TRANSPLACENTAL PHARMACOLOGY AND TOXICOLOGY OF MORPHINE IN HUMANS: IN VITRO AND IN VIVO STUDIES

by

Ernest Albert Kopecky

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Pharmaceutical Sciences, Faculty of Pharmacy at the University of Toronto

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Transplacental Pharmacology and Toxicology of Morphine in Humans: *In Vitro* and *In Vivo* Studies, Doctor of Philosophy, 1999, Ernest Albert Kopecky, Graduate Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto.

**ABSTRACT**

Purpose: To investigate the effects of morphine on the placenta and on fetal response.

Methods: Placentas from uncomplicated term pregnancies were obtained after vaginal delivery or cesarean section. The treatment groups were morphine and morphine-naloxone. A chorionic vein and artery supplying a single, peripheral cotyledon were cannulated for perfusion. Morphine, antipyrine, lactate, glucose, human chorionic gonadotropin, oxygen consumption, perfusion pressures, and pH gradient were measured.

Women with singleton pregnancies, requiring fetal blood sampling (FBS), between 21 and 39 weeks gestation, were assigned to the study or control groups based on placental location, fetal position, and pre-FBS extent of fetal movement. Only study patients received 10 or 15 mg of morphine. Pre- and post-procedure maternal vital signs, biophysical profile score (BPS), and Doppler indices were measured. Maternal and cord blood was drawn for morphine analysis.

Results: The rate of morphine transfer across the placenta was $0.73 \pm 0.44$ and $0.69 \pm 0.26$ ng/mL/min in the morphine ($n=4$) and morphine-naloxone ($n=5$) perfusion experiments, respectively ($p = 0.89$). The mean fetal-to-maternal transfer ratio was $0.31 \pm 0.27$ (morphine) and $0.27 \pm 0.24$ (morphine-naloxone) ($p = 0.66$). The clearance of morphine was $0.89 \pm 0.40$ (morphine) and $0.87 \pm 0.27$ mL/min (morphine-naloxone) ($p = 0.92$). The morphine:antipyrine clearance index was $0.63 \pm 0.08$ (morphine) and $0.64 \pm 0.26$ (morphine-naloxone) ($p = 0.99$). Morphine was retained by and released from all placentas.

The mean morphine dose ($n=10$) was $0.16 \pm 0.02$ mg/kg. Mean maternal and cord morphine levels were $41.8 \pm 14.6$ and $25.5 \pm 11.6$ ng/mL, respectively. The fetal-to-maternal ratio was $0.61 \pm 0.20$. After morphine exposure, there was a mean 3.2 point decrease in the BPS.
A significant positive trend was measured when biophase morphine was correlated with the S/D ratio, RI, and PI.

Conclusions: Morphine transfers across the placenta,不影响 placental viability or barrier integrity. Naloxone does not alter morphine transplacental kinetics. Placental retention and release of morphine prolongs fetal exposure. Morphine affects fetal response as indicated by a decreased BPS. The correlation between the biophase morphine and the Doppler indices suggests that morphine functions as a vasoconstrictor of the placental vasculature.
ACKNOWLEDGEMENTS

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I fervently believe that it is also paramount to extend my appreciation to the patients who so graciously volunteered to participate in my clinical trials, to the nurses and Fellows at both Mt. Sinai and Women’s College Hospitals for their help with scanning study patients, and the Medical Research Council of Canada for the financial support without which this thesis would not have been possible to complete.

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antibody
antipyrine
amniotic fluid volume
blood pressure
biophysical profile
central nervous system
counts per minute
cerebrospinal fluid
chorionic villous sampling
Da
Doppler velocimetry
end-diastolic
ethylenediaminetetraacetic acid
fetal artery
fetal breathing movements
fetal blood sampling
fetal heart rate
fetal movement
fetal tone
fetal vein
fetal-to-maternal
human chorionic gonadotropin
human follicle stimulating hormone
luteinizing hormone
human thyroid stimulating hormone
high performance liquid chromatography
intrahepatic vein
intramuscular
intrauterine growth retardation
intravenous
lactate dehydrogenase
maternal artery
6-monoacetylmorphine
maximum binding
milli-international units
middle cerebral artery
millimeters of mercury
morphine
medium 199
morphine-3-glucuronide
morphine-3,6-diglucuronide
morphine-6-glucuronide
maternal vein
1 normal unit
naloxone-3-glucuronide
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<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced)</td>
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<tr>
<td>NAL</td>
<td>naloxone</td>
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<tr>
<td>NBS</td>
<td>National Bureau of Standards</td>
</tr>
<tr>
<td>NM</td>
<td>normorphine</td>
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<td>NM-6G</td>
<td>normorphine-6-glucuronide</td>
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<td>NSB</td>
<td>non-specific binding</td>
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<td>NST</td>
<td>non-stress test</td>
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<td>OD</td>
<td>optical density</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PI</td>
<td>pulsatility index</td>
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<td>PK-PD</td>
<td>pharmacokinetic-pharmacodynamic</td>
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<td>PS</td>
<td>peak systolic</td>
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<td>RI</td>
<td>resistance index</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<td>RR</td>
<td>respiratory rate</td>
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<td>SC</td>
<td>subcutaneous</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>S/D ratio</td>
<td>systolic/diastolic ratio</td>
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<td>SIDS</td>
<td>sudden infant death syndrome</td>
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<td>S:P ratio</td>
<td>saliva:plasma ratio</td>
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<td>t_{1/2}</td>
<td>half-life</td>
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<td>TCA</td>
<td>trichloroacetic acid</td>
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<td>UA</td>
<td>umbilical artery</td>
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<td>UV</td>
<td>umbilical vein</td>
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PUBLICATIONS

The following publications have arisen from the research presented in this doctoral dissertation.

Manuscripts


Book Chapters


Abstracts


To my mother, Professor Dagmar Ledlová-Kopecky, and grandmother, the late Vera Čejková (Mamka), for their unconditional support and inspiration ... with appreciation and deepest of love.
Chapter 1 – Background
1. Introduction

The human body is exposed to xenobiotics with alarming frequency each day we interact with the environment, especially in those individuals residing in busy urban centers. It was thought that the human body possesses innate defense mechanisms that render the fetus completely protected from all xenobiotic exposure. The thalidomide disaster of the late 1950s and early 1960s was the first large-scale evidence that the fetus is not unconditionally exempt from xenobiotic exposure.\(^1\)\(^2\) Initially developed specifically for use by gravid women for nausea and vomiting during pregnancy, thalidomide was removed from the pharmaceutical market after the emergence of major malformations in children born to women who used thalidomide during their pregnancy. The thalidomide disaster is a classic example of the danger associated with extrapolating animal research to humans; animal data from the thalidomide experiments yielded results that did not indicate the impending harm that the drug was going to cause to humans.

Once the notion of a susceptible fetus was introduced into the scientific and medical literature, our views of pregnancy and the caring for gravid women were changed permanently. Research into the susceptibility of the fetus began to emerge and as data were acquired, a new specialized field of research was established: placentology. Placentology is a relatively new branch of medical research aimed at resolving placental function, placental pathology and the ensuing effects on the fetus, placental metabolism of xenobiotics, and the transfer characteristics of xenobiotics across the human placenta. As a result of the data generated from placental studies and pharmacokinetic and pharmacodynamic studies involving fetal exposure to xenobiotics, during the past ten to fifteen years, counseling services have been established for gravid women, such as the Motherisk Program at the Hospital for Sick Children in Toronto. These services disseminate information to gravid women and physicians caring for pregnant women pertaining to drug exposure and medically managed drug use during pregnancy. Furthermore, with the advent, within the last five years, of improved specialized diagnostic tools for fetal assessment
and the ability to treat the fetus in utero, maternal-fetal assessment and treatment units have been established at many tertiary care facilities that offer full obstetric services.

There are two principal sources of drug exposure during pregnancy. First, exposure may occur through appropriately managed pharmacologic care of the mother and the fetus by a physician and secondly, illicit drug use by the expectant mother. Illicit drug use may be in the form of abusing drugs prescribed for legitimate medical management of the patient, abusing another’s prescription medications with the sole intent of eliciting a euphoric effect, and/or consuming “recreational” drugs purely for their psychotropic effects.

2. Demographics: Drug Exposure During Pregnancy – Therapeutic and Illicit Use
   i. Therapeutic Drug Exposure During Pregnancy

Morphine is the most widely used opioid analgesic currently available. Its use continues to increase as our understanding of and need for adequate pain control increases. Moreover, it is recommended by the World Health Organization as the drug of choice for the treatment of severe pain attributable to cancer.³

For obstetrical patients, morphine has been recommended as the drug of choice to be used during pregnancy to treat maternal pain attributable to: vasoocclusive crises in sickle cell disease;⁴ chronic pain syndromes;⁵,⁶ special procedures such as fetal blood sampling (FBS), chorionic villous sampling (CVS), and fetal tissues biopsies; and nonobstetric surgery. Nonobstetric surgical procedures include surgery for the treatment of cervical incompetence, complications of ovarian cysts, trauma, and removal of breast tumors and other malignancies.⁷ Estimates of the frequency of nonobstetric surgery performed during pregnancy range from 0.75 to 2.0%.⁸,⁹ In the United States, this amounts to approximately 75,000 gravid women who may be clinically exposed to anesthetics and analgesics each year. Moreover, this figure may be an underestimate because many pregnancies may be unrecognized at the time of surgery. Morphine
has also been used during pregnancy to ameliorate fetal pain attributable to special procedures, such as FBS, CVS, and fetal tissue biopsies. As well, morphine may be used for pain management during open fetal surgery; fetal surgery performed in utero to correct such life-threatening anatomic malformations as bilateral obstructive hydronephroses, congenital diaphragmatic hernias, sacrococcygeal teratomas, and obstructions of the ventricular outflow tract.  

The prevalence of the use of morphine to ameliorate fetal pain may continue to increase as a result of the works of Giannakouloupolous et al. and Radunovic et al. who have shown that the fetus mounts a pain response when subjected to needle insertion during FBS. These groups have shown that fetal plasma cortisol and β-endorphin levels rise significantly in response to intrauterine needling. Given this data, not providing adequate fetal pain management may be viewed as inflicting unnecessary pain on a patient, and hence, medical mismanagement.

Currently, there are no reports of adverse fetal outcomes as a result of clinically managed maternal exposure to opioids during pregnancy. Moreover, the majority of therapeutic agents given to pregnant women do not pose significant reproductive hazards when these agents are used in recommended doses, with correct medical management.

ii. Illicit Drug Exposure During Pregnancy

Although babies are being exposed to opioids during pregnancy as part of therapeutic regimens, fetal exposure due to illicit drug use carries a far more serious risk to fetal health and outcome.

Illicit drug use (substance abuse) is defined as an excessive self-administration of licit and illicit chemicals to alter one's perception of one's own cognitive status. The most frequently abused substances include alcohol, cocaine, cannabis, tobacco, psychotherapeutic...
agents, and opioids. Among the opioids, morphine, heroin, and methadone appear to be most commonly used by women of childbearing age.15

There have been numerous attempts to describe the nature and extent of maternal drug abuse during pregnancy and its potential impact on the fetus and the neonate. The magnitude of the problem is illustrated by the number of infants exposed to drugs in utero. The National Institute on Drug Abuse’s National Pregnancy and Health Survey15 showed that 5.5% (221,000) of the approximately 4,000,000 women who delivered live babies in the United States in 1992 had used some illicit drug during pregnancy. Moreover, of all the infants born in the United States and exposed in utero to maternal substance abuse in 1992, 18.8% (756,900) were exposed to alcohol, 1.1% (45,100) to cocaine, 0.1% (3,600) to heroin, 3.0% (118,700) to cannabis, 0.1% (3,400) to methadone, 20.5% (819,700) to cigarette smoke, and 1.5% (61,200) to psychotherapeutic agents without physician orders.15 Neonates exposed in utero to either heroin or methadone account for 3% to 5% of the total number of live births in municipal hospitals of New York City.16

Maternal drug abuse during pregnancy is further complicated because evidence suggests only a trend among mothers to decrease the rate of drug consumption from 3 months before pregnancy and throughout gestation, rather than to achieve complete discontinuation of drug use.15 In addition, the tabulated figures provided by the National Pregnancy and Health Survey may actually be underestimates because of the underreporting of live neonates born to substance-abusing women.

