Evaluation of Cannabidiol (CBD) and Cannabinol (CBN) Toxicity in the Human Quad-Culture Liver-Chip

Jacob D Larson[1], James Velez[2], Sushma Jadalannagari[2], Jake T Chaff[2], Josiah Sliz[2], Lorna Ewart[2], Donna Webster[1], Jiang Hu[1].

[1]Herbalife Nutrition, Torrance, CA, [2]Emulate Inc., Boston, MA.

Abstract

As cannabinoid use expands there is a need to evaluate for toxicity potential. Considering the rising need to reduce animal use in toxicology testing, this study was conducted to compare CBD and CBN hepatotoxicity using the Human-Quad Culture Liver-Chip, consisting of upper and lower channels seeded with primary human hepatocytes and non-parenchymal cells, respectively. Dosing concentrations were adjusted to account for compound distribution in the Chip (~80% for CBD and 70% for CBN), which was assessed using a specially designed assay to achieve target dosing concentrations of 0.24, 3, or 4.7μM CBD and CBN. Both channels of Liver-Chips were continuously dosed for 7 days with CBD or CBN. Effluent samples were collected and brightfield images were taken on days 1, 3, 5, and 7. CBN showed greater cytotoxicity to primary human hepatocytes through a decline in healthy morphology compared to CBD. No major changes were observed in albumin, ALT, and AST release for CBD and CBN relative to the control. At 4.7μM, CBD significantly increased IL-6, IL-8, and MCP-1 cytokine and LDH levels in top-channel effluents on day 7. No changes in IL-6 and MCP-1 were observed for CBN, a decrease of IL-8 was observed at 3 and 4.7μM CBN on day 7, and an increase of LDH at 4.7μM on day 1 and 0.24μM on day 5. These results indicated differences in CBD and CBN toxicity profiles while demonstrating the sensitivity and specificity of the Liver-Chip model as an alternative tool for liver toxicity screening.

Introduction

- The hepatotoxic effects from oral consumption of purified CBD in the form of the drug EPIDIOLEX® have been well documented in animal and human studies[1-3]. This has raised concerns regarding lower doses of CBD used in food and health products, along with potential concerns regarding the safety of structurally related cannabinoids such as Cannabinol (CBN).
- Given the rising need to reduce animal use in toxicology testing, the Human Quad-Culture Liver-Chip is an *in vivo* relevant three-dimensional model consisting of upper and lower channels seeded with primary human hepatocytes and non-parenchymal cells (NPCs), respectively, and with multicellular architecture, tissue-tissue interface, and fluid flow (Figure 1). It has been shown to meet the IQ MPS guidelines with a sensitivity of 80% and specificity of 100% after the testing of 27 known hepatotoxic and non-toxic drugs[4].
- The objective of this study was to compare CBD and CBN hepatotoxicity potential using the Human-Quad Culture Liver-Chip model.

1. Top Channel
2. Extracellular Matrix
3. Hepatocytes
4. Porous Membrane
5. Stellate Cells
6. Kupffer Cells
7. Endothelial Cells
8. Bottom Channel

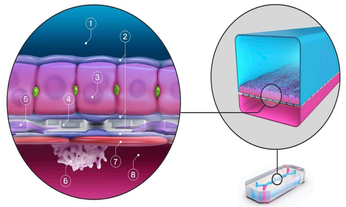
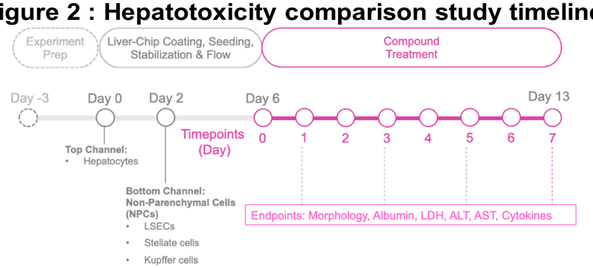


Figure 1: Human Quad-Culture Liver-Chip Schematic
The Human Liver-Chip (Emulate, MA) is made of polydimethylsiloxane (PDMS) and split into two channels. The upper channel (1mm high x 1mm wide) contains hepatocytes while the bottom channel (0.2mm x 1mm) contains NPCs including human stellate, Kupffer and endothelial cells. Human hepatocytes are in a sandwich culture between ECM components (bovine fibronectin, rat tail collagen I) and Matrigel®. A porous membrane separates the top channel from the bottom channel and media specific to hepatocytes and NPCs is flown in their respective channels at a flow rate of 400 μL/h.

