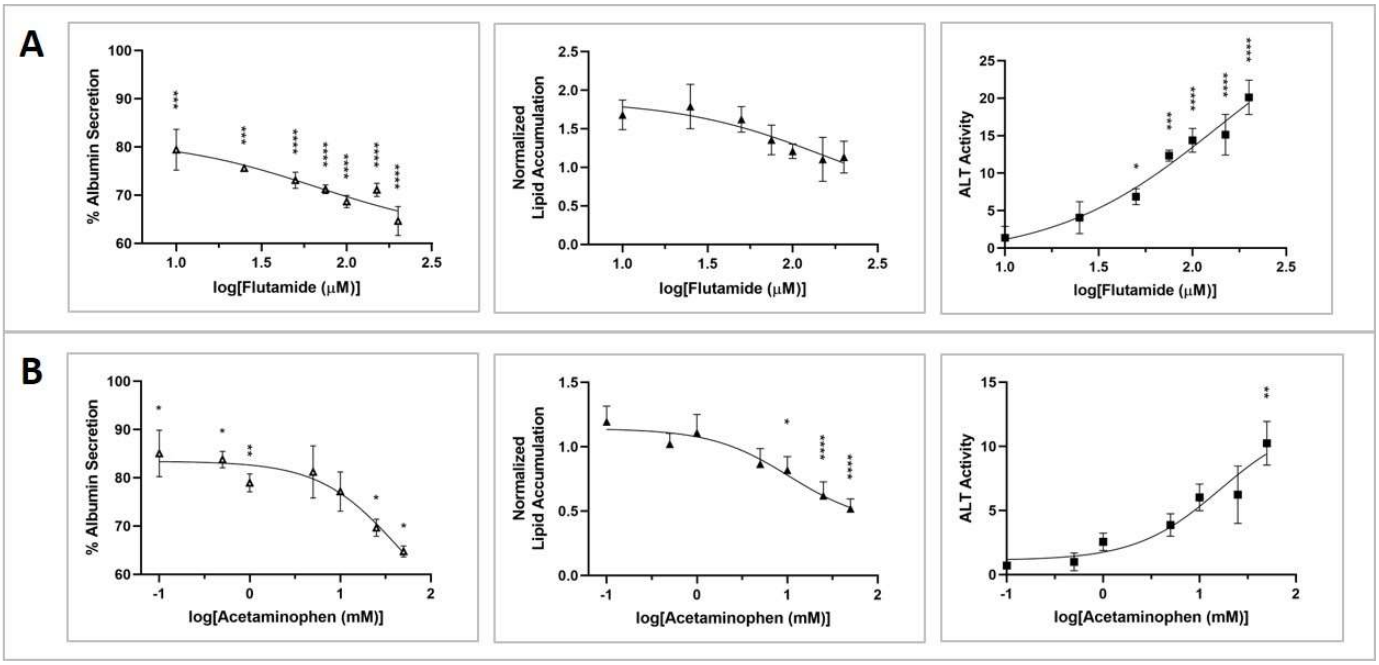

Figure 2. Analysis of albumin secretion, lipid accumulation and ALT activity after addition of FLU (A) and APAP (B) was performed using 3 independent assays. Results indicate a dose-dependent cytotoxic response in bioprinted mini livers. Values were normalized to untreated controls.

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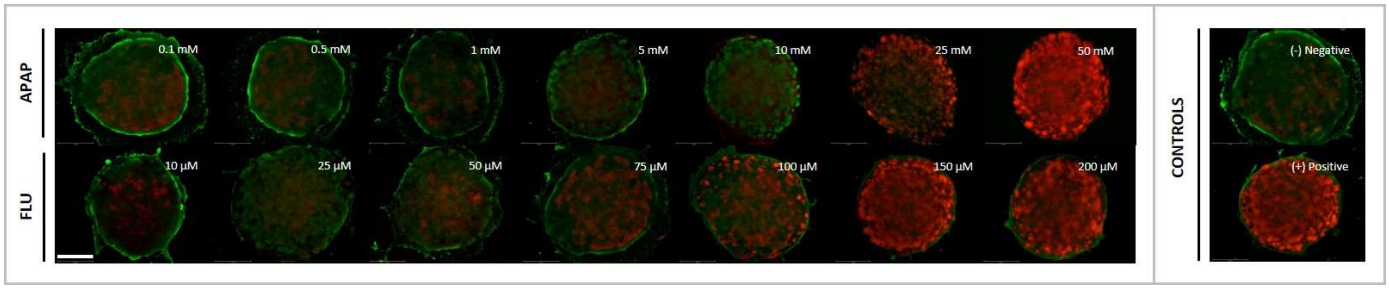

Figure 3. LIVE/DEAD images of mini livers following drug treatment depict decreasing viability for increasing doses of APAP and FLU. A combination of APAP and FLU was used as the positive control. Scale bar = 1000 µm.

