

CRYOPRESERVED FISH HEPATOCYTES AS A MODEL FOR BIO-ACCUMULATION AND CYTOTOXICITY EVALUATION OF AQUATIC POLLUTANTS

Aruna Koganti^{1*}, Aarti Uzgare, Andrew S. Kane², James C. Hardy¹, and Paul M. Silber^{1*}

¹BioreclamationIVT, 1450 South Rolling Road, Baltimore, MD and ²Department of Epidemiology and Preventive Medicine, University of Maryland, School of Medicine, Baltimore, MD



Abstract

The objective of this study is to develop an *in vitro* model to examine the bioaccumulation and cytotoxic effects of aquatic and waste-water related pollutants. In support of this objective, we developed a method for the isolation of rainbow trout hepatocytes (*Oncorhynchus mykiss*) in which viability and metabolic activities are maintained. Using a two-step perfusion technique, we successfully isolated hepatocytes with viabilities ranging from 80% to 95% as assessed by Trypan blue exclusion and flow cytometry. These hepatocytes were cryopreserved in an automated controlled rate freezer with very little loss in viability. On thawing, these cells were >80% viable as assessed by Trypan blue exclusion with vial-to-vial variation of only 6% as assessed by flow cytometry. The biotransforming ability of trout hepatocytes was assessed using the following nine specific substrates: dextromethorphan, tolbutamide, phenacetin, testosterone, mephenytoin, chlorzoxazone, coumarin, 7-ethoxycoumarin, and 7-hydroxycoumarin. While the specific activities of several CYP enzymes were detected, significant CYP3A-like-Phase I activity was observed during the conversion of testosterone to 6 β - and 16 β -hydroxytestosterone. Similarly, significant activity of the phase II enzyme, glucuronide transferase, was evident by the glucuronidation of 7-hydroxycoumarin. Using the above mentioned panel of nine substrates, we also demonstrated the maintenance of enzyme activities following cryopreservation. For these studies, cryopreserved cells were compared to freshly isolated cell suspensions. Taken together, these results demonstrate that cryopreserved trout hepatocytes are viable and exhibit xenobiotic metabolizing enzyme activities. Hence, they represent a suitable model for screening a large number of hazardous chemicals for their cytotoxicity and ability to be either metabolized or accumulated.

Introduction

It is widely recognized that the production of organic substances for various applications (industrial, medical, horticultural) have continuously increased over the years, resulting in widespread contamination of the environment. Some of these chemicals, such as DDT, PCBs, and dioxins, are known to persist in the environment and bioaccumulate into aquatic organisms and into other species via the food chain. Thus, persistence and bioaccumulation of these chemicals pose eco-toxicological problems as well as human health hazards.

The existing chemical hazard-identification-classification systems utilize acute toxicity evaluations based on L(E)C₅₀ values in fish, crustaceans or algae and information on persistence and bioaccumulation potential of the chemicals (1, 2).

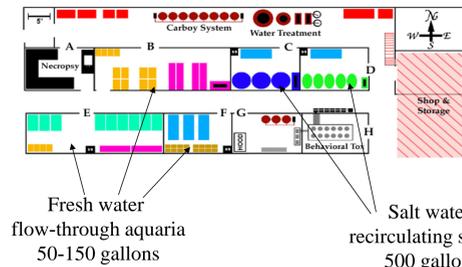
In vitro methods, in particular cell- or cell line-based methods, are scientifically relevant mechanistic models (i.e., possess biotransformation, uptake, and loss processes) and therefore provide information regarding acute toxicity as well as persistence and bioaccumulation (3). In addition, *in vitro* methods are amenable to high throughput assaying of chemicals, amenable to testing mixtures rather than individual chemicals, and less expensive. Thus, our objective was to develop fish hepatocytes as an *in vitro* model for testing chemicals.

Materials and Methods

Preparation of Fish Hepatocytes. Hepatocytes were isolated from rainbow trout (*Oncorhynchus mykiss*) livers. The trout, which were hatchery-raised, triploid, non-reproductive, and phenotypically male. The trout were obtained from the Aquatic Pathobiology Center at the University of Maryland. Trout livers were perfused through the portal vein using a two-step collagenase (collagenase II) perfusion method. The isolated cells were centrifuged at 50xg in *InVitroGRO*TM CP medium for 5 minutes at room temperature. Hepatocytes were then centrifuged through a Percoll gradient at 100xg for 10 minutes at room temp. The cells were then resuspended in CP medium with DMSO and cryopreserved.



Rainbow Trout



Aquatic Pathobiology Center, U. of MD



Living Stream System for Trout

Preparation of Cryopreserved Hepatocyte Suspensions. Vials were rapidly thawed in a 37°C water bath and diluted in *InVitroGRO*TM CP medium at room temperature. Cells were pelleted and resuspended in supplemented Krebs-Henseleit buffer. Cell counts and viability were determined by a Guava flow cytometer. The cell suspension was then diluted to 2,000,000 viable cells per mL with *InVitroGRO*TM supplemented Krebs-Henseleit buffer.