Materials and Methods

The Liver-Chips were coated with a mixture of rat tail collagen type I and bovine fibronectin. Primary human hepatocytes were seeded at a density of 3.5 million cells/mL in the upper parenchymal channel and later overlaid with Matrigel® and incubated at 37°C with 5% CO_2. In the lower vascular channel on the opposite side of the porous membrane, NPC's such as human liver sinusoidal endothelial cells (LSECs) (3 million cells/mL), human liver Kupffer cells (2 million cells/mL) and stellate cells (0.1 million cells/mL) were seeded. One day later, the Chips were connected to the Zoë® Culture Module, and both the Chip channels were perfused with maintenance media at a constant flow rate of 400 μL/h. On Day 6 post-hepatocyte seeding, the Liver-Chips were treated with acetaminophen (APAP; 1, 3, 10 mM as positive control), CBD (0.24, 3, 4.7 μM) or CBN (0.24, 3, 4.7 μM) and vehicle (0.01% DMSO in media as negative control) continuously for 7 days. Imaging and effluent collection from top and bottom channels were carried out on Days 1, 3, 5 and 7 (Figure 2). All brightfield images were blind scored by independent investigators and assigned a cytotoxicity score (0-4) based on cell morphology. The effluent was assayed for Albumin (Abcam; ab179887), Alanine Transaminases (ALT)(Abcam; ab234578), Aspartate Transaminase (AST)(Abcam; ab263881), Lactate Dehydrogenase (LDH)(Promega; J2381) and Cytokines (IL-6, IL-8, IP-10, and MCP-1) (Meso Scale Diagnostics). Due to the high flow rate, the effluent was concentrated using 3kDa spin columns (ThermoFisher, 88526). Statistical comparisons were done by 2-way ANOVA.

Figure 2 : Hepatotoxicity comparison study timeline

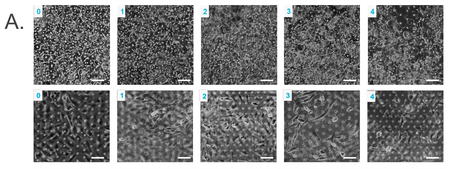


Results & Discussion

APAP treatment displayed a dose and time dependent increase in cytotoxicity in the hepatocytes and NPCs, as observed through cell morphology. Albumin was significantly decreased after treatment with APAP at 3 and 10 mM compared to vehicle control. APAP treatment at 10 mM led to statistically significant increases in ALT, AST, and LDH in the top channel . APAP did not cause significant changes in cytokine release.
CBD treatment did not cause any morphological changes as compared to the vehicle control, except for minimal toxicity (score of 1) in the 3 and 4.7 μM dosed hepatocytes by Day 7 post-treatment. There was a statistically significant increase in LDH caused by 4.7 μM CBD, but no increases in ALT nor AST were seen and albumin levels were in line with the vehicle. This dose of CBD did, however, cause an increase in proinflammatory cytokines by Day 7 post-treatment as increases in IL-6, IL-8 and MCP-1 were observed.
CBN led to a dose- and time-dependent toxicity in the hepatocytes as observed through morphology. The NPCs, however, maintained healthy morphology for the entirety of the study, except for the 4.7 μM treated NPCs which displayed minimal toxicity (score of 1) on Day 7. No toxicity was observed through effluent-based readouts as no decrease in albumin were observed along with no increase in ALT, AST, LDH nor the cytokines.

