



A cocktail probe approach to evaluate the effect of hormones on the expression and activity of CYP enzymes in human hepatocytes with conditions simulating late stage of pregnancy

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Abstract

Purpose Pregnancy-mediated physiological and biochemical changes contribute to alterations in the pharmacokinetics of certain drugs. There is a paucity of data on the systematic evaluation of the underlying mechanisms. The objective of the current study was to examine the impact of changes in circulating and tissue hormonal concentration during the late stage of pregnancy on the activity and expression of hepatic cytochrome P450 (CYP) enzymes using a cocktail probe approach.

Methods Freshly isolated primary human hepatocytes were incubated with third trimester physiologic (plasma) and projected liver (ten-fold higher) concentrations of female hormones: progesterone (2 μM), estradiol (0.3 μM), estriol (0.8 μM), estrone (0.2 μM), 17α -hydroxyprogesterone (0.1 μM), and human growth hormone (0.005 μM). The metabolic activity of the hepatocytes was assessed using a cocktail of isozyme-specific P450 probe substrates (CYP1A2 (phenacetin), CYP2C9 (diclofenac), CYP2C19 (S-mephenytoin), CYP2D6 (dextromethorphan), and CYP3A4 (testosterone)). A validated LC–MS/MS assay was used to measure the corresponding metabolite concentrations. CYP450 protein and mRNA levels were measured using western blot and qRT-PCR, respectively.

Results Female hormones at projected third-semester hepatic concentrations significantly enhanced mRNA and protein expression and increased the metabolic activity of CYP3A4. The expression and activity of other CYP450 enzymes studied were not affected by mixtures of female hormones at concentrations used.

Conclusion The increased activity of CYP3A4 is consistent with the clinically observed increase in clearance of CYP3A4 substrates during pregnancy. Overall expression and activity of CYP450 isozymes are differentially regulated during pregnancy.

Keywords Pregnancy · Female hormones · Primary human hepatocytes · Cocktail probes · Cytochrome P450 · Liquid chromatography-tandem mass spectrometry (LC–MS/MS) · Quantitative real-time polymerase chain reaction (qRT-PCR)

Abbreviations

CYP	Cytochrome P450
P	Progesterone
17α -OHP	17α -Hydroxyprogesterone
E1	Estrone
E2	Estradiol
E3	Estriol
hGH	Human growth hormone
LC–MS/MS	Liquid chromatography-tandem mass spectrometry
qRT-PCR	Quantitative real-time polymerase chain reaction

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Introduction

Pregnant women often use several medications to manage chronic health conditions and/or treat ailments. Dosing regimen of most of these drugs in pregnant women is based on data derived from studies in men or non-pregnant women. Recent reports show that the clearance of CYP2C9, CYP2D6, and CYP3A4 substrates increases during pregnancy [1–5], whereas the clearance of CYP1A2 and CYP2C19 substrates decreases during pregnancy [2, 6]. However, the underlying physiological and biochemical mechanisms leading to the observed changes in the pharmacokinetics of many drugs used during pregnancy are not completely characterized. Therefore, studies that shed light on the underlying mechanism(s) of pregnancy-mediated changes in drug metabolism provide better understanding of pregnancy-mediated effect and pave the way to improve drug therapy during pregnancy.

Several physiological changes observed in pregnancy are essential to facilitate the growth and development of the fetus [7, 8]. These changes may directly or indirectly impact the pharmacokinetics of certain drugs. One of the noticeable changes is the significant increase in the concentrations of steroid hormones throughout gestation. In humans, concentration of both estrogens and progestins increase during the course of pregnancy. A report from Tulchinsky et al. shows a substantial increase in progesterone, estradiol, estriol, estrone, and 17 α -hydroxyprogesterone levels during normal pregnancy [9]. Studies in animal models provide information about the role of steroid hormone levels on the regulation of P450 enzymes. Female rats have 2–threefold higher levels of hepatic Cyp2c7 than male rats. The differences in hepatic Cyp2c7 expression can be nullified with neonatal gonadectomy in male and female rats. Further, estradiol treatment restored Cyp2c7 expression in ovariectomized female rats; on the other hand, estradiol induced the Cyp2c7 expression in male rats. Testosterone does not affect Cyp2c7 levels in female rats but decreases Cyp2c7 expression in neonatally castrated male rats treated during adulthood. Neonatal gonadectomy abolished the expression of Cyp2c11 in male rats, and testosterone treatment during adulthood induced the hepatic levels of Cyp2c11 in male rats [10]. Progesterone treatment caused an increase in liver size and total protein and enhanced its levels in the liver. Additionally, Cyp2d1 activity was increased threefolds in the liver microsomes, but the activity of Cyp3a and Cyp2e was unchanged indicating a differential effect on specific drug metabolizing enzymes [11]. Glucocorticoid treatment partially restored cyp3a enzymes in hypophysectomized mice. The combination of glucocorticoid and growth hormone treatment shows strong induction of cyp3a enzyme in mice hepatocytes as well as hypophysectomized mice [12]. Growth hormone treatment

in hypophysectomized male rats induced liver Cyp2c11, Cyp2a2, and Cyp3a2 activity, protein, and mRNA. Though Cyp2c11 expression was stimulated by growth hormone in hypophysectomized female rats, Cyp2a2 and Cyp3a2 were not induced. Growth hormone deficiency in female rats may lead to the loss of factors other than growth hormone that are required for full expression of growth hormone stimulated by Cyp2a2, 3a2, and 2c11 [13].

Despite few similarities in enzyme regulation, data from animal studies cannot be directly extrapolated to humans owing to mechanistic differences between animals and humans. It is important to evaluate the effect of various hormones in a clinically relevant system such as primary cultures of human hepatocytes. Most of the reported studies focused on using one or two hormones, but the levels of multiple hormones change continuously during the course of pregnancy. A small number of studies have addressed the effect of the combination of hormones on the expression and activity of drug-metabolizing enzymes [14–17].

Hepatocytes adjust to a new equilibrium with pregnancy-mediated physiological changes and are exposed to combinations of hormones and growth factors during the course of gestation. A myriad of changes over the duration of pregnancy may show synergistic or antagonistic effects on the pharmacokinetics of drugs, and dissecting individual mechanisms and regulators requires more *in vivo* studies during pregnancy and more detailed *in vitro* studies [15]. The effects of hormonal changes on the regulation and activity of CYP450 enzymes have been observed in different cells such as neurons [18], hepatocytes, HepG2 cells, and g2car-3 cells [19]. Progesterone inhibits estrogen-mediated constitutive androstane receptor (CAR) transactivation in HepG2 cells [19], and high concentrations of progesterone and estradiol synergistically regulate the expression and activity of mouse cyp2b10 [18]. Differential regulation and expression of CYPs by estrogens and progesterone have been studied in human hepatocytes [14]. Papageorgiou et al. studied the effect of the combination of hormones on CYP450 activity and expression using *in vitro* models and reported that cortisol by itself and in combination with growth hormone increased CYP3A4 activity and expression [16]. However, the effect of these hormones in combination with other female hormones, such as estrone, estriol, and 17 α -hydroxyprogesterone, on the expression and activity of P450 enzymes remains unknown.

