1521-009X/46/11/1608–1616$35.00
Drug Metabolism and Disposition

https://doi.org/10.1124/dmd.117.079657
Drug Metab Dispos 46:1608–1616, November 2018

Special Section – New Models in Drug Metabolism and Transport

A Novel In Vitro Experimental System for the Evaluation of Drug Metabolism: Cofactor-Supplemented Permeabilized Cryopreserved Human Hepatocytes (MetMax Cryopreserved Human Hepatocytes)[S]

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Received November 22, 2017; accepted January 22, 2018

ABSTRACT

We report here a novel experimental system, cryopreserved MetMax human hepatocytes (MMHHs), for in vitro drug metabolism studies. MMHHs consist of cofactor-supplemented permeabilized cryopreserved human hepatocytes. The use procedures for MMHHs are significantly simplified from that for conventional cryopreserved human hepatocytes (CCHHs): 1) storage at −80°C instead of in liquid nitrogen and 2) usage directly after thawing without centrifugation and microscopic evaluation of cell density and viability and cell density adjustment. In this study, we compared MMHHs and CCHHs in CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP2J2, monoamine oxidase A, aldehyde oxidase, flavin-containing monooxygenase, UDP-glucuronyl transferase, SULT, *N*-acetyltransferase 1, and acetaminophen glutathione (GSH) conjugation activities based on liquid chromatography–tandem mass spectrometry quantification of substrate metabolism. MMHHs were prepared from CCHHs consisting of hepatocytes pooled from 10 individual donors. The drug metabolizing enzyme activities of both CCHHs and MMHHs were cell concentration and time dependent, with specific activities of MMHHs ranging from 27.2% (carboxylesterase 2) to 234.2% (acetaminophen GSH conjugation) of that for CCHHs. As observed in CCHHs, sequential oxidation and conjugation was observed in MMHHs for coumarin, 7-ethoxycoumarin, and acetaminophen. 7-Hydroxycoumarin conjugation results showed that metabolic pathways in MMHHs could be selected via the choice of cofactors, with glucuronidation but not sulfation observed in the presence of UDP-glucuronic acid and not 3-phosphoadenosine-5-phosphosulfate, and vice versa. Results with noncytotoxic and cytotoxic concentrations of acetaminophen showed that drug metabolism was compromised in CCHHs but not in MMHHs. Our results suggest that the MMHHs system represents a convenient and robust in vitro experimental system for the evaluation of drug metabolism.

Introduction

Successful cryopreservation of human hepatocytes is a major reason for the routine application of this experimental system for preclinical assessment of human-specific drug properties including metabolic fate, drug-drug interactions, and drug toxicity (Li, 2007; Godoy et al., 2013; Jones et al., 2017b; Wood et al., 2017). Since the functional performance of cryopreserved human hepatocytes can be significantly compromised by cellular damage, use of cryopreserved human hepatocytes requires practitioners to have extensive experience with handling procedures, including thawing, centrifugation, resuspension, microscopic quantification of cell viability, adjustment of cell concentration, and the ultimate delivery of the cells into experimental vessels for experimentation. Furthermore, the use of cryopreserved hepatocytes requires liquid nitrogen cryogenic freezers for storage, which may not be readily available in laboratories where in vitro drug metabolism studies are routinely performed.

Before the development of cryopreserved hepatocyte technologies, hepatic subcellular fractions such as human liver microsomes and postmitochondrial supernatants (S9 or S10), were used exclusively in drug metabolism studies (Iwatsubo et al., 1997; Gombar et al., 2003). These subcellular systems, especially human liver microsomes, continue to be practical and useful experimental systems in pharmaceutical industrial laboratories, including screening of new chemical entities for metabolic stability (Halladay et al., 2007; Choi et al., 2015), estimation of in vivo hepatic clearance (Obach, 2011; Chen et al., 2017), evaluation of cytochrome P450–related drug properties (Dinger et al., 2014), and UDPGT–mediated drug metabolism (Walsky et al., 2012; Joo et al., 2014). Compared with cryopreserved hepatocytes, the use of subcellular fractions requires relatively simple application procedures, and they are relatively robust and are not readily subjected to functional damages due to handling. A major drawback of the use of subcellular fractions is the incompleteness

https://.doi.org/10.1124/dmd.117.079657.
[S]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: CCHH, conventional cryopreserved human hepatocyte; DME, drug metabolizing enzyme; LC/MS-MS, liquid chromatography–tandem mass spectrometry; MMHH, MetMax human hepatocyte; PAPS, 3-phosphoadenosine-5-phosphosulfate; SULT, sulfotransferase; UDPGA, UDP-glucuronic acid.

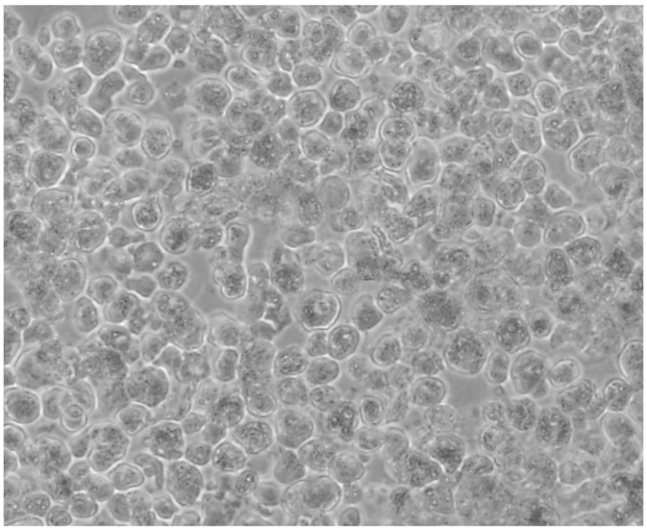

Fig. 1. Phase-contrast microscopy of MMHHs. The hepatocytes were plated into a 24-well plate and allowed to settle for approximately 10 minutes. The cells exhibited intact plasma membranes similar to CCHHs.

