Comparison of Metabolic Capacities of Fresh and Cryopreserved Human Hepatocytes Isolated from the Same Donor: Metabolic Stability, Plated Metabolism, Metabolite ID Applications

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Introduction

Primary hepatocytes are utilized to address a wide array of ADME issues including drug-drug interactions, metabolism and hepatotoxicity. Cryopreserved hepatocytes have emerged as a favored model due to commercial availability and convenience (Laine). The present study assessed the metabolic function of cryopreserved hepatocytes by direct comparison with fresh hepatocytes isolated from the same donor tissue. Endpoints included metabolic activity, predicted clearance and metabolite ID. These studies help establish cryopreserved hepatocytes as a robust and effective in vitro model for drug disposition studies.

Methods

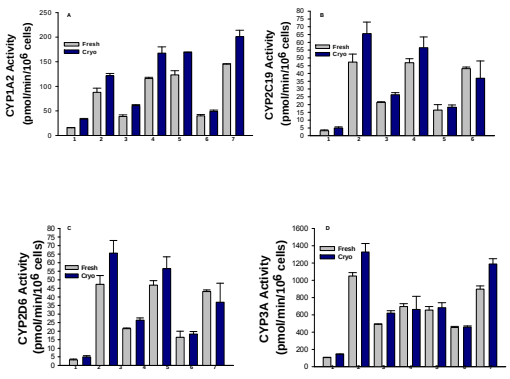
Hepatocyte Isolation and Cryopreservation. Primary human hepatocytes were isolated from resected liver tissue or whole liver tissue by a two-step collagenase perfusion method and subsequently cryopreserved (LeCluyse). Freshly isolated hepatocytes were stored in HTS®-FRS for <24 hr post isolation prior to use. Cryopreserved hepatocytes were stored at cryogenic temperatures for a minimum of two weeks prior to use. Cryopreserved hepatocytes were thawed in Williams' E Medium (WEM). Cell viability was assessed by Trypan blue exclusion.

Metabolic Activity. Enzymatic activities were determined in hepatocytes suspended to 0.5 x10^6 cells/mL in WEM with the following probe substrates: phenactin (CYP1A2), bupropion (CYP2B6), paclitaxel (CYP2C8), diclofenac (CYP2C9), S-mephenytoin (CYP2C19), dextromethorphan (CYP2D6), testosterone and midazolam (CYP3A), benzydamine (FMO), 7-ethoxycoumarin (CYP) and 7-hydroxycoumarin (UGT and SULT). Metabolites were identified by LC/MS/MS analysis.

Intrinsic Clearance. Hepatocytes were incubated in WEM with six substrates at low concentrations (0.5 or 1 μM). Substrates used were phenacetin (PHN), midazolam (MDZ), dextromethorphan (DXT), tolbutamide (TLB), diazepam (DZP) and benzydamine (BNZ). Assays were performed both in suspensions (0.5 x 10^6 cells/mL) and cultured cells. Cultures were seeded in 48-well plates at 0.8 x10^6 cells/mL and incubations proceeded the following day. The disappearance of parent was monitored by LC/MS/MS. Clearances were predicted using the well-stirred liver model with scaling factors, human liver weight = 22 g/kg, hepatocellularity = 120 x 10^6 cells/g liver, hepatic blood flow = 20 mL/min/kg (McGinnity; Obach).

Metabolite ID. Incubations in suspensions proceeded for two hours to allow for adequate metabolite formation. Metabolites were identified by HPLC and a sensitive fast-cycle-time-hybrid tandem Qq-LIT mass spectrometer operated in positive ESI mode.

Figure 1 – Enzymatic activities of fresh and cryo human hepatocytes isolated from same donor tissue



Specific activities were determined in fresh and cryo hepatocytes isolated from the same donor tissue. Representative specific activities, CYP1A2, CYP2C19, CYP2D6 and CYP3A (testosterone) are depicted in panels A-D. Bars represent average values from replicates of 3 with associated standard deviations. Averages from duplicates are shown in cases where the error bar is absent.

