Use of Plateable Cryopreserved Rat Hepatocytes as a Model to Assess Hepatotoxicity and Drug-Drug Interactions

Timothy A. Moeller, Scott Lloyd, Paul M. Silber, and Neil S. Jensen
BioreclamationIVT, 1450 South Rolling Road, Baltimore, MD

BioreclamationIVT
The complete resource for all biologicals

Abstract

Freshly isolated rat hepatocytes are widely used to study drug metabolism, toxicity, and drug-drug interactions. However, the preparation of fresh rat hepatocytes is labor- and time-intensive and can be subject to interday variability with respect to both the quantity and quality of the isolated hepatocytes. We have recently identified cryopreserved rat hepatocytes (PCRH) that form monolayers on collagen-coated plates, and which can be used to assess long-term (>24 hour) toxicity and drug-drug interactions. To evaluate the PCRH model, hepatocytes from SD rats were thawed, and allowed to attach to collagen-coated plates overnight in a 37°C, 5% CO_2 incubator. Plates were then washed to remove nonadherent cells. After washing, all hepatocyte monolayers were >70% confluent. Hepatotoxicity was determined by treating hepatocyte monolayers with tamoxifen (0-100 µM), followed by measurement of MTT reduction and intracellular ATP levels at 24- and 48-hour time points following dosing. Tamoxifen demonstrated hepatotoxicity that was both dose and time dependent with an LD_{50} of 23 µM and 15 µM at 24 and 48 hours, respectively when measured by MTT reduction. The LD_{50} as measured by ATP levels was 30 µM and 18 µM at 24 and 48 hours, respectively. These results demonstrated the increased sensitivity of these hepatocytes to hepatotoxicants with increasing exposure time. PCRH were also evaluated for their ability to support induction of cytochrome P450 1A activity. Hepatocytes were dosed with ß-naphthoflavone (10 µM) on days 3 and 4 post plating. The metabolism of ethoxyresorufin was measured on day 5. An 8-fold increase in ethoxyresorufin O-deethylase (EROD) activity was observed following treatment with ß-naphthoflavone as compared to the untreated vehicle controls. Viability as measured by MTT reduction remained high throughout the five-day culture. In summary, PCRH combine the benefits of fresh and cryopreserved rat hepatocytes, and allow the long-term study of drug toxicity, metabolism, and drug-drug interactions in a reproducible model.

Introduction

Hepatocytes from different breeds of rats have been successfully isolated and cultured for over 30 years. Rat hepatocytes have been used in a broad range of studies including drug metabolism, drug-drug interaction, compound toxicity, virus propagation, and experiments for liver assist devices. Freshly isolated rat hepatocytes have been the gold standard for many of these studies. This is due to their ability to actively metabolize compounds when incubated in cell suspension, or to attach to collagen-coated tissue culture plates where they can be cultured for more than 5 days. Traditionally, cryopreserved rat hepatocytes have only been used for cell suspension studies. They have not been shown to reliably attach to collagen-coated plates in numbers sufficient for use in multi-day studies. Because of this, the use of rat hepatocyte monolayers has depended on the availability of freshly isolated rat hepatocytes. In the work presented here, we demonstrate the use of plateable cryopreserved rat hepatocytes. These hepatocytes can be thawed and cultured in collagen-coated plates where they attach in sufficient numbers (> 70% confluence) to be used for long-term toxicity studies and induction studies. The availability of cryopreserved rat hepatocytes with these characteristics can provide researchers with a consistent adherent rat hepatocyte model that avoids the burdensome requirement of preparing fresh hepatocytes.

Materials and Methods

Plateable cryopreserved rat hepatocytes. Plateable rat cryopreserved hepatocytes (PCRH) were obtained from BioreclamationIVT. Hepatocytes were isolated from female SD rats (lots MJA and RQY), female Wistar rats (lot WSW) and male Wistar rats (lot ODQ).

Plating of plateable cryopreserved rat hepatocytes. Vials were thawed in a 37°C water bath, and cells were transferred resuspended in *InVitro*GRO™ CP medium. Viability and cell counts were determined by Trypan blue exclusion. The cell suspension was diluted to 700,000 viable cells per ml with *InVitro*GRO™ CP medium and transferred to a collagen coated 48-well plate (140,000 cells per well). Plates were incubated overnight in a 37°C, 5% CO_2, humidified incubator to allow attachment of the hepatocytes. Medium was replaced after 24 hr.

Induction of CYP1A and CYP3A. At 48 hours, the medium was removed and ß-naphthoflavone (10 µM), 3-methylcholanthrene (1 µM), or dexamethasone (50 µM) in *InVitro*GRO™ HI medium was added to the PCRH, along with vehicle control dosing solution (1% DMSO). At 72 hours, new medium inducers or vehicle controls were added to the PCRH and cells were incubated an additional 24 hr.

Metabolism of Testosterone. Medium was removed and testosterone (100 µM) in modified Krebs Henseleit Buffer was added to PCRH induced with dexamethasone (50 µM) or vehicle control. Incubations were performed in a 37°C, 5% CO_2, humidified incubator for one hour, and stopped by addition of an equal volume of cold methanol. Metabolites were identified by HPLC.

Metabolism of Ethoxyresorufin. Medium was removed and ethoxyresorufin (10 µM) in modified Krebs Henseleit Buffer was added to PCRH induced with ß-naphthoflavone (10 µM), 3-methylcholanthrene (1 µM), or vehicle control. Incubations were performed in a 37°C, 5% CO_2, humidified incubator for one hour, and stopped by addition of an equal volume of cold methanol. The amount of metabolite was determined by fluorescence on Wallac Victor² multilabel counter at excitation 530 nm and emission 590 nm and compared to a standard curve of resorufin.

