THE USE OF PRIMARY CRYOPRESERVED HUMAN HEPATOCYTES AS A MODEL FOR NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) RELATED CHANGES IN PHASE I AND PHASE II ENZYME ACTIVITIES

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Abstract

Primary human hepatocytes are the current gold standard for *in vitro* predictions of *in vivo* drug metabolism. Inter-individual variability in activity of drug metabolizing enzymes based on age, BMI, and gender have been assessed, however, it is not known if activity changes in complex liver disorders such as non-alcoholic fatty liver disease (NAFLD) can be accurately modeled using primary hepatocytes. NAFLD is a liver disease characterized by hepatic steatosis, which can progress to non-alcoholic steatohepatitis (NASH). To investigate changes in enzyme activities associated with this disease state we utilized isolated cryopreserved human hepatocytes from donor livers. Metabolic activity for both cytochrome p450(CYP) (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) and conjugating enzymes (UGT and SULT) was generated by assessing metabolite formation with prototypic substrates in suspension hepatocyte incubations. In parallel, formalin fixed paraffin embedded (FFPE) tissue samples from the same donor livers were blindly examined by a board-certified pathologist and given an NAFLD Activity Score (NAS) comprised of graded assessment of hepatic steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2), with NAS ≥ 5 commonly used as a histologic diagnosis of NASH. Additionally, a graded assessment of fibrosis (0-4) was performed. Donors with NAS ≥ 5 had significantly lower activity in several enzymes as compared to those with a NAS of 0. To determine if individual pathological features contribute to metabolic changes, we next evaluated enzyme activity in donor hepatocytes separated only by their steatosis, lobular inflammation, ballooning, and fibrosis scores. We found that separating by steatosis scores alone uncovered a host of changes in metabolic enzyme activities, including some which were not seen when total NAS was evaluated. No changes in enzymatic activity were seen based on inflammation score, while minimal changes were seen based on ballooning score (significantly reduced CYP2E1 and ECOD activity with trending reduction in CYP2C19 (P=0.10) activity (not shown)). Additionally, examination of metabolism based on donor fibrosis score showed significant reductions in activity of numerous metabolic enzymes in hepatocytes from donors with stage 2 or higher fibrosis as compared to those with no fibrosis. These results suggest that, although inflammation and hepatocyte ballooning contribute to the complex pathological manifestations of NASH, steatosis and fibrosis may be the most prominent factors leading the changes in drug metabolizing enzymes. Therefore, when assessing potential drug-drug interactions in a NASH population, utilization of hepatocytes from livers with high levels of steatosis and fibrosis may give a more accurate prediction for severe cases.

Methods

Liver Pathology Assessment

Tissue biopsies from donor livers were taken prior to generation of hepatocyte lots. Tissues were formalin fixed and paraffin embedded (FFPE). FFPE blocks were sectioned and H&E stained. H&E stained sections were examined by a board-certified pathologist and a detailed pathological assessment was performed, including scoring of fibrosis stage (0-4) and levels of steatosis (0-3), hepatocyte ballooning (0-2), and lobular inflammation (0-3). These criteria were used to give a total NAS score (0-8), which is a common scoring method for determining NAFLD and NASH in patient liver biopsies.

Hepatocyte Metabolism

Human cryopreserved hepatocytes corresponding to pathologically examined donor livers were thawed at 37°C for 90 seconds, transferred into INVITROGRO™ CP Media and spun at 50xg for 5 minutes to pellet cells. Cell pellets were resuspended in INVITROGRO™ KHB, counted, and cell concentration adjusted to 2x10^6 cells/ml. Metabolite formation assays were run by aliquoting 0.2x10^6 cells into 48 well plates along with Phase I and Phase II enzyme substrates (table 1). After a 60-minute incubation, reactions were stopped by the addition of an equal volume of methanol. Substrate formation was quantitated by LC/MS/MS and calculated as pmol/min/10^6 cells.

Statistical Analysis

Metabolite formation in hepatocyte lots was assessed according to pathological features from the corresponding donor liver biopsies (NAS score, steatosis score, lobular inflammation score, hepatocyte ballooning score, or fibrosis stage). Hepatocyte lots were excluded from analysis if patient records indicated that the donor drank >2 alcoholic drinks/day or if the donor was classified as pediatric (age<18 y/o). Mean ± SEM of metabolic rates for each enzyme in all groups were determined using GraphPad Prism. A one-way ANOVA was used to compare means between groups, with P≤0.05 being considered a statistically significant difference.