Studies in literature used female hormones similar to either unbound or total concentrations in plasma observed during pregnancy either individually or combinations of two or three hormones, and the metabolic studies were conducted using one probe at a time. The current study aimed to comprehensively study the effect of the combination of multiple important female steroidal hormones and human growth hormone on primary human hepatocytes at physiologically

Table 1 Hepatocytes donor demographics

Donor ID	Age	Sex	Race	BMI	Smoking	Alcohol use
HU1522	67	F	Caucasian	25	No	No
HU1527	31	F	Caucasian	21	Yes	Yes
*HU12-010	31	F	Caucasian	ND	ND	ND
HU1593	31	F	Caucasian	29	No	Yes
HU1632	34	F	Caucasian	38	No	No

ND no data

*This batch was not treated with human growth hormone

relevant third-trimester total plasma concentrations as well as the predicted maternal hepatocyte concentrations calculated from log P and plasma concentrations. Further, a cocktail of 5 probe substrates was used to study the activities of five CYP450 enzymes, simultaneously.

Material and methods

Chemicals

Progesterone (P), 17 α -hydroxyprogesterone (17 α -OHP), estrone (E1), estradiol (E2), estriol (E3), human growth hormone (hGH), bovine serum albumin (BSA), phenacetin, S-mephenytoin, dextromethorphan hydrobromide monohydrate, testosterone, 6 β -hydroxy testosterone, acetaminophen, and all solvents of the highest purity LC–MS/MS grade were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Diclofenac sodium salt and dextrorphan-d-tartrate were purchased from MP Biomedical Inc. (Solon, OH, USA). 4-Hydroxy diclofenac, (S)-4-hydroxy mephenytoin, and deuterated internal standards for the respective metabolites acetaminophen-D4, 4-hydroxy diclofenac-D4, (\pm)-4-hydroxy mephenytoin-D3, dextrorphan-D3 tartrate salt, and 6 β -hydroxy testosterone-D3 were procured from Toronto Research Chemicals Inc. (Ontario, Canada). Cell lysis buffer was purchased from Roche Diagnostics Corporation (Indianapolis, IN). Rabbit anti-human primary monoclonal antibodies against CYP3A4 and β -actin and anti-rabbit IgG linked with horseradish peroxidase were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA). Enhanced chemiluminescence substrate was purchased from Thermo Fisher Scientific (Rockford, IL).

Human hepatocyte incubation and treatments

Freshly isolated primary human hepatocytes from female donors ($n = 5$) were obtained from Life Technologies Corporation (Carlsbad, CA). Hepatocytes were plated in collagen-coated 6-well plates as monolayers (1.5×10^6 cells/

well) and shipped in hepatocyte maintenance medium (HMMTM) (Lonza Pharma & Biotech, Morristown, NJ). The viability in all batches of human hepatocytes was greater than 87%. Hepatocyte donor demographics are presented in Table 1 indicating that 80% of the donors were in reproductive age. Upon arrival, the media was replaced with HMMTM supplemented with 1 μ M dexamethasone, 4 μ g/mL insulin, and 10,000 U/mL penicillin/streptomycin. Hepatocytes were observed under a microscope upon arrival and every day before changing media for any significant morphological changes. The hepatocytes were maintained at 37 °C in a humidified atmosphere with 5% CO₂. One plate in each batch of hepatocytes was treated with vehicle (0.1% v/v dimethyl sulfoxide (DMSO)), prototypical CYP3A4 inducer (rifampin (10 μ M)), and CYP3A4 inhibitor (ketoconazole (10 μ M)), and CYP3A4 activity was determined using testosterone as substrate. For the hormone studies, cells were treated with vehicle (DMSO), a mixture of low or high concentration of hormones corresponding to plasma (low) or liver (high) levels resembling the third trimester of pregnancy for 72 h. The hormone combinations used for hepatocyte treatments are presented in Table 2. Predicted liver exposure levels were calculated using logP values and observed plasma levels of the respective hormone. The treatment medium containing hormone treatments was replaced every 24 h for 3 days. On day 4, the hepatocyte monolayer was rinsed with HMMTM and was

Table 2 Composition of mixtures of low and high concentrations of female hormones

Hormone	Low conc. (μ M)	High conc. (μ M)
Progesterone	2	20
E1	0.2	2
E2	0.3	3
E3	0.8	8
17- α OHP	0.1	1
hGH	0.005	0.05

E1 estrone, E2 estradiol, E3 estriol, 17- α OHP 17- α hydroxyprogesterone, hGH human growth hormone

replaced with fresh HMM™ containing a cocktail of five CYP450 substrates (100 µM phenacetin (CYP1A2), 90 µM diclofenac (CYP2C9), 50 µM S-mephenytoin (CYP2C19), 20 µM dextromethorphan (CYP2D6), and 250 µM testosterone (CYP3A4)) and incubated for 60 min. After incubation, the culture medium was collected to measure the metabolites levels of the CYP450 substrates. Hepatocyte pellets were collected for measuring mRNA and protein levels. Total RNA was isolated using the TRIzol method. The mRNA expression of selected CYP450 enzymes was assessed using qRT-PCR. Protein levels were determined using western blot analysis.

Sample preparation and estimation of CYP450 activities

The hepatocyte medium collected after 60-min incubation with a cocktail of substrates was used to estimate CYP450 activities. Briefly, in a micro-centrifuge tube, 200 µL incubation medium, 20 µL internal standards mixture (IS), and 500 µL water were added and vortex mixed for 30 s. The mixture was passed through Waters Oasis HLB 1 mL (30 mg) solid-phase extraction (SPE) cartridge, previously conditioned with 1 mL methanol followed by 1 mL water. The SPE column was washed with 1 mL 5% methanol, and the analytes retained on the column were eluted with 1 mL of 100% methanol. The eluent was evaporated for dryness under an air stream, and the residue was reconstituted in 100 µL of 50% methanol. Twenty microliters of the reconstituted sample was injected onto the column and analyzed by LC–MS/MS. The samples were estimated to quantitate the metabolites using a previously validated LC–MS/MS assay with minor modifications [20].

