REGULATION OF PHASE I, II, AND III PATHWAYS BY PREGNANCY RELATED HORMONES

by

Ali Alshabi

B.S., King Saud University, 2004

MS, University of Pittsburgh, 2011

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SCHOOL OF PHARMACY

This dissertation was presented

by

Ali Alshabi

It was defended on

[June 17, 2015]

and approved by

Michael A. Zemaitis, Professor, Department of Pharmaceutical Sciences

Steve N. Caritis, Professor, Magee-Womens Hospital

Xiang-Qun (Sean) Xie, Professor, Department of Pharmaceutical Sciences

Jan H. Beumer, Associate Professor, Department of Pharmaceutical Sciences

Dissertation Advisor: Raman Venkataramanan, Professor, Department of Pharmaceutical Sciences

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Clinical studies have indicated that pregnancy alters the pharmacokinetics profiles of several medications. Results from previous studies have suggested that physiological changes that occur in pregnancy, including increased plasma concentrations of female hormones may play an important role in the observed changes in drug metabolism. Despite this general awareness, the mechanisms underlying the regulation of major drug metabolizing enzymes such as cytochrome P450 (CYP450) and UDP-glucuronosyltransferase (UGT) during pregnancy remain unknown. The aim of this dissertation was to characterize the impact of pregnancy related hormones on hepatic CYPs, UGTs and transporters.

The effect of female hormones on the expression and activity of selected CYP, UGT, and transporters was measured in four independent studies in primary cultures of human hepatocytes. A mixture of female hormones at projected hepatic concentrations in the third trimester of pregnancy significantly increased mRNA expression, activity, and protein expression of CYP3A4 but limited impact on other CYPs. Female hormones however, differentially altered the expression and activity of various UGT enzymes. Progesterone and estradiol increased the activities of UGT1A1 and UGT1A4, respectively. Conversely, human chorionic gonadotropin decreased the activity of UGT1A4. A significant increase in the expression of UGT1A6 and UGT1A9 was observed by progesterone compared to the control. Human growth hormone

enhanced the mRNA expression of UGT2B7. Expression of UGT1A1, UGT1A3 and AhR were significantly increased by estradiol. Progesterone significantly increased the expression of BCRP. Estradiol also enhanced the expression of NHERF1. Together, these findings provide support for the role of female hormones in the altered specific drug metabolism and transport during pregnancy.

Using physiological based pharmacokinetics (PBPK) modeling, we predicted that pregnancy will increase the clearance of buprenorphine (a drug that is simultaneously metabolized by CYP and UGT). This was supported by preliminary clinical observations. However, female hormones did not have any significant impact on the metabolism of buprenorphine in primary cultures of human hepatocytes, implicating additional factors to be responsible for pregnancy-mediated changes in pharmacokinetics of buprenorphine.

In conclusion, our findings indicate that pregnancy related hormones contribute to some of the observed changes in drug metabolism and transport during pregnancy.

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ABBREVIATIONS

AAG	α-1-acid glycoprotein
17OHP	17α-hydroxyprogesterone
4MU	4-methylumbelliferone
4MUG	4-methylumbelliferone glucuronide
ABC	ATP-binding cassette
AhR	Aryl hydrocarbon receptor
AUC	Area under the concentration-time curve
BCA	Bicinchoninic acid assay
BCRP	Breast cancer resistance protein
BSEP	Bile salt export pump
BUP	Buprenorphine
BUPG	Buprenorphine-glucuronide
CAR	Constitutive androstane receptor
CL	Clearance
CL_{int}	Intrinsic clearance
CL_{sub}	Sublingual clearance
Cmax	Maximum plasma concentration

CYP450	Cytochrome P450
E1	Estrone
E2	Estradiol
E3	Estriol
ER	Estrogen receptor
ETOP	Etoposide
F	Bioavailability
fa	Fraction absorbed
fub	Fraction unbound
HCG	Human chorionic gonadotropin
hGH	Human growth hormone
HPLC	High performance liquid chromatography
ka	Absorption rate constant
LCMS	Liquid chromatography-tandem mass spectrometry
LTG	Lamotrigine
LTGG	Lamotrigine glucuronide
MRP2	Multidrug resistance associated proteins
NBUP	Norbuprenorphine
NBUPG	Norbuprenorphine-glucuronide
NHERF-1	Na/H exchange regulatory factor 1
P-gp	P-glycoprotein
PBPK	Physiologically based pharmacokinetic

PCR	Polymerase chain reaction
PPARα	Peroxisome proliferator-activated receptor alpha
PXR	Pregnane X receptor
TCA	Taurocholic acid
Tmax	Time to reach maximum concentration.
UGT	UDP-glucuronosyltransferase

1.0 INTRODUCTION

1.1 DRUG USE DURING PREGNANCY

Women represent 50.8% (157 million) of the population in the United States. About 40% (62 million) women are of childbearing age between 15 and 44 years, and about 6.5% (4 million) of them get pregnant every year (Dye *et al*., 2013). More than 50% of pregnant women take at least one prescription medication (Andrade *et al*., 2004; Choi *et al*., 2013; Glover *et al*., 2003). Medication use during pregnancy is very common. A prospective and observational study reported that about 96% of pregnant patients received at least one medication during their pregnancy, and more than 62% of them used over-the-counter medications (Refuerzo *et al*., 2005). Andrade *et al*., 2014, reported that during the 270 days before delivery, the percentage of pregnant women who used a drug other than over-the-counter vitamins or minerals from the United States Food and Drug Administration category A, B, C, D, and X were 2.4%, 50.0%, 37.8%, 4.8%, and 4.6%, respectively (Figure 1-1-A) (Andrade, 2004). Moreover, 3.4% and 1.1% of pregnant women received a drug from category D and X, respectively, after the first prenatal visit (Andrade, 2004). Commonly used prescription medications during pregnancy include anti-hypertensives (labetalol and methyldopa), anti-diabetics (insulin, glyburide, and metformin), and anti-retrovirals (ritonavir), in addition to antivirals, antibiotics, and medications that minimize nausea. The most common over-the-counter medications used during different trimesters in the National Birth Defects Prevention Study are shown in (Figure 1-1-B) (Werler *et al*., 2005; Yoon *et al*., 2001). Improper dosing of these medications during pregnancy can cause treatment failure or drug toxicity, leading to serious complications to the mother and fetus (Tatum *et al*., 2004). For example, using angiotensin-converting enzyme (ACE) inhibitors in pregnancy may cause fetal hypertension as well as fetal death (Pryde *et al*., 1993). Moreover, inadequate treatment of some diseases during pregnancy can lead to disease complications, and increase prematurity and

perinatal death. Therefore, health care professionals are still concerned about using drug therapy in pregnant women since it plays an important role in maternal and fetal health. That is why determining the suitable dosage according to the changes in drug disposition in pregnancy is very critical. The current labeling of medications only provides safety information about whether or not the drug can be used in pregnancy according to the FDA pregnancy classification, which is based on safety studies in animals or humans. However, labeling usually does not provide information about drug dosing (based on changes in pharmacokinetics and pharmacodynamics) during pregnancy. Therefore, physicians usually prescribe doses used in non-pregnant population for pregnant women, due to the lack of pharmacokinetic information. As a result, the standard adult dose could be inadequate or toxic. For example, (Webb *et al*., 2004) reported on the lack of antipsychotic treatment guidelines during pregnancy, where the treatment is based on the prescriber's clinical judgment. With limited information about the influence of pregnancy on the efficacy of these medications, these findings emphasize the significant need for studying the effect of pregnancy on drug disposition in order to determine the appropriate dose and optimize pharmacotherapy in this special population, as well as to avoid any unexpected harm to the pregnant woman and the fetus.

Altered pharmacokinetics during pregnancy has been hypothesized in several studies based on what has been revealed as gender differences in pharmacokinetic parameters in animals and humans (Anger and Piquette-Miller, 2008; Curry, 2001; Czerniak, 2001). Pregnancy is a very unique period that has its own physiological changes starting from conception and increasing gradually and linearly until the end of gestation (Anger, 2008). The aim of this review is to discuss the current data on altered pharmacokinetics of medications in pregnancy.

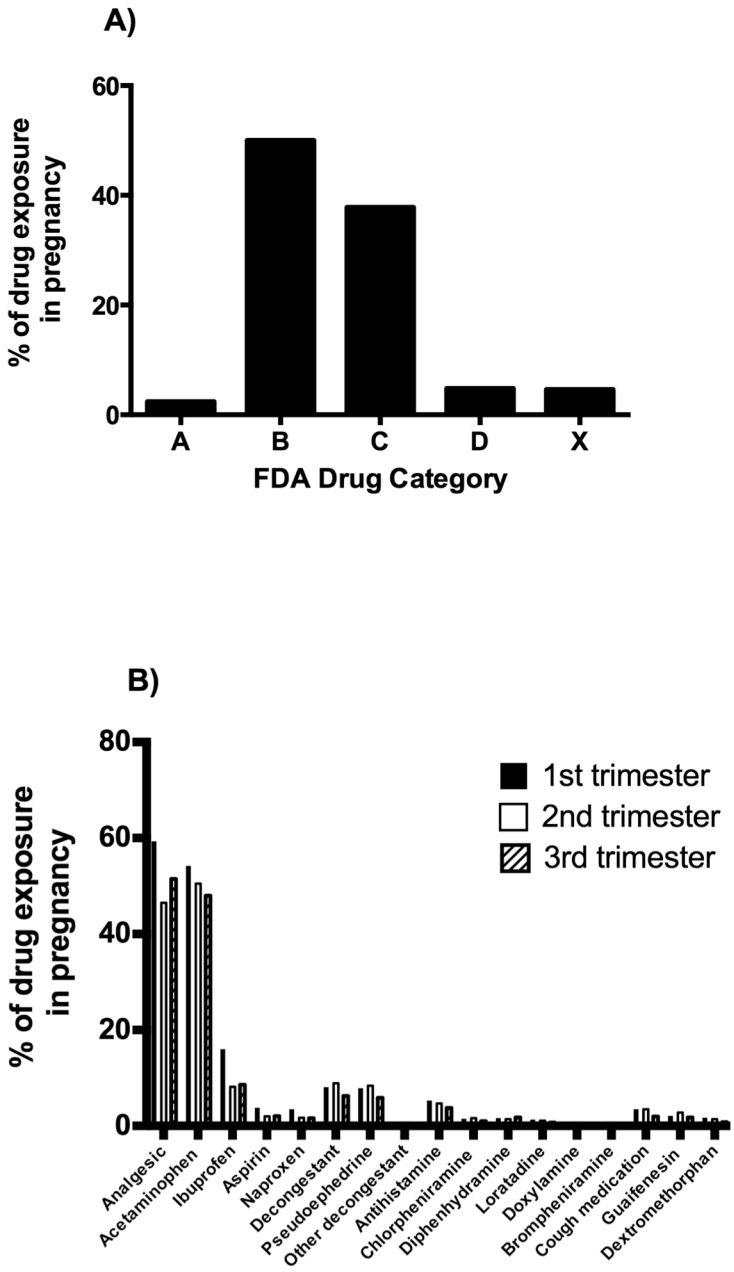

Figure 1-1 Percentage of drug exposure during pregnancy.

(A) Drug exposure during pregnancy based on FDA pregnancy risk category (Andrade, 2004).

(B) Percentage of using over-the-counter medications during pregnancy (Werler, 2005).

1.2 ALTERED DRUG PHARMACOKINETICS IN PREGNANCY

Pharmacokinetic data in pregnant women is limited due to the difficulty in conducting clinical studies in pregnant women. Some of the challenges are small sample sizes and use of different control population to compare the results (pregnant women at different stages of pregnancy versus non-pregnant subjects or male controls).

Changes in hepatic drug metabolism by specific isoforms of CYP450 enzymes during pregnancy has been reported in several clinical pharmacokinetic studies (Table 1-1). For example, increases in the clearance of CYP2C9, CYP2D6, and CYP3A4 substrates in the third trimester have been noted, compared with non-pregnant control subjects or during post partum (McGready *et al*., 2003; Tracy *et al*., 2005; Yerby, 2001). On the other hand, the metabolism of CYP1A2 and CYP2C19 substrates is decreased by 50% and 40%, respectively (McGready, 2003; Tracy, 2005). Results from clinical pharmacokinetic studies indicate that clearance of UGT1A1, UGT1A4, and UGT2B7 substrates are increased (Table 1-1) (Chen *et al*., 2009; Fischer *et al*., 2014; Watts *et al*., 1991). The pharmacokinetics of lamotrigine, an antiepileptic drug that is metabolized mainly by UGT1A4, is significantly changed during pregnancy with a 360% increase in its clearance in the third trimester compared to pre-pregnancy, by an unknown mechanism (Pennell *et al*., 2004). Studies also reported that serum concentration-to-dose ratio of lamotrigine is decreased by 27% and 66% in the first and third trimester, respectively, compared to the postpartum period, suggesting the need for lamotrigine dosage adjustments in pregnant women (de Haan *et al*., 2004). Changes in drug clearance during pregnancy can influence steady-state drug concentrations significantly (Mosekilde *et al*., 2012). Consequently, healthcare professionals are at a crossroad in prescribing medications to pregnant women, because changes in therapeutic efficacy plays an important role in both maternal and fetal health. Therefore,

understanding drug pharmacokinetics during pregnancy is very important in accomplishing the desired therapeutic outcomes and avoiding drug toxicity by determining the adequate dose and achieving the therapeutic drug plasma concentration.

Table 1-1 Change of drug metabolizing activities and drug clearance *in vivo*.

↑ increase, ↓ decrease

Enzyme	Activity change	Pharmacokinetics change	Reference
CYP1A2	↓	Decreased the apparent oral clearance of caffeine	(Tracy, 2005)
CYP2A6	↑	Increased clearance of nicotine	(Dempsey *et al*., 2002)
CYP2C9	↑	Increased clearance of phenytoin	(Yerby *et al*., 1990)
CYP2C19	↓	Decreased the metabolic ratio of proguanil	(McGready, 2003)
CYP2D6	↑	Decreased the urinary ratio of dextromethorphan/dextrorphan	(Tracy, 2005)
CYP3A4	↑	Increased oral clearance of midazolam Increased oral clearance of glyburide Increased oral clearance of methadone	(Hebert *et al*., 2008) (Hebert *et al*., 2009) (Wolff *et al*., 2005)
UGT1A1	↑	Increased labetalol oral clearance	(Fischer, 2014)
UGT1A4	↑	Increased lamotrigine apparent clearances	(Pennell, 2004)
UGT2B7	↑	Increased oral clearance of zidovudine	(Watts, 1991)

Pregnant women undergo several physiological and metabolic changes throughout gestation. These changes have been known to alter drug pharmacokinetics such as absorption, distribution, metabolism, and excretion (Table 1-2). However, the underlying mechanism for these changes is poorly understood.

Table 1-2 Physiological changes during pregnancy and related alterations in drug pharmacokinetics

Physiological changes during pregnancy	Possible pharmacokinetic changes	Reference
Reduced stomach motility and delay in gastric emptying	Decreased drug absorption	(Dawes and Chowienczyk, 2001)
Increased plasma volume	Increased volume of distribution	(Lund and Donovan, 1967)
Decreased albumin (20–30%) and α 1-acid glycoprotein	Increased drug free fraction Increased clearance of low clearance drugs	(Frederiksen, 2001) (Clark *et al*., 1989) (Honda *et al*., 1990)
Increased cardiac output	Increased hepatic clearance for high clearance drugs	(Hodge and Tracy, 2007)
Increased expression of drug-metabolizing enzymes	Increased hepatic clearance of low clearance drugs	(Hill and Pickinpaugh, 2008)
25-50 % increased of renal plasma flow	Increased renal clearance of renally secreted drugs	(Baylis, 1982) (Koren, 2011)
50% increased of glomerular filtration rate	Increased renal clearance of renally filtered drugs	(Davison and Hytten, 1974)

1.2.1 Drug Absorption

Gastrointestinal symptoms such as nausea and vomiting are very common in pregnancy (Bassey, 1977; Shah *et al*., 2000). Due to these conditions, drug absorption can be decreased, resulting in low drug concentrations in plasma (Koren, 2011). It has been reported that increased levels of progesterone is linked to reduced stomach motility, delayed gastric emptying (Dawes, 2001), as well as extension of small bowel transit time by about 30–50% (Clark, 1989). Moreover, during pregnancy, the ionization of weakly acid drugs is increased due to increased gastric pH (Carter *et al*., 1981). Consequently, these changes have the potential to alter drug absorption and drug bioavailability (Parry *et al*., 1970). Huston *et al*., 1989 reported decreased gastric emptying of solid meals in postmenopausal women who had been treated with hormonal replacement therapy compared to men. This indicates that female steroidal hormones may play a role in reducing the rate of gastric emptying, which may decrease rate of drug absorption and maximum drug concentration, and increase the time to reach maximum drug concentration (Dawes, 2001). On the other hand, absorption of certain drugs, such as inhaled medications, can increase because of elevated cardiac output and alveolar uptake (Dawes, 2001).

1.2.2 Drug Distribution.

Plasma volume increases during pregnancy, starting from the first trimester up to the third trimester, leads to increased total body water (8 liters intravascularly and extravascularly), and increases the volume of drug distribution particularly for hydrophilic drugs (Frederiksen, 2001; Lund, 1967). Plasma albumin concentration decreases from 4.2 gm/dL in non-pregnant women

to 3.6 gm/dL in pregnant women during the second trimester of pregnancy (Frederiksen, 2001). Moreover, levels of α-1-acid glycoprotein (AAG) are decreased during pregnancy (Honda, 1990), leading to an increase of fraction unbound (f_{ub}) of certain drugs. As a result of lower plasma protein levels, the protein binding of the drugs will be reduced, leading to increased free fraction, especially for highly protein bound drugs such as midazolam and digoxin (Costantine, 2014). For example, the oral clearance of valproic acid in pregnant women was higher, and this was reported to be due to an increased free fraction of the drug (Pennell, 2003). Extracellular fluid space is also increased as the weight of the pregnant woman increases (Frederiksen, 2001; Plentl and Gray, 1959). An increase in the pregnant women's body weight can lead to an increased volume of distribution for lipophilic drugs and decrease the dose per kilogram when a fixed dose is administered, which may result in decreased steady state concentrations (Koren, 2011). A combination of these factors is expected to increase the volume of distribution and lower the plasma concentrations of most drugs and therefore justifies a need for dosing adjustment (Frederiksen, 2001; Frederiksen *et al*., 1986; Hill and Pickinpaugh, 2008; Hodge, 2007; Mendenhall, 1970).

1.2.3 Drug Metabolism.

Pregnancy alters drug clearance by either increasing or decreasing the activity of hepatic drug-metabolizing enzymes. Clinical studies in pregnant women have shown that clearance of CYP2C9, CYP2D6, CYP3A4, UGT1A1, UGT1A4, and UGT2B7 substrates were increased compared with non-pregnant controls (Fischer, 2014; Hebert, 2008; Pennell, 2004; Tracy, 2005; Watts, 1991; Yerby, 1990). On the other hand, the clearance of CYP1A2 and CYP2C19 substrates were decreased (McGready, 2003; Tracy, 2005). For instance, several medications that

are used in pregnant women are metabolized by CYP3A4, such as nifedipine and midazolam. Studies performed in pregnant women reported that nifedipine clearance increased four-fold during the third trimester of pregnancy compared to controls (Prevost *et al*., 1992). Recently, changes in the drug-metabolizing activity during first, second, and third trimester of pregnancy compared to the postpartum period have been characterized using caffeine metabolism as a marker for CYP1A2 activity, dextromethorphan O-demethylation as a marker for CYP2D6 activity, and dextromethorphan N-demethylation as a marker for CYP3A activity (Tracy, 2005). Activity of CYP1A2 was significantly reduced by 32%, 48%, and 65% during the first, second, and third trimester, respectively (Tracy, 2005). On the other hand, activity of CYP2D6 increased significantly by 25% in first trimester, 34% in second trimester, and 47% in third trimester compared to the postpartum period (Tracy, 2005). Also, CYP3A activity significantly increased by 35-38% during all pregnancy trimesters compared to the same subjects during the postpartum period (Tracy, 2005).

Results from *in vitro* experiments demonstrated significant changes (induction or inhibition) in specific isoforms of CYP450 and UGT enzymes by estradiol, progesterone, human growth hormone, pituitary growth hormone, cortisol, placental lactogen, and prolactin in primary cultured human hepatocytes (Choi, 2013; Papageorgiou *et al*., 2013). This implies an alteration in the clearance of drugs that are metabolized by these enzymes. However, the mechanism of how these changes occur has not been fully evaluated.

1.2.4 Drug Elimination.

The cardiovascular system also changes physiologically during pregnancy with 30-60% increase in cardiac output during the third trimester which leads to an increase in blood flow to the liver,

kidneys, uterus, and placenta (Hodge, 2007). Due to the increase in cardiac output, the total hepatic blood flow increases by 160% in the third trimester compared to non-pregnant women, which can significantly enhance the metabolic capacity of phase I and II enzymes, leading to increased drug exposure for hepatic metabolism, as well as influencing the clearance of high extraction ratio drugs (Anger, 2008; Hodge, 2007; Nakai *et al*., 2002). Clinical studies have shown that clearances of intravenously administered nicotine (high extraction ratio drug) is increased by 60–70% during pregnancy as compared to the postpartum period (Dempsey, 2002). Increasing plasma volume also results in increases in the glomerular filtration rate and increase in renal blood flow of up to 50%, leading to increased drug renal clearance and decreased elimination half-life (Baylis, 1982). Examples of drugs that are renally excreted are digoxin, atenolol, ampicillin, cefazolin, and aminoglycosides (Baylis, 1982; Hebert *et al*., 2005). Increases in the renal clearance of atenolol by 36% have been reported during pregnancy compared to the 3-month postpartum period (Hebert *et al*., 2005). Moreover, renally eliminated active metabolites, such as morphine-6-glucuronide (the active metabolite of morphine), were reported to have high clearance in late pregnancy, resulting in a lower therapeutic effect (Koren, 2011).

In summary, absorption, distribution, metabolism and elimination can be altered during pregnancy. In the following section, we will discuss hormonal regulation of drug metabolism.

1.3 HORMONAL REGULATION OF DRUG METABOLIZING ENZYME EXPRESSION

Drug metabolism can be classified into two phases. Phase I reactions include primarily cytochrome P450 (CYP450) enzymes, which convert drugs to more polar metabolites through oxidation and chemical reduction. CYP1, CYP2, and CYP3 families are the major CYP enzymes responsible for most phase I reactions (Shimada *et al*., 1994). Among all 18 CYP enzymes that have been identified in humans, CYP3A and CYP2C are the most abundant hepatic subfamilies, which account for 30% and 20%, respectively, of the total amount of hepatic CYP enzymes (Shimada, 1994). CYP 3A4, 2C9, 2C8, 2E1, and 1A2 have the highest expression in the liver, while CYP 2A6, 2D6, 2B6, 2C19, and 3A5 are the lowest expressed P450 enzymes (Zanger and Schwab, 2013). CYP3A is responsible for about 60% of the P450-mediated metabolism of currently available drugs (Michalets, 1998).

Phase-II conjugation reactions are responsible for the metabolism of many endogenous and exogenous compounds and comprise of glucuronidation, sulfation, methylation, acetylation, glutathione conjugation, amino acids conjugation, and sulfoxidation (King *et al*., 2000). Glucuronide conjugation is one of the major detoxification and elimination pathways for endogenous and exogenous chemicals. It accounts for more than 35% of all phase II drug metabolism pathways (Refuerzo, 2005; Tukey and Strassburg, 2000). Glucuronide conjugates of drugs are normally excreted through biliary membrane transporters. UGT1A and UGT2B (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, and UGT2B15) are the major subfamilies responsible for glucuronidation of many dietary chemicals, drugs, and their metabolites (Mackenzie *et al*., 2005). Expression of these enzymes is influenced by multiple

factors including xenobiotics, cytokines, hormones, sex, age, as well as genetic polymorphisms (Zanger, 2013).

Pregnancy is characterized by dramatic increases in the concentration of female hormones (Figure 1-2). Pregnancy is known as an estrogenic condition with a 100-fold increase in estradiol, the hormone responsible for maturation and growth of fetal organs (Buster and Abraham, 1975). Progesterone, the hormone that plays a key role to maintain gestation, increases significantly during pregnancy from 30-40 ng/ml to 100-200 ng/ml (Tulchinsky and Hobel, 1973). It has been also reported that concentrations of estradiol and progesterone in normal, healthy women are 0.2−1 nM and 5-50 nM, respectively (Stricker *et al*., 2006). However, during pregnancy, estradiol and progesterone levels increase gradually up to 100 nM and 1 mM, respectively (Tulchinsky *et al*., 1972). Other estrogen hormones such as estrone and estriol, as well as 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin, are also dramatically increased during pregnancy, especially in the third trimester (Soldin *et al*., 2005; Tulchinsky, 1972).

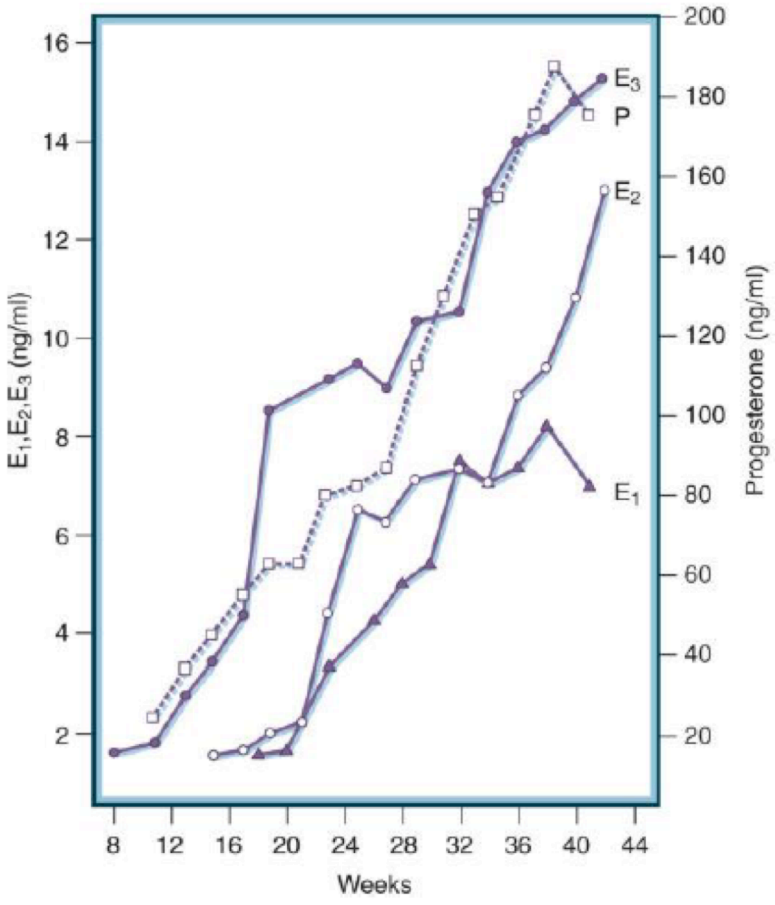

Figure 1-2 Plasma concentrations of female hormones during pregnancy. Adapted from (Tulchinsky, 1972).

Several studies reported that elevated levels of estradiol and progesterone are associated with many changes that occur throughout the gestational period (Moyer and Felix, 1998; Nilsen and Brinton, 2002). These high levels of estradiol and progesterone have been suggested to be responsible for the alteration in hepatic enzymatic expression and activity, such as CYP450 and UGT, by several studies. Data from a recent *in vitro* study reported that estradiol can increase the expression of CYP2A6, CYP2B6, CYP2C9, CYP3A4, and CYP2E1, while progesterone enhances the expression of CYP2A6, CYP2B6, CYP2C8, CYP3A4, and CYP3A5 in primary human hepatocytes (Choi, 2013). In addition, estradiol up-regulates the expression of UGT1A4 while progesterone up-regulates UGT1A1 expression in a concentration-dependent manner (Jeong *et al*., 2008).

Several assumptions have been made by many authors in supporting the role of female hormones in regulating drug-metabolizing enzymes. These assumptions are based on the evidence that the type and levels of female hormones may play a major role in modulating the expression and activity of many hepatic drug-metabolizing enzymes. It has been reported that changes in drug metabolism are different between females and males. For instance, the rate of elimination of drugs that are metabolized by CYP2A6, CYP2B6, and CYP3A4 are faster in females than in males (Anderson, 2005; Lamba *et al*., 2003). On the other hand, drugs that are metabolized by CYP1A2 have slower elimination rates in females than in males (Anderson, 2005). Since these alterations in drug metabolizing enzymes occur similarly in pregnancy, and due to the differences in the hormonal and physiological characteristics between females and males, this may indicate that female hormones can play a potential role in altering hepatic enzyme expression and activity. In addition, similar changes in the activity of CYP1A2, CYP2A6, CYP2C19, and UGT1A4 have been reported between pregnant women and oral

contraceptive users (Hodge, 2007). Also, high concentrations of progesterone up-regulate the expression of UGT1A1 (the major enzyme for labetalol metabolism) through activation of the pregnane X receptor (PXR) (Jeong, 2008). Together, this suggests that fluctuations of female hormone levels such as progesterone and estradiol could mediate changes in the clearance of drugs that are metabolized by these enzymes during pregnancy.

