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Title page

A novel in vitro experimental system for the evaluation of drug metabolism: Cofactor-supplemented permeabilized cryopreserved human hepatocytes (MetMax™ Cryopreserved Human Hepatocytes)

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Running Title Page

Running title: MetMax™ Cryopreserved Human Hepatocytes

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2. Introduction: 582
3. Discussion: 827

Non-standard abbreviations: HQM (Hepatocyte Incubation Medium); CCHH (conventional cryopreserved human hepatocytes); MMHH (MetMax™ cryopreserved human hepatocytes)

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Abstract

We report here a novel experimental system – MetMax™ cryopreserved human hepatocytes (MMHH), for in vitro drug metabolism studies. MMHH consist of cofactor-supplemented permeabilized cryopreserved human hepatocytes. The use procedures for MMHH are significantly simplified from that for conventional cryopreserved human hepatocytes (CCHH): 1. Storage at -80^{o} C instead of in liquid nitrogen; 2. Usage directly after thawing without centrifugation and microscopic evaluation of cell density and viability and cell density adjustment. In this study, we compared MMHH and CCHH in CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, CYP2J2, monoamine oxidase A, aldehyde oxidase, flavin-containing monooxygenase, UGT, SULT, NAT-1, and acetaminophen glutathione (GSH) conjugation activities based on LC/MS-MS quantification of substrate metabolism. MMHH were prepared from CCHH consisted of hepatocytes pooled from 10 individual donors. The DME activities of both CCHH and MMHH were cell concentration and time-dependent, with specific activities of MMHH ranged from 27.2% (CES2) to 234.2% (acetaminophen GSH conjugation) of that for CCHH. As observed in CCHH, sequential oxidation and conjugation was observed in MMHH for coumarin, 7-ethoxycoumarin, and acetaminophen. 7-hydroxycoumarin conjugation results showed that metabolic pathways in MMHH could be selected via the choice of cofactors, with glucuronidation but not sulfation observed in the presence of UDPGA and not PAPS and vice versa. Results with non-cytotoxic and cytotoxic concentrations of acetaminophen showed that drug metabolism was compromised in CCHH but not in MMHH. Our results suggest that the MMHH system represents a convenient and robust in vitro experimental system for the evaluation of drug metabolism.

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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, Hewitt et al. 2013; Jones, Srivastava et al. 2017; Wood, Houston et al. 2017). As the functional performance of the cryopreserved human hepatocytes can be significantly compromised by cellular damage, use of cryopreserved human hepatocytes requires the 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 the 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 (HLM) and post-mitochondrial supernatants (S9 or S10), were used exclusively in drug metabolism studies(Iwatsubo, Suzuki et al. 1997; Gombar, Silver et al. 2003). These subcellular systems, especially HLM, remain to be practical and useful experimental systems in pharmaceutical industrial laboratories including screening of new chemical entities for metabolic stability (Halladay, Wong et al. 2007; Choi, Furimsky et al. 2015), estimation of in vivo hepatic clearance (Obach 2011; Chen, Prieto Garcia et al. 2017), evaluation of P450-related drug properties (Dinger, Meyer et al. 2014), and UGT-mediated drug metabolism (Walsky, Bauman et al. 2012; Joo, Lee et al. 2014). As compared to cryopreserved hepatocytes, the use of subcellular fractions requires relatively simple application procedures, and are relative robust and are not readily subjected to functional damages due to handling. A major drawback of the use of subcellular fractions is the incompleteness of the drug

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metabolizing enzyme pathways, thus the data obtained may not allow an accurate assessment of in vivo hepatic metabolic fates. In contrast, cryopreserved hepatocytes contain complete, undisrupted drug metabolizing enzymes and cofactors, and are considered the "gold standard" for the evaluation of in vitro human drug metabolism (Fabre, Combalbert et al. 1990; Ulrich, Bacon et al. 1995; Hewitt, Lechon et al. 2007). For this reason, in spite of the relatively more complicated experimental procedures, cryopreserved human hepatocytes have been applied in studies routinely performed with subcellular fractions including hepatic clearance (Di, Atkinson et al. 2013; Peng, Doshi et al. 2016) and P450 inhibition (Doshi and Li 2011; Li and Doshi 2011; Kazmi, Barbara et al. 2015) in order 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 drug metabolizing enzyme pathways in hepatocytes, and the robustness and simplicity of the application procedures of subcellular fractions. Towards this goal we have developed a novel experimental system, the MetMax™ human hepatocytes (MMHH). MMHH are permeabilized cryopreserved human hepatocytes supplemented with key drug metabolizing enzyme cofactors. Similar to subcellular fractions such as human liver microsomes, MMHH can be stored at -80^{o} C in contrast to the conventional cryopreserved human hepatocytes (CCHH) which require liquid nitrogen storage. Furthermore, similar to subcellular fractions, MMHH can be used directly after thawing without centrifugation and microscopic evaluation of viability and cell concentration as required for CCHH.

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

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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, Pennsylvania). Benzydamine N-oxide, 7-hydroxycoumarin sulfate potassium salt, kynuramine hydrobromide, and N-acetyl sulfamethazine were obtained from Santa Cruz Biotechnology (Dallas, Texas). 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 drug metabolizing enzyme substrates were obtained from Sigma Aldrich (St. Louis, MO).

CCHH and MMHH preparations. Cryopreserved human hepatocytes previously prepared from 10 individual donors were used in the preparation of the pooled donor CCHH and MMHH. Pooled CCHH were prepared by thawing of cryopreserved human hepatocytes from individual lots, pooling of the individual lots, and re-cryopreserving the pooled multiple donor hepatocytes at a cell density of approximately 5 x 10^6 cells/mL using a patented technology (US Patent Number 9078430 B2). For the preparation of CCHH, hepatocytes from each donor were isolated via collagen digestion of human livers and cryopreserved in liquid nitrogen (1). Pooled MMHH was prepared from the same lot CCHH 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 x 10^6 cells/mL. The human livers used for hepatocyte isolation were obtained from

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International Institute for the Advancement of Medicine (IIAM, Edison, NJ), and National Disease Research Exchange (NDRI, Philadelphia, PA). The demographics of the 10 donors are shown in Supplement material section; Table 1.

