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Characterization of Rainbow Trout Hepatic 3D Spheroids for Next Generation Ecotoxicity Testing

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INTRODUCTION & AIM

Conventional animal testing methods for chemical risk assessment pose ethical and economic challenges. So, there is a growing demand for New Approach Methods (NAMs) for toxicity testing in line with the principles of the 3Rs (refinement, reduction, and replacement). While many established *in vitro* tests rely on primary and continuous cell cultures, there are limited *in vitro* systems available for evaluating long-term chemical toxicity in fish. The 3-dimensional (3D) hepatic spheroid model derived from primary hepatocytes ~~of~~ from rainbow trout (*Oncorhynchus mykiss*) (RT-HEP) is a promising model, as it has demonstrated its potential by maintaining morphological, physiological, and biochemical properties for weeks post-formation[1][2] . Being a novel model, it necessitates comprehensive morphological and physiological characterization.

Multiphoton fluorescence microscopy (MFM) offers advanced imaging capabilities, including high-resolution images, deeper tissue penetration, superior light detection, reduced phototoxicity, and improved spectral accessibility and flexibility[3] . Additionally, RNA sequencing (RNA-seq) is an advance technique in transcriptomics, enabling detailed analysis of gene expression patterns and regulatory mechanisms within cells and tissues[4].

The study aimed to characterize the morphology of RT-HEP 3D spheroids, assess cellular and subcellular responses to DMSO, pyrene, and Copper(II) sulfate pentahydrate using MFM and FM, and analyze spheroid transcriptomics after exposure to 17α-Ethynyl estradiol.

METHOD

- Spheroids were exposed to sublethal concentration (25 nM) of pyrene for 24h, then visualized under MFM (Bruker Ultima IV) to detect viability (metabolic activity), reactive oxygen species (ROS) activity and hypoxia using the appropriate fluorochromes. The fluorescence signals generated by MFM 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 sublethal concentration (1500 µM) of copper for 30 min, then visualized under FM.
- Spheroids were exposed to sublethal concentration (0.1µM) of 7α-ethinylestradiol (EE2) for 48h, and RNA-seq analysis was performed.

RESULTS & DISCUSSION

Cytotoxicity - Metabolic activity

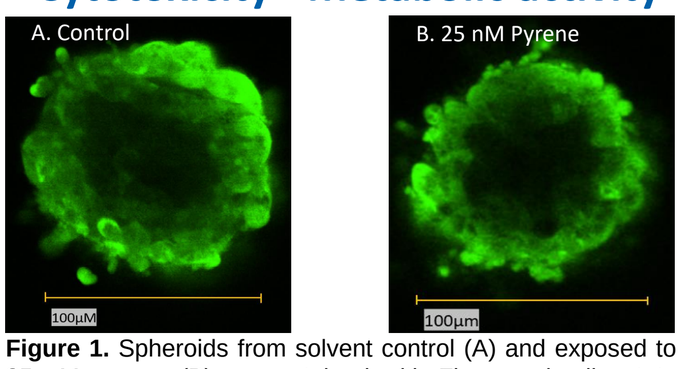

Figure 1. Spheroids from solvent control (A) and exposed to 25 nM pyrene (B) were stained with Fluorescein diacetate (37.5 µM) for 30 min. Images taken at the middle layer, transverse section (λ =800 nm).Pyrene showed no cytotoxic effects at tested concentrations.

Hypoxia

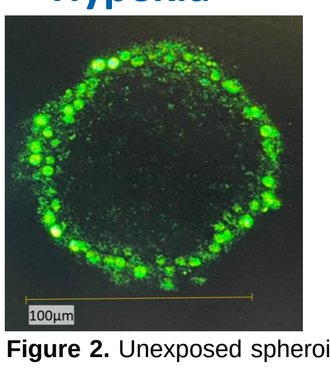

Figure 2. Unexposed spheroids stained with Image-iT green hypoxia reagent (5 µM) for 1hr. Images taken at the middle layer, transverse section (λ =800 nm). No hypoxia at core.