1.4 ALTERATION OF BILIARY DRUG TRANSPORTERS DURING PREGNANCY

Transporters are large proteins located in the biological membrane of different tissues such as liver, kidney, intestine, brain, and placenta (DeGorter *et al*., 2012). Drug transporters play an important role movement of drugs in and out of cell and in drug-drug interactions as well as in determining drug efficacy and safety (Feghali and Mattison, 2011). Based on their location and physiological function of how they transport drugs through the cell membrane, they can be classified as uptake or efflux transporters. Influx transporters expressed at the sinusoidal (basolateral) membrane of hepatocytes belong to the solute carrier (SLC) family, such as organic cation transporters (OCT), multidrug and toxin extrusion transporters (MATE), organic anion transporters (OAT), and organic anion transporting polypeptides (OATP), which are important in transporting substrates into the cells (Russel., 2010). On the other hand, efflux transporters expressed at the apical (canalicular) membrane of hepatocytes belong to the ATP-binding cassette (ABC) family (Russel., 2010). Efflux transporters facilitate the transfer of drugs from the intracellular to the extracellular environment by using energy, which results from ATP hydrolysis (Russel., 2010). Examples of ABC transporters are P-glycoprotein (P-gp), multidrug resistance associated proteins (MRP), breast cancer resistance protein (BCRP), and bile salt

export pump (BSEP) (International Transporter, 2010). Both influx and efflux transporters are important key factors in drug biotransformation and excretion pathways by regulating the drug access to the metabolizing enzymes (Shugarts and Benet, 2009). The therapeutic role of drug transporters in pregnancy and how they modify drug pharmacokinetics has not been studied (Feghali, 2011). Drug-drug interactions can occur at the site of transport proteins by either induction or inhibition (Feghali, 2011). For example, co-administering antiretroviral drugs together, such as saquinavir (P-gp substrate) and ritonavir (P-gp inhibitor), results in inhibition of Pgp by ritonavir, increasing the bioavailability of saquinavir (Alsenz *et al*., 1998; Eagling *et al*., 1999; Feghali, 2011; Huisman *et al*., 2001).

Several studies have focused on drug transporters during pregnancy, particularly placental transporters (Keitel *et al*., 2006). However, there is a great need for studying the regulation of other transporters in other organs during pregnancy, such as the ABC(Fenech *et al*., 2011) transporters at the canalicular side of hepatocytes, which is important elimination of glucuronide conjugates. Biliary excretion plays an important role in the detoxification and elimination of many drugs. Therefore, inhibition of biliary transporters may result in pathologic complications such as intrahepatic cholestasis of pregnancy (ICP) (Fattinger *et al*., 2001). Physiological changes during pregnancy and inherent genetic variations have been linked to variations in transporter expression and activity (Fenech, 2011). For example, hepatic uptake of steroid glucuronides and biliary secretion of glucuronides and dibromosulfophthalein were significantly altered in pregnant rats (Wen *et al*., 2013). MRP2 protein levels have been reported to be reduced significantly during pregnancy compared to the postpartum period (Cao *et al*., 2001). This suggests a regulation of these transporters during pregnancy. Progesterone levels increase significantly during pregnancy and have been reported to inhibit BSEP transporters

(Sharma *et al*., 2013). BSEP is expressed on the canalicular membranes and play an important role in transporting bile acids such as taurocholic acid (Sharma, 2013). Additionally, high serum concentrations of progesterone metabolites have been known to be associated with impaired biliary excretion and inducing trans-inhibition of BSEP, which leads to accumulation of bile acids and eventually causes toxicity (Fattinger *et al*., 2001). Recent studies demonstrated that 17α-hydroxyprogesterone caproate (17α-OHPC), a synthetic analog of progesterone used for preventing pre-term delivery, inhibited the taurocholate efflux with sandwich cultured human hepatocytes in a concentration-dependent manner (Sharma, 2013).

1.5 NUCLEAR MECHANSIM FOR REGULATION OF DRUG METABOLIZING ENZYMES AND BILIARY DRUG TRANSPORTERS EXPRESSION

It has been known that the binding of inducers such as rifampin and phenobarbital to certain nuclear receptors (e.g. pregnane X receptor (PXR), estrogen receptor (ER), and constitutive androstane receptor (CAR)) in the cytosol leads to homo-dimerization or translocation from the cytoplasm into the nucleus. These transcriptional regulators bind to a specific sequence of DNA or different response elements in the upstream region of the target gene, resulting in a nuclear receptor/DNA complex (Figure 1-3). Then DNA is transcribed downstream into messenger RNA and finally translated into proteins that will alter cell function. The underlying mechanisms or factors responsible for alterations in drug-metabolizing enzymes and transporters during pregnancy remain unclear. Therefore, understanding transcriptional regulation and metabolic activity of drug-metabolizing enzymes and transporters during pregnancy is very important in maintaining optimal therapeutic efficacy.

The effect of pregnancy on the mRNA and protein expressions of drug-metabolizing enzymes and transporters has been studies in mice and rats. These studies showed down-regulation of various cyps (except cyp2da1, cyp2d, and cyp3a13), ugts, uptake (except oat2) and efflux transporters (Table 1-3) and (Table 1-4). In mice and rats, it has been also reported that expression of hepatic nuclear receptors are decreased during pregnancy and is associated with down-regulation of many metabolizing enzymes and hepatobiliary transporters (Milona *et al*., 2010; Wen, 2013). Recent studies reported the association between transporters, like MDR1 and MRP2, and the metabolizing enzymes that can be regulated by the pregnane X receptor (PXR) (Jigorel *et al*., 2006). For example, rifampicin activates PXR and subsequently induces the MRP2 expression in human liver cells (Jigorel, 2006).

Table 1-3 mRNA Expression of Drug-metabolizing enzymes and transporters in pregnant mice

[a]Hepatic mRNA expression. [b]Renal expression

Increased expression	Decreased expression	Unchanged expression	Reference
Cyp26a1[a], cyp2d[a], Cyp3a13[a], ugt1a5[a]	Cyp1a2[a], cyp2c37[a], cypc50[a], cyp2c54[a], cyp2d22[a], cyp3a11[a], ugt1a1[a], ugt1a6[a], ugt1a9[a], ugt2a3[a], ugt2b1[a], ugt2b34[a], and ugt2b35[a]	Cyp2b10[a]	(Fortin *et al*., 2013; Topletz *et al*., 2013; Wen, 2013)
Oat2	Abca1[a], Abcg5[a], Abcg8[a], Atb8b1[a], Bcrp[a], Bsep[a], Mate 1[b], Mdr1b[b], Mdr2[a], Mrp2[b], Mrp4[b], Ntcp[a], Oatp1b2[a], Oct1[a],	Mrp1[b], Mrp3[b], Mrp5[b], Mrp6[b], Oatp1a4[a], Oat2b1[a]	(Aleksunes *et al*., 2012; Yacovino *et al*., 2013)

Table 1-4 Hepatic Protein Expression of Drug-metabolizing enzymes and transporters in pregnant rats

Increased expression	Decreased expression	Unchanged expression	Reference
-------	Ugt1a, ugt1a1, ugt1a5, ugt1a6, ugt2b1	-------	(Luquita *et al*., 2001)
-------	Mrp2, Mrp3, Mrp6, Oatp2	Mrp1, Oatp1	(Cao *et al*., 2002)

High concentrations of female hormones have been suggested to have the capability of influencing the expression of drug metabolizing enzymes via activation of nuclear receptors such as estrogen receptor α (ERα), PXR, and CAR (Chen, 2009; Jeong, 2008). Accumulating data shows that estradiol is an activator of ERα and CAR while progesterone is an activator of PXR (Jeong, 2008; Lehmann *et al*., 1998; Masuyama *et al*., 2003). Recent *in vitro* studies have shown that estradiol up-regulated the expression of CYP2A6 and CYP2B6, and this was mediated by the activation of ERα and CAR, respectively, in human hepatocytes (Choi, 2013; Koh K, 2009). Altogether, the findings from *in vitro* studies indicated that estradiol enhances the expression of UGT1A4 via ERα activation, and progesterone enhances UGT1A1 expression by PXR activation (Chen, 2009; Jeong, 2008). This suggests a potential mechanism for the increased clearance of lamotrigine (UGT1A4 substrate) and labetalol (UGT1A1 substrate) during pregnancy. This data indicates that female hormones may have the potential to regulate the expression and activity of drug-metabolizing enzymes and biliary drug transporters through certain nuclear receptors.

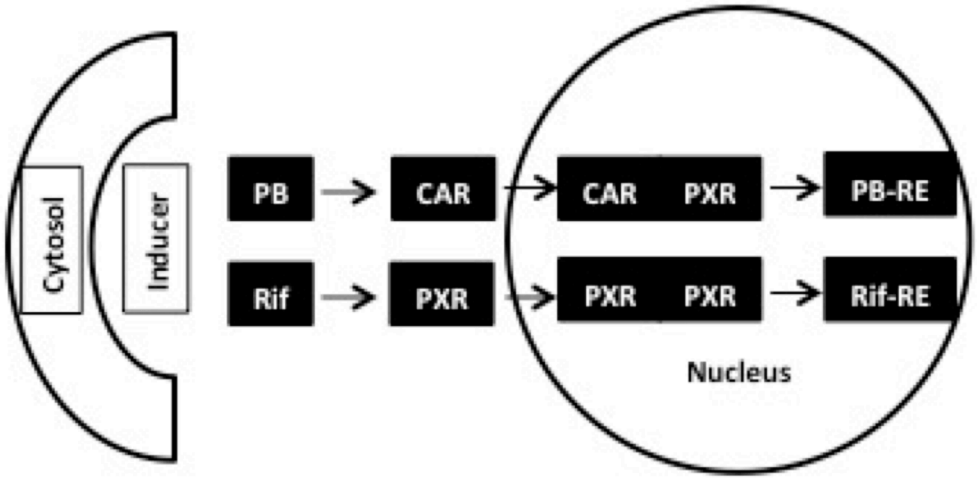

Figure 1-3 Nuclear mechanism of drug-metabolizing enzyme expression

PB: Phenobarbital; Rif: Rifampin; PXR: Pregnane X receptor; CAR: constitutive androstane receptor; PB-RE: Phenobarbital response element; Rif-RE: Rifampin response element.

1.6 HYPOTHESIS AND OBJECTIVES

Several health conditions and complications such as gestational diabetes, preeclampsia, epilepsy, and human immunodeficiency virus (HIV) exist during pregnancy. However, there is a lack of information about the drug disposition in pregnancy to optimize appropriate treatment of these disorders. As previously discussed, physiological changes during pregnancy are associated with alterations in drug pharmacokinetics. Multiple studies have demonstrated that elevated levels of female hormones, such as estradiol and progesterone, play an important role in regulating the expression and activity of hepatic CYP enzymes. However, these studies were mainly limited to estradiol and progesterone and their individual effect on CYP expression and activity. Human growth hormone, 17α-hydroxyprogesterone, and human chorionic gonadotropin also are very important steroidal hormones, and their concentrations also increase during pregnancy. Combining these hormones together will mimic the human physiological condition much closer than individual hormones.

Glucuronide conjugation is one of the major phase II drug metabolism pathways. Lamotrigine and labetalol are commonly used medications in pregnant women for treatment of epilepsy and hypertension, respectively. Data from clinical studies indicated that clearance of these medications is significantly increased in pregnant women, affecting the efficacy while the underlying mechanism has not yet been determined. A few animal studies have shown that biliary membrane transporters are altered in pregnant rodents. These findings are difficult to extrapolate to pregnant women due to the difference in expression of these transporters between animals and humans. Several nuclear receptors regulate the expression of different drug-metabolizing enzymes. Information about the effect of female hormones on the expression of these transcriptional receptors is very limited. Therefore, our primary aim was to characterize

how pregnancy influences the expression and activity of hepatic phase I and II drug metabolism, biliary drug transporters, and nuclear receptors. This will improve our understanding about how pregnancy alters drug disposition. We hypothesize that pregnancy related hormones have the potential to change the expression and activity of drug metabolizing enzymes and transporters, causing altered pharmacokinetics during pregnancy.

We used a two-dimensional of primary cultures of human hepatocytes to study the regulation of CYPs, UGTs and biliary drug transporters by pregnancy related hormones. Primary cultures of human hepatocytes express most of the phase I and phase II drug metabolizing enzymes, and many uptake and efflux transporters. Over the past several years, hepatocytes have been widely used to study drug metabolism in different aspects such as evaluating the drug-drug interactions, drug toxicity and induction or inhibition studies. Human hepatocytes used in our studies were isolated from premenopausal and postmenopausal female donors. Recent studies have shown that induction of CYP3A4 by growth hormone and cortisol is similar in premenopausal and postmenopausal donors (Papageorgiou, 2013), suggesting that hepatocytes model can be used from both donors to evaluate the regulation of drug metabolizing enzymes by pregnancy related hormones. In addition, some of the hepatocytes were isolated from female donors who had a history of chemotherapy. Several studies have reported that previous chemotherapy does not affect the function and integrity of hepatocytes (Hewes *et al*., 2006; Vondran *et al*., 2008).

To test our hypothesis, we first evaluated the effect of female hormone mixtures on the expression and activity of major CYP450 isoforms using primacy cultures of human hepatocytes isolated from human female donors. In this work, we used a validated CYP cocktail assay of enzyme-specific probe drugs (**chapter 2**). Our secondary aim was to examine the regulation of

UGT enzymes and the major nuclear receptors by individual and mixture treatment of female hormones. This was accomplished using primary cultures of human hepatocytes isolated from human female donors (**chapter 3**). Our third objective was to validate a physiologically based pharmacokinetics (PBPK) model for buprenorphine in non-pregnant women in order to evaluate the effect of pregnancy on buprenorphine pharmacokinetics using this model, and to compare the predicted and observed pharmacokinetic parameters in pregnant subjects (**chapter 4**). The fourth aim was to examine the effect of female hormones on both CYP and UGT metabolism together using buprenorphine as one of the medications used in pregnant women to treat pain and opioid addiction and simultaneously metabolized by both CYP and UGT pathways. In this study, we evaluated the effect of female hormones on the major pharmacokinetics of buprenorphine using primary cultures of human hepatocytes (**chapter 5**). The final aim was to characterize the influence of female hormones on the expression and activity of biliary drug transporters using sandwich cultured human hepatocytes isolated from human female donors (**chapter 6**).

2.0 EFFECT OF GESTATIONAL HORMONES ON THE EXPRESSION AND ACTIVITY OF HEPATIC CYP450 USING PRIMARY CULTURES OF HUMAN HEPATOCYTES

2.1 ABSTRACT

Pregnancy alters the pharmacokinetics of many drugs, but the underlying mechanism for these alterations is still unclear. The objective of this study was to examine the impact of gestational hormones on the activity and expression of hepatic cytochrome P450 (CYP450) enzymes. Primary human hepatocytes from five different female donors were treated for 72 hours with physiologic concentrations of gestational 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). Projected hepatic concentrations (10-fold higher) of the same hormones were also used in combination. The activities of five major cytochrome P450 enzymes were determined using a validated CYP cocktail assay with enzyme-specific probe drugs, CYP1A2 (phenacetin), CYP2C9 (diclofenac), CYP2C19 (S-mephenytoin), CYP2D6 (dextromethorphan) and CYP3A4 (testosterone). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for measuring the respective metabolite concentrations and the mRNA expression of the major CYPs was evaluated by qRT-PCR. Enzyme protein concentration was determined by western blot analysis. Our results showed that mixture of female hormones at projected hepatic concentrations (predicted concentrations of female hormones accumulated in the liver at third trimester of pregnancy) significantly enhanced mRNA expression, activity, and protein expression of CYP3A4. This observation is consistent with increased clearance of CYP3A4 substrate observed in pregnancy. However, female hormones did not have any effect on the expression and activity of other CYP450 enzymes tested (CYP1A2, CYP2C9, CYP2C19, and CYP2D6). These observations suggest that additional regulatory mechanisms might be responsible for the observed alteration in the *in vivo* effect of pregnancy on certain CYP enzymes.

2.2 INTRODUCTION

Many pregnant women use several medications. Pharmacokinetics of some of these drugs are altered during pregnancy. For example, clearance of drugs that are substrates of CYP2C9, CYP2D6, and CYP3A4 are reported to be increased (Hebert, 2008; Hebert, 2009; Tracy, 2005; Wolff, 2005; Yerby, 1990), while clearance of drugs that are metabolized by CYP1A2 and CYP2C19 are decreased (McGready, 2003; Tracy, 2005). However, the underlying physiological and biochemical mechanism of changes in the pharmacokinetics of these drugs remain unknown. Therefore, a better understanding of the underlying mechanism of pregnancy-mediated changes in drug metabolism is needed to improve drug therapy during pregnancy.

Several physiological changes occur during pregnancy. These are essential to facilitate the growth and development of the fetus (Granger, 2002; Hill, 2008). These changes have an impact on drug metabolism and pharmacokinetics of certain drugs. One of these changes is the significance increase in steroid hormones throughout the gestation. Progesterone and estradiol concentrations are substantially increased during pregnancy. Increased levels of other steroid hormones such as estriol, estrone, and 17α-hydroxyprogesterone have also been reported (Tulchinsky, 1972). Therefore, we hypothesized that pregnancy-related hormones have the capability to modulate the expression and activity of various drug-metabolizing enzymes during pregnancy. Previous studies have shown that estradiol, progesterone and growth hormone are the most important steroid hormones that alter the expression and activity of certain CYP450 enzymes (Bandiera and Dworschak, 1992; Ochs *et al*., 1986; Sakuma *et al*., 2004; Waxman *et al*., 1995). Estradiol increased the expression of Cyp3a41 and Cyp3a44 in mouse (Sakuma, 2004) and increased the expression of CYP2C7 in rat (Bandiera, 1992). Waxman *et al*., 1995 reported that growth hormone regulated the expression of CYP2A2 and CYP3A2 in

hypophysectomized rats. Progesterone also changed the rate of hepatic drug metabolism in rat (Ochs, 1986). However, due to the differences between animal and human in terms of expression and regulation of hepatic drug metabolizing enzymes, it is difficult to extrapolate data from animal species to humans.

Hepatocytes are exposed to a combination of female hormones (Isoherranen and Thummel, 2013). The synergistic effect of progesterone and estrogen has been reported in different tissues such as the brain (Nilsen, 2002). Additionally, progesterone inhibits the estrogen-mediated constitutive androstane receptor **(**CAR) transactivation in HepG2 cells (Kawamoto *et al*., 2000). It has been also suggested that high concentration of progesterone and estradiol may have a synergistic effect in regulating the expression and activity of certain CYP450 enzymes (Nilsen, 2002). The individual effect of estradiol and progesterone on the expression of certain CYP enzymes has been previously evaluated in hepatocytes (Choi, 2013; Papageorgiou, 2013). However the combination effect of these hormones and other female hormones such as estrone, estriol, 17α-hydroxyprogesterone and human growth hormone on the expression and activity of other major CYP enzymes are lacking.

Our aim is to comprehensively examine the effect of multiple important female hormones known to increase dramatically during pregnancy using a cocktail of probes for various CYP enzymes on the expression and activity of major CYP450 enzymes in primary human hepatocytes system.

2.3 MATERIAL AND METHODS

2.3.1 Chemicals

Progesterone (P), 17α- hydroxyprogesterone (17α-OHP), estrone (E1), estradiol (E2), estriol (E3), human growth hormone (hGH) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). CYP450 enzymes substrates, corresponding metabolites and internal standards (IS) were obtained from Sigma-Aldrich (St. Louis, MO) or Toronto Research Chemicals (Toronto, Ontario, Canada). Cell lysis buffer was purchased from Roche (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 (Danvers, MA). Polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad (Hercules, CA). Enhanced chemiluminescence substrate was purchased from Thermo Fisher Scientific (Rockford, IL).

2.3.2 Human Hepatocyte Incubation of Female Hormones and Treatment with a Cocktail of CYP450 Substrates

Freshly isolated primary human hepatocytes in maintenance media from five different female donors were purchased from Life Technologies (Carlsbad, CA) as monolayers (1.5×10^6 cells/well) in 6-well plates. The viability of human hepatocytes was greater than 87%. Hepatocyte donor demographics are shown in (Table 2-1**)**. Upon arrival, the media was replaced with Hepatocytes Maintenance Medium Medium (HMM^{TM}) (Lonza, Allendale, NJ) containing 1 μM dexamethasone, 4 μg/mL insulin, and 10000 U/mL penicillin/streptomycin. The hepatocytes

were maintained at 37°C in a humidified atmosphere with 5% CO_2. The baseline activity was determined by treating hepatocytes with 0.1% (v/v) of dimethyl sulfoxide (DMSO) as vehicle control. The response of the hepatocytes to 10 μM of rifampin as CYP3A4 inducer and 10 μM of ketoconazole as CYP3A4 inhibitor was also evaluated. Cells were also treated with vehicle (DMSO) as a control, or a mixture of low and 10 times higher concentration of a mixture of female hormones corresponding to the third trimester plasma concentration of these female hormones in pregnancy as listed in (Table 2-2**)** for 72 hours. Low hormone concentrations simulated the concentration of these hormones observed in plasma at third trimester of pregnancy, while high hormone concentrations simulated those in the liver during the third trimester according to the log p values of these hormones. Treatment medium was replaced every 24 hours for the 72-hour period of incubation. On day 4, the medium was replaced with fresh HMM^{TM} containing a cocktail of CYP substrates for 60 min: [100 μM phenacetin (CYP1A2), 90 μM diclofenac (CYP2C9), 50 μM S-mephenytoin (CYP2C19), 20 μM dextromethorphan (CYP2D6) and 250 μM testosterone (CYP3A4). After incubation, culture medium supernatant was collected to measure the metabolites of the selected CYP450 substrates. Hepatocyte total RNA was extracted using Trizol. Expression of mRNA of the selected CYP450 enzymes was measured using qRT-PCR. Protein levels of CYP3A4 in all hepatocytes were determined by western blot analysis. Under the microscope, there were no significant morphological changes observed in the hepatocyte culture over the 72 hours incubation period.

Table 2-1 Hepatocyte donors 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

* This batch was not treated with human growth hormone
ND: no data

Table 2-2 Female hormone concentrations at third trimester of pregnancy in humans

Hormone	Low conc. (µM)	High conc. (µM)
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

2.3.3 CYP450 Cocktail Activity Measurement and Sample Preparation

A validated method of LC-MS/MS assay was used to quantitate the metabolites formed from the substrates. In a micro-centrifuge tube, a mixture of 200 µL of cell medium, 20 µL of internal standard (IS), and 500 µL of water was added and contents passed through Waters Oasis HLB 1 Ml (30mg) extraction cartridge, previously conditioned with 1 mL methanol and 1 mL water. After washing with 1 mL of 5% methanol, the analytes retained on the column were eluted with 1 mL of methanol and the eluent was evaporated for dryness. The residue was reconstituted with 100 µL of 50% methanol. 20 µL of the solution was finally injected into a LC-MS/MS system.

2.3.4 Chromatographic and Separation Condition

Sample analysis was performed using Micromass Quattro triple quadrupole mass spectrometer interfaced with electrospray ionization probe (Waters 2759 LC model). Chromatographic condition was performed using a Luna C8 column (150 x 3.0 mm, 5µm). A mobile phase consisted of solvent A (95% H_2O, 5% methanol containing 0.02% ammonium formate) and solvent B (methanol containing 0.02% ammonium formate). Gradient elution (delivered at 0.3 mL/min) was performed using 1-minute initial increase of B from 3 to 100%, followed by maintaining B at 100% for 4 minutes in order to achieve the baseline. The retention times of acetaminophen, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrophan, and 6β-hydroxytestosterone were 6.0, 7.5, 6.6, 6.1, and 7.0 min, respectively. The precursor ions and their daughter ions products used for selected reaction monitoring in the positive-ion ESI mode for acetaminophen, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrophan, and 6β-hydroxytestosterone were *m/z* 152 ➔ 110, 312 ➔ 230, 235 ➔ 150, 257 ➔ 157, 305 ➔ 269,

respectively. The lower limit of quantification for acetaminophen, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, dextrophan, and 6β-hydroxytestosterone were 0.1, 1, 2, 0.1 and 0.1 ng/mL, respectively. The assay was validated with precision (coefficients of variation ≤15%), specificity and accuracy (≥85%).

2.3.5 Determination of mRNA Expression

Total RNA was extracted from the cells using Trizol reagent. Briefly, 0.2 mL of chloroform for each one mL of Trizol reagent was added. The clear supernatant layer was then transferred to new eppendorf tube and RNA was precipitated by adding 1.5 volume of isopropanol. The sample was centrifuged at12000 rpm at 4 °C for 30 minutes, and supernatant was removed. Ethanol 75% was added to wash out remaining solvent. Sample was then centrifuged. After completely drying the sample, RNA was dissolved in 30 μL of RNase-free water. The concentration of RNA was determined using nanodrop spectrophotometer at 260 nm. Pure RNA was used to synthesize the first strand of cDNA by reverse-transcription reaction using iScript TM Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). The following primers for each CYP450 enzymes were used: aggtcaaccatgacccagag and agggcttgttaatggcagtg for CYP1A2, cctctggggcattatccatc and atatttgcacagtgaaacatagga for CYP2C9, cctcgggactttattgattgct and ccagctccaagtaagtcagc for CYP2C19, acaccatactgcttcgacca and cagcccattgagcacgac for CYP2D6, agagctcttcagaacttctcct and tctggttgaagaagtcctcct for CYP3A4, and ctcaagggcatcctgggctaca and tggtcgttgagggcaatgcc for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). PCR reaction mixture was prepared by mixing 2 μL cDNA sample, 1 μL GAPDH or CYP gene (primer of interest), 10 μL SYBR Green master mix and 7 μL water. After initial denaturation at

95°C for 10 minutes, 40 cycles of amplification were performed with denaturation at 95°C followed by annealing and extension performed at 60°C for 1 minute. To identify PCR products, dissociation curves were used in the reaction. The relative levels of mRNA of CYP450 genes were normalized with the copy number of GAPDH. The relative levels of mRNA fold changes of all genes were quantified using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001).

2.3.6 Western Blot Analysis

Hepatocyte pellets were collected in phosphate buffered saline (PBS) and then centrifuged at (10,000 G at 4ºC for 10 minutes). The cells were lysed as per the procedures reported earlier (Pillai *et al*., 2013). Briefly, 125 µL cell lysis buffer was added to the cell pellet, sonicated to lyse the cells, and total protein levels were measured using bicinchoninic acid assay (BCA) (Smith *et al*., 1985). Twenty-five micrograms of proteins were loaded onto 10% SDS-polyacrylamide gel and electrophoresis was used for separation. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 90V for 1.5 hours. The membrane was 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 primary monoclonal antibody against CYP3A4 (1:1000 dilution) or rabbit anti-human monoclonal antibody against β-actin (1:1000 dilution) in 5% BSA. After washing with TTBS, the membrane was incubated with anti-rabbit IgG linked with horseradish peroxidase (1:3000 dilution) for 1 hour 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 1.48V (http://imagej.en.softonic.com).

2.3.7 Prediction of Female Hormones Interactions with CYP3A4

A computational approach to predict the interactions of female hormones (ligands) with CYP3A4 was utilized. The crystal structures of esrtone, estradiol, estriol, progesterone and 17α-hydroxyprogesterone were obtained from protein data bank (PDB). SYBYL® X1.3 software from Tripos Inc. was used to generate the binding pockets by the reported co-crystalized binding pockets. Binding pockets were confirmed by extracting the ligands and re-docking the crystalized ligands with the protein virtually. Important amino acid interactions with the ligands have been checked. The docking scores were calculated utilizing the CScore calculation methods. Final image refinements were performed using PyMol (Schrodinger).

2.3.8 Statistical Analysis

All the experiments were carried out in duplicate in each of the five sets of hepatocytes. The data was expressed as the mean ± SEM. Activity and expression of CYP450 enzymes in treated groups were expressed relative to the vehicle (control group). The differences in activity and expression between the incubation with low hormone concentrations or high hormone concentrations with control group were compared using student's *t*-test. The statistical significance was considered if the p value < 0.05.

2.4 RESULTS

2.4.1 Effect of Low and High Concentrations of Female Hormones Mixtures on the Activity of CYP450

Hepatocyte metabolic capacity was examined by treating cells with prototypical inducer (rifampin) and inhibitor (ketoconazole), and the magnitude of change in CYP3A4 enzyme activity was determined by measuring testosterone 6β-hydroxylation. Rifampin increased CYP3A4 activity by 5-fold, while ketoconazole decreased CYP3A4 activity to 30-50% of control (Figure 2-1). Treatment with female hormone combinations at predicted liver concentrations significantly enhanced the activity of CYP3A4. Contrary to the reduced activity of CYP1A2 during pregnancy (Tracy, 2005), our data showed no effect of female hormones on the activity of CYP1A2. Additionally, there was no change in the activity of CYP2C9, CYPC19, or CYP2D6.

