Evaluating the Hepatotoxicity of Cannabidiol, Cannabinol, Cannabichromene and Cannabigerol Using a Human Quad-Culture Liver-Chip

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Abstract (313)

As the popularity of hemp-derived products grows, understanding the prospective hepatotoxicity of cannabinoids becomes crucial for personal safety. Despite conflicting evidence from limited human clinical studies, a safety gap persists, and no standard threshold has been established for these various cannabinoid-containing products. Furthermore, the hepatotoxicity potential of other cannabinoids like Cannabinol (CBN), Cannabichromene (CBC), and Cannabigerol (CBG) remains largely unexplored.

The current study was designed to address some of these gaps by utilizing microphysiological systems (MPS), such as the Emulate Quad-Culture Liver-Chip, which adhere to IQ MPS consortia guidelines, as an alternative to animal testing. Here, pressure-driven flow for compound delivery was used with primary human cells in an *in vivo*-like arrangement, separated by a semi-porous membrane to allow for crosstalk between the hepatocytes and nonparenchymal cells (NPCs). Hepatotoxicity was compared among CBD, CBN, CBC, and CBG, in parallel with the known hepatotoxic compound acetaminophen (APAP), in a three-point concentration response evaluation (0.24, 3, or 4.7 µM). The assessment encompassed morphological effects, hepatocyte function, and potential mechanisms of action over a 7-day continuous dosing period. These evaluations included live imaging for mitochondrial dysfunction, total reactive oxygen species (ROS), and inflammatory cytokines derived from effluent-based sampling in the Emulate platform.

Morphological analysis revealed 3 or 4.7 µM CBD impacting hepatocytes by Day 7, while CBG exhibited no visible changes compared to the control. Endpoints evaluating hepatocyte function and viability indicated that LDH release increased only with 4.7 µM CBD, CBN, and CBC. CBD, CBN, and CBG did not significantly affect albumin, ALT, or AST, while 4.7 µM CBC significantly decreased albumin production. Inflammatory cytokines increased at high concentrations of CBD, CBC, and CBG. ROS and mitochondrial function displayed different responses among the cannabinoids affecting the NPCs more versus the hepatocytes. Up until now, the majority of what is known regarding these compounds is mostly murine-based. Hence, this study was an effort to leverage the use of advanced, human-relevant MPS to carefully assess and compare the hepatotoxicity of CBD and other cannabinoids, shedding light on their safety in foods and health products.

Introduction

- Hepatotoxic effects from oral consumption of purified CBD, as in the drug EPIDIOLEX®, have been well documented in animal and human studies. These effects raise concerns about the safety of lower CBD doses used in food and health products, as well as structurally related cannabinoids, such as Cannabichromene (CBC) and Cannabigerol (CBG).[1-3]
- Given the increasing push to reduce animal use in toxicology testing, the Human Quad-Culture Liver-Chip presents an *in vivo*-relevant three-dimensional model. This model consists of upper and lower channels seeded with primary human hepatocytes and non-parenchymal cells (NPCs), respectively, featuring multicellular architecture, tissue-tissue interface, and fluid flow (Figure 1). It meets the IQ MPS guidelines with a sensitivity of 87% and specificity of 100% after testing 27 known hepatotoxic and non-toxic drugs.[4]
- **The objective of this study was to compare the hepatotoxicity potential of CBD, CBN, CBC, and CBG 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

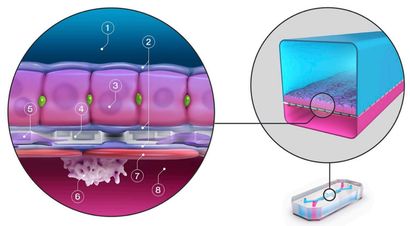
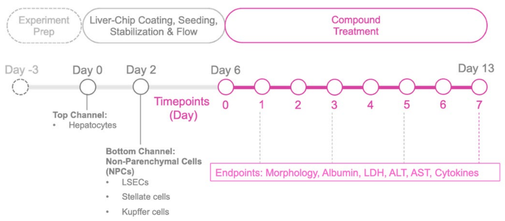


