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METHADONE METABOLISM IN PLACENTAS FROM PRETERM PREGNANCIES

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METHADONE METABOLISM IN PLACENTAS FROM PRETERM PREGNANCIES

by
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METHADONE METABOLISM IN PLACENTAS FROM PRETERM PREGNANCIES

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The aim of this investigation is to identify and characterize the enzyme system responsible for the N-demethylation of methadone in human preterm placentas. The metabolism of methadone revealed typical Michaelis-Menten saturation kinetics. Methadone was N-demethylated to EDDP, only, by human placental aromatase. This was confirmed through the use of chemical inhibitors and monoclonal antibodies raised against specific CYP isoforms. The affinity of methadone to CYP19 remained relatively unchanged throughout gestation. However, the activity of the enzyme increased as gestation progressed, but showed wide variations between individual placentas. Taken together, it was shown that aromatase is the major enzyme responsible for the biotransformation of methadone throughout pregnancy. The variability in activity should affect the concentration of the drug within the fetal circulation. Accordingly, this might be an important factor affecting the occurrence and intensity of neonatal abstinence syndrome.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

THE PLACENTA

The placenta is a highly specialized organ, consisting of a complex system of membranes that surround the developing fetus, in all viviparous vertebrates. It separates the maternal and fetal circulation system, while being simultaneously perfused by both. The placenta provides the fetus with water, oxygen, and its vital nutrients, as well as a clearance route for excretory products. Additionally, it produces a large number of proteins and hormones that are essential to the development of the fetus and the maintenance of pregnancy.

Anatomical barrier

There are generally four different types of membranes involved in the development of the placenta. The chorion is an epithelial layer derived from the trophoblast and completed by an inner layer of mesenchyme. This layer is derived from the embryo and soon develops its own capillary system. In humans, as in most species, the chorion is the exchange barrier between the mother and the fetus (Leiser and Kaufmann, 1994). The amnion is another epithelial layer derived from the folding of the embryonic ectoderm. It forms an additional inner surrounding layer and possibly an extra exchange barrier. The vitelline sac, or yolk sac, develops as an accessory structure of the embryonic midgut. However, in humans, the vitelline sac is a rudimentary structure that does not participate in transplacental exchange (Leiser and Kaufmann, 1994). The allantois is an extraembryonic sac that develops from the hindgut and acts as an embryonic bladder. It is highly vascularized and fuses with the chorionic capillary bed. In humans, the allantois is barely involved in maternal-fetal exchange and usually regresses (Leiser and Kaufmann, 1994).

All placental types form interdigitations of the fetal and maternal surfaces to increase the surface area of both, facilitating increased exchange. This area is composed of chorioallantoic placental tissue, but is not the only area of transplacental exchange. There are several different types of interdigitation seen throughout the mammalian

species. A diffuse placenta describes the infolding of the entire surface of the chorion. While this type does occur, most mammalian placentas exhibit some type of localization of the interdigitation. The cotyledonary placenta is typified by many concentrated regions of interdigitation separated by areas of smooth membranes. When the membrane infolding is localized around the middle of the chorion sac, it is referred to as a zonary placenta. Most primates exhibit a bidiscoidal placenta, meaning the interdigitation is concentrated in two distinct regions (Kaufmann et al., 1985). However, humans have a discoidal placenta, containing only one placental disc, which exhibits the highest degree of concentration of exchange tissue (Boyd and Hamilton, 1970).

Additionally, mammals exhibit different patterns of the interdigitation between maternal and fetal membranes. This refers to the spatial arrangement of membranes to form the placenta. The simplest pattern is the folded type in which folds of uterine membranes an chorion interdigitate. The lamellar type involves a more complex composition of folds. Humans exhibit the villous type of placentation (Boyd and Hamilton, 1970). In this type, the fetal membrane forms branching villi which come in contact with the surrounding maternal tissues. The human villi are directly bathed in maternal blood. The most complex type of interdigitation is the labyrinthine placenta. The chorion is penetrated by arranged channels; half of which are perfused by maternal blood and half containing fetal capillaries.

The Grosser classification is the most prominent classification system for placental types. This system involves the description of the type and number of tissue layers separating the maternal and fetal circulations. In epitheliochorial placentas, the chorion is attached to intact uterine tissue and forms the most complete barrier. There is little endometrial invasion, and the circulations are separated by six tissue layers. This arrangement provides added safety since the maternal and fetal circulations are completely isolated (Enders and Carter, 2004). However, there is the greater difficulty in the transfer of materials between the two organisms. The synepitheliochorial placenta is similar to the first classification, but some trophoblastic cells fuse with maternal epithelial cells. Following invasion through the uterine epithelium and maternal

connective tissue, the trophoblast is in direct contact with maternal endothelial cells, leading to the formation of an endotheliochorial placenta. The presence of the endothelium may help reduce the transfer of fetal cells into the mother (Enders and Carter, 2004). Hemochorial placentas results from further invasion of maternal tissues and destruction of fetal capillaries, leading to the direct contact between trophoblastic cells and maternal blood. Humans exhibit this form of placentation. This condition is advantageous because it provides direct access to facilitate transplacental transfer, such as glucose, amino acids, and oxygen – carbon dioxide exchanges (Enders and Carter, 2004). However, the invasiveness of this placentation can create disadvantages, including the increased possibility of the passage of cells between the fetus and the mother, and extensive maternal bleeding at birth, due to disruption of maternal vessels. This category is further subdivided by the number of trophoblastic layers: hemotrichorial, three layers; hemodichorial, two layers; and hemomonochorial, one layer. Humans are hemotrichorial at the beginning of gestation, but as the placenta grows, it begins to thin, resulting in a hemomonochorial placenta at term (Boyd and Hamilton, 1970).

The human placenta is comprised of 20-40 vascular units, known as cotyledons (Syme et al., 2004). Each cotyledon contains a chorionic villus tree, which is the site of transplacental exchange between the maternal and fetal circulation. Each villus tree is surrounded by the basal plate on the maternal side, the chorionic plate on the fetal side, and laterally by decidual septa. The villus consists of a central fetal capillary, villus stroma, and an outer trophoblast layer. The outer trophoblastic layer consists of the cytotrophoblasts and the multi-nucleated syncytiotrophoblast, formed by the fusion of the mononucleated cells. Early in pregnancy, this layer is thick with a complete cytotrophoblast layer under the syncytiotrophoblasts. However, as the placenta grows and matures, the outer layer begins to thin, due to the partial disappearance of the cytotrophoblast layer (Enders and Blankenship, 1999). The circulations of the two organisms are further separated by the trophoblastic basement membrane, connective tissue, the endothelial basement membrane, and the endothelium of the fetal capillaries. Within the villus stroma, there are placental tissue macrophages, also known as Hofbauer

cells. The villus trees project into the intervillus space and are directly surrounded by maternal blood, which circulates in this space. However, maternal blood does not perfuse the placenta during the embryonic period. In fact, the feto-placental-maternal circulation is not established until the tenth week of pregnancy (Syme et al., 2004).

Blood flow of the maternal and fetal circulation is a physiological aspect of placental development used for classification purposes. This classification system is based on the efficiency of the systems, stressing the importance of the fetal oxygen supply. The most inefficient system is the concurrent flow exchanger (Leiser and Kaufmann, 1994). Capillaries from both organisms are arranged parallel to each other and flow in the same direction. However, due to its inefficiency, this exchanger is not represented in mammalian placentas (Martin, 1981). In countercurrent flow, maternal and fetal capillaries are arranged in parallel, but blood flow is in opposite directions. This is the most efficient exchanger arrangement for passive diffusion, and therefore, allows for smaller placental size, compared to fetal weight (Leiser and Kaufmann, 1994). The crosscurrent flow and multivillous flow systems represent exchangers of intermediate efficiencies. In the crosscurrent system, fetal capillaries lie perpendicular to the maternal capillaries and interface with several capillaries, allowing for transfer with all vessels. The multivillous flow exchanger represents the system used in humans. In this system, fetal capillaries bathed in maternal blood.

Functional barrier

Despite the presence of the anatomical barriers, transfer of drugs and xenobiotics does occur. Transplacental transfer occurs by passive diffusion, facilitated diffusion, active transport, and to a lesser extent, phagocytosis and pinocytosis (Pacifici and Nottoli, 1995). However, the latter two processes are not efficient enough to have a significant impact on the transfer of drugs (Syme et al., 2004). These mechanisms provide a functional barrier for transplacental transfer of drugs, and with efflux pumps and saturable systems, are able to regulate the amount that crosses the placenta.

Passive diffusion is the primary form of exchange (Syme et al., 2004) and is determined by the drug's concentration gradient. The amount of a drug that crosses is

dependent on the concentration in the maternal circulation, the chemical properties of the drug, and the properties of the placenta. This type of transport is ideal for low molecular weight, lipophilic, non-ionized drugs. Drugs with a molecular weight ≤ 600 Daltons pass readily across the placenta (Ward, 1993). Since the placenta represents a lipid bilayer membrane, drugs that are lipid soluble will transfer easily, while hydrophilic drugs face more hindrance. Transfer of lipophilic drugs is limited by the blood flow within the placenta and is considered "flow-limiting" (Syme et al., 2004). However, if the drug expresses a high affinity for the placental tissue, the drug will not be readily released in the fetal circulation and accumulates in the placenta. However, due to their lipid insolubility, hydrophilic drugs are transferred by extracellular pores and are generally limited by their molecular size. Since most drugs have low molecular weights, their transfer is restricted by the concentration gradient and is considered "diffusion-limited". Many drugs are weak acids or bases and dissociate at physiological pH. An ionized drug is charged and cannot pass readily through the placental membrane (Syme et al., 2004). However, the fetal circulation is slightly more acidic than the maternal. Drugs that are weak bases become more ionized in the fetal circulation, creating a concentration gradient of free, unionized drug toward the fetus, which is also known as ion trapping (Reynolds and Knott, 1989). Drugs bound to plasma proteins are too large for transplacental transfer; therefore, plasma protein binding is another important factor. During gestation, the placenta grows and the membranes separating the maternal and fetal circulation begin to thin. These changes can influence the transplacental transfer of drugs and xenobiotics.