of the drug metabolizing enzyme (DME) pathways, thus the data obtained may not allow accurate assessment of in vivo hepatic metabolic fate. In contrast, cryopreserved hepatocytes contain complete, undisrupted DMEs and cofactors, and are considered to be the gold standard for the evaluation of in vitro human drug metabolism (Fabre et al., 1990; Ulrich et al., 1995; Hewitt et al., 2007). For this reason, despite the relatively more complicated experimental procedures, cryopreserved human hepatocytes have been applied in studies routinely performed with subcellular fractions, including hepatic clearance (Di et al., 2013; Peng et al., 2016) and cytochrome P450 inhibition (Doshi and Li, 2011; Li and Doshi, 2011; Kazmi et al., 2015), to obtain data that are more likely to reflect in vivo events.

It would be desirable to have an experimental system that combines the completeness of the DME pathways in hepatocytes, and the robustness and simplicity of the application procedures of subcellular fractions. Toward this goal we have developed a novel experimental system that uses cryopreserved MetMax human hepatocytes (MMHHs). MMHHs are permeabilized cryopreserved human hepatocytes supplemented with key DME cofactors. Similar to subcellular fractions such as human liver microsomes, MMHHs can be stored at −80°C in contrast to conventional cryopreserved human hepatocytes (CCHHs) which require liquid nitrogen storage. Furthermore, similar to subcellular fractions, MMHHs can be used directly after thawing without centrifugation and microscopic evaluation of viability and cell concentration as required for CCHHs.

We report here a comparison of MMHHs and CCHHs in phase 1 oxidation and phase 2 conjugation DME activities as part of our ongoing research program to evaluate the applicability of this novel experimental system to study drug metabolism.

Materials and Methods

Chemicals. Dextrorphan tartrate, diclofenac sodium salt, 4-hydroxydiclofenac, *S*-mephenytoin, 4-hydroxyquinoline, paclitaxel, and testosterone were purchased from Cayman Chemical (Ann Arbor, MI). 7-Hydroxycoumarin was purchased from Chem Service (West Chester, PA). Benzydamine *N*-oxide, 7-hydroxycoumarin sulfate potassium salt, kynuramine hydrobromide, and *N*-acetyl sulfamethazine were obtained from Santa Cruz Biotechnology (Dallas, TX). 4-Acetamidobenzoic acid, *p*-acetamidophenyl β-D-glucuronide sodium salt, 4-aminobenzoic acid, benzydamine hydrochloride, chlorzoxazone, coumarin, dextromethorphan hydrobromide, 6β-hydroxytestosterone, 7-hydroxycoumarin β-D-glucuronide sodium salt, 7-ethoxycoumarin, paracetamol sulfate potassium, phenacetin, and sulfamethazine were purchased from Sigma Aldrich (St. Louis, MO). Carbazeran, 4-hydroxycarbazeran, 6-hydroxychlorzoxazone, 6α-hydroxypaclitaxel, acetaminophen glutathione disodium salt, midazolam, 1′-hydroxymidazolam, and 4-hydroxy-*S*-mephenytoin were obtained from Toronto Research Chemicals (Toronto, Canada). All other DME substrates were obtained from Sigma Aldrich.

CCHH and MMHH Preparations. Cryopreserved human hepatocytes previously prepared from 10 individual donors were used in the preparation of

