The effect of multiple cryopreservation cycles on drug-metabolizing enzymes in human hepatocytes.

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ABSTRACT

Cryopreserved hepatocytes provide a convenient *in vitro* test system to study the phase-1 and phase-2 metabolism of new chemical entities; however cryopreservation (freeze-thaw cycles) can damage the cells. Cryoinjury is associated with rapid dehydration of cells (osmotic effects) and formation of intracellular ice during the freezing process, which in turn leads to a disruption of cellular membranes, changes in protein conformation and nucleic acid damage (single and double DNA strand breaks). This study characterized the effects of multiple cryopreservation cycles on the activities of drug-metabolizing enzymes in individual and pooled samples of human hepatocytes. Hepatocytes isolated from four donors were cryopreserved once, twice or three times according to a stepwise protocol. Pooled hepatocytes (n=5) were prepared by two protocols: one involving the thawing of individual lots of frozen hepatocytes, followed by pooling and re-freezing, and the other by pooling cryopreserved hepatocyte pellets (CryostaX™) without thawing or re-freezing the cells. All cells were stored in the vapor phase of liquid nitrogen and were thawed under the same conditions. Viable hepatocytes were separated from non-viable cells by Percoll® gradient centrifugation. The following enzyme activities were measured *in situ*: CYP3A4 (testosterone 6β-hydroxylation), CYP1A2 (phenacetin *O*-dealkylation), CYP2B6 (bupropion hydroxylation), FAD-containing monooxygenase (FMO, benzydamine *N*-oxidation), UDP-glucuronosyltransferase (UGT), and sulfonotransferase (SULT, 7-hydroxycoumarin sulfonation). Compared with individual lots of hepatocytes that were cryopreserved once, hepatocytes that were cryopreserved twice lost 23% of SULT, 18% of CYP3A4, 7-13% of UGT, and 6% of FMO activity. Hepatocytes that were cryopreserved three times lost even more enzyme activity (at least twice as much as the cells cryopreserved twice). Compared with pooled hepatocytes that were cryopreserved once (CryostaX™), pooled hepatocytes that were cryopreserved twice lost 33% of SULT, 30% of UGT, 24% of CYP1A2, 14% of FMO, 13% of CYP3A4/5, and 10% of CYP2B6 activity. In summary, multiple cryopreservation cycles have a deleterious effect on the activity of drug-metabolizing enzymes in human hepatocytes. In general, the loss of UGT and SULT activity was more extensive than that of CYP and FMO activity.

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INTRODUCTION

Pooled human hepatocytes are a desirable test system for phase-1 and phase-2 drug metabolism and metabolic stability studies. Fresh pooled hepatocytes are rarely available; therefore cryopreserved cells are commonly used to support *in vitro* studies of drug metabolism.

During the cryopreservation process, cells can become damaged (*i.e.*, they sustain cryoinjury) while at a temperature zone between -15 and -60°C. The risk of cell damage is repeated during thawing when the cells transverse the same temperature zone (Mazur, 1963). The mechanisms of cryoinjury include osmotic effects associated with water rapidly leaving or entering the cytoplasm as well as intracellular and extracellular ice formation, which can alter the native conformation of proteins and disrupt cellular membranes (Harris *et al.*, 1991; Pikal-Cleland *et al.*, 2000). In hepatocytes, cryopreservation has the potential to cause single- and double-stranded DNA breaks, reduce intrahepatic engraftment, reduce post-transplantation albumin production, diminish cell attachment in culture and alter mitochondrial respiration (Mueller *et al.*, 2002; David *et al.*, 2001; Stéphenne *et al.*, 2007).

Pooled human hepatocytes are currently prepared by two protocols: one involving the thawing of individual lots of frozen hepatocytes, followed by pooling and re-freezing (multiple cryopreservation cycles), and the other by pooling cryopreserved hepatocyte pellets (CryostaX™) without thawing or re-freezing the cells (single cryopreservation cycle). This study compared the effects of single- and multiple-cryopreservation cycles on the CYP- and FMO-mediated-oxidation, glucuronidation and sulfonation of drugs in individual lots and pooled lots of human hepatocytes.