Incubation with DME substrates. All incubations were performed in 96-well cell culture plates (Falcon, obtained from VWR Inc.). For CCHH, 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 (UCRM, In Vitro ADMET Laboratories Inc. (IVAL), Columbia, MD) in a 50 mL conical cell culture tube (Falcon, obtained from VWR Inc.) and centrifuged at 100 x g for 10 min. The cell pellet from each conical tube was resuspended in 4 mL of Hepatocyte Incubation Medium (HQM, IVAL) for viability determination (Trypan blue exclusion) and cell concentration determination. The cell suspension was then adjusted with HQM to 2X of the final cell density. For MMHH, the hepatocytes were thawed and used directly without centrifugation or viability/cell concentration determination. All DME substrates were prepared in HQM at 2X of the final concentrations and added at a volume of 50 μL per well in a 96-well cell culture plate. CCHH, MMHH and substrate plates were pre-warmed to 37° C for 15 minutes in a cell culture incubator before the initiation of the incubation by pipetting 50 μL of CCHH or MMHH 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). At the end of each incubation, 100 μL of acetonitrile was added into each well to terminate metabolism. The plates after termination were stored in a -80° C freezer for later LC/MS-MS quantification of metabolite formation. All DME activity studies were performed at a final cell density of 1 x 10^6 cells/mL except for the evaluation of cell density versus metabolite formation where CCHH and MMHH were firstly prepared at cell concentrations of 4 x 10^6 cells per mL, followed by dilution to 2 x 10^6, 1 x 10^6, and 0.5 x 10^6 per mL before addition to the substrate plates, thereby yielding final concentrations of 2 x 10^6, 1 x 10^6, 0.5 x 10^6 and 0.25 x 10^6 mL.

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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 an addition of 100 µL of acetonitrile solution containing the internal standard Tolbutamide (250nM) and mixing. All samples were centrifuged at 3,500 rpm for 5 minutes. An aliquot of 100 µL of supernatant from each was transferred to a 96 well plate and was 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 6b-hydroxylation), ECOD, UGT, SULT, GST, FMO, MAO, AO, NAT1 and NAT2 metabolites were quantified performed by using API 4000 QTRAP mass spectrometer with an electrospray ionization source (AB SCIEX, Framingham, MA) connected to Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA) using LC/MS/MS MRM mode, monitoring the mass transitions (parent to daughter ion) (Supplement material section, Table 2). An Agilent Zorbax Eclipse Plus C18 column (4.6 x 75 mm i.d., 3.5 µm; Agilent Technologies, Santa Clara, CA) at a flow rate of 1 mL/min was used for the 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 the positive ion mode operation was programed as: 0 to 2.5 min, increase B from 5 to 95%; 2.5 to 3.5 min, 95% B; 3.5 to 3.6min, decrease B to 5%; run-time 5 min. The gradient program for the negative ion mode was: 0 to 3 min, increase B from 5 to 95%; 3 to 4 min, 95% B; 4 to 4.2 min, decrease B to 5%; run-time 6 min. Data acquisition and data procession were performed with the software Analyst 1.6.2 (AB SCIEX, Framingham, MA).

Data Analysis. Data are presented as mean and standard deviation of triplicate incubations derived using the Microsoft Excel 6.0 software. Statistical analysis was performed using 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 (pmol/min/million hepatocytes) of each drug metabolizing enzyme pathway was determined by dividing the total metabolite formed by the incubation time and normalized to cell concentration.

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Results

Cell morphology. When placed into a cell culture plate, the hepatocytes from MMHH appeared as intact cells which rapidly settled on the bottom of the wells. Phase contrast microphotography showed that MMHH were intact (not lysed) cells with distinct cell membranes (Fig. 1), similar to CCHH (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: MMHH were incubated for 30 min with various P450 isoform-selective substrates at 0.25, 0.5, 1.0 and 2.0 x 10^6 cells/mL. Metabolite formation was found to increase with cell concentration for all the substrates evaluated (Fig. 2). From the results, 1.0 x 10^6 cells/mL was chosen as the cell concentration for subsequent studies.

Incubation duration versus metabolite formation: MMHH and CCHH 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: MMHH and CCHH were incubated with 7-ethoxycoumarin. 7-ethoxycoumarin is firstly oxidation 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 MMHH and CCHH, with 7-hydroxycoumarin found to be extensively conjugated to the glucuronide and sulfate conjugates (Fig. 4).

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2. Acetaminophen metabolism: Acetaminophen metabolism by MMHH and CCHH was evaluated at the non-cytotoxic concentration of 10 mM at the incubation durations of 30, 60, 120, and 240 minutes (Fig. 5; Table 1). Time-dependent formation of glucuronide, sulfate and glutathione conjugates was observed in both MMHH and CCHH. The glutathione conjugate of acetaminophen formed in MMHH ranged from approximately 200% at 30 minutes to approximately 600% of that formed in CCHH at 240 minutes. In a separate study, MMHH and CCHH were incubated with 100 mM and 200 mM of acetaminophen (Table 2). The results confirmed the observation made with 10 mM acetaminophen in that higher levels of acetaminophen glutathione were formed in MMHH. Significant reduction of acetaminophen metabolism was observed at 200 mM in CCHH which was contributed to the cytotoxic effects at this drug concentration. No apparent reduction in metabolite formation was observed in MMHH at 200 mM of acetaminophen .