Facilitated diffusion requires the presence of a carrier substance within the placenta, but does not require energy. Transport by this system can become saturated at high drug concentrations. However, only a few drugs have been shown to be transported across the placenta by this system (Henderson et al., 1993; Kudo et al., 1989; Fant et al., 1983). Facilitated diffusion is generally reserved for initial uptake of drugs into the syncytiotrophoblast.

Active transport across the placenta requires energy and is generally against a concentration gradient. The energy is supplied by the hydrolysis of adenosine triphosphate (ATP) or the transmembrane electrochemical gradient of ions, such as Na^+, K^+, and Cl^-. Active drug transporters are protein pumps and are commonly located in the apical and basal membranes of the syncytiotrophoblasts. P-glycoprotein (P-gp) is a member of the ATP-binding cassette (ABC) transporter family and is a product of the multidrug resistance (MDR1) gene. There are two subclasses of P-gp: class I, which is MDR1 in humans, and class II, which is MDR2 and 3 in humans (Gottesman et al., 1995). These transporters are large multispanning membrane proteins, where they function as ATP-driven efflux pumps, limiting the intracellular accumulation of drugs and xenobiotics (Gottesman and Pastan, 1993). Within the placenta, P-gp is generally located in the apical membrane of the syncytiotrophoblast (St.Pierre et al., 2000). It functions by transporting amphiphilic and hydrophobic drugs from the intracellular space to the extracellular area. Studies have shown that p-glycoprotein plays a major functional role in the placental barrier, providing protection for the fetus from xenobiotics and environmental toxins, but is negligible in the removal of compounds from the fetal circulation (Molsa et al., 2005).

Another group of ABC transporters that is expressed in the human placenta is the multidrug resistance protein (MRP) family. Within the human body, there are seven members of this family and are typically larger than other ABC transporters. However, only three members of the MRP family; MRP1, MRP2, and MRP3, were found to be expressed in the human placenta at term (St.Pierre et al., 2000; Flens et al., 1996). All three transporters are expressed in the apical syncytiotrophoblast, while MRP1 and MRP3 are particularly abundant in the endothelial cells of the fetal capillaries (St.Pierre et al., 2000). MRP1 and MRP2 are responsible for the transfer of lipophilic compounds conjugated to glucuronide, glutathione, and sulfate from the fetal to maternal compartment, while MRP3 is responsible for the fetal to maternal transfer of anionic conjugates (Syme, et al., 2004). The physiological role of MRP transporters in the

placenta remains speculative, but it has been proposed that there is an involvement in the removal of metabolic end products from the fetus to the mother (St.Pierre et al., 2000).

The placenta-specific ABC transporter, also known as the breast cancer resistance protein (BCRP), is highly expressed in the human placenta (Allikmets et al., 1998). Currently, its physiological function and location in the placenta has yet to be defined. However, it is believed to be located on the apical membrane of the syncytiotrophoblast.

There are several transporters which facilitate the transfer of drugs and xenobiotics to the fetal compartment. Monoamine transporters, such as the serotonin transporter (SERT), norepinephrine transporter (NET), and the extraneuronal monoamine transporter (OCT3), have been identified in the placenta (Ramamoorthy et al., 1993 a&b; Kekuda et al., 1998). In addition to normal monoamine transfer, these transporters have been shown to actively transport several drugs from the maternal side into the syncytiotrophoblast. The novel Na+-driven organic cation transporter (OCTN) is expressed in the basal membrane of the syncytiotrophoblast. This transporter is responsible for the transplacental transfer of carnitine from the maternal circulation to the fetal circulation (Ohashi et al., 1999). However, OCTN also transports several drugs, which compete for the transporter, affecting carnitine transfer (Ohashi et al., 1999; Wu et al., 1998).

Metabolic barrier

The placenta also acts as a metabolic barrier, functioning as an extra-hepatic site for the metabolism of drugs and xenobiotics. The phase I enzymes, particularly cytochrome P450 (CYP) isoforms, and a few phase II enzymes have been identified in human placentas. Maternal and environmental factors affect the level and activity of drug metabolizing enzymes in the placenta.

CYP enzymes participate in the synthesis of steroid hormones and the metabolism of vitamins and fatty acids (Marray, 1992). These enzymes are responsible for the metabolism of many medicinal drugs and toxic chemicals, by converting the parent drug to a more polar metabolite. CYP enzymes are primarily expressed in the liver and small intestine. However, the majority of hepatic metabolic enzymes are present in the

placenta, although their expression levels and activity are lower than in the liver (Juchau, 1980; Pasanen and Pelkonen, 1994; Pasanen et al., 1996). CYP enzymes are present as early as the first trimester, but their expression varies depending on the period of gestation. In fact, there is a greater variety of CYP enzymes in the first trimester than at term (Hakkola et al., 1996 a&b). It has been postulated that this was due to the susceptibility of the fetus to the effects of teratogens early in gestation; meaning, later in pregnancy, the CYP enzymes that are not needed can be turned off (Syme et al., 2004).

Despite metabolism by the phase I enzymes, many metabolites are not readily excreted. Therefore, these products must undergo subsequent conjugation to an endogenous substrate, such as glucuronic acid, glutathione, or sulfate. These phase II reactions produce highly polar molecules which are rapidly eliminated. Uridine diphosphate glucuronosyltransferase (UGT) is involved in phase II reactions, by conjugating glucuronic acid to a metabolite. These enzymes are present in the placenta throughout pregnancy, and UGTs might play a major role in placental metabolic activity (Collier et al., 2002; Collier et al., 2000). Glutathione S-transferase (GST) catalyzes the conjugation of glutathione to electrophilic compounds. Placental GST is active throughout gestation, and its activity relates more to hormone metabolism than drug detoxification (Pasanen and Pelkonen, 1990). GSTP1-1 has been the only form of GST isolated from the placenta and accounts for at least 85% of the total GST activity in the placenta (Guthenburg and Mannervick, 1981).

Transplacental transfer

Transplacental transfer of drugs and xenobiotics does occur and affects normal fetal growth and development despite all of these barriers. Teratogenic effects of xenobiotics on the fetus include spontaneous abortion, defects in development and malformations, intrauterine growth restriction, and mental retardation (Briggs et al, 2004). There are direct effects on the fetus, due to the presence of the parent drug and/or its metabolite in the fetal circulation. Furthermore, there are also indirect effects on placental physiology. The presence of a drug can alter placental functions, e.g. changes in hCG release, glucose utilization, and oxygen consumption; all of which can adversely

affect the fetus. Drugs compete with endogenous compounds for access to transporters and receptors, interfering with transplacental transfer of essential molecules and consequently its functions.

Several factors determine the degree of teratogenicity a drug may have on the fetus (Gurnee and Sylvestri, 2002). The main factor is the amount of the drug that reaches the fetal circulation (Young and Koda-Kimble, 1995). The gestational age of the fetus at the time of exposure is also an important determinant. During the first few weeks of pregnancy, there is little effect of drugs on the fetus, due to the lack of placental perfusion by the maternal circulation. Drugs can cause the most congenital malformations during the period of organogenesis; while exposure during the second and third trimester has the most effect on neurological development, physiological functions and growth (Gurnee and Sylvestri, 2002). Duration of exposure, as well as environmental factors present during exposure, might influence the amount of harm a drug causes. The susceptibility of the fetus and placenta can also determine the degree of teratogenicity of xenobiotics. The multitude of factors makes it difficult to accurately predict the effects a drug will have on fetal development and growth.

PHARMACOKINETIC CHANGES DUE TO PREGNANCY

Normal pharmacokinetic parameters

Pharmacokinetic properties describe the processes affecting drug concentration in the body. Drug absorption, distribution, and elimination all affect the plasma concentration of a drug. Plasma concentration is important because it determines the therapeutic action of the drug. Many drugs have a concentration range necessary to produce the desired effects, while minimizing its adverse effects at higher concentrations.

The basic parameters for pharmacokinetics of a drug are absorption, clearance and the volume of distribution of a drug. The extent and rate of drug absorption is influenced by site of administration and formulation of the drug. Drugs are usually administered orally, so absorption is usually achieved by passive diffusion in the small intestine. However, due to first pass metabolism and incomplete absorption, bioavailability is a

more accurate measure of systemic drug concentration. Bioavailability of intravenous drugs is generally 100%, but bioavailability of an orally administered drug is typically less than 100%, and can be as low as 5% (Dawes and Chowienczyk, 2001). Clearance refers to the measure of the body's ability to eliminate the drug, while volume of distribution describes the measure of space in the body available to contain the drug. Clearance of a drug is the rate of elimination in relation to drug concentration in the body. Total systemic clearance refers to the elimination of a drug by all available processes in the body. Elimination can occur by several routes, but the kidneys and the liver are the two major sites (Holford, 2004). Renal clearance occurs with elimination of the unchanged drug in the urine, while hepatic clearance occurs by biotransformation of the drug into metabolite(s) or excretion of the drug into the bile. The volume of distribution correlates the amount of drug in the body to the concentration of the drug in the blood. Drugs in the systemic circulation are distributed at varying degrees within the intravascular, interstitial, and intracellular compartments. The distribution of a drug within these spaces depends on the water and lipid solubility of the drug (Dawes and Chowienczyk, 2001). Lipophilic drugs diffuse through cell membranes and are more widely distributed, while polar drugs are located primarily in the extracellular spaces.