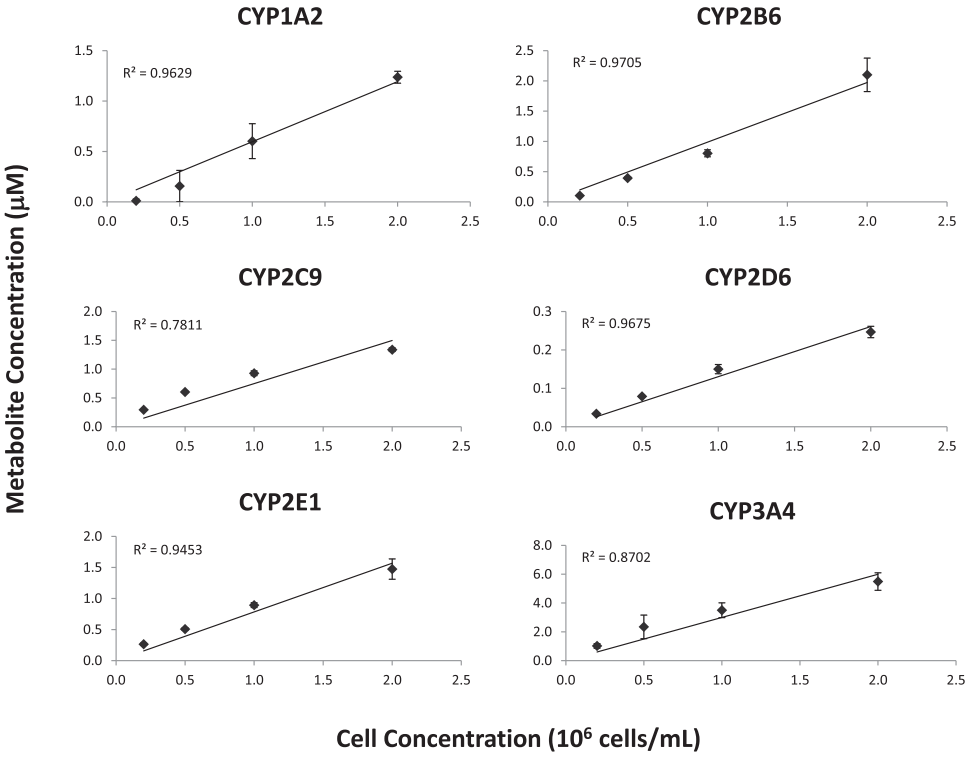

Fig. 2. Metabolite formation as a function of cell concentration in MMHHs. Cytochrome P450 isoform-selective pathways evaluated were CYP1A2 (phenacetin *O*-deethylation), CYP2B6 (bupropion hydroxylation), CYP2C9 (diclofenac 4′-hydroxylation), CYP2D6 (dextromethorphan *O*-demethylation), CYP2E1 (chlorzoxazone 6-hydroxylation), and CYP3A4 (midazolam 1′-hydroxylation). The cell concentrations evaluated were 0.25, 0.5, 1, and 2×10^6 cells/ml. (Error bars: S.D. of triplicate determinations).

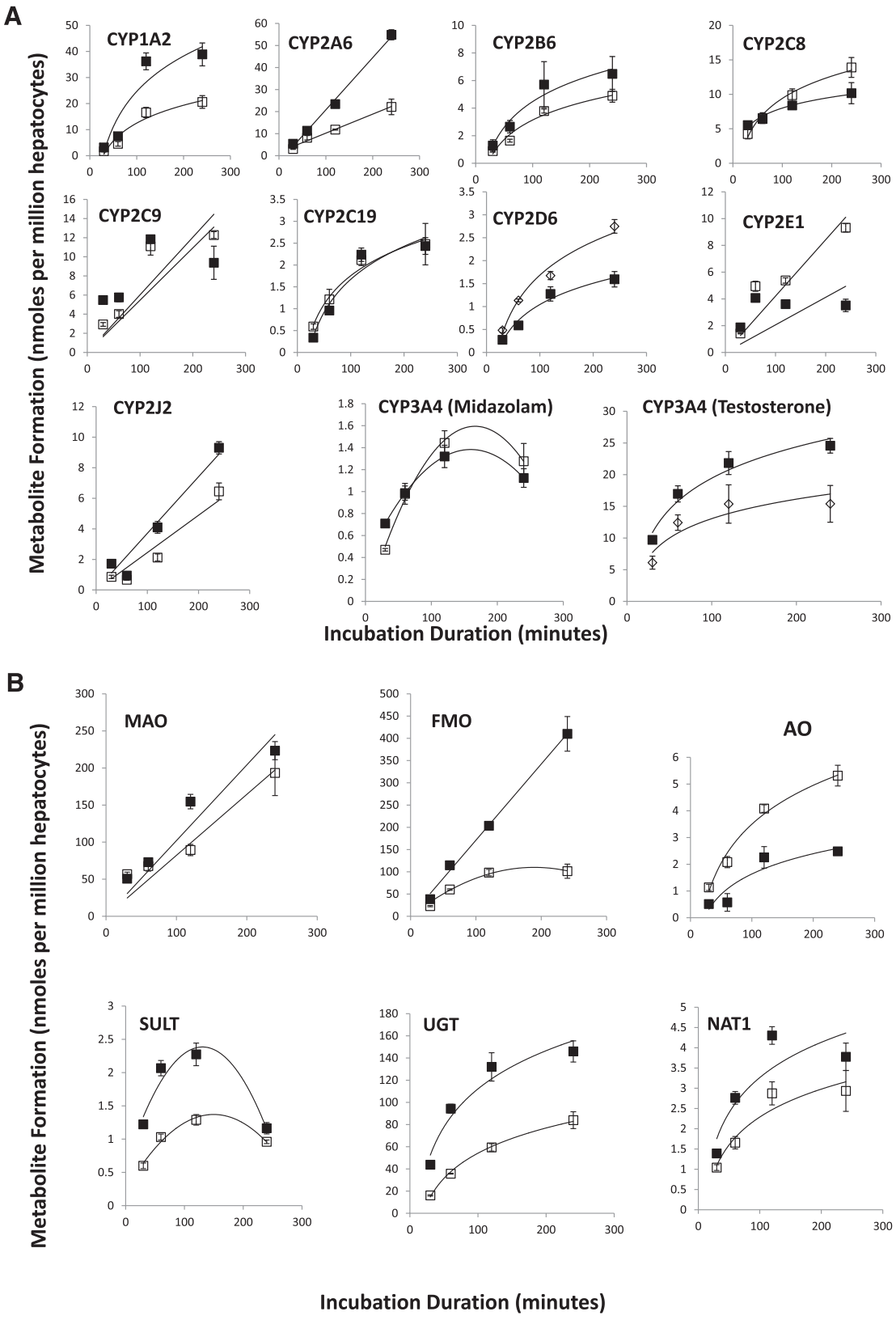

Fig. 3. Metabolite formation as a function of incubation durations in CCHHs (open squares) and MMHHs (filled squares). The time durations evaluated were 30, 60, 120, and 240 minutes. The cell concentration used was 1×10^6 cells/ml. Cytochrome P450 (A) and non-cytochrome P450 (B) pathway-selective substrate metabolism are shown. (Error bars: S.D. of triplicate determinations).

the pooled donor CCHHs and MMHHs. Pooled CCHHs were prepared by thawing of cryopreserved human hepatocytes from individual lots, pooling of the individual lots, and recryopreserving the pooled multiple donor hepatocytes at a cell density of approximately 5×10^6 cells/ml using a patented technology (Li, 2015a). For the preparation of CCHHs, hepatocytes from each donor were isolated via collagen digestion of human livers and cryopreserved in liquid