Cofactor-mediated selection of metabolic pathways in MMHH: MMHH was prepared without cofactors and then incubated with coumarin with the following cofactor compositions: 1. NADPH (without UDPGA nor 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 but were observed under condition 2. 7-hydroxylation and 7-sulfate but not the 7-glucuronide metabolites were observed under condition 3. (Fig. 6)

Activity comparison between MMHH and CCHH: A comparison of the rates of metabolism of the various drug metabolism enzyme-selective substrates in MMHH and is shown in Fig. 7 with MMHH activities expressed as percentages of that for CCHH (Fig. 7A) and with MMHH activities plotted versus CCHH (Fig. 7B). Numerical data for drug metabolizing enzyme activities for MMHH and CCHH are presented in the

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supplementary materials section (Supplement Table 3). The results demonstrate that MMHH were active in all the enzyme pathways evaluated, with activities in general similar to that observed in CCHH.

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, Li et al. 1989; Li 2015) and more recently, human enterocytes(Ho, Ring 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. MMHH was 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 MMHH retain cellular integrity and subcellular structures and thereby contain complete enzyme pathways akin to CCHH. As the drug metabolizing enzyme activities of MMHH are driven by the cofactors and therefore not affected by viability, MMHH, like subcellular fractions, are not subjected to handling damages which can drastically affect the functions of CCHH. The presence of all the cellular organelles and the associated drug metabolizing enzymes are the reasons that CCHH are considered the "gold standard" in vitro drug metabolism system. These are also the attributes of MMHH, but not subcellular fractions which contain only a portion of the organelles and therefore lacking the drug metabolizing enzymes associated with the missing organelles (Table 3).

A comparison of MMHH and CCHH in the activities of key drug metabolizing enzyme pathways showed no apparent deficiencies in these activities in MMHH. Both experimental systems were competent in phase 1 oxidation and phase 2 conjugation, including sequential events with oxidation followed by

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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 MMHH and CCHH in key phase 1 oxidation and phase 2 conjugation drug metabolism enzyme activities, with MMHH competent in all pathways evaluated. Activities observed in MMHH in general are within 2 fold of that for CCHH (Fig. 7). Activities observed in MMHH as percent of that in CCHH based on the rate of metabolite formation after an incubation duration of 30 minutes are: UGT (271%), GST (234%), SULT (204%), CYP1A2 (180%), CYP2A6 (177%), CYP2B6 (146%), CYP2C8 (131%), CYP2C9 (187%), CYP2E1 (132%), CYP3A4 (midazolam 1'hydroxylation: (151%); testosterone 6b-hydroxylation: (159%); CYP2J2 (100%); ECOD (179%); FMO (169%); NAT1 (134%); CYP2C19 (57%); CYP2D6 (86%); MAO (89%); AO (34%); CES2 (27%); acetaminophen glucuronidation (80%); and acetaminophen sulfation (43%).

Our results therefore show that MMHH are active in all drug metabolizing enzyme pathways evaluated with activities comparable to that for CCHH, thereby suggesting that MMHH can be used for drug metabolism studies that routinely employ CCHH. The advantages MMHH over CCHH include storage at -80^{o} C instead of liquid nitrogen to retain viability and functions, used directly after thawing without a need for centrifugation cell recovery and microscopy examination of cell viability and cell concentration determination. We have estimated that the time required from thawing to incubation for drug metabolism evaluation was approximately 7 – 10 minutes for MMHH versus 30.5 – 45 minutes for CCHH (Table 4). Further, unlike CCHH in which cofactor compositions cannot be readily controlled, specific metabolic pathway can be evaluated with MMHH based on the composition of the added cofactor mixtures. Selection of metabolic pathways based on cofactor compositions in MMHH 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.

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Another potential advantage of MMHH over CCHH 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 CCHH but not in MMHH. This property of MMHH should be useful in the evaluation of metabolic fate of highly toxic drugs such as anticancer therapeutics.

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

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Authorship Contributions

Participated in research design: Albert P. Li, Ming-Chih David Ho, Kirsten Amaral, and Carol Loretz

Conducted experiments: Ming-Chih David Ho, Kirsten Amaral, and Carol Loretz

Performed data analysis: Albert P. Li, Ming-Chih David Ho

Wrote or contributed to the writing of the manuscript: Albert P. Li, Ming-Chih David Ho, Kirsten Amaral, and Carol Loretz

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Legends for Figures

Figure 1. Phase contrast microscopy of MMHH. 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 conventional cryopreserved human hepatocytes.

Figure 2. Metabolite formation as a function of cell concentration in MMHH. 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) , CYP3A4 (midazolam 1'-hydroxylation). The cell concentrations evaluated were 0.25, 0.5, 1 and 2 x 10^6 cells/mL. (Error bars: standard deviation of triplicate determinations)

Figure 3. Metabolite formation as a function of incubation durations in CCHH (open squares) and MMHH (filled squares). The time durations evaluated were 30, 60, 120, and 240 minutes. The cell concentration used was 1 x 10^6 cells/mL. P450 (Fig. 3A) and non-P450 (Figure 3B) pathway-selective substrate metabolism are shown. (Error bars: standard deviation of triplicate determinations)

Figure 4. A comparison of CCHH (top) and MMHH (bottom) in the formation of secondary metabolites from 7-ethoxycoumarin (C, D). 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 x 10^6 cells/mL. (Error bars: standard deviation of triplicate determinations)

Figure 5. Time-dependent formation of acetaminophen conjugates in CCHH (open bars) and MMHH (filled bars) at the non-cytotoxic concentration of 10 mM. The metabolites quantified were glucuronide (A), sulfate (B), and GSH (C) conjugates. (Error bars: standard deviation of triplicate determinations)

Figure 6. Effects of cofactor composition on metabolite formation from coumarin in MMHH. MMHH was 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); 4. NADPH and PAPS (horizontally shaded bars). (Error bars: standard deviation of triplicate determinations)

Fig. 7. A comparison of CCHH and MMHH in drug metabolizing enzyme activities: Drug metabolizing enzyme activities of MMHH expressed as percentages of that for CCHH (A) (calculated using the following equation: [Activity (MMHH)/Activity (CCHH)] x 100%) and correlation of MMHH and CCHH activities (B). Each data point in Fig. 7B represents activity data for each of the drug metabolizing enzymes identified in Fig. 7 A. Numerical data for the figures are presented in Table 3 of the Supplementary Materials. (Error bars: standard deviation of triplicate determinations)

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Tables

Table 1: A comparison of conventional cryopreserved human hepatocytes (top table) and MetMax™ cryopreserved human hepatocytes (bottom table) in the metabolism of acetaminophen at the noncytotoxic concentration of 10 mM. Mean and standard deviations (sd) of triplicate samples are shown.