Percent Induction. The concentration of metabolites from the induced PCRH were divided by the concentration of metabolites from the vehicle control PCRH and multiplied by 100 to determine the percent of induction. If the vehicle control was below the lower limit of detection, the lowest point of the standard curve was used for calculations instead of the vehicle control value.

Tamoxifen Incubation. At 24 hours after plating, tamoxifen (0 – 100 µM) or vehicle controls (1% DMSO) were prepared in *InVitro*GRO™ HI medium as dosing solutions. Medium was removed from PCRH and dosing solutions were added. At 24 hours and 48 hours after addition of solutions, MTT reduction and ATP levels were determined to assess viability.

MTT Assay. MTT (10X) was added to each well containing drug, VC and background controls (final concentration: 0.5 mg/ml). Plates were incubated in a 37°C, 5% CO_2, humidified tissue culture incubator for three hours, then medium from all wells was removed, and 0.2 ml of acidified isopropanol was added to each well to dissolve the MTT formazan. Absorbance of MTT formazan was measured at 572 nm and 690 nm on a Wallac Victor² multilabel counter. The corrected absorbance was determined by subtracting the 690 nm value from the 572 nm value. The average background blank was subtracted from the average of the other experimental groups to derive the adjusted absorbance. The dosing groups were compared to VC by dividing adjusted absorbance of the dosing group by the adjusted absorbance of the VC and multiplying by 100 to get the percentage of VC.

ATPlite™ Assay. ATPlite (Perkin Elmer, Boston, MA) was used to determine ATP levels. Components of the homogeneous solution were added to the PCRH as indicated in the kit's instructions, and incubated at ambient temperature in the dark for 30 minutes. Luminescence was measured with a Wallac Victor² multilabel counter. The dosing groups were compared to VC by dividing adjusted absorbance of the dosing group by the adjusted absorbance of the VC and multiplying by 100 to get the percentage of VC.

Results

Long-term toxicity. Tamoxifen induced toxicity in a dose- and time-dependent manner as measured by MTT reduction and ATP levels at 24- (Fig. 4) and 48-hour (Fig. 5) time points with lot MJA. The LD_{50} of 23 µM and 15 µM at 24 and 48 hours respectively was determined by MTT reduction. LD_{50} as measured by ATP levels was 30 µM and 18 µM at 24 and 48 hours, respectively.

Viability and CYP1A and CYP3A induction and metabolism. MTT was added to PCRH incubated with inducers or vehicle control to determine viability (Fig. 1). An absorbance of 0.45 – 0.6 indicated healthy monolayers of PCRH were maintained throughout the induction experiment. Induction of CYP3A with dexamethasone (50 µM) resulted in an increase in testosterone 6ß-hydroxylase activity of 200% to 1500% (Fig. 2). Induction of CYP1A with either ß-naphthoflavone (10 µM) or 3-methylcholanthrene (1 µM) showed an increase in ethoxyresorufin O-deethylase activity of 1100% to 4000% (Fig. 3).

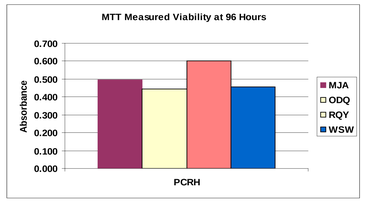

Figure 1. Viability of PCRH at 96 hours after plating as determined by MTT reduction.

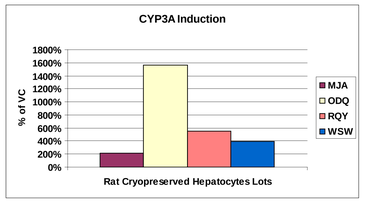

Figure 2. Dexamethasone (50 µM) induction of CYP3A as measured by the metabolism of testosterone to 6ß-hydroxytestosterone in four lots of plateable cryopreserved rat hepatocytes.

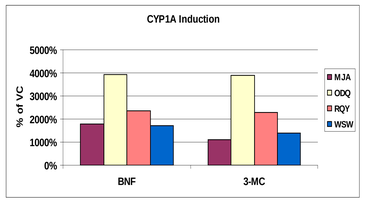

Figure 3. ß-naphthoflavone (10 µM) or 3-methylcholanthrene (1 µM) induction of CYP1A as measured by the metabolism of ethoxyresorufin to resorufin in four lots of plateable cryopreserved rat hepatocytes.

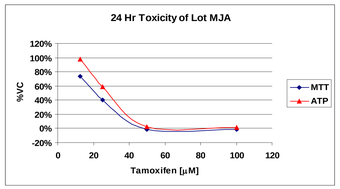

Figure 4. Toxicity of tamoxifen at 24 hours as measured by MTT reduction and ATP levels using plateable cryopreserved rat hepatocytes.

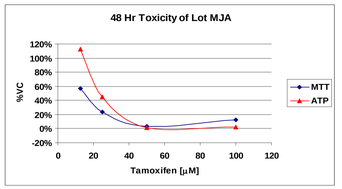

Figure 5. Toxicity of tamoxifen at 48 hours as measured by MTT reduction and ATP levels using plateable cryopreserved rat hepatocytes.

Conclusion

- PCRH have the ability to maintain a viable monolayer for at least five days *in vitro*.
- Induction of CYP1A and CYP3A activities can be demonstrated in PCRH.
- Toxicity of compounds can be assessed by multiple day incubations using PCRH.
- PCRH offer a reliable and convenient alternative to freshly plated rat hepatocytes.