The impact of fetal exposure to illicit chemicals and/or abused therapeutic medications is profound.17 The mechanisms responsible for the effects have not been completely elucidated, but may involve direct drug effects on the fetus or detrimental effects on placental development and function, thereby facilitating abnormal fetal outcomes. Significantly lower birth weight, shorter length, and smaller head circumference have been observed in neonates who have been exposed
to heroin or methadone in utero when compared with nonexposed neonates.\textsuperscript{18} The effects on variables of infant growth are thought to be a direct result of fetal opioid exposure. A 20% incidence of Apgar scores less than 7 at 1 and 5 minutes postpartum has been documented for neonates of drug-dependent or heroin- or methadone-addicted women, representing a twofold higher incidence of such findings than for the control group.\textsuperscript{19,20} In addition, a twofold increase in premature deliveries over a 10-year period has been observed in 384 neonates born to heroin-addicted mothers.\textsuperscript{21}

In the first 3 days of neonatal life, a less common, severe hyperbilirubinemia occurs in neonates of opioid-addicted women.\textsuperscript{21,22} Sudden infant death syndrome (SIDS) increases 5- to 10-fold in neonates born to opioid-addicted women.\textsuperscript{23,24} Despite the consensus linking maternal opioid use to SIDS, no proof of causation has been developed. The most plausible hypothesis suggests that morphine and related opioids reduce brain-stem responsiveness to carbon dioxide\textsuperscript{25} and depress the pontine and medullary centers that control respiratory rhythmicity.\textsuperscript{26} Most of these data, however, are derived from studies in adults. Studies of the prolonged effects of opioids on infants after in utero exposure are scarce.

No homogeneous pattern of malformations has been established. The results of one study\textsuperscript{19} indicated that 37 among 830 opioid-exposed neonates had congenital malformations. Of these 37 neonates, 20 exhibited major malformations such as hydrocephalus, heart anomalies, and genitourinary defects; an incidence of 2.4% compared with 0.5% in controls. However, the rates of major malformations in the general population have been shown to range between 1% to 3\%.\textsuperscript{14} Therefore, more data are required in this area before any conclusions regarding in utero opioid exposure and the incidence of fetal major malformations may be drawn.

Unlike the controlled in utero exposure to opioids during medical management of pregnant women, the ramifications of the nonmedical use of opioids during pregnancy are serious, leading to children with significant adverse outcomes.
3. Drugs

i. Morphine

Because of the widespread use of morphine for therapeutic purposes, it is important to have a thorough understanding of the drug's pharmacokinetics. Currently, the pharmacokinetics of morphine are not completely understood nor is the relationship between plasma morphine concentrations and pharmacodynamic effects. This is due to many reasons, the most notable of which are listed in Table 1-1.

Table 1-1. Reasons for the Incomplete Understanding of Morphine Pharmacokinetics

1. The limited relevance of pain management studies in animals and normal human volunteers to acute and chronic pain.
2. The presence of contaminants in biological samples that cause poor inter-assay reliability by decreasing the specificity of the assay.
3. The incomplete understanding of the role of the active metabolites of morphine in ameliorating pain.
4. Difficulty in standardizing the assessment of pain and in assessing the influence of nonphysical factors on pain perception, such as mood.
5. The influence of route of administration, dose, and the clinical characteristics of different patient populations.
6. The wide interindividual variations in the pharmacokinetics of morphine, which generate conflicting data between and within studies.

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a. Chemistry of Morphine

Morphine, a naturally occurring alkaloid, is isolated from the opium poppy plant, *Papaver somniferum.* Although discovered in 1805, the correct chemical structure was not determined until 1927. The drug was not completely synthesized until 1952.

Morphine (7,8-didehydro-4,5-epoxy-17-methyl-(5α,6α)-morphinan-3,6-diol) consists of five fused ring systems (Figure 1-1). The carbon atoms are number 1 to 16. There are five asymmetrical carbon atoms, conferring strong levorotation; the levo isomer being pharmacologically active while the dextro isomer is inactive.
The nitrogen atom forms a tertiary amine that can exist in either of two forms, depending on whether or not it is protonated. The nitrogen usually is unprotonated, making morphine a base. The pKa for the morphine base is 7.9 – 8.1. Even in the unionized form, the drug is relatively water-soluble and poorly lipid-soluble because of the hydrophilic –OH groups. This poor lipid solubility limits the transfer of morphine from plasma to the tissues and into the biophase (effect compartment; refer to section 4). The salt form of the drug (morphine sulfate) is used clinically due to the greater solubility in an aqueous medium and the increased dissolution rate.\textsuperscript{34} Since the salt form of morphine is more soluble in an aqueous medium, the dissolution rate for morphine sulfate is greater than the dissolution rate of the unionized form of the drug. Because the dissolution rate is the rate-limiting step in absorption, then it can be anticipated that the bioavailability of the salt form of the drug will increase as solubility increases.\textsuperscript{34}

b. Antinociception

Supraspinal antinociception has been postulated to occur primarily, although not exclusively, through the $\mu_1$ receptor (high affinity receptors for morphine and enkephalins),
whereas respiratory depression occurs mainly through the \( \mu_2 \) receptor subtype.\textsuperscript{35} The molecular characterization of the \( \mu \) receptor subtypes remains to be elucidated. It is the high affinity \( \mu_1 \) receptors that have been localized in brain regions involved in opioid analgesia, such as the periaqueductal gray matter, medial thalamus, and the median raphe.\textsuperscript{36} The relative contribution of each subtype to spinal and supraspinal analgesia is not known.\textsuperscript{37,38} In addition, the mechanism involved in the interaction of an agonist with the opioid receptor and the ensuing neuronal electrophysiological response remains to be determined.\textsuperscript{39}

It has been hypothesized that both \( \mu \) and \( \delta \) receptors are coupled to potassium channels which, when opened, facilitate hyperpolarization of the membrane and inhibition of neuronal conductance.\textsuperscript{39} The \( \mu \), \( \delta \), and \( \kappa \) receptors are also coupled to voltage-dependent calcium channels. Stimulation of these receptors closes the calcium channel thereby inhibiting the release of neurotransmitter(s).\textsuperscript{39} Activation of ion channels by opioids involves interaction with G-proteins.\textsuperscript{40} Coupling between the receptors and the channels is thought to occur by one or more guanosine triphosphate (GTP)-binding proteins that are closely associated with the receptors.\textsuperscript{39} All opioid receptor types appear to be regulated by GTP-binding proteins because high affinity agonist binding can be diminished by exogenous guanine nucleotides.\textsuperscript{41} The binding of morphine to all three receptors also inhibits adenyl cyclase activity via inhibitory G-proteins which, through additional messengers, may also control ion conductance.\textsuperscript{39} Morphine-type agonists have been able to block neuronal firing in several brain regions suggesting that \( \mu \) receptors may be coupled to ion channels. It is the control of ion conductance that may be the primary mechanism responsible for the analgesic effects of opioids.

Pain perception occurs through the transmission of sensory information by ion conductance from nociceptors to motor neurons. Morphine elicits its analgesic effect by depressing the transmission of sensory information from nociceptors to motor neurons and by
depressing the nociceptive stimulation of neurons that project into the supraspinal region.\textsuperscript{38,42}

Depression of sensory information is mediated by $\mu$ receptors located in the dorsal horn of the spinal cord. Morphine also modulates nociception by blocking sensory information propagating to interconnecting neurons located in the midbrain and medulla.\textsuperscript{38,42}

Sedation is a physiologic effect of $\kappa$ agonists. Morphine has also been shown to exhibit moderate $\kappa$ receptor specificity,\textsuperscript{43} thereby eliciting sedation in addition to its analgesic effects.

c. Pharmacokinetics of Morphine

1. Absorption

Morphine is absorbed to some extent through all mucosae and diffuses across the spinal dura. Therefore, multiple routes of administration can be used to deliver morphine. Transdermal application, however, is not available because morphine does not penetrate intact skin.\textsuperscript{44} Peak plasma morphine levels following intravenous (IV) injection depend on the sampling time. After a single 10 mg dose of morphine, blood drawn within five minutes of injection yields plasma levels in the range of 200 – 400 ng/mL in young volunteers.\textsuperscript{45} The dose-concentration relationship for morphine is linear over a wide range of doses (20 – 750 mg/day).\textsuperscript{46,47,48}

Factors that influence absorption after intramuscular (IM) and subcutaneous (SC) injections of morphine include variation in blood pressure and tissue perfusion, site of the injection, pH of the injection site, and drug lipophilicity.\textsuperscript{49,50} IM absorption is rapid exhibiting an absorption $t_{1/2}$ of 2 – 8 minutes; the peak occurring at 10 – 20 minutes.\textsuperscript{51,52} The systemic bioavailability is essentially complete.\textsuperscript{52} Peak plasma concentrations occur at approximately 15 minutes. Plasma levels after IM and SC administration are equivalent to those achieved using the IV route.\textsuperscript{50,51} As with IV administration, significant variability in steady-state morphine levels has been reported among patients receiving continuous infusions of SC morphine.\textsuperscript{50,53}
2. Distribution and Protein Binding

Morphine rapidly diffuses from the blood stream after absorption. The distribution phase half-life for IV morphine is 0.9 – 2.5 minutes.\textsuperscript{54,55} Morphine is distributed to highly perfused tissues such as the lungs, kidney, liver, spleen, and muscle.\textsuperscript{51,52} Morphine and its highly polar metabolites cross the blood-brain barrier.\textsuperscript{56} After systemic administration, morphine concentrations in the cerebrospinal fluid (CSF) are 4 to 60% of plasma morphine levels.\textsuperscript{49,57,58}

In addition, studies have shown that morphine appears in the fetus and also appears in breast milk.\textsuperscript{46} However, the specific transplacental exchange characteristics of morphine and whether or not the drug is stored by the placenta are unknown.

Various studies report that morphine has a large volume of distribution, 1 to 6 L/kg, with high variability between individuals.\textsuperscript{59,60,61} The volume of distribution may be affected by the hemodynamic status of a patient, alterations in plasma protein binding, pregnancy, and variations in tissue blood flow, each of which may be significantly altered in patients receiving morphine.

Morphine binds to albumin and $\alpha_1$-acid glycoprotein. Binding is independent of dose but dependent on the plasma protein concentration.\textsuperscript{44} At therapeutic doses, protein-binding of morphine is only 20 to 40%,\textsuperscript{62,63} with an average frequently reported value of 35%.\textsuperscript{64} Because of the low extent of morphine protein-binding, major changes in binding would be required to influence plasma levels of the free drug.

3. Biotransformation

Morphine is primarily metabolized by glucuronidation, quantitatively the most significant biotransformation mechanism, and oxidation reactions (Figure 1-2);\textsuperscript{27} phase-2 conjugation reactions that convert morphine into readily excretable water-soluble compounds. In humans, the principal site of morphine metabolism is the liver, albeit that the conjugating enzyme UDP-glucuronyltransferase is also found in the gastrointestinal tract, kidney, and the brain.\textsuperscript{27} The
metabolic role of extrahepatic sites such as the kidney, brain, and placenta is controversial. Morphine glucuronidation is proportional to dose during chronic therapy, even with increasing dose titration. This indicates that the conjugation pathway is not saturable even after months of continuous high-dose therapy.65

M3G is quantitatively the major morphine metabolite.27 However, the metabolite is not pharmacologically active because opioid receptor-binding is thought to require an unoccupied 3-OH position on the morphine molecule.66

M6G is quantitatively a minor metabolite. However, it is pharmacologically active exhibiting an analgesic potency of up to 45 times that of the parent compound.27,67

Factors controlling the extent to which either glucuronide is produced are unknown. It is also unknown if there are isoenzymes of UDP-glucuronyltransferase that exhibit genetically determined differences in activity, which could explain the wide interindividual variability in systemic bioavailability seen with oral morphine. The relationship between dose and plasma concentrations of morphine and its metabolites appears to be linear and independent of dose or duration of treatment.46,68

NM is also a quantitatively minor metabolite exhibiting pharmacological activity. It is formed in larger amounts after oral morphine dosing than after IV administration of morphine.51 This may account for the lower urinary free morphine levels after PO and IV administration. NM is formed in the liver by the microsomal oxidation system involving cytochromes C and P450.27 NM-6-glucuronide (NM-6G) is also thought to be pharmacologically active.27

There is limited information about M3,6DG in the medical literature. The metabolite should be inactive because of the occupied 3-OH position of the morphine ring. M-3-ethereal sulfate is also inactive although quantitatively more abundant than M3,6DG.27
Fig. 1-2. Morphine Biotransformation Pathways

* - Active metabolites; M3G - morphine-3-glucuronide; M6G - morphine-6-glucuronide; NM-6G - normorphine-6-glucuronide; M3,6DG - morphine-3,6-diglucuronide
4. Excretion

Morphine undergoes biphasic elimination following distribution; an early, major phase and a slow minor phase. Morphine is primarily excreted renally by glomerular filtration of the water-soluble conjugates produced by phase-2 metabolic conjugation reactions in the liver. Most of the drug is excreted during the first 24 hours. Up to 85% of an administered dose of morphine is recovered from the urine as free morphine, 50 - 60% as M3G, and approximately 15% as M6G and NM. Approximately 10 - 20% of a dose is unaccounted for by renal elimination and is presumably excreted in urine as unidentified metabolites or excreted via alternate routes of elimination. To a small extent, the enterohepatic circulation may contribute to morphine excretion in the bile or feces. These results are similar in both healthy adult volunteers and in cancer patients, as well as in situations of acute and chronic administration of morphine.