Incubation Time (minutes)	**Total Metabolite Formation in CCHH (nmoles per million hepatocytes)**					
	APAP Glucuronide		APAP Sulfate		APAP Glutathione	
	mean	*sd*	*mean*	*sd*	*mean*	*sd*
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 Time (minutes)	**Total Metabolite Formation in MMHH (nmoles per million hepatocytes)**					
	APAP Glucuronide		APAP Sulfate		APAP Glutathione	
	mean	*sd*	*mean*	*sd*	*mean*	*sd*
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

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Table 2: A comparison of conventional cryopreserved human hepatocytes (CCHH)and MetMax™ cryopreserved human hepatocytes (MMHH) in acetaminophen metabolism at the less cytotoxic concentration of 100 mM and the cytotoxic concentration of 200 mM. Mean and standard deviations (sd) of triplicate samples are shown.

Substrate Conc. (µM)	**Marker Metabolite**	**Total Metabolite Formation (pmol per million hepatocytes)**			
		CCHH		MMHH	
		Ave.	Std.	Ave.	Std.
100 mM	Acetaminophen Glutathione	206.50	3.91	856.72	71.28
200 mM		99.81	7.49	676.70	54.43
100 mM	Acetaminophen Glucuronide	572.55	27.12	1298.77	64.29
200 mM		170.28	16.38	1361.84	83.36
100 mM	Acetaminophen Sulfate	105.55	3.36	150.22	6.68
200 mM		52.58	1.40	156.36	11.86

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Table 3. Organelle compositions of the various in vitro drug metabolism experimental systems.

MetMax™ cryopreserved human hepatocytes (MMHH), conventional cryopreserved human hepatocytes (CCHH), human liver microsomes (HLM) and post-mitochondrial supernatant (PMS). The mark "X" indicates presence of the organelles in the specified experimental systems. Organelle contents in various in vitro systems have been previously reviewed (Brandon, Raap et al. 2003).

Organelles	Experimental Systems			
	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		

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Table 4. A comparison of the procedures for drug metabolism studies with CCHH and MMHH. The time requirement for CCHH will increase significantly if multiple individual lots (e.g. comparison of different hepatocyte donors) are used as 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 MMHH.

Procedures	**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° waterbath	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 2X of the final desired cell density	5 minutes	not required
Addition to vessels containing 2X test compounds (e.g. 50 μL of hepatocytes to a 96 well with 50 μL of test articles at 2x of final concentrations), followed by incubation for the desired time duration at 37 C: 1 - 3 minutes		
Total time from freezer to incubation	**30.5 – 45 minutes**	**7 - 10 minutes**