Table 1

Enzyme	substrate	[µM]	Metabolite(s)
CYP450 1A2	Phenacetin	1.5	Acetaminophen
CYP450 2A6	Coumarin	0.8	7-HCG, 7-HCS, 7-HC
CYP450 2B6	Bupropion	25	Hydroxybupropion
CYP450 2C8	Amodiaquine	2	N-Desethylamodiaquine
CYP450 2C9	Tolbutamide	15	4'-Methylhydroxytolbutamide
CYP450 2C19	S-mephenytoin	2	4'-Hydroxymephenytoin
CYP450 2D6	Dextromethorphan	0.8	Dextrorphan
CYP450 2E1	Chlorzoxazone	10	6-Hydroxychlorzoxazone
CYP450 3A4	Testosterone	5	6ß-Hydroxytestosterone
CYP450 3A4	Midazolam	1.5	1-Hydroxymidazolam
UGT 1A1	Estradiol	1	Estradiol 3-glucuronide
SULT	7-Hydroxycoumarin	3	7-Hydroxycoumarin sulfate
UGT	7-Hydroxycoumarin	3	7-Hydroxycoumarin glucuronide
ECOD (multiple CYP450s)	7-Ethoxycoumarin	7.5	7-HCG, 7-HCS, 7-HC

Data

Table 2

Group	Percent Female	Percent Male	Mean Age	SEM Age	Mean BMI	SEM BMI	sample size (n)
NAS Score							
Score = 0	40%	60%	46.7	2.32	26.5	1.11	30
Score = 1-2	37%	63%	50.3	2.81	28.3	0.94	19
Score = 3-4	42%	58%	45.2	2.78	27.4	0.87	26
Score ≥ 5	40%	60%	51.4	2.19	30.25 *	1.27	25
Steatosis Score							
Score = 0	39%	61%	45.3	1.94	26.6	0.82	44
Score = 1	45%	55%	51.3	2.62	27.7	0.93	22
Score = 2	24%	76%	50.2	3.13	29.3	1.15	17
Score = 3	53%	47%	49.4	2.92	30.66 *	1.68	17
Hepatocyte Ballooning Score							
Score = 0	39%	61%	47.5	1.73	27.6	0.83	49
Score = 1	42%	58%	50.8	2.63	29.1	1.12	26
Score = 2	40%	60%	46.3	2.63	27.6	0.98	25
Lobular Inflammation Score							
Score = 0	47%	53%	46.2	2.58	27.9	1.16	30
Score = 1	38%	62%	49.3	1.80	27.5	0.71	50
Score ≥ 2	35%	65%	48.2	2.29	29.5	1.23	20
Fibrosis Stage							
Stage = 0	45%	55%	42.9	3.13	27.3	1.48	22
Stage = 1	42%	58%	50.7 *	1.56	28.1	0.70	50
Stage ≥ 2	32%	68%	47.8	2.45	28.4	1.05	28

Table 2: Donor Demographics
Donor sex distribution, mean age, and mean BMI were determined from each score group when sorted by total NAS score as well as by each distinct pathological feature. Generally, male donors represented a higher percentage of assessed lots compared to female donors. Mean age was similar among all groups except for fibrosis stage, where stage 0 donors were younger than stage 1 donors (P≤ 0.05). BMI did not differ in each score group when donors were sorted by hepatocyte ballooning scores, lobular inflammation scores, or fibrosis stage. When donors were sorted by total NAS score, or by steatosis score the mean BMI was significantly increased in the highest scored group as compared to the lowest scored group (P≤ 0.05). Differences in age and BMI were assessed via a one-way ANOVA with * indicating P≤ 0.05.

Figure 1

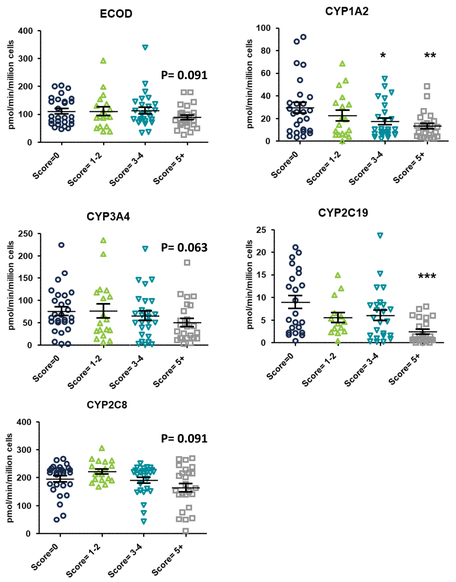

Figure 1: Activity Changes Based on Total NAS Score
Hepatocyte lots were separated based on total NAS score of their donor liver and activity of Phase I and II enzymes (table 1) was examined from each group. Primary hepatocytes from donors with NAS of 3-4 and greater than 5 had significantly decreased activity of CYP1A2 (P≤ 0.01), while NAS ≥ 5 had decreased CYP2C19 (P≤ 0.001) as compared to those having a score of 0. Hepatocytes from donors with NAS ≥ 5 had a trending reduction (P≤ 0.10) in ECOD (P=0.091), CYP3A4 (P=0.063), and CYP2C8 (P=0.091). * Denotes P≤0.05, **indicates P≤0.01, *** indicates P≤0.001.

