3D CELL MODELS TO EVALUATE THE EFFECTS OF SELECTED ENVIRONMENTAL TOXICANTS ON CHRONIC LIVER DISEASES

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BACKGROUND

Nowadays, there is growing evidence that exposures to common environmental toxicants, including **endocrine-disrupting chemicals (EDCs)** or selected natural toxins (e.g. **hepatotoxic cyanotoxins**), are associated with **metabolic diseases**, including prevalent **chronic liver diseases** such as **Non-Alcoholic Fatty Liver Disease (NAFLD)** and **Non-Alcoholic Steatohepatitis (NASH).** However, there is a lack of toxicological information regarding the effects of most EDCs on metabolic diseases, which would allow for inadequate human health risk assessment and eventual regulatory decisions.

NAFLD is the **most common cause of chronic liver disease** in Western countries with continually increasing incidence. NAFLD has the potential to **progress** through the inflammatory phase of **NASH** to **fibrosis, cirrhosis** (20%), and in some cases (9%) to **liver failure** or **hepatocellular carcinoma** (HCC) (1%). NAFLD is characterized by **accumulation of lipids** (triglycerides) inside the hepatocytes. NASH increases **hepatocyte death** via apoptosis and is associated with **inflammation**, a hallmark of NASH, which might be induced/exacerbated by further liver injury, or *second hit*. **Oxidative stress** and **proinflammatory cytokines** are believed to play an important role in the progression of liver damage.

HYPOTHESIS

- Selected **environmental toxicants** contribute to the development of chronic liver diseases, such as **NAFLD/NASH.**
- **Hepatotoxicity and steatogenic** potential of toxicants can be assessed/predicted by **human liver 3D *in vitro* models.**

AIMS

- Develop and optimize human liver 3D *in vitro* model(s) and set of biomarkers of NAFLD/NASH.
- Validate the model by the set of steatogenic drugs.
- Evaluate hepatotoxic and steatogenic effects of the selected contaminants.
- Mechanistic assessment of further molecular and cellular events and AOP key events for NAFLD/NASH.
- Toxicokinetic studies.

METHODS

SPHEROID PREPARATION

- ✓ **Human HCC cell line HepG2** -> Hepatocyte-like properties induced by 3D culture and microenvironment.

CHEMICAL EXPOSURES

- ✓ **EDCs** – e.g. bishpenols (BPA, BPS, BPF), phthalates (DEHP, DBP), fluorinated fatty acids (PFOA, PFOS), cadmium, DDE, butyl-paraben
- ✓ **Natural toxins** – Microcystin-LR, Cylindrospermopsin & degradation products, other prominent Cyanopeptides
- ✓ **Steatosis/steatohepatitis inducers:** Amiodarone, Valproate, Palmitic-Oleic acid mixture, Chloroquine

IMAGE ACQUISITION

- ✓ **Spheroid size, shape, and integrity** evaluated from bright field images obtained by Cytation 5, Gen 5.0 software (4× objective) and assessment of size (diameter, area), perimeter and circularity.

HEPATOCYTOTOXICITY

- ✓ Hepatospheroid viability evaluated by **resazurin conversion** (dehydrogenases), **CFDA-AM cleavage** (esterase activity); **ATP content** (measurement by HTS ATP kit); and **LDH release** in the culture medium

LIPID ACUMULATION

- ✓ **BODIPY 493/505** as a marker of neutral lipid accumulation.
- ✓ **Fluorescence imaging** by Cytation 5, Gen 5.0 software (4x objective) along the focal axis (z-stack)

GENES CONTROLLING LIPID HOMEOSTASIS AND METABOLISM

- ✓ **qRT-PCR** of genes involved in lipid homeostasis and metabolism, such as ACC, FASN, DGAT1/2, FAT/CD36, APOB, CPT1A ….

DISCUSSION

- Mature HepG2-derived spheroids showed increased expression of key hepatospecific genes, e.g. albumin, connexin32, drug-transporting proteins (OATP1B1, 1B3), or drug-metabolizing enzymes (CYP1A2).
- Spheroids with initial seeding density 1000 cell/spheroid reaching 450-500 diameter after 15 days maturation were used for chemical treatments.
- This setup was compatible with (semi-)automated workflow (liquid handling, automated readouts/imaging, image analysis) and suitable for (semi-) high throughput screening.
- The present study demonstrates that such 3D hepatic spheroid model can has potential to be further developed and used for *in vitro* assessment of key molecular and cellular events of AOPs for hepatic steatosis and steatohepatitis, and for evaluation of steatogenic potential of EDCs or other environmental toxicants.

ACKNOWLEDGMENTS

MUNI | RECETOX SCI
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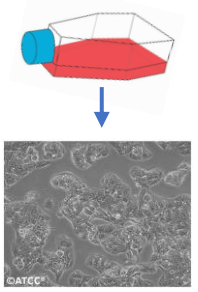
OBERON

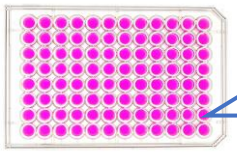
EXPERIMENTAL DESIGN

Human hepatocellular carcinoma cell line
HepG2 (ATCC)