Figure 1

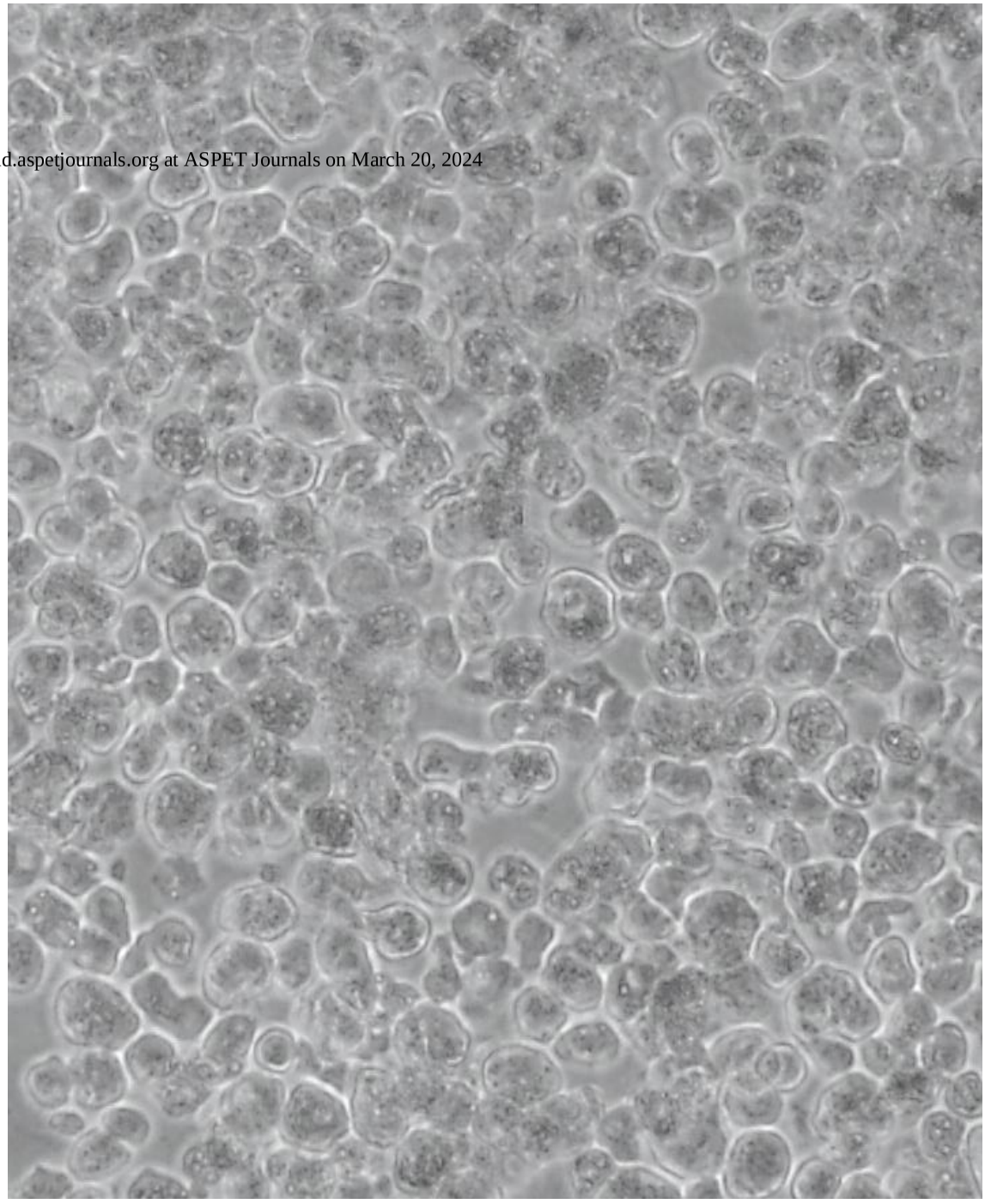


Figure 2

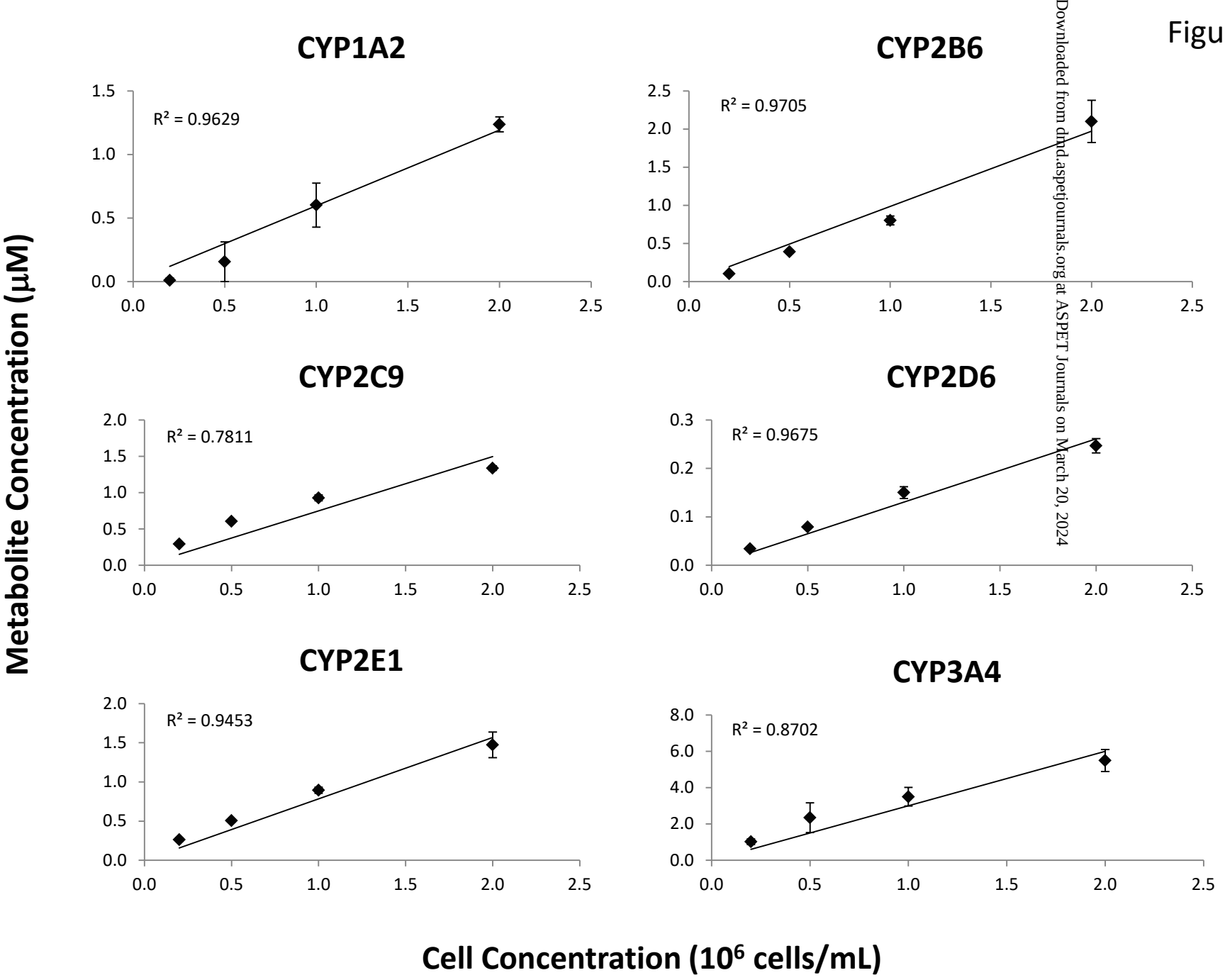


Figure 3A