Chromatography and separation conditions

The samples were separated, using Waters 2695 HPLC system, on Phenomenex Luna C8 column (150×3.0 mm, 5 µm) with a C8 Security Guard cartridge (4.0 mm×2.0 mm). The column was maintained at 40 °C, and the auto sampler temperature was set at 4 °C. A gradient mobile phase system, at a flow rate of 0.3 mL/min, consisted of solvent A (95% water + 5% methanol + 0.1% formic acid + 2 mM ammonium acetate) and solvent B (methanol + 0.1% formic acid + 2 mM ammonium acetate) with a gradient starting from 97% solvent A to 0% solvent A over 0.2 min, held until 6 min, followed by returning to the initial condition of 97% solvent A over 0.1 min and held until 11 min to achieve the baseline. The total run time was set to 11 min.

Mass spectrometry conditions

Sample analysis was performed using the Micromass Quattro triple quadrupole mass spectrometer (Waters Corporation, MA, USA) interfaced with an electrospray ionization probe using multiple reaction monitoring (MRM). Analytes and internal standards were estimated using the following MRM settings: capillary voltage 2.0 kV; source and desolvation temperatures 100 °C and 300 °C, respectively; cone and desolvation gas flows 50 L/h and 300 L/h, respectively; argon pressure 20 ± 10 psig; nitrogen pressure 100 ± 20 psig. The MS conditions for the metabolites and internal standards are presented in Table 3. The LC and MS systems were controlled by the MassLynx® software version 4.1, and the data were collected with the same software and processed using QuanLynx software (Waters Corporation, MA, USA). The precursor and daughter ions used for selected reaction monitoring in the

Table 3 Mass spectrometry conditions for CYP metabolites and internal standards

Enzyme	Metabolite	Parent ion mass (m/z)	Daughter ion mass (m/z)	Ionization mode	Cone voltage (V)	Collision energy (eV)	Retention time (min)
CYP 1A2	Acetaminophen	151.5	110.05	ESI+	32	16	5.95
CYP2C9	4-Hydroxy diclofenac	311.5	229.96	ESI+	25	31	7.45
CYP2C19	4-Hydroxy mephenytoin	234.53	149.93	ESI+	33	19	6.56
CYP2D6	Dextrorphan	257.48	156.93	ESI+	34	38	6.10
CYP3A4	6β-Hydroxy testosterone	304.56	269.06	ESI+	30	15	6.56
Enzyme	Internal standard	Parent ion mass (m/z)	Daughter ion mass (m/z)	Ionization mode	Cone voltage (V)	Collision energy (eV)	Retention time (min)
CYP 1A2	Acetaminophen-d4	155.5	113.5	ESI+	28	16	5.95
CYP2C9	4-Hydroxy diclofenac-d4	315.6	234.0	ESI+	25	32	7.45
CYP2C19	4-Hydroxy mephenytoin-d3	237.8	150.03	ESI+	33	19	6.56
CYP2D6	Dextrorphan-d3	261.0	156.8	ESI+	38	40	6.07
CYP3A4	6β-Hydroxy testosterone-d3	307.8	272.3	ESI+	30	17	6.56

positive ion ESI mode were previously reported by our group [20]. The lower limit of quantification for acetaminophen, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrophan, and 6 β -hydroxytestosterone were 0.1, 1, 2, 0.1, and 0.1 ng/mL, respectively. The assay was validated for specificity, precision (coefficients of variation $\leq 15\%$), and accuracy ($\geq 85\%$).

mRNA analysis

Total RNA was extracted from each batch of hepatocytes using TRIzol reagent. Briefly, the cell pellet was homogenized in one mL TRIzol reagent. After 5 min, 0.2 mL of chloroform was added to the homogenate, shaken vigorously for 15 s, and centrifuged at 12,000 g for 15 min at 4 °C. The clear supernatant layer was transferred to a new micro-centrifuge tube, and isopropanol (1.5-fold volume of sample) was added to precipitate RNA. The mixture was centrifuged at 12,000 g for 30 min at 4 °C, and the supernatant was discarded. The resultant pellet was washed with 75% ethanol to remove remnant solvent by centrifugation, and the supernatant was discarded. The pellet was dried completely, and total RNA was reconstituted in 30 μ L RNase-free water. The concentration of RNA was determined by reading the absorbance of the sample against water reference at 260 nm using a NanoDrop spectrophotometer. The isolated RNA was used to synthesize the first strand of cDNA by reverse-transcription reaction using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). The following primers were used: aggtcaaccatgaccagag and agggcttggtaatggcagtg for CYP1A2, cctctggggcattatccatc and atatttcacagtgaaacatagga for CYP2C9, cctcgggactttattgattgct and ccagctc-caagtaagtcagc for CYP2C19, acaccatactgcttcgacca and cagccattgagcagcagc for CYP2D6, agagctcttcagaactctctcct and tctggttgaagaagtctcct for CYP3A4, and ctcaaggcctcctggctaca and tggctgttgaggccaatgcc for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). IDT primer design tool (PrimerQuest®) was used to design primers with specificity, to avoid cross reactivity and primer dimerization. PCR reaction mixture was prepared by mixing 2 μ L cDNA sample, 1 μ L primer (GAPDH or CYP gene of interest), 10 μ L SYBR Green master mix, and 7 μ L water. After initial denaturation at 95 °C for 10 min, 40 cycles of amplification were performed with denaturation at 95 °C followed by annealing and extension performed at 60 °C for 1 min. To identify PCR products, dissociation curves were used in the reaction. The relative levels of each CYP450 mRNA were normalized with the copy number of GAPDH. The relative levels of mRNA fold changes for all genes were quantified using the $2^{-\Delta\Delta C_T}$ method [21].

Western blot analysis

Hepatocytes collected in phosphate-buffered saline (PBS) were centrifuged (10,000 g for 10 min at 4 °C), and cells were lysed as per procedures previously reported by our group [20]. Briefly, 125 μ L cell lysis buffer was added to the cells and sonicated for 30 s. Total protein levels in cell lysate was measured using bicinchoninic acid assay (BCA) [22]. Twenty-five micrograms of proteins were loaded onto 10% SDS–polyacrylamide gel and separated by electrophoresis. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 90 V for 90 min. The non-specific binding sites on the membrane were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TTBS). Then PVDF membrane was incubated overnight at 4 °C with rabbit anti-human CYP3A4 primary monoclonal antibody (1:1000 dilution) or rabbit anti-human β -actin monoclonal antibody (1:1000 dilution) in 5% BSA. The membrane was washed with TTBS and incubated with anti-rabbit IgG linked with horseradish peroxidase (1:3000 dilution) for 1 h at room temperature. The membrane was treated with enhanced chemiluminescence substrate (ECL), and the luminescence was captured on films and developed. The difference in the band intensities was determined by densitometry using ImageJ Software 1.48 V (<http://imagej.en.softonic.com>).

Statistical analysis

All the experiments were carried out in duplicate in each batch ($n=5$) of hepatocytes. The average of data obtained from two cells with same treatment in each batch was considered as one data point, and % difference is calculated relative to vehicle-treated cells in the same batch. The % change observed with different treatments is pooled and expressed as the mean \pm SEM. The differences in activity and expression between treatments were compared using one-way ANOVA followed by Tukey's post hoc test. The expression and activity of the enzyme was correlated using Spearman's correlation. $p < 0.05$ was considered statistically significant.