Pharmacokinetic parameters during pregnancy

The physiological changes that accompany pregnancy influence the pharmacokinetics of drug absorption, distribution, and elimination. Some drugs may reach higher concentrations in pregnant patients, increasing the potential for toxicity and suggesting the need to reduce the dose (Amon and Huller, 1984). Other drugs may have decreased levels during pregnancy, calling for a higher dose to maintain effective therapeutic levels. However, this raises additional concerns about potential toxicity to the fetus and mother. Drug treatment during pregnancy is a careful balance between achieving plasma concentrations that are sufficient to produce desired therapeutic effects, while minimizing the risk of dose-related adverse effects to the mother and fetus.

Absorption of drugs, especially those taken orally, is influenced by the increase in plasma progesterone levels (Dawes and Chowienczyk, 2001). The elevation of

progesterone causes a reduction in gastric emptying and small intestine motility, which can affect the time of peak plasma concentration (Parry et al., 1970). A reduction in gastric acid secretions, together with an increase in mucus production, can cause an increase in gastric pH, affecting the ionization of weak acids and bases and their subsequent absorption (Hume and Killam, 1990). These changes may have little effect on repeated dosing, but would be significant for a single dose of an oral medication. More importantly, the major problem affecting absorption is the nausea and vomiting associated with pregnancy. This usually requires changes in dosing regimens to times when nausea is minimal, such as the evening (Dawes and Chowienczyk, 2001).

During pregnancy, the intravascular and extravascular water content increases by up to 8 liters, creating a larger volume of distribution (Loebstein et al., 1997). The expansion creates a larger compartment within which hydrophilic drugs may distribute, reducing the peak plasma concentration of these drugs. Theoretically, the dosage of these drugs should be adjusted to maintain therapeutic levels, but the effect is compensated by changes in protein binding (Dawes and Chowienczyk, 2001). The increase in albumin production is not enough to compensate for the increase in plasma volume, creating hypoalbuminemia (Dean et al., 1980). The increased steroids and placental hormones cause displacement of the drug from the protein-binding sites. The overall effect is an increase in the concentration of free drug in plasma. However, as the increased unbound drug is available for distribution, metabolism, and excretion, the net effect on total free drug concentration remains unchanged (Loebstein et al., 1997). In addition, body fat increases during pregnancy, leading to a larger volume of distribution for lipophilic drugs. Nevertheless, the increase is insignificant and has little clinical importance (Dawes and Chowienczyk, 2001).

Enzymes involved in metabolism, a major route of drug clearance, vary considerably during gestation. Hepatic cytochrome P450 enzymes, such as CYP3A4 and CYP2D6, are induced by estrogen and progesterone, resulting in higher metabolism of their respective drugs. However, other enzymes (CYP1A2) are down-regulated by progesterone and estradiol, leading to reduced metabolism of some drugs. Additionally,

some extrahepatic enzymes, such as cholinesterase, have reduced activity during pregnancy (Little, 1999). The placenta and developing fetal liver express some CYP enzymes, but their effect on maternal pharmacokinetics is low. However, the reduced elimination capacity of these enzymes, coupled with the fact that approximately half of the fetal circulation bypasses the fetal liver and flows directly to the brain, may cause drug accumulation and a pronounced effect on the fetus (Dawes and Chowienczyk, 2001).

Elimination of drugs and xenobiotics is driven by several factors. Renal blood flow increases by as much as 60-80% and glomerular filtration rate rises by approximately 50% during gestation (Dunlop, 1981). This enhances elimination of drugs that are normally excreted unchanged in the urine and creates lower steady-state plasma concentrations. Tubular secretion and reabsorption also increase, but by a more moderate rate of 20% (Berg, 1997). However, these changes are clinically insignificant and rarely necessitate dosage adjustments (Loebstein et al., 1997). Pulmonary function increases by half during gestation, making this a more important route of elimination during pregnancy. Initially, elimination from the fetus is controlled by diffusion of the drug back to the maternal compartment. However, metabolites tend to be more polar than the parent drug and less likely to cross the placental membranes, leading to their accumulation in the fetus. As pregnancy progresses, the fetal kidneys mature, excreting more drug metabolites into the amniotic fluid (Loebstein et al., 1997).

METHADONE AND NEONATAL ABSTINENCE SYNDROME

Methadone

Methadone is a synthetic opioid synthesized in 1937 by German scientists, who were searching for an analgesic that would be easier to use during surgery, but with a low addiction potential. Heroin binds the opioid receptor, causing release of excessive dopamine in the brain. Consequently, addicts require continuous occupation of these receptors. Although structurally different from morphine and heroin, methadone also acts on the μ opioid receptor. It produces many of the same effects of other opiates,

including analgesia, and it prevents withdrawal symptoms, and reduces cravings (Wang, 1999). However, it does not produce the same euphoric high as heroin and morphine. Therefore, methadone has been extensively used for several decades to treat opiate addiction.

Methadone is administered orally and exhibits a high bioavailability. In fact, it is almost equally effective when administered orally or by injection (Wikipedia, 2005). Methadone has high lipid solubility and undergoes slow metabolism, making it longer acting than the morphine-based opiates. It is metabolized by CYP isozymes, primarily hepatic CYP3A4 and to a lesser extent CYP2D6, CYP1A2, CYP2B6, CYP2C9, and CYP2C19, to the inactive metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (Moody et al., 1997; Foster et al., 1999; Iribarne et al., 1996; Oda & Kharasch, 2001 a&b; Ferrari et al., 2004; Gerber et al., 2004). This compound is sequentially metabolized to 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP), by human intestinal microsomes (Oda & Kharasch, 2001b). Methadone has also been shown to be metabolized to EDDP by CYP19 in microsomes from human term placentas (Nanovskaya et al., 2004). The half-life of methadone is approximately 24 hours, allowing for once daily administration. This is especially beneficial in the methadone maintenance programs for opiate addicts. However, like heroin, tolerance and dependence can develop with methadone. Withdrawal symptoms associated with methadone administration are generally less severe, but more prolonged (Wikipedia, 2005). Therefore, treatment in a methadone maintenance program is intended to be for an indefinite amount of time.

Methadone administration during pregnancy

Estimations on the prevalence of opiate use among pregnant women ranges from 1% to 21% (Brown et al., 1998). This patient population experiences an increase in maternal complications during pregnancy, as well as significant neonatal complications. Maternal complications include preterm labor, placental abruption, third trimester bleeding, and the presence of sexually transmitted diseases. Neonates exposed to opiates can experience intrauterine growth retardation, low birth weight, prematurity, fetal

distress, and meconium aspiration. Infants can also face postnatal growth deficiency, microcephaly, withdrawal from the narcotic, and an increased risk of sudden infant death syndrome (Beauman, 2005; Dattel, 1990).

Over the past few decades, treatment with methadone has been the standard of care for pregnant women addicted to opiates. In fact, methadone is currently the only drug approved in the United States for such treatments. However, there is concern over the appropriate dose that benefits the mother and does not adversely affect the fetus (Allen, 1991). In most methadone programs, a daily dose ranges between 10 and 90 mg/day (Warner et al., 1997). However, due to pharmacokinetic changes throughout gestation, higher doses of methadone could be required later in pregnancy to achieve trough levels necessary to prevent maternal and fetal withdrawal symptoms (Drozdick et al., 2002). Maternal withdrawal leads to simultaneous withdrawal in the fetus, leading to an increased risk of fetal distress.

Treatment with methadone provides a steady concentration in the maternal circulation, due to its longer half-life. This prevents the adverse effects of repeated withdrawal on the fetus. In addition, methadone causes a potential reduction in drug-seeking behaviors, which benefits the fetus (Wang, 1999). However, studies outlining the benefits of these treatment programs have met recent opposition. Many patients in the methadone maintenance programs received enhanced prenatal care, compared to heroin addicts with little or no prenatal care. Therefore, several researchers argue that improved neonatal outcome is a result of increased care during pregnancy, rather than a direct effect of the methadone (Suffet and Brotman, 1984; Chang et al., 1992).

Neonatal abstinence syndrome

Symptoms and diagnosis

Infants exposed to certain drugs in utero may become dependent on them and suffer withdrawal symptoms after birth. These symptoms, collectively termed neonatal abstinence syndrome, are characterized by dysfunction of the central nervous system, gastrointestinal tract, and the respiratory system (Johnson et al., 2003). Effects on the nervous system lead to high-pitch crying, irritability, sleep difficulties, tremors,

hyperreflexia, myoclonic jerks, and seizures. Gastrointestinal disturbances include excessive sucking, poor feeding, vomiting, and diarrhea. Additionally, affected infants experience sweating, low grade fevers, mottling, frequent yawning and sneezing, and respiratory distress (Beauman, 2005). Due to the long half-life of methadone, NAS symptoms may occur 48 hours after birth and up to 7-14 days later.

Several factors can affect the onset and severity of neonatal abstinence syndrome (NAS). The type of drug influences the appearance of symptoms, due to variations in half-life and pharmacokinetics of the drug. The amount and frequency of maternal dosing, as well as the timing of the last dose before delivery, can affect the time to onset of NAS. The character of labor, such as the length or difficulty, may alter the manifestation of NAS. Finally, the health and maturity of the neonate can influence the onset of NAS. Oddly enough, preterm infants experience a lower incidence and intensity of neonatal abstinence syndrome, compared to full-term babies (Doberczak et al., 1991).