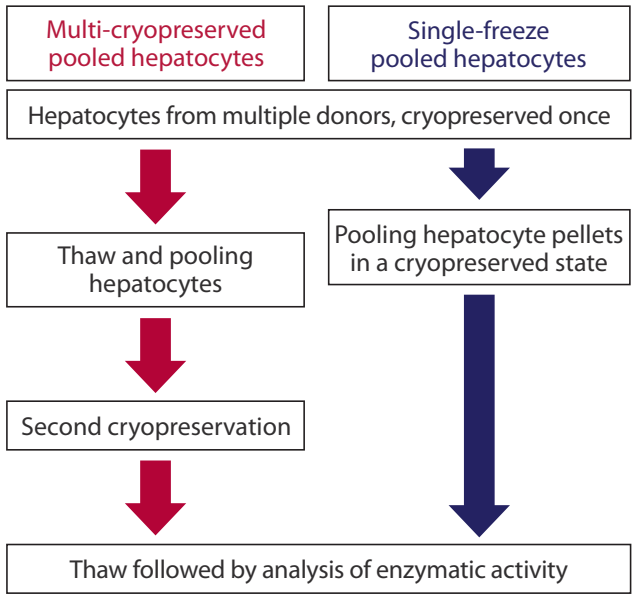
MATERIALS & METHODS

Hepatocytes were isolated with a two-step liver perfusion method followed by Percoll-gradient centrifugation to separate viable and non-viable cells (Parkinson and Mudra, 2001). Cells were frozen in a temperature-controlled chamber in medium containing cryopreservation agents (Loretz *et al.*, 1989; Madan *et al.*, 1999).

The effects of multiple freeze-thaw cycles on the activity of drug-metabolizing enzymes were evaluated in hepatocytes from four individual donors that were cryopreserved once, twice or three times.

Multi-cryopreserved and single-freeze pooled hepatocytes were prepared from five donors, according to the procedure outlined in **Figure 1**.

Figure 1. Cryopreservation flow chart



For measurement of enzymatic activity, cells were rapidly thawed at 37°C, transferred to Dulbecco's-modified essential medium (DMEM) containing 21.6% Percoll® and subjected to centrifugation at 80 x g for 5 minutes. Following a rinse spin in DMEM at 60 x g for 3 minutes cell, viability was measured by Trypan Blue exclusion.

Details of the analytical methods are given in **Table 1**.

Table 1.

Incubation and analytical conditions

Enzyme	Substrate (µM)	Metabolite	Incubation Time (min)	Cells per Incubation	Ionization Mode	Mass Transitions
CYP1A2	Phenacetin, 100	Acetaminophen	30	500,000	ESI -	150 → 107 amu
CYP2B6	Bupropion, 500	Hydroxybupropion	30	500,000	ESI +	256 → 238 amu
CYP3A4/5	Testosterone, 250	6β-hydroxytestosterone	30	500,000	ESI -	303 → 287 amu
Multiple sulfonotransferases	7-hydroxycoumarin, 100	7-hydroxycoumarin sulfate	30	500,000	ESI -	241 → 161 amu
Multiple UDP-glucuronosyltransferases	7-hydroxycoumarin, 100	7-hydroxycoumarin glucuronide	30	500,000	ESI -	337 → 161 amu
Multiple UDP-glucuronosyltransferases	4-methylumbelliferone,1000	Methylumbelliferone-4-glucuronide	30	500,000	ESI +	353 → 177 amu
UGT1A1	17β-Estradiol, 100	Estradiol-3-glucuronide	15	500,000	ESI -	447 → 271 amu
UGT1A4	Trifluoperazine, 25	Trifluoperazine glucuronide	30	500,000	ESI +	584 → 408 amu
UGT1A6	1-Naphthol, 500	Naphthol glucuronide	7.5	125,000	ESI -	319 → 143 amu
UGT1A9	Propofol, 50	Propofol glucuronide	15	125,000	ESI -	353 → 177 amu
UGT2B7	Morphine, 1000	Morphine-3-glucuronide	15	500,000	ESI +	462 → 286 amu
FAD-containing monooxygenase	Benzydamine, 500	Benzydamine *N*-oxide	30	500,000	ESI +	326 → 102 amu