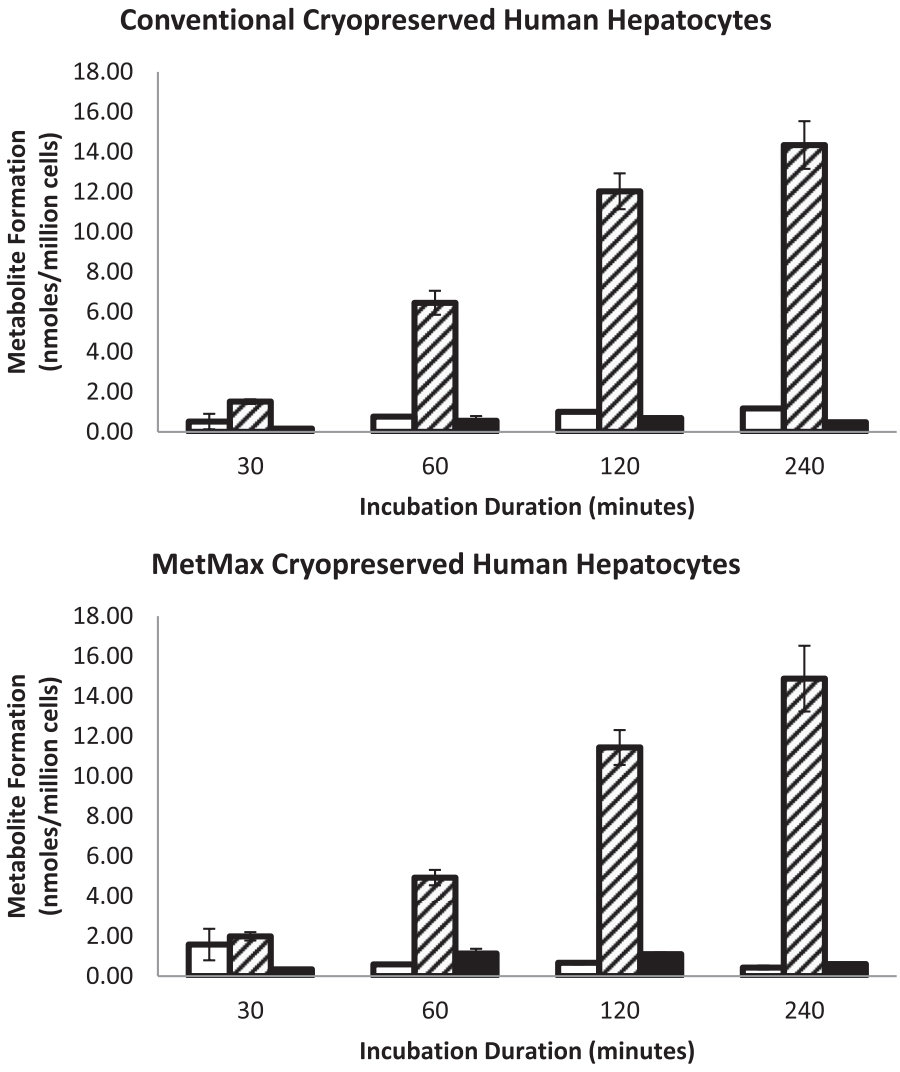

Fig. 4. A comparison of CCHHs (top) and MMHHs (bottom) in the formation of secondary metabolites from 7-ethoxycoumarin. The metabolites quantified were 7-hydroxycoumarin (7HC; open bars) and its glucuronide (7HCG; shaded bars) and sulfate (7HCS; filled bars) conjugates. The incubation durations were 30, 60, 120, and 240 minutes. The cell concentration used was 1×10^6 cells/ml. (Error bars: S.D. of triplicate determinations).

nitrogen (Li, 2017). Pooled MMHHs were prepared from the same lot of CCHHs used in the study via thawing and recovery of the CCHH lot, recovery of the hepatocytes followed by permeabilization of the cells with a proprietary technology (patent pending), and re-cryopreserved at a cell density of 2×10^6 cells/ml. The human livers used for hepatocyte isolation were obtained from the International Institute for the Advancement of Medicine (Edison, NJ) and the National Disease Research Exchange (Philadelphia, PA). The demographics of the 10 donors are given in Supplemental Table 1.

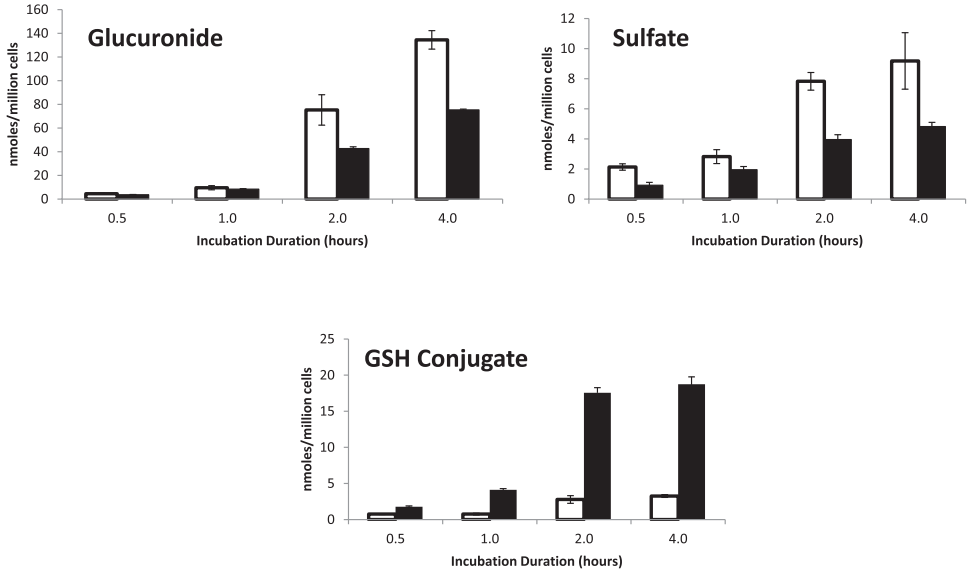

Fig. 5. Time-dependent formation of acetaminophen conjugates in CCHHs (open bars) and MMHHs (filled bars) at the noncytotoxic concentration of 10 mM. The metabolites quantified were glucuronide (A), sulfate (B), and glutathione (GSH) (C) conjugates. (Error bars: S.D. of triplicate determinations).