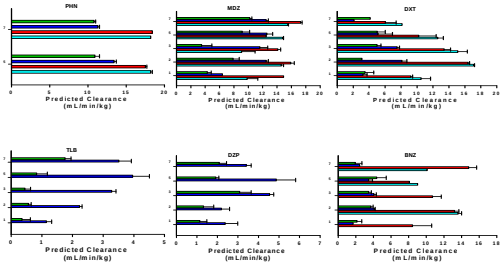
Table 1 – Enzymatic activities in fresh and cryo human hepatocytes isolated from several donor tissues

Specific Activity		Fresh (pmol/min/10^6 cells)		Cryo (pmol/min/10^6 cells)	
Enzyme	N	Average	Median	Average	Median
CYP1A2	7	80.9 ± 49.8	87.8	115 ± 67	121
CYP2B6	7	42.5 ± 20.2	35.1	48.0 ± 22.6	39.5
CYP2C8	6	9.51 ± 5.97	8.70	10.1 ± 5.7	7.90
CYP2C9	5	94.6 ± 51.3	80.5	159 ± 57	172
CYP2C19	6	30.6 ± 25.5	34.4	32.4 ± 24.2	34.6
CYP2D6	7	26.6 ± 18.9	21.4	30.8 ± 23.6	26.4
CYP3A-TEST	7	622 ± 310	655	728 ± 409	663
CYP3A-MDZ	7	58.3 ± 36.2	49.0	110 ± 75	95.3
FMO	3	271 ± 41	275	275 ± 22	278
ECOD	7	25.7 ± 8.8	25.4	34.4 ± 8.6	32.6
UGT	7	606 ± 298	471	666 ± 333	523
SULT	7	45.4 ± 27.5	45.2	43.9 ± 20.0	48.6

Table 2 – Viabilities of fresh and cryo hepatocytes from same preparation

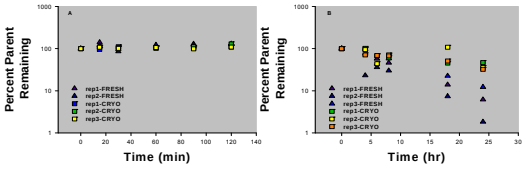
	Fresh		Cryo	
Hepatocyte Preparation	Viability (%)	Viability Stability (%)	Viability (%)	Viability Stability (%)
1	76	68	71	62
2	91	80	83	80
3	77	67	77	66
4	72	59	72	60
5	77	73	82	77
6	81	77	78	78
7	74	63	74	64

Figure 2 – Predicted clearances from suspensions and plated fresh and cryo human hepatocytes from same donor tissue



Clearance values for six substrates were predicted using fresh and cryo hepatocytes isolated from the same donor tissue. Bars represent average values from replicates of 3 with associated standard deviations. Where error bars are missing, averages are from duplicates. Each bar color is representative of the following: green cryo culture, blue fresh culture, red cryo suspensions, light blue fresh suspensions. Predicted clearances were calculated as described in methods.

Figure 3 – Disappearance of parent vs time profiles in suspensions and plated fresh and cryo human hepatocytes from same donor tissue



Intrinsic clearance assays were performed in fresh and cryo human hepatocytes isolated from the same donor tissue. Representative disappearance vs time profiles for tolbutamide are depicted from preparation 5 in (A) suspensions and (B) cultured cells. Incubation times were 0, 15, 30, 60, 90, and 120 min and 0, 2, 4, 6, 8, 18 and 24 hr for suspensions and cultured hepatocytes, respectively.

