Characterization of rainbow trout hepatic 3D spheroids for next generation ecotoxicity testing

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INTRODUCTION

Conventional animal testing for chemical risk assessment exerts both ethical and economic challenges, driving the demand for New Approach Methodologies (NAMs), endorsing the 3Rs principles. While various *in vitro* acute assays rely on primary and continuous cell cultures, there is a lack of such systems tailored for evaluating chronic chemical toxicity in fish. The three-dimensional (3D) hepatic spheroid model, derived from primary hepatocytes of rainbow trout (*Oncorhynchus mykiss*) (RT-HEP), exhibits considerable potential, as suggested by its capacity to maintain morphological, physiological, and biochemical characteristics for extended periods of time following their formation[1][2]. Given its novelty, further detailed morphological and physiological characterization is imperative. In-depth characterization may be performed using advanced imaging techniques like Multiphoton Fluorescence Microscopy (MFM & FM) due to its resolution, tissue penetration, light detection, reduced phototoxicity, and enhanced spectral accessibility and flexibility[3]. Furthermore, knowledge about 3D RT-HEPs gene expression patterns and regulatory mechanisms within the 3D structure by using RNA sequencing (RNA-seq) is required to fully understand the physiological differences and properties between 3D- and monolayer cell formats[4]. This study aimed to characterize the morphology of RT-HEP 3D spheroids, evaluate cellular and subcellular responses to solvent control (Dimethyl sulfoxide), Benzo[a]pyrene (B[a]P) and Copper(II) sulfate pentahydrate (Cu) using MFM and FM, and map spheroid transcriptomics following exposure to 17α-Ethinyl estradiol (EE2).

METHODS

- Spheroids were exposed to 0.03 µM, 1 µM and 10 µM of B[a]P (48 h) and 0.31 µM, 1 mM and 10 mM of Cu (48 & 96 h) followed by FM imaging (Olympus inverted microscope IX71) using multiple fluorophores.
- Spheroids were exposed to a sublethal concentration (1500 µM) of Cu for 30 min, then visualized under FM to detect induction of reactive oxygen species (ROS).
- Spheroids were visualized under MFM (Bruker Ultima IV) to detect core hypoxia. The fluorescence signals generated were split into a green and a red channel using two optical bandpass filters (CWL 525 nm/FWHM 70nm and CWL 593nm/FWHM 45nm) in front of photomultiplier tubes (PMTs).
- Spheroids were exposed to a sublethal concentration (0.1 µM) of EE2 for 48 h, and further subjected to RNA-seq analysis.

RESULTS AND DISCUSSION

B[a]P showed concentration dependent increase in cytotoxicity at 48h

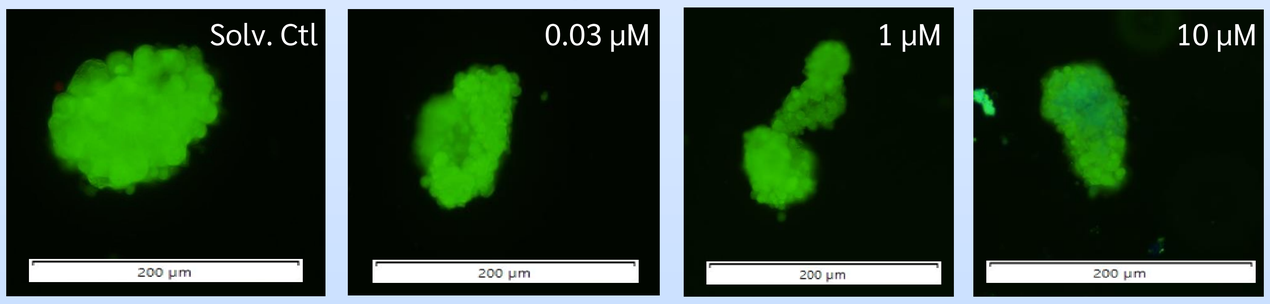

Figure 1: Cytotoxicity (cell membrane integrity) in spheroids exposed to solvent control and 0.03 µM, 1 µM and 10 µM of B[a]P for 48 h and stained with Fluorescein Diacetate (FDA, 10 µM) for 30 min, followed by FM imaging.