There have been a variety of tools developed to assess the occurrence and severity of NAS, with the most popular being the Finnegan scale (Fike, 2003). It allows a semiquantitative measure of the degree to which the newborn is experiencing symptoms of withdrawal. The decision of the practitioner to begin scoring is based on the observation of symptoms or a positive drug screen for the mother or neonate. The symptoms are divided into physiological categories; such as neurological, state, respiratory, and gastrointestinal. Each symptom is assigned a value depending on severity, similar to pain assessment tools. If there is any indication or suspicion of drug exposure in utero, it is recommended to begin scoring early and continue every 4 hours until the baby is stable. A score of 8 or greater dictates the need for treatment and more frequent monitoring. Other scoring systems that have been developed include the Lipsitz tool and the Neonatal Withdrawal Inventory, which each utilize a similar scoring system for specific symptoms. These tools offer common parameters for evaluation, in addition to a guide for clinical treatment (Beauman, 2005).

Treatment

Between 30% and 80% of neonates exposed to opiates during gestation require treatment for NAS (Johnson et al., 2003). Treatment includes management of withdrawal symptoms, as well as administration of medications to help alleviate symptoms. Inadequate feeding and weight loss are a common problem for these infants. Frequent, smaller feedings may aid digestion and prevent vomiting, providing ample calories. In some cases, nutrition is provided through gavage or infusion of dextrose and electrolytes. Reduction of energy expenditure is also important in prevention of weight loss during NAS management. Furthermore, many drug-exposed infants experience diarrhea, causing loss of fluid and possible dehydration. Administration of fluids and anti-diarrheic medication is used to alleviate this symptom (Beauman, 2005). Additional medications used in the treatment of NAS include phenobarbital, diazepam, methadone, tincture of opium, and paregoric. Phenobarbital is used to control the neurological symptoms but has little effect on the gastrointestinal symptoms. Dosing is adjusted according to the withdrawal score and testing for phenobarbital levels is important throughout treatment. Diazepam has been used for treatment of NAS, but is more effective in combination with another drug, such as an opiate. Methadone has been shown to be effective for treatment of withdrawal symptoms, but hospitalization is prolonged due to slower weaning off of the drug. Tincture of opium is effective, but is highly contraindicated. Small dosing errors may occur, and this can lead to a significant overdose in the patient. Paregoric is very effective for controlling the diarrhea associated with NAS, but is no longer recommended because it contains benzoic acid, which interferes with the binding of bilirubin (Beauman, 2005).

The drug-exposed neonate usually recovers from the symptoms of neonatal abstinence syndrome after treatment. However, some studies have found delays in language abilities and alterations in sleep patterns in these infants. Furthermore, impaired motor behavior and attention deficit hyperactivity disorder may present themselves later in childhood. However, the home environment and continued maternal drug use seem to have more impact on the outcome of the infant than prenatal drug exposure. The

probability of a positive outcome for the infant hinges on the mother entering a treatment program and remaining drug-free (Beauman, 2005).

EXPERIMENTAL INTRODUCTION

Methadone treatment programs have been recommended for pregnant opiate addicts for several decades. These programs have been successful in improving maternal and neonatal outcome. However, due to maternal physiological changes and the development of the fetus, practitioners are confronted with the challenge of determining the dose necessary for the pregnant patient that prevents maternal and fetal withdrawal while reducing negative effects on the fetus (Frederiksen, 2001).

Neonatal abstinence syndrome continues to be a problem afflicting drug-exposed neonates, and there are currently no indicators to predict the occurrence or intensity of this condition. Current reports debate the existence (Doberczak et al., 1993; Dashe et al., 2002) or absence (Rosen et al., 1975; Kandall et al., 1977; Mack et al., 1991; Berghella et al., 2003) of a correlation between maternal methadone dose and the incidence of NAS. Furthermore, there are reports correlating NAS to umbilical cord blood concentration or maternal excretion of metabolites. However, these measures are difficult to obtain and are generally not accurate predictors of NAS. The incidence and intensity of NAS is probably more closely related to the concentration of methadone in the fetal circulation, which is determined by placental bio-disposition of the drug. The current investigations are focused to better understand placental bio-disposition of opioids, specifically methadone, used for the treatment of the pregnant opiate addict.

Despite metabolism by other hepatic and intestinal cytochrome P450 systems, recent reports have shown that CYP19, or aromatase, is responsible for the N-demethylation of methadone to EDDP in human term placentas (Nanovskaya et al., 2004). Normally, aromatase is the enzyme which catalyzes the conversion of androgens to estrogens, by aromatization of ring A. However, it has been shown that levels of placental xenobiotic-metabolizing CYP isoforms vary throughout gestation, with more P450 enzymes expressed in the placenta during the first trimester than at term (Hakkola

et al., 1996 a&b). Given the changes in placental enzyme expression throughout gestation, it is entirely possible that other enzyme(s) may be involved in the N-demethylation of methadone to EDDP. Consequently, the activity of these enzyme(s) may be different than that by term placental aromatase.

Data on the N-demethylation of methadone in placentas from earlier gestational ages is scarce. Therefore, the aim of this investigation is to identify and characterize the major enzyme(s) responsible for the biotransformation of methadone to EDDP in human preterm placentas. The information obtained from this investigation will provide a better understanding of the factors contributing to the bio-disposition of methadone, and ultimately the concentration in the fetal circulation.

CHAPTER 2: EXPERIMENTAL PROCEDURES

PLACENTAL PREPARATIONS

All placentas were obtained from the labor and delivery ward of John Sealy Hospital at the University of Texas Medical Branch, Galveston, TX, according to a protocol approved by the Institutional Review Board. Placentas from women who abused drugs and/or tobacco during pregnancy, as well as multiple births (twins), were excluded from this investigation. Placentas were divided based on the gestational age of the fetus at parturition and arbitrarily placed into the following groups: late 2nd trimester (17 – 27 weeks), early 3rd trimester (28 – 33 weeks), and late 3rd trimester (34 – 40 weeks). Villus tissue was removed at random from the placenta and rinsed with ice-cold saline. The tissue was minced and homogenized in two volumes of cold 0.1 M potassium phosphate buffer, pH 7.4, using a Tekmar SDT-1810 Tissumizer (Cincinnati, OH). The homogenate was subjected to differential centrifugation. The microsomal subcellular fraction was separated at 104,000 × g and was resuspended in 0.1 M potassium phosphate buffer (pH 7.4). Protein content of each preparation was determined by a protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. Aliquots of the microsomal fractions were stored at -80°C until analysis. Enzymatic activity was not affected by these storage conditions.

PRELIMINARY EXPERIMENTS

Time course analysis and protein concentration experiments were conducted to ensure optimal incubation time and adequate protein concentrations, respectively, for the experimental conditions. The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.4), methadone (750 μM), and increasing concentrations of microsomal proteins (0.25 – 1 mg/mL), in a final volume of 1 mL. The reaction mixture was preincubated for 5 minutes at 37°C, and initiated by the addition of an NADPH-regenerating system. The reaction was incubated at 37°C for 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes. Termination of the reaction was achieved by the addition of 100 μL of

15% (w/v) trichloroacetic acid (TCA). LAAM (1 μg/mL) was added as an internal standard. The reaction mixture was centrifuged at 12,000 × g for 15 minutes to separate the precipitated proteins. Controls contained TCA- denatured proteins at a time point of 0. Analysis was achieved by HPLC as described below. Results were compared to a standard curve of known concentrations of EDDP, ranging from 0.05 – 2 μg/mL, and optimal reaction conditions were determined.

N-DEMETHYLATION OF METHADONE

The activity of placental microsomal fractions in catalyzing the N-demethylation of methadone to EDDP was determined. The reaction components contained 100 mM potassium phosphate buffer (pH 7.4), 1 mg protein of the microsomal fraction, and methadone (concentration range of 50–1500μM), in a total volume of 1 mL. The reaction mixture was preincubated for 5 minutes at 37°C. Then, initiated by the addition of the NADPH-regenerating system, composed of the following: 0.4 mM NADP, 4 mM glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, and 2 mM $MgCl_2$. This system utilizes glucose-6-phosphate dehydrogenase, in the presence of glucose-6-phosphate, to convert $NADP^+$ to NADPH, which is required for the oxidase enzyme. The reaction was carried out for 20 minutes at 37°C, and then terminated by the addition of 100 μL of 15% (w/v) TCA. Addition of 1 μg/mL LAAM was used as an internal standard. The reaction mixture was centrifuged at 12,000 × g for 15 minutes, and the supernatant was analyzed by HPLC as described below. The control reaction was carried out utilizing TCA-denatured placental microsomal fractions. The effect of methadone, within its concentration range, on the velocity of the reaction was used to construct the saturation curve and calculate the apparent K_m and V_{max} values for the reaction.

IDENTIFICATION OF THE ENZYME(S)

The enzyme(s) responsible for catalyzing the N-demethylation of methadone to EDDP was investigated. Chemicals known as inhibitors for CYP isoforms, as well as antibodies specific for CYP isoforms, were utilized.