TABLE 1

Comparison of CCHHs in the metabolism of acetaminophen at the noncytotoxic concentration of 10 mM

Mean and S.D. of triplicate samples are shown.

Incubation Time	Total Metabolite Formation in CCHHs (nmoles per Million Hepatocytes)					
	APAP Glucuronide		APAP Sulfate		APAP Glutathione	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
min						
30	4.51	0.46	2.13	0.21	0.76	0.06
60	9.57	1.76	2.82	0.46	0.76	0.12
120	75.26	12.84	7.83	0.59	2.78	0.53
240	134.46	7.78	9.18	1.88	3.26	0.20

Incubation with DME Substrates. All incubations were performed in 96-well cell culture plates (Falcon; obtained from VWR Inc., Bridgeport, NJ). For CCHHs, the hepatocytes were thawed in a 37°C water bath, with 1 ml of the thawed suspension added to 50 ml of universal cryopreservation recovery medium (In Vitro ADMET Laboratories Inc., Columbia, MD) in a 50 ml conical cell culture tube (Falcon; obtained from VWR Inc.) and centrifuged at 100*g* for 10 minutes. The cell pellet from each conical tube was resuspended in 4 ml of HQM Hepatocyte Incubation Medium (In Vitro ADMET Laboratories Inc.) for viability determination (Trypan blue exclusion) and cell concentration determination. The cell suspension was then adjusted with HQM to 2× the final cell density. For MMHHs, the hepatocytes were thawed and used directly without centrifugation or viability/cell concentration determination. All DME substrates were prepared in HQM at 2× the final concentrations and added at a volume of 50 μl per well in a 96-well cell culture plate. The CCHHs, MMHHs, and substrate plates were prewarmed to 37°C for 15 minutes in a cell culture incubator before initiation of the incubation by pipetting 50 μl of CCHHs or MMHHs into each well of the 96-well plates containing the substrates and returned to the cell culture incubator without shaking for the desired durations (30, 60, 90, 120, and 240 minutes). After incubation, 100 μl of acetonitrile was added into each well to terminate metabolism. After termination, the plates were stored at −80°C in a freezer for later liquid chromatography–tandem mass spectrometry (LC-MS/MS) quantification of metabolite formation. All DME activity studies were performed at a final cell density of 1×10^6 cells/ml except for the evaluation of cell density versus metabolite formation where CCHHs and MMHHs were first prepared at cell concentrations of 4×10^6 cells per ml, followed by dilution to 2×10^6, 1×10^6, and 0.5×10^6 per ml before addition to the substrate plates, thereby yielding final concentrations of 2×10^6, 1×10^6, 0.5×10^6, and 0.25×10^6 ml.

LC-MS/MS Analysis. Upon thawing, an aliquot of 200 μl of each sample was transferred from each well into a labeled 96-well plate followed by addition of 100 μl of acetonitrile solution containing the internal standard Tolbutamide (250 nM) and mixing. All samples were centrifuged at 3500 rpm for 5 minutes. An aliquot of 100 μl of supernatant from each was transferred to a 96-well plate and diluted with 200 μl of deionized water with mixing before LC-MS/MS analysis. CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 (midazolam 1′-hydroxylation), CYP3A4 (testosterone 6β-hydroxylation), 7-ethoxycoumarin *O*-deethylation, UDP-glucuronyl transferase, sulfotransferase (SULT), glutathione *S*-transferase, flavin-containing monooxygenase, monoamine oxidase, aldehyde oxidase and, *N*-acetyltransferase 1 metabolites were quantified by an API 4000 QTRAP mass spectrometer with an electrospray ionization source (AB SCIEX, Framingham, MA) connected to an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA) using the LC-MS/MS multiple reactions monitoring mode and monitoring the mass transitions (parent-to-daughter ion) (Supplemental Table 2). A Zorbax Eclipse Plus C18 column (4.6 × 75 mm i.d., 3.5 μm; Agilent Technologies) at a flow rate of 1 ml/min was used for chromatography separation. The mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The gradient for positive ion mode operation was programmed as: 0–2.5 minutes, increase B from 5% to 95%; 2.5–3.5 minutes, 95% B; 3.5–3.6 minutes, decrease B to 5%; run time, 5 minutes. The gradient program for the negative ion mode was: 0–3 minutes, increase B from 5% to 95%; 3–4 minutes, 95% B; 4–4.2 minutes, decrease B to 5%; run time 6 minutes. Data acquisition and data procession were performed with the software Analyst 1.6.2 (AB SCIEX).

Data Analysis. Data are presented as mean and S.D. of triplicate incubations derived using the Microsoft Excel 6.0 software. Statistical analysis was performed using the Student's *t* test with the Microsoft Excel 6.0 software, with the probability of $P < 0.05$ to be considered statistically significant. Specific activity (picomoles per minute per million hepatocytes) of each DME pathway was determined by dividing the total metabolite formed by the incubation time and normalized to cell concentration.