The early, major phase plasma elimination half-life of morphine is approximately 2 hours and is independent of the route of administration, dosing interval, age, or clinical status of the patient. There is, however, a large interindividual variation in the plasma elimination half-life, with reported values in the range of 1 to 8 hours. The half-life of M3G and M6G is 3 to 4 hours after IV administration and 9 to 10 hours after oral administration. These values are significantly prolonged in patients with renal failure, indicating accumulation.

The total body clearance of morphine is mostly accounted for by metabolic clearance. Renal clearance has been estimated at 100 mL/min and is independent of drug dose or urinary flow. There is wide variation in the values obtained for total clearance, ranging between 5 to 35 mL/kg/min, although similar for normal volunteers, surgical patients, and cancer patients. Studies have reported the clearance of morphine at approximately 20 mL/kg/min, approximating hepatic blood flow. The wide variation in morphine clearance values is attributable to age and physical status of the patient, effects of surgery, anesthesia, and disease, and the type of
assay used. There is limited information regarding the details about the effects of the aforementioned factors on morphine clearance.  

\[ \text{Formula} \]

\[ \text{Equation} \]

\[ \text{Diagram} \]

\[ \text{Figure} \]

d. Pharmacodynamics and the Plasma Concentration-Effect Relationship

Because equivalent pain control in different patients necessitates the administration of a wide range of morphine doses, therapeutic drug level monitoring of morphine may yield clinically useful data. Identical doses (mg/kg) are typically associated with a wide range of plasma concentrations among patients, although the dose-concentration effect is linear within patients. In addition, the plasma concentration is influenced by pharmacokinetic factors that are also highly variable between and within individuals. To date, there is no definite evidence of a direct link between morphine levels and effect.

In summary, the following observations can be made regarding the concentration-effect of morphine. First, the dose-concentration relationship of morphine is linear over a wide range of doses (20 to 750 mg/day). Secondly, the peak analgesic effect of morphine appears to be related to the peak plasma concentration of morphine, although there is a lag time between peak level and effect. The lag time may be due to active metabolite formation and distribution into the biophase (effect compartment). Thirdly, the area under the time-effect curve varies with the route and formulation. Fourthly, satisfactory analgesia is achieved by a broad range of steady-state morphine and M6G concentrations. Fifth, the concentration-adverse effect relationship has not been examined for morphine or its active metabolites except for some conflicting data regarding respiratory function. Establishment of a therapeutic window above which an unacceptable incidence of major side-effects may occur would be very useful. And finally, with repeated administration, tolerance develops to both effects and side-effects. The mechanism is probably functional (pharmacodynamic) rather than dispositional (pharmacokinetic), albeit that there are few evidence-based pharmacokinetic data in the literature.
ii. Naloxone

Naloxone prevents or reverses the effects of opioids including respiratory depression, sedation, and hypotension. The drug is a pure opioid antagonist that does not possess the agonistic or morphine-like properties characteristic of other opioid antagonists. Naloxone does not produce respiratory depression, psychotomimetic effects, or pupillary constriction. In the absence of opioids, naloxone exhibits no pharmacologic activity. Conversely, in the presence of psychological dependence (addiction), the drug will produce withdrawal symptoms.

The mechanism of action is not completely understood. The preponderance of evidence suggests that naloxone antagonizes the effects of opioids and endogenous opiate-like peptides such as endorphins and enkephalins by competitive inhibition of the same receptor sites. Naloxone has been shown to cross the placenta, appearing in the fetal blood within 2 minutes after maternal administration; the plasma concentration gradually increasing over 10 to 30 minutes.

a. Chemistry of Naloxone

Naloxone hydrochloride is a synthetic congener of oxymorphone with the chemical name (-)-17-allyl-4, 5α-epoxy-3,14-dihydroxymorphinan-6-one hydrochloride. Structurally, it differs from oxymorphone in that the methyl group on the nitrogen atom is replaced by an allyl group (Figure 1-3).

b. Pharmacokinetics of Naloxone

After IV administration, the onset of action occurs within one to two minutes. The onset of action is only slightly less rapid in adults when the drug is administered SC or IM. However, in neonates, the onset of action after SC or IM administration ranges from 15 to 40 minutes probably due to erratic absorption attributable to respiratory depression-induced poor perfusion,
hypotension, and peripheral vasoconstriction. The duration of action is dependent on the dose and route of administration. IM administration produces a prolonged effect compared with IV administration. The necessity for repeated doses will also be dependent on the amount, type, and route of administration of the opioid being antagonized. Following parenteral administration, naloxone is rapidly distributed in the body.

Naloxone is absorbed from the gastrointestinal tract and is subject to significant first-pass metabolism. It is primarily metabolized by the liver via glucuronide conjugation, N-dealkylation, and reduction of the 6-keto group (Figure 1-4). The major metabolite is naloxone-3-glucuronide (N3G) and is excreted in the urine. The serum half-life in adults ranges from 30 to 81 minutes (mean 64 ± 12 minutes). In neonates, the mean plasma half-life is 186 ± 30 minutes and ranges between 26 to 122 minutes in preterm neonates. In adults, the systemic clearance is approximately 21.7 mL/kg/min and the volume of distribution is approximately 2 to 3 L/kg. In premature infants, calculations from plasma concentration versus time data have shown that the elimination rate constant is 0.75 ± 0.39/hour, the systemic clearance is 39.1 ± 14.5 mL/kg/min, and the apparent volume of distribution is 3.52 ± 1.20 L/kg. Single SC doses
as high as 24 mg/70 kg (0.343 mg/kg) and multiple doses of 90 mg daily for two weeks were administered to normal volunteers and produced no behavioral or psychologic changes, yet the antagonistic activity to subsequent morphine challenge was maintained.75 In neonatal patients, doses of 0.4 mg (0.16 mg/kg) and constant IV infusion of 0.16 mg/kg/hour for five days have not been associated with side effects.83

4. Biophase (Effect Compartment)

Plasma drug concentrations have traditionally been used in the study of drug level versus effect. However, when studying the relation between pharmacokinetics and pharmacodynamics, a lag period is frequently observed between a plasma drug level and the onset of the desired effect. This lag period is due to the time that the drug requires to equilibrate at its site of action, otherwise known as the effect compartment or biophase.

The effect of a drug may not be directly related to its plasma concentration. The lag time seen between the plasma drug concentration and the effect may be attributable to various factors such as a peripheral site of action, an indirect effect mediated through an endogenous compound, or active metabolite(s). Most commonly, a delayed effect is modeled by an effect compartment approach,84,85 that is, a system analysis approach using biophase (effect compartment) principles.86,87

The ability to concurrently measure pharmacodynamic effects and opioid concentrations in biofluids confers the opportunity to study the relationship between the disposition of an analgesic and its pharmacodynamic effects in patients experiencing pain.88 The biophase approach has been employed to relate PK-PD drug data for such agents as morphine and methadone88 and fentanyl and alfentanil.89

It has been shown that morphine analgesia lags behind the time course of decline in
Fig. 1-4. Naloxone Biotransformation Pathways
plasma morphine concentration. That is, in non-steady state conditions, the equilibrium between the plasma morphine concentration and the biophase at the CNS opioid receptors as well as binding to the receptors, is not instantaneous. Conversely, at steady state, the plasma drug concentration and effect appear to be in phase; no disequilibrium exists because when a constant proportionality exists between the plasma drug concentration and the biophase concentration, hysteresis is eliminated.

5. Determinants of Drug Transfer Across Biological Membranes

i. Transport Mechanisms

The principal mechanism of drug transfer across the human term placenta is simple diffusion. The net transfer of a substance is governed by Fick's equation: rate of diffusion = K x A(Cm - Cf)/X, where K is the diffusion constant of the drug, A is the available surface area for transfer, Cm is the maternal blood concentration, Cf is the fetal blood concentration, and X is the thickness of the placental membrane. The key factors that contribute to the rate of drug transfer across the placenta include the surface area of the membrane, the thickness of the layers to be traversed, and the physiochemical properties of the drug.

Most drugs are not transported actively unless they are analogues of endogenous compounds such as α-methyldopa and 5-fluorouracil. Pinocytosis has been proposed as a transport mechanism to get compounds across the human placenta. Because this mechanism is not a major means of transplacental transport, it is not thought to significantly contribute to fetal drug exposure.

ii. Factors Influencing Opioid Transfer

Chemical, maternal, and fetal factors also affect the rate of drug transfer across the placenta. The physiochemical properties of the drug, such as lipid solubility, polarity, and the
pKa, can influence drug transport. A substance that is highly lipid-soluble, low molecular weight, minimally ionized at physiologic pH, and apolar will transfer readily.94

Water-soluble and lipid-soluble compounds with a molecular weight of less than 100 Da cross the placenta readily.94 Substances with molecular weights between 600 and 1000 Da transfer at slower rates. Substances with a molecular weight of greater than 1000 Da are relatively unable to cross the placenta.96

The placental transfer of drugs is also influenced by the difference in pH between the maternal and fetal circulations. The pH of the umbilical vessel blood is normally 0.1 to 0.15 pH units lower than the pH of the maternal blood. This is especially important for weakly basic drugs such as morphine (pKa 7.9-8.1).97,98 As morphine transfers from the maternal to the fetal circulation, the drug becomes more ionized. The drug's ionization causes a net transfer to and accumulation in the fetal circulation. This effect is known as ion trapping and is enhanced by fetal acidosis. The opposite is true for weakly acidic drugs.

It is the unbound fraction of a drug that diffuses across the placental membranes to enter organs and organelles for receptor-binding, metabolism, and excretion.90 Therefore, binding drugs to macromolecules inhibits the transfer of a drug across the placenta. Highly protein-bound drugs do not readily cross the placenta. Since the maternal plasma albumin concentration is lower in gravid compared with nongravid women, unbound morphine concentrations are higher in pregnant women; therefore more morphine can be transferred across the placenta.90 Fetal protein has a lower binding affinity for drugs than maternal protein and is present in lower amounts early in gestation compared with later in gestation. The result is a higher free-morphine fraction in the fetus.

The placenta binds and retains compounds, thus effectively acting as a reservoir for drugs. The placenta may, therefore, leach drugs out into the fetal circulation after drug concentrations are no longer detectable in the maternal circulation. This has the effect of
prolonging fetal exposure to a drug. The placental-tissue retention of drugs such as cocaine has been previously shown in our laboratory. 99

The rate-limiting step determining placental drug transfer is blood flow. 93 Changes in blood flow on either side of the placenta may influence the rate of morphine transfer. Under normal physiologic conditions, blood flow increases to meet the demands of the growing fetus for nutrients and oxygen. This increased blood flow with advancing pregnancy means increased delivery of morphine present in the mother’s circulation. 90 Blood flow can also be altered by maternal morphine use. For example, vasoconstriction leading to decreased placental perfusion may partially explain the retardation of intrauterine growth that occurs with a mother’s cigarette smoking and concomitant use of cocaine. 100

A normal, pregnancy-attributable decrease in gastric motility in the mother delays drug absorption and decreases peak drug concentrations in the blood of the mother. 101 Drugs that require a large concentration gradient across the placenta to transfer to the fetus’s blood cross the placenta to a lesser extent when gastric motility is decreased. There is also an approximate 30% expansion of the mother’s blood volume and an increase in the mother’s body fat content during pregnancy. 102 These changes alter the distribution of lipophilic and hydrophilic drugs and produce lower plasma morphine concentrations than in the nonpregnant woman. The percentage of the total body water in the fetus decreases from the beginning to the end of gestation, 103 causing changes in the distribution of hydrophilic compounds in fetal tissues throughout pregnancy. In addition, the fetus’s body fat increases during gestation. 101 Hence, throughout fetal development, lipophilic drugs exhibit a changing distribution into fetal tissue and varying concentrations in the blood.
6. Placental Structure and Function

The term human placenta is a discoidal mass, thickest at the center, with an approximately circular outline. The human placenta is hemomonochorial meaning that the maternal and fetal circulations are separated by only one cell layer. The placenta has an average volume of 500 mL (range: 200-950 mL), weight of 500 g (range: 200-800 g), a diameter of 185 mm (range: 150-200 mm), a thickness of 23 mm (range: 10-40 mm), and a surface area of approximately 30,000 mm². The total villous surface area exceeds 10 m². Macroscopically, the fetal side of the placenta is covered by a membranous sac, the amnion, which is smooth, shiny, and transparent (Figure 1-5). The maternal surface is granular and subdivided into approximately 15 to 30 cotyledons by a series of placental septa (Figure 1-6).