Cu showed concentration dependent increase in cytotoxicity at 48h

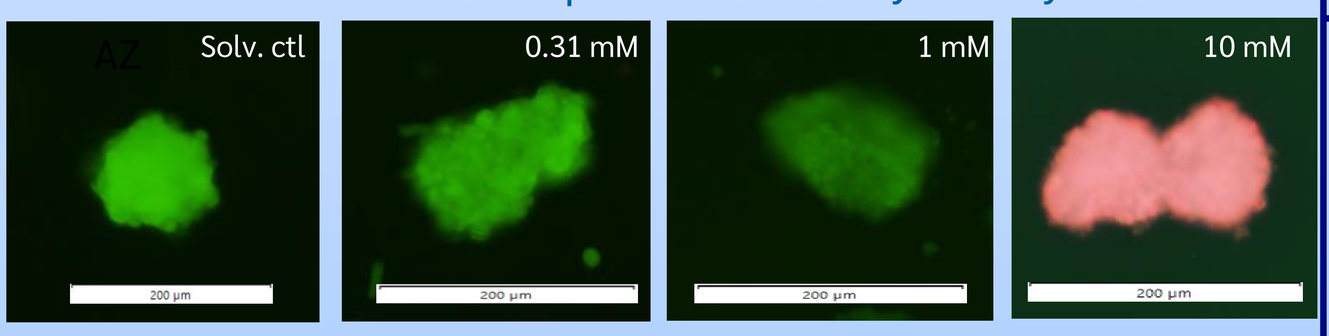

Figure 2: Cytotoxicity (cell membrane integrity) in spheroids exposed to solvent control and 0.31 mM, 1 mM and 10 mM of Cu for 48 h and stained with FDA (10 µM) for 30 min, followed by FM imaging.

Cu showed concentration dependent cytotoxicity at 96h

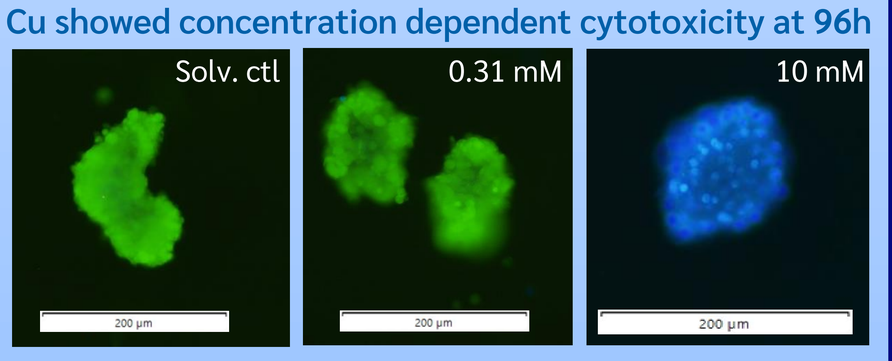

Figure 3: Cytotoxicity (cell membrane integrity) in spheroids exposed to solvent control 0.31 mM and 10 mM of Cu for 96 h and stained with FDA (10 µM) and 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/ml) for 30 min, followed by FM imaging.

No hypoxia in the core

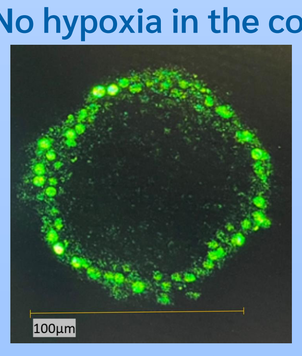

Figure 4: Unexposed spheroids stained with Image-iT green hypoxia reagent (5 µM) for 1h. Images taken with MFM at the middle layer, transverse section (λ =800 nm).