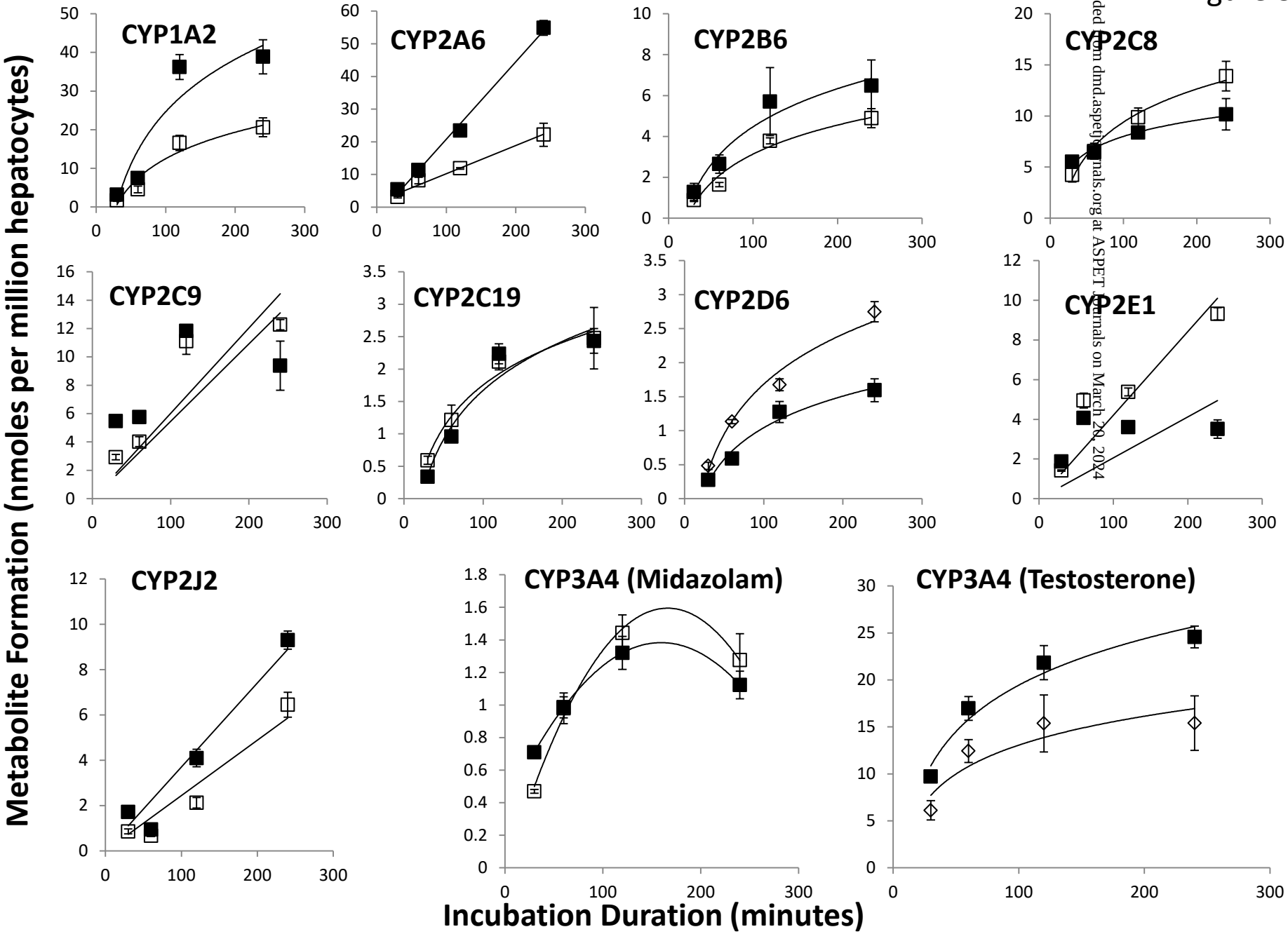


Figure 3B

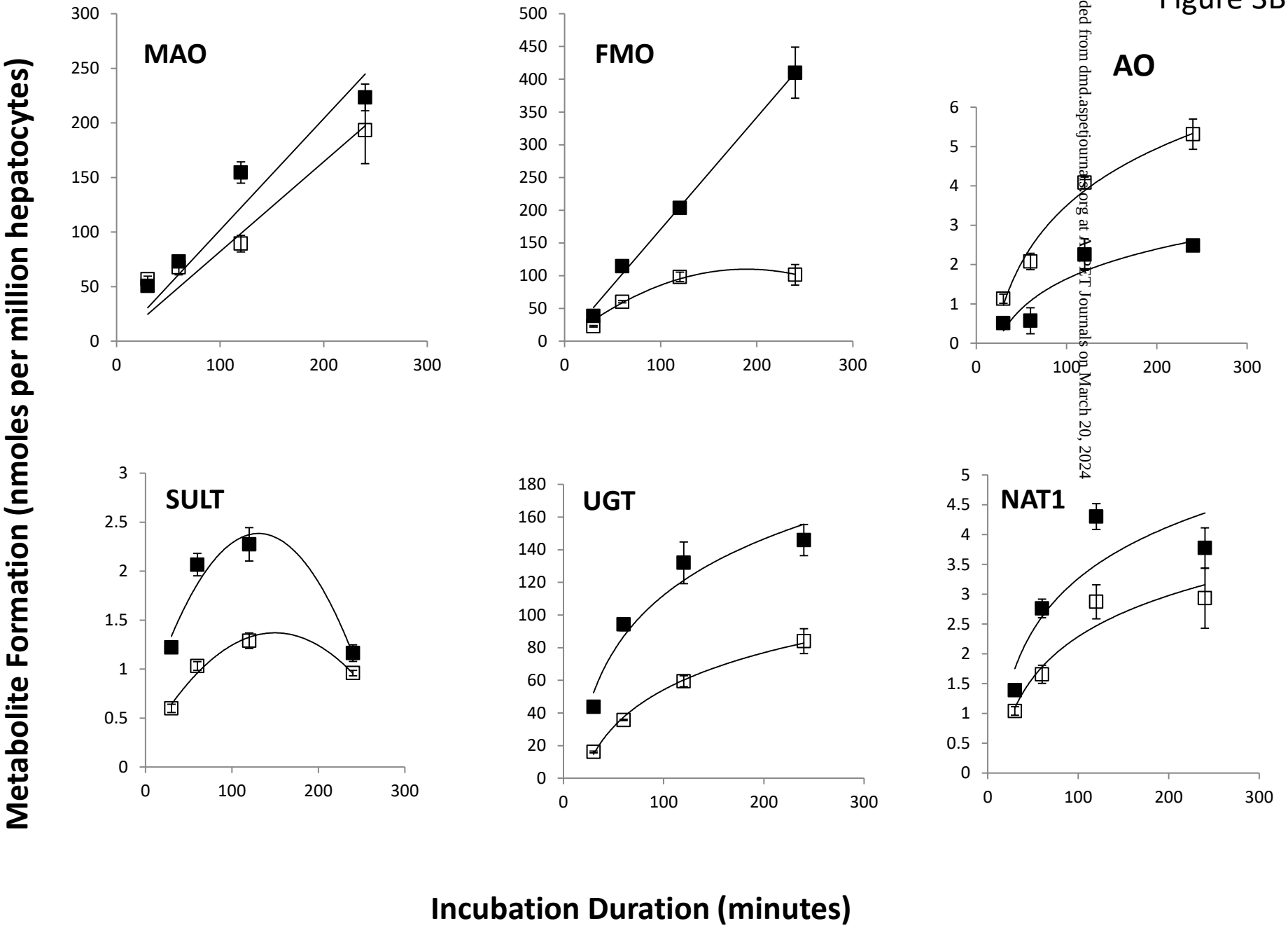


Figure 4