Results

Cell Morphology

When placed into a cell culture plate, the hepatocytes from the MMHHs appeared as intact cells, which rapidly settled on the bottom of the wells. Phase contrast photomicroscopy showed that the MMHHs were intact (not lysed) cells with distinct cell membranes (Fig. 1), similar to the CCHHs (data not shown). Trypan blue exclusion evaluation, however, showed that 100% of the cells would include the dye (data not shown), demonstrating that their plasma membranes were permeabilized.

Cell Concentration versus Metabolite Formation

MMHHs were incubated for 30 minutes with various cytochrome P450 isoform-selective substrates at 0.25, 0.5, 1.0, and 2.0×10^6 cells/ml.

TABLE 2

Comparison of MMHHs in the metabolism of acetaminophen at the noncytotoxic concentration of 10 mM

Mean and S.D. of triplicate samples are shown.

Incubation Time	Total Metabolite Formation in MMHHs (nmoles per Million Hepatocytes)					
	APAP Glucuronide		APAP Sulfate		APAP Glutathione	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
min						
30	3.62	0.29	0.92	0.19	1.78	0.10
60	8.40	0.41	1.95	0.21	4.12	0.17
120	42.66	1.46	3.95	0.32	17.56	0.70
240	75.32	0.68	4.82	0.28	18.74	1.01

TABLE 3

Comparison of CCHHs and MMHHs in acetaminophen metabolism at the lesser cytotoxic concentration of 100 mM and the cytotoxic concentration of 200 mM

Mean and S.D. of triplicate samples are shown.

Substrate Concentration (μM)	Marker Metabolite	Total Metabolite Formation (pmol per Million Hepatocytes)			
		CCHH		MMHH	
		Average	S.D.	Average	S.D.
mM					
100	Acetaminophen Glutathione	206.50	3.91	856.72	71.28
200	Acetaminophen Glutathione	99.81	7.49	676.70	54.43
100	Acetaminophen Glucuronide	572.55	27.12	1298.77	64.29
200	Acetaminophen Glucuronide	170.28	16.38	1361.84	83.36
100	Acetaminophen Sulfate	105.55	3.36	150.22	6.68
200	Acetaminophen Sulfate	52.58	1.40	156.36	11.86

Metabolite formation was found to increase with cell concentration for all of the substrates evaluated (Fig. 2). From the results, 1.0×10^6 cells/ml was chosen as the cell concentration for subsequent studies.

Incubation Duration versus Metabolite Formation

MMHHs and CCHHs were incubated with various pathway-selective substrates for 30, 60, 120, and 240 minutes. In general, similar relationships between metabolite formation and incubation duration were observed for the two hepatocyte systems, with linear or curve-linear time course (Fig. 3).

Secondary Metabolite Formation.

1. 7-Ethoxycoumarin metabolism: MMHHs and CCHHs were incubated with 7-ethoxycoumarin. 7-Ethoxycoumarin is first oxidized to 7-hydroxycoumarin, which in turn is subjected to metabolism by UDPGT and SULT to 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate, respectively. Similar metabolite profiles were observed for MMHHs and CCHHs, with 7-hydroxycoumarin found to be extensively conjugated to the glucuronide and sulfate conjugates (Fig. 4).
2. Acetaminophen metabolism: Acetaminophen metabolism by MMHHs and CCHHs was evaluated at the noncytotoxic concentration of 10 mM at incubation durations of 30, 60, 120, and 240 minutes (Fig. 5; Tables 1 and 2). Time-dependent formation of glucuronide, sulfate, and glutathione conjugates was observed in both MMHHs and CCHHs. The glutathione conjugate of acetaminophen formed in MMHHs ranged from approximately 200% at 30 minutes to approximately 600% of that formed in CCHHs at 240 minutes. In a separate study, MMHHs and CCHHs were incubated with 100 and 200 mM of acetaminophen (Table 3). The results confirmed the observation made with 10 mM acetaminophen, in that higher levels of acetaminophen glutathione were formed in MMHHs. Significant reduction of acetaminophen metabolism was observed at 200 mM in CCHHs, which was contributed to the cytotoxic effects at this drug concentration. No apparent reduction in metabolite formation was observed in MMHHs at 200 mM of acetaminophen.

Cofactor-Mediated Selection of Metabolic Pathways in MMHHs

MMHHs were prepared without cofactors and then incubated with coumarin with the following cofactor compositions: 1) NADPH [without UDP-glucuronic acid (UDPGA) or 3-phosphoadenosine-5-phosphosulfate (PAPS)]; 2) NADPH plus UDPGA (without PAPS); and 3) NADPH plus PAPS (without UDPGA). 7-Hydroxycoumarin was formed under all three cofactor compositions. 7-Hydroxy and 7-glucuronide (but not the 7-sulfate metabolites) were observed under condition 2; 7-hydroxylation and 7-sulfate (but not the 7-glucuronide metabolites) were observed under condition 3 (Fig. 6).