Figure 1: Human Quad-Culture Liver-Chip Schematic
The Human Quad-Culture Liver-Chip (Emulate, MA) is made of polydimethylsiloxane (PDMS) and split into two channels. The upper channel (1mm high x 1mm wide) contains hepatocytes while the bottom channel (0.2mm x 1mm) contains NPCs including human stellate, 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

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. The next day, the chips were connected to Zoë® Culture Module, and both 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 a positive control), CBD, CBN, CBC, or CBG (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), 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, effluent was concentrated using 3kDa spin columns (ThermoFisher, 88526). Statistical comparisons were done by 2-way ANOVA.

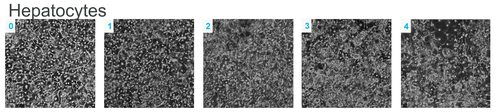
Figure 2: Hepatotoxicity Comparison Study Timeline



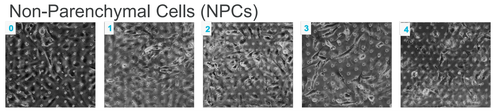
Results

APAP treatment displayed a dose- and time-dependent increase in cytotoxicity in 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 AST in the top channel. CBD treatment did not cause any morphological changes as compared to vehicle control, except for minimal toxicity (score of 1) in the 3 and 4.7 µM dosed hepatocytes by Day 7 post-treatment. No increases in AST were seen and albumin levels were in line with the vehicle. No changes were observed in hepatocyte ROS compared to vehicle. MitoTracker signal decreased for hepatocytes treated with 0.24 and 4.7µM CBD. CBN led to a dose- and time-dependent toxicity in hepatocytes as observed through morphology. The NPCs, however, maintained healthy morphology throughout 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 decreases in albumin were observed along with no dose-dependent increases in AST. No changes were observed in hepatocyte ROS compared to vehicle. MitoTracker signal decreased for hepatocytes treated with 4.7µM CBN. CBC also led to a dose- and time-dependent toxicity in the hepatocytes as observed through morphology. 4.7 µM CBC significantly decreased albumin production and increased AST release. ROS increased with 4.7 µM CBC compared to vehicle. MitoTracker signal decreased for hepatocytes treated with 3 µM CBC. CBG remained healthy compared to vehicle by morphology and did not significantly affect albumin or AST. No changes in hepatocyte ROS compared to vehicle. MitoTracker signal decreased for hepatocytes treated with 3 and 4.7 µM CBG. NPC ROS-induced oxidative stress was seen with every treatment group at all concentrations. Mitochondrial activity was mostly unaffected by any treatment in the NPCs.

A. Hepatocytes



Non-Parenchymal Cells (NPCs)



B.

Treatment	Corrected Concentration	Top Channel (Hepatocytes)				Bottom Channel (NPCs)			
		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
CBG	0.24 µM	0	0	0	0	0	0	0	0
	3 µM	0	0	0	0	0	0	0	0
	4.7 µM	0	0	0	0	0	0	1	1
CBC	0.24 µM	0	0	0	0	0	0	0	0
	3 µM	0	1	2	2	0	0	0	1
	4.7 µM	0	1	2	3	0	1	1	2

Healthy 0 1 2 3 4 Damage

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. Morphology scoring summary of vehicle control and treated groups for timepoint Days 1, 3, 5, and 7.