RESULTS

As shown in **Figure 2**, compared with human hepatocytes that were cryopreserved only once, hepatocytes from four individual donors that were cryopreserved twice lost, on average, 23% of sulfonotransferase (SULT), 18% of CYP3A4, 10% of UDP-glucuronosyltransferase (UGT) and 6% of FAD-containing monooxygenase (FMO) activity. Hepatocytes that were cryopreserved for a third time lost 57% of SULT, 30% of CYP3A4, 54% of UGT and 17% of FMO activity compared with cells that were cryopreserved only once.

As shown in **Figure 3**, pooled hepatocytes that were cryopreserved twice lost 33% of SULT, 30% of UGT, 24% of CYP1A2, 14% of FMO, 13% of CYP3A4/5 and 10% of CYP2B6 activity compared with pooled human hepatocytes that were cryopreserved only once (CryostaX™).

The significant decline in 7-hydroxycoumarin and 4-methylumbelliferone glucuronidation in multi-cryopreserved hepatocytes prompted an analysis of the effects of cryoinjury on the activity of various individual UGT enzymes in pooled human hepatocytes. As shown in **Figure 4**, the activity of UGT1A1, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 declined 37, 26, 43, 34 and 41% in multi-cryopreserved hepatocytes compared with single-cryopreserved pooled hepatocytes (CryostaX™).

Figure 2.

The effects of multiple freezes on the activity of phase-1 and phase-2 enzymes in human hepatocytes: Average of four donors frozen and refrozen individually