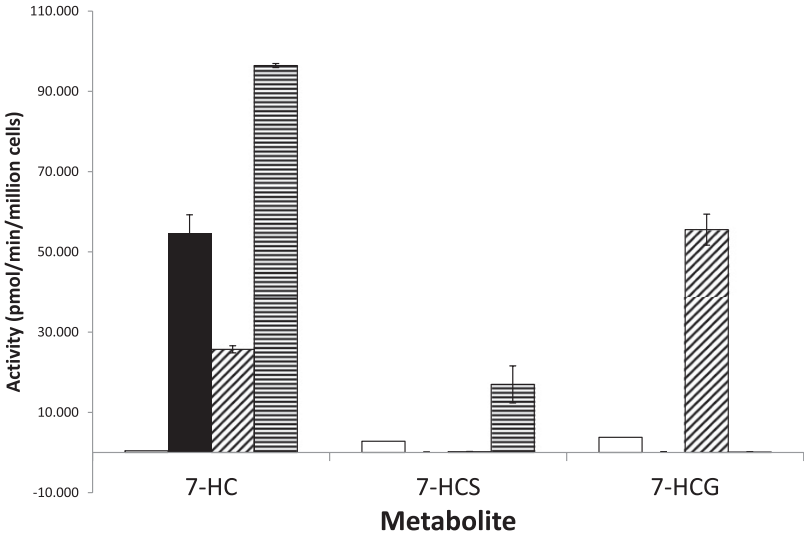

Fig. 6. Effects of cofactor composition on metabolite formation from coumarin in MMHHs. MMHHs were incubated with coumarin under four cofactor conditions: 1) no cofactor supplements (open bars), 2) NADPH only (filled bars), 3) NADPH and UDPGA (slanted shaded bars), and 4) NADPH and PAPS (horizontally shaded bars). (Error bars: S.D. of triplicate determinations).

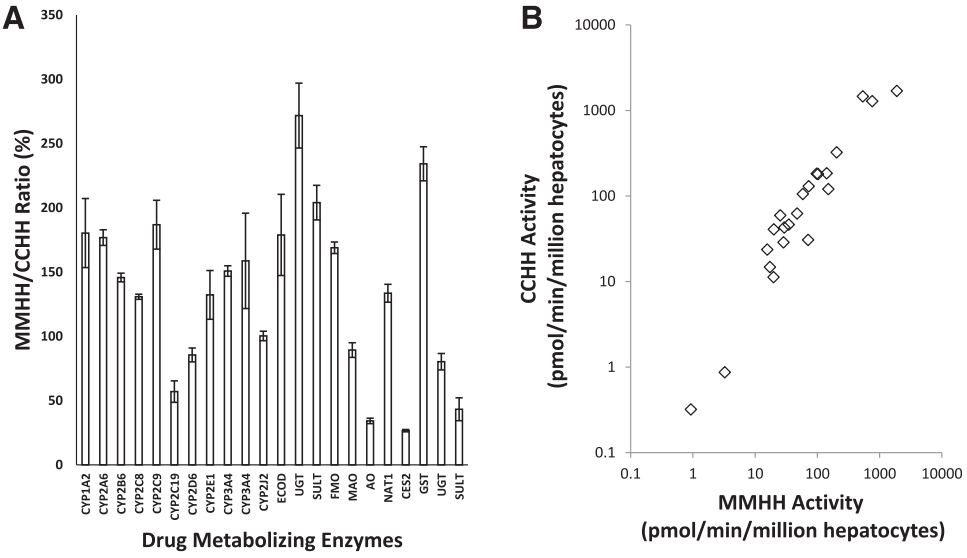

Fig. 7. A comparison of CCHHs and MMHHs in DME activities: DME activities of MMHHs expressed as percentages of that for CCHHs (A), calculated using the following equation: [Activity (MMHH)/Activity (CCHH)] × 100%, and correlation of MMHH and CCHH activities (B). Each data point in (B) represents activity data for each of the DMEs identified in (A). Numerical data are presented in Supplemental Table 3. (Error bars: S.D. of triplicate determinations).

Activity Comparison between MMHHs and CCHHs

A comparison of the rates of metabolism of the various drug metabolism enzyme-selective substrates in MMHHs is shown in Fig. 7, with MMHHs activities expressed as percentages of that for CCHHs (Fig. 7A) and MMHH activities plotted versus CCHH activities (Fig. 7B). Numerical data for DME activities for MMHHs and CCHHs are presented in Supplemental Table 3. The results demonstrate that MMHHs were active in all of the enzyme pathways evaluated, with activities in general similar to that observed in CCHHs.

Discussion

The major focus of our laboratory is the development and application of in vitro human-based experimental systems to allow accurate assessment of human drug properties. Our laboratory was one of the first to demonstrate successful cryopreservation of human hepatocytes (Loretz et al., 1989; Li, 2015b), and more recently human enterocytes (Ho et al., 2017), to retain viability and drug metabolism activities. We report here the characterization of a novel experimental system, namely, the MMHH experimental system —permeabilized, cofactor-supplemented cryopreserved human hepatocytes. MMHHs were developed in our laboratory to be an in vitro tool for drug metabolism studies with the desirable properties of both hepatocytes and subcellular fractions. As permeabilized, not lysed, cells, the MMHHs retain cellular integrity and subcellular structures and thereby contain complete enzyme pathways akin to CCHHs. Since the DME activities of MMHHs are driven by the cofactors—and therefore are not affected by viability—MMHHs, like subcellular fractions, are not subjected to handling damages, which can drastically affect the functions of CCHHs. The presence of all of the cellular organelles and the associated DMEs are the reasons that CCHHs are considered to be the gold standard in vitro drug metabolism system. These are also the attributes of MMHHs, but not subcellular fractions, which contain only a portion of the organelles, and therefore lack the DMEs associated with the missing organelles (Table 4).