Figure 2

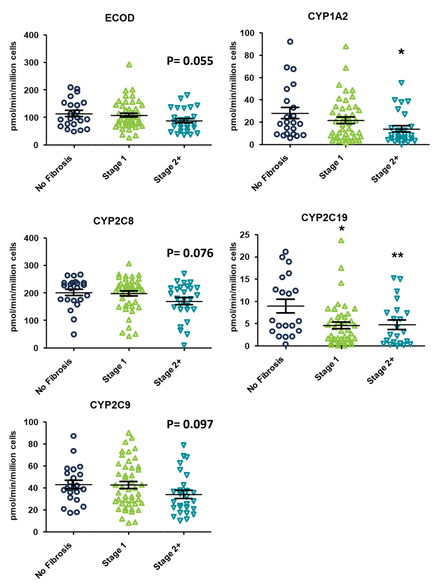

Figure 2: Activity Changes Based on Fibrosis Stage
Assessment of metabolic activity rates in primary hepatocytes sorted by fibrosis stage showed significantly decreased activity of CY2C19 from donors with a fibrosis stage of 1 or higher as compared to donors with no fibrosis (stage 1 P≤0.05, stage 2+ P≤0.01). Donors with a fibrosis stage of 2 or greater also had significantly decreased activity of CYP1A2 (P≤0.05). Additionally, hepatocytes from donors with stage 2 or higher fibrosis also had a trending decrease in metabolic rate of ECOD (P=0.055), CYP2C8 (P=0.076), and CYP2C9 (P=0.097). * Denotes P≤0.05, **indicates P≤0.01.

Figure 3

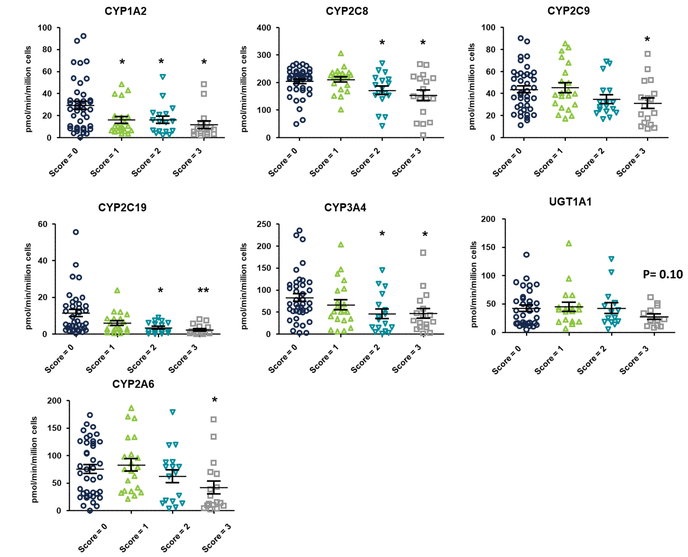

Figure 3: Activity Changes Based on Steatosis Score
The amount of liver steatosis is one component of the total NAS score. Primary hepatocytes were separated based only on the steatosis score (0-3) of their donor livers. Hepatocytes from donors across all scores ≥ 1 had significantly decreased CYP1A2 activity (P≤0.05). Hepatocytes from livers with steatosis scores of 2 or 3 also had significantly decreased metabolic rates of CYP2C8 (P≤0.05), CYP2C19 (score=2 P P≤0.05, score=3 P≤0.01), and CYP3A4 (P≤0.05). Hepatocytes from donors with a steatosis score of 3 also had significantly reduced CYP2A6 and CYP2C9 activity as compared to those with a score of 0 (P≤0.05). Additionally, hepatocytes from donors with steatosis scores of 3 had a trending reduction in metabolic activity of UGT1A1 (P=0.10). * Denotes P≤0.05, **indicates P≤0.01.

Conclusions

Sorting primary hepatocytes into groups based on their donor livers' NAS score and fibrosis stage exposes activity differences in several drug metabolizing enzymes with higher pathological scores, however, using steatosis score alone as the sorting criteria uncovers changes in additional drug metabolizing enzymes. Sorting by hepatocyte ballooning showed minimal changes between high scoring and low scoring livers (significantly reduced ECOD and CYP2E1 activity, trending CYP2C19 activity reduction), while no changes were seen when hepatocytes were sorted by lobular inflammation levels in donor livers. CYP2B6, CYP2D6, SULT, and UGT did not demonstrate any pathology induced changes in metabolic activity when hepatocytes were sorted by any criteria. Currently, the molecular underpinnings responsible for these activity changes are not known. Enzymes showing reduced activity in this study have been previously reported to be under constitutive androstane receptor (CAR) and/or pregnane x receptor (PXR) transcriptional regulation [1-6], which are known to be modulated by polyunsaturated fatty acids [7-9].

- ❑ **Activity changes observed here in primary hepatocytes largely replicate reported findings on drug metabolism changes in NASH [10, 11].**
- ❑ **These findings may further assist in developing *in silico* models for drug metabolism in patients with NASH.**
- ❑ **Additionally, these data can be used to help develop an *in vitro* model of NASH having a similar metabolic profile to both patients with NASH and primary hepatocytes from donor livers having characteristics of NASH.**

References

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