The placenta may be regarded as the most important fetal organ. The placenta and fetal membranes separate the fetus from the mother, yet allow the exchange of nutrients, xenobiotics, and waste products between the separate maternal and fetal circulations. The fetal membranes are composed of the chorion, amnion, yolk sac, and allantois.

The placenta connects the mother and baby through the umbilical cord; it consists of two components: first, a fetal portion developed from the chorionic sac, and secondly, a maternal portion formed by the endometrium and known as the decidua in a gravid woman. The placenta and fetal membranes perform the following functions that are essential for sustaining fetal life: protection, nutrition, respiration, excretion, and hormone production.

The functional layer of the gravid endometrium is called the decidua. Many of the decidual cells degenerate at the syncytiotrophoblast of the blastocyst and, in conjunction with maternal blood and uterine secretions, provide a rich source of nutrition for the embryo. The syncytiotrophoblast is a multinucleated protoplasmic mass with finger-like processes that extends into the endometrial epithelium and invades the endometrial stroma. The syncytiotrophoblast produces maternal tissue-eroding substances that enable the blastocyst to
implant in the endometrium. It has also been suggested that the decidual cells may protect maternal tissues from uncontrolled syncytiotrophoblast invasion and may be involved in hormone production. The decidua is divided into three main regions based on the relation of the decidual cells to the implantation site of the conceptus. First, the decidua basalis is that portion of the decidua, which lies deep to the conceptus and forms the maternal component of the placenta. Secondly, the decidua capsularis is the superficial portion of the decidua overlying the conceptus, and thirdly, the decidua parietalis defines the remaining mucosal lining of the uterus.

Fig. 1-5. Fetal Aspect of the Human Placenta
i. Placental Development

The anatomical structure necessary for physiological exchanges between mother and embryo are established by the beginning of the fourth week of gestation. The chorionic villi cover the entire surface of the chorionic sac up to approximately the eighth week of gestation. As the sac develops, the decidua capsularis villi are compressed and their blood supply is reduced. Subsequently, these villi degenerate producing a relatively avascular area known as the smooth chorion. As the villi of the decidua capsularis degenerate, those of the decidua basalis rapidly proliferate, branch, and enlarge, thereby creating the villous chorion\(^{105}\) (Figure 1-7). Figure 1-7 is a diagrammatic representation of the uterus of a pregnant woman showing a normal placenta in situ. Magnified area “A” shows the following structures, from fetal to maternal aspect; amnion, chorion, and myometrium. Magnified area “B” illustrates, from fetal to maternal aspect, the chorionic plate, decidua basalis, and myometrium.
The fetal component of the placenta is formed by the wall of the chorion (chorionic plate) and the chorionic villi that originate from it and project into the intervillous spaces that contain maternal blood. The maternal component of the placenta is formed by the decidua basalis and encompasses all of the endometrium deep to the fetal component of the placenta. As the chorionic villi invade the decidua basalis, several wedge-shaped areas of decidual tissue (placental septa) are formed. These placental septa divide the fetal part of the placenta into cotyledons; irregular convex areas, each consisting of two or more stem villi and their numerous branches (Figures 1-7, 1-8).

The maternal blood-filled intervillous spaces, derived from lacunae developed in the syncytiotrophoblast during the second week of gestation, enlarge as the chorionic villi branch. Collectively, these spaces form a large blood sinus (the intervillous space) that is bounded by the chorionic plate and the decidua basalis. The intervillous space is sectioned into compartments by placental septa. There is communication between intervillous space compartments because the placental septa do not extend to reach the chorionic plate. Maternal blood enters the intervillous space via the spiral arteries of the decidua basalis and is drained by the endometrial veins that are found over the entire surface of the decidua basalis (Figures 1-7, 1-8). Maternal blood continuously circulates throughout the intervillous spaces, sustaining embryonic and fetal development by delivering nutrients and taking away waste products of fetal metabolism.

The cytotrophoblastic shell anchors the fetal portion of the placenta (villous chorion) to the maternal portion of the placenta (decidua basalis). Some chorionic villi (anchoring villi) are firmly attached to the decidua basalis, through the cytotrophoblastic shell, thus anchoring the chorionic plate to the decidua basalis.

ii. Placental Circulation

The anatomical structure of the placental membrane greatly increases the surface area, between the fetal and maternal circulations, that is available for transplacental exchange. The
maternal and fetal circulations are separated by the placental membrane; the syncytiotrophoblast which is of fetal origin. As the pregnancy progresses, the membrane becomes thinner and larger effectively increasing the surface area.

Oxygen-poor blood flows from the fetus to the placenta through the umbilical arteries. At the cord root insertion into the placenta, the umbilical arteries divide into radially oriented vessels that branch freely in the chorionic plate before entering the villi. The extensive arterio-capillary-venous system within the villus causes fetal blood to flow very close to maternal blood (Figure 1-8). This anatomical design provides a very large surface area for the exchange of
metabolic and gaseous products between the maternal and fetal circulations. Normally, there is no gross mixing of fetal and maternal blood, however small amounts of fetal blood may enter the maternal circulation through very small gaps in the placental membrane. Oxygenated fetal blood flows into thin-walled veins that follow the placental arteries back to the cord root insertion, where they converge to form the umbilical vein. This large vessel carries oxygenated blood to the fetus.

Fig. 1-8. Placental Circulatory System

Maternal blood flows into the intervillous space through 80 to 100 spiral arteries. The volume of maternal blood circulating through the intervillous space has been estimated to be 500 mL per minute. Blood flow in the spiral arteries is pulsatile and is therefore propelled by maternal blood pressure. The entering blood is at a significantly higher pressure than that in the intervillous space; hence the maternal blood spurts toward the chorionic plate, which is often referred to as the roof of the intervillous space. As the pressure dissipates, blood flows slowly
around the surface of the villi, enabling the transfer of metabolic and gaseous products within the fetal blood to diffuse into the maternal blood. The maternal blood eventually empties into the endometrial veins.

The health of the embryo and fetus depends primarily on adequate bathing of the chorionic villi by maternal blood. Pathologic changes or acutely induced reductions in the uteroplacental circulation result in fetal hypoxia and growth retardation, or even fetal death. Chronic reductions in the uteroplacental circulation have been shown to disrupt growth and development resulting in intrauterine growth retardation.

The intervillous space of a mature placenta contains an approximate blood volume of 150 mL that is replenished 3 or 4 times per minute. Intermittent uterine contractions during pregnancy slightly decrease uteroplacental blood flow, but do not pump significant amounts of blood out of the intervillous space. As a result, oxygen transfer to the fetus is decreased during uterine contractions.

The placental membrane separates the maternal and fetal circulations. At term, the human placental membrane consists of a thin syncytiotrophoblast and a discontinuous cytotrophoblast. The placental membrane is regarded as the placental “barrier”; a misleading term because there are few compounds, endogenous or exogenous, that are unable to cross the placental membrane. The placental membrane functions as a true barrier only if the substance has a certain molecular mass, physiochemical properties, and charge (refer to section 5. ii.). Electron micrographs show that the free surface of the syncytiotrophoblast has many microvilli that increase the surface area available for nutrient, drug, or metabolic waste product transfer between the maternal and fetal circulations.

iii. Placental Function

The functions of the placenta that are essential for maintaining pregnancy and sustaining
normal embryonic and fetal development include metabolism, transfer of xenobiotics, nutrients, and waste products, and endocrine secretion. The placenta synthesizes glycogen, cholesterol, and fatty acids and serves as a source of nutrition and energy for the embryo.\textsuperscript{105}

The syncytiotrophoblast synthesizes various hormones using precursors derived from the fetus and/or the mother. Protein hormones produced by the placenta include human chorionic gonadotropin (hCG), human placental lactogen (hPL), human chorionic thyrotropin (hCT), and human chorionic corticotropin (hCACTH). The placenta also produces progestins and estrogens.
Principal Objectives
Morphine is one of the most widely used opioid analgesics in medicine. The non-medical use of morphine, directly or through the abuse of heroin and/or codeine, also facilitates exposure to this drug. Particularly disturbing are the high rates of morphine exposure during pregnancy. Although there are two means of fetal morphine exposure, maternal therapeutic use and maternal abuse, the latter potentially exposing the unborn child to much higher morphine levels, this thesis will focus on steady-state concentrations of morphine seen and bolus doses administered in clinical practice during pregnancy.

There is little information in the medical literature about adverse fetal outcome when morphine is administered therapeutically during pregnancy. However, there are many documented adverse fetal outcomes resulting from the recreational use of opioids by gravid women; a situation that is further complicated by polydrug abuse – the concomitant use of more than one illicit or medicinal xenobiotic. Severe perinatal complications that occur with greater frequency in opioid-dependent women include intrauterine growth retardation, antepartum hemorrhage, anemia, uterine irritability, myometrial excitation, premature labor, short-duration labor, extrauterine pregnancies, miscarriages, spontaneous abortions, decreased head circumference, decreased Apgar scores, meconium staining of the amniotic fluid, premature rupture the membranes, chorioamnionitis, infections, and fetal death. Many of the mechanisms responsible for these conditions remain unclear or are poorly understood. Despite its widespread use, there is little information regarding morphine pharmacokinetics and pharmacodynamics especially pertaining to the fetus and neonate. Large variations within and between patients in morphine-associated kinetics and dynamics and the difficulty of obtaining serial blood samples from neonates and children make morphine particularly difficult to study. It has been suggested that therapeutic drug monitoring of morphine may yield helpful therapeutic information in patients that may be exhibiting an atypical response(s) to morphine.
Currently, plasma is used in the laboratory determination of morphine concentration. However, there are numerous reports in the literature showing good correlation between saliva and plasma concentrations of other drugs. Because of the difficulties in obtaining (serial) blood samples from neonates, children, and some adults, especially those adults with difficult venous access attributable to poor veins or difficulty in physically accessing venipuncture sites during special procedures such as FBS, I wanted to determine whether morphine concentrations in saliva could be used in the therapeutic drug monitoring of morphine (Appendix 1).

Fetal exposure to drugs is primarily dependent upon the placenta. The key functions of the human placenta are to deliver nutrients and also medications to the fetus and to remove metabolic waste products from the fetal circulation. Impairing these processes can have a detrimental impact on fetal development and outcome. Morphine has been shown to function as a vasoconstrictor in the placental vasculature in in vitro tissue sections and in vivo animal experiments. Vasoconstriction can impair blood flow and hence alter the placental transfer of substances subject to flow-dependent diffusion.

Naloxone is a morphine antagonist that has been administered to gravid women to reverse the pharmacodynamic effects of morphine. Naloxone may elicit its antagonistic effects by vasodilating placental blood vessels, thereby returning vascular resistance and consequently blood flow to pre-morphine levels. Because morphine transfer across the human placenta is primarily blood-flow dependent, changes in vascular resistance may elicit changes in blood flow and therefore alter the rate of morphine transfer across the placenta. This potential increase in drug transfer may increase fetal exposure to morphine. The effect of naloxone on morphine transfer across the placenta and clearance by the placenta has not been determined to date.

The determinants of transplacental exchange, such as rate of transfer and clearance of morphine, and the effects of morphine on placental physiology in humans have not been previously described. The in vitro placental perfusion model was employed to study the transfer
kinetics of morphine across the term human placenta and to study the effects of morphine on placental viability, barrier integrity, and the retention-release characteristics of the tissue. The *in vitro* perfusion model, using human placentas, affords advantages over animal placentas, tissue homogenates, and subcellular preparations such as eliminating interspecies differences and maintaining structural integrity and cell-to-cell organization. Furthermore, the *in vitro* model eliminates the ethical considerations associated with conducting *in vivo* morphine pharmacokinetic-pharmacodynamic experiments in gravid women.

**Objectives (Refer to Chapter 2)**

i. To measure the transfer rate and clearance of morphine across the term human placental cotyledon perfused *in vitro* as a means of estimating fetal exposure to morphine *in vivo*.

ii. To determine if naloxone alters the transfer rate and clearance of morphine across the human placenta as a means of showing the potential antagonistic effect of naloxone on morphine-attributed placental vascular constriction.

iii. To determine if the placenta functions as a storage depot for morphine and if the placenta releases morphine into the fetal circulation in order to explain the pharmacodynamic effects of morphine seen in the newborn child that may be due to prolonged fetal morphine exposure.

Although determining the transplacental transfer rate, clearance, and placental tissue storage-release characteristics of morphine *in vitro* will provide an estimate of fetal morphine exposure *in vivo*, it is necessary to quantitate the effects of morphine, once in the fetus, on fetal
response so as to avoid misinterpretation of normal pharmacodynamic effects for a hostile uterine environment, culminating in the unnecessary, preterm delivery of the baby.

