Long Term Storage Effects on the Metabolic Activity of Cryopreserved Primary Human and Rat Hepatocytes

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ABSTRACT

Cryopreserved hepatocytes represent the current "state of the art" model system for studying xenobiotic metabolism and drug-drug interaction potential. Advances in cryopreservation technology have allowed for the long-term storage of hepatocytes and provided a consistent supply for research applications. Although several laboratories have demonstrated that cell attachment in culture and metabolic activity are not significantly impacted by cryopreservation, little is known in regards to the functionality of hepatocytes which have been stored cryopreserved for extended periods. To test the hypothesis that cell morphology, viability, viability stability, metabolic activity and attachment are sustained after 'long-term' storage, human and rat hepatocytes stored in cryogenic conditions for 4 - 5 years (long-term) were assessed and compared to those stored for 1 - 2 years (short-term). Cell viability and yield were determined by trypan blue exclusion. Metabolic activities CYP1A2, CYP2C9, CYP2D6 and CYP3A were determined using probe substrates phenacetin, diclofenac, dextromethorphan and testosterone, respectively and analyzed by LC-MS-MS. Phase II activities glucuronidation (UGT) and sulfation (SULT) were assessed by 7-hydroxycoumarin incubations and HPLC. Human cryopreserved hepatocytes were seeded on collagen-coated plates, overlaid and monitored over several days for attachment. Data from each individual lot was compared to its own historical data obtained post cryopreservation. Viability, yield and viability stability were maintained in both groups with less than 3 percent difference noted between historical and current data. The human lots previously characterized as plateable, maintained this quality, even after long-term storage. CYP activities were sustained also. For example average (± S.E.) CYP3A activities were 631 ± 224 pmol/min/10^{-6} cells (historical) and 516 ± 198 pmol/min/10^{-6} cells (current) for stored 4 - 5 year lots and 631 ± 142 pmol/min/10^{-6} cells (historical) and 552 ± 119 pmol/min/10^{-6} cells (current) for stored 1 - 2 year lots. Likewise, SULT activities were not significantly different between historical and current values in either group. Interestingly, a significant decrease ($p < 0.05$) in UGT activity was observed in the 4 – 5 year old human lots, with average values of 574 ± 54 pmol/min/10^{-6} cells versus 256 ± 7 pmol/min/10^{-6} cells. A non-significant decrease in UGT activity was observed for the 4 - 5 year old rat lots. UGT activities did not significantly change in the 1 – 2 year old human lots [432 ± 8 pmol/min/10^{-6} cells (historical) versus 498 ± 40 pmol/min/10^{-6} cells (current)]. In conclusion, no significant changes in viability, viability stability, attachment and analyzed CYP activities were observed in long-term cryogenically stored human and rat hepatocytes. The observed decreases in UGT activity in lots stored for 4 – 5 years warrant additional investigation to further characterize the impact of long-term storage on phase II activity.

MATERIALS AND METHODS

Materials Cell culture reagents Williams' Medium E (WME), Cryopreserved Hepatocytes Recovery Medium (CHRM®) and Fetal Bovine Serum (FBS) were supplied by Life Technologies. Trypan blue solution and all chemicals were purchased from Sigma-Aldrich.

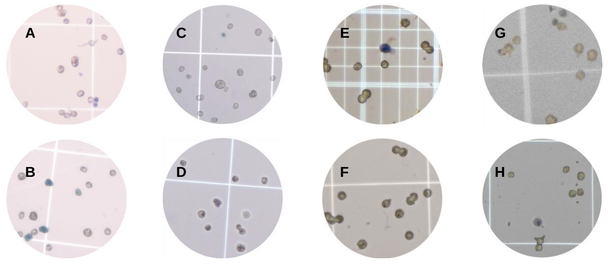
Hepatocyte Isolation and Cryopreservation Liver tissues were obtained from Research Tissue Organizations and participating medical centers. Life technologies adhered to HIPAA and ethical rules and regulations in the procurement and handling of human tissues. Hepatocytes were isolated as previously described by a two-step collagenase perfusion process (1) and cryopreserved by modification of previously reported methods (2). Cells were counted by Trypan blue exclusion. Viability Stability was determined by measurement of viability after an incubation period of 2 h.