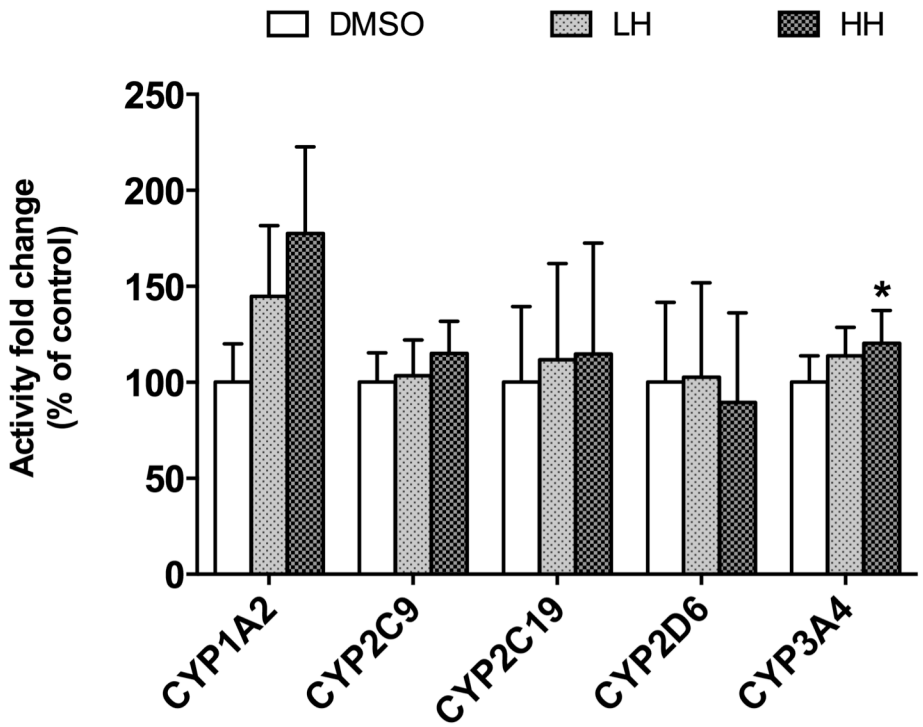

Figure 2-1 Effect of female hormones on the activity of CYP450 enzymes

Activity of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A was measured by quantifying their corresponding metabolites. Experiments were conducted in duplicate for each hepatocyte (n=5), and results are expressed as mean ± SEM. *$p < 0.05$ compared with control.

2.4.2 Effect of Low and High Female Hormone Mixtures on the Protein Level of CYP3A4

To examine the impact of female hormones on the expression of CYP3A4, the protein level of CYP3A4 was quantified after treatment with low and high hormonal mixtures in five different primary cultures of human hepatocytes (Figure 2-2). Protein level increased significantly by 1.4-fold after treatment with high concentrations of the hormones combination ($p<0.005$). The influence of high hormone concentrations on the protein expression of CYP3A4 was compared with rifampin, a known CYP3A4 inducer and PXR activator, in three different primary cultures of human hepatocytes. Rifampin significantly induced CYP3A4 protein expression by 2.6 fold ($P<0.05$). Based on the studies of Pharmaceutical Research and Manufacturers of America (PhRMA) in determining the extent of inducers in *in-vitro* and *in-vivo* drug-drug interactions (Bjornsson *et al*., 2003), a mixture of female hormones at accumulated liver concentrations seems to be an inducer of CYP3A4 by increasing the protein expression of CYP3A4 by more than 50% relative to rifampin induction.

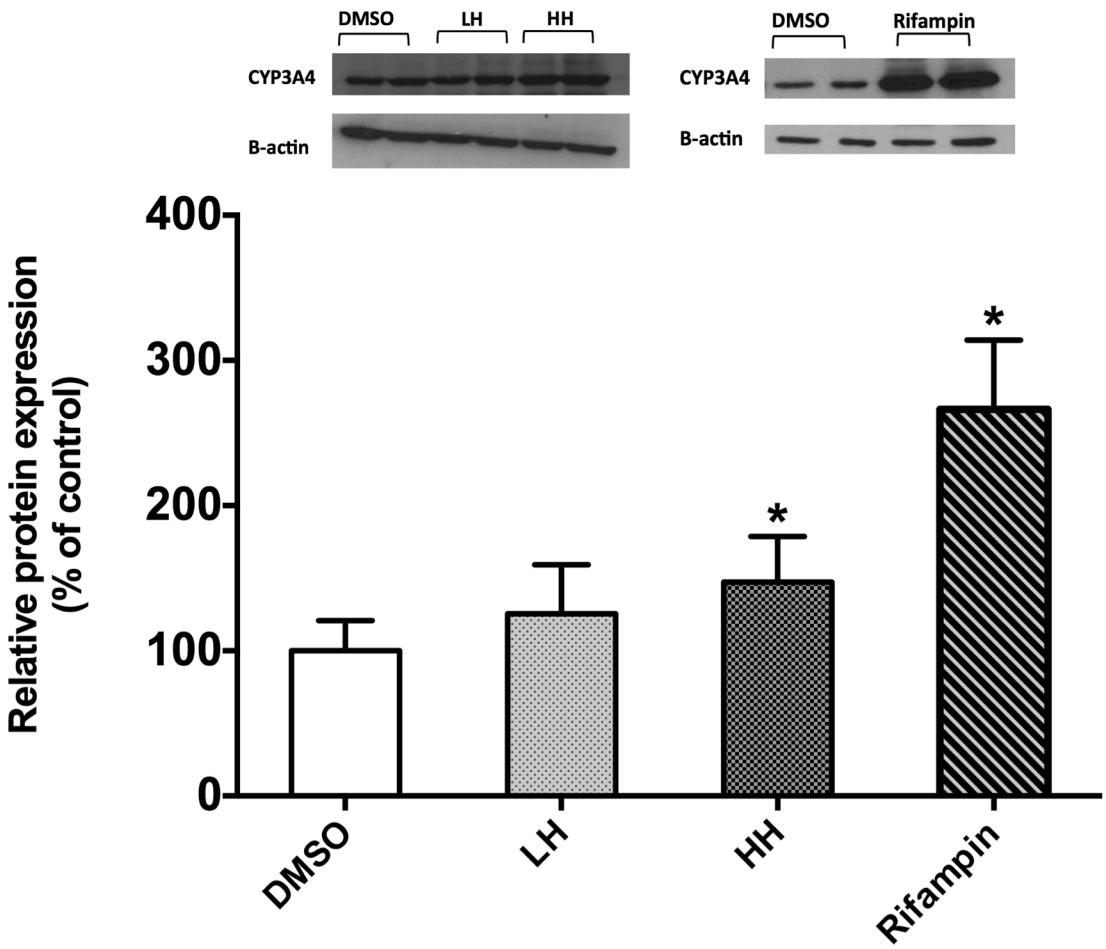

Figure 2-2 Effect of female hormones and rifampin on CYP3A4 protein expression.

Protein expression of CYP3A4 in three human hepatocytes treated with DMSO (control), low and high hormone concentration mixtures, and rifampin. Densitometry readings of CYP3A4 protein expression were normalized to β-actin and control group was arbitrarily defined as 100%. A representative western blot performed in duplicate is shown. (Experiments were conducted in duplicates for each hepatocyte, and results are expressed as mean ± SEM. *p<0.05 compared with control.

2.4.3 Effect of Low and High Female Hormones Mixtures on the mRNA Expression of CYP450

The impact of low and high hormone concentrations on mRNA expression of the various CYP enzymes was measured using qRT-PCR (Figure 2-3). The expressions of CYP1A2, CYP2C19, and CYP2D6 were not changed. The mRNA expression of CYP2C9 was increased by 1.4- and 1.8-fold at low and high concentrations, respectively. CYP3A4 mRNA expression was increased approximately 2-fold at low concentrations and 4-fold ($p < 0.05$) at high concentrations of gestational hormones. (Figure 2-4) shows that the protein level of CYP3A4 was correlated with the mRNA expression of CYP3A4 after treatment with high hormone mixture ($r^2 = 0.8$, $p<0.001$).

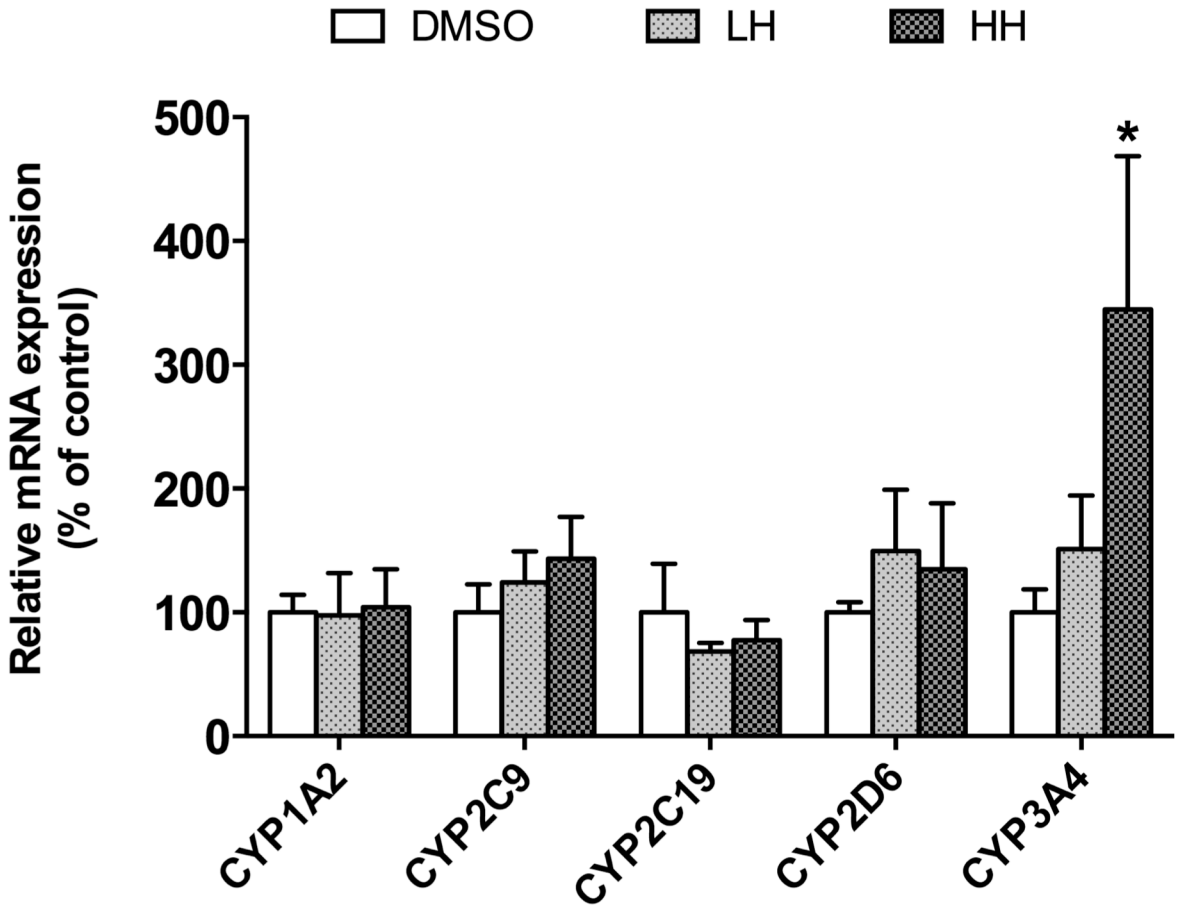

Figure 2-3 Effect of female hormones on CYP450 mRNA expression.

Expression of CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A was measured by qRT-PCR and normalized to the mRNA expression of GAPDH. Experiments were conducted in duplicate for each hepatocyte, and results are expressed as mean ± SEM. *$p < 0.05$ compared with control.

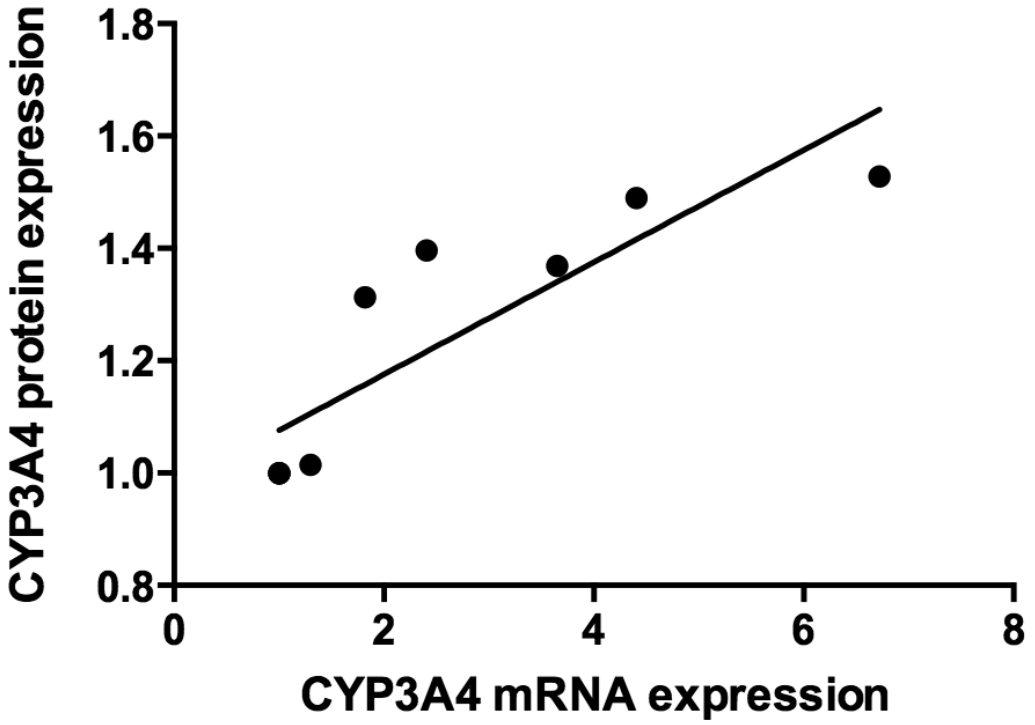

Figure 2-4 Effect of female hormones on CYP450 mRNA expression.

Correlation between CYP3A4 protein and mRNA expression in primary culture of human hepatocytes (r=0.8, *p* value= 0.002).

2.5 DISCUSSION

Results from clinical studies indicate that clearance of CYP3A4 substrates is increased during pregnancy and was found to be higher in females than in males (Anderson, 2005; Hebert, 2008; Hebert, 2009; Tracy, 2005; Wolff, 2005). indicating the potential role of female hormones in altering drugs metabolized by CYP3A4 during pregnancy. We hypothesized that female hormones may be responsible for the increased expression and activity of various CYP450 enzymes in the liver and would contribute to the altered pharmacokinetics of several drugs in pregnant women. The concentrations of female hormones used throughout the study were tested in hepatocytes and presented similar concentrations as those in the third trimester of pregnancy (Choi, 2013; Tulchinsky, 1972).

Our results showed that pregnancy-related hormones at predicted liver concentrations similar to that at the end stage of pregnancy increased the activity, protein, and mRNA expressions of CYP3A4 significantly. This was similar to the observed increase in CYP3A4 activity using dextromethorphan N-demethylation as a marker of CYP3A4 activity during the third trimester of pregnancy (Tracy, 2005). A positive correlation between the protein and the mRNA expressions of CYP3A4 indicating that the observed increase in the CYP3A4 protein levels by high concentrations of female hormones mixture might be driven by the increased expression of CYP3A4. It has been reported that pregnancy significantly increases cyp3a protein levels in mice (Zhang *et al*., 2008), which is consistent with our findings. It has also been reported that high concentrations (similar to level of these hormones in the third trimester of pregnancy) of estradiol, progesterone, and a combined treatment of placental growth hormone, growth hormone, and cortisol increases the expression of CYP3A4 significantly in human hepatocytes, which is also in agreement with our data (Choi, 2013; Papageorgiou, 2013). The

findings in our study may have important clinical implications, as we considered the effect of a mixture of six important female hormones and evaluated their effect on human CYP activities using a simultaneous measurement of specific and sensitive CYP cocktail assay. We also measured the mRNA and protein expression of CYP enzymes.

CYP3A4 and CYP3A5 are the major forms of CYP3A expressed in human liver. CYP3A4 is the most abundant CYP450 isoform accounting about 30% of the total hepatic P450 enzyme in humans (Fahmi *et al*., 2010). It is responsible for about 60% of P450-mediated metabolism of currently available drugs (Michalets, 1998). CYP3A4 has a wider substrate range than CYP3A5 (Flockhart and Rae, 2003). Known substrates for CYP3A4 during pregnancy include midazolam, amlodipine, cyclosporine, clarithromycin, dexamethasone, diltiazem, erythromycin, hydrocortisone, methadone, nifedipine, verapamil, saquinavir, and ritonavir. Clinical observations demonstrated that clearance of midazolam, methadone, and nifedipine is increased during pregnancy (Hebert, 2008; Prevost, 1992; Wolff, 2005). Our data suggested that induction of CYP3A4 by female hormones might be responsible for the observed increase in the clearance of CYP3A4 substrates during pregnancy.

The underlying mechanisms or factors responsible for altering drug-metabolizing enzymes during pregnancy remain unclear. Therefore, understanding the transcriptional regulation and metabolic activity of drug-metabolizing enzymes during pregnancy is very important. Pregnane X receptor (PXR) and constitutive androstane receptor **(**CAR) are activated by different endogenous ligands such as steroid hormones (Hernandez *et al*., 2009). Estradiol has been reported to activate CAR and estrogen receptor (ER) in mouse and human hepatocytes, respectively (Chen, 2009; Kawamoto, 2000). Moreover, results from previous studies indicated that estradiol and progesterone at concentrations corresponding to that during pregnancy

increased the activities of PXR and CAR-driven promoters, respectively (Jeong, 2008; Lehmann, 1998). Ligand-activated transcription factors such as PXR and CAR are known to up-regulate the expression of CYP3A4 (Burk *et al*., 2004). This data suggested that female hormone-mediated increase of CYP3A4 expression is likely to be a PXR-and CAR-dependent mechanism.

Our findings showed that female hormones have no effect on the expression or activity of CYP1A2, CYP2C9, CYP2C19, and CYP2D6. Several studies indicate that estradiol and progesterone are rapidly eliminated from the body through hepatic metabolism (Goldzieher and Brody, 1990; Kuhl, 1990). It has also been reported that estradiol and progesterone at third trimester concentrations are rapidly depleted from hepatocytes maintaining medium (Choi, 2013; Koh *et al*., 2012; Papageorgiou, 2013), which may explain the absence effect of female hormones on the expression and activity of CYP1A2, CYP2C9, CYP2C19 and CYP2D6. We used phenacetin as a substrate for CYP1A2. CYP1A2 activity is known to be decreased during pregnancy (Tracy, 2005). This observation can be explained by the fact that both estradiol and phenacetin are substrates for CYP1A2, and this can lead to a competition between these two substrates, which can also influence the impact of estradiol on the activity of CYP1A2. Eugster *et al*., 1993 reported that estradiol did not inhibit CYP1A2 activity in microsomes using caffeine as a CYP1A2 substrate.

In conclusion, we evaluated the impact of pregnancy-related hormones on the expression and activity of major CYP450 enzymes in primary cultures of human hepatocytes. Our results showed that these female hormones at third trimester-estimated liver concentrations increased the enzyme activity, mRNA and protein expression of only CYP3A4, but did not change the expression or activity of other P450 enzymes. This data provides a biologically plausible mechanism for the alterations observed in hepatic CYP450 enzyme activity during pregnancy.

3.0 REGULTION OF HEPATIC UDP-GLUCURONOSYLTRANSFERASE ENZYMES BY FEMALE HORMONES USING PRIMARY CULTURES OF HUMAN HEPATOCYTES

3.1 ABSTRACT

Clinical studies have shown that pharmacokinetics of drugs that undergo glucuronide conjugation such as lamotrigine, labetalol, and zidovudine, are altered during pregnancy. However, the underlying mechanism remains unknown. Increased plasma concentrations of pregnancy related hormones, including estradiol and progesterone, have been suggested to influence the expression and activity of major cytochrome P450 (CYP450) enzymes. However, information regarding the regulation of UDP-glucuronosyltransferase (UGT) enzymes by female hormones is limited. The aim of this study is to evaluate the impact of estrogens (estrone, estradiol, and estriol), progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin individually and in combination for the expression and activity of major UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B7) as well as the expression of pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptor alpha (PPARα), and aryl hydrocarbon receptor (AhR) using primary cultures of human hepatocytes. Our results indicated that progesterone increases the expression and activity of UGT1A1. A mixture of female hormones at liver concentration also increased activity of UGT1A1. Estradiol induces the expression and activity of UGT1A4. Induction of UGT1A4 activity was also observed in human hepatocytes treated with a combination of female hormones at concentrations predicted in the human liver during the third trimester of pregnancy. On the other hand, human chorionic gonadotropin reduced the activity of UGT1A4. Progesterone induced mRNA level of UGT1A6 and UGT1A9, and human growth hormone enhanced the mRNA expression of UGT2B7. Expression of UGT1A3 and UGT2B7 was also increased by female hormone combinations at concentrations observed in the plasma during the third trimester of pregnancy. Human growth hormone also enhanced the expression of

UGT2B7. Female hormones have different effects on the expression of other UGTs and nuclear receptors. Our findings show that estradiol increased the expression of UGT1A1, UGT1A3, UGT1A9, and UGT2B7. Female hormones did not change the expressions of PXR, CAR and PPARα. AhR mRNA was up-regulated by estradiol and human growth homorne, suggesting that AhR can be a candidate-signaling pathway responsible for the observed changes in UGT1A, UGT1A3 and UGT2B7 (Goodwin *et al*., 1999; Lankisch *et al*., 2008; Yueh *et al*., 2003). These findings provide an important basis for understanding the mechanism of altered glucuronidation pathway during pregnancy.

3.2 INTRODUCTION

Phase II drug metabolizing enzymes play an important role in the detoxification of several endobiotics and xenobiotics to form more water soluble conjugates that can be easily excreted into the bile or urine (Jancova *et al*., 2010). Phase II enzymes include UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), and N-acetyltransferases (NATs) (Kohalmy and Vrzal, 2011). Glucuronidation reactions catalyzed by UGTs are considered to be the most important metabolic detoxification pathways among phase II reactions. Previous studies have indicated that UGTs are responsible for glucuronidation of about 40-70% of medications in human (Wells *et al*., 2004). Studies in pregnant women revealed that the pharmacokinetics of medications such as lamotrigine (Tran *et al*., 2002), labetalol (Fischer, 2014), and zidovudine (Watts, 1991) are significantly altered. However, the underlying mechanism has not been identified.

Regulation of UGT enzymes has been linked to sex-specific hormones in mice (Nicolson *et al*., 2010). For instance, hepatic UGT1A1 and UGT1A5 enzymes are expressed mostly in female mice. However, hepatic UGT2B1 is predominantly expressed in male mice (Buckley and Klaassen, 2009). These findings highlight the role of sex hormones in regulating the expression of UGTs.

Elevated levels of female hormones are some of the physiological changes seen during pregnancy. Plasma concentrations of estrogens (estrone, estradiol, and estriol) increase during pregnancy (Loriaux *et al*., 1972). Compared with estrone and estriol, plasma concentrations of estradiol are double that of estrone and four times that of estriol during human gestation (Loriaux, 1972). As pregnancy progresses, plasma concentrations of progesterone and 17α-hydroxyprogesterone increase (Seren *et al*., 1981). Plasma concentrations of human chorionic

gonadotropin reach its peak in the first ten weeks of pregnancy. After that, it declines to a plateau at 19 weeks of pregnancy (Braunstein *et al*., 1976; Korhonen *et al*., 1997).

Regulation of UGTs by pregnancy has been evaluated in pregnant mice and indicated that mRNA expression of Ugt1a1, 1a6, 1a9, 2a3, 2b1, 2b34, and Ugt2b35 are decreased by 40-80%. However, mRNA levels of UGT1A5 increased by 50-100% compared with virgin controls (Wen, 2013). It is however difficult to extrapolate these findings to humans due to specie differences in drug-metabolizing enzymes.

The effects of estradiol and progesterone on the expression and activity of hepatic UGTs have been evaluated in different human cell systems. In HepG2 cells, progesterone is shown to induce the expression of UGT1A1 by activating the pregnane X receptor (PXR) (Jeong, 2008). In HepG2 and MCF7 cells, estradiol up-regulates the expression of UGT1A4, mediated by estrogen receptor α (ERα) (Chen, 2009). The effects of other female hormones on the expression of other UGTs in human hepatocytes have not been evaluated.

This study aimed to examine the effects of multiple female hormones individually and in combination on the activity and expression of major UGTs in primary cultures of human hepatocytes. Effects of female hormones on the mRNA expression of UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B7 were evaluated. The activities of UGT1A1 and UGT1A4 were studied utilizing etoposide (Wen *et al*., 2007) and lamotrigine (Rowland *et al*., 2006), respectively, as specific probe substrates.

3.3 MATERIAL AND METHODS

3.3.1 Chemicals

Progesterone (P), 17α-hydroxyprogesterone (17α-OHP), estrone (E1), estradiol (E2), estriol (E3), human growth hormone (hGH), human chorionic gonadotropin (HCG), 4-methylumbelliferone (4MU), 4-methylumbelliferone glucuronide (4MU-G), etoposide (ETOP), etoposide glucuronide (ETOPG) and were purchased from Sigma-Aldrich (St. Louis, MO). TaqMan Primers for UGTs and nuclear receptors were purchased from Life Technologies (Carlsbad, CA) .

3.3.2 Incubation of Primary Cultures of Human Hepatocytes with Female Hormones and Treatment with Etoposide and Lamotrigine Substrates

Freshly isolated primary human hepatocytes in a cold maintenance media (1.5×10^6 cells/well) in 6-well plates were purchased from Life Technologies (Carlsbad, CA). Hepatocyte donor demographics are shown in (Table 3-1). Upon receipt, the media was replaced with ice-cold hepatocyte maintenance media (HMM™) (Lonza, Allendale, NJ) containing 0.35 mg/mL Geltrex™ for overnight incubation. The cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. The extent of baseline activity and response of the primary cultures of human hepatocytes were determined by treatment for 72 hours with 0.1 % dimethyl sulfoxide (DMSO) as vehicle control and 2 mM phenobarbital as a prototypical inducer of UGT1A6/9 activity. Cells were also treated with DMSO as a control, a low and 10-times higher concentration of female hormone mixture, high concentration of estradiol, progesterone, human growth hormone, and human chorionic gonadotropin (Table 3-2) for 72 hours to evaluate the

effect of these hormones on the expression and activity of different UGTs. Final concentration of DMSO was 0.1% (v/v). Treatment medium was replaced every 24 hours for the 72-hour period. On day 4, the medium was replaced with fresh HMM™ medium containing 100 μM of 4MU (UGT1A6/1A9 substrate) for 30 minutes, 400 μM of ETOP (UGT1A1 substrate) for 60 minutes and 40 μM of LTG (UGT1A4 substrate) for 24 hours. After the incubation time, the culture medium and cell lysate were collected to measure the formation of 4-methylumbelliferone glucuronide, etoposide glucuronide and lamotrigine-*N*-glucuronide. The total hepatocyte RNA was extracted using Trizol reagent. There were no significant morphological changes observed in hepatocyte culture. Each experiment was performed in triplicate and repeated twice. Statistical differences between control and treated groups were determined using student's *t*-test. A *p* value of less than 0.05 was considered statistically significant. Data is expressed as mean ± SEM.

Table 3-1 Hepatocyte donors demographic.

Donor ID	Age	Sex	Race	BMI	Smoking	Alcohol use
HU1718	59	F	Caucasian	39	YES	NO
HU1743	56	F	Caucasian	19	YES	YES
HU14016	82.	F	Caucasian.	ND	ND	ND

ND: No data

Table 3-2 Female hormones concentrations at third trimester of pregnancy in humans

Hormone	Low conc (μM)	High conc (μM)
Progesterone	2	20
E1	0.2	2
E2	0.3	3
E3	0.8	8
17-α OHP	0.1	1
hGH	0.0005	0.005
HCG	0.0009	0.009

3.3.3 4-methylumbelliferone glucuronide, Etoposide and Lamotrigine Glucuronidation

β-glucuronidase was used to hydrolize etoposide glucuronide as described in (Reder-Hilz *et al*., 2004). Samples were diluted with 1 mL methanol and then evaporated under a stream of nitrogen at 40°C and then dissolved in either 100 μL of sodium acetate buffer (100 mM, pH 5.0) and 50 μL β-glucuronidase solution (2000 U/ml) or 150 μL sodium acetate buffer (100 mM, pH 5.0). All samples were incubated in a water bath at 37°C overnight. 1.5 μL of 70% perchloric acid was added to 150 μL of samples and centrifuged at 13,000 rpm for 5 minutes. 50 μL of supernatant was injected into the HPLC system. For 4-methylumbelliferone and lamotrigine glucuronidation assay, 1.5 μL of 70% perchloric acid was added to 150 μL of all samples and centrifuged at

13,000 rpm for 5 minutes. Then, 20 µL and 100 µL of 4-methylumbelliferone and lamotrigine glucuronides supernatant samples, respectively were applied into the HPLC system.