Incubations. Various human CYP-specific substrates were prepared in water or acetonitrile and diluted with supplemented Krebs-Henseleit buffer to dosing concentrations. Aliquots (150 μ L) of the dosing solutions were transferred to uncoated 48-well plates. Hepatocyte suspensions (150 μ L) were added to the wells and the mixtures were incubated at room temperature for 1 hour. The final concentration of the chemicals in the incubation mixtures was 5 μ M, in an incubation volume of 300 μ L containing 0.3 million viable hepatocytes. At each time-point, a 250- μ L aliquot of incubation mixture was harvested and mixed with an equal volume of methanol. The samples were stored at -70°C until analysis.

Sample Analysis. The samples were analyzed by HPLC-UV or LC/MS methods to quantify the amount of metabolite formation.

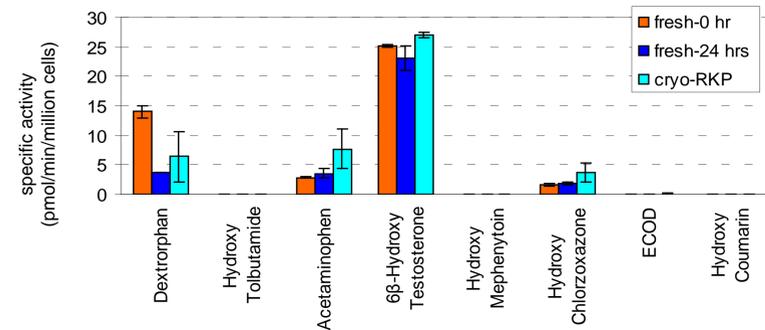


Figure 1: Phase I Metabolism in Freshly Isolated vs. Cryopreserved Trout Hepatocytes

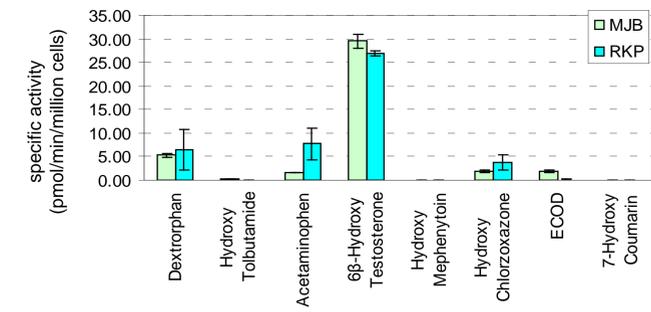


Figure 2. Phase I Metabolism in Different Lots of Cryopreserved Hepatocytes.

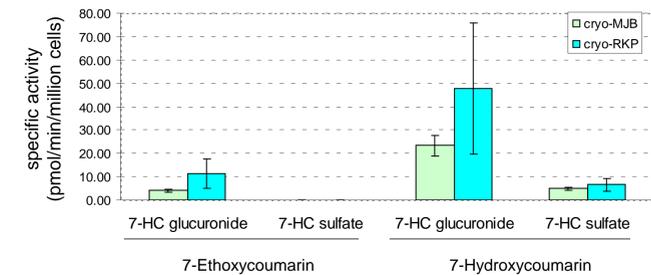


Figure 3. Phase II Metabolism in Different Lots of Cryopreserved Hepatocytes.

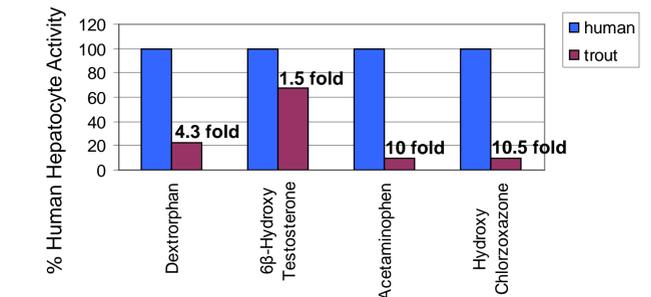


Figure 4. Phase I Metabolism in Trout Hepatocytes vs. Human Hepatocytes.

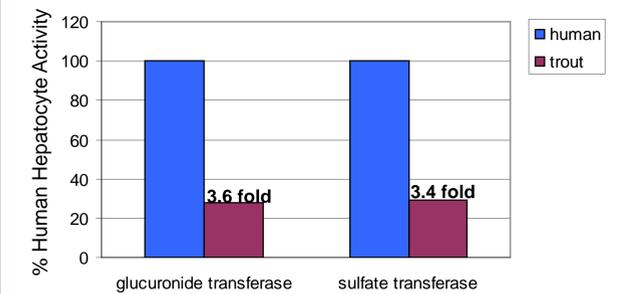


Figure 5. Phase II Metabolism in Trout Hepatocytes vs Human Hepatocytes.

Results

- Methods were established to isolate and cryopreserve hepatocytes from rainbow trout.
- No significant differences in Phase I or Phase II metabolic capacities were observed between freshly isolated trout hepatocytes or cryopreserved trout hepatocytes.
- The two lots of cryopreserved trout hepatocytes generated appeared to have similar Phase I and Phase II enzyme activities.
- Metabolism of known human CYP and Phase II enzyme substrates was lower in trout hepatocytes compared with human hepatocytes.
- Cryopreserved trout hepatocytes may provide a suitable model for screening of compounds for ecological-toxicity and bioaccumulation potential as well as for species comparison of toxicity potentials.
- Further studies will be conducted to compare toxicity potentials of known environmental chemicals to validate the model.

References

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