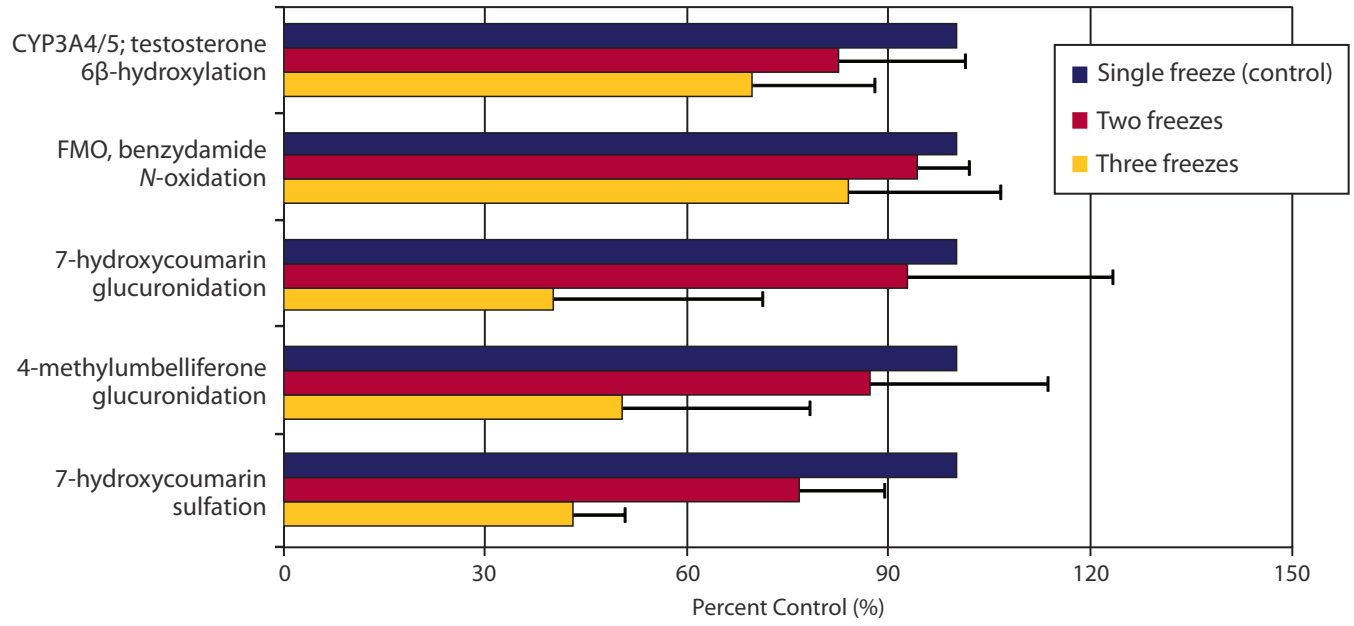


Figure 3.

The effects of multiple freezes on the activity of phase-1 and phase-2 enzymes in human hepatocytes (pools contain cells from the same donors)