Chemical Inhibition

The effect of chemical inhibitors, selective for the CYP isoforms, on the formation of EDDP by microsomal fractions was determined by addition of each to the reaction components. The inhibitors used (followed by its concentration and CYP isoform that it is selective for) are listed as follows: α-naphthoflavone (0.1 μM), CYP1A; sulfaphenazole (10 μM), CYP2C; quinidine (5 μM), CYP2D6; 4-methylpyrazole (25 μM), CYP2E1; ketoconazole (2.5 μM), CYP3A4; 4-hydroxyandrostenedione (1 and 10 μM), CYP19, and aminoglutethimide (1 and 10 μM), CYP19. The concentration of each inhibitor used was equal to its reported IC_{50}, K_i, or apparent K_m values for its specific CYP isoform. The final concentration of methadone used in the reaction was 500 μM, approximately equal to its apparent K_m value of 424 μM. Each inhibitor was added, with methadone and the microsomal proteins, to the reaction mixture and pre-incubated for 5 minutes at 37°C. The reaction was initiated by the addition of the NADPH-regenerating system, and incubation was continued for 20 minutes. The reaction was terminated by the addition of 100 μL of 15% (w/v) TCA, followed by the addition of the internal standard (LAAM). All stock solutions of the inhibitors were prepared in methanol, and the final concentration of methanol in the reaction was 0.5%. The control reactions included all components, except the inhibitor, but in the presence of 0.5% methanol (v/v). The reaction mixture was analyzed using the HPLC-UV as described below.

Monoclonal antibodies

Monoclonal antibodies, raised against human specific CYP isoforms, were used to identify the enzyme responsible for the bioconversion of methadone to EDDP by placental microsomes. The CYP isozymes targeted were human liver CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, and 3A4, as well as human placental aromatase (CYP19). Each antibody was added to the reaction at the concentration which causes 80% inhibition of the CYP isoform. Antibodies were preincubated with 0.2 mg of microsomal protein, in 100 mM potassium phosphate buffer (pH 7.4), for 15 minutes at room temperature. After the addition of methadone, at its final concentration of 1.25 mM, the components were incubated for an additional 5 minutes at 37°C. The reaction

was initiated by the addition of the NADPH-regenerating system and incubated for 20 minutes at 37°C. Termination of the reaction was achieved by the addition of 15% (w/v) TCA, and LAAM was added as an internal standard. The control reactions contained mouse IgG, instead of monoclonal antibodies. The reaction mixture was analyzed using HPLC-UV, as described below.

EXPRESSION OF AROMATASE IN PLACENTAL MICROSOMES

The level of aromatase expression in microsomal fractions was determined using Western Blot analysis. The amount of placental microsomal protein loaded on each electrophoresis well was 10 µg. Resolution of the samples was achieved using a 7.5% SDS-PAGE and electro-transferred to nitrocellulose membranes overnight at 4^0C, using a constant potential of 25 V. The blots were probed, at room temperature, with rabbit polyclonal antiserum against human aromatase (Hauptman-Woodward Medical Research Institute, Buffalo, NY), at a dilution of 1:2000 for 90 min. Goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was the secondary antibody at a dilution of 1:5000. Stained membranes were incubated with Super Signal Chemi-luminescent Substrate (Pierce, Rockford, IL) for 5 minutes, and the resulting signal was exposed and quantitated by spot densitometry using a Multi-Imager instrument (Alpha Innotech Corp., San Leandro, CA). Standards were prepared using defined concentrations of cDNA-expressed aromatase supersomes (Gentest, Woburn, MA). The membranes were reprobed with monoclonal rabbit anti-actin antibody (Sigma, Dallas, TX), followed by incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase. The amount of actin detected was used as an internal control to ensure equal loading of the samples.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ULTRAVIOLET (HPLC-UV)

The HPLC-UV system employed in these experiments was composed of a Waters 600E multi-solvent delivery system with a Waters 2487 dual wavelength absorbance detector and a Waters 717 autosampler, all of which were controlled by Waters

Millennium[32] chromatography manager (Waters, Milford, MA). The stationary phase was a 250 mm × 4.6 mm Luna 5 μM C-18 column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile: water (36:64 v/v). Triethylamine (0.1 % v/v) was added, and the pH was adjusted to 2.8, using orthophosphoric acid. Isocratic elution was carried out at a flow rate of 1 mL/min, and the elutants were monitored at a wavelength of 210 nm. Controls containing known concentrations of methadone, its metabolite (EDDP), and the internal standard (LAAM) were used to determine the elution time for each standard. Eluted peaks were integrated using the software of Waters Millennium[32] chromatography manager. The concentration of EDDP formed was determined using a standard curve, composed of six concentrations of EDDP, ranging from 0.05 – 2 μg/mL. The chromatographic conditions for the standards were identical to the samples and all analyses were carried out in the linear range of the standard curve prepared for each experiment.

CHEMICALS

All chemicals were purchased from Sigma Chemical Co. (Dallas, TX) unless otherwise noted. Acetonitrile was purchased from Fisher Scientific Co. (Pittsburgh, PA). Methadone, its metabolite (EDDP), and L-α-acetylmethadol (LAAM) were a gift from the National Institute on Drug Abuse drug supply unit. Monoclonal antibodies to CYP isoforms were purchased from Xenotech LLC (Kansas City, KS). Rabbit antiserum to human placental aromatase was purchased from Hauptman-Woodward Institute (Buffalo, NY). Properties of the antibodies and their effect on the activity of placental aromatase have been previously reported (Kitawaki et al., 1989). The cDNA-expressed CYP 19 supersomes are commercially available from Gentest (San Jose, CA), and their characterization has been described (McNamara et al., 1999).

DATA ANALYSIS

All data are represented as mean ± standard deviation. Values for the apparent K_m and V_{max} were calculated from the saturation curves using the Michaelis-Menten equation and non-linear regression (SPSS 13.0 for Windows, SPSS Inc., Chicago, IL.).

Statistical analysis of the data on the effect of the inhibitors on EDDP formation was carried out using one-way ANOVA with Tukey's comparison. Values were deemed significant if the *P* value was < 0.05.

CHAPTER 3: RESULTS

INITIAL EXPERIMENTS

Assay of enzyme activity

Microsomal fractions from placentas of different gestational ages were used to establish conditions for the enzyme-catalyzed metabolism of methadone and identify the metabolite(s) formed. Preliminary experiments were conducted to determine the appropriate incubation time and concentration of microsomal protein. The rate of EDDP formation was linear with incubation time, up to 20 minutes (Fig. 1). In addition, the rate of EDDP formation was linear with concentration of microsomal protein, up to 3 mg/mL. Therefore, an incubation time of 20 minutes and a protein concentration of 1 mg/mL were used.

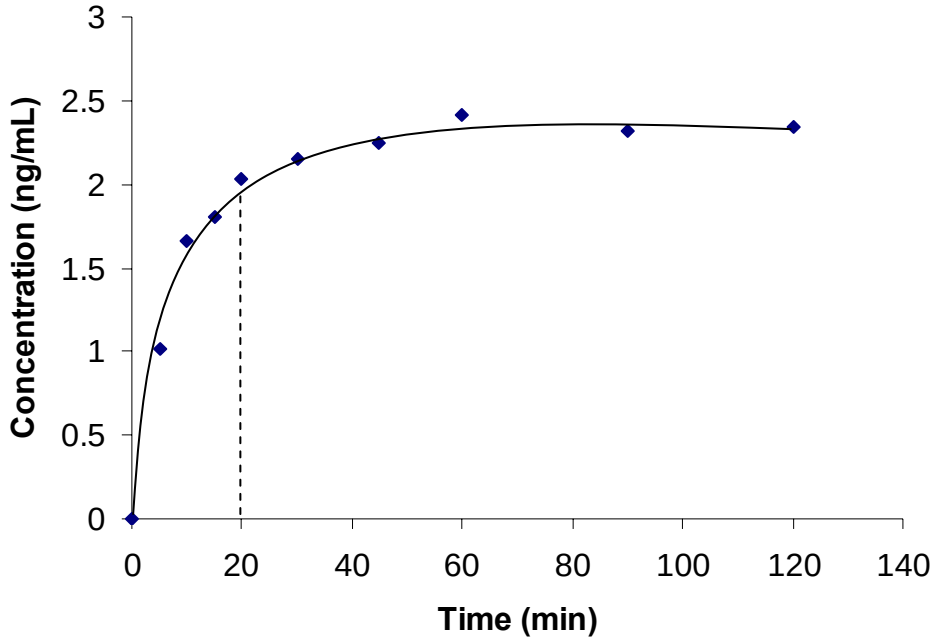

Figure 1. Effect of incubation time on EDDP formation. Reaction samples, containing 1 mg/mL microsomal protein were incubated for varying incubation times.

HPLC-UV

The retention time of each component of the reaction mixture was determined by injecting standards, containing 1 µg/mL EDDP, 1.25 mM methadone, and 1 µg/mL LAAM. Separation of methadone, its metabolite (EDDP), and the internal standard, LAAM, was achieved by the HPLC methods outlined. A chromatogram showing the retention times of the standards is presented in Figure 2A. The retention times were 14 minutes (EDDP), 18 minutes (methadone), and 24.5 minutes (internal standard, LAAM). A chromatogram of the reaction components, containing methadone and the microsomal fraction, in absence and presence of the NADPH-regenerating system (Fig 2B and C, respectively), revealed that product formation occurred only in the presence of NADPH. This suggests the involvement of a cytochrome P450 monooxygenase system. The product formed was identified by comparing its retention time with standards of EDDP and EMDP. The analysis of HPLC chromatograms revealed that incubation of