Cytosolic Reactive Oxygen Species (ROS) activity

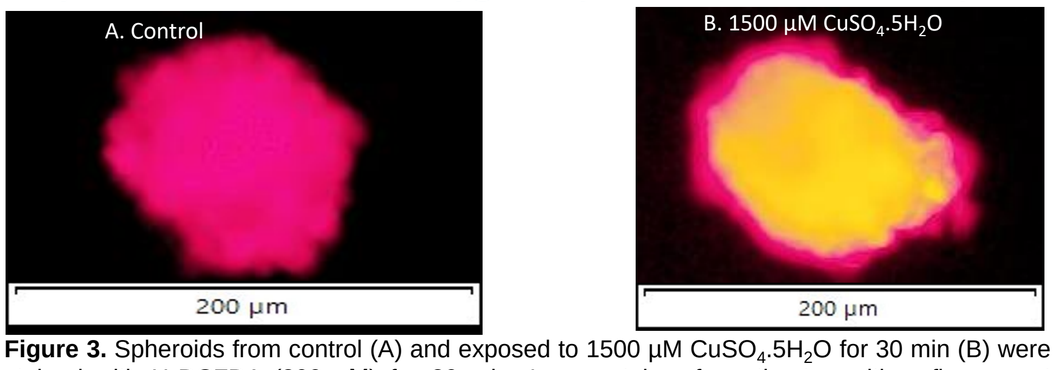

Figure 3. Spheroids from control (A) and exposed to 1500 µM $CuSO_4.5H_2O$ for 30 min (B) were stained with H_2DCFDA (200 µM) for 30 min. Images taken from the top with a fluorescent microscope (Olympus inverted microscope IX71) . Copper showed a notable increase in cytosolic ROS at the tested concentration.

Gene Ontology Enrichment Analysis

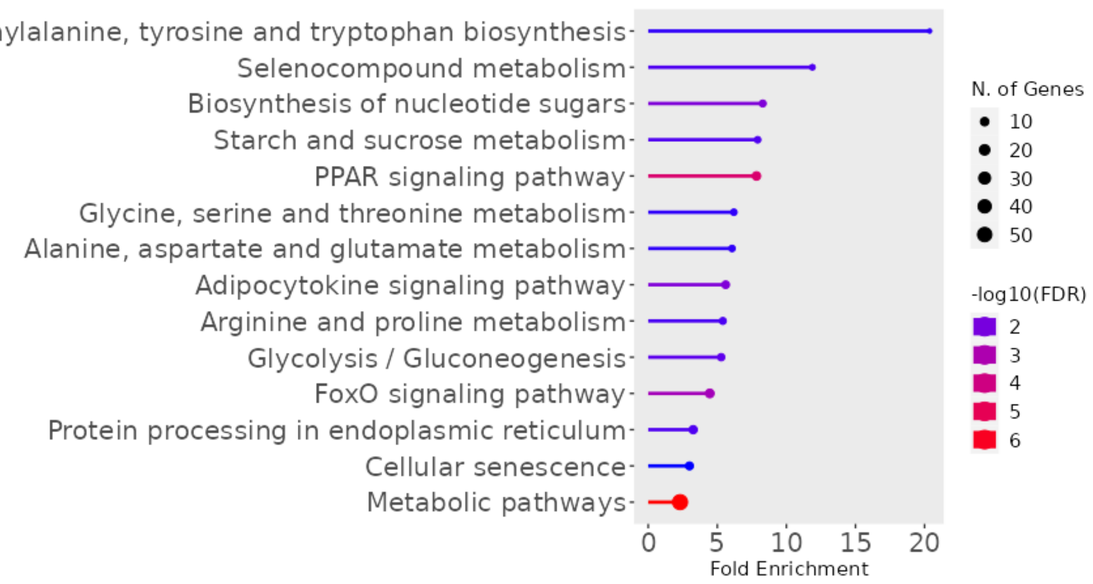

Figure 4. Top enriched KEGG pathways in spheroids after short-term exposure to EE2.