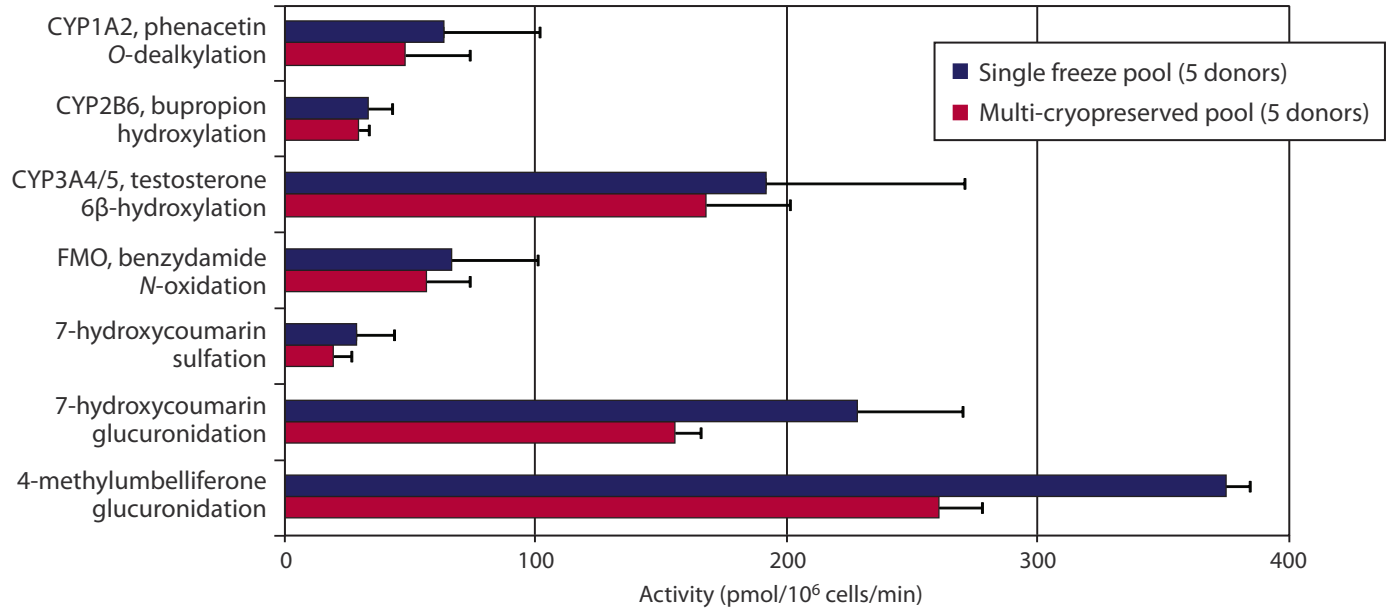
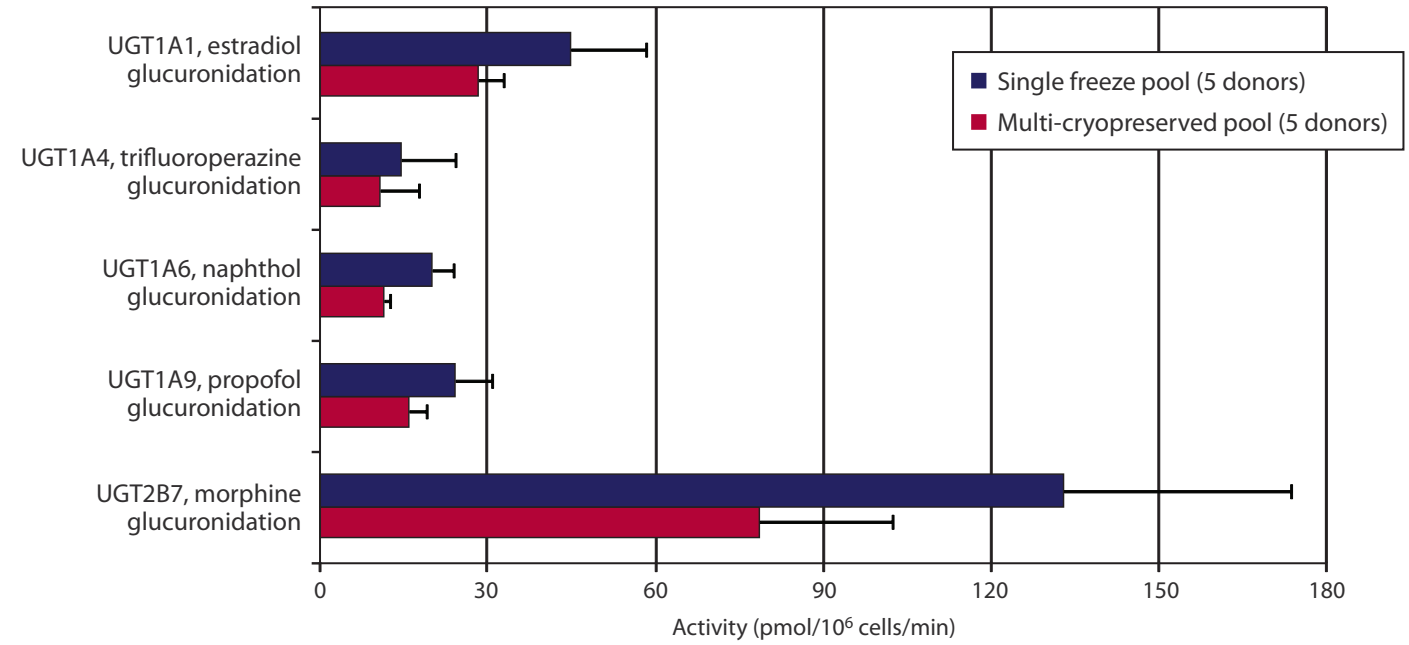


Figure 4.

The effects of multiple freezes on the activity of UDP-glucuronosyl transferases in human hepatocytes (pools contain cells from the same donors)



CONCLUSIONS

- A decline in both phase-1 and phase-2 drug-metabolizing enzyme activity is one of the consequences of preparing pooled human hepatocytes by the traditional pooling process that involves two freeze-thaw cycles.
- Repeated thawing and cryopreservation resulted in a more pronounced decline in SULT and UGT activity compared with CYP and FMO activity.
- Pooled human hepatocytes that had been cryopreserved only once (CryostaX™) had phase-1 and phase-2 enzyme activities that were up to 41% higher than those in multi-cryopreserved cells.

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