Results

Cell cultures

Hepatocytes were observed under a microscope upon arrival and then every 24 h to check for changes in morphology, contamination, loss of adherence, and/or cell death. All batches of hepatocytes were maintained without any contamination. There was no detachment of the cells during

incubation with hormones or with CYP450 substrate cocktail. Hepatocytes appeared healthy under the microscope. Cell viability was >87% in all the batches of hepatocytes.

Effect of mixtures of low and high concentrations of hormones on CYP450 activity

Responsiveness of each batch of hepatocytes to induction and inhibition was examined by treating cells with the prototypical inducer (rifampin) and inhibitor (ketoconazole), and the magnitude of change in CYP3A4 enzyme activity was estimated by measuring the extent of 6 β -hydroxylation of testosterone. Treatments and incubation with substrates for assessing CYP450 activities were carried out in duplicate and in each batch of hepatocytes, and the reaction rates between duplicate incubation were within 10% of each other. Despite the wide range in donor's age and variability in absolute activity rates for CYP3A4 between donors, all batches of hepatocytes responded to both induction and inhibition treatments, and the data from hormone studies were used for further analysis. The mean increase for CYP3A4 activity due to rifampin treatment was seven-fold, where as ketoconazole decreased CYP3A4 activity to 30–50% of the control. Fold change in metabolite formation by different CYP450 substrates in hepatocytes treated with a mixture of hormones is presented in Figs. 1 and 2. The mixture of hormones at predicted liver concentrations during third trimester of pregnancy showed a 25% increase in the activity of CYP3A4 which was statistically significant ($p < 0.05$)

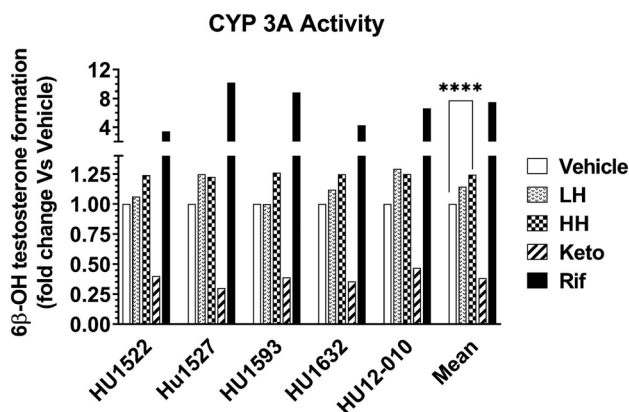


Fig. 1 CYP3A4 activity in freshly isolated human hepatocyte cultures treated with Vehicle (0.1% DMSO), third trimester plasma (LH) and predicted liver concentrations of female hormones (HH), Rifampin (Rif, 10 μ M) and Ketoconazole (Ket, 10 μ M). Fold induction values are expressed relative 6 β -hydroxylation of testosterone formation rates compared to vehicle treated hepatocytes in the respective batch of hepatocytes. HU1552, HU1527, HU1593, HU1632 and HU12-010 represent CYP3A4 activity in individual batch of hepatocytes and Mean represents the average of activity in 5 batches of hepatocytes. Treatments were compared using ANOVA followed by Tukeys' multiple comparison test; **** $p < 0.0001$

(Fig. 1). The activity of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 was not affected with mixture of either low or high levels of hormones (Fig. 2). The CYP activity data obtained from individual batches of hepatocytes and the mean change in activity after different treatments are also presented.

Effect of mixture of low and high concentrations of hormones on CYP3A4 expression

To study the effect of hormones on the expression of CYP3A4, hepatocytes ($n = 5$) treated with mixtures of hormones at third-trimester plasma and the predicted liver concentrations were analyzed for CYP3A4 protein levels using western blotting. Densitometric analysis of band intensities for CYP3A4 presented in Fig. 3 shows a 1.4-fold increase in CYP3A4 protein levels in hepatocytes treated with a mixture of predicted liver concentrations of the hormones, and the observed increase was statistically significant ($p < 0.005$). In addition, rifampin-treated hepatocytes showed a significant ($p < 0.05$) 2.6-fold increase in CYP3A4 protein.

Effect of low and high levels of female hormones on P450 mRNA expression

The impact of physiological and predicted liver concentrations of hormones on gene expression of various CYP450 enzymes was evaluated by quantitating mRNA levels using qRT-PCR. mRNA expression data presented in Fig. 4 shows that the expression of CYP1A2, CYP2C19, and CYP2D6 mRNA was not affected. Though CYP2C9 mRNA showed a 1.4- and 1.8-fold increase with low and high concentrations, respectively, the observed increases were not statistically significant. CYP3A4 mRNA expression was increased by 2- and four-fold ($p < 0.05$) at low and high concentrations of female hormones, respectively (Fig. 5). Furthermore, the correlation of CYP3A4 protein to mRNA in respective batches of hepatocytes showed a strong and statistically significant correlation ($p < 0.001$) with a $r^2 = 0.7532$ (Fig. 6).

Discussion

Pregnancy causes a multitude of physiological and biochemical changes in the women's body. The levels of estrogens, progestins, growth hormones, and other factors needed for fetal growth increase with the progress in gestational age. The profound increase in the hormonal levels and the exposure of various organs to these hormones could affect both the expression and activity of several proteins involved in drug disposition. We studied the effects of mixture of female hormones at both circulatory and projected liver levels on drug-metabolizing enzymes using human hepatocytes and a cocktail approach.