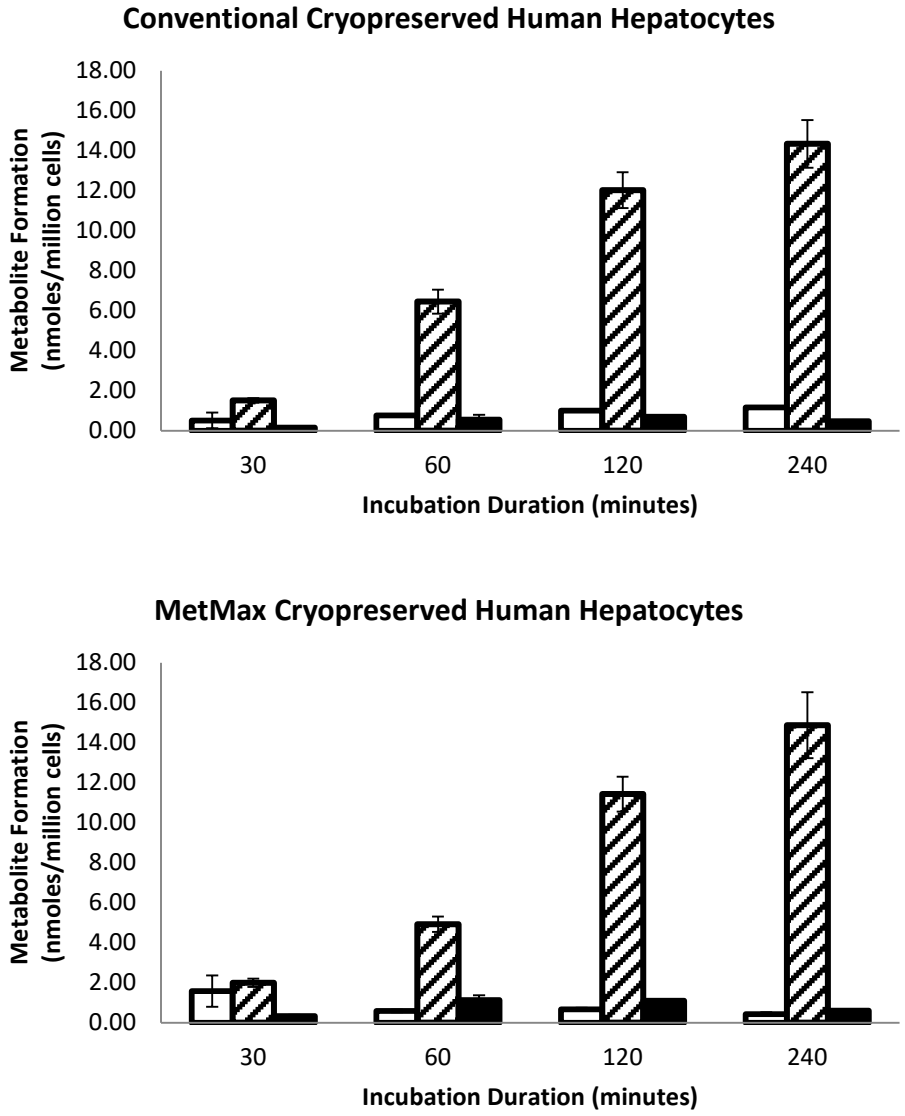
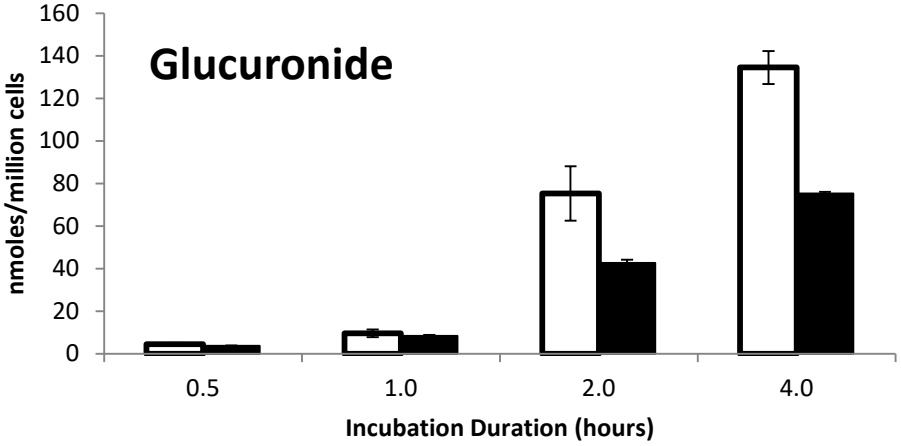
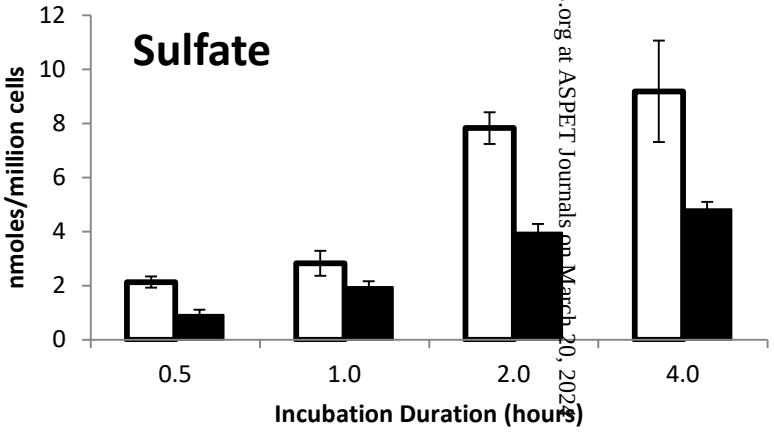