Cytotoxicity of Cu at 96h

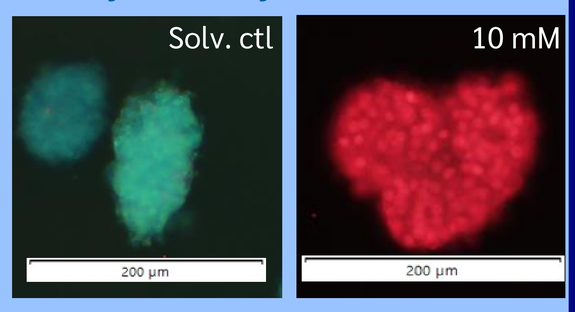

Figure 5: Cytotoxicity (cell membrane integrity) in spheroids exposed to solvent control and 10 mM of Cu for 96h and stained with FDA (10 µM) and Propidium Iodide (PI, 30 µM) for 30 min, followed by FM imaging.

Cu causes oxidative stress

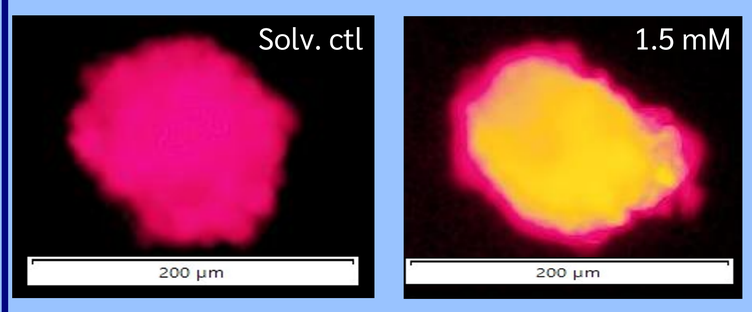

Figure 6: ROS induction (cytosolic) in spheroids exposed to solvent control and 1.5 mM of Cu for 30 min and stained with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, 200 µM) for 30 min, followed by FM imaging.

Gene Ontology Enrichment Analysis

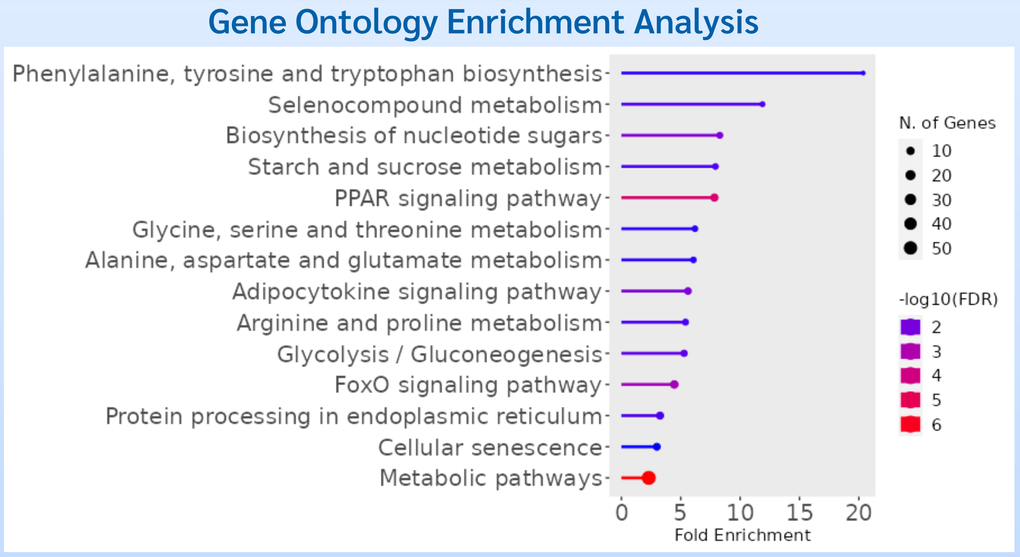

Figure 7: Top enriched KEGG pathways in spheroids after 48 h exposure to EE2.