Figure 3 : Morphology through Brightfield Imaging

A. Cytotoxicity classification of hepatocytes and NPCs with corresponding representative images. A score of 0 denotes healthy morphology while a score of 4 denotes severe toxicity.
B. Representative brightfield images of Day 1 & 7 of vehicle control hepatocytes and NPCs and those treated with , CBD, CBN, and APAP (10 mM only). Blue arrows indicate areas with healthy cell morphology and white arrows indicate areas of toxicity. Scale bars represent 50 μm
C. Morphology scoring summary of vehicle control and treated groups for timepoint days 1, 3, 5, and 7.



Treatment	Dose	Top Channel				Bottom Channel			
		Day 1	Day 3	Day 5	Day 7	Day 1	Day 3	Day 5	Day 7
Vehicle	-	0	0	0	0	0	0	0	0
APAP	1 mM	0	0	1	1	0	0	0	1
	3 mM	0	1	2	3	0	1	2	3
	10 mM	0	2	4	4	0	2	3	4
CBD	0.24 μM	0	0	0	0	0	0	0	0
	3 μM	0	0	0	1	0	0	0	0
	4.7 μM	0	0	0	1	0	0	0	0
CBN	0.24 μM	0	0	1	2	0	0	0	0
	3 μM	0	0	1	2	0	0	0	0
	4.7 μM	0	0	2	2	0	0	0	1

Healthy 0 1 2 3 4 Damage

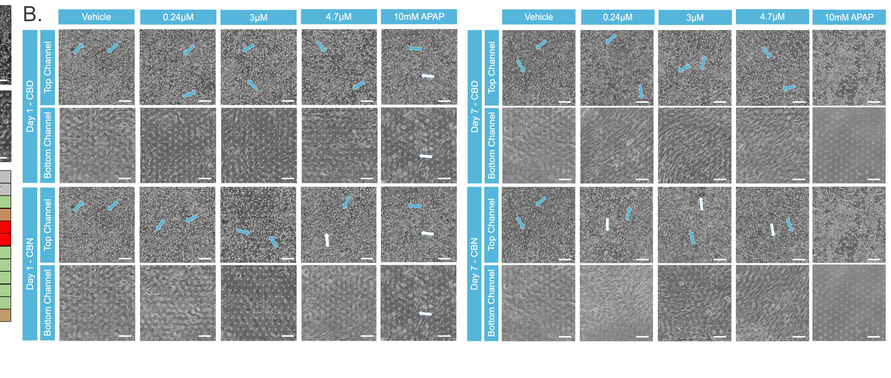


Figure 4: Modelling toxicity and inflammation on the Liver-Chip
Measurements of Albumin, ALT, AST, LDH and cytokine release obtained from effluent collected from the top channel on Days 1, 3, 5 & 7 post-treatment.

Conclusions

- The Human Quad-Culture Liver-Chip was able to identify and distinguish toxicity between CBD, CBN, vehicle control, and APAP treated groups.
- Toxicity in hepatocytes was confirmed at higher doses, but not at the lowest dose of CBD. This effect may be associated with increase in pro-inflammatory cytokines.
- Based on morphology CBN showed greater cytotoxicity than CBD at similar doses. Toxicity of CBN may not be associated with pro-inflammatory cytokines.

References: 1) FDA-CDER (2018). Epidiolex: Pharmacology review. Application Number 210365. 2) Devinsky, O., et al. (2018). "Randomized, dose-ranging safety trial of cannabidiol in Dravet syndrome." Neurology **90**(14): e1204-e1211. 3) Thiele, E. A., et al. (2018). "Cannabidiol in patients with seizures associated with Lennox-Gastaut syndrome (GWPCARE4): a randomized, double-blind, placebo-controlled phase 3 trial." The Lancet **391**(10125): 1085-1096. 4) Ewart, et al. (2022). "Qualifying a human Liver-Chip for predictive toxicology: Performance assessment and economic implications." Biorxiv 2021.12.14.472674.