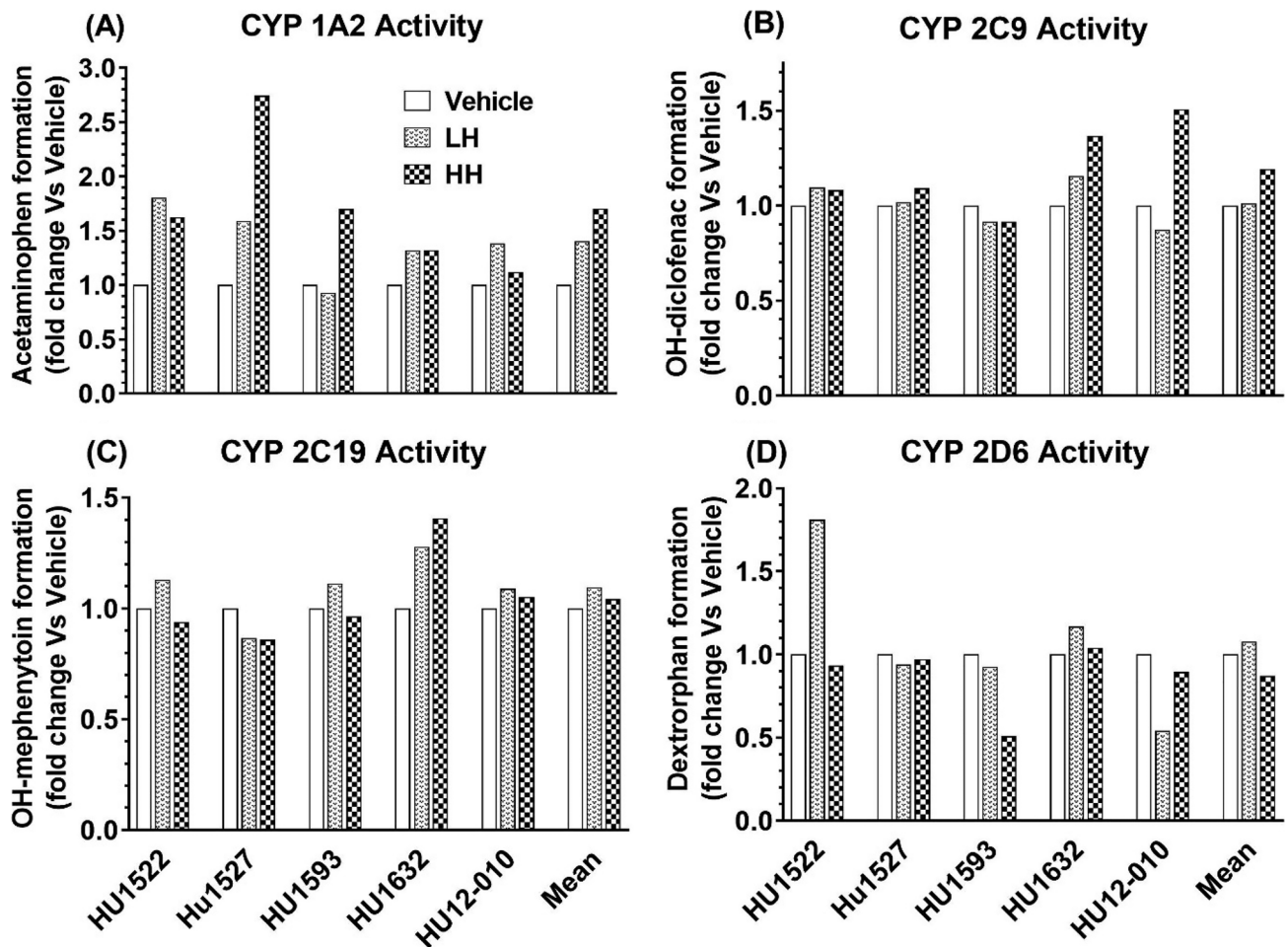


Fig. 2 Activity of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 in freshly isolated human hepatocyte cultures treated with 0.1% DMSO (Vehicle), third trimester plasma (LH) and predicted liver (HH) concentrations of female hormones. Fold induction values are expressed relative rates of acetaminophen, 4-hydroxy diclofenac, 4-hydroxy

mephenytoin and dextropran formations compared to vehicle treated hepatocytes. HU1552, HU1527, HU1593, HU1632 and HU12-010 represent respective CYP isoenzyme activity in individual batch of hepatocytes and Mean represents the average of activity in 5 batches of hepatocytes

Primary cultures of human hepatocytes are valuable resources to evaluate the impact of chemicals and various physiological and pathological conditions on the activity and expression of drug-metabolizing enzymes and transporters [23]. All the currently published studies typically use one substrate at a time to study the activities of drug-metabolizing enzymes using primary cultures of human hepatocytes. Given the difficulties associated with obtaining primary cultures of human hepatocytes, and the high cost associated with procuring them for studies, our group has developed a sensitive and specific CYP450 cocktail assay using human hepatocytes for simultaneously assessing the activity of several major CYP450 enzymes involved in the metabolism of almost 95% of drugs in clinical use [20]. We have performed incubations with substrates using primary cultures of freshly isolated human hepatocytes in our previously published studies and observed that the reaction rates

remained linear for more than 4 h for several drugs tested [24, 25]. In the current report, the incubations were performed for 60 min only, and we utilized a cocktail of substrates to investigate the modulation of drug-metabolizing enzymes due to hormonal changes observed during pregnancy using primary cultures of human hepatocytes.

Both in vitro and animal models have been employed to elucidate the potential role of changes in hormone levels in altering the metabolism of multiple CYP450 substrates, but most of the studies used either one or two hormones and may not predict the overall outcomes observed in pregnancy [15, 18, 19]. Pregnancy is a dynamic state with many altered physiologic and metabolic functions where maternal hepatocytes are exposed continuously to changing combinations of hormones and growth factors. These combinations may act in a synergistic or antagonistic manner. Our