The specific effects of morphine on measures used to assess fetal responses have not been previously described. Obstetricians commonly use fetal assessment tools such as the biophysical profile and Doppler examinations to assess fetal health and circulatory status. Morphine and other opioids may be administered to gravid women prior to special procedures such as FBS or CVS to ameliorate maternal and fetal pain and anxiety. In addition, some obstetricians prefer to administer morphine with the specific intent of sedating the fetus to restrict fetal movements during the procedure. Currently, there is no evidence-based data to support this practice. Although morphine and other opioids are being used for these purposes, the effects of these agents on the fetal assessment tools have not been characterized. The results of this study will identify and quantitate the extent to which the biophysical profile and Doppler studies may be affected by morphine. This study will also clarify the role for this drug facilitating such special procedures and help avoid unnecessary hospital admissions for fetal monitoring and/or inappropriate interventions based on the misinterpretation of the BPS and Doppler scores.

Objectives (Refer to Chapter 3)

i. To determine if morphine diminishes fetal movements by sedating the fetus as a means of minimizing the risk of unexpected fetal movements during FBS that may occur and cause the sampling needle to shear the target vessel causing the fetus exsanguinate.

ii. To examine the effect of intramuscular morphine administered to the mother on measures of fetal health and circulatory status in order to
quantify the effects attributable to morphine rather than to a hostile uterine environment.

iii. To determine if there is a correlation between fetal biophase morphine concentrations and the fetal biophysical profile score and the UA Doppler S/D ratio, RI, and PI so as to clarify the dose-response effect of morphine on the fetal and the placental vascular responses.
Chapter 2 - Transfer of Morphine Across the Human Placenta and its Interaction with Naloxone
Abstract

Objective: The purpose of this investigation was to measure the transfer rate and clearance of morphine across the placenta with and without naloxone.

Methods and Materials: Term human placental cotyledons were perfused in vitro. The placenta was perfused with 50 ng/mL of morphine in the absence (n=4) and presence (n=5) of 100 ng/mL of naloxone. Maternal and fetal samples were collected. Student's t-test or one-way repeated measures ANOVA were used for all comparisons.

Results: The maternal-to-fetal morphine transfer rate was 0.73 ± 0.44 ng/mL/min in the morphine and 0.69 ± 0.26 ng/mL/min in the morphine-naloxone experiments (p = 0.89). The clearance of morphine was 0.89 ± 0.39 mL/min without naloxone and 0.87 ± 0.27 mL/min with naloxone (p = 0.92). Final morphine concentrations in the second control period of the morphine perfusion experiments were 9.78 ± 6.17 ng/mL (maternal) and 3.43 ± 2.14 ng/mL (fetal); they were 10.04 ± 3.89 ng/mL (maternal) and 4.16 ± 1.64 ng/mL (fetal) in the morphine-naloxone experiments.

Conclusions: Morphine readily crosses the term human placenta. Naloxone does not alter placental transfer or clearance of morphine, suggesting that transfer across the syncytiotrophoblast of the placenta is not altered by changes in vascular resistance. Placental retention of morphine prolongs fetal exposure to morphine.
Introduction

Prenatal exposure to morphine has been associated with diminished fetal breathing efforts, delayed fetal lung maturation, and/or with deleterious neurologic sequelae such as, for example, neonatal withdrawal symptoms and increased irritability, tremors, and tone. Nevertheless, morphine continues to be administered to pregnant women in situations of sickle cell crises, severe migraine headaches, severe pain, and to alleviate the pain during parturition.

For obstetrics, morphine may be used in special procedures, such as cordocentesis, to sedate the fetus and thereby minimize fetal movements at the time of needle insertion into the umbilicus or intrahepatic vein.

Non-medical use of opiates and opioids occurs in a large number of women during pregnancy. Many women using or abusing heroin and/or codeine will also expose the unborn child to morphine following metabolic conversion of these drugs to morphine.

Naloxone HCl (Narcan™) is a pure morphine μ receptor antagonist, which does not possess the partial agonist properties of other opioid antagonists. It prevents or reverses central nervous system (CNS) depression, respiratory depression, and hypotension produced by opioids such as morphine and by endogenous opiate-like peptides such as endorphins or enkephalins, which may play a role in controlling ventilation and circulation, by competitive binding to μ receptors. Naloxone is administered to a patient in emergency situations to reverse the pharmacologic effects of morphine; severe maternal or fetal respiratory and/or CNS depression due to medication error, deliberate opioid overdose, or opioid hypersensitivity.

Cord blood sampling in humans and animals has shown that morphine rapidly crosses the placenta. Naloxone also crosses the human placenta, appearing in the fetal blood in as little as 2 minutes after maternal administration. Morphine has been shown to dilate the peripheral vasculature causing an increase in blood flow and a decrease in vascular resistance.
Naloxone elicits its antagonistic effects by constricting the peripheral vasculature causing blood flow and vascular resistance to return to pre-morphine levels. However, in the placental vasculature, morphine causes vasoconstriction potentially reducing blood flow through the placenta and subsequently to the fetus, by increasing vascular resistance. Decreasing the blood flow to the developing child can decrease the amount of nutrients, amino acids, vitamins, electrolytes, and drugs delivered to the fetus; it can also decrease the removal of waste products such as carbon dioxide and urea from the fetus. As naloxone may be administered in the presence of morphine, the aim of this investigation was to measure and compare the placental transfer and clearance of morphine across the term human placental cotyledon, in the presence and absence of naloxone. The aim of this study was to determine if naloxone alters the rate of transfer and clearance of morphine across the human placenta. In addition to the clinical implications of such an interaction, this study aimed at elucidating whether the transfer of morphine across the placenta may be decreased due to the added effect of morphine-attributed placental vascular constriction.

Methods and Materials

Perfusion Technique

The in vitro placental perfusion method (Appendix 2) has been previously described by our laboratory. Briefly, placentas from uncomplicated term pregnancies were obtained after vaginal delivery or cesarean section and transported in ice-cold heparinized saline solution to the laboratory. Placentas were taken from mothers who did not smoke, consume alcohol, or use prescription medications (including opioids) during the course of the pregnancy. A chorionic vein and artery supplying a single, peripheral placental cotyledon were identified and cannulated. The maternal circuit was established by piercing the decidual plate and cannulating the intervillous space with two maternal arterial catheters. Flow rates of approximately 13-15
mL/min (maternal circuit) and 4-5 mL/min (fetal circuit) were established within 45 minutes of placental delivery.

The perfusate was a tissue culture medium (Appendix 3) (M199, Sigma, St. Louis) containing heparin (2000 U/L), kanamycin (100 mg/L), glucose (1.0 g/L), antipyrine (0.2 g/L) and 40,000 MW dextran (maternal 7.5 g/L, fetal 30 g/L) maintained at 37°C. The maternal perfusate was equilibrated with 95% oxygen and 5% carbon dioxide while the fetal perfusate was equilibrated with 95% nitrogen and 5% carbon dioxide. The physiological pH gradient, between the maternal and fetal circulations, was maintained by the addition of sodium bicarbonate or hydrochloric acid throughout the perfusion experiments to sustain a maternal pH of 7.40 ± 0.05 and a fetal pH of 7.35 ± 0.05.

In ‘closed’ circuit experiments, the perfusates were recirculated through the maternal and fetal circulations. The volume of the maternal and fetal reservoirs was 250 mL and 150 mL, respectively. In ‘open’ circuit experiments, the perfusates were not recirculated through the maternal and fetal circulations and the venous effluent was discarded.

Experimental Design

Parameters Measured Throughout the Perfusion Experiment

Perfusate samples were collected from the maternal and fetal reservoirs throughout the perfusion experiment to measure hCG, lactate, glucose, antipyrine, and morphine. Additional arterial samples were taken to measure pH, oxygen tension (pO₂), and carbon dioxide tension (pCO₂) with a blood gas analyzer (Radiometer ABL 330, Copenhagen).

In order to emulate the physiologic conditions necessary to reproduce morphine transfer in vitro, a maternal-to-fetal pH gradient was maintained throughout the morphine and morphine-naloxone perfusion experiments. The maternal and fetal reservoirs were replaced with fresh perfusate at the start of each control and experimental period.
Initial Control Period

The placenta was initially perfused for 2 hours in a closed system without drug (control period) in order to assess the integrity of the preparation. Measures used to assess placental integrity, defined as sustained metabolic and barrier functions, included fetal artery inflow pressure, fetal artery and vein oxygenation, maternal-to-fetal pH gradient, and fetal perfusate volume loss. In addition, hCG secretion into the maternal compared to the fetal circulation was used to assess the barrier function of the placenta. Lactate production and glucose and oxygen consumption were measured to test the metabolic viability of the placental cotyledon throughout the perfusion setup.

The fetal perfusion pressure was recorded and the fetal circuit volume loss was measured. Fetal perfusate volume losses in excess of 3 mL/hour indicated a fetal-to-maternal compartment perfusate leak and constituted a criterion for terminating the perfusion experiment.

Morphine Transfer Experiments

Following the control period, the placenta was perfused for 4 hours with 50 ng/mL of morphine sulfate (Morphine HP®, Sabex Inc., Boucherville) in the absence (n=4) or presence (n=5) of 100 ng/mL of naloxone (Narcan™, Du Pont Merck Pharma Inc., Dorval) in a closed circuit system.

Samples were obtained from maternal and fetal reservoirs every 5 minutes for 30 minutes, every 15 minutes for 30 minutes, and every 30 minutes thereafter.

Morphine Clearance Experiments

Morphine clearance was measured during a second experimental period in an open circuit. In the morphine perfusion experiments (n=4), samples were taken every 10 minutes for 60 minutes from the fetal artery and vein and the maternal artery and vein.
In the morphine-naloxone perfusion experiments (n=5), the maternal side of the placenta was perfused with morphine for one hour, after which 100 ng/mL of naloxone was added and the perfusion experiment continued for a second hour. Samples were taken every 10 minutes for 120 minutes from the fetal artery and vein and the maternal artery and vein.

Final Control Period

A final 1-hour 'closed' circuit, post-experimental control experiment was conducted to measure morphine wash-out from the placenta and to measure the sustained viability and physical integrity of the placental preparation.

Samples were taken from the maternal and fetal reservoirs. All measured parameters were compared to those of the initial control period to ensure that the placenta was viable and maintained its integrity throughout the perfusion experiment.

Sample Analysis

Perfusate samples were kept at -20°C until analysis. Morphine sulfate (Appendix 4) and hCG (Appendix 5) were analyzed using a modified commercially available radioimmunoassay (RIA) kit (Coat-A-Count, Diagnostic Products, Los Angeles and Tandem-R, Hybritech Inc., San Diego, respectively). The Coat-A-Count serum morphine RIA kit antiserum is highly specific for morphine and its principal metabolites, morphine-3-glucuronide and morphine-6-glucuronide. There is no detectable cross-reactivity with naloxone even at concentrations of 10,000 ng/mL.\textsuperscript{125} The sensitivity (detection limit) of the assay for morphine is 0.8 ng/mL. The Tandem-R hCG immunoradiometric assay is a solid phase, two-site immunoradiometric assay with a minimum detectable hCG concentration of 1.5 mIU/mL.\textsuperscript{126} Antipyrine levels were measured using a previously published assay (Appendix 6).\textsuperscript{127}

Lactate concentrations (Appendix 7) were determined by measuring the reduction of nicotinamide adenine dinucleotide (NAD) by lactate dehydrogenase (Sigma Lactate Procedure
44
No. 826-UV), causing the reversible conversion of lactate to pyruvate. In the presence of excess NAD, all lactate present in a sample will be converted to pyruvate. The lactate concentration was determined by quantifying the increase in absorbance at 340 nm using a Shimadzu UV160U-VIS Recording Spectrophotometer (Shimadzu Corporation, Tokyo) as NAD was reduced to NADH. Glucose concentrations were measured with a YSI 23A Glucose Analyzer (Yellow Springs Instrument Company, Inc., Yellow Springs) (Appendix 8).

Calculations

The rate of morphine extraction (disappearance) from the maternal circulation and the rate of morphine transfer (appearance) to the fetal circulation were calculated by linear regression analysis of the linear portion of the morphine versus time curve; rates of hCG accumulation, lactate production, and glucose consumption were calculated similarly. Morphine clearance was calculated using the equation \( \left( \frac{FV - FA}{MA - FA} \right) \times Q_r \), where FV is the fetal vein drug concentration, FA is the fetal artery drug concentration, MA is the maternal artery drug concentration, and \( Q_r \) is the fetal flow rate.\(^{128} \) In the transfer and clearance experiments, the fetal artery did not contain any morphine. Thus, the clearance equation simplified to \( \frac{FV}{MA} \times Q_r \).