Metabolic Activity in Hepatocyte Suspensions Hepatocytes were thawed in serum-containing WME, spun for 76 *x g* for 6 min (human) and 55 *x g* for 3 min (rodent) and re-suspended in serum-free WME prior to initiation of experiments in non-coated multi-well plates. Final suspended hepatocyte concentrations were 0.5 x 10^6 cells/mL and I substrate incubation times were15 to 30 min. Metabolites for prototypical CYP substrates phenacetin, diclofenac, dextromethorphan and testosterone were detected by LC-MS-MS. Metabolites for generic substrates 7-ethoxycoumarin (ECOD) and 7-hydroxycoumarin were assessed by HPLC. All metabolic activity rates are expressed as pmol/min/10^6 cells.

Enzyme Induction Assays Cryopreserved human hepatocytes were thawed in CHRM®, allowed to attach for 4 to 6 hr prior to overlay with Geltrex™. Cell cultures were exposed to inducers omeprazole (OMP), phenobarbital (PB) and rifampicin (RIF) for 72 hours. Metabolic activity assays were performed in situ on the fifth day in culture and activities determined by LC-MS-MS analyses.

RESULTS

Figure 1. Images of Cryopreserved Hepatocytes Original and Current Thaws



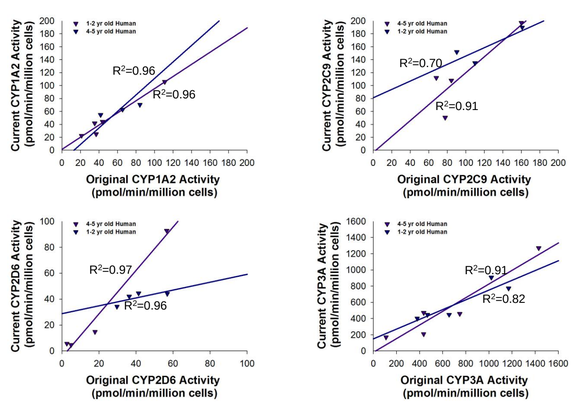
Cryopreserved hepatocytes were thawed in serum-containing WME and typical microscopic images depicted for A) original thaw human cryo'd April 2007, B) current thaw human cryo'd April 2007, C) original thaw human cryo'd May 2010, D) current thaw human cryo'd May 2010, E) original thaw rodent cryo'd December 2007, F) current thaw rodent cryo'd December 2007, G) original thaw rodent cryo'd September 2009, H) current thaw rodent cryo'd September 2009

Table 1. Cell Characteristics of Cryopreserved Hepatocytes from Original and Current Thaws

Cryo Lot Category	Original Viability (%)	Current Viability (%)	Original Viability Stability(%)	Current Viability Stability (%)	Original viable cell yield (x10^6 cells)	Current viable cell yield (x10^6 cells)
Human (4-5 yr old)	74.5 ± 3.7	71.3 ± 4.9	65.3 ± 4.6	62.0 ± 6.0	6.0 ± 0.8	6.1 ± 0.8
Human (1-2 yr old)	74.3 ± 1.5	74.3 ± 3.7	60.5 ± 3.7	60.8 ± 5.1	5.3 ± 0.9	5.5 ± 1.3
Rat (4-5 yr old)	78.7 ± 8.9	75.3 ± 9.2	65 ± 4.9	62 ± 7.4	5.3 ± 0.8	5.7 ± 1.1

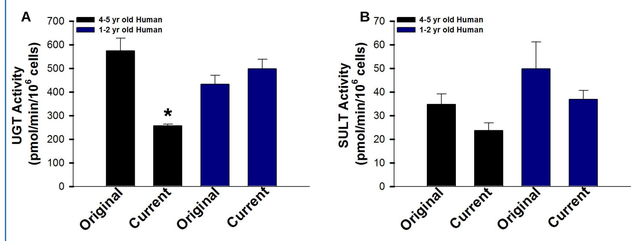
Cryopreserved hepatocytes were thawed in serum-containing WME and viability ,viability stability and yield determined by Trypan blue exclusion. All data is represented by averages ± S.E. of at least four separate lots.

Figure 2. Correlation Analyses of Original and Current CYP Activities in Cryopreserved Human Hepatocytes



Prototypical CYP Activities were determined in cryopreserved human hepatocytes originally and currently. Data points represent the averages of three replicates. The solid lines represent correlation trend lines of the original CYP activity and the current CYP activity.