PPAR signalling pathway

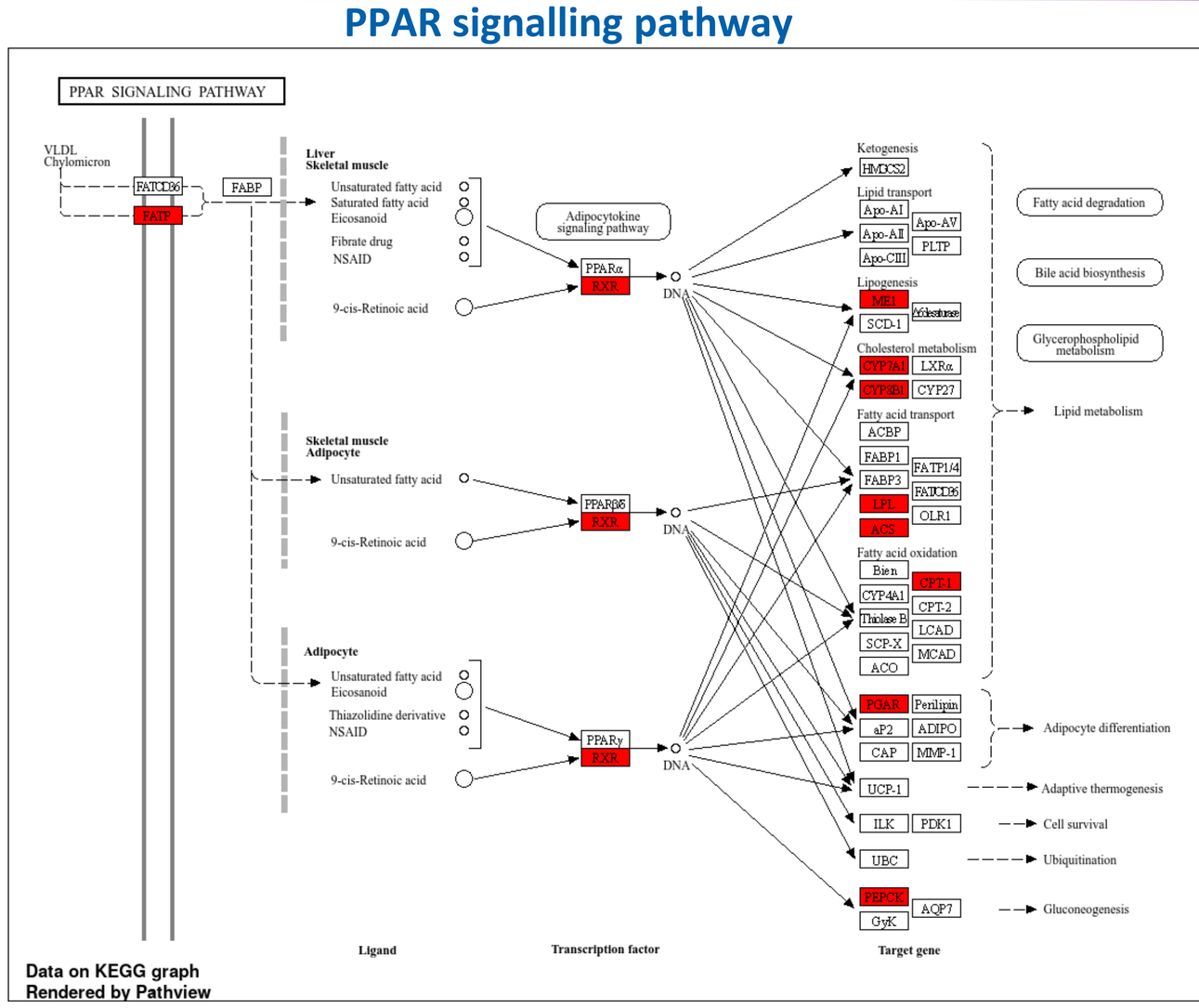

Figure 5. Enrichment of the peroxisome proliferator-activated receptor (PPAR) signaling pathway (differentially expressed genes highlighted in red) in spheroids after short-term exposure to EE2.

CONCLUSION

- MFM and MF appear suitable techniques for investigating cytotoxicity, cell membrane integrity, ROS activity, and hypoxia in spheroids.
- Results show no cytotoxicity with pyrene, ROS induction with copper, and no hypoxia, consistent with previous fish spheroid findings[5][6].
- Exposure to EE2 affected various endocrine and metabolic pathways as revealed by RNA sequencing, demonstrating the capability of the 3D spheroids as an alternative to in vivo toxicity testing. For example, the GO biological process on estrogen signaling, and the KEGG pathway of PPAR pathway, a key metabolic pathway that cross-talks with ER, were highly enriched [7] .

FUTURE WORK / REFERENCES

Further refinement of MFM will explore morphological structures and physiological properties of 3D hepatic rainbow trout spheroids for toxicity assessment. Ongoing analysis of transcriptomic and metabolomic data aims to uncover spheroids' physiological complexity and their potential role in chemical toxicity assessment.

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] Yazdani M. Drug chem toxicol 71-78 (2018); [6] Farmen, E., Toxicol pharmacol 431-438 (2010); [7] Lopes, C, Environ toxicol pharmacol, 328-336 (2016)

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