Dynamic Intracellular Responses to Drug Treatment

Having seen the cytotoxic effects of both drugs on bioprinted mini livers, we next investigated how APAP and FLU regulate intracellular fat content. ORO staining of hepatic tissue is often used to identify various stages of fibrosis or steatosis in response to fatty acids or drugs (Pingitore, 2019). In our study, ORO staining of treated mini livers revealed minimal fat accumulation in samples treated with high drug doses and significant fat accumulation in samples that were left untreated or treated with low drug doses (Figure 4A). Here, one explanation is that APAP and FLU have been linked to lipid peroxidation, in which oxidative stress caused by toxic drug levels could have triggered lipid degradation and membrane damage (Behrends, 2019). Additionally, given that there is still some fat content in highly dosed samples, it is possible that the lack of perfusion and macrophages leave a graveyard of dead cells and uncleared lipids. A closer look at the untreated sample in Figure 4B provides a cross-section view of our droplet-in-droplet model. This image shows a large pool of cells migrating toward the outer droplet shell and producing fats, possibly indicating the presence of a nutrient- and oxygen-gradient, and validating patterns of cell reorganization and spheroid polarization within collagen.

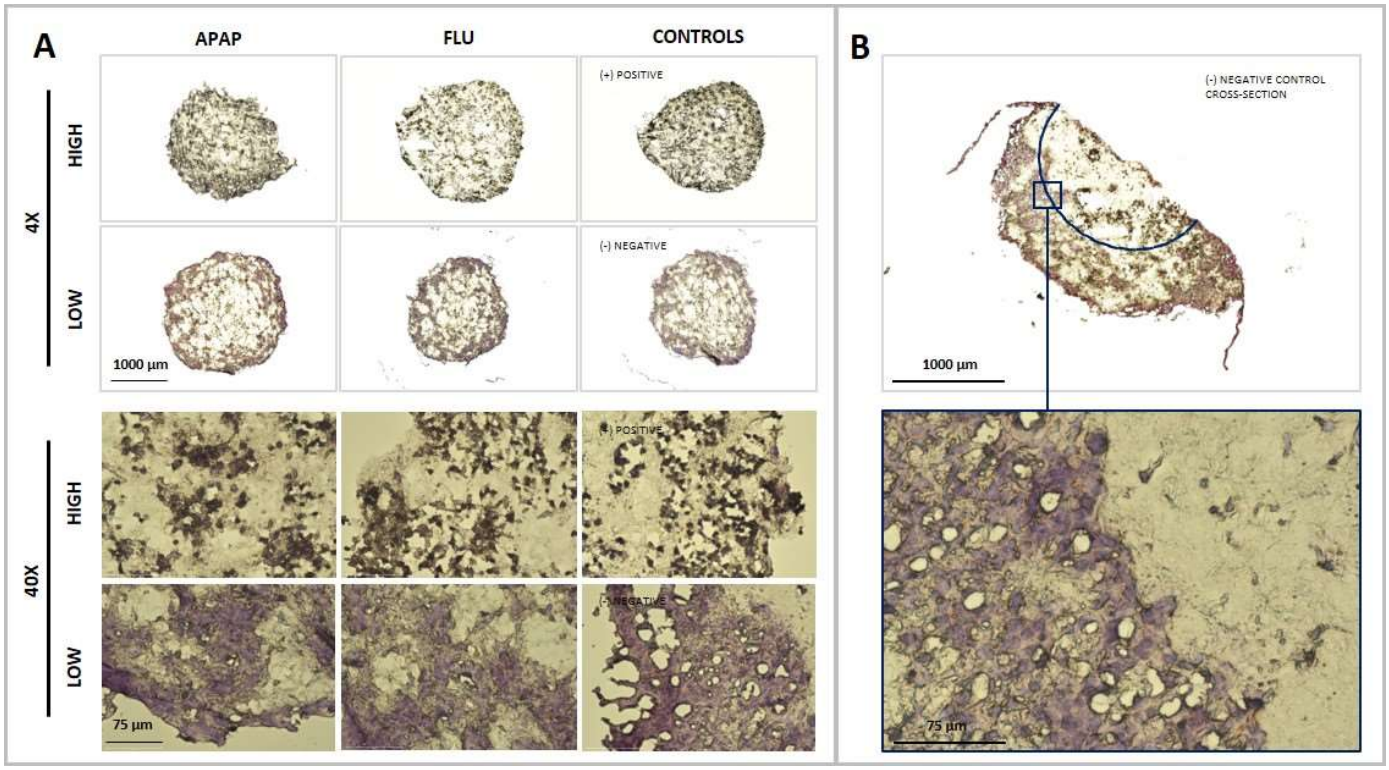

Figure 4. ORO staining of mini livers treated with high and low doses of APAP and FLU (A) or untreated (B) display patterns of lipid accumulation. A combination of APAP and FLU was the positive control. Scale bar = 1000 µm (4x) and 75 µm (40x).

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Conclusions

- As a reliable alternative to 2D cell culture systems, animal studies and lab-on-a-chip prototypes, the BIO X can be used as a medium- to high-throughput tool for producing functional 3D bioprinted liver models that enable drug screening and analysis, and relieve the costs of drug attrition.
- The use of CELLINK Coll I as scaffold for DID models provides a stable, tunable and highly compatible environment with abundant binding sites for hepatic cellular rearrangement and spheroid formation.
- Our findings, based on evidence of lipid peroxidation, reduced albumin secretion and upregulated ALT activity suggest that DID mini livers are functional and demonstrate a dose-dependent and cytotoxic response to APAP and FLU.
- A DID model allows for cell-cell interactions between layers of tissue and provides a unique opportunity to investigate migration patterns between layers of different stiffnesses. Future toxicity studies can adopt this model to replicate the various stages of fibrosis or investigate the regenerative capabilities of liver tissue following drug treatment.

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