Figure 5





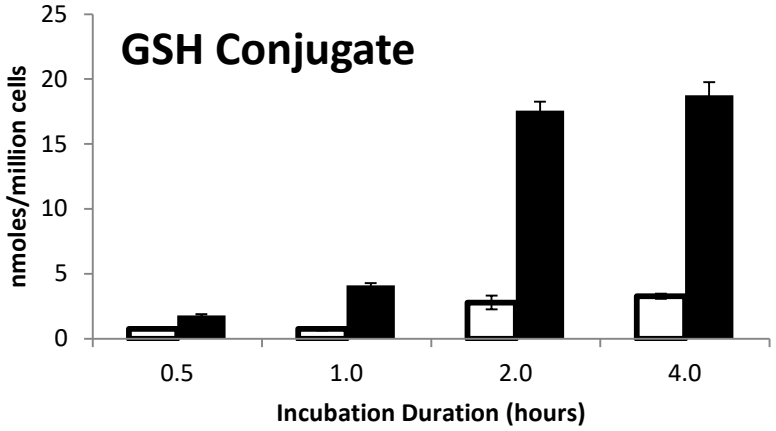


Figure 6

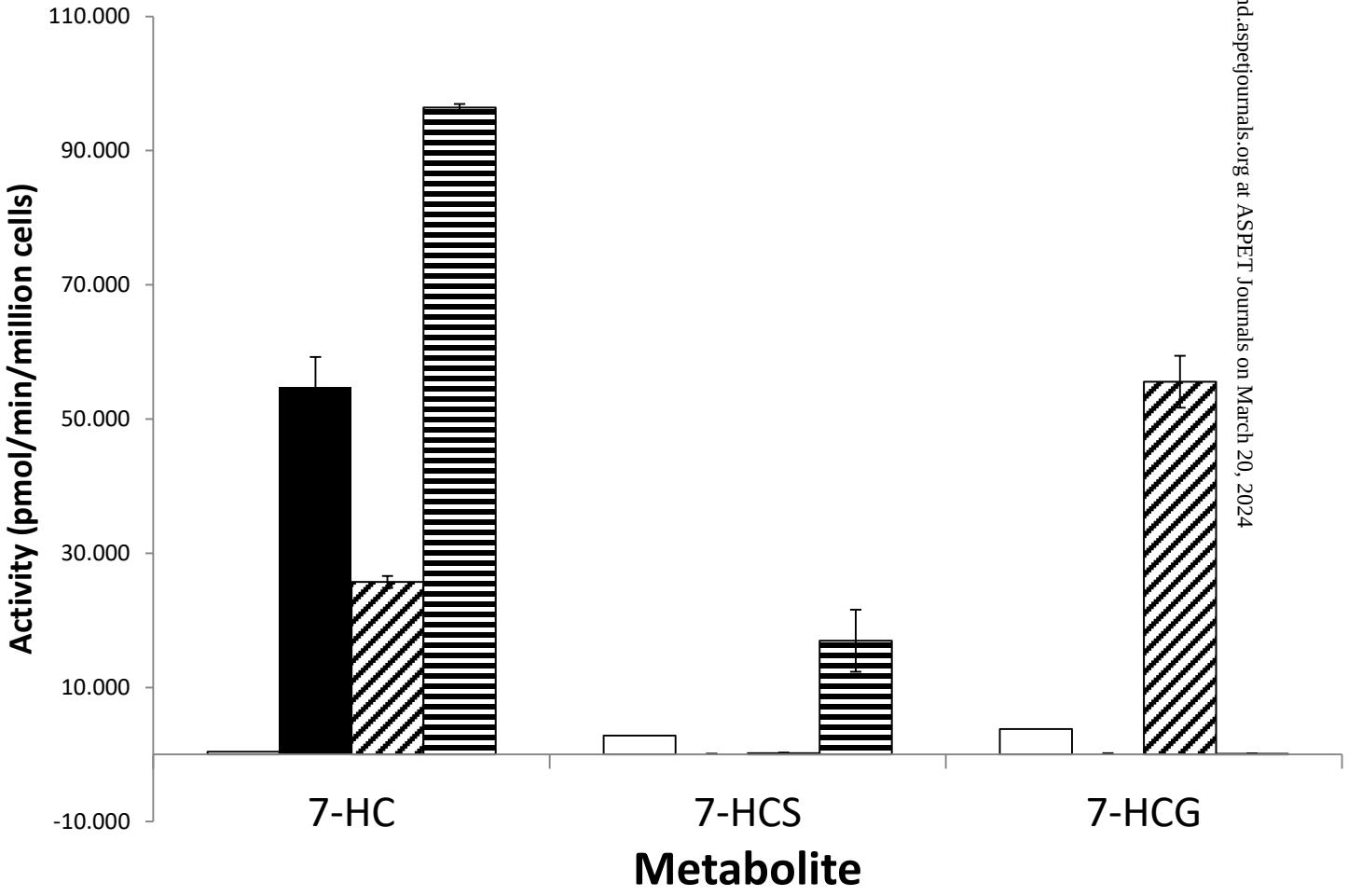


Figure 7