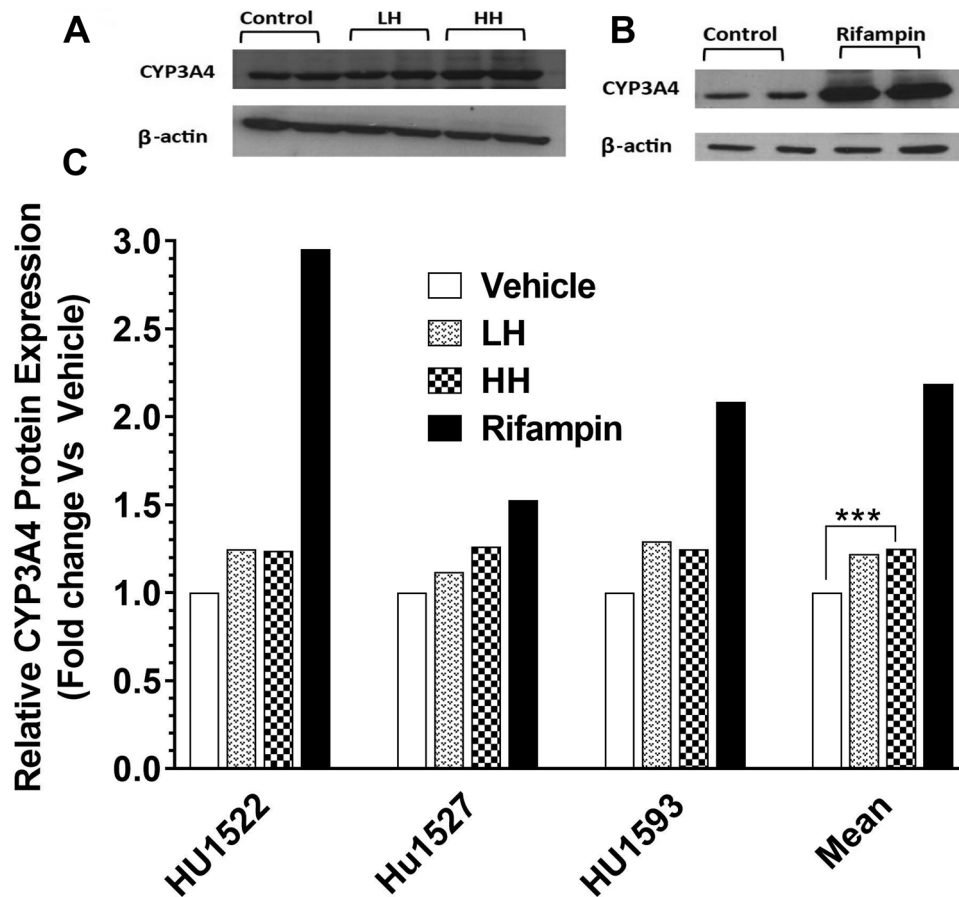


Fig. 3 Western blot analysis of cell lysate obtained from hepatocytes treated with 0.1% DMSO (Vehicle), third trimester plasma (LH) and predicted liver (HH) concentrations of female hormones and, Rifampin (Rif, 10 μ M). **A** Representative Western blot of CYP3A4 protein in hepatocytes collected after treatment with vehicle (DMSO), mixture of low (LH) and high (HH) concentrations of female hormones and expression of β -actin in the corresponding samples is shown as loading control. **B** Representative Western blot of CYP3A4 protein in hepatocytes collected after treatment with vehicle (DMSO) and Rifampin (10 μ M) for 72 hours. Expression of β -actin in the cor-

responding samples is shown as loading control. **C** Densitometric analysis of CYP3A4 immunoblots corrected for protein loading using β -actin band density. Expression is based on corrected densitometric values are expressed as fold increase over vehicle treatment. HU1522, HU1527, HU1593 represent protein expression in individual batch of hepatocytes and Mean represents the average of protein expression in 3 batches. Columns represent mean ($n=3$). Treatments were compared using ANOVA followed by Tukey's multiple comparison test; **** $p < 0.0001$

studies were designed to evaluate the net effect of multiple hormones resembling hormone levels observed in plasma and projected liver concentrations to study the effects in primary cultures of freshly isolated human hepatocytes.

The mean of clinically observed plasma levels of female hormones reported in literature [9] were used for treatment at plasma levels. Previous studies in hepatocytes have used similar concentrations of hormones and used individual substrates for assessing the activity of drug-metabolizing enzymes [14]. We hypothesized that the combined effects of the increased levels of female hormones may be responsible for the alterations in expression and activity of various CYP450 enzymes in the liver and would contribute to

the changes in the pharmacokinetics of several drugs in pregnant women.

The metabolism in mother and fetus and the growth and development of fetus are regulated by human growth hormone (hGH) which belongs to human placental lactogen (hPL) gene family. During early gestation, maternal circulation of growth hormone originates from the anterior pituitary gland, but during the second trimester onwards, pituitary growth hormone levels decline, and placental growth hormone levels increase gradually and reach a maximum of 15–25 ng/mL in the third trimester [26]. Placental growth hormone (pGH) is traditionally not detected in fetal serum during pregnancy. Recent findings by Mittal et al. report that pGH in fetal circulation is around 1% of the maternal

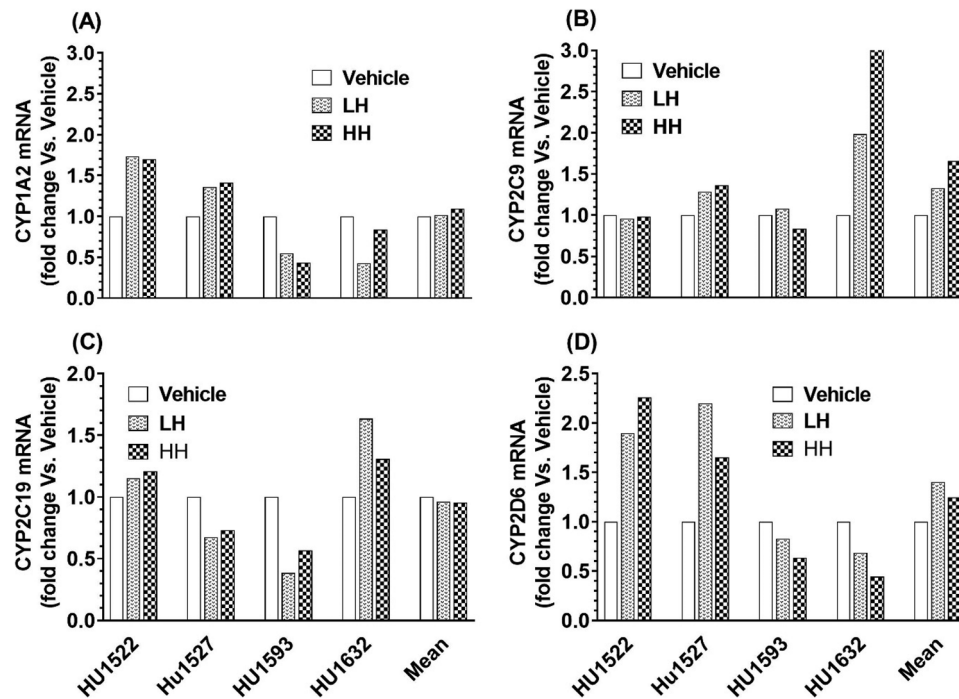


Fig. 4 mRNA levels of CYP isozymes measured by qRT-PCR and normalized to mRNA expression of GAPDH. Corrected mRNA expression values are expressed as fold increase over vehicle treatment. mRNA expression of (A) CYP1A2, (B) CYP2C9, (C) CYP2C19 and (D) CYP2D6 in hepatocytes from four donors (HU 1522, HU1527, HU1593 and HU1632) treated with 0.1% DMSO (Vehicle), third trimester plasma (LH) and predicted liver (HH) concentrations of female

hormones and collected after 72 hr treatment with 0.1% DMSO (Vehicle), third trimester plasma (LH) and predicted liver (HH) concentrations of female hormones (with media change every 24 hours). HU1552, HU1527, HU1593 and HU1632 represent mRNA levels in individual batch of hepatocytes and Mean represents the average of mRNA levels in 4 batches of hepatocytes

circulation [27]. hGH differs from placental growth hormone by 13 amino acids and is differentially glycosylated [28], and differences in proteins may be related to localizing the activity in placental tissue. Despite high circulatory levels of pGH in late stages of pregnancy, the physiological role of placental growth hormone is not understood completely. Both hGH and pGH are expected to have similar pharmacological effects on the maternal liver, but the uptake of pGH in maternal hepatocytes is unknown. Our studies were designed to study the effect of different hormones on hepatocytes; it was reasonable to use hGH as a part of the hormone mixture in our studies in the absence of data about the localization and effect of pGH on hepatocytes in the mother.