2D monolayer cultures

Liquid agarose overlay

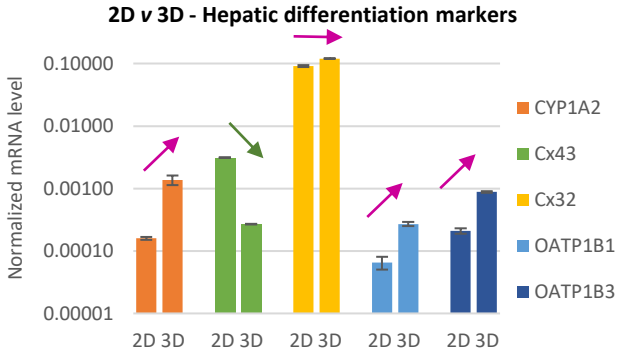


3D hepatospheroid cultures

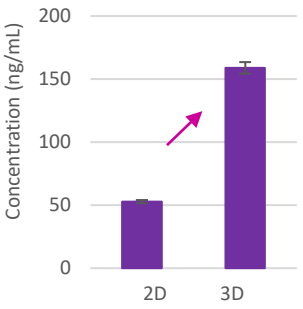
EpMotion 5075

BioSpa8G&Cytation 5
Automated imaging of up to 8 microplates

2D *v* 3D - Hepatic differentiation markers

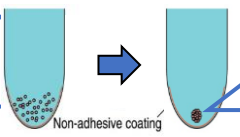


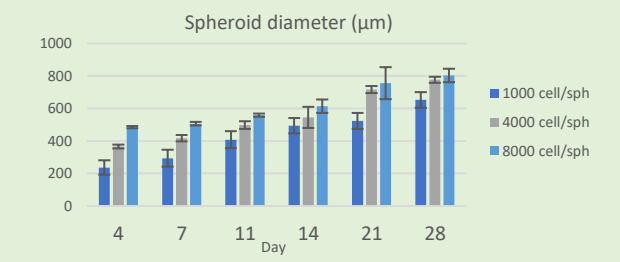
2D *v* 3D - Albumin secretion



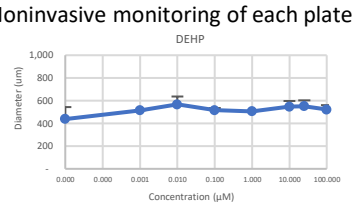
1000 cell/sph
15 days maturation

- Increased expression of hepatocyte-specific markers (CYP1A2, OATP1B1/1B3)
- Decreased expression of liver progenitor/biliary duct cells (Connexin43)
- Increased hepatocyte-specific functions (Albumin secretion)
- Other markers will be further evaluated (e.g. inducibility and activity of CYP1A2, 3A4, 2B6)

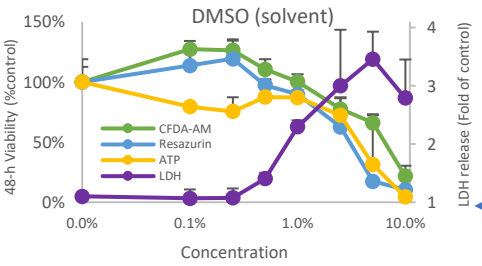




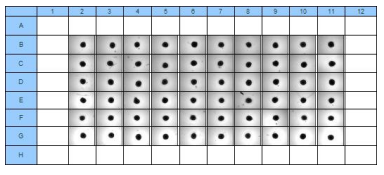
Spheroid growth, shape, integrity
Noninvasive monitoring of each plate

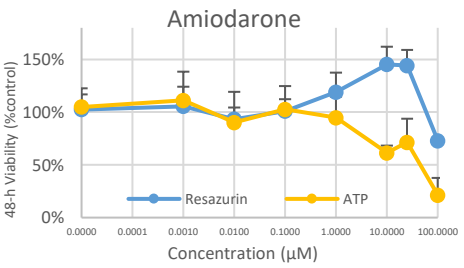


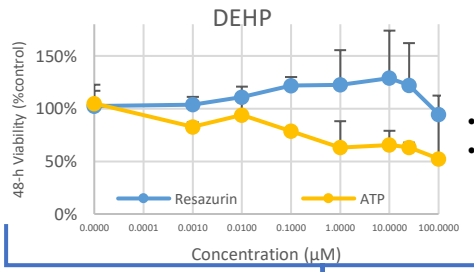
Hepatospheroid viability
Resazurin, CFDA-AM, ATP, LDH release



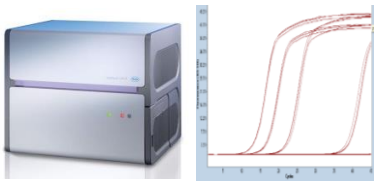
14 d maturation
Exposure to EDCs or model compounds



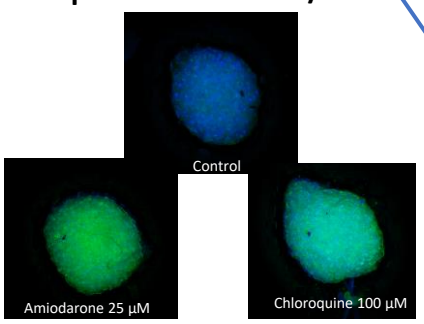




Genes controlling lipid homeostasis (qPCR)



Lipid accumulation / HCA



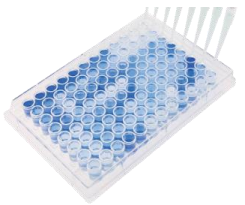
48-h exposure: **Green: BODIPY** (neutral lipids)
Blue: Hoechst (nuclei)

- Image-based assessment of lipid accumulation
- Other fluorophores can be used for HCA assessment of multiple endpoints: **phospholipid accumulation, oxidative stress, apoptosis/necrosis.**

Immuno/biochemical assays of medium:

- ELISA: Cytokine release
- Albumin secretion
- Lactate/Glucose

+ Instrumental analysis
(toxicokinetics *in vitro]*



Hepatocytotoxicity?
Disruption of hepatic lipid metabolism?
& possibly other cellular and metabolic events
(inflammation, oxidative stress, metabolic activity)

Liver Steatosis
Steatohepatitis
Toxicokinetic *in vitro*
?

- **Hazard identification**
- **QIVIVE**
- **Mechanisms**