The clearance index (CI) is a measure of drug clearance efficiency where the clearance of a test compound is compared against the clearance of a test molecule such as antipyrine. Antipyrine was selected as the reference molecule because of its rapid rate of diffusion, minimal protein binding, and negligible metabolism. The CI was calculated according to the equation \( \left( \frac{\text{Mean T Drug}_{CI}}{\text{Mean AP}_{CI}} \right) \times 100\% \), where \( \text{Mean T Drug}_{CI} \) is the average test drug clearance and \( \text{Mean AP}_{CI} \) is the average antipyrine clearance.
Statistics

Student's t-test was used to compare the rate of maternal drug extraction to fetal drug transfer, morphine clearance, antipyrine transfer and clearance, cotyledon mass, and final morphine concentrations in the post-experimental period. One-way repeated-measures ANOVA (or the equivalent non-parametric statistical test - Friedman repeated-measures ANOVA on ranks) was used for comparisons made within and between the morphine and morphine-naloxone treatment groups for 1. hCG production; 2. glucose consumption; 3. lactate production; and 4. oxygen delivery, transfer, and consumption. Within treatment group analyses encompassed comparisons made between the initial control period experiments, the transfer and clearance experiments, and the final control period experiments in each of the treatment groups. Differences were significant at \( p \leq 0.05 \). All results are reported as the mean ± standard deviation (SD). The SigmaStat for Windows statistical software package (Jandel Scientific, San Rafael, California) was used for all statistical calculations.

Results - Physical Parameters

The mean mass of the cotyledons were 11.2 ± 2.56 g and 12.6 ± 5.57 g in the morphine and morphine-naloxone perfusion experiments, respectively \( (p = 0.67) \). The fetal arterial perfusion pressures were 38.0 ± 5.48 mmHg and 32.2 ± 1.64 mmHg in the morphine and morphine-naloxone groups, respectively \( (p = 0.06) \). The mean fetal-to-maternal (F/M) morphine transfer ratio was 0.31 ± 0.27 and 0.27 ± 0.24 for the morphine and morphine-naloxone treatments, respectively \( (p = 0.66) \).

Placental Viability and Physical Integrity

hCG accumulation, lactate production, and glucose consumption in the morphine and morphine-naloxone perfusion experiments are provided in Table 2-1. There was no difference in the small amount of hCG detected in the fetal circuits of both the morphine-only and morphine-
naloxone perfusion transfer experiments (p > 0.05). The preferential appearance of hCG in the maternal circuit compared with the fetal circuit reflects the expected secretion characteristics of the hormone and indicates physical integrity of the placental preparation. There was no difference between the two treatment groups in terms of hCG accumulation in the maternal circuit and secretion of hCG (Table 2-1, E2 data) into the maternal and fetal circuits (p > 0.05). There was also no significant difference in lactate production, glucose consumption, (Table 2-1) and oxygen delivery, transfer, and consumption (Table 2-2) between the initial control and transfer experiments in each perfusion experiment (p > 0.05).

TABLE 2-1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morphine (n=4)</th>
<th>Morphine-Naloxone (n=5)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hCG Accumulation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>0.44 ± 0.19 mIU/mL/g/min</td>
<td>0.42 ± 0.09 mIU/mL/g/min</td>
<td>0.46</td>
</tr>
<tr>
<td>E1</td>
<td>0.32 ± 0.25 mIU/mL/g/min</td>
<td>0.30 ± 0.23 mIU/mL/g/min</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0.12 ± 0.08 mIU/mL/g/min</td>
<td>0.23 ± 0.26 mIU/mL/g/min</td>
<td></td>
</tr>
<tr>
<td><strong>hCG Concentration - E2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Vein</td>
<td>10.13 ± 9.61 mIU/mL/g</td>
<td>9.97 ± 5.16 mIU/mL/g</td>
<td>0.97</td>
</tr>
<tr>
<td>Fetal Vein</td>
<td>0.13 ± 0.09 mIU/mL/g</td>
<td>0.24 ± 0.19 mIU/mL/g</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Total Lactate Production</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>0.02 ± 0.01 mg/dL/g/min</td>
<td>0.02 ± 0.01 mg/dL/g/min</td>
<td>0.84</td>
</tr>
<tr>
<td>E1</td>
<td>0.02 ± 0.01 mg/dL/g/min</td>
<td>0.02 ± 0.01 mg/dL/g/min</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0.01 ± 0.01 mg/dL/g/min</td>
<td>0.02 ± 0.01 mg/dL/g/min</td>
<td></td>
</tr>
<tr>
<td><strong>Total Glucose Consumption</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>0.03 ± 0.01 mg/dL/g/min</td>
<td>0.03 ± 0.01 mg/dL/g/min</td>
<td>0.19</td>
</tr>
<tr>
<td>E1</td>
<td>0.02 ± 0.01 mg/dL/g/min</td>
<td>0.02 ± 0.02 mg/dL/g/min</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0.04 ± 0.01 mg/dL/g/min</td>
<td>0.04 ± 0.03 mg/dL/g/min</td>
<td></td>
</tr>
</tbody>
</table>

C1 - Initial control period; E1 - Drug transfer perfusion experiments; E2 - Drug clearance perfusion experiments (analyzed by t-Test); C2 - Final control period; * - Maternal circuit. Statistical test used: one-way repeated-measures ANOVA. Results are reported mean ± SD.
TABLE 2-II

Oxygenation of the Placental Cotyledon

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morphine (N=4)</th>
<th>Morphine-Naloxone (N=5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen Delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>0.023 ± 0.005 mL O₂/min/g</td>
<td>0.023 ± 0.013 mL O₂/min/g</td>
<td>0.99</td>
</tr>
<tr>
<td>E1</td>
<td>0.022 ± 0.005 mL O₂/min/g</td>
<td>0.021 ± 0.013 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0.022 ± 0.007 mL O₂/min/g</td>
<td>0.020 ± 0.010 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>Oxygen Transfer</td>
<td></td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td>C1</td>
<td>0.001 ± 0.001 mL O₂/min/g</td>
<td>0.001 ± 0.001 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.003 ± 0.002 mL O₂/min/g</td>
<td>0.002 ± 0.001 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0.002 ± 0.001 mL O₂/min/g</td>
<td>0.001 ± 0.001 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>Oxygen Consumption</td>
<td></td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td>C1</td>
<td>0.004 ± 0.002 mL O₂/min/g</td>
<td>0.003 ± 0.001 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.002 ± 0.001 mL O₂/min/g</td>
<td>0.003 ± 0.002 mL O₂/min/g</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>0.003 ± 0.001 mL O₂/min/g</td>
<td>0.003 ± 0.001 mL O₂/min/g</td>
<td></td>
</tr>
</tbody>
</table>

C1 – Initial control period; E1 – Drug transfer perfusion experiments; C2 – Final control period. Statistical test used: one-way repeated measures ANOVA. Results are reported mean ± SD.

Closed-Circuit Morphine Transfer Experiments

Comparing the morphine to the morphine-naloxone perfusion experiments, there were no significant differences between the two treatments groups for any of the studied parameters (Table 2-3).

TABLE 2-III

Perfusion Across the Term Human Placenta

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morphine (n=4)</th>
<th>Morphine-Naloxone (n=5)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Closed-Circuit Perfusion Experiments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine Extraction (Maternal) (ng/mL/min)</td>
<td>0.73 ± 0.44</td>
<td>0.69 ± 0.26</td>
<td>0.89</td>
</tr>
<tr>
<td>Morphine Transfer (Fetal) (ng/mL/min)</td>
<td>0.30 ± 0.19</td>
<td>0.16 ± 0.05</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Open Circuit Perfusion Experiments</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine Clearance (mL/min)</td>
<td>0.89 ± 0.40</td>
<td>0.87 ± 0.27</td>
<td>0.92</td>
</tr>
<tr>
<td>Antipyrine Clearance (mL/min)</td>
<td>1.44 ± 0.72</td>
<td>1.48 ± 0.48</td>
<td>0.93</td>
</tr>
<tr>
<td>Clearance Index</td>
<td>0.63 ± 0.08</td>
<td>0.64 ± 0.26</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Mean data reported as ± SD.
TABLE 2-IV

Maternal and Fetal Circuit pH Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Morphine (N=4)</th>
<th>Morphine-Naloxone (N=5)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Circuit (MA)</td>
<td>7.43 ± 0.01</td>
<td>7.44 ± 0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>Fetal Circuit (FA)</td>
<td>7.37 ± 0.02</td>
<td>7.39 ± 0.04</td>
<td>0.73</td>
</tr>
</tbody>
</table>

MA – maternal artery; FA – fetal artery. Mean data reported as ± SD.

The maternal and fetal circulations equilibrated within 225 minutes of the start of the perfusion experiments (Figures 2-1a and 2-1b). Maternal-to-fetal morphine transfer (extraction) occurred within 5 minutes of morphine or morphine-naloxone administration.

Fig. 2-1a.

a. Morphine Placental Transfer (Morphine Perfusion Experiments)
b. Morphine Placental Transfer (Morphine-Naloxone Perfusion Experiments)

Concentration versus time profiles in the maternal and fetal circulations obtained from 'closed' circuit drug transfer perfusion experiments in human placental cotyledons. Maternal to fetal transplacental transfer of morphine (n=4) and morphine-naloxone (n=5) commenced almost immediately after drug administration. Within 225 minutes, the two circulations reached equilibration. The rate of extraction from the maternal circuit was greater than the rate of morphine appearance in the fetal circulation. Each data point is the mean ± SD.
Open-Circuit Morphine Clearance Experiments

In open circuit experiments, the steady-state clearance of morphine and morphine in the presence of naloxone was measured. The difference in the mean drug clearance between the two treatments was not statistically significant (Table 2-3). The clearance index was not different between the two treatment groups. There was a difference between the rates of morphine extraction from the maternal circulation and transfer of the drug to the fetal circulation within each perfusion experiment in both treatment groups.

Post-Experimental Control Period

After the morphine and morphine-naloxone perfusion experiments, a final morphine concentration in the maternal and fetal circulations was measured, over 60 minutes (Figure 2-2), to determine if morphine was released from the placental tissue after maternal administration of the drug ceased. The difference between the final morphine concentrations in the two treatment groups was not statistically significant in neither the maternal (p = 0.88) nor the fetal circulations (p = 0.29). The maternal circulations in both treatment groups exhibited a significantly higher final morphine concentration compared to the final fetal circulation concentration of morphine, morphine p = 0.001 and morphine-naloxone p < 0.0001 perfusions.

Tissue Retention

In both the morphine and morphine-naloxone perfusion experiments, the rate of morphine extraction from the maternal circulation was higher compared to the rate of morphine appearance in the fetal circulation indicating retention of morphine by the placenta (Table 2-3). The retained morphine was subsequently washed-out during the course of the post-experimental 60 minute control period of the perfusion experiments (Figure 2-2). No preferential morphine release from the placenta to either the maternal or fetal circulations was evident.
Fig. 2-2. Morphine Release from the Placenta. Morphine concentration versus time measured in the maternal and fetal circulations (closed system) during the final control period when morphine was not administered into the maternal circulation. Morphine levels indicate continued release of morphine from the placenta. Morphine is not preferentially secreted into either the maternal or fetal circulation. Each data point is the mean ± SD of n=2 morphine and n=5 morphine-naloxone perfusion experiments. *Morphine perfusion experiments had a n=2 due to a technical failure of the pump that circulated the perfusate through the maternal circulation during experiments 1 and 2.

Discussion

Exposure to morphine during pregnancy may necessitate the administration of naloxone to reverse the effects of morphine-induced maternal respiratory depression, deliberate overdose, medication error, or extreme fetal sedation. Fetal sedation can be assessed by completing an ultrasound biophysical profile (BPP). The BPP is a composite score assessing fetal breathing movements, gross fetal body movements, fetal tone, amniotic fluid volume, and the non-stress test (combination of fetal heart rate accelerations and movement), parameters used to discern the extent of fetal sedation. Naloxone elicits several pharmacodynamic effects such as increasing the number, duration, and amplitude of fetal heart rate accelerations, the number of fetal body movements, the percentage of time spent breathing, and the number of active wake cycles. To
the best of our knowledge, this is the first report on morphine and morphine-naloxone transfer across the human placental cotyledon perfused *in vitro*.

The placental perfusion model\textsuperscript{123} enables the noninvasive collection of information regarding human placental transfer and clearance of drugs. The model is suited for measuring the clearance and transfer of drugs across an intact tissue, which approximates the *in vivo* situation more closely than subcellular or cell culture systems.\textsuperscript{90} Furthermore, the technique negates the need to consider confounding extraplacental metabolic and physiologic factors of maternal and fetal origin.