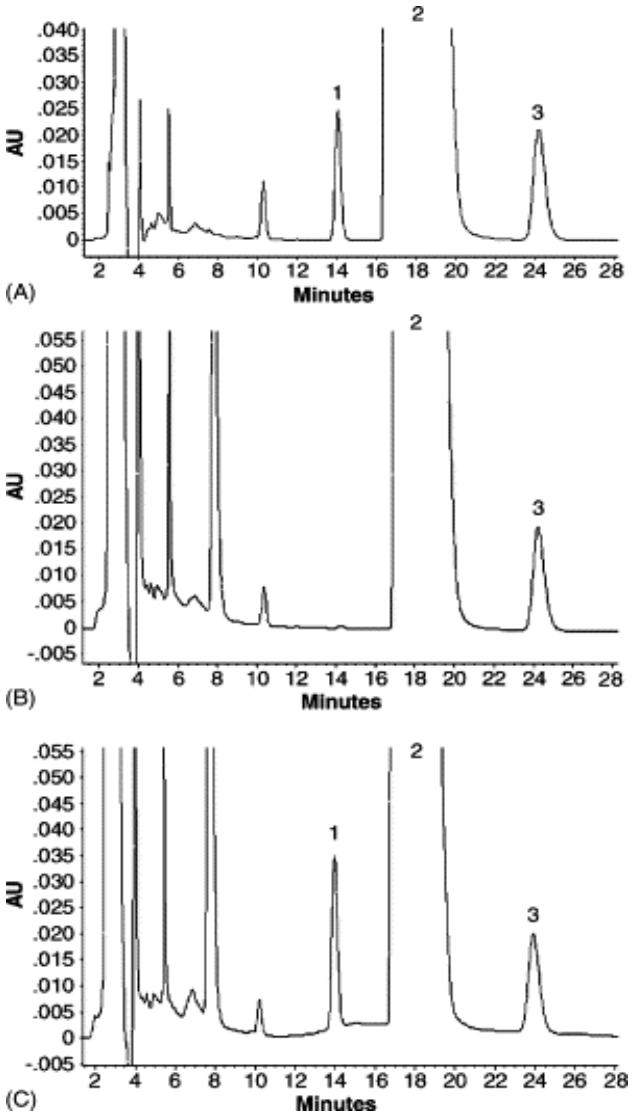

Figure 2. Representative chromatograms showing retention times and experimental conditions. (A) shows the retention times of the standards. Chromatograms of the reaction mixture in the absence (B) and presence (C) of the NADPH regenerating system are also shown.

methadone with microsomal fractions obtained from several placental preparations of different gestational ages results in formation of EDDP only. EMDP was not detected under our experimental conditions.

N-DEMETHYLATION OF METHADONE

The rate of EDDP formation in the presence of microsomal fractions, irrespective of placenta gestational age, was dependent on methadone concentration and exhibited typical Michaelis-Menten saturation kinetics. Representative saturation curves for placentas from each gestational age group are shown in Figure 3. Microsomal fractions prepared from 36 placentas (12 per group) were utilized to determine the apparent Km and Vmax values for the demethylation of methadone (Table 1). Analysis of the saturation curves obtained from the three different groups of placentas revealed that the

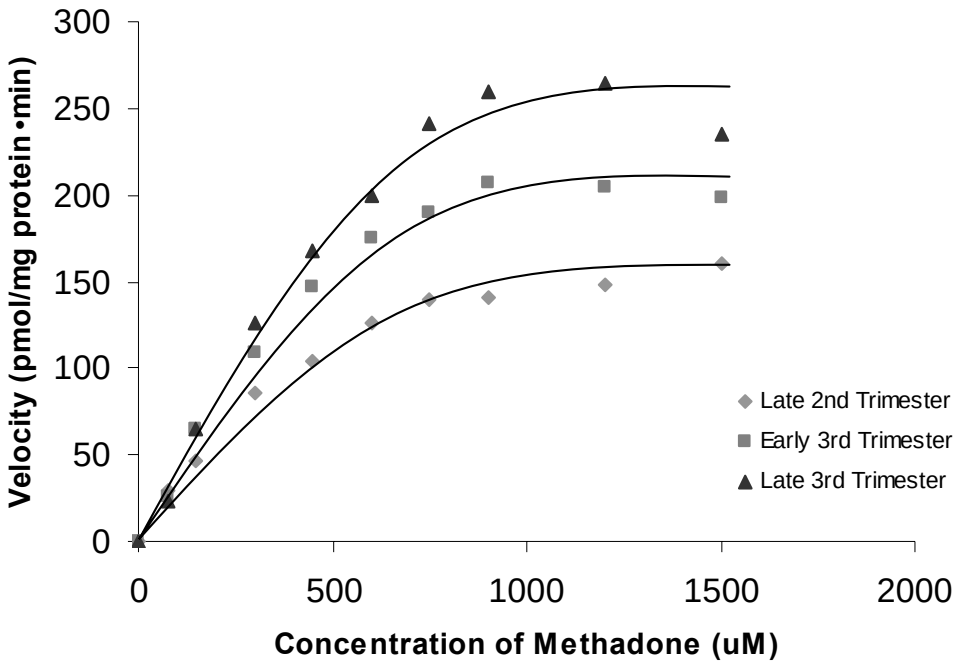

Figure 3. Representative saturation curves of the rate of EDDP formation for placentas from late 2nd, early 3rd, and late 3rd trimester deliveries. The rate of EDDP formation was dependent on methadone concentration and exhibited typical Michaelis-Menten saturation kinetics.

Table 1. Apparent Km, Vmax, and intrinsic clearance values for the conversion of methadone to EDDP by placental microsomal fractions of different gestational ages.

Gestational Period (weeks)	K_m (μM)	V_{max} (pmol· mg.protein^{-1}min^{-1})	Intrinsic Clearance (ml· mg·protein^{-1} min^{-1})
Late 2nd Trimester (17-27 weeks) n = 12	514 ± 187	192 ± 88**	0.42 ± 0.25**
Early 3rd Trimester (28-33 weeks) n = 12	519 ± 154	271 ± 88*	0.57 ± 0.26*
Late 3rd Trimester (34-40 weeks) n = 12	503 ± 120	385 ± 129	0.79 ± 0.28

Values represented as mean ± SD. * $p < 0.05$, ** $p < 0.001$.

apparent Km value (514 ± 187 μM) obtained from placentas of early gestational age did not differ from the apparent Km value (503 ± 120 μM) obtained from term placentas, suggesting that the affinity of the enzyme catalyzing the reaction remained unchanged throughout gestation.

However, there were differences in the activity of the enzyme between placentas from the gestational age groups. The activity of the enzyme during late 2nd trimester (192 ± 88 pmol mgprotein^{-1}min^{-1})and early 3rd trimester (271 ± 88 pmol mgprotein^{-1}min^{-1}) were significantly lower than at late 3rd trimester (385 ± 129 pmol mgprotein^{-1}min^{-1}), $p < 0.001$ and $p < 0.05$, respectively. Scatter plot of apparent Vmax values (Fig. 4) revealed that activity of the enzyme(s) in each experimental group varied over a wide range. Since each point on the scattered plot represents a Vmax value obtained from an individual placenta, these data indicate the existence of inter-individual variability in the activity of the placental enzyme metabolizing methadone at all gestational ages.

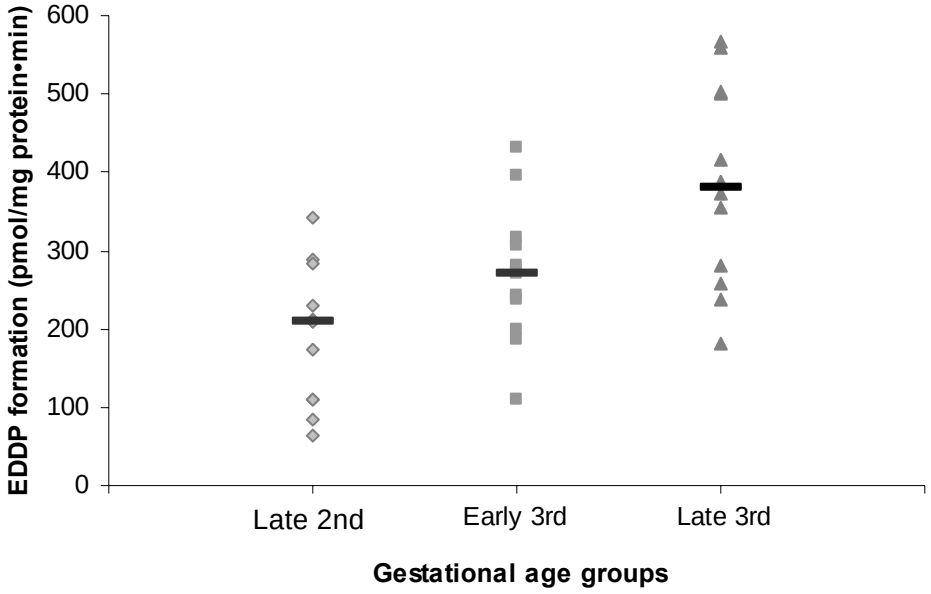

Figure 4. Activity of the enzyme catalyzing the N-demethylation of methadone at different gestational ages (scatter plot). Vmax values are grouped according to the gestational age: late 2nd trimester (17-27 weeks), early 3rd trimester (28-33 weeks), and late 3rd trimester (34-40 weeks). Cross bars represent the median for each gestational age.

IDENTIFICATION OF THE ENZYME(S) SYSTEM

Chemical inhibition

The effect of compounds, which are selective inhibitors of CYP isoforms, on EDDP formation were investigated, utilizing two pools (12 placental preparations per pool) obtained from late second and early third trimester placentas. The effect of these compounds on the EDDP formation in term placentas, under the same experimental conditions, was already determined in our laboratory (Nanovskaya et al., 2004). In each reaction, the final concentration of methadone was 500 μM and each inhibitor was 10x its reported IC50 value for its specific CYP isozyme. Compounds were considered effective inhibitors of EDDP formation if its addition to the reaction mixture decreased its formation by 25% or more.