PPAR signalling pathway

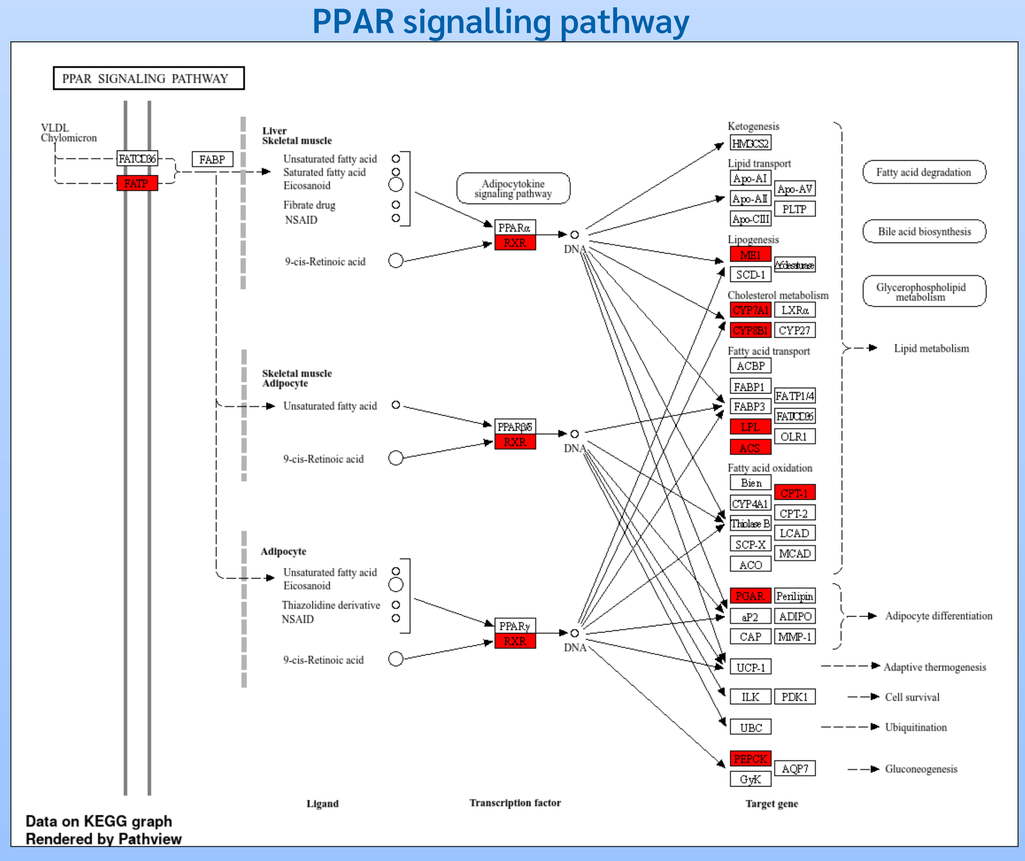

Figure 8: Enrichment of the peroxisome proliferator-activated receptor (PPAR) signaling pathway (mapped- and differentially expressed genes highlighted in red) in spheroids after 48 h exposure to EE2.

CONCLUSIONS

- MFM and MF are effective techniques for studying qualitative cytotoxicity, cell membrane integrity, ROS activity and hypoxia in 3D RT-HEP.
- Concentration-dependent cytotoxicity seen in 3D RT-HEP exposed to B[a]P and Cu, with Cu exposure inducing ROS production[5][6].
- No hypoxia observed at the core, aligning with fish spheroid studies[7].
- EE2 exposure impacts endocrine and metabolic pathways, shown through RNA sequencing.
- **3D RT-HEP holds promise as an alternative to *in vivo* testing.**

FUTURE WORK

Further refinement of MFM will explore additional morphological structures and physiological properties of 3D RT-HEP. Ongoing analysis of transcriptomic and metabolomic data aims to further uncover spheroids' physiological complexity and their potential role in chemical toxicity assessment.

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References: 1] Baron, M.G., Ecotox. 21, 2419-2429(2012); [2] Hultman, M.T., Environ toxicol chem 38,1738-1747 (2019); [3] Wang, X., Technol cancer res tret 21, 1533-1541 (2022); [4] Bähler, J., Cell mol life sci 569-579(2010); [5]Michalak, S., Environ sc and tech, 3091-3100 (2018 [6] Yazdani M. Drug chem toxicol 71-78 (2018); [7] Farmen, E., Toxicol pharmacol 431-438 (2010)