In order to assess the metabolic viability and physical integrity of the placenta throughout the perfusion experiments, several parameters were measured, including hCG production, lactate production, glucose consumption, and oxygen delivery, transfer, and consumption. Comparing the control period to the experimental period concentrations of each parameter allowed the assessment of the physiological condition of the placenta during the experiments, which is a direct reflection of its functional state.

hCG production is a normal, physiologic function of a viable placenta. The ability of the placenta to synthesize hCG and secrete it preferentially into the maternal circuit of the in vitro perfusion system is an indication of the viability of the placental tissue and the sustained physical integrity of the placental barrier. Our data show that hCG accumulation in the maternal circulation continued throughout the perfusion experiment and that hCG did not accumulate in the fetal circulation, thereby verifying the viability and maintained integrity of the placental maternal-fetal interface. Glucose consumption during the control and experimental periods, in both treatment groups, was not significantly different indicating that the placenta maintained its ability to consume glucose for energy metabolism for up to 10 hours of in vitro perfusion.

Lactate is produced by the placenta as a by-product of placental glucose metabolism. There was no significant difference in lactate production between the control and experimental periods in
each of the perfusion experiments, in either treatment group. There was no significant difference in oxygen delivery, transfer, and consumption between the control and experimental periods in each of the perfusion experiments, in both treatment groups. These observations indicate that oxygen tensions remained stable and the placental preparations sustained their metabolic function and barrier integrity during perfusions lasting up to 10 hours. These results also indicate that the anatomy and, therefore, the physiologic functions of the placenta were not damaged by the administration of either morphine or the combination of morphine-naloxone.

Although the experimental conditions were kept constant, the actual surface area of the placenta, utilized in the transfer/exchange of all of the aforementioned measures of metabolic viability and barrier integrity of the placenta, will undoubtedly vary from one experiment to another, depending upon such factors as the size of the cotyledon being perfused and the degree of overlap between the maternal and fetal circulations. Therefore, it is not unusual in this model to see large standard deviations associated with the parameters in Table 2-1. By comparing the pre-drug levels of hCG, lactate, glucose, and oxygen to the post-drug levels, each placenta effectively functions as its own control. It is, therefore, the trend that is of interest in the comparisons presented in Table 2-1, allowing the assessment of metabolic viability and sustained barrier integrity of the placental tissue before and after exposure to the drug of interest.

The therapeutic plasma concentration range for morphine is 10-100 ng/mL while the minimum effective concentration has been estimated to be between 15-65 ng/mL. Pharmacokinetic studies have shown that a plasma concentration of 50 ng/mL is required to produce moderate analgesia. The morphine concentrations measured in the maternal circulations were well within the range of plasma concentrations of morphine measured after the clinical administration of 10 mg of morphine IM to ameliorate moderate pain. Therefore, the morphine concentrations used in the perfusion experiments were representative of those used clinically.
Morphine glucuronidation, by UDP-glucuronyl-transferase, yields both morphine-6-glucuronide and morphine-3-glucuronide. Various studies have shown that glucuronidation by the placenta is negligible.\textsuperscript{131,132} Thus, we chose to measure total morphine levels by radioimmunoassay because we did not anticipate detecting significant levels of the glucuronide metabolites.

As illustrated in Figures 2-1a and 2-1b, equilibration between the maternal and fetal circulations occurred within 225 minutes, with morphine being detected in the fetal circulation within 5 minutes of administration. The physiochemical properties of morphine that facilitate rapid transplacental diffusion of the drug from the mother to the fetus are lipophilicity (pKa 7.9) and low molecular weight (285 Da).\textsuperscript{115,116,117,133} Although binding of morphine to plasma-proteins is dependent on the protein concentration,\textsuperscript{44} at therapeutic doses, morphine is only about 35% protein-bound in the adult.\textsuperscript{63} It has been shown that as pregnancy progresses, there is a reduction in both albumin and $\alpha_1$-acid glycoprotein concentrations,\textsuperscript{134,135} thereby facilitating a potential reduction in morphine plasma-protein binding during pregnancy. Moreover, an increase in endogenous compounds such as free fatty acids facilitates competition with drugs for binding to plasma albumin, thereby further increasing the free-fraction of drug.\textsuperscript{136} Because of the low extent of protein-binding and the decrease in protein concentrations during pregnancy, large changes in binding would be necessary to alter the free morphine concentration in plasma and transplacental kinetics. It is therefore placental blood flow and not protein binding that is the rate-limiting process for the transfer of morphine across the placenta. Since morphine exhibits low plasma-protein binding in human plasma,\textsuperscript{63} protein-free maternal and fetal perfusates were used and did not undermine the use of this model to determine the \textit{in vivo} kinetics of morphine. In addition, the use of protein-free perfusates ensured that all of the administered morphine into the maternal circuit was available to equilibrate with the fetal circulation.
During the perfusion experiments, a physiologic pH gradient of approximately 0.05 pH units was maintained between the maternal and fetal circulations because the pH in the fetal circulation is typically 0.05 units lower than that of the maternal circulation and because morphine is a weak base and transfer is sensitive to changes in the maternal-to-fetal pH gradient (Table 2-4). The transfer of morphine and hence, the results of the study may vary if tight regulation of the maternal-to-fetal pH gradient in the in vitro perfusion system is not maintained, as in clinical situations of maternal or fetal metabolic acidosis or alkalosis; physiologic conditions that can alter the placental transfer characteristics of morphine.

Morphine rapidly crosses the placenta in its unionized state. Due to the maternal-to-fetal pH gradient, morphine undergoes increased conversion to the ionized form on the fetal side that in turn leads to ion-trapping, where the ratio of ionized to unionized drug increases as the pH decreases. Thus, the transplacental transfer of morphine, by passive diffusion, is influenced by pH.

Increasing the concentration of morphine on the maternal side should not affect the obtained clearance index, unless either carrier-mediated or active-transport processes are involved. There is no evidence to date to support carrier-mediated or active transport of morphine across the human placenta.

Transfer of morphine across the term human placenta occurs by simple diffusion, which is flow dependent. Perfusate flow through the placenta is partially dependent upon vascular resistance. Therefore, vasoconstriction caused by morphine can decrease perfusate flow by increasing vascular resistance. The lack of an effect of naloxone on morphine transfer indicates that any vasodilatation attributable to naloxone on the placental vasculature is not sufficient to elicit a change in the rate of transfer of morphine from the maternal-to-fetal circuits. Thus, naloxone likely elicits its antagonistic effects on morphine by direct actions on fetal µ-receptors.
Morphine is washed-out of the placental cotyledon, which functions as a storage depot for morphine, into both the maternal and fetal circulations for at least 60 minutes after the administration of morphine into the maternal reservoir has ceased. The higher morphine concentration during the final control period in the maternal circulation is not due to preferential release but, rather, reflects the higher flow rate in the maternal circuit, which facilitates drug wash-out (Figure 2-2). As size of the perfused cotyledon will influence the amount of retained morphine available for wash-out, the large standard deviations of morphine concentration, are due to the wide range in cotyledon sizes used in the perfusion experiments (range: 8.49 g – 14.17 g (morphine experiments) and 5.10 g – 20.53 g (morphine-naloxone experiments). Determining the size of a cotyledon necessitates dissection of the placenta, which cannot be done until the perfusion experiment has been completed.

In vivo, morphine has been shown to decrease pH and base excess in the fetus. This effect is exacerbated by the placental storage of morphine and elicits an increase in ion-trapping. Consequently, prolonged fetal exposure to morphine may potentially extend the time for fetal effects to manifest long after the maternal dose of the drug has been eliminated.

The elimination half-life of naloxone is shorter (64 ± 12 minutes) than the half-life of morphine (114 ± 30 minutes) necessitating repeated dosing to elicit the desired clinical effect. It remains to be seen whether or not the placenta retains naloxone. The placental retention of naloxone may have the effect of maintaining prolonged opiate antagonism. This would have the clinical effect of counteracting any prolonged fetal exposure to morphine, due to the placental release of the drug, while negating the need for repeated maternal naloxone dosing.

In summary, our experiments showed that morphine readily crosses the term human placenta perfused in vitro. Naloxone does not alter the transfer or clearance of morphine across the placenta. Morphine and morphine-naloxone do not change the rate of hCG production, lactate production, glucose consumption, and oxygen transfer and consumption. The placenta
retains morphine, which is released for at least 60 minutes after maternal administration of the
drug ceases, effectively prolonging fetal exposure to morphine. These results indicate that the
transfer of morphine across the human placenta is not altered by changes in placental vascular
resistance caused by naloxone. We conclude that naloxone likely antagonizes the effects of
morphine by direct actions on fetal μ receptors.
Chapter 3 – Fetal Response to Morphine Administered to the Mother
Abstract

Objective: To determine the effects of morphine administered to the mother on the human fetus. The primary outcome measure was the BPS and the secondary outcome measures were the Doppler indices measured in the umbilical artery (UA); systolic/diastolic (S/D) ratio, resistance index (RI), and the pulsatility index (PI).

Patients and Methods: Singleton pregnancies, requiring fetal blood sampling (FBS), between 21 and 39 weeks of gestation, were assigned to the study or control groups by the attending obstetrician on the basis of placental location, fetal position, and pre-FBS extent of fetal movement. Study patients were given 10 mg, if < 64 kg, or 15 mg, if ≥ 64 kg, of morphine intramuscularly, to elicit fetal sedation, 30 minutes prior to commencing FBS. All aspects of the study procedure were the same for both groups except that the control group did not receive morphine. Pre-procedure maternal vital signs, fetal heart rate (FHR), BPS, and Doppler indices were completed. The same parameters were measured immediately after FBS. The BPS and Doppler examinations were recorded on videotape for analysis after the procedure. In the study group, before- and after-FBS comparisons were made to determine the effect of morphine on fetal behavior. In the control group, before- and after-FBS comparisons were made to determine if any of the outcome measures exhibited procedure-related changes. Maternal and cord blood samples were drawn simultaneously. Plasma morphine concentrations were measured in both the maternal and cord samples to determine the drug transfer ratio across the placenta. The following variables of the BPS and Doppler indices were compared in the analysis: fetal breathing movements (FBM), fetal tone (FT), fetal movements (FM), amniotic fluid (AF), the nonstress test (NST), FHR, and UA S/D ratio, RI, and PI.

Results: There were 10 patients in the study group and 6 patients in the control group. The mean dose of morphine administered to the study group was 0.16 mg/kg (0.12 – 0.19 mg/kg). The mean maternal plasma morphine concentration was 41.8 ± 14.6 ng/mL. The mean cord plasma
morphine concentration was 25.5 ± 11.6 ng/mL. The fetal-to-maternal ratio was 0.61 ± 0.20.

There were no significant differences in the maternal vital signs, FHR, UA S/D ratio, RI, and PI before- and after-FBS, in both groups. An average of a 3.2 point lower BPS was observed in the study group when the fetus was exposed to morphine (p = 0.001). The most sensitive variables of the BPS to morphine were FBM and the NST. Gross and fine fetal movements were unaffected. A significant, positive trend was measured when the biophase morphine concentration was correlated with the S/D ratio, RI, and PI.

Conclusions: Morphine transferred across the human placenta causes a significant decrease in the BPS. Absent FBM and non-reactive NSTs decreased the composite BPS upon fetal exposure to morphine, thereby necessitating careful interpretation of the BPS. There were no significant changes in the Doppler indices upon exposure to morphine. A positive correlation between the biophase morphine concentration and the UA S/D ratio, RI, and PI was calculated. These data support previous in vitro tissue and in vivo animal experiments showing that morphine acts as a vasoconstrictor of placental blood vessels but do not support the use of IM morphine to suppress fetal movement.
Morphine is an opioid analgesic that exerts its primary effect on the central nervous system (CNS) and organs containing smooth muscle. The drug acts as an agonist at specific, saturable opioid μ receptors in these regions. Pharmacological effects include analgesia, decreased gastric motility, suppression of the cough reflex, respiratory depression, CNS depression, nausea and vomiting, euphoria and dysphoria, (fetal) sedation, mental clouding, and alterations of the endocrine and autonomic nervous systems. Morphine is readily absorbed by the gastrointestinal tract and undergoes significant 'first-pass' metabolism in the liver. Glucuronic acid conjugation of the phenolic hydroxyl group by glucuronyl transferase produces two primary metabolites, morphine-3-glucuronide and morphine-6-glucuronide, that are predominantly excreted in the urine. Morphine sulfate is 35% plasma-protein bound to α₁-acid glycoprotein. It has been reported that α₁-acid glycoprotein is decreased by 30% as a pregnancy approaches term. This leads to decreased binding of basic drugs during pregnancy, effectively increasing the levels of free drug. This fact is significant for highly protein-bound drugs, but is not considered a significant factor for morphine due to its low protein binding.

In pregnancy, morphine is used to alleviate pain associated with parturition. Morphine is also sometimes used in invasive procedures, such as FBS, to alleviate maternal anxiety and procedure-related pain, to alleviate any procedure-related pain that the fetus may feel, and to sedate the fetus prior to FBS. Presently, there is no evidence-based data to support the practice of fetal sedation to restrict fetal movements at the time of needle insertion. Very little information exists on fetal effects attributable to morphine.