3.3.4 HPLC analysis

4MUG, ETOPG and LTGG were quantified with an HPLC (Waters 2695 Separations Module, Alliance Analytical Inc.) and UV-detector (2998 Photodiode Array, Waters Corporation). A C8 column was used to seprate various components (Zorbax Eclipse XBD-C8 analytical column (4.6 X 15 cm; Rockland Technologies INC).

For quantification of 4MUG, the mobile phase consisted of solvent A (10 mM of phosphoric acid, pH 2.9) and solvent B (20% acetonitrile). UV absorption was measured at 316 nm and the flow rate was 1 mL/min. The following gradient was used: 90 % A for 0-4 min; then, mobile phase B increased to 50% over 4 min, and then the ratio returned back to the initial conditions for 3 min. Retention time for 4MUG was 6.8 minutes and the standard curves were linear from 0-20 µg/mL.

For etoposide quantification, the mobile phase consisted of solvent A (0.1 % formic acid) and solvent B (100% acetonitrile). UV absorption was measured at 254 nm and the flow rate was 1 mL/min. The following gradient was used: 90 % A for 0-3 min; then, mobile phase B increased to 40% over 5 min, and then the ratio returned back to the initial conditions for 1 min. Retention time for etoposide was 7.5 min. Peak areas of the authentic etoposide prepared from a concentration range of 1 to 300 µg/mL were used to quantify etoposide glucuronide formation.

Lamotrigine-*N*-glucuronide were analyzed using mobile phase consisting of solvent A (25 mM of phosphate buffer, pH 7.4) contains 200 µL of triethylamine for 1 L of total volume) and solvent B (100% acetonitrile). UV absorption was detected at 254 nm and the flow rate was

1 mL/min with using the following gradient: 91 % A for 0-3 min and held for 4 min, 91 % A for 7-8 min, 63 % A for 9-15 min and 91 % A for 16-30 min. Retention time for lamotrigine-*N*-glucuronide was 10.3 min. Peak areas of standard curve concentration of LTGG prepared from a concentration range of 0 to 20 μg/mL were used to quantify lamotrigine formation.

3.3.5 RNA Sample preparation and Quantitative Real-Time PCR Assay

1 mL of Trizol reagent was added to the plated cells to extract total RNA. Then, 0.2 mL of chloroform was added to separate each sample into two phases. RNA was precipitated by adding isopropanol (1.5 volume of the supernatant layer) to the supernatant layer. Then the sample was centrifuged at 12,000 rpm at 4°C for 30 minutes, and then the supernatant was removed. Thirty microliters of RNase-free water was added to the dried samples to dissolve the RNA. The concentration of RNA was determined using nanodrop spectrophotometer at 260 nm. Pure RNA was used to synthesize the first strand cDNA by reverse-transcription reaction using iScript TM Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA). The following primers were obtained for quantitative RT-PCR (qRT-PCR) analysis from Applied Biosystems (Foster City, CA): UGT1A1 (Hs02511055), UGT1A3 (Hs04194492), UGT1A4 (Hs01655285), UGT1A6 (Hs01592477), UGT1A9 (Hs02516855), UGT2B7 (Hs00426592), and GAPDH (Hs02758991) genes. The PCR amplifications were performed on the ABI Prism 7300 system (Applied Biosystems, Foster City, CA). The reactions were performed in 20 μL: 10 μL of TaqMan Master Mix, 1 μL of each TaqMan probe, 4 μL of cDNA (1 μg/ml), and 5 μL of RNase-free water. The reactions were performed under the following conditions: After initial denaturation at 95°C for 10 minutes, 40 cycles of amplification were performed with denaturation at 95°C for 15 seconds followed by annealing and extension performed at 60°C for

one minute. Dissociation curves were used in the reaction in order to identify PCR products. The relative levels of mRNA of all genes were normalized with the copy number of GAPDH. The relative levels of mRNA fold changes of all genes were quantified using the $2^{-\Delta\Delta C_T}$ method (Livak, 2001).

3.4 RESULTS

3.4.1 Effect of Female Hormones on UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 Expression

To investigate the role of female hormones on the regulation of UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B7 expression, mRNA expression of these UGTs was measured using qRT-PCR after treating the hepatocytes with 2 mM phenobarbital, low and high female hormones mixtures (estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin) as well as with individual treatments of high concentrations of estradiol, progesterone, human growth hormone, and human chorionic gonadotropin for 3 days. GAPDH was used as a housekeeping gene.

3.4.1.1 Effect of Female hormones on UGT1A1 Expression

Phenobarbital, estradiol, and progesterone induced UGT1A1 expression by 4-fold ($p < 0.005$), 1.7-fold ($p < 0.05$) and 1.6-fold ($p < 0.05$), respectively. Other hormones did not change the expression of UGT1A1 compared with control (Figure 3-1).

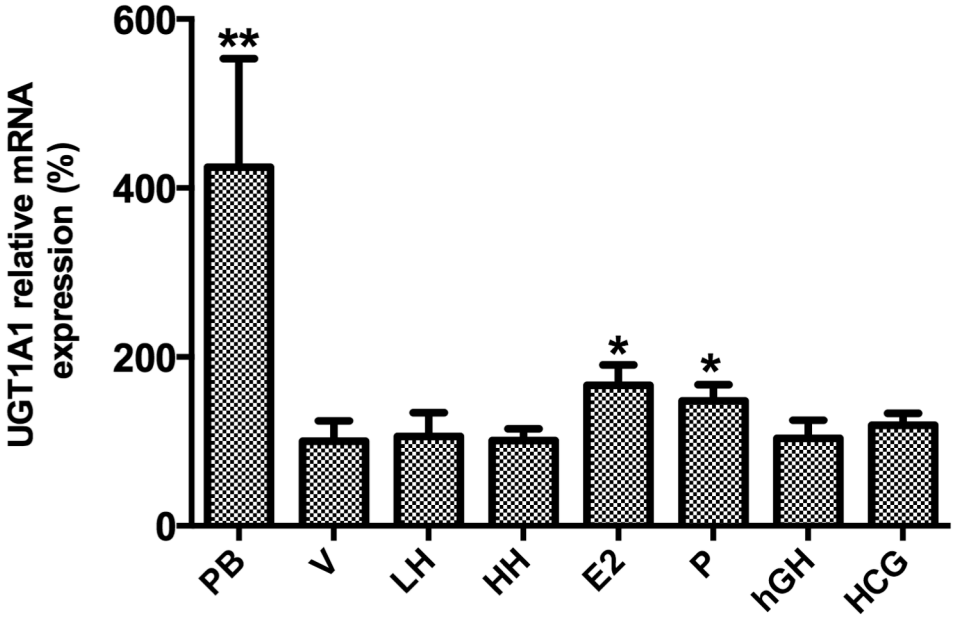

Figure 3-1 Effect of female steroid hormones on UGT1A1 expression.

The mRNA expression of UGT1A1 was determined after treating the primary cultures of human hepatocytes with female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) and ** ($p < 0.01$) compared to the control group.

3.4.1.2 Effect of Female hormones on UGT1A3 Expression

Phenobarbital and estradiol enhanced the expression of UGT1A3 by 3-fold ($p < 0.005$) and 1.9-fold ($p < 0.05$), respectively. Expression of UGT1A3 increased by 1.4-fold in the presence of low concentrated female hormone mixture ($p < 0.005$). However, other hormones did not affect the expression of UGT1A3 when compared to control (Figure 3-2).

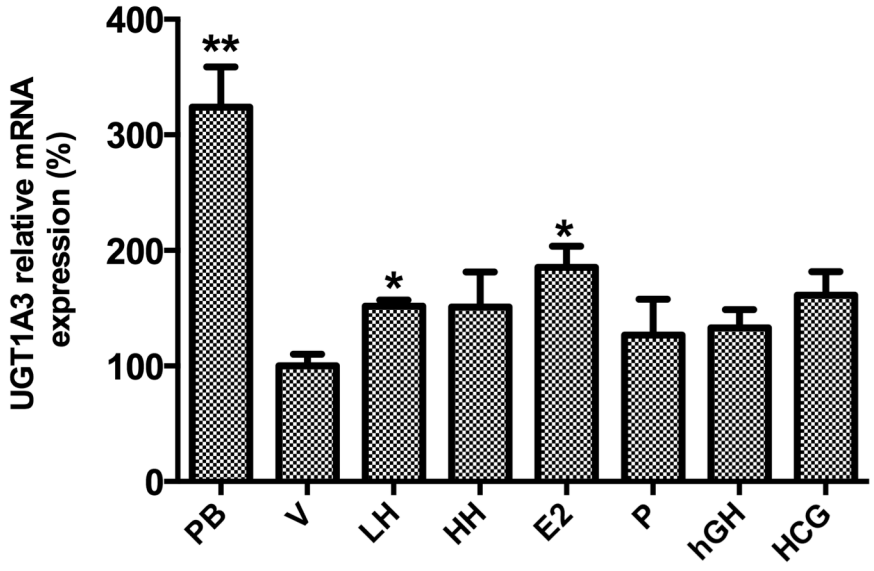

Figure 3-2 Effect of female steroid hormones on UGT1A3 expression.

The mRNA expression of UGT1A3 was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) and ** ($p < 0.01$) compared to the control group.

3.4.1.3 Effect of Female hormones on UGT1A4 Expression

UGT1A4 exhibited increased mRNA expression: 12-fold increase by phenobarbital ($p < 0.005$) and 1.4-fold increase ($p < 0.05$) by estradiol. UGT1A4 expression is not influenced by other female hormones (Figure 3-3).

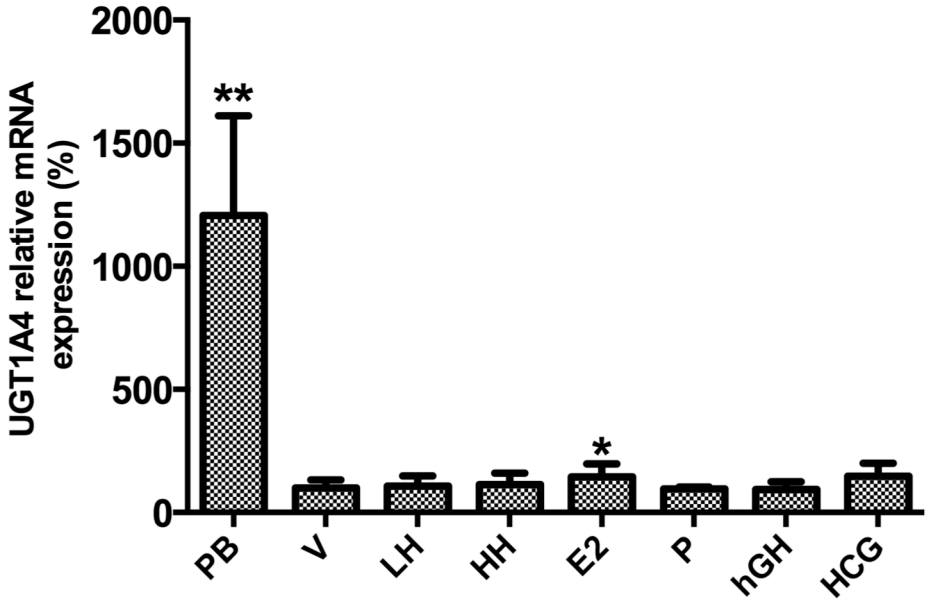

Figure 3-3 Effect of female steroid hormones on UGT1A4 expression.

The mRNA expression of UGT1A4 was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) and ** ($p < 0.01$) compared to the control group.

3.4.1.4 Effect of Female hormones on UGT1A6 Expression

As shown in (Figure 3-4), phenobarbital caused a 2-fold increase in the mRNA levels of UGT1A6 ($p < 0.05$). Progesterone and mixture of female hormones at low concentrations increased the expression of UGT1A6 by 1.2-fold and 1.4-fold, respectively ($p < 0.05$), whereas other female hormones showed no change in mRNA expression.

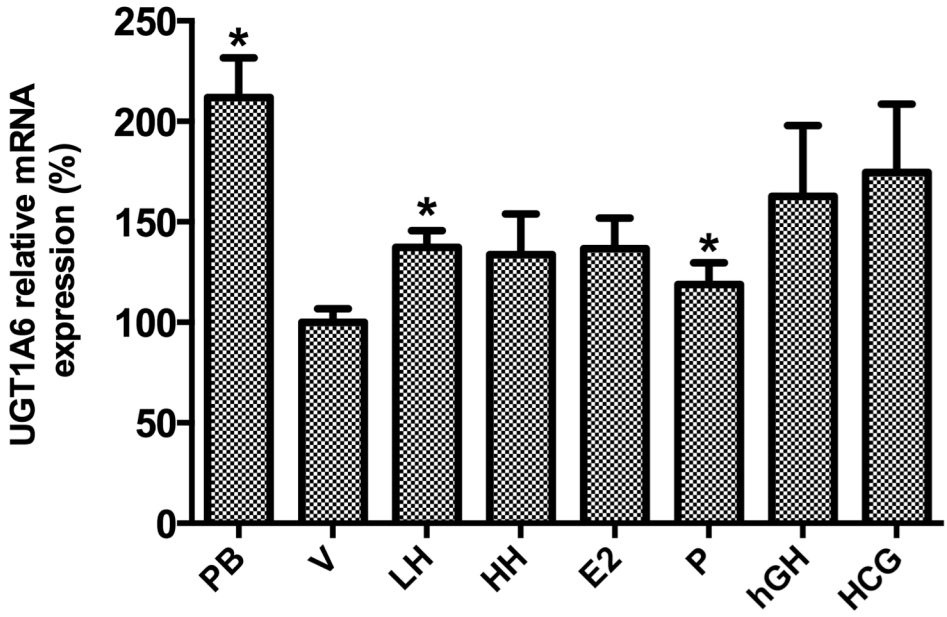

Figure 3-4 Effect of female steroid hormones on UGT1A6 expression.

The mRNA expression of UGT1A6 was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) compared to the control group.

3.4.1.5 Effect of Female hormones on UGT1A9 Expression

Only estradiol and progesterone showed significant increase in the expression of UGT1A9 by 1.5-fold ($p < 0.005$) and 1.4-fold ($p<0.0005$), respectively (Figure 3-5).

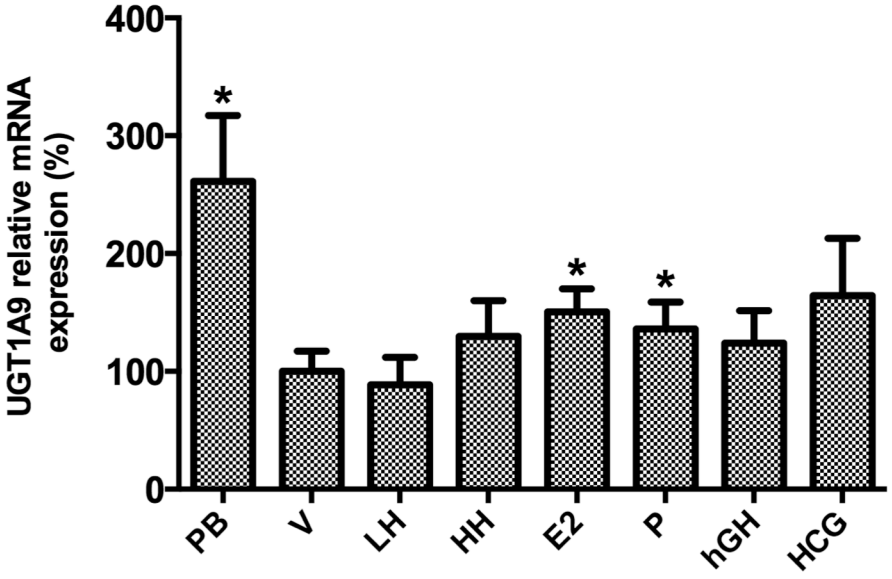

Figure 3-5 Effect of female steroid hormones on UGT1A9 expression.

The mRNA expression of UGT1A9 was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. ** ($p < 0.01$) and *** ($p < 0.001$) compared to the control group.

3.4.1.6 Effect of Female hormones on UGT2B7 Expression

The combination of female hormones and individual treatment with estradiol and human growth hormone increased the mRNA levels of UGT2B7 (Figure 3-6). Low concentration of female hormone mixtures increased expression of UGT2B7 by 1.3-fold ($p < 0.05$, and high concentrations of female hormone mixtures increased expression of UGT2B7 by 1.4-fold ($p < 0.05$). Estradiol and human growth hormone increased expression of UGT2B7 by 1.7-fold ($p < 0.05$) and 1.5-fold ($p < 0.05$).

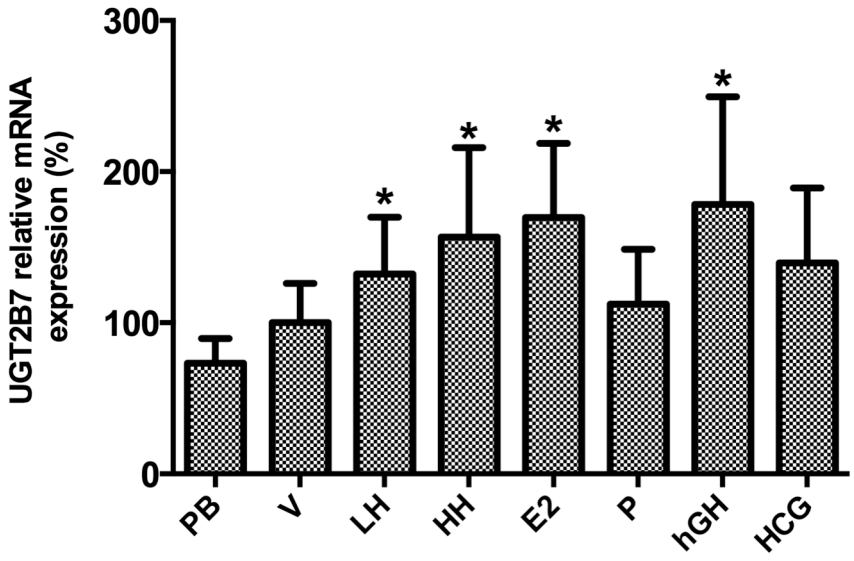

Figure 3-6 Effect of female steroid hormones on UGT2B7 expression.

The mRNA expression of UGT2B7 was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) compared to the control group.

3.4.2 Hormonal Regulation of UGT1A1 and UGT1A4 Activities

To examine the effect of female hormones on the activity of UG1A1, we initially validated our system by treating the cells with 2 mM phenobarbital as a positive control for 3 days. On day 4, we replaced the media with 100 μM of 4-methylumbelliferone for 30 minutes. We measured the activity of UGT1A6/1A9 by quantifying the concentration of 4-methylumbelliferone glucuronide (Figure 3-7)**.** Phenobarbital induced UGT1A6/1A9 activity by 85%, which confirms that our model system is responsive to external stimuli.

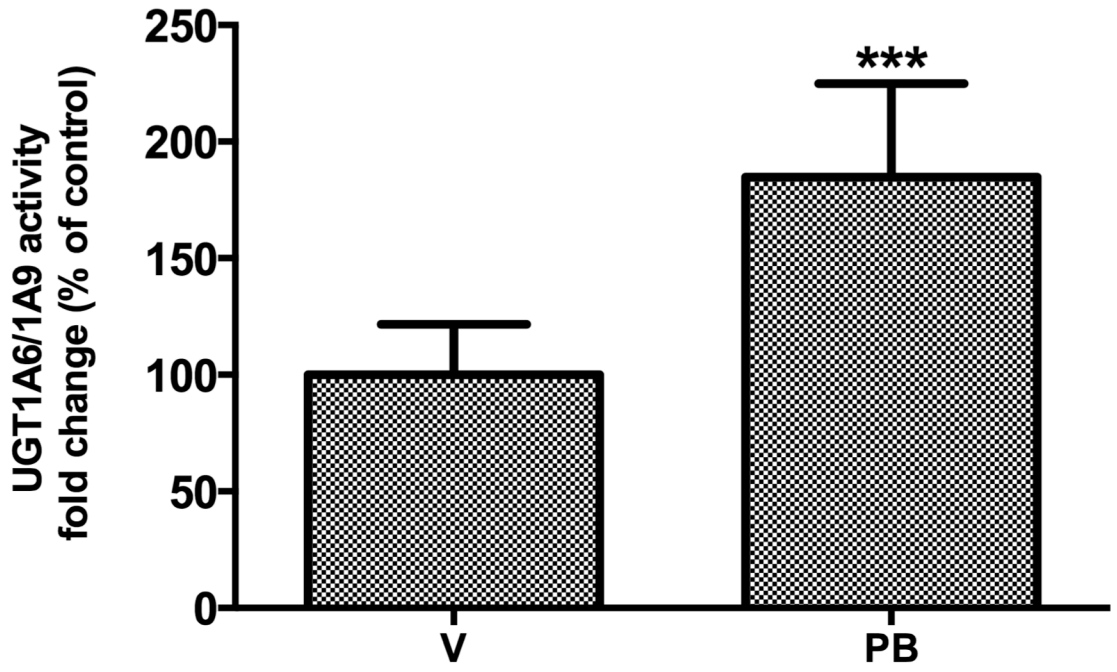

Figure 3-7 Effect of phenobarbital on UGT1A6/1A9 induction

Concentration of 4-methylumbelliferone glucuronide was measured to test the functional activity of UGT1A6/1A9. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. *** ($p < 0.001$) compared to the control group.

3.4.2.1 UGT1A1 Activity

Female hepatocytes were treated with mixtures of female hormones (estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin) at low and high concentrations as well as with high concentrations of estradiol, progesterone, human growth hormone, and human chorionic gonadotropin for 3 days. On day 4, hepatocytes were incubated with 400 μM of ETOP as UGT1A1 substrate for 60 minutes. The concentration of ETOP glucuronide was determined (Figure 3-8). Progesterone and mixture of female hormones at high concentrations significantly increased the activity of UGT1A1 by 2-fold ($p < 0.05$).

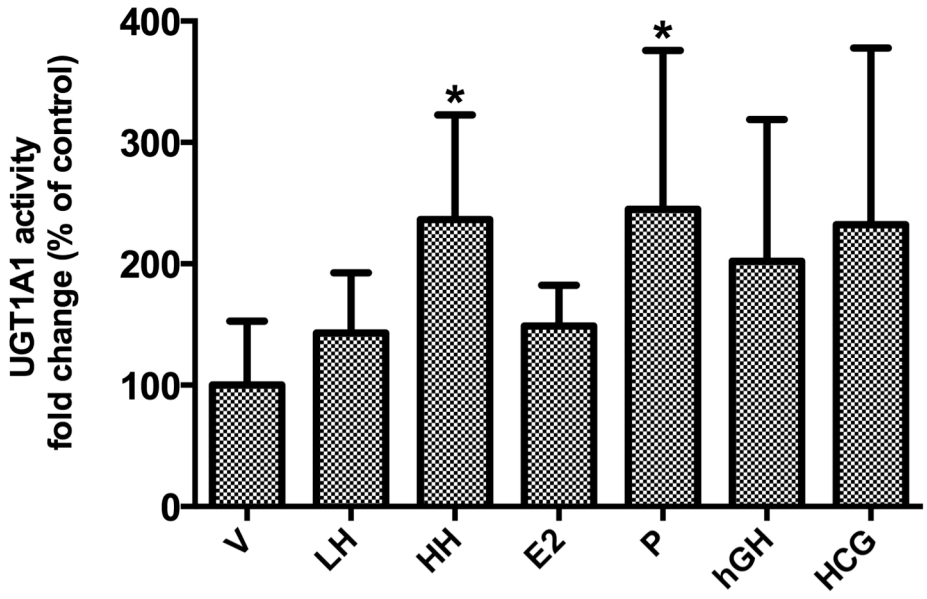

Figure 3-8 Effect of female steroid hormones on UGT1A1 activity.

Activity of UGT1A1 was measured by quantifying the formation of etoposide glucuronide after treating the primary cultures of human hepatocytes with female hormones chronically. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) compared to the control group.

3.4.2.2 UGT1A4 Activity

Female hepatocytes were treated with mixtures of female hormones (estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin) at low and high concentrations as well as with individual treatments of high concentrations of estradiol, progesterone, human growth hormone, and human chorionic gonadotropin for 3 days. On day 4, the media was replaced with 40 μM of LTG as UGT1A4 substrate for 24 hours. Then the concentration of LTGG was determined (Figure 3-9). High levels of female hormone mixtures increased the activity of UGT1A4 significantly by 1.3-fold ($p < 0.05$). However, human chorionic gonadotropin suppressed 65% of UGT1A4 activity significantly ($p < 0.05$). Estradiol and progesterone at high concentrations did not significantly change the activity of UGT1A4.

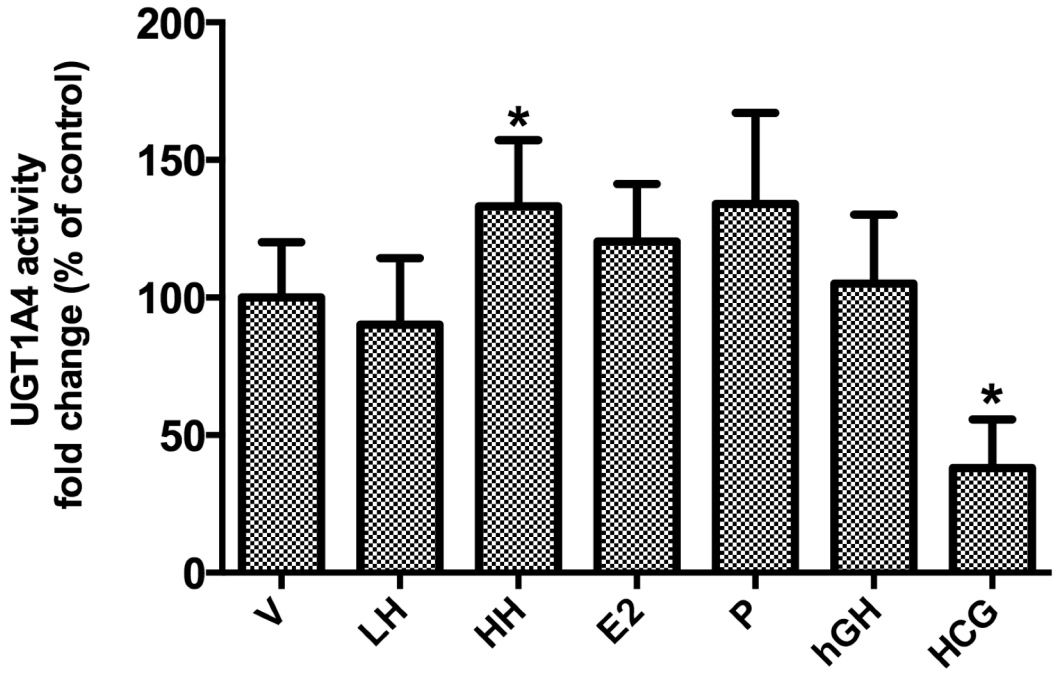

Figure 3-9 Effect of female steroid hormones on UGT1A4 activity.

Activity of UGT1A4 was measured by quantifying the formation of lamotrigine glucuronide after treating the primary cultures of human hepatocytes with female hormones chronically. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) compared to the control group.

3.4.3 Hormonal Regulation of Nuclear Receptors

It is known that several CYPs are regulated by PXR and CAR. UGTs are also known to be regulated via PXR, CAR, AhR, and PPARα (Zhou *et al*., 2005). To determine the role of these nuclear receptors in regulation of CYP450, UGTs, and transporters by female hormones, we measured the expression of PXR, CAR, AhR, and PPARα after treating the primary culture of hepatocytes with 2 mM phenobarbital, low and high female hormone mixtures (estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin), as well as with individual treatments of high concentrations of estradiol, progesterone, human growth hormone, and human chorionic gonadotropin for 3 days. GAPDH was used as a housekeeping gene.

3.4.3.1 Effect of Female hormones on AhR Expression

Phenobarbital, low hormone combinations, high hormone combinations, and human chorionic gonadotropin did not significantly induce AhR expression (1.7-, 1.6-, 1.3-, 2- and 1.6-fold, respectively) (Figure 3-10). Estradiol and human growth hormone increased the expression of AhR significantly by 1.7-fold and 1.9-fold, respectively ($p < 0.05$).