A comparison of MMHHs and CCHHs in the activities of key DME pathways showed no apparent deficiencies in these activities in MMHHs. Both experimental systems were competent in phase 1 oxidation and phase 2 conjugation (including sequential events with oxidation followed by conjugation of the oxidative metabolites), as demonstrated using 7-ethoxycoumarin and acetaminophen as substrates. Similar cell density dependency (from 0.25 to 2 million cells/ml), time course (up to 4 hours of incubation), and overall specific activities were observed for MMHHs and CCHHs in key phase 1 oxidation and phase 2 conjugation DME activities, with MMHHs competent in all pathways evaluated. Activities observed in MMHHs in general are within 2-fold of that for CCHHs (Fig. 7). Activities observed in MMHHs as percentage of that in CCHHs based on the rate of metabolite formation after an incubation duration of 30 minutes are: UDP-glucuronosyltransferase (271%), glutathione *S*-transferase (234%), SULT (204%), CYP1A2 (180%), CYP2A6 (177%), CYP2B6 (146%), CYP2C8 (131%), CYP2C9 (187%), CYP2E1 (132%), CYP3A4 (midazolam 1′hydroxylation: (151%); testosterone 6β-hydroxylation: (159%); CYP2J2 (100%); 7-ethoxycoumarin *O*-deethylation (179%); flavin-containing monooxygenase (169%); *N*-acetyltransferase 1 (134%); CYP2C19 (57%); CYP2D6 (86%); monoamine oxidase (89%); aldehyde oxidase (34%); carboxylesterase 2 (27%); acetaminophen glucuronidation (80%); and acetaminophen sulfation (43%).

Our results, therefore, show that MMHHs are active in all DME pathways evaluated with activities comparable to that for CCHHs, thereby suggesting that MMHHs can be used for drug metabolism studies that routinely employ CCHHs. The advantages of MMHHs over CCHHs include storage at −80°C instead of liquid nitrogen to retain viability and functions, direct use after thawing without a need for the time-consuming procedures of centrifugation cell recovery microscopy

TABLE 4

Organelle compositions of the various in vitro drug metabolism experimental systems

MMHHs, CCHHs, human liver microsomes (HLM) and postmitochondrial supernatant (PMS). The "X" indicates the presence of the organelles in the specified experimental systems. Organelle contents in various in vitro systems have been previously reviewed (Brandon et al., 2003).

Organelle	Experimental System			
	CCHH	MMHH	HLM	PMS
Plasma membranes	X	X		
Cytosol	X	X		X
Mitochondria	X	X		
Lysosomes	X	X		
Endoplasmic reticulum	X	X	X	X
Nucleus	X	X		

TABLE 5

Comparison of the procedures for drug metabolism studies with CCHHs and MMHHs

The time requirement for CCHHs will increase significantly if multiple individual lots (e.g., comparison of different hepatocyte donors) are used since each lot will require efforts in cell recovery, viability and cell concentration determination, and preparation of cell suspensions at the desired cell density. These additional steps are not required for MMHHs.

Procedure	CCHH	MMHH
Vial retrieval of from storage	From liquid nitrogen storage (3–5 minutes)	From −80°C storage (3–5 minutes)
Thawing of the cryopreserved hepatocytes in a 37°C water bath	1.5–2 minutes	1.5–2 minutes
Addition of the thawed hepatocytes to recovery medium followed by centrifugation for 10 minutes for cell recovery	10–15 minutes	Not required
Microscopic examination of thawed hepatocytes for the quantification of viability and cell concentration	10–15 minutes	Not required
Adjustment of cell suspension to contain 2× the final desired cell density	5 minutes	Not required
Addition to vessels containing 2× test compounds[a]		
Total time from freezer to incubation	30.5–45 minutes	7–10 minutes

[a]For example, addition of 50 μl of hepatocytes to a 96-well plate with 50 μl of test articles at 2× the final concentration, followed by incubation for the desired time duration at 37°C: 1–3 minutes.

examination of cell viability, and cell concentration determination. We have estimated that the time required from thawing to incubation for drug metabolism evaluation is approximately 7–10 minutes for MMHHs versus 30.5–45 minutes for CCHHs (Table 5). Furthermore, unlike CCHHs in which cofactor compositions cannot be readily controlled, specific metabolic pathways can be evaluated with MMHHs based on the composition of the added cofactor mixtures. Selection of metabolic pathways based on cofactor compositions in MMHHs is demonstrated with coumarin metabolism where the use of UDPGA in the absence of PAPS led to formation of coumarin 7-glucuronide with no sulfate conjugate and vice versa.

Another potential advantage of MMHHs over CCHHs is that drug metabolism can be evaluated at cytotoxic drug concentrations. This was demonstrated with acetaminophen where drug metabolism activity is significantly decreased by drug cytotoxicity in CCHHs but not in MMHHs. This property of MMHHs should be useful in the evaluation of metabolic fate of highly toxic drugs such as anticancer therapeutics.

Our experimental findings suggest that MMHHs may represent a convenient hepatocyte-based experimental system that can be used for drug metabolism studies. Studies have been initiated in our laboratory on the application of MMHHs in the evaluation of metabolic stability and metabolite profiling, as well as reversible and mechanism-based DME inhibition. It needs to be emphasized, however, that CCHHs remain valuable and cannot be replaced by MMHHs in the evaluation of a number of key drug properties. These include uptake and efflux transport (Shitara et al., 2003; Bi et al., 2017; Izumi et al., 2017; Zhang et al., 2017), which require intact plasma membranes; enzyme induction (Fahmi et al., 2008; Zhou et al., 2011; Jones et al., 2017a), which requires RNA and protein synthesis; and in vitro hepatotoxicity (Zhang et al., 2016; Andersson, 2017; Proctor et al., 2017), which requires intact, viable hepatocytes. We believe that MMHHs should be complementary to current in vitro systems for the evaluation of human drug metabolism.

Acknowledgments

We gratefully acknowledge the technical assistance of Nick Ring in hepatocyte incubations and analytical chemistry sample preparation.

Authorship Contributions

Participated in research design: Li, Ho, Amaral, Loretz.

Conducted experiments: Ho, Amaral, Loretz.

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