Our laboratory has shown, using the placental perfusion model, that morphine crosses the term human placenta. Morphine has also been shown to appear in the fetal circulation, in as little as 5 minutes, after a single, maternal IV injection of morphine prior to commencing
The objective of this study was to determine the effects of morphine on the fetus as measured by the BPS and Doppler indices.

Fetal blood sampling (FBS), also called ultrasound-guided percutaneous umbilical cord blood sampling or cordocentesis, is a technique where a needle is inserted into the pregnant uterus until entry into the lumen of the umbilical or intrahepatic vein is achieved thereby permitting access to the fetal circulation and sampling of pure fetal blood. The technique has become a widely used diagnostic procedure since its introduction in 1983. No other fetal tissue, villous tissue, or amniotic fluid can yield as much combined cytogenetic, biochemical, and hematological diagnostic information as pure fetal blood. The main indications for FBS include the diagnosis of chromosomal abnormalities by rapid karyotyping, investigation of intrauterine infections such as rubella and toxoplasmosis by serology, investigation of hematological abnormalities such as thalassaemia and sickle cell disease, management of rhesus and platelet alloimmunization, investigation of fetal blood gas and acid-base values, platelet counts, and fetal hemoglobin levels, and determining biochemical abnormalities and the twin-twin transfusion syndrome. The procedure has been performed as early as 12 weeks gestation, but is commonly utilized beyond 17 - 18 weeks gestation.

The biophysical profile score (BPS) is a method of evaluating fetal health and wellbeing. The resolution and frame rates of real time ultrasound images have vastly improved during the last decade, thereby effectively increasing the number of measurable fetal actions.

The BPS consists of a composite score using the following five variables: fetal breathing movements (FBM), sustained for 30 seconds, fetal movements (FM), 3 movements are required, fetal tone (FT), 1 episode of flexion-extension of either limbs or trunk is required, an amniotic fluid (AF) measurement of 1 pocket > 2 cm x 2 cm without cord, and a nonstress test (NST), ≥ 2 fetal heart rate (FHR) accelerations of at least 15 beats/minute, each lasting at least 15 seconds. Each variable is arbitrarily assigned a score of 2 (normal) or 0 (abnormal)
according to previously defined criteria. The BPS is reported as a composite score encompassing the aforementioned variables determined by ultrasound and the NST, for a total BPS out of 10. A reported score ≤ 6/10 indicates the need for further fetal evaluation. The BPS is evaluated over a minimum of 30 minutes because fetuses are known to exhibit up to a 40-minute sleep interval. The 30-minute observation period effectively rules out variations in results attributable to normal fetal sleep-wake cycles. These coordinated, complex parameters have been shown to be very useful in antepartum assessment of fetal risk.

These dynamic aspects of fetal behavior do not occur as random events but rather are initiated and regulated by the fetal CNS and the placenta. Hence, the presence of these variables is indirect evidence of a functioning and intact fetal CNS and placenta. In human pregnancies, for example, the fetal CNS responds to maternal hypoxemia and cigarette smoking by reducing or abolishing FBM. In comparison, animal study results show that nicotine injection or exposure to tobacco smoke resulted in hypoxemia and a corresponding decrease in FBM.

The advantages of the composite BPS include: immediate results, perturbation of the uterine environment is not required, it is independent of gestational age, and the results are specific to the fetus being examined. Many antepartum examinations rely on a single outcome variable. The BPS yields results from five fetal tests combined which effectively results in a significant reduction in the false positive rate, yet does not alter or may even reduce the false negative rate.

Umbilical artery (UA) Doppler velocity waveform analyses have been shown to be valuable in the assessment of the compromised fetus. It has been shown that abnormal UA velocity waveforms are associated with blood flow redistribution in the compromised fetus. These changes in fetal blood flow velocity waveforms have also been documented to exhibit a direct relationship with abnormal biochemical results obtained from fetal blood, collected by FBS, and unfavorable perinatal outcome. Altered fetal blood flow velocity also
facilitates flow-dependent changes in the rate of fetal drug exposure, oxygen delivery, and nutrient delivery; parameters fundamentally related to fetal health.

A Doppler signal can be detected as early as the 15th week of gestation and even earlier if color Doppler technology is used. The normal fetal UA velocity waveform has a relatively slow systolic rise, a slow diastolic fall, and a large end-diastolic velocity reflecting a low resistance circulation. At least 3 measurements of 3 to 4 waveforms are required to compensate for differences in the angle of insonation in the umbilical cord. The number used in each examination should be the average of 9 to 12 waveforms.\textsuperscript{164}

The UA has a specific waveform pattern that allows easy differentiation from that of the fetal aorta, heart, uterine artery, and umbilical vein. Dividing the systolic peak velocity by the end-diastolic velocity generates the systolic/diastolic (S/D) ratio. The use of a ratio overcomes potential errors caused by the tortuosity of the umbilical arteries, the angle of insonation, and the direction of flow. The ratio serves as a measure of fetal circulatory status. The UA S/D ratio is an inverse proportion. In normal pregnancies, the S/D ratio shows a steady decline with gestation, with a statistical break at 28 to 30 weeks when the majority of fetuses have a ratio of less than 3.0.\textsuperscript{164} A S/D ratio value of less than 3.0 can be considered as evidence that the UA circulation is normal.\textsuperscript{165} In an abnormal pregnancy, where the diastolic flow is reduced, most likely due to an increase in vascular or interstitial resistance, the S/D ratio increases inversely. As the fetus becomes more severely compromised, there is the possibility of absent or reversed end-diastolic velocity in the UA. The S/D ratio becomes less useful as it becomes infinite.

The pulsatility index (PI) score is the most valuable ratio to evaluate pathological pregnancies with low diastolic flow velocities. The PI is a more comprehensive calculation relating vascular elasticity and flow volume; the S/D ratio utilizes only two points on the waveform, whereas the PI considers the entire waveform and calculates the mean flow across an entire fetal cardiac cycle. When the resistance is very high, the diastolic velocities decrease or
become absent; the S/D ratio becomes infinite and, thus, mathematically less practical. In such a situation, the PI may offer more information than the S/D ratio as it reflects the area under the curve and continues to increase with increasing degree of absent end-diastolic flow velocity. At 30 weeks gestation, the majority of fetuses have a PI < 1.0.\textsuperscript{165} The PI of the flow velocity waveform is calculated by: (peak systolic velocity (PS) – end diastolic velocity (ED))/mean velocity.

The resistance index (RI) is a measure of fetal vascular impedance; opposition to flow in a pulsatile circulation.\textsuperscript{166} At 30 weeks gestation, the majority of fetuses have a RI < 0.7.\textsuperscript{164,167} The RI of the flow velocity waveform is calculated by: (PS velocity – ED velocity)/PS velocity.

Since the BPS and the UA Doppler indices are used as measures of fetal health and circulatory status, the studies in this chapter examined the effect of IM morphine administered to the mother on these measures and specifically, which variables may be affected.

The results of this study will identify and quantitate the extent to which the BPS and Doppler indices may be affected by morphine given prior to FBS. This will clarify the role for this drug facilitating such procedures and help avoid unnecessary hospital admissions for fetal monitoring and/or inappropriate interventions based on the misinterpretation of the BPS and Doppler scores.

Patients and Methods

The clinical research protocol was approved by the University of Toronto’s Research Ethics Review Board as well as the Research Ethics Committees at both participating hospitals; Women’s College and Mt. Sinai Hospitals in Toronto. All procedures conformed to the "Declaration of Helsinki", revised in Hong Kong, 1989, and the PMAC commentary on guidelines for the "Conduct of Clinical Investigations" of Health and Welfare Canada, July,
1989. Prior to enrollment into the study, the investigator (EAK) fully informed each patient of the nature and purpose of the study and obtained written consent (Appendix 9).

The number of patients required in each arm of the experimental protocol was calculated based on a ≥ 2 point decrease in the post-morphine BPS, which was defined a priori as a clinically significant change in the BPS. A total of 10 study and 6 control patients were enrolled to achieve a power of 0.80 and an α of 0.05. Patients enrolled into the study were healthy, pregnant women with a singleton pregnancy, > 18 weeks gestation, requiring FBS for diagnostic or therapeutic purposes (Appendix 13, Table A12-1). Patients removed from the study for reasons such as protocol violations or incomplete data sets were replaced until the required sample size was achieved. Study participants were not taking any opioid medications. Other than morphine, no other opioid medications were administered prior to or during the study.

Patients were assigned to the control or study groups by the obstetrician, based on clinical presentation. Women with anterior placentas were assigned to the control group because fetal movement was less likely to interfere with the procedure while patients with posterior placentas were assigned to the study group. The control and study protocols differed only in that study patients received an IM injection of morphine; 10 mg for patients weighing < 64 kg and 15 mg for patients weighing ≥ 64 kg.

The study was designed so as not to interfere with the clinical procedure, effectively minimizing any risks to the fetus. Each patient was admitted to hospital approximately 2 hours prior to FBS. Each patient had her vital signs taken by the unit nurse: temperature, blood pressure (BP), pulse, and respirations. The patient was then taken to a procedure room or an operating room where the FBS was to be performed. Prior to FBS (Figure 3-1), a BPS (Appendix 10), FHR, and UA Doppler S/D ratio, RI, and PI (Appendix 11) were obtained and recorded. These measures were used as pre-morphine, pre-procedure baseline scores for each patient. All ultrasound examinations were videotaped thereby producing a permanent study record for
Figure 3-1. Schematic Representation of the FBS Experimental Protocol

The experimental procedure commenced with a pre-FBS BPS and UA Doppler assessment after which morphine was administered to the mother. After allowing 20–30 minutes for the drug to distribute in the mother, transfer across the placenta, and distribute to the fetus, FBS was started. Maternal and cord blood samples were taken at almost the same time. In 1 study and 1 control patient, a fetal transfusion was performed adding another 15 minutes to the procedure time. The post-procedure BPS and UA Doppler indices were done immediately after FBS was completed.

Future analysis. Maternal vital signs, a BPS, and a Doppler score were obtained and recorded following the procedure to serve as post-morphine, post-procedure scores for each patient.

Patients in the study group received an IM injection of morphine immediately after the first BPS and Doppler examinations were completed. The obstetricians waited 20 to 30 minutes before commencing FBS to facilitate distribution of the drug into the maternal central compartment, transfer across the placenta, and distribution into the biophase in the placental vasculature and fetus. Dimenhydrinate (Gravol™), 50 mg, was co-administered with all morphine injections. Control patients did not receive morphine or dimenhydrinate.

FBS (Appendix 12) was performed by two obstetricians; one obstetrician operated the ultrasound transducer and inserted the needle (single-operator approach) while the other withdrew the blood samples. Waveforms were obtained by means of a duplex Doppler technique utilizing a curved linear array 3.5 MHz transducer connected to an Acuson XP 10 (Acuson, Mountainview, USA) or an ATL Ultramark 9 (ATL, Bothell, USA) ultrasound machine. The needles were inserted either freehand or using a needle guide based on operator preference. The
patient’s abdomen was prepared with an antiseptic solution (Hibatane™) as for any surgical procedure and gloves, gowns, and a simple sterile drape were used to prevent unnecessary contamination. Lidocaine HCl 2% without CO₂ (Xylocaine™) was used to anesthetize the needle insertion site. Needles used for sampling were 20 or 22 gauge, 6 to 9 cm in length. The entire procedure was completed under continuous ultrasound guidance. The sampling site was the umbilical vein (UV) at the placental cord root insertion; the intrahepatic vein (IHV) was sampled in one study and one control patient because fetal positioning made the cord root insertion inaccessible. Approximately 3 to 4 mL of blood were aspirated into heparinized syringes from the fetus for all clinical testing and research purposes (measurement of fetal plasma morphine concentrations). At almost the same time as the fetal samples were being drawn, 2 mL of blood was drawn from the mother for determination of the morphine concentration.

Maternal and fetal blood samples were immediately centrifuged; plasma samples were stored at -20°C until analysis, which was done using a commercially available radioimmunoassay (RIA) kit (Coat-a-Count, St. Louis).

Each maternal and fetal plasma sample was assayed for free morphine using a solid phase RIA (Appendix 4). Free morphine refers to unconjugated morphine available to bind with the corresponding receptor to elicit an effect. The usual RIA method requires only 25μL of serum per run to measure total morphine; it is sensitive and specific for morphine and has a coefficient of variation <10% for concentrations between 2.5 and 250 ng/mL. The assay was run in duplicate.

Each patient was subsequently monitored for several hours to ensure maternal and fetal wellbeing after FBS. A patient completed the study after the post