Our studies were conducted in accordance with the guidelines prescribed by Pharmaceutical Research and Manufacturers of America (PhRMA) directives in determining the extent of inducers in in vitro and in vivo DDI [29]. All the hepatocytes used in our studies showed response to positive control for induction (rifampin 10 μ M) with a ~seven-fold increase CYP3A4 activity where a ~two-fold increase is considered acceptable to evaluate the quality of hepatocytes. Additionally, a 50% reduction in CYP3A4 activity was observed with ketoconazole treatment also (Fig. 1). Our

previous studies in hepatocytes also show a 30–50% reduction which is similar to the inhibition observed in current studies [24, 25, 30]. The response to positive control for both induction and inhibition from all donors is in agreement with the reported observations by Papageorgiou et al. [16]. The positive controls rifampin (induction) and ketoconazole (inhibition) were used in the current investigation to examine the response of the hepatocytes modulation of CYP3A4. Additionally, it was not practical to use a positive control for each and every enzyme tested. Positive control for CYP3A represents the overall response of the hepatocytes to treatments.

Observations from clinical studies show that clearance of multiple CYP3A4 substrates was increased during pregnancy. Changes in drug dosing are needed for pregnant women to maintain optimal therapeutic effects [2–5, 31]. The clearance of midazolam and digoxin, CYP3A4 substrates, was significantly higher during pregnancy compared to postpartum [3], and glyburide dose-normalized plasma concentrations were ~50% lower in pregnant women than in non-pregnant subjects [4]. The pregnancy-mediated changes in the pharmacokinetics of drugs metabolized by CYP450 enzymes could be attributed to changes in the expression

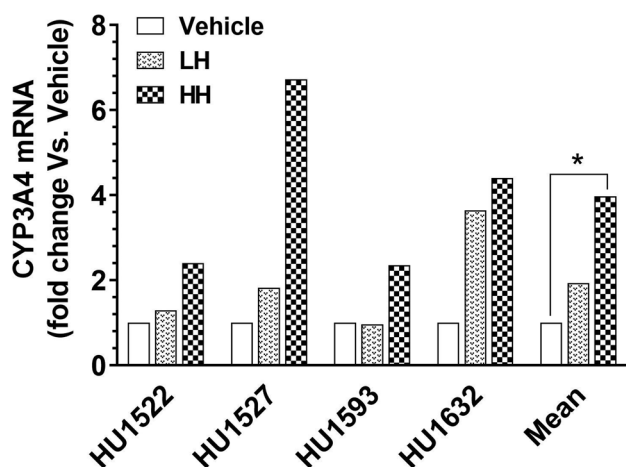


Fig. 5 mRNA levels of CYP3A4 measured by qRT-PCR and normalized to mRNA expression of GAPDH. Corrected mRNA expression values are expressed as fold increase over vehicle treatment. CYP3A4 mRNA expression in hepatocytes from four donors (HU 1522, HU1527, HU1593 and HU1632) treated with 0.1% DMSO (Vehicle), third trimester plasma (LH) and predicted liver (HH) concentrations of female hormones and collected after 72 hr treatment (with media change every 24 hours). HU1522, HU1527, HU1593 and HU1632 represent mRNA levels in individual batch of hepatocytes and Mean represents the average of mRNA levels in 4 batches of hepatocytes. Treatments were compared using ANOVA followed by Tukeys' multiple comparison test; * $p < 0.05$

and activity of the respective drug-metabolizing enzymes. Because of its unusually poor substrate selectivity, CYP3A4 is sensitive to reversible or irreversible inhibition by a range of medications. Mechanism-based CYP3A4 inhibition is defined by NADPH, time, and concentration-dependent enzyme inactivation. Additionally, other characteristics associated with medications and patients can also affect the clinical outcomes resulting in difficulty to predict drug-drug interactions involving CYP3A4 inactivation.

Results from our studies show a statistically significant increase in CYP3A4 mRNA and protein expression as well as activity at predicted concentrations in the liver. The four-fold increase in CYP3A4 mRNA observed in our studies is in concurrence with reported values by Choi et al., using similar concentrations of progesterone [14]. Additionally, the observations in our study are consistent with results from our clinical studies in pregnant women during the third trimester where dextromethorphan N-demethylation was used as a marker of CYP3A4 activity [2]. The extent of midazolam exposure (AUC) was reduced by 50% during pregnancy compared to postpartum [3], and our results show a 25% increase in CYP3A4-mediated metabolism in the simulated pregnancy state. A corresponding change in CYP3A4 protein and mRNA levels is observed. Our study model and design allowed incubation of human hepatocytes with hormone mixtures for 72 h; but during pregnancy, hepatocytes are exposed

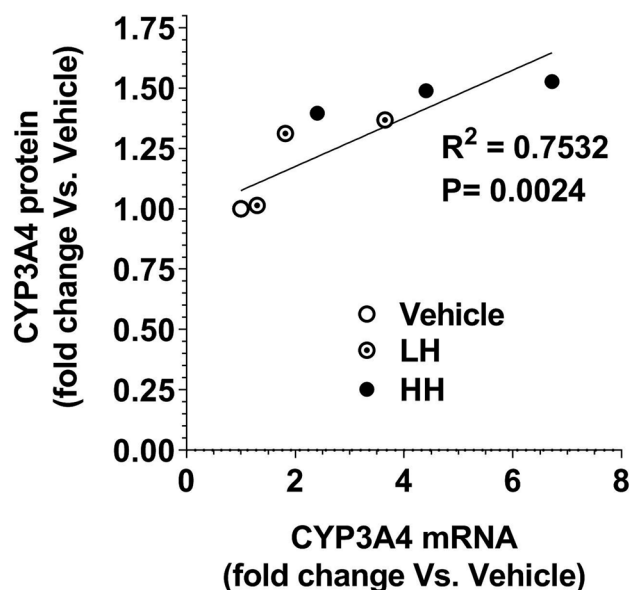


Fig. 6 Spearman correlation of CYP3A4 protein and mRNA expression in freshly isolated human hepatocytes treated with 0.1% DMSO (Vehicle), third trimester plasma (LH) and predicted liver (HH) concentrations of female hormones (with media change every 24 hours). CYP3A4 protein levels were determined by Western blot followed by densitometric analysis and CYP3A4 mRNA expression was estimated using qRT-PCR. Symbols and line represent individual treatment and regression line respectively

to increased levels of hormones. With maternal hepatocytes getting sustained exposure of higher levels of hormones, the observed difference in magnitude of effect between clinical studies and human hepatocyte studies is reasonable.