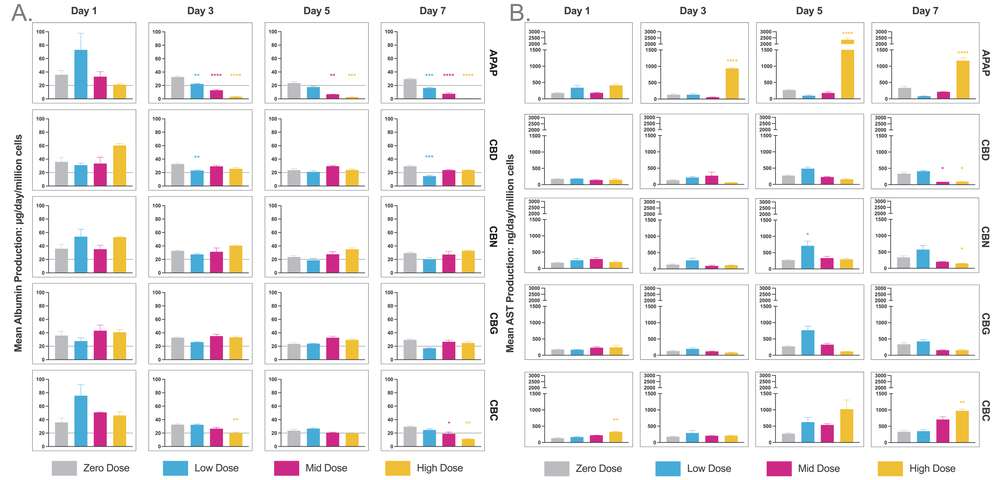


Figure 4: Modeling toxicity through Liver-Chip biomarkers
Measurements of Albumin (A) and AST (B), obtained from top channel effluent collected on Days 1, 3, 5 & 7 post-treatment.

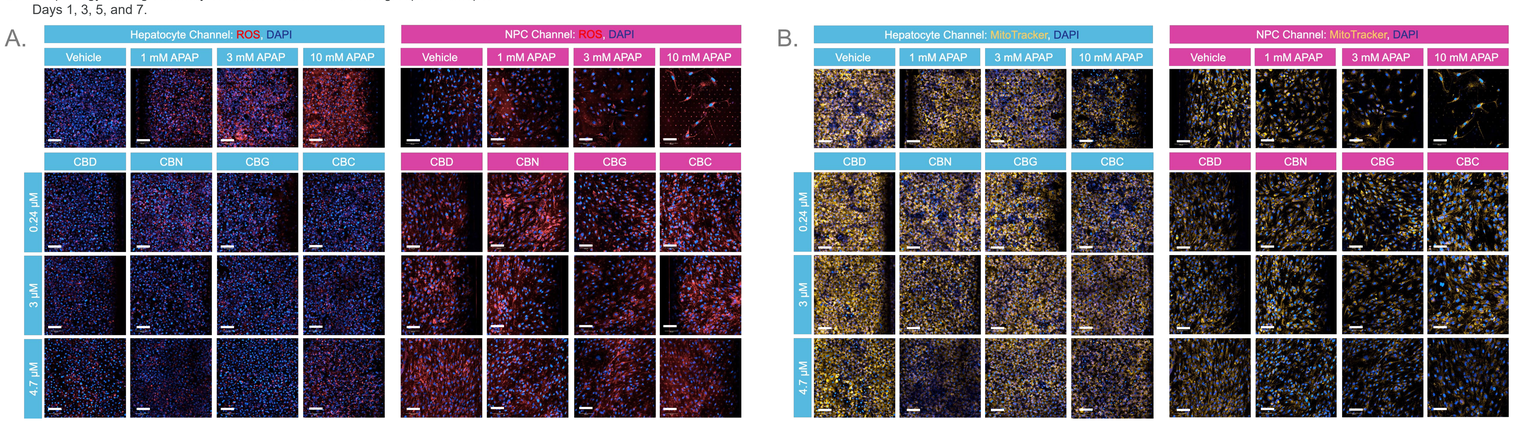


Figure 5: Investigating mechanism of toxicity
Measurements of ROS accumulation for oxidative stress (A) and mitochondrial activity, as shown by live imaging for MitoTracker (B) Scale bar = 100 µm.

Conclusion

- The Human Quad-Culture Liver-Chip was able to identify and distinguish toxicity between CBD, CBN, CBC, CBG, vehicle control, and APAP treated groups.
- Toxicity in hepatocytes was confirmed at higher doses, but not at the lowest dose of CBD and CBC. This effect may be associated with increased pro-inflammatory cytokines.
- Based on morphology, CBN and CBC 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, L. et al. (2022). "Performance assessment and economic analysis of a human Liver-Chip for predictive toxicology." Communications Medicine **2** (154).