In the pool of late second trimester microsomal preparations, EDDP formation was inhibited by ketoconazole, aminoglutethimide and 4-hydroxyandrostenedione (Figure 5A). The maximum inhibition of EDDP formation was achieved in the presence of 10 μM aminoglutethimide (95 ± 1.9% of control, $p < 0.001$) or 10 μM 4-hydroxyandrostenedione (85 ± 1.4% of control, $p < 0.001$). Both compounds are selective inhibitors of human placental aromatase (CYP19). Ketoconazole (2.5 μM), a CYP3A inhibitor, caused 63 ± 1.8% inhibition of EDDP, $p < 0.01$, whereas the other compounds α-naphthaflavone (CYP1A), sulfaphenazole (CYP2C9), quinidine (CYP2D6), and 4-methylpyrazole (CYP2E1) did not affect EDDP formation in the concentration tested.

The pool of microsomal preparations of early third trimester showed similar results in the presence of the chemical inhibitors (Figure 5B). Maximal inhibition was observed with 10 μM aminoglutethimide (CYP19) (95 ± 0.5% of control, $p < 0.001$), followed by 10 μM 4-hydroxyandrostenedione (CYP 19) (82 ± 1.9% of control, $p < 0.001$). Ketoconazole (CYP3A), at a concentration of 0.5 μM, caused 69 ± 2.2%

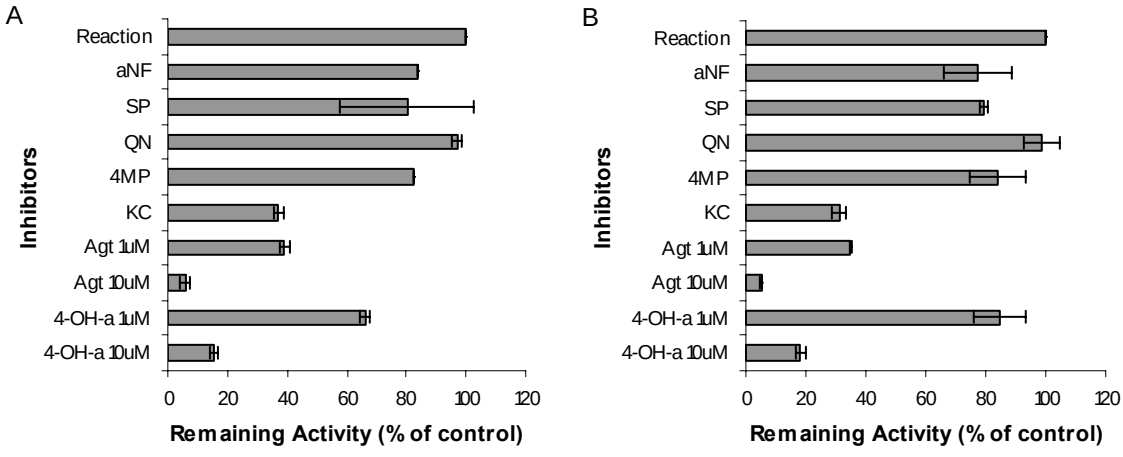

Figure 5. The effect of chemical inhibitors on methadone N-demethylation by placental microsomes from (A) late 2nd and (B) early 3rd trimester. The inhibitors are as follows: α-naphthaflavone, aNF; sulfaphenazole, SP; quinidine, QN; 4-methylpyrazole, 4MP; ketoconazole, KC; aminoglutethimide, Agt; and 4-hydroxyandrostenedione, 4-OH-a. The rates of metabolite formation are expressed as percent of control (absence of inhibitors). KC, Agt, and 4-OH-a caused a significant inhibition of EDDP formation in both sample groups.

inhibition, $p < 0.001$. These values were comparable to the data obtained from late 2nd trimester placentas. Sulfaphenazole (CYP2C9) and α-naphthaflavone (CYP1A) showed 21% and 23% inhibition, respectively, while quinidine (CYP2D6) and 4-methylpyrazole (CYP2E1) did not cause any inhibition. Taken together, this data suggests that CYP19 might be the major enzyme catalyzing the N-demethylation of methadone to EDDP in placentas from late second and early third trimesters.

Monoclonal Antibodies

Antibodies raised against specific human CYP isoforms were used to confirm the identity of the microsomal enzyme(s) involved in the biotransformation of methadone to EDDP. Both pools (from late second and early third trimesters) were tested in the presence of the antibodies at their concentrations which cause 80% inhibition of their selected isoform. Again, the effect of these antibodies on EDDP formation in term placentas, under the same experimental conditions, was determined in our laboratory (Nanovskaya et al., 2004).

In the late second trimester pool, CYP19 antibodies were the most effective, causing 85 ± 13.6% inhibition of EDDP formation, $p < 0.001$ (Figure 6A). Antibodies against 2C8 ($p < 0.05$), 2C19 ($p < 0.05$), and 2E1 ($p < 0.05$) caused ~30% inhibition, while the remaining antibodies had no effect (< 20%). In early third trimester pool, antibodies raised against CYP19 caused more than 95 ± 3.0% ($p < 0.001$) inhibition of EDDP formation (Figure 6B). In addition, antibodies against 2C8 ($p < 0.05$) caused 36% inhibition, while antibodies against 2B6 ($p < 0.05$) and 2E1 ($p < 0.05$) exhibited ~25% inhibition. All remaining antibodies did not inhibit EDDP formation.

Therefore, antibodies against CYP19 were at least three times more potent than any of the others tested. This confirms the effect of chemical inhibitors on EDDP formation. Taken together, it is likely that aromatase is the major enzyme responsible for the N-demethylation of methadone to EDDP in placentas from late second and early third trimesters.

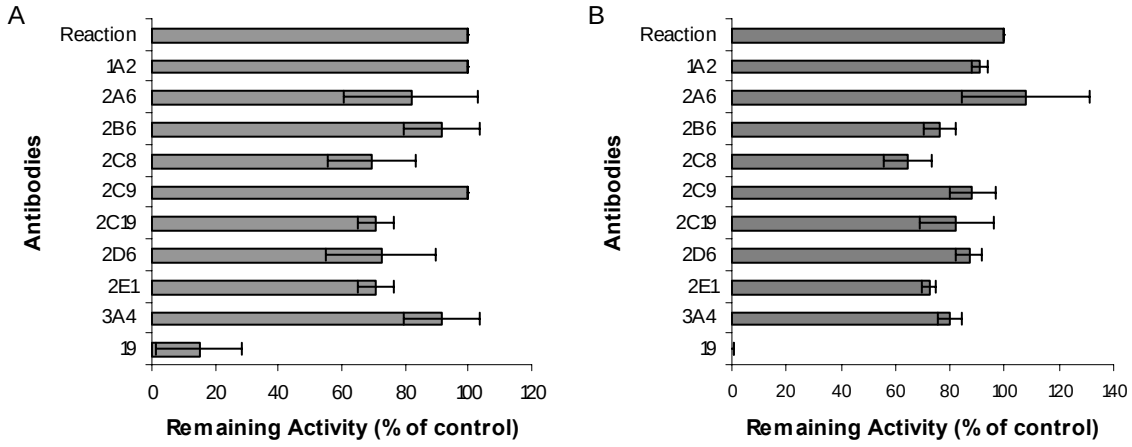

Figure 6. The effect of antibodies raised against human CYP isozymes on the N-demethylation of methadone by placental microsomes from (A) late 2nd and (B) early 3rd trimester. The rates of metabolite formation are expressed as percent of control (absence of antibodies). Antibodies raised against CYP19 caused >80% inhibition of EDDP formation in both sample groups.

EXPRESSION OF AROMATASE IN PLACENTAL MICROSOMES

The levels of aromatase expressed in the microsomal preparations of placentas at all gestational ages appeared to be similar (Figure 7). The amount of CYP19 expression was lowest (48.2 μg/μL) during the early second trimester and gradually increased to (49.8 μg/μL) during the early third trimester and reached (51.0 μg/μL) in the late third trimester. The fact that the differences in the levels of CYP 19 were not statistically significant, coupled with the uncertainty associated with the accuracy in determining protein concentration by Western blots, the data can only suggest that the levels of CYP19 do not appear to change during gestation.

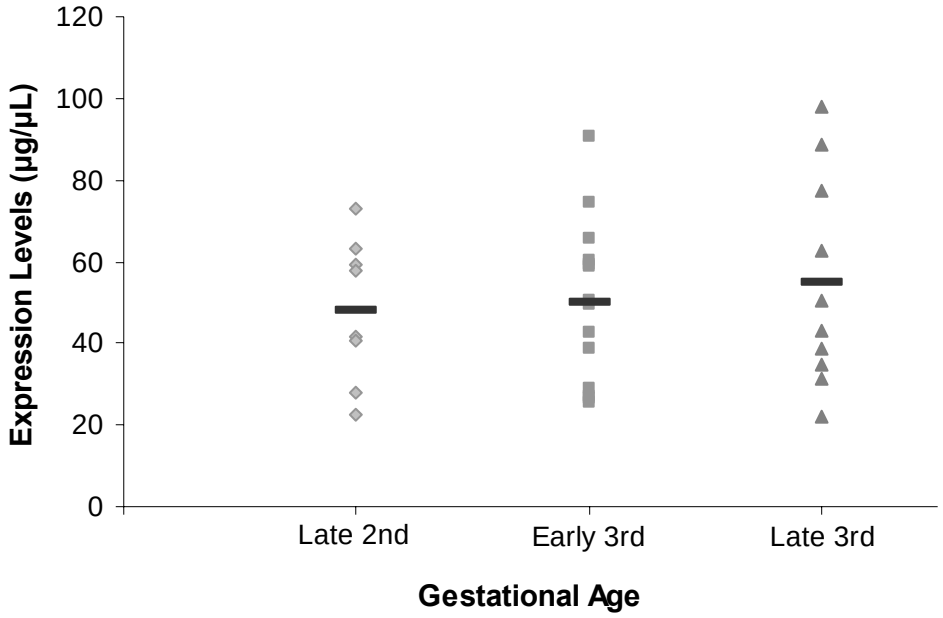

Figure 7. Levels of aromatase expression in microsomal samples from placentas from different gestational ages (scatter plot). Expression levels are grouped based on gestational age: late 2nd trimester (17-27 weeks), early 3rd trimester (28-33 weeks), and late 3rd trimester (34-40 weeks). Cross bars represent the mean for each gestational age.