Figure 3. Comparisons of Phase II Activities determined in Cryopreserved Human Hepatocytes Originally and Currently



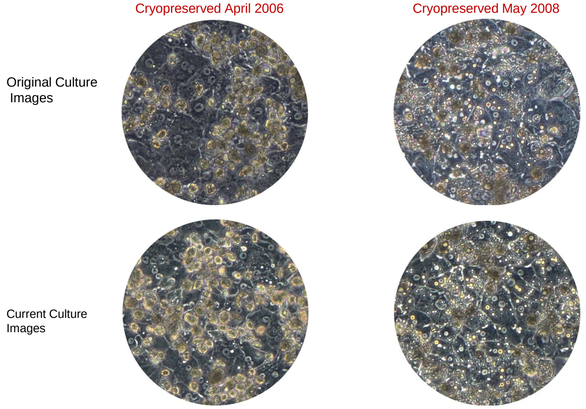
Phase II Activities, UGT (A) and SULT (B) were determined in human cryopreserved hepatocytes after cryopreservation (original) and recently (current). Bars represent averages ± S.E. of at least four separate lots with statistical significance ($p<0.05$) denoted by an asterisk.

Table 2. Phase I and Phase II Metabolic Activities in Cryopreserved Rodent Hepatocytes Determined Originally and Currently

Activity (pmol/min/10^6cells)	Original (4-5 yr old Rodent)	Current (4-5 yr old Rodent)	Original (1-2 yr old Rodent)	Current (1-2 yr old Rodent)
ECOD	33.7 ± 1.6	40.9 ± 7.3	21.5	29.8
UGT	800 ± 251	564 ± 137	664	654
SULT	150 ± 68	217 ± 75	286	330

Phase I Activity (ECOD) and phase II Activities UGT and SULT were determined in rodent cryopreserved hepatocytes after cryopreservation (original) and recently (current). Where indicated, data is represented as averages ± S.E. of at least four separate lots.

Figure 4. Images of Attached Cryopreserved Hepatocytes stored for 3 to 5 yr and Recently Thawed and Cultured



Two lots of human hepatocytes were cryopreserved at different time periods. Images depict plateable cryopreserved human lots which were cultured with Geltrex™ or similar overlay for 5 days shortly after cryopreservation (original) and recently (current).

Table 3. Enzyme Induction Data of Cryopreserved Human Lots stored for 3 to 5 yr and Recently Thawed and Cultured

Cryopreservation Date	Basal CYP1A2	Induced CYP1A2	Fold CYP1A2	Basal CYP2B6	Induced CYP2B6	Fold CYP2B6	Basal CYP3A	Induced CYP3A	Fold CYP3A
April 2006	5.16 ± 1.36	94.0 ± 6.2	18	1.73 ± 0.36	10.1 ± 0.75	6	60.9 ± 12.5	769 ± 36	13
September 2007	1.46 ± 0.08	9.64 ± 1.66	7	0.621 ± 0.043	9.64 ± 1.66	4	10.7 ± 1.7	455 ± 53	42
May 2008	3.84 ± 0.30	351 ± 27	91	1.52 ± 0.20	17.4 ± 2.0	12	74.6 ± 6.9	612 ± 22	8

Enzyme induction assays were performed recently in three different plateable human hepatocytes cryopreserved lots stored for 3 to 5 yr. Cultures were induced with OMP (CYP1A2), PB (CYP2B6) and RIF (CYP3A) and enzyme activities determined in situ using appropriate CYP substrates. Data points are shown as averages ± s.d. of quadruplicate measurements.

CONCLUSIONS

- Post-thaw viabilities, viability stabilities and viable cell yields were relatively stable for both the 4-5 yr old lots and the 1-2 yr old lots.
- Percent differences for any given cell characteristic were < 3% between original and current thaws.
- CYP activities remained stable for both the human 4-5 yr old lots and the human 1-2 yr old lots as indicated by strong correlations ,R^2 ranged 0.7 to 0.97 between original activities determined and analysis of current activities.
- UGT activities dropped ~ 2-fold in the 4-5 yr old human group and the difference was statistically significant. UGT activities remained relatively stable in the 1-2 yr old human group.
- There was not a statistical difference in SULT activity for either the 4-5 yr old human lots or the 1-2 yr old human lots.
- Phase I and Phase II activities determined in rodent lots were stable for both the 4-5 yr old group and the 1- 2 yr old group. A drop in UGT activity was observed in the 4-5 yr old rodent lots, however this difference was not statistically significant.
- Plateable cryopreserved human lots stored for 3 to 5 yr retained functionality and enzyme induction assays revealed that these lots were still attachable after 5 days in culture and responsive to CYP inducers.

REFERENCES

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2. Madan A, et al., (1999) Effect of cryopreservation on cytochrome P-450 enzyme induction in cultured rat hepatocytes. *Drug Metab Dispos* **27:**327-335.

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TRADEMARKS/LICENSING