In our studies, hepatocytes were treated with hormones every 24 h, but physiological regulation of hormones during pregnancy is continuous and maintained throughout pregnancy. Studies reporting the use of high concentrations of these hormones such as estradiol, progesterone individually or in combination with placental growth hormone, growth hormone, and cortisol in human hepatocyte systems reported a significant increase in the expression of CYP3A4 activity, which is in agreement with our data [14, 16]. A positive correlation ($R^2 = 0.7532$) was observed between CYP3A4 protein and mRNA expressions in hepatocytes treated with predicted liver concentrations of hormones. The increase in mRNA expression is consistent with higher CYP3A4 protein levels and a corresponding increase in CYP3A4 activity.

CYP3A4 and CYP3A5 are the major forms of CYP3A expressed in the human liver and gut. Though CYP3A4 is the most abundant CYP450 isoform accounting for about 30% of the total hepatic P450 enzyme in humans [32], it is responsible for about 60% of the P450-mediated metabolism of currently available drugs [33] and has a wider substrate range than CYP3A5 [34]. It is difficult to tease out the contributions of 3A4 and 3A5 individually due to the

large overlap in their substrate specificities. Both proteins contribute to the metabolism of different classes of drugs. Furthermore, observations from our study are in accordance with clinical observations demonstrating that clearance of CYP3A4 substrates such as midazolam, methadone, and nifedipine was increased during pregnancy [3, 5, 35].

Additionally, our findings showed that projected hormone levels experienced by the liver during the late stage of pregnancy did not affect the expression or activity of CYP1A2, CYP2C9, CYP2C19, and CYP2D6. Though CYP2C9 activity showed an increasing trend with hormone treatment, the increase was not statistically significant. A longer duration of exposure and the continued presence of hormones throughout the entire culture period might have resulted in a significant increase in CYP2C9 activity. Estradiol and progesterone are rapidly eliminated from the body through hepatic metabolism [36, 37], and previous reports in hepatocyte studies show a rapid depletion of hormones within 4–8 h from hepatocyte maintenance medium [14, 38]. In our studies, hormones combination was replenished every 24 h, and the exposure of hepatocytes to hormones may not be similar to circadian modulation of physiological conditions. In pregnant women, liver cells are continuously exposed to hormones at higher levels [39] which may have a significant impact on the expression and activity of CYP450 enzymes. Overall, our results are consistent with the reports from Choi et al. where progesterone, the most abundant hormone during pregnancy, did not show significant effect on mRNA expression or enzyme activities of CYP1A2, CYP2C9, CYP2C19, and CYP2D6 [14].

Our study aimed to get the maximum information from each batch of freshly isolated primary human hepatocytes as they are a valuable resource and are sparsely available. We studied the impact of a mixture of pregnancy-related hormones in observed plasma and predicted liver concentrations during the third trimester of pregnancy on the expression and activity of major CYP450 enzymes and used a cocktail of CYP450 substrates to measure changes in enzyme activities. Our results show that female hormones in the third trimester predicted liver concentrations that increased the enzyme activity, mRNA, and protein expression of CYP3A4. The present study is in line with previous studies to utilize human hepatocyte model with cocktail of CYP450 probes to study the effect of hormones on drug metabolism. Our observations agree with data from previously reported *in vitro* and clinical studies [3, 16]. Though the results are consistent with clinical data for CYP3A4 metabolism, the discrepancies in the expression and activity of other CYP450 enzymes could be related to the rapid decline of the hormone levels during each 24 h of incubation, and the presence of dexamethasone in the medium could alter the activity of some P450 enzymes and an incubation period of 72 h that may not be long enough for inducing observable alterations

in other enzymes using our current protocol. Ongoing studies in our lab are focused on evaluating the effect of more frequent media changes to replenish the hormones and better maintenance of hormones at constant levels using a flow through microphysiological system with human hepatocytes [40].

The outcomes of the research can be extrapolated to *in vivo* scenario as well as for the development of prediction models, in that the clearance of the medications metabolized by CYP3A4 will be higher during pregnancy. We characterized the effect of pregnancy on the changes in the expression and activity of drug metabolizing enzymes in the liver. Our data is critical in understanding the effects of medications such as nifedipine (antihypertensive), clindamycin (antibiotic), oxycodone (opioid analgesic), escitalopram (antidepressant), venlafaxine (anxiolytic), oseltamivir (antiviral), midazolam (anesthetic/sedative), indinavir (anti-HIV), ondansetron (prevent nausea), rosuvastatin (antihyperlipidemic), trazodone (antidepressant), and remdesivir (antiviral) that are used during and after pregnancy and are metabolized by CYP3A4. The pregnancy-mediated increase in hormonal concentration will significantly impact the concentration of these drugs, and plasma concentrations of female hormones should be considered as a covariate in computational models to explain variable impact of pregnancy on CYP3A activity. Further studies are recommended to evaluate the effect of pregnancy on the clearance of these medications *in vivo*. CYP3A4 is the enzyme that breaks down the most drugs in humans, so it is an important area to study when it comes to enzyme-based drug interactions during pregnancy. Some of these medications are sedatives such as diazepam, antidepressants such as amitriptyline, antiarrhythmics such as quinidine, antihistamines such as loratidine, and calcium channel antagonists such as nifedipine. Human hepatocyte studies were used to assess the change in clearance in the presence of drug-drug interactions, and the magnitude of change in clearance can be used to adjust the dose of affected drug. We have used this approach in dose optimization for anti-HIV drugs and anti-cancer drugs [24, 30].

Limitations

There are a few limitations worth addressing. In the current investigation, freshly isolated primary human hepatocytes were incubated with female hormone concentrations that were physiologic for the third trimester (plasma concentration) and projected liver (tenfold higher) concentrations. It is important to note that the degree of induction seen cannot be directly translated into steady-state alterations in CYP450 activities *in vivo*, and that hepatocyte activities under the culture conditions utilized in the present investigation are much lower than their activities *in vivo*. Variables other than changes in CYP450 metabolic activity, such as membrane

transporter activity, drug- and patient-related factors, and culture conditions, may affect the results of incubations of primary hepatocytes with CYP450 selective substrates and the determination of the concentrations of their specific metabolites in the cultivation media.

Further studies with long-term hepatocyte cultures can be performed to assess the modulation of CYP450 activities at steady-state condition which represent in vivo conditions. Newer models such as microphysiological systems or Hep-aRG cell cultures which can sustain the CYP450 activity of hepatocytes for longer times can be utilized. The expression and activity of different drug transporters may be altered during pregnancy, and studies evaluating the role of transporters are also needed.

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Author contribution Participated in research design: Alshabi, Zhao, Pillai, and Venkataramanan. Conducted experiments: Alshabi, Shaik, and Zhao. Contributed new reagents or analytic tools: Caritis and Venkataramanan. Performed data analysis: Alshabi and Shaik. Wrote or contributed to the writing of the manuscript: Alshabi, Shaik, Caritis, and Venkataramanan.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The freshly isolated human hepatocytes were obtained from commercial supplier, and the study is an exempt study.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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