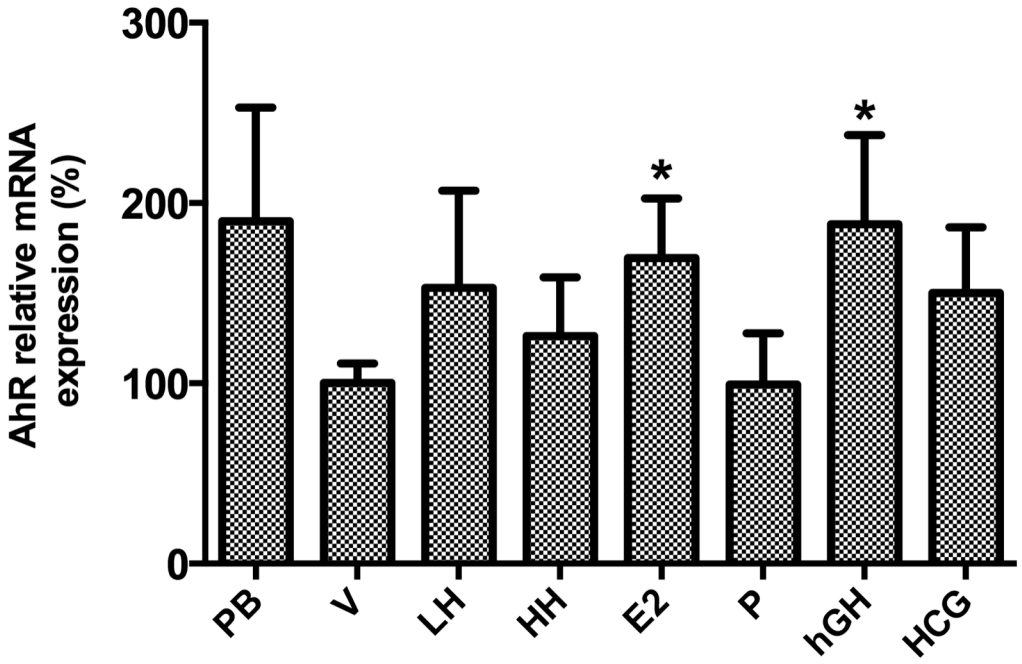

Figure 3-10 Effect of female steroid hormones on AhR expression.

The mRNA expression of AhR was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) compared to the control group.

3.4.3.2 Effect of Female hormones on PXR Expression

Our data showed that PXR mRNA levels were not changed by female hormones (Figure 3-11). This may indicate that the observed increase in the expressions of UGTs by these hormones is independent of PXR.

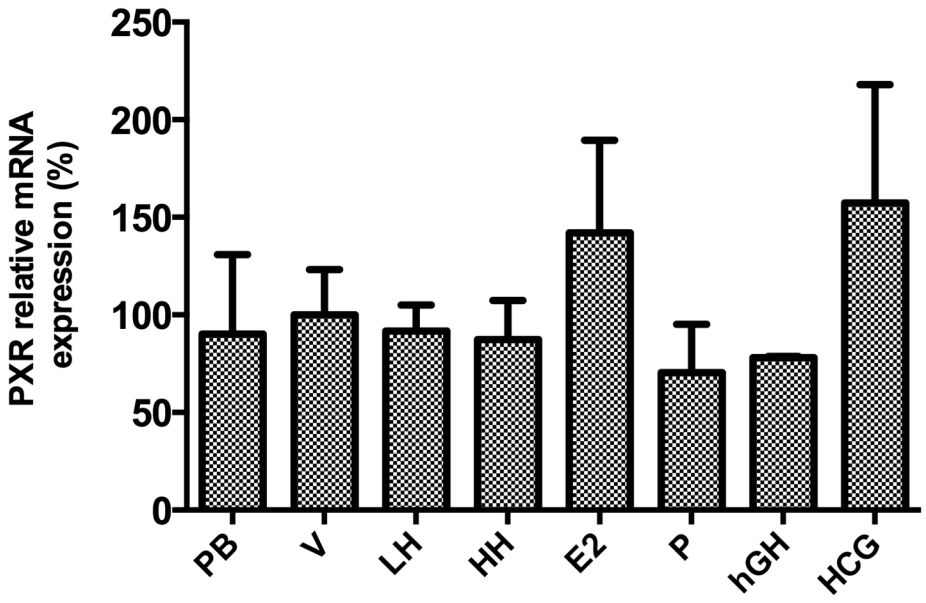

Figure 3-11 Effect of female steroid hormones on PXR expression.

The mRNA expression of PXR was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * ($p < 0.05$) compared to the control group.

3.4.3.3 Effect of Female hormones on CAR Expression

Female hormones did not show any affect on the mRNA expressions of CAR (Figure 3-12).

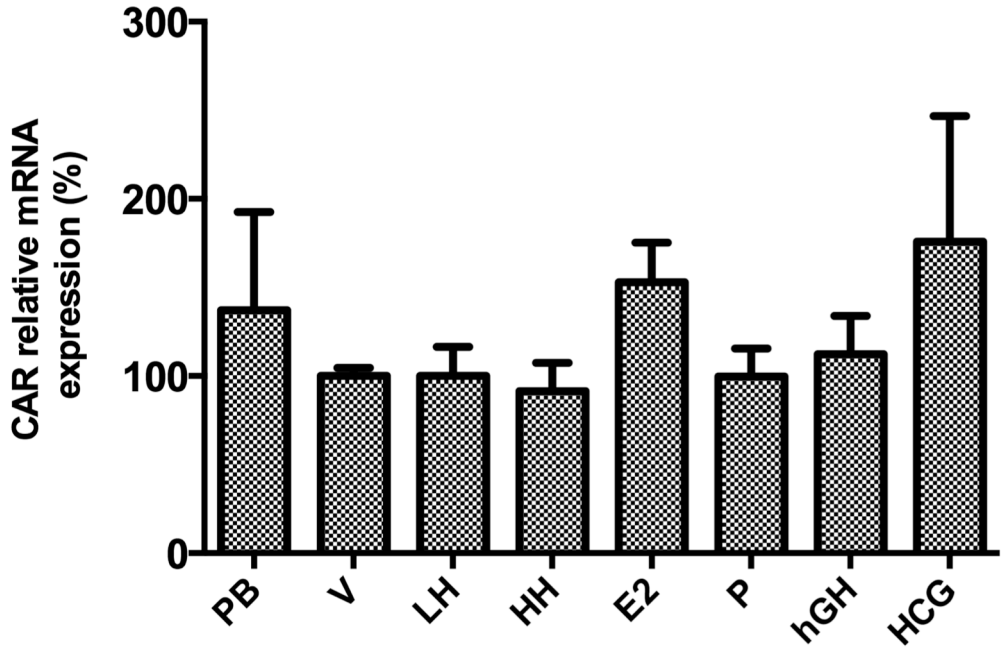

Figure 3-12 Effect of female steroid hormones on CAR expression.

The mRNA expression of CAR was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM.

3.4.3.4 Effect of Female hormones on PPARα Expression

Female hormones did not show any affect on the mRNA expressions of PPARα (Figure 3-13).

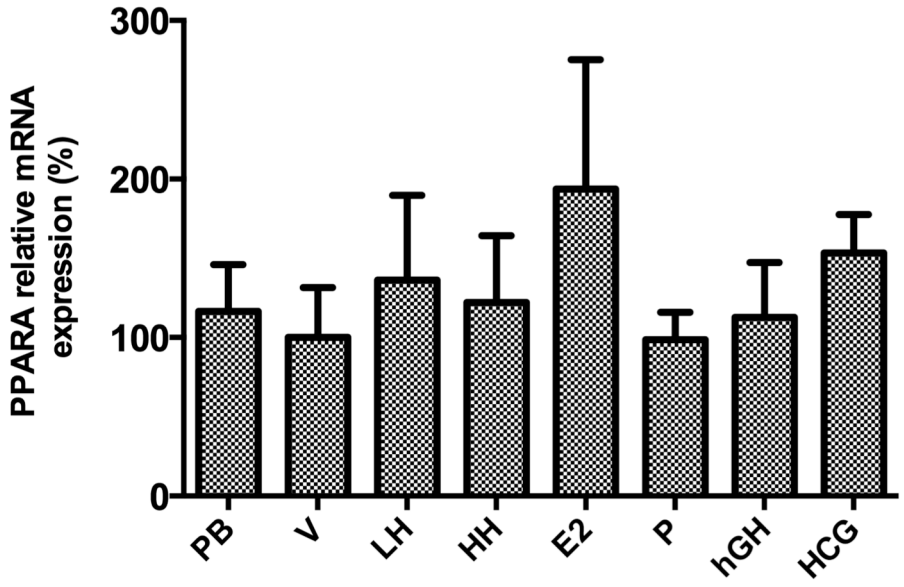

Figure 3-13 Effect of female steroid hormones on PPARα expression.

The mRNA expression of PPARα was determined after treating the primary cultures of human hepatocytes with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM.

3.5 DISCUSSION

Results from clinical studies have demonstrated that clearance of labetalol and lamotrigine is altered during pregnancy by 60% (Fischer, 2014) and 200% (Tran, 2002), respectively, indicating the need for dosage adjustments for these medications during pregnancy. Similar changes in lamotrigine clearance in pregnant women and women using oral contraceptives have been reported (Ohman *et al*., 2008), suggesting that female hormones such as estradiol and progesterone are responsible for the altered lamotrigine pharmacokinetics during pregnancy. Labetalol and lamotrigine are primarily metabolized by UGT1A1 and UGT1A4 enzymes. This indicates that UGT1A1 and UGT1A4 are induced during pregnancy, but the underlying mechanism of this alteration remains unknown. In this study, we examined the regulation of activity and expression of phase II enzymes, particularly UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B7) by pregnancy-related hormones. We also evaluated the impact of female hormones on the major transcriptional nuclear receptors including AhR, PXR, CAR, and PPARα that are known to regulate the expression of these UGTs.

To evaluate the impact of female hormones on the activity and expression of UGTs, we used the physiological plasma concentrations of these female hormones reached in pregnancy as a low concentration and at 10-times higher, comparable to concentrations of these hormones in the liver (Table 3-1).

At ten times higher than the progesterone plasma concentration (20 μM), activity and mRNA expression of UGT1A1 was significantly increased. This increase is in agreement with another study where 10 μM progesterone increased the activity and mRNA expression of UGT1A1 in HepG2 cells (Jeong, 2008). A previous microsomal study indicates that UGT2B7 also contributes to the glucuronidation of labetalol (Jeong, 2008). In our study, we also examined

the effect of female hormones on the expression of UGT2B7. Our results showed that progesterone and phenobarbital (a known inducer) did not increase UGT2B7 expression. These results are also in consistent with another study where progesterone and rifampin (known inducers) did not show induction in the mRNA level of UGT2B7 (Jeong, 2008). On the other hand, expression of UGT2B7 was significantly increased by a mixture of female hormones at low and high concentrations as well as by the high concentrations of estradiol and human growth hormone. This can explain the increased oral clearance of morphine (UGT2B7 substrate) in pregnant women by 70% more than in non-pregnant women (**Figure 3-6**) (Gerdin *et al*., 1990; Jeong, 2008).

Lamotrigine is a substrate of UGT1A4. Lamotrigine clearance has been known to increase significantly in pregnant women (Tran, 2002). Therefore, increased oral clearance of lamotrigine can be attributed to increased expression and activity of UGT1A4. Our results showed that a mixture of female hormones and estradiol induced the activity and mRNA expression of UGT1A4, respectively, at concentrations 10 times higher than their plasma concentration in primary cultures of hepatocytes. Our data agreed well with previously reported results where estradiol up-regulated the expression of UGT1A4 in HepG2 and MCF7 cells (Chen, 2009). Contrary to the induction of UGT1A4 activity and expression by female hormones, our findings indicate that high concentrations of human chorionic gonadotropin reduced the activity of UGT1A4. It has been reported that activity of hepatic CYP2A and CYP2E1 decreased in porcine liver after treatment with human chorionic gonadotropin decreased (Zamaratskaia *et al*., 2008). Activity of major drug metabolizing enzymes is known to be similar and has a comparable level between pig and human livers (Anzenbacher *et al*., 1998).

We have further evaluated the expression of other UGT isoforms such as UGT1A3, UGT1A6, and UGT1A9. Our results indicate that mixture of female hormones at lower concentrations and high concentrations of estradiol increase the expression of UGT1A3 in human hepatocytes. UGT1A3 is the major hepatic UGT enzyme that conjugates chenodeoxycholic acid (CDCA), the major bile acid in the liver that controls cholesterol homeostasis, to chenodeoxycholic acid-24 glucuronide (CDCA-24G) (Trottier *et al*., 2006). During cholestasis, the concentration of CDCA-24G increased by 50-fold (Trottier, 2006). Therefore, increased expression of UGT1A3 can be clinically relevant by increasing the metabolism of CDCA leading to cholestasis. Intrahepatic cholestasis of pregnancy (ICP) has been associated with increased levels of estrogen (Schreiber and Simon, 1983). In the present study and based on the *in-vitro* and *in-vivo* drug-drug interaction studies conducted by the Pharmaceutical Research and Manufacturers of America (PhRMA) (Bjornsson, 2003), estradiol seems to be an inducer of UGT1A3 by 2-fold compared to control and has more than a 40% increase relative to phenobarbital.

The expression of UGT1A6 was increased by phenobarbital and high concentrations of estradiol. The induction by estradiol was only about 1.2-fold compared to control, suggesting that estradiol does not alter expression of UGT1A6. Similarly, UGT1A9 expression was increased by estradiol and progesterone by 50% and 40%, respectively. However, this increase was not more than 40% of phenobarbital's effect on the expression of UGT1A9. Therefore, they are not considered to be inducers of UGT1A9.

In the present study, we evaluated the impact of female hormones on the expression and activity of several major UGTs that are known to be regulated by transcription receptors such as AhR, PXR, CAR, and PPARα. Therefore, we examined the effect of female hormones on the

expression of these specific transcription receptors. Our study indicated that estradiol induced AhR expression. Several studies have shown that AhR regulates the expression of UGT1A1 and UGT1A3 (Chen *et al*., 2005; Fujiwara *et al*., 2012; Lankisch, 2008; Senekeo-Effenberger *et al*., 2007; Yueh, 2003). Our data also revealed that estradiol induces the expression of UGT1A1, suggesting that estradiol-mediated UGT1A1 induction is attributed to an increase in AhR expression. In addition, there was no change in the expression of PXR, CAR and PPARα. This suggests that PXR, CAR, and PPARα do not play a major role in female hormone-mediated UGT induction in human hepatocytes.

In summary, we have characterized the regulation of major UGTs by pregnancy related hormones in primary cultures of human hepatocytes. Activity and expression of UGT1A1 is regulated by progesterone, whereas activity and expression of UGT1A4 is regulated by high hormone mixtures and estradiol, respectively. This indicates that progesterone and estradiol are likely to be responsible for the altered metabolism of labetalol and lamotrigine, respectively, in pregnancy. Estradiol and progesterone are the only hormones that regulate the expression of UGT1A6 and UGT1A9. Mixtures of female hormones regulate the expression of UGT1A3 and UGT2B7. AhR seems to be the probable pathway for female hormone-mediated UGT induction. Our findings provide a possible mechanism for the alteration in drug metabolism during pregnancy.

4.0 BUPRENORPHINE DISPOSITION DURING PREGNANCY: OBSERVED AND PBPK PREDICTION

4.1 ABSTRACT

Physiological changes during pregnancy are expected to alter the pharmacokinetics of medications. Limited pharmacokinetic studies, however, have been performed in pregnant subjects. Due to the lack of drug dosing guidelines during pregnancy, and since conducting clinical studies in pregnant women is challenging, there is a need to develop and validate physiologically based pharmacokinetic (PBPK) models that can predict the systemic exposure of medications used during pregnancy. Recently, PBPK has been used to facilitate clinical studies and fast-track drug development processes. In this study, our aims are to validate a PBPK model (SIMCYP) for buprenorphine in non-pregnant women, to evaluate the effect of pregnancy on buprenorphine pharmacokinetics using this model, and to compare predicted and observed parameters in pregnant subjects. We validated a PBPK model using a digitized buprenorphine concentration-time curve in 17 non-pregnant women obtained from the literature. We then incorporated various major physiological changes that occur during pregnancy in the PBPK model and predicted the pharmacokinetic parameters during pregnancy. Due to the lack of a sublingual route of buprenorphine administration in the current SimCYP version, we used the oral route as a surrogate and refined our model by optimizing the fraction absorbed and absorption rate constant in order to achieve similar exposure of the observed data. Then, we validated the PBPK model prediction for buprenorphine in pregnant women by comparing with our observed data in pregnant women. Our data shows that the predicted concentration-time curves of buprenorphine in all trimesters of pregnancy agreed well with the observed concentrations-time curves. The prediction fold error for the area under the concentration-time curve (AUC), maximum plasma concentration (Cmax), and oral clearance (CL) are between 0.82

to 1.3, indicating that pharmacokinetics of buprenorphine can be reasonably predicted using a pregnancy PBPK model.

4.2 INTRODUCTION

Clinical observations during pregnancy suggest that pharmacokinetics of certain drugs are changed, indicating the need for designing a new dosing regimen in pregnant women. Due to ethical considerations and the difficulty in conducting clinical studies in pregnant women, a physiologically based pharmacokinetics (PBPK) model can be used to predict the exposure of medications during different trimesters of pregnancy.

Multiple literature observations indicated that drug-metabolizing enzymes, including CYPs or UGTs, are altered during pregnancy (Chen *et al*., 2009; Choi *et al*., 2013; Jeong *et al*., 2008; Papageorgiou *et al*., 2013). Pregnancy PBPK models have been used recently to predict the pharmacokinetics of drugs that are metabolized by different CYP450 enzymes (Ke *et al*., 2014; Xia *et al*., 2013) by incorporating the major pregnancy related physiological changes. However, there is limited information on the application of PBPK model for drugs that are cleared by hepatic UGTs or by a combination of CYPs and UGTs. Therefore, we proposed to evaluate the effect of pregnancy on a drug that is simultaneously cleared by both CYP and UGT enzymes such as buprenorphine. Similar to other medications used in pregnancy, manufacture labeling of buprenorphine does not include any information on the pharmacokinetics during pregnancy.

To develop and validate the pregnancy PBPK model, it is required that predicted data be comparable to the observed data. Therefore, we first conducted a clinical pharmacokinetic study in pregnant women who were on chronic buprenorphine treatment and compared the generated data from pregnancy PBPK with those observed in pregnant women. Buprenorphine is administered sublingually to treat acute pain and opioid addiction during pregnancy (Jones *et al*., 2008). The sublingual bioavailability of buprenorphine is approximately 51% (Kuhlman *et al*.,

1996). Buprenorphine is highly bound to plasma protein, mainly alpha-1 glycoprotein (AAG) (Welsh and Valadez-Meltzer, 2005) and has a long half-life (37 hours) (Ducharme *et al*., 2012). It is metabolized to its active metabolite, norbuprenorphine, by CYP 3A4, and both buprenorphine and norbuprenorphine undergo glucuronidation by UGT11A1, UGT1A3, and UGT2B7 (Ducharme, 2012; Rouguieg *et al*., 2010). Buprenorphine is excreted primarily in the feces, and 10%-30% of buprenorphine is excreted in urine (Welsh, 2005). To the best of our knowledge, using a PBPK model to predict the impact of pregnancy on the pharmacokinetics of drugs that are metabolized simultaneously by CYPs and UGTs has not yet been reported. In the current study, we applied a pregnancy PBPK model to predict the exposure of buprenorphine in pregnant women in different stages of pregnancy. We verified the model using observations of buprenorphine pharmacokinetics in pregnant women.

4.3 MATERIAL AND METHODS

4.3.1 Reagents and Software

Buprenorphine and buprenorphine-D4 were purchased from Cerilliant (Round Rock, TX). Strata X-C cartridge used for buprenorphine extraction was obtained from Phenomenex (Torrance, CA). Version 13 of SimCYP (SimCYP, Sheffield, UK) was used for PBPK simulations.

4.3.2 PBPK Model Structure

Anatomical and physiological parameters for both non-pregnant and pregnant populations were incorporated in the SIMCYP 13 as a compartment full PBPK model. These parameters include body weight, organ volumes, blood flows, plasma protein cardiac output, serum creatinine, glomerular filtration rate, renal function and the metabolic enzyme activity. The pregnancy PBPK model was extended by adding the fetoplacental unit, which occurs between arterial and venous blood compartments. Fetoplacental unit represents fetus, placenta, and uterus, as well as amniotic fluid (Figure 4-1).

4.3.3 PBPK Model of Buprenorphine in Non-pregnant and Pregnant Subjects

(Figure 4-2) shows the approach that was used in this project. SIMCYP was used to predict plasma concentration versus time profile of buprenorphine in non-pregnant women utilizing the

literature information about the physicochemical properties and the pharmacokinetics parameters of buprenorphine such as log P, pka, B:P ratio, f_{up}, CL, and Clint (Table 4-1). Since the current SimCYP version does not incorporate a sublingual route of administration, we selected the oral route of administration for optimizing the model. We further optimized the rate constant of absorption (ka=1.7 h-1) and fraction absorbed (fa=0.26) in order to mimic sublingual administration and to improve the predictions of Cmax and AUC. Renal clearance (CLr=7.192 L h−1) was also adjusted in order to improve the adaptation of the elimination phase. The predictions were compared to the observed buprenorphine pharmacokinetic profile in 17 healthy female subjects who received 16 mg of sublingual buprenorphine (Compton *et al*., 2007)

After verifying the PBPK model in non-pregnant subjects, the mean concentration time profiles of buprenorphine and pharmacokinetics data were simulated at first trimester (14-18 weeks), second trimester (24-28 weeks) and third trimester (28-40 weeks) of pregnancy and compared with the observed results in pregnant subjects studied. Briefly, human female population size (n=100) of the PBPK model under fasting conditions was used by selecting 10 trials and having 10 subjects in each trial. The PBPK model incorporated the changes in physiological parameters during pregnancy such as body weight, cardiac output, serum creatinine, renal function and the metabolic changes related to the CYP450 and UGT enzymes. In pregnancy, the activity of CYP2C9, CYP2D6, CYP3A4, UGT1A1, and UGT1A4 increases while the activity of CYP1A2 and CYP2C19 decreases. The glomerular filtration rate and renal blood flow also increases in pregnancy (Baylis, 1982; Davison, 1974; Koren, 2011). The mean predicted buprenorphine plasma concentration and the pharmacokinetic profiles in different tissue compartments, such as fetoplacental (fetus and placenta) and brain were also obtained by simulations.

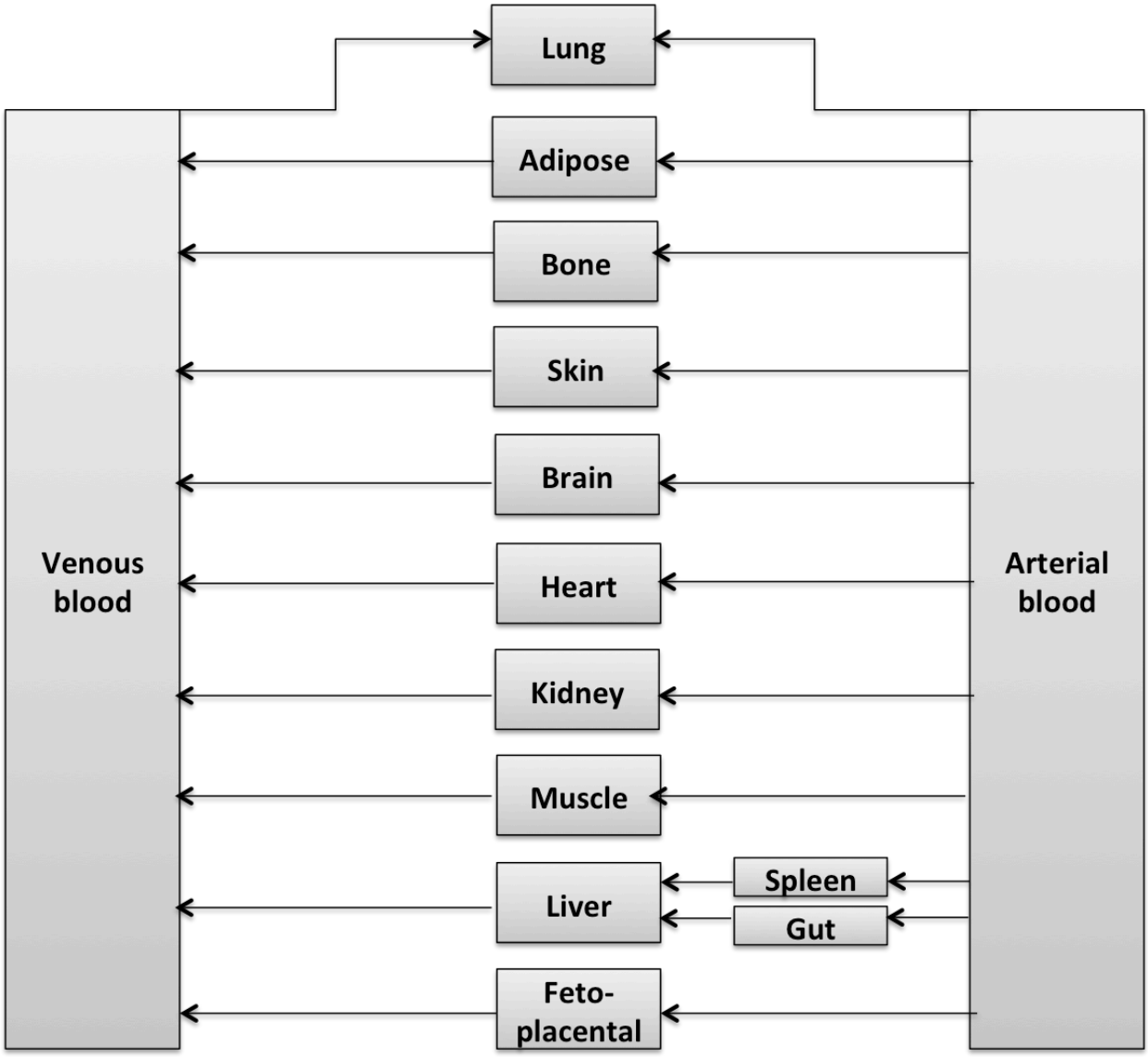

Figure 4-1 Pregnancy PBPK model structure

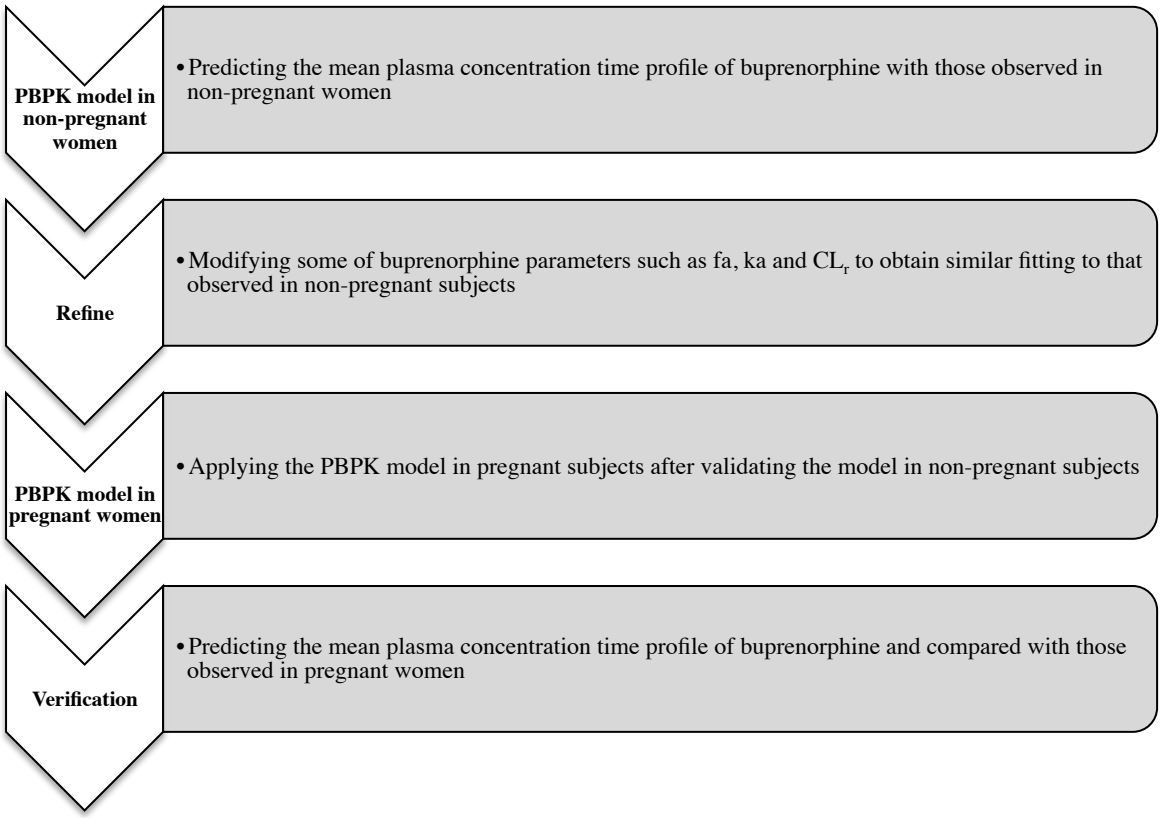

Figure 4-2 PBPK model general workflow of buprenorphine in pregnancy

Table 4-1 Physicochemical properties and pharmacokinetic parameters of buprenorphine.