Table 3 – Metabolite ID

Drug /RT	Metabolite /RT	Relative Abundance Fresh/Cryo
Midazolam /7.3	(N-)? Oxidation + Glucuronidation/7.3	220
Midazolam /7.3	Glucuronidation /6.53	120
Midazolam /7.3	Glucuronidation /6.62	120
Midazolam /7.3	Oxidation /6.8	100
Midazolam /7.3	N-Oxidation /8.5	90
Midazolam /7.3	Di Oxidation /7.8	100
Phenacetin /8.6	De-ethylation to APAP /6.9	120
Phenacetin /8.6	Demethylation ? /8.7	250
Phenacetin /8.6	Demethylation /6.8	85
Phenacetin /8.6	De-ethylation + Demethylation + Glucose /7.3	250
Phenacetin /8.6	Oxidation /8.2	100
Dextramethorphan /8.7	DRR? Demethylation /7.1	105
Dextramethorphan /8.7	DRR? Demethylation /9.1	100
Dextramethorphan /8.7	2x Demethylation /7.4	100
Dextramethorphan /8.7	Oxidation /7.8	90
Dextramethorphan /8.7	Demethylation Glucuronidation /6.4	120
Testosterone /11.2	Dehydrogenation /10.9	105
Testosterone /11.2	Oxidation + Dehydrogenation /8.5	125
Testosterone /11.2	Dehydrogenation Oxidation /9.2	105
Testosterone /11.2	Dehydrogenation Oxidation /9.0	110
Testosterone /11.2	Dehydrogenation Oxidation /8.1	120
Testosterone /11.2	Dehydrogenation Oxidation /7.5	170
Testosterone /11.2	Oxidation + Glucuronidation /7.6	140
Testosterone /11.2	Oxidation /9.6	115
Testosterone /11.2	Oxidation /9.3	115
Testosterone /11.2	Oxidation /9.1	110
Testosterone /11.2	Oxidation /8.5	105
Testosterone /11.2	Oxidation /8.1	105
Testosterone /11.2	Oxidation /7.95	110
Testosterone /11.2	Oxidation /7.4	140
Testosterone /11.2	Oxidation /7.2	130
Testosterone /11.2	Oxidation + Hydrogenation /8.2	140
Testosterone /11.2	Glucuronidation /8.8	110
Testosterone /11.2	Di Oxidation /7.0	130
Testosterone /11.2	Di Oxidation /6.6	130
Testosterone /11.2	Di Oxidation /6.12	160
Benzydamine /9.1	Oxidation + Glucuronidation /6.2	115
Benzydamine /9.1	Di Oxidation + Glucuronidation /5.5	125
Benzydamine /9.1	Di Oxidation + Glucuronidation /6.4	120
Benzydamine /9.1	Oxidation /7.4	105
Benzydamine /9.1	Glucose /8.5	90
Benzydamine /9.1	Glucuronidation /8.8	105
Benzydamine /9.1	Di Oxidation /7.71	140
Benzydamine /9.1	Di Oxidation /7.60	105
Benzydamine /9.1	N-Oxidation /9.3	100
Bupropion /7.7	N-Oxidation /7.8	125
Bupropion /7.7	Oxidation /6.6	130
S-Mephenytoin /8.3	Oxidation /6.1	95
S-Mephenytoin /8.3	Demethylation /7.3	100
Paclitaxel /9.7	Oxidation /9.3	100
Paclitaxel /9.7	Oxidation /8.6	100

Results and Conclusions

- No statistically significant differences between fresh and cryopreserved metabolic activities were observed for all isoforms tested.
- Predicted clearances for the six substrates were not different between fresh and cryo suspensions (1.6-fold at most). Exceptions to this general observation include 3-fold differences in BNZ clearances from preparation 3.
- Predicted clearances for moderate to high clearance compounds (PHN, MDZ, DXT, BNZ), were not very different between fresh and cryo cultured hepatocytes. Exceptions were 3.3-fold and 2.6-fold differences for MDZ in preparation 3 and DXT in preparation 2, respectively.
- Fresh cultured hepatocytes generally predicted 4-fold and 2-fold higher than those from cryo cultures for TLB and DZP, respectively.
- Cultured hepatocytes are more robust than suspensions for assessments of low-turnover compounds.
- No metabolites analyzed from met ID studies were exclusive to either the fresh or cryo group.
- Cryopreserved human hepatocytes are an effective model system to use for metabolism, met ID and metabolic profiling of drug candidates.

References

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LeCluyse, E.L. et al. (2005) *Methods Mol Biol* **290:**207-229McGinnity, D.F. (2004) *Drug Metab Dispos* **32:**1247-1253

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