CHAPTER 4: DISCUSSION

Methadone has been used for treatment of the opioid-dependent patient for over five decades. However, treatment of pregnant opiate addicts has been a particular challenge due to the effects of the drug on the mother and her developing fetus. Ideally, the dose of methadone administered to the pregnant women should prevent both maternal and fetal withdrawal (Allen, 1991), while minimizing the toxic effects to the fetus. Stress due to fetal withdrawal can increase the risk of meconium aspiration, fetal hypoxia and death (Kaltenbach et al., 1998). Doses of methadone as high as 150 mg/day have been used to achieve adequate maternal serum trough levels that prevent maternal and fetal withdrawal symptoms (Drozdick et al., 2002). The variations in methadone dosing of maintenance programs during pregnancy raised the following observations: first, the controversy on the correlation between maternal dose of methadone and development of NAS (Doberczack et al., 1993; Dashe et al., 2002; Kandall et al., 1977; Berghella et al., 2003); second, the incidence and intensity of NAS in preterm newborns was less than in term (Doberczak et al., 1991); and third, there is the occasional need for higher doses of methadone toward the end of pregnancy or during the third trimester (Drozdick et al., 2002). Taken together, it appears that disposition of methadone changes with gestational age and stage of placental development. Placental disposition of methadone potentially determines its concentration in the fetal circulation, and ultimately, the incidence and intensity of NAS. A better understanding of the changes in disposition of methadone by placental tissue throughout gestation might lead to a better understanding of the incidence and severity of NAS in infants exposed to the drug in utero.

The disposition of drugs by placental tissue is regulated by three processes; namely, simple diffusion, transporter –mediated efflux of the drugs from the tissue, and bio-transformation of the drugs by placental metabolic enzyme(s). However, the structure and functions of the placental tissue undergoes continuous changes during gestation that may affect the transfer of drugs, including methadone, to fetal circulation. These include changes in anatomical structure of placenta as well as in the activity and expression of its metabolic enzymes (Hakkola et al., 1996 a&b). Therefore, the goal of the current

investigation is to obtain information on the placental metabolism of methadone at different stages of gestation.

In a previous report from our laboratory, CYP19 / aromatase was identified as the major enzyme responsible for N-demethylation of methadone to EDDP in term placentas (Nanovskaya et al., 2004). On the other hand, more xenobiotic-metabolizing CYP isoforms are expressed in the placenta during the first trimester than at term (Hakkola et al., 1996 a&b). The concentration of placental microsomal aromatase increased with gestation by 4 times at term, but its activity in metabolizing its substrates, for estrogen formation, only doubled (Kitawaki et al., 1992). Therefore, the aim of this investigation is to identify and determine the activity of the major enzyme(s) responsible for N-demethylation of methadone in placentas obtained from different gestational ages.

The microsomal preparations used in this study were prepared from placentas obtained after vaginal or abdominal deliveries with gestational ages ranging from 17 to 40 weeks. After microsomal fractions were prepared, samples were divided into three groups: the first group (late 2^{nd} trimester) 17 to 27 weeks of gestation; the second group (early 3^{rd} trimester) 28 to 33 weeks of gestation; and the third group (late 3^{rd} trimester) 34 - 40 weeks of gestation.

In our initial experiments to identify the enzyme(s) responsible for the metabolism of methadone in human placentas of different gestational age, it was assumed that the enzyme(s) involved in the N-demethylation of methadone after 17 weeks of gestation until term was a microsomal CYP450 isozyme, since the presence of an NADPH-regenerating system was required. In all three groups of placentas, under our experimental conditions, EDDP was the only metabolite detected by utilizing the HPLC-UV method; EMDP was not detected. Therefore, no sequential demethylation of methadone was observed, irrespective of the gestational age of the placenta.

The effects of increasing concentrations of methadone on EDDP formation exhibited typical Michaelis-Menten saturation kinetics. The data obtained for late 2^{nd} and early 3^{rd} trimesters was compared to values obtained for late 3^{rd} trimester placentas. Analysis of the data revealed that apparent K_m values of 514 ± 187 μM and 519 ± 154μM

for placentas from late 2nd trimester and early 3rd trimesters, respectively, were not statistically different from the K_m value of 503 ± 120 μM obtained for placentas of the late 3rd trimester (Table 1). These data indicate that the affinity of the enzyme(s) responsible for N-demethylation of methadone was not affected by changes in gestational age. On the other hand, the activity of microsomal preparations in metabolizing methadone was the lowest in placentas from late 2nd trimester (192 ± 88 pmol mg protein^{-1}min^{-1}) and increased toward the end of gestation (385 ± 129 pmol mgprotein^{-1}min^{-1}, $p < 0.001$). The values obtained for intrinsic clearance (Table 1) in placentas obtained from late 2nd trimester was half of that obtained for placentas of late 3rd trimester ($p < 0.001$). Taken together, these data demonstrate that the metabolism of methadone by human placenta can begin as early as 17 weeks of gestation and that the activity of the enzyme(s) responsible for its metabolism increases with gestational age. It is important to note here that the activity of the enzyme(s) metabolizing methadone varies by as much as 4-6 times between placentas of the same gestational age group (Figure 4). These data suggest high interplacental variations in the metabolism of methadone for placentas obtained from different individuals.

Two approaches were used to identify the major placental enzyme responsible for the N-demethylation of methadone to EDDP: the effect of chemical inhibitors selective for specific CYP isoforms and the effect of monoclonal antibodies raised against human CYP isoforms. All chemicals were used at the concentration that is most selective for their respective CYP isoforms. Maximal inhibition was seen with aminoglutethimide and 4-hydroxyandrostenedione (95% and ~85%, respectively) in both the late 2nd and early 3rd trimester microsomal pools (Fig. 5 A&B). In the pool of term placentas the maximum inhibition of EDDP formation was also observed with aminoglutethimide (88%) and 4-hydroxyadrostenedione (70%) (Nanovskaya et al., 2004). These data suggest that CYP19/aromatase is the major placental enzyme responsible for the N-demethylation of methadone. In our investigation, ketoconazole caused a 66% inhibition of EDDP formation in late 2nd and early 3rd trimester placental pools, although in the pool of term placentas, ketoconazole did not have any effect on metabolite formation (Nanovskaya et

al., 2004). In early publications from our laboratory utilizing two cDNA preparations expressing CYP3A4 and CYP19, we reported that ketoconazole inhibited the metabolism of buprenorphine by CYP19 (Deshmukh et al., 2003). The inhibitory effect of ketoconazole on the CYP19 metabolism of androstenedione and testosterone has also been demonstrated (Ayub and Levell, 1988).

The use of chemical inhibitors does not provide unequivocal identification of the enzyme because of the cross inhibition with other CYP isoforms. Therefore, monoclonal antibodies raised against CYP isoforms were used to confirm the identification of the enzyme. The effects of monoclonal antibodies on the biotransformation of methadone to EDDP revealed that antibodies raised against human placental aromatase were the most effective. The antibodies raised against CYP19 caused 85% and >96% inhibition of EDDP formation in the late 2nd and early 3rd trimester placentas, respectively (Fig.6 A&B) as well as 80% in term placentas (Nanovskaya et al., 2004). Antibodies against CYP2C8 and 2E1 caused more than 30% inhibition of methadone biotransformation in placentas from late 2nd and early 3rd trimesters, respectively. Other antibodies, namely, 2C19 and 2D6 in late 2nd trimester and 2B6 in early 3rd trimester, caused 30%, 30%, and 25% inhibition, respectively, while all other antibodies had no effect. On the other hand, the same antibodies (CYP2C8, 2C19, 2B6, and 2E1) caused approximately 20% inhibition in the pool of term placentas (Nanovskaya et al., 2004). The involvement of these enzymes, to a lesser extent, at early stages of gestation can not be rule out. In summary, all data obtained on the effects of chemical inhibitors, monoclonal antibodies, and kinetics of the N-demethylation of methadone confirm that the major enzyme catalyzing this reaction in human placental microsomes obtained between 17 weeks to 40 weeks of gestation is CYP19.

Our data revealed that the activity of term placental preparations in metabolizing methadone is higher than activity of placentas obtained from earlier gestational ages. Activity of placental preparations in the formation of EDDP from methadone varies by four- to six- times between different individuals, irrespective of the gestational age. These inter-individual variations, together with increased metabolism of methadone, during

third trimester might influence placental disposition of methadone and could be one of the contributors to the reported dosage adjustments of methadone for pregnant patients during third trimester of pregnancy (Drozdick et al., 2002).

There are two principal changes associated with pregnancy that can have an effect on pharmacokinetics of methadone, and consequently adjustments of its dosing: first, the pregnancy-induced maternal physiological changes, and second, the development of the placental-fetal compartment. These changes can affect the disposition of methadone and consequently, its concentration in the fetal circulation. The latter should be one of the factors affecting the incidence and intensity of neonatal abstinence syndrome. A better understanding of the role of the placenta in disposition of methadone and how changes in placental structure and function could affect this disposition will enhance the current knowledge concerning methadone treatment of the pregnant opiate addict and provide maximal protection for the mother and fetus.

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