Parameter	Value	Reference
Molecular weight	467	(Likar, 2006)
Log P	4.98	(Avdeef *et al*., 1996)
pKa	8.31	(Avdeef, 1996)
B : P ratio	0.6	(Mistry and Houston, 1987)
f_{up}	0.07	(Mistry, 1987)
fa	0.26	*
Ka (h^{-1})	1.7	*
CL (L h^{-1})	610.86	Predicted
CL_r (L h^{-1})	7.192	*
$CL_{int,CYP}$ (µL/min/mg - microsomal protein)	$CL_{int, CYP3A4}$ = 38.3	(Picard *et al*., 2005)
	$CL_{int, CYP2C8}$ = 14.2	(Picard, 2005)
$CL_{int,UGT1A1}$ (µL/min/mg - microsomal protein)	279	(Kilford *et al*., 2009)
Buprenorphine administration		
Dose (mg)	8	
Route	Oral	
Dosing interval (h)	12	

*fa, ka and CL_r were optimized in order to predict the best fitting curve and similar predicted PK parameters compared with observed data.

4.3.4 Subjects and Study Design

Subjects between the ages of 18 and 45 years old and on buprenorphine therapy were eligible for the study. The study was approved by the institutional review board at the University of Pittsburgh. All study subjects were provided with a written, informed consent before participation in the clinical study. The exclusion criteria included subjects who are on HIV medications or taking medications known to induce CYP3A4 activity (rifampin and phenobarbital) or inhibit CYP3A4 activity such as ketoconazole and other antifungal medications. Eligible subjects were enrolled into four pharmacokinetic studies: first trimester (n=1), second trimester (n=5), third trimester (n=3). Subject demographics such as age, race, body weight, height, and vital signs were recorded. Buprenorphine (8 mg) was administered. Blood samples (7 mL) were collected at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 10, and 12 hours. Plasma samples were then stored at -80^{o}C until analysis.

4.3.5 Sample Preparation

Plasma sample (500 µL) mixed with 25 µL of buprenorphine (BUP)-D4 as an internal standard was extracted with 1 ml of 0.1% perchloric acid (HClO4). Samples were then centrifuged at 15000 rpm for 10 minutes. Then, buprenorphine and its metabolites were extracted by solid phase extraction. The extraction cartridge was conditioned twice with 1 mL methanol and twice with 1 mL 0.1% HClO4. Then, the sample was loaded onto the cartridge. After washing twice with 1 mL of 2% HCOOH in H2O and two times with 1 mL of 2% HCOOH in methanol, the

analytes were eluted three times with 60% acetonitrile, and 40% isopropanol containing 5% ammonium hydroxide. The eluent was air-dried. The residue was then reconstituted with 100 µL of the initial mobile phase (10% acetonitrile, 90% water, containing 0.1%HCOOH), and 7.5 µL of the sample solution was injected directly into the LC-MS/MS system.

4.3.6 Liquid Chromatographic and Mass Spectrometry

Sample analysis was performed on a Thermo TSQ Quantum Ultra-Triple quadrupole mass spectrometer. Chromatographic conditioning was performed using a C18 column (1.7 µM, 2.1 x 100mm). A mobile phase consisting of solvent A (98% H2O, 2% methanol containing 0.1% HCOOH) and solvent B (acetonitrile containing 0.1% HCOOH) was used. The following gradient was used: 87 % A for 0-0.5 minute and then 35% A for 0.5-4.5 minutes and then held for 0.5 minutes, and 87 % A for 5-7 minutes. The flow rate was 0.3 ml/min. The ion transitions for BUP were m/z 468.3 ⇒ 396.2. The standard curve for BUP ranged from 0.50 to 50 ng/ml.

4.3.7 Pharmacokinetic Analysis

The area under the concentration time curve (AUC) of BUP from 0-8 hours was calculated using the linear trapezoidal rule. Apparent oral clearance of BUP was calculated as follows:

CL = F*Dose/ (AUC) **(Equation 4-1)**

We used the reported sublingual bioavailability (F) of buprenorphine as 51% (Kuhlman, 1996). Cmax was defined as the highest plasma concentration.

4.4 RESULTS

4.4.1 Prediction of Buprenorphine Pharmacokinetics in Non-pregnant Women

To evaluate the adequacy of prediction of the simulated plasma buprenorphine concentration during pregnancy, we first compared the simulated plasma concentration versus time profiles of buprenorphine in non-pregnant women to the observed data in non-pregnant women (Figure 4-3). Simulated plasma concentration versus time profile was similar to the observed concentrations time profile during non-pregnancy. As shown in (Table 4-2), the area under the concentrations-time curve (AUC) and the sublingual clearance (CL_{sub}) of predicted (11.9 ng/mL.h and 343 L/h) and observed data (11 ng/mL.h and 343 L/h) were similar. The prediction fold error for AUC and CL_{sub} were 1.1 and 0.9, respectively.

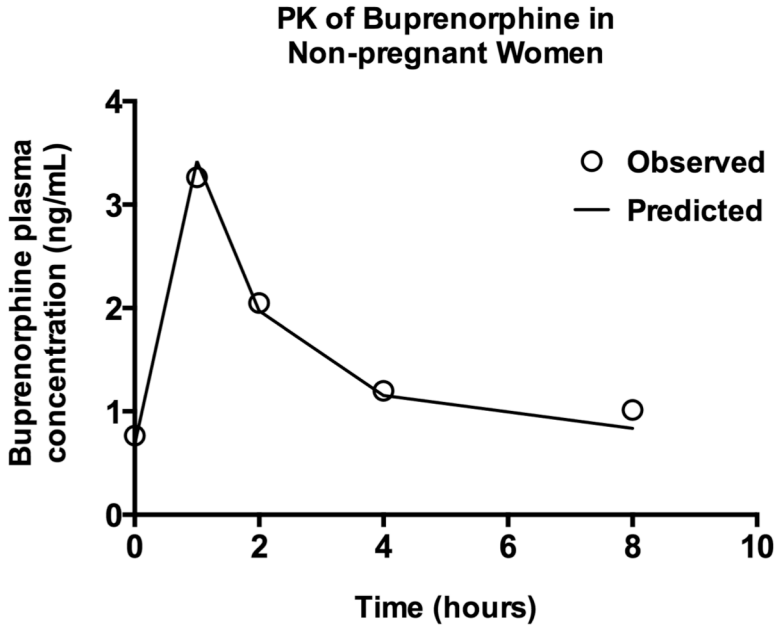

Figure 4-3 Predicted and observed mean plasma concentration time curve of buprenorphine (ng/mL) in non-pregnant women

4.4.2 Prediction of Buprenorphine Pharmacokinetics in Pregnancy Versus Non pregnancy

After we verified the PBPK model in non-pregnant women, we predicted buprenorphine pharmacokinetics in pregnancy and compared that to buprenorphine pharmacokinetics in non-pregnant women (Figure 4-4). The predicted AUC of buprenorphine in non-pregnant women, and in pregnant women during the first trimester, second, trimester, and third trimester is 11.9, 10.5, 9.4 and 8.7 (ng/mL.h), respectively. The predicted AUC decreased overall by 20% in pregnancy versus non-pregnancy. The predicted CL_{sub} of buprenorphine in non-pregnant women, and in pregnant women during the first trimester, second, trimester, and third trimester is 343, 389, 434 and 469 (L/h), respectively. The changes in AUC and CL_{sub} seemed to be trimester-dependent.

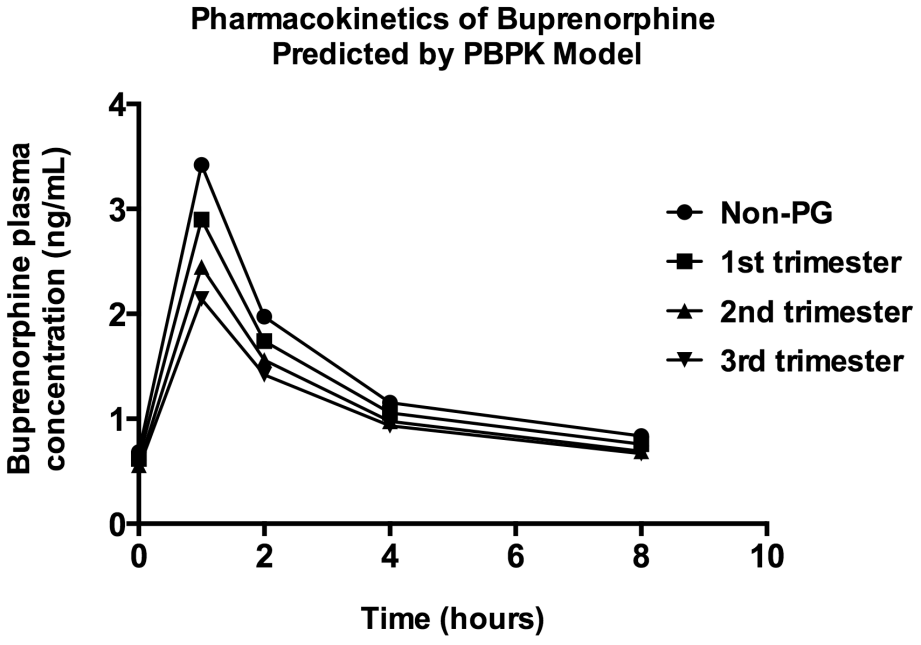

Figure 4-4 Predicted plasma concentration time curve of buprenorphine (ng/mL) in pregnant women

4.4.3 Predicted versus Observed Pharmacokinetics of Buprenorphine in Pregnancy

Nine pregnant women on buprenorphine treatment participated in the study. One subject was in the first trimester, five subjects were in the second trimester, and three subjects were in the third trimester of pregnancy. The observed buprenorphine plasma concentration versus time profiles in different gestational periods in the pregnant women are presented in (Figure 4-5). Compared to the control group (non-pregnant women), the mean area under the concentration-time curve (AUC_{0-8}) decreased approximately 25% and 30% at second trimester and third trimester, respectively. However, there was no change in the AUC between the first trimester group and the control group, which reflect low number of subjects studied in the first trimester. The observed maximum concentration (Cmax) in the third trimester was 30% lower than the observed Cmax in non-pregnant control population. Our data showed that the simulated profile fitted the observed profile during all the three trimesters. We also compared the PBPK model predicted pharmacokinetic parameters with the observed buprenorphine pharmacokinetics in non-pregnant and pregnant women (Table 4-2). The predicted and observed Cmax, AUC_{0-8}, Tmax, and sublingual clearance (CL_{sub}) of buprenorphine are similar in all the trimesters, and their prediction fold errors are between 0.82 and 1.2. This indicates that the PBPK model is able to adequately predict the pharmacokinetics of buprenorphine during pregnancy.

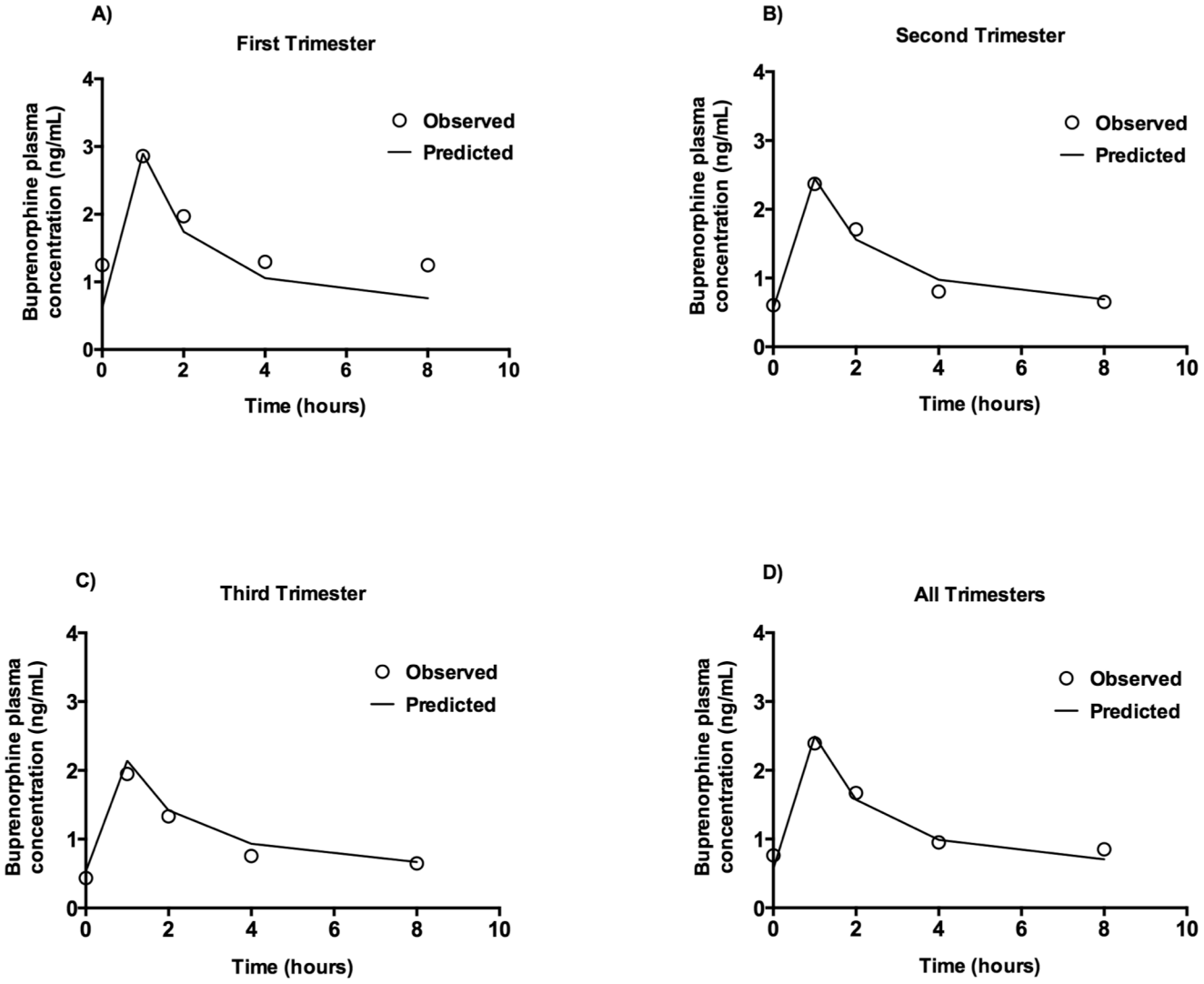

Figure 4-5 Predicted and observed AUC of buprenorphine during pregnancy.

Predicted versus observed area under the concentration-time curve of buprenorphine (ng/mL) in first trimester (A), second trimester (B), third trimester (C) and overall pregnancy (E).

The pharmacokinetics parameters of buprenorphine in non-pregnant women and in pregnant women at each trimester are presented in (**Table 4-2**). Compared to non-pregnant women, the observed buprenorphine apparent oral clearance was increased by 1.2- and 1.4-fold in second and third trimester of pregnancy, respectively.

Table 4-2 Observed and predicted pharmacokinetic parameters of buprenorphine.

	Non-PG			**1st trimester**			**2nd trimester**			**3rd trimester**			**All trimesters (mean)**		
	Pred	**Obs**	**PFE**	**Pred**	**Obs**	**PFE**	**Pred**	**Obs**	**PFE**	**Pred**	**Obs**	**PFE**	**Pred**	**Obs**	**PFE**
Cmax (ng/mL)	3.42	2.65	1.3	2.9	2.9	1	2.4	2.4	1	2.1	1.9	1.1	2.5	2.4	1
Tmax (h)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
AUC0-8 (ng/mL.h)	11.9	11	1.1	10.5	12.8	0.82	9.4	8.9	1.1	8.7	7.7	1.1	9.5	9.8	0.97
CL (L/h)	672	727	0.9	762	625	1.2	851	899	0.95	920	1039	0.89	842	816	1
CL/F (L/h)	343	371	0.9	389	319	1.2	434	458	0.95	469	530	0.89	429	416	1

Pred: predicted. Obs: observed. PFE: prediction fold error. Cmax: maximum concentration. Tmax: time to reach maximum concentration.
AUC: area under the concentration time curve. CL: clearance, F: bioavailability

4.4.4 Predication of Buprenorphine Exposure in Maternal Brain and Fetoplacental Unit

We applied our PBPK model to predict the effect of pregnancy on buprenorphine exposure in major physiological organs such as brain and fetoplacental unit (Figure 4-6**).** Feto-placental unit represents fetus, placenta, and uterus, as well as amniotic fluid (Xia, 2013). Brain buprenorphine AUC during pregnancy appeared to be lower than that in non-pregnant women (Table 4-3). During pregnancy, brain-to-plasma and feto-placental-to-plasma ratios of buprenorphine were 50:1 and 10:1, respectively resulting in an overestimating of the buprenorphine concentrations compared to a reported exposure of buprenorphine in in these organs (Coles *et al*., 2009; Pontani *et al*., 1985).

Table 4-3 Simulated AUC of buprenorphine in maternal plasma, brain and feto-placental unit.

AUC (ng/mL.h)	Non-PG	1st trimester	2nd trimester	3rd trimester
Plasma	11.9	10.5	9.4	8.7
Brain	592	557	535	547
Fetoplacental		102	101	107

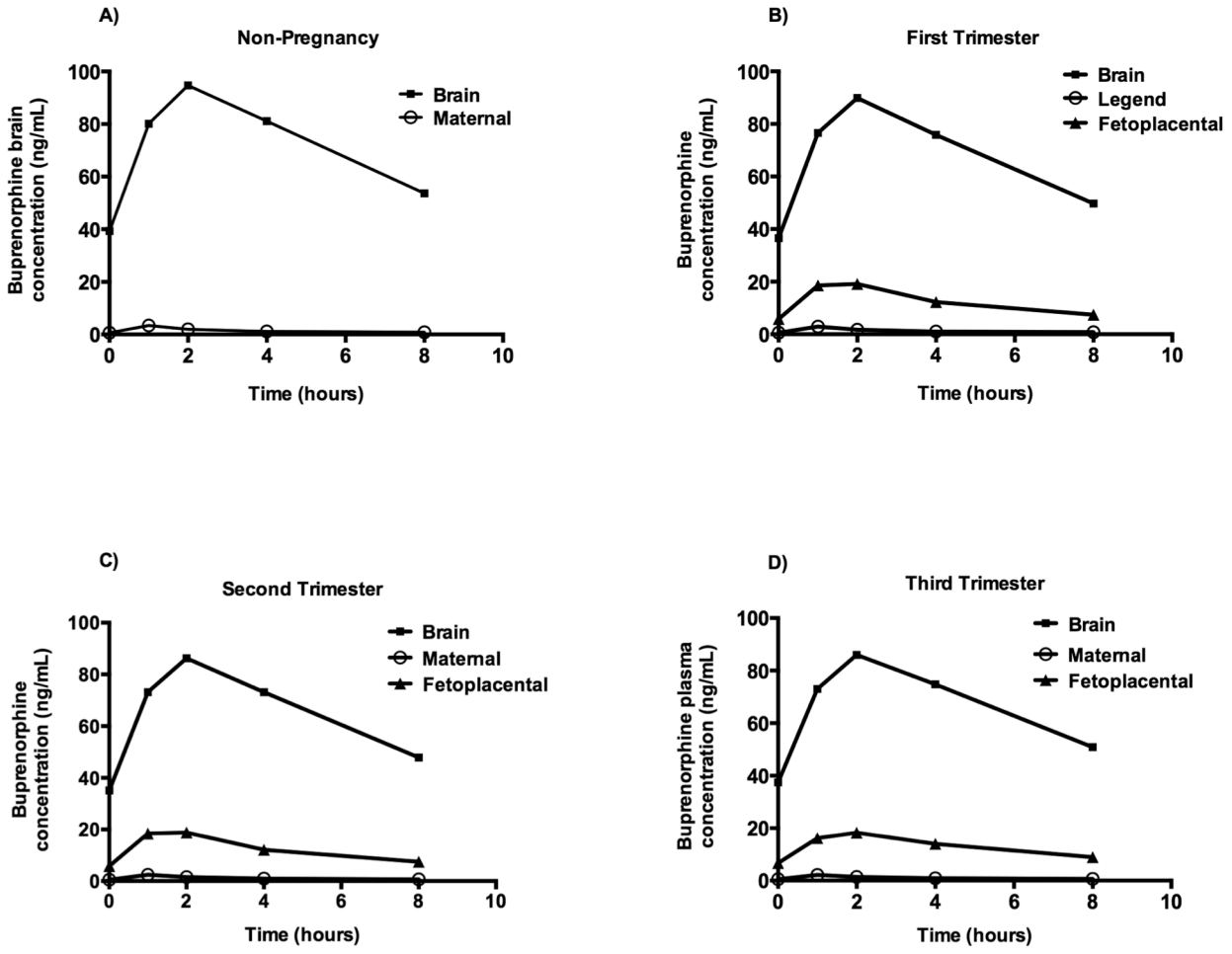

Figure 4-6 Simulated mean plasma concentrations-time curve of buprenorphine (ng/mL) in plasma, brain, and feto-placental unit.

The predicted mean concentration of buprenorphine (ng/mL) versus time curve in plasma and brain of non-pregnant women **(A)**, maternal plasma, brain, and feto-placental unit in first trimester **(B)**, second trimester **(C)**, and third trimester **(D)**.

4.5 DISCUSSION

A Physiologically based pharmacokinetics (PBPK) model is a very valuable tool for predicting drug exposure in different physiological conditions. Due to limitations in conducting clinical studies in pregnant women as well as the limited availability of data on drug dosing in pregnancy, PBPK is a valuable tool to predict drug exposure in plasma and other organs including liver, kidney, brain, and fetal in pregnant women. PBPK model can also facilitate the prediction of drug exposure in different tissues, which cannot be readily measured in human subjects. The PBPK model has been used mostly for simulating the disposition of multiple xenobiotics in pregnant women particularly for evaluating the maternal and fetal disposition and mainly in the third trimester (Gaohua *et al*., 2012; Ke *et al*., 2014). In this study, predicted and observed pharmacokinetics parameters of caffeine, metoprolol and midazolam were compared during the third trimester of pregnancy using pregnancy PBPK model. Their prediction fold change of the pharmacokinetics of these compounds was about 2-fold compared to the observed data. However, the impact of gestational age on the plasma and tissues pharmacokinetics is lacking. Therefore, we successfully developed and verified the pregnancy PBPK model for predicting the pharmacokinetics of buprenorphine incorporating all trimesters, pregnancy physiological changes, renal clearance, fraction absorbed, and absorption rate constant. Buprenorphine is a synthetic opioid used to treat pain and opiate addiction (Brown *et al*., 2011; Picard, 2005). It is also recommended in opioid-dependent pregnant patients (Jones, 2008). It is metabolized to its inactive metabolite, nor-buprenorphine, by the *N*-dealkylation pathway (Picard, 2005). Multiple CYP450 enzymes are involved in buprenorphine metabolism including CYP2C8, CYP2C9, CYP2C18, CYP2C19, and CYP3A4. CYP3A4 accounts for 65% of nor-buprenorphine formation (Picard, 2005). Buprenorphine also undergoes glucuronidation, mainly

by UGT1A1, UGT1A3, and UGT2B7 (Rouguieg, 2010). Activity of CYP3A4, UGT1A1, and UGT2B7 is known to be altered significantly during pregnancy (Fischer *et al*., 2014; Hebert *et al*., 2008; Watts *et al*., 1991). Due to limited information about the pharmacokinetics of buprenorphine in pregnant women, we predicted for the first time the effect of pregnancy on the pharmacokinetics of buprenorphine using a pregnancy based PBPK model and compared predicted data with the observed data in pregnant subjects. A successful application of PBPK model for buprenorphine in pregnancy indicates the possibility of applying a PBPK model for predicting the pharmacokinetics of other medications that have similar metabolic pathways in the pregnant population. Our model predicted AUC, Cmax, and CL of buprenorphine similar to those in observed data, indicating that our pregnancy PBPK model is appropriate. As we have previously shown in chapters 2 and 3, the metabolic activity of CYP3A4 and UGT1A1 increased during pregnancy. Therefore, our assumption of increased observed and predicted clearance of buprenorphine in pregnant women was due to increased activities of these metabolizing enzymes, suggesting the need for increasing buprenorphine doses during pregnancy for achieving the optimal therapeutic effect.

The brain-to-plasma ratio of buprenorphine concentration has been reported in rats to range from 3.0 to 10.5 after administration of 0.2 mg/kg of buprenorphine intravenously (Pontani *et al*., 1985). Fetoplacental-to-plasma ratio (2:1) of buprenorphine concentration has also been reported in pregnant mice after a single intravenous dose (120 mCi/kg) of [3H] buprenorphine (Coles *et al*., 2009). Our pregnancy PBPK model predicted the exposure of buprenorphine in maternal brain and fetal compartments. Our data showed that brain-to-plasma and feto-placental-to-plasma ratios of buprenorphine were 50:1 and 10:1, respectively. This indicates that our model overestimates the concentrations of buprenorphine in brain and feto-placental unit. Our

PBPK simulations were performed at steady state in contrast to the published single dose studies. This can explain the higher brain exposure of buprenorphine predicted by the PBPK model. Multiple dose studies in animals should be performed to confirm the predictions. The lower brain-to-plasma and fetoplacental-to-plasma ratios of buprenorphine in animals may also be related to efflux of buprenorphine by certain transporters that are yet to be identified. Results from *in vitro* and *in vivo* studies indicated that the efflux transporter P-glycoprotein (P-gp) mediates norbuprenorphine access to the brain and plays an important role in the antinociceptive of norbuprenorphine (Alhaddad *et al*., 2012; Brown *et al*., 2012). In this study, we predicted the pharmacokinetics of buprenorphine only. Future studies should be performed to evaluate the effect of pregnancy on the exposure of norbuprenorphine in plasma and brain during pregnancy. In summary, based on the reported data of buprenorphine in healthy women volunteers, we have shown the ability of our pregnancy PBPK model to predict the observed data in pregnant women during different trimesters of pregnancy. Our data also suggests the need for dosing adjustments of buprenorphine during pregnancy. The pregnancy PBPK model can be further refined once there is more data available from additional subjects, especially during the first trimester stage.

5.0 EFFECT OF PREGNANCY HORMONES ON HEPATIC BUPRENORPHINE METABOLISM

5.1 ABSTRACT

It has been known that pregnancy affects hepatic drug metabolism. However, the underlying mechanism responsible for this alteration is unknown. Regulation of hepatic cytochrome P450 (CYP) and UDP-glucuronsyltransferases (UGT) by female hormones–estradiol and progesterone–have been reported using different probes for CYP450 and UGT enzymes. An example of a medication that is simultaneously metabolized by both CYP and UGT is buprenorphine. Buprenorphine is a partial μ-opioid receptor agonist used to treat pain and opiate addiction in pregnant women. It is metabolized to norbuprenorphine mainly by CYP3A4 and CYP2C8, and both buprenorphine and norbuprenorphine undergo glucuronidation by UGT1A1, UGT1A3, and UGT2B7. Physiological based pharmacokinetics (PBPK) modeling indicated increased clearance of buprenorphine during pregnancy. Our goal in this study was to characterize the impact of pregnancy hormones on the pharmacokinetics of buprenorphine and its metabolites. Plasma (low) and liver (high) concentrations of female hormone mixtures were incubated with primary cultures of human hepatocytes and treated with buprenorphine (100 ng/mL) for 0-2 hours. Concentrations of buprenorphine and its metabolites were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Buprenorhine-glucuronide was the predominant metabolite formed in human hepatocytes. Both plasma and liver concentrations of female hormones did not influence the metabolism of buprenorphine in human hepatocytes. Female hormones mixtures did not change the exposure of any of the metabolites. Intrinsic clearance, hepatic clearance, and sublingual clearance of buprenorphine were not altered after treatment with female hormones at both concentrations. These observations indicate that pregnancy related hormones effect on the liver may not be responsible for the predicted and observed increase in the clearance of buprenorphine during pregnancy. Given that buprenorphine

can also be metabolized by small intestine, alterations in gut metabolism of buprenorphine, should be evaluated in future studies.

5.2 INTRODUCTION

The activity of hepatic enzymes such as cytochrome P450 (CYP) and UDP-glucuronsyl transferases (UGT) may be altered during pregnancy, leading to changes in pharmacokinetics, necessitating dose-adjustment of certain drugs (Concheiro *et al*., 2011; Fischer, 2014; Tracy, 2005; Tran, 2002). Clinical findings in pregnant women have indicated that clearance of methadone, a full opioid agonist, decreased during pregnancy (Pond *et al*., 1985). It has also been reported that clearance of morphine is greater in pregnant women compared to non-pregnant women (Gerdin, 1990). These findings support the hypothesis that pregnancy alters drug metabolism. However, the mechanisms that lead to such alterations are not yet to be completely understood. Buprenorphine is an example of an opioid medications used during pregnancy. Buprenorphine is a synthetic partial μ-opioid receptor agonist derived from the morphine alkaloid, thebaine, used to treat pain and opiate addiction during pregnancy (Brown *et al*., 2011; Jones *et al*., 2012; Picard, 2005).

The primary metabolic pathway of buprenorphine is *N*-dealkylation of its cyclopropyl group at the 17-position to nor-buprenorphine (Figure 5-1) (Brown, 2011; Cone *et al*., 1984; Huang *et al*., 2001; Kobayashi *et al*., 1998; Picard, 2005). CYP3A4 is the primary enzyme that mediates *N*-dealkylation of buprenorphine metabolism (Brown, 2011; Kobayashi, 1998). Out of thirteen P450 isoforms tested in human liver microsome, CYP2C8, 3A4, 3A5, and 3A7 have been identified to produce nor-buprenorphine in human liver microsome (Picard, 2005). The majority of nor-buprenorphine is formed by CYP3A4, accounting for about 65% and CYP2C8 with 30% (Picard, 2005). CYP 2C9, 2C18, 2C19 and CYP3A also produce additional oxidative products in human liver microsome or CYP450 transfected cells (Picard, 2005). A study in human liver microsome also reported that both buprenorphine and nor-buprenorphine formed hydroxy-

buprenorphine and hydroxy-nor-buprenorphine, respectively (Picard, 2005). CYP3A was the major CYP isoform that formed buprenorphine to hydroxy-buprenorphine (Picard, 2005). Chang *et al*, 2006, reported that buprenorphine undergoes hydroxylation in human liver microsome to produce M1 and M2 following by *N*-dealkylation to produce M3 and M4 or M5. M1 was mainly formed by 2C8, 3A4, 3A5, and 3A7. M3 and M5 were produced by 3A4, 3A5, and 3A7 (Chang *et al*., 2006). On the other hand, M1 (conjugated) and M3 (60 to 70% unconjugated) have been identified in human urine in subjects taking buprenorphine (Chang, 2006). In addition, the free buprenorphine has not been detected in human urine after a sublingual administration of buprenorphine (Cone, 1984). Buprenorphine and nor-buprenorphine (the active metabolite) undergo glucuronidation by UDP-glucuronsyl transferases (UGT) (Cone, 1984). Glucuronidation of buprenorphine and nor-buprenorphine has been tested in human liver microsome (Rouguieg *et al*., 2010). Buprenorphine and nor-buprenorphine glucuronides are formed only by UGT1A1, UGT1A3, and UGT2B7 among other six hepatic UGT isoforms (Rouguieg, 2010). UGT1A3 and UGT2B7 are the most predominant UGT isoforms that produce buprenorphine glucuronides (Rouguieg, 2010). On the other hand, UGT1A1 and UGT1A3 were primarily responsible for formation of nor-buprenorphine glucuronide (Rouguieg, 2010). Recent studies identified that *N*-dealkylation and glucuronidation are predominant in small intestine and human liver microsomes, respectively, suggesting that both gut and liver are involved in the metabolism of buprenorphine (Moody *et al*., 2009).

To the best of our knowledge, there is no data available related to the impact of pregnancy on buprenorphine metabolism. During pregnancy, pharmacokinetics parameters such as hepatic clearance, may be altered due to the effect of pregnancy on hepatic blood flow, drug protein binding and, intrinsic clearance (CL_{int}) (Jeong, 2010). PBPK modeling and preliminary

data form out lab indicated that clearance of buprenorphine is increased during pregnancy (chapter 5). One of the suggested potential mechanisms responsible for modifications in drug pharmacokinetics during pregnancy is the increased levels of major female hormones such as estradiol, progesterone and growth hormone. Therefore, in this study, we examined the influence of pregnancy hormones at plasma and accumulated liver concentrations on the pharmacokinetics of buprenorphine and its metabolites using primary cultures of human hepatocytes.

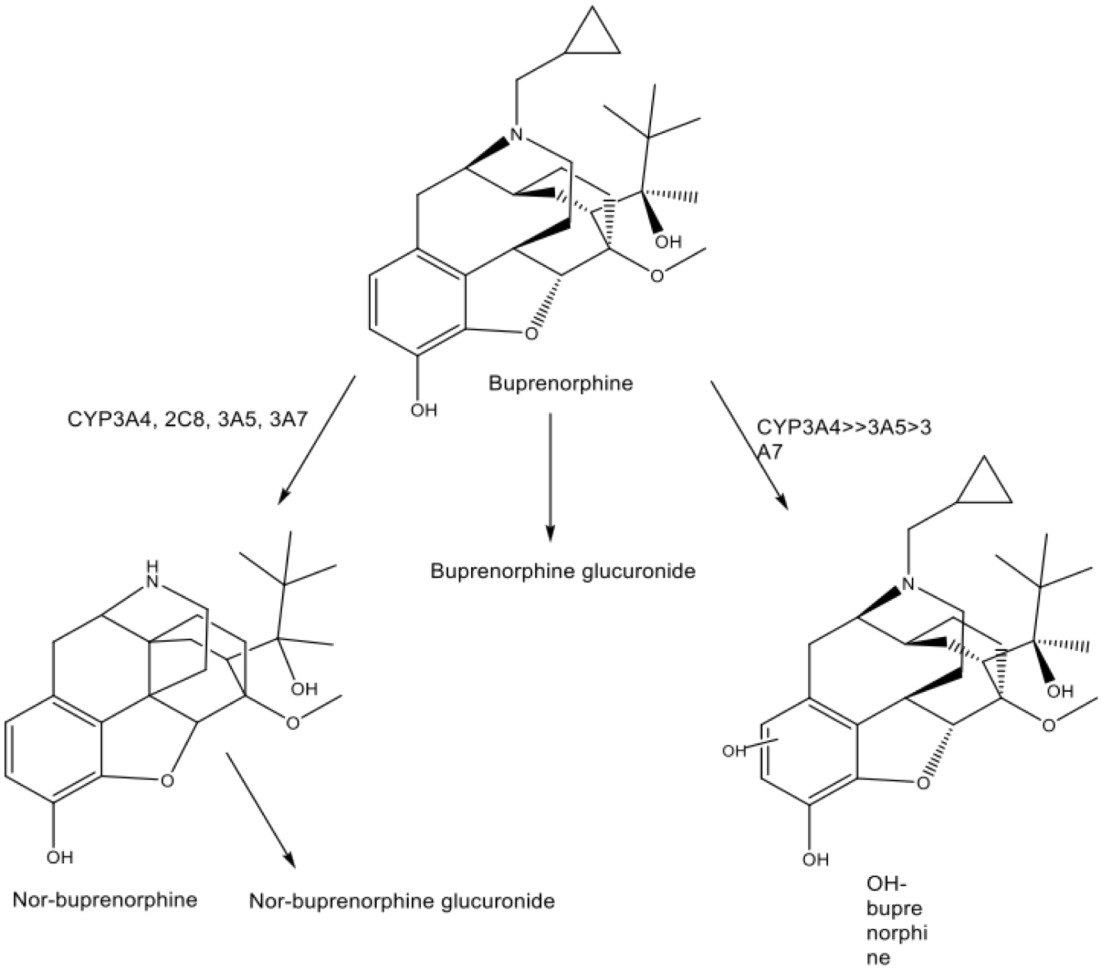

Figure 5-1 Buprenorphine metabolic pathway.

Adapted from (Picard, 2005).

5.3 MATERIAL AND METHODS

5.3.1 Chemicals

Progesterone (P), 17α-hydroxyprogesterone (17α-OHP), estrone (E1), estradiol (E2), estriol (E3), human growth hormone (hGH), and human chorionic gonadotropin (HCG) were purchased from Sigma-Aldrich (St. Louis, MO). Buprenorphine and buprenorphine-D4 were purchased from Cerilliant (Round Rock, TX). MCX cartridge used for buprenorphine extraction was obtained from Waters Corp. (Milford, MA).

5.3.2 Incubation of Primary Cultures of Human Hepatocytes with Female Hormones, and Treating with Buprenorphine

Freshly isolated primary human hepatocytes (1.5×10^6 cells/well) in 6-well plates were purchased from Life Technologies (Carlsbad, CA) in a cold maintenance media. Hepatocytes donor demographics are shown in (Table 5-1). Upon receipt, the media was replaced with ice-cold hepatocyte maintenance media (HMM™) (Lonza, Allendale, NJ) containing 0.35 mg/mL Geltrex™ and incubated overnight. The cell cultures were maintained at 37°C in a humidified atmosphere at 5% CO_2. Cells were treated with DMSO (vehicle), low hormones mixture, or high hormones mixtures for 72 h. The concentrations of female hormones used are shown in (Table 5-2). Low and high concentrations of female hormones mixtures correspond to the levels of these hormones in plasma and liver, respectively. Final concentration of DMSO in the incubation medium was 0.1% (v/v). Treatment medium was replaced every day. On day 4, the medium was

replaced with fresh HMM™ medium containing buprenorphine (100 ng/ml) for 0, 30, 60, and 120 minutes. The culture medium and cell lysate were collected to measure the concentration of buprenorphine and its metabolites using LC-MS/MS analysis. There were no significant morphological changes observed in the hepatocyte cultures. Each experiment was conducted in triplicate. Statistical differences were determined using student's *t*-test. *P* value <0.05 was considered statistically significant. Data is expressed as mean ± SEM.

Table 5-1 Hepatocyte donors demographic.

Donor ID	Age	Sex	Race	BMI	Smoking	Alcohol use
HU14016	82	F	Caucasian	n.d.	n.d.	n.d.
HU14017	57	F	Caucasian.	n.d.	n.d.	n.d.
HU1745	57	F	Caucasian	17	No	No

Table 5-2 Female hormones concentrations at third trimester of pregnancy in humans

Hormone	Low conc (µM)	High conc (µM)
Progesterone	2	20
E1	0.2	2
E2	0.3	3
E3	0.8	8
17-α OHP	0.1	1
hGH	0.0005	0.005
HCG	0.0009	0.009

5.3.3 Samples Preparation

300 µL of supernatant sample was pre-treated with 700 µL of 0.1% perchloric acid (HClO4) and 25 µL of mixture of buprenorphine-d4 and norbuprenorphine-d3 were used as internal standards. The mixture was then loaded onto MCX cartridge, Waters Corp. (Milford, MA), initially conditioned two times with 1 mL methanol and 1 mL 0.1% $HClO_4$. One mL of samples was then loaded onto the cartridge. After washing two times with 1 mL of 2% HCOOH in H_2O, the analytes were eluted two times with 1 mL of (60% acetonitrile, 40% isopropanol containing 5% ammonium hydroxide). Then the eluent was dried down under air. The residue was then

reconstituted with 100 μL of the initial mobile phase (10% acetonitrile, 90% water, containing 0.1%HCOOH) and 7.5 μL of the solution was injected directly into the LC-MS/MS system.

5.3.4 Determination of Buprenorphine Concentration

Sample analysis was performed on a Thermo TSQ quantum ultra-triple quadrupole mass spectrometer. Chromatographic condition was performed using a C18 column (1.7 μM, 2.1x100 mm). A mobile phase consisted of solvent A (98% H_2O, 2% acetonitrile containing 0.1% HCOOH) and solvent B (acetonitrile containing 0.1% HCOOH). The flow rate was 0.3 ml/min. The ion transitions for buprenorphine, norbuprenorphine, buprenorphine-glucuronide, norbuprenorphine-glucuronide, buprenorphine-d4 and norbuprenorphine-d3 were *m/z* 468.3 ➔ 396.2, 414.1 ➔ 340, 644.3 ➔ 468.3, 590.1 ➔ 414.1, 472.3 ➔ 400.2, and 417.1 ➔ 343. The standard curve range for buprenorphine was 1-100 ng/ml and quality controls were 2, 10, and 50 ng/ml. The following gradient was used: 87 % A for 0-0.5 minutes, and then 35% A for 0.5-4.5 minutes, held for 0.5 min, and 87 % A for 5-7 min.

5.3.5 Data Analysis

The apparent intrinsic clearance of buprenorphine was calculated using the following equation (Pillai *et al*., 2014):

$Cl_{int,app}$(mL/min/kg) = (0:693/$t_{1/2}$)(1mL incubation/1.5X10^6 cells)

(130X10^6/g liver)(20 g liver/kg per body weight) **(Equation 5-1)**

Based on the intrinsic clearance, the hepatic clearance (CL_h) was calculated as following equations:

$$CL_h = Q_h(1-F_h) \quad \textbf{(Equation 5-2)}$$

Where the Q_h is the hepatic blood flow and equal to 21 mL/min/kg, and F_h is the hepatic availability.

F_h was calculated based on the following equations:

$$F_h = Q_h/(Q_h + f_{u,inc}XCl_{int,app}) \quad \textbf{(Equation 5-3)}$$

Where $f_{u,inc}$ is the fraction unbound in the hepatocytes incubation media and was calculated:

$$f_{u,inc} = 1/(1+K_p(V_c/V_m) \quad \textbf{(Equation 5-4)}$$

Where K_p is the partition coefficient and equal to 4.98 (Avdeef, 1996), V_c is the volume for the cell adhesion and equal to 0.0051 (Pillai, 2014), and V_m is the volume of the media = 1 mL.

Sublingual clearance of buprenorphine was predicted based on the hepatic clearance using the following equation:

$$CL_{sub} = CL_h/F$$

Were F = 0.51 is the reported sublingual bioavailability of buprenorphine obtained form literature (Kuhlman *et al*., 1996).

5.4 RESULTS

The average concentration of buprenorphine, norbuprenorphine, buprenorphine-glucuronide and norbuprenorphine-glucuronide are shown in (**Figure 5-2**A-D). There was a continuous loss of buprenorphine over time from the culture media. The formation of norbuprenorphine was small in all hepatocytes and the norbuprenorphine-glucuronide was detected only in one donor. Buprenorphine-glucuronide was the most predominant detected metabolite, accounting for most of buprenorphine metabolism. (**Table 5-3**) and (**Table 5-4**) show the mean area under the concentrations-time curves ($AUC_{0\text{-}120\ min}$) and the mass balance for buprenorphine, norbuprenorphine, buprenorphine-glucuronide and norbuprenorphine-glucuronide after treatment with buprenorphine (100 ng/mL). Exposure of buprenorphine did not change with treatment of low or high concentrations of female hormones mixture. Three was no significance difference in $AUC_{0\text{-}120\ min}$ or the mass balance of norbuprenorphine, buprenorphine-glucuronide and norbuprenorphine-glucuronide between low hormones treated group and control group. Compared to DMSO, there was no effect of female hormones on the formation of norbuprenorphine, buprenorphine-glucuronide and norbuprenorphine-glucuronide.

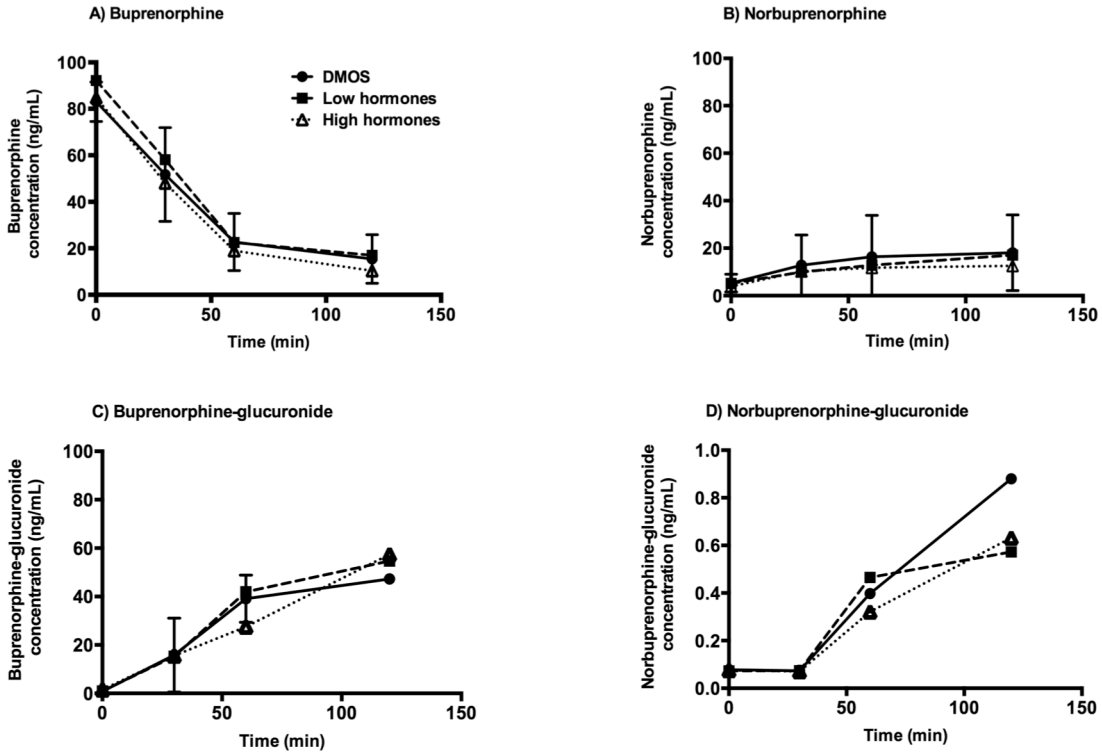

Figure 5-2 Buprenorphine elimination time course.

Time course of (A) Buprenorphine, (B) Norbuprenorphine, (C) Buprenorphine-glucuronide and (D) Norbuprenorphine-glucuronide after low and high concentrations of female hormones, following incubation with 100 ng/mL of buprenorphine in primary cultures of human hepatocytes. Data are expressed as mean ± SEM.

Table 5-3 Area under the concentrations-time curves for buprenorphine and its metabolites.

The mean area under the concentrations-time curves ($AUC_{0\text{-}120\ min}$ (µg*min/mL)) for Buprenorphine (BUP), Norbuprenorphine (NBUP), Buprenorphine-glucuronide (BUP-G) and Norbuprenorphine-glucuronide (NBUP-G) after incubation with low and high concentrations of female hormones, following incubation with 100 ng/mL of buprenorphine in primary cultures of human hepatocytes. Data are expressed as mean ± SEM.

Treatment	BUP	NBUP	BUP-G	NBUP-G
Vehicle	4.3 ± 1.6	1.74± 1.7	3.67± 0.02	0.048
Low hormones	4.6 ± 1.2	1.46 ± 1.42	4 ± 0.55	0.041
High hormones	3.9 ± 0.9	1.27± 1.29	2 ± 2.26	0.037

Table 5-4 Mass balance of buprenorphine and its metabolites.

Mass balance of Buprenorphine (BUP), Norbuprenorphine (NBUP), Buprenorphine-glucuronide (BUP-G) and Norbuprenorphine-glucuronide (NBUP-G) after incubation with DMSO, low and high concentrations of female hormones, following incubation with 100 ng/mL of buprenorphine in primary cultures of human hepatocytes.

Treatment	BUP	NBUP	BUP-G	NBUP-G
Vehicle	17.1	14.4	72.9	1.5
Low hormones	25.7	13.5	94.3	0.97
High hormones	15.9	5.5	101.9	1.1

Pharmacokinetics parameters of buprenorphine, including area under the concentration-time curve ($AUC_{0\text{-}120\ min}$), elimination half-life ($t_{1/2}$), intrinsic clearance ($Cl_{int,\ app}$), and predicted sublingual clearance (CL_{sub} (mL/min) are shown in (**Table 5-5**). Compared to vehicle (control), there was no significant change in buprenorphine half-life, intrinsic clearance, hepatic clearance and predicted sublingual clearance after treatment with female hormones mixture at low concentrations. There was no difference in the half–life, intrinsic clearance, hepatic clearance and predicted sublingual clearance of buprenorphine after treatment with female hormones mixture at high concentrations relative to control (DMSO). Overall, both concentrations of female hormones mixtures did not affect buprenorphine pharmacokinetics.

Table 5-5 Pharmacokinetics parameters of buprenorphine in hepatocytes treated with female hormones.

The mean pharmacokinetics parameters of buprenorphine after incubation with low and high concentrations of female hormones, following incubation with 100 ng/mL of buprenorphine in primary cultures of human hepatocytes. Data are expressed as mean ± SEM.

Treatment	Half-life (min)	Bup metabolized after 2 hours (%)	$Cl_{int, app}$ (mL/min/kg)	CL_h (mL/min)	CL_{sub} (mL/min)
Vehicle	47 ± 15	79.6 ± 3.5	26.9 ± 8.6	11.5 ± 1.7	22.6 ± 3.3
Low hormones	48.3 ± 7.1	83 ± 7.1	25.1 ± 3.7	11.3 ± 0.8	22.1 ± 1.5
High hormones	38.9 ± 6.1	89.8 ± 3.9	31.2 ± 4.9	12.4 ± 0.8	24.3 ± 1.6

5.5 DISCUSSION

PBPK simulation and preliminary clinical observations indicated increase clearance of buprenorphine during pregnancy. It is known that buprenorphine undergoes hepatic metabolism mainly via *N*-dealkylation pathway by CYP3A4 to its active metabolite (norbuprenorphine) (Cone, 1984; Picard, 2005). Buprenorphine and norbuprenorphine undergo glucuronidation mainly by UGT1A1, UGT1A3 and UGT2B7 (Rouguieg, 2010). Our observations in chapter 1 and 2 have indicated that a mixture of female hormones at third trimester concentrations corresponds to what is rejected in the liver of pregnant women enhanced significantly the expression and activity of CYP3A4, and the expression of UGT2B7 in primary cultures of human hepatocytes. Our data in chapter 2 also showed that progesterone significantly increased the expression and activity of UGT1A1 and expression of UGT1A3 increased significantly by estradiol and low hormones mixture. As a result, we expected that mixtures of female hormones at similar concentrations would have the potential to increase the metabolism of buprenorphine in primary cultures of human hepatocytes. In this study, we evaluated the magnitude change in the buprenorphine metabolism after treatment with female hormones mixtures at plasma (low) and liver (high) concentrations during the third trimester of pregnancy.

Our data showed that buprenorphine-glucuronide was the predominant metabolite in the hepatocytes. These findings are in agreement with the theory that buprenorphine is metabolized by *N*-dealkylation, mainly in the small intestine and glucuronidated primarily by the liver (Moody, 2009). It has been also reported that norbuprenorphine metabolite is excreted more in the urine than the buprenorphine glucuronide (Cone, 1984). On the other hand, buprenorphine

and its glucuronide metabolite are excreted in the bile more than norbuprenorphine after sublingual administration of buprenorphine (Cone, 1984). Norbuprenorphine-glucuronide formation was detected only in one subject, and this is consistent with other reported data where glucuronidation was detected only for buprenorphine (Moody, 2009). Our results demonstrated that plasma concentrations of female hormones mixture did not affect the exposure of buprenorphine, norbuprenorphine, buprenorphine-glucuronide and norbuprenorphine-glucuronide.

We also evaluated the impact of female hormones on the pharmacokinetic parameters of buprenorphine (i.e. half-life, intrinsic clearance, hepatic clearance, and sublingual clearance). Our results indicated that both concentrations of female hormones did not significantly change the pharmacokinetics of buprenorphine. This indicates that combination of female hormones may not be responsible for the observed change in the clearance of sublingual buprenorphine that was observed during pregnancy. The formation of norbuprenorphine, buprenorphine-glucuronide and norbuprenorphine-glucuronide was investigated in human liver microsomes and human small intestine microsomes incubated with therapeutic concentrations of buprenorphine (Moody, 2009). In their study, buprenorphine concentration was higher in gut more than in the liver due to the deconjugation of buprenorphine-glucuronide by bacterial glucuronidases (Moody, 2009). Moreover, *N*-dealkylation was the major pathway of buprenorphine metabolism in human small intestine microsomes and buprenorphine-glucuronide was the predominant metabolite in human liver microsomes (Moody, 2009). Their findings indicated that hepatocyte plays an important role in buprenorphine glucuronidation, however it has a minimal contribution to *N*-dealkylate buprenorphine metabolism. Therefore, this suggested that both small intestine and liver play important role in the metabolism buprenorphine of in humans.

In summary, our findings indicate that female hormones did not affect the metabolism of buprenorphine in primacy cultures of human hepatocytes. Therefore, further studies are needed to explain the contribution of both small intestine and hepatocyte in the metabolism of buprenorphine.

6.0 EFFECT OF FEMALE HORMONES ON THE EXPRESSION AND ACTIVITY OF BILIRY DRUG TRANSPORTERS

6.1 ABSTRACT

Physiological changes during pregnancy alter the pharmacokinetics of several medications, resulting in either lowered drug efficacy or drug toxicity. Biliary elimination is a very important pathway for elimination of endogenous and exogenous compounds. The objective of our study is to evaluate the impact of pregnancy on the expression and activity of biliary drug transporters. Sandwich cultured human hepatocytes (SCHH) from three independent livers were incubated with third trimester plasma and liver-extrapolated concentrations of female hormones including estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin individually, and in combination. Liver transporter function was examined using sodium taurocholate as a substrate of bile salt export pump (BSEP). mRNA expression levels of P-glycoprotein (P-gp), multidrug resistance associated proteins (MRP2), breast cancer resistance protein (BCRP), BSEP, and Na/H exchange regulatory factor 1 (NHERF1) were assessed using qRT-PCR. The functional activity of the hepatic transporter was confirmed by calculating the biliary excretion index (BEI) of taurocholate. Female hormones did not play a major role in altering the activity of BSEP. Progesterone significantly decreased the mRNA expression of BCRP, and estradiol significantly increased the mRNA expression of NHERF1. No significant changes in the expression of P-gp, MRP2, and BSEP were observed compared with the relative control. Taken together, this data may provide limited impact of pregnancy related hormones on the expression and activity of drug transporters.

6.2 INTRODUCTION

Liver is the primary organ responsible for the metabolism and elimination of several endogenous and exogenous compounds (Yang *et al*., 2013). Many drugs that undergo glucuronidation, cross the canalicular space and get excreted into the bile via the ATP-binding cassette (ABC) transporters superfamily (Liu *et al*., 1999). The most highly expressed biliary drug transporters at the canalicular membrane are P-glycoprotein (P-gp), multidrug resistance associated proteins (MRP2), breast cancer resistance protein (BCRP), and the bile salt export pump (BSEP) (Ghibellini *et al*., 2006).

P-gp is a transmembrane protein encoded by MDR1 and MDR2 in humans and is composed of 1280 amino acids (Dong *et al*., 1998). The MDR1 gene is the most common gene reported to transport drugs such as digoxin, antitumor agents, and opiates (Fardel *et al*., 2001). A study in mice showed that phospholipid secretion is controlled by MDR2 (Frijters *et al*., 1999). Schondorf *et al*., 2002 reported that MDR1 transcription is potentially induced by antineoplastic agents (cisplatin, doxorubicin, paclitaxel) in ovarian cancer cell lines.

MRP2 is a 13-transmembrane protein that contains 1545 amino acids (Fardel, 2001; Sun *et al*., 2010). MRP2 is expressed primarly in the liver at the canalicular space with low expression in renal proximal tubules, gut enterocytes, placenta, and blood brain barrier (Kruh and Belinsky, 2003). MRP2 is responsible for the elimination of conjugated bilirubin, xenobiotics, as well as conjugated metabolites (Keppler and Arias, 1997). It plays an important role in the drug disposition of several glucuronide metabolites. Different physiopathological conditions contribute in the modulation of MRP2 (Payen *et al*., 2002). For example, MRP2 expression is down-regulated during cholestasis and increased in some tumor tissues (Payen, 2002).

BSEP is predominantly localized in liver canaliculi (Stieger *et al*., 2007). It has a narrow substrate spectrum and is responsible mainly for the canalicular transport of conjugated bile acids such as taurocholic acid (TCA) (Stieger, 2007). BSEP is also involved in the transport of some antineoplastic drugs such as vinblastine (Lecureur *et al*., 2000). Expression of BSEP can be controlled by different physiological factors. It has been reported that inhibiton of BSEP can affect bile salt excretion (Ghibellini, 2006). Steroid hormones such as estrogen and progesterone play an important role in regulating the hepatic expression of these transporters (Fardel, 2001). For example, estrogen metabolites have been reported to produce BSEP trans-inhibition leading to cholestasis in rat liver (Stieger *et al*., 2000). Moreover, progesterone has been shown to inhibit BSEP (Barnes *et al*., 1996), which may lead to a condition similar to intrahepatic cholestasis of pregnancy (ICP).

BCRP is localized and highly expressed in different human tissues including liver (canalicular membrane of hepatocytes), breast, placenta, and intestine (Yang *et al*., 2014). It has a broad substrate specificity and is involved in the biliary elimination different substrates such as chemotherapeutic drugs (Kock and Brouwer, 2012). Studies in human placenta BeWo cells indicated that progesterone, estriol, human placental lactogen, and human prolactin up-regulated the expression of BCRP during pregnancy (Wang *et al*., 2008; Wang *et al*., 2006). However, estradiol down-regulated BCRP expression in human placenta BeWo cells (Wang, 2006). Estradiol in combination with progesterone has been found to increase the expression of BCRP (Wang, 2006).

Multiple studies have reported the regulation of drug transporters in a pregnant mouse model. Hepatic mRNA expression of MDR2, MRP2, BSEP, and BCRP were down-regulated in pregnant mice from day 14 to day 17 of gestation compared with the postnatal period (Aleksunes

et al., 2012). Although much has been learned, the role of pregnancy-related hormones in regulating the human hepatic expression of biliary drug transporters is still poorly understood. Due to the continuous increase in the plasma levels of female hormones during pregnancy, we hypothesize that female hormones will potentially regulate the expression of biliary drug transporters in human hepatocytes. Therefore, the goals of our study were: 1) to examine the impact of pregnancy-related hormones on the activity of BSEP using sodium taurocholate as a substrate of BSEP with sandwich culture human hepatocytes, and 2) to evaluate the effect of female hormones on the hepatic expression of P-gp, MRP2, BSEP, BCRP, and NHERF-1 in sandwich cultured human hepatocytes.

6.3 MATERIAL AND METHODS

6.3.1 Chemicals

Progesterone (P), 17α-hydroxyprogesterone (17α-OHP), estrone (E1), estradiol (E2), estriol (E3), human growth hormone (hGH), and human chorionic gonadotropin (HCG were purchased from Sigma-Aldrich (St. Louis, MO). Taurocholic acid and sodium salt (24-14C) were purchased from Perkin Elmer. TaqMan Primers for transporters were purchased from Life Technologies.

6.3.2 Female Hormones Incubation in SCCH and Transporter Study

Freshly isolated, sandwich cultured human hepatocytes (1.5×10^6 cells/well) overlaid in sandwich fashion into 6-well plates were purchased from Life Technologies (Carlsbad, CA) in cold maintenance media. Hepatocyte donor demographics are shown in (Table 6-1). Upon receipt, media was replaced with ice-cold hepatocyte maintenance media (HMM™) (Lonza, Allendale, NJ) containing 0.35 mg/mL Geltrex™ for overnight incubation. The cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

The effect of female hormones on the functional activity of BSEP was studied in sandwich cultured human hepatocytes (SCHH) was described by Sharma *et al*., 2013. Briefly, freshly isolated SCHHs from three female livers were incubated with 1 μM of taurocholic acid (TCA) containing DMSO as a vehicle control, or a mixture of high concentration female hormones (Table 6-2), or 40 μM of cyclosporine in standard Hank's balanced salt solution (HBSS) containing Ca^{2+} at 37°C for 20 minutes. The uptake of taurocholate was stopped by washing cells three times with ice-cold media. The taurocholate efflux from canalicular space

was initiated by adding standard HBSS (containing DMSO, or a mixture of high concentration female hormones, or 40 μM of cyclosporine) to maintain the integrity of tight junctions or by adding Ca^{2+} free HBSS (containing DMSO, or a mixture of high concentration female hormones or 40 μM of cyclosporine) to open the bile canaliculi tight junctions. Culture supernatants and cell lysates were collected and 200 μL aliquots were subjected to liquid scintillation counting to determine the efflux of TCA [14C]. Cell lysis buffer was prepared as described by (Liu and Unadkat, 2013). Briefly, 1 mM EDTA was mixed with 10-M Tris HCl, pH 8, and 0.5% Triton-X 100. Total protein was measured using the Bradford method (Bradford, 1976) in order to normalize data with total amount of protein. Efflux of TCA into canalicular space and intrinsic biliary clearance were calculated using the following equations (Liu, 1999):

Biliary excretion index BEI (%) =

[Accumulation (cell+bile) - Accumulation (cell)]/ Accumulation (cell+bile) * 100 (**Equation 6-1**)

Intrinsic biliary clearance (Bililary CL_{int}) =

[Accumulation (cell+bile) - Accumulation (cell)] / (AUC_{medium}) (**Equation 6-2**)

Where accumulation (cell+bile) is the amount of radioactivity in standard HBSS containing Ca^{2+}, accumulation (cell) is the amount of radioactivity in HBSS without Ca^{2+}, and AUC is the product of the initial concentration in the medium multiplied by the incubation time.

In the absence of cyclosporine or female hormones, taurocholic acid efflux in canaliculi is defined to be 100% based on the difference in radioactivity between the standard HBSS

containing Ca^{2+} or Ca^{2+}-free HBSS. In the presence of cyclosporine or female hormones, taurocholic acid efflux in canaliculi becomes less and was used to represent the percent of inhibition of taurocholic acid efflux into the canalicular space. Data is representative of three independent livers and repeated twice. Statistical differences were determined using student's *t*-test. *P* value <0.05 was considered statistically significant. Data is expressed as mean ± SEM.

Table 6-1 Hepatocyte donors demographic.

Donor ID	Age	Sex	Race	BMI	Smoking	Alcohol use
HU1743	56	F	Caucasian	19	YES	YES
HU14016	82	F	Caucasian	ND	ND	ND
HU14017	57	F	Caucasian.	ND	ND	ND

ND: No data

Table 6-2 Female hormones concentrations at third trimester of pregnancy in humans

Hormone	Low conc (μM)	High conc (μM)
Progesterone	2	20
E1	0.2	2
E2	0.3	3
E3	0.8	8
17-α OHP	0.1	1
hGH	0.0005	0.005
HCG	0.0009	0.009

6.3.3 RNA Sample preparation and Quantitative Real-Time PCR Assay

Isolation of total RNA and quantification of mRNA expression levels were described in chapter 2. Briefly, Trizol reagent (Invitrogen, San Diego, CA) was used to extract total RNA from hepatocytes. The purity and concentration of the extracted RNA were determined using NanoDrop spectrometers at absorbance of 260 nm. cDNA was synthesized using 1 μg of total RNA by reverse-transcription using iScript TM Reverse Transcription Supermix (Bio-Rad, Hercules, CA). Taqman Master Mix and the following primers were obtained for quantitative RT-PCR (qRT-PCR) analysis from Applied Biosystems: ABCB1 (Hs00184500), ABCB11 (Hs00184824), ABCC2 (Hs00166123), ABCG2 (Hs01053790) and GAPDH (Hs02758991) genes. The PCR amplifications were performed in an ABI Prism 7300 system (Applied

Biosystems, Foster City, CA, USA). Expressions of mRNA levels were normalized to the housekeeping gene (GAPDH). The relative levels of mRNA fold changes of all genes were quantified using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak, 2001).

6.4 RESULTS

6.4.1 Effect of Female Hormones on the Expression of Biliary Drug Transporters

To evaluate the influence of female hormones on the expression of biliary drug transporters, three independent SCHHs were treated with 2 mM phenobarbital, low and high female hormone mixtures (estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin) as well as with individual treatment of high concentration estradiol, progesterone, human growth hormone, and human chorionic gonadotropin for 3 days. mRNA levels of BSEP, P-GP, MRP2, and BCRP were measured by quantitative real-time PCR (Figure 6-1**A-D**). The relative mRNA expression was normalized to GAPDH. Phenobarbital decreased the mRNA expression of BSEP by about 80 % ($p < 0.05$). Progesterone at high concentration significantly suppressed the expression of BCRP by 33% ($p < 0.05$). Female hormones did not alter the expression of other biliary transporters.

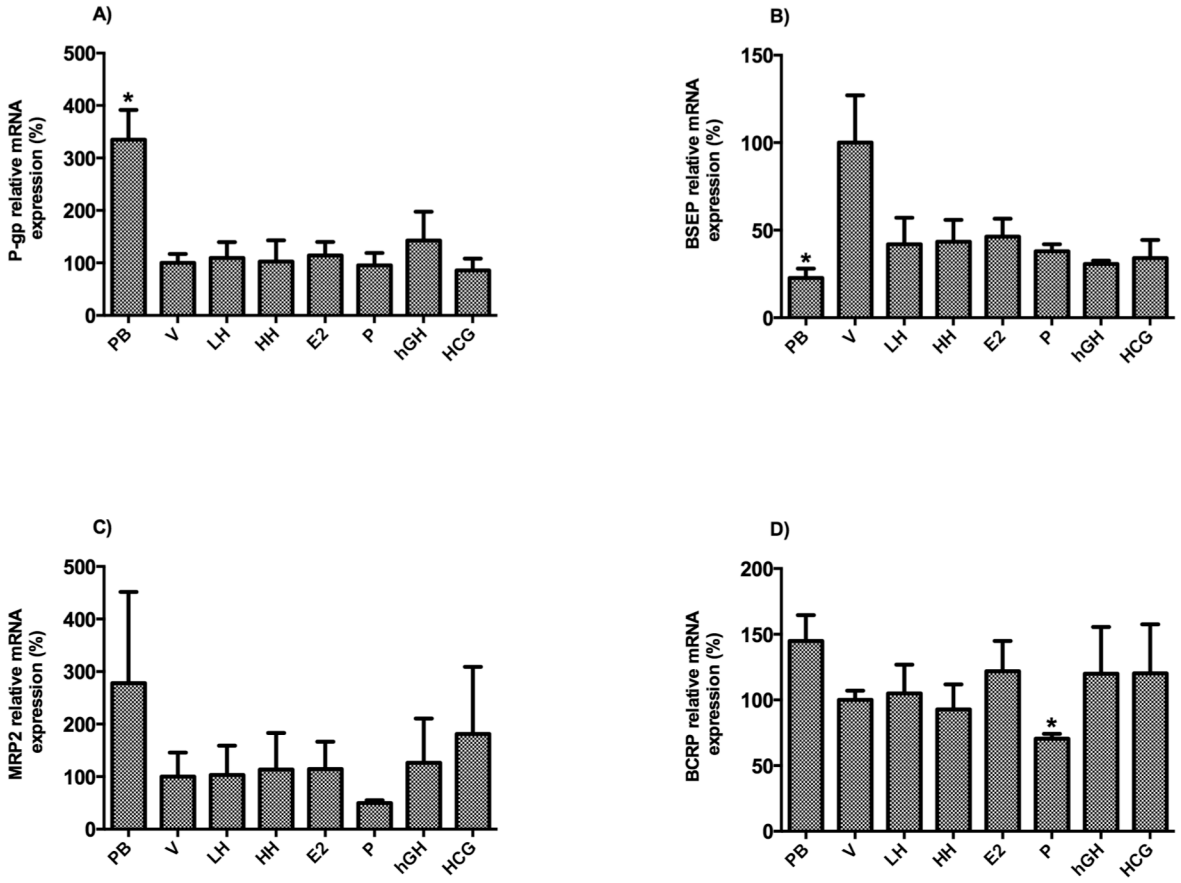

Figure 6-1 Effect of female hormones on hepatic mRNA expression of biliary drug transporters.

The mRNA expression of **(A)** P-gp, **(B)** BSEP, **(C)** MRP2, and **(D)** BCRP were determined after treating SCHH (n=3) with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * (P<0.05) compared to the control group.

6.4.2 Effect of Female Hormones on TCA Transport

To determine the impact of female hormones on the inhibition of TCA efflux, we incubated SCHH with female hormones at the predicted concentrations in the liver corresponding to the third trimester of pregnancy (Figure 6-2). High concentrations of female hormone combinations decrease the efflux of TCA. However, compared to control, this was not statistically significant. Significant inhibition in TCA efflux ($p < 0.05$) was observed after treatment with 40 μM of cyclosporine (positive control inhibitor).

The average (±SD) accumulation in the presence of calcium (cells+bile) for cyclosporine, and female hormones was 1.3 ± 0.08, and 7.3 ± 0.3 (pmol/mg protein), respectively. However, the average (±SD) accumulation in the absence of calcium (cells) for cyclosporine, and female hormones was 0.95 ± 0.1, and 1.5 ± 0.06 (pmol/mg protein), respectively. The mean BEI and intrinsic biliary clearance for taurocholate, cyclosporine, and female hormones were 71%, 27%, and 77%, respectively, and 12.3, 3.1, and 11.9 μL/min/mg protein, respectively.

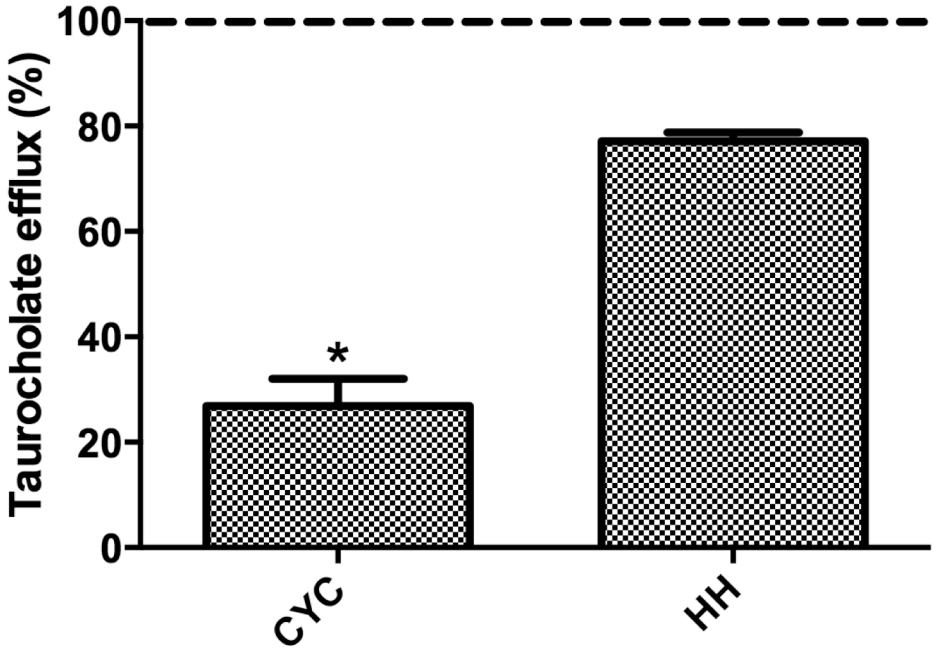

Figure 6-2 Effect of female hormones on TCA efflux.

Percent of taurocholate efflux in canaliculi after treatment with female hormones and cyclosporine (positive control) was calculated based on equation (1) relative to the control. Experiments were conducted in duplicate in three groups hepatocytes, and results are expressed as mean ± SEM. *($p < 0.05$) compared to the control group.

6.4.3 Expression of NHERF-1 after Female Hormones Treatment

To understand the regulation of NHERF1 by female hormones, we treated three different SCHHs with 2 mM phenobarbital, low and high female hormone mixtures (estrone, estradiol, estriol, progesterone, 17α-hydroxyprogesterone, human growth hormone, and human chorionic gonadotropin) as well as with individual treatment of high concentration estradiol, progesterone, human growth hormone, and human chorionic gonadotropin for 3 days. Expression levels of NHERF1 were measured by quantitative real-time PCR (Figure 6-3). The relative mRNA expression was normalized to GAPDH. Estradiol is the only hormone that regulates the expression of NHERF1 by significantly enhancing its mRNA level ($p < 0.05$).

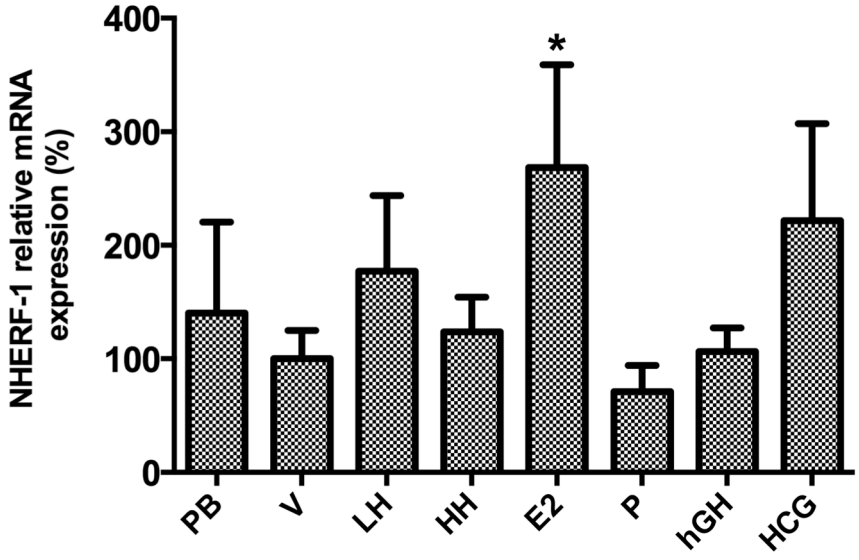

Figure 6-3 Effect of female hormones on NHERF-1 expression.

The mRNA expression of NHERF-1 was determined after treating SCHH (n=3) with multiple female hormones chronically. The ratio of the treated group (PB or female hormones)/control group (DMSO or vehicle) was normalized to GAPDH and control group was arbitrarily defined as 100%. Experiments were conducted in duplicate for all hepatocytes, and results are expressed as mean ± SEM. * (P<0.05) compared to the control group.

6.5 DISCUSSION

Biliary drug transporters play an important role in the elimination of several drugs. Bile salt export pump (BSEP, ABCB11) is a very important transporter responsible for excretion of bile acids such as taurocholic acid (TCA) from the hepatocytes into the bile (van Beusekom *et al*., 2013). Intrahepatic cholestasis of pregnancy (ICP) is a common medical condition that has been linked to the inhibition of BSEP, presumably due to the accumulation of toxic bile acids in the liver (Woodhead *et al*., 2014). Information about the regulation of biliary transporters by female hormones is limited. To examine the impact of female hormone combinations on the activity of BSEP, we first calculated the percentage of taurocholate BEI to determine the functional expression of substrates into the canalicular space (Liu, 1999). Our findings showed that BEI of taurocholate is 71%, consistent with other reported BEI: 41-63% ((Bi *et al*., 2006) and 53-71% (Abe *et al*., 2009). We also calculated the intrinsic biliary clearance of taurocholate (12.3 μL/min/mg protein), which was in agreement with the reported intrinsic biliary clearance from (Abe, 2009): taurocholate, 12-25 μL/min/mg protein. We used cyclosporine as a positive control (known inhibitor of BSEP). The BEI and biliary intrinsic clearance in presence of cyclosporine was 27% and 3.1 μL/min/mg, respectively, indicating a significant inhibition of BSEP and confirming the functional activity of the BSEP transporter. Since the BEI in the presence of female hormones is higher than the BEI of taurocholate (substrate of BSEP), this suggests that female hormones play a limited role in regulating the biliary excretion pathway.

In this study we evaluated for the first time the combined effect of female hormones on the expression of various biliary drug transporters in SCHH. Our data showed the inhibitory effect of female hormones on the mRNA expression of the bile salt export pump (BSEP). It also demonstrated the role of progesterone in regulating the mRNA expression of breast cancer

resistance protein (BCRP). Moreover, the regulation of NHERF1 by estradiol has been also examined in SCHH. Our overall findings indicate the potential role of female hormones in regulating the expression of BSEP, BCRP, as well as NHERF1. We also evaluated the regulation of other efflux transporters by female hormones. Our findings indicated that only progesterone has the potential to decrease the expression of BCRP. However, other female hormones did not change the expressions of P-gp and MRP2. BCRP is highly expressed in different tumor cells (Carcaboso *et al*., 2010; Huss *et al*., 2005; Kawabata *et al*., 2003; Wu *et al*., 2013). Recently it has been shown that progesterone regulated BCRP by down-regulating BCRP-mRNA levels (Wu, 2013). For the regulation of P-gp, our data indicated that female hormones did not alter the expression of P-gp and MRP2 at the mRNA level, which is in part consistent with other studies that demonstrated the unchanged expression of P-gp during pregnancy in mouse liver (Zhang *et al*., 2008). However, it has been reported that expression of MRP2 is decreased in pregnant rats (Cao, 2002). The reason for this discrepancy may be due to the interspecies differences between humans and rodents in regard to the expression of MRP2.

Na/H exchange regulatory factor 1 (NHERF1) is a cytoplasmic protein that plays an important role in recruiting different ion transporter proteins to the plasma membrane in various cells (Wang *et al*., 2008). (Cuello-Carrion *et al*., 2010) reported that expression of NHERF1 is increased by estrogen in rat colon during the estrous cycle. It was also reported that estrogen enhanced the mRNA expression of NHERF1 in breast cancer cell lines (Katzenellenbogen *et al*., 2000) as well as in the epithelial cells in the bile duct of rat liver (Fouassier *et al*., 2009). As levels of estrogen increased during pregnancy, we expected increases in the expression of NHERF1 by estrogen. Based on this, we measured the mRNA expression of NHERF1 after treatment with various female hormones including estradiol in SCHH. Our data indicate that

estradiol significantly increased the expression of NHERF1. Accumulation studies indicated that NHERF1 interacts with MRP2 and plays an important role in the expression and function of MRP2 at the canalicular space (Li *et al*., 2010). We also observed a slight increase in the expression of MRP2 by estradiol (17%), which may be due to increased NHERF1 expression, which is consistent with the reported decrease of the function of MRP2 in NHERF1 deficient mice (Li, 2010).

In summary, the present study indicated that progesterone significantly inhibits the expression of BCRP. Given the fact that BCRP has a large number of substrates (Krishnamurthy and Schuetz, 2006; Mao and Unadkat, 2005; Zhang *et al*., 2009), these findings may explain the altered pharmacokinetics of BCRP substrates such as sulfasalazine in pregnant women. Our data also suggested that female hormones do not play a major role in modulating the expression of BSEP, MRP2 and P-gp.

7.0 CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

Pregnancy is associated with several physiological changes that are expected to alter the pharmacokinetics of medications. Limited studies about the effect of pregnancy on drug pharmacokinetics. These studies indicated that clearance of certain drugs metabolized by CYPs and UGTs is increased during pregnancy. However, limited studies have been performed to characterize reasons for the observed increase in clearance of certain drugs in pregnancy. The overall purpose of this dissertation was to evaluate the effect of pregnancy on phase I, II, and II pathways. First, the effect of major pregnancy hormones on the expression and activity of hepatic CYP450 enzymes was evaluated. Second, the effect of major female hormones on the expression and activity of hepatic UGT enzymes was analyzed. Third, the effect of major female hormones on the expression and activity of biliary drug transporters was assessed. Fourth, we developed and validated a pregnancy PBPK model of buprenorphine. Finally, the impact of pregnancy-related metabolism of buprenorphine–as this drug is cleared by both CYP and UGT–was appraised.

In the first study, the effect of a mixture of female hormones at concentrations corresponding to plasma concentrations and projected liver concentrations on the expression and activity of major hepatic CYP enzymes was investigated in primary cultures of human hepatocytes. Findings of this investigation indicated that pregnancy-related hormones significantly increased the mRNA, activity, and protein levels of CYP3A4 but did not alter the

expression of CYP1A2, CYP2C9, CYP2C19, or CYP2D6. This observation provided a mechanistic understanding of increased clearance of substrates of CYP3A4 during pregnancy. Results supported the need for a dosage adjustment of CYP3A4 substrates used in pregnancy is recommended.

In the second study, the effect of various individual female hormone mixtures on the expression and activity of major hepatic UGT enzymes was evaluated in primary cultures of human hepatocytes. Results indicated that expression and activities of UGT1A1 and UGT1A4 are increased in the presence of female hormones, which explained the observed increase clearance of labetalol and lamotrigine during pregnancy. However, increased expression and activity of UGT1A4 substrate (lamotrigine) is only partially explained by female hormones. In this study, our findings demonstrated that different female hormones increased the expressions of UGT1A3, UGT1A6, UGT1A9, and UGT2B7. The expressions of nuclear receptors were also measured. Data showed that female hormones have an effect only on AhR by up-regulating its mRNA expression. These findings demonstrated that female hormones increased the expression of UGT1A1 and UGT1A3 perhaps through the effect on AhR.

The new study evaluated the effect of pregnancy on buprenorphine pharmacokinetics using a PBPK model. A PBPK model in non-pregnant women was first validated. Then the model was used to evaluate the effect of pregnancy on buprenorphine pharmacokinetics by comparing the SIMCYP-predicted data to the observed data in pregnant subjects. Findings demonstrated that prediction of buprenorphine pharmacokinetic parameters in pregnant women is in agreement with observed results, suggesting that buprenorphine pharmacokinetics can be reasonably predicted using a pregnancy PBPK model. Both predicted and observed data of buprenorphine in pregnancy demonstrated that clearance of buprenorphine was increased by

about 25%. These results support making dosage adjustments of buprenorphine in pregnant women and illustrate value of PBPK modeling in predicting pharmacokinetic of drugs during pregnancy.

In the fourth study, the metabolism of buprenorphine was evaluated after treatment of human hepatocytes with plasma and projected liver concentrations of female hormones. This study was conducted to evaluate whether pregnancy related hormones play a major role in the observed increased clearance of buprenorphine during pregnancy using primary cultures of human hepatocytes. Buprenorphine-glucuronide was the predominant metabolite. This study demonstrated that both plasma and liver concentrations of female hormones did not influence the metabolism of buprenorphine or generation of the metaboltes. The observed increase in clearance of buprenorphine in pregnancy may be due to altered gut metabolism of buprenorphine.

In the study of regulation of biliary drug transporters by female hormones in sandwich cultured human hepatocytes, the effect of predicted liver concentrations of female hormones on the activity of BSEP transporter was characterized. Findings indicated that female hormones did not play a major role in altering the activity of BSEP. Expressions of BSEP, P-gp, MRP2, and BCRP were measured. Female hormones had a different effect on the mRNA levels of various biliary drug transporters. Progesterone decreased the mRNA expression of BCRP significantly while estradiol increased the mRNA expression of NHERF1 substantially.

The overall findings provide some specifics on the mechanistic understanding of how pregnancy influences drug disposition. The important role that pregnancy-related hormones have in altering the expression and activities of hepatic CYPs, UGTs, and biliary transporters was identified. Several substrates of these enzymes and transporters are commonly used in pregnant

women. Therefore, these results indicate that dosing of these medications should be carefully considered. *In vitro* and *in vivo* studies were also conducted to predict the altered pharmacokinetics during pregnancy. Incorporating this data, together with knowledge of physiological changes in pregnancy, can be used to develop more accurate models, which ultimately can help us optimize drug therapy in pregnant women.

Primary contribution of the current work:

- Studies were performed in human relevant tissues (primary cultures of female human hepatocytes).
- Effect of female hormones mixture at plasma and liver concentrations was tested.
- CYP cocktail assay was used for measuring CYP activity.
- Provided potential mechanism of increased clearance of CYP3A4 substrates during pregnancy.
- Provided potential mechanism of increased UGT-mediated clearance of lamotrigine and labetalol during pregnancy.
- Applied and validated PBPK model of buprenorphine in pregnancy and predicted fetal and brain exposure of buprenorphine during pregnancy.
- *In vitro*, *in silico*, and *in vivo* studies were performed to understand the impact of pregnancy on metabolism and transport.

Limitations of the current work:

There were some limitations to the current studies:

- Three sets human hepatocytes were utilized in the UGT and transporter regulation studies. Availability and cost were the primary reasons for use of only three sets of hepatocytes. The small sample size could be the reason that significant differences in the expression of some of the other enzymes, transporters, and transcriptional receptors studied between the control group and the group treated with female hormones were not observed.
- Most of the human hepatocytes that were utilized were isolated from subjects who had a history of chemotherapy. This may have confounded the results observed. Unfortunately this is the primary source of human hepatocytes available currently.
- Some of the hepatocytes utilized in transporter regulation and buprenorphine metabolism studies were isolated from postmenopausal women. This could be a possible reason for the lack of effect of female hormones on transporters expression and on buprenorphine pharmacokinetics.
- Hepatocytes are not a suitable model for evaluating the activation of nuclear receptors. We were only able to measure m-RNA expression of certain nuclear receptors.
- The impact of pregnancy hormones on the expression and activity of CYPs, UGTs, and transporters was evaluated. The protein level of CYP3A4 after treatment with female hormones was also measured. Future measurements of protein expression of UGTs and transporters could expand the understanding and interpretation of these findings.
- As previously described, female hormones did not significantly alter the metabolism of buprenorphine in human hepatocytes. Because the small intestine has been shown in

previous studies to contribute to the metabolism of buprenorphine, future studies might investigate the effect of female hormones on the metabolism of buprenorphine in the small intestine.

- The PBPK model used in these studies predicted the pharmacokinetics of buprenorphine in pregnant women. Future studies could be performed to validate the exposure of buprenorphine metabolites using this model, which could contribute to a greater understanding of the adequacy of this modeling approach.
- Female hormones only partially explained changes in expressions and activities of certain drug metabolizing enzymes and transporters. Other factors that alter pharmacokinetics of drugs are not addressed in this study.
- Addition of serum from pregnant women to the human hepatocyte culture in order to evaluate the effect of various components was not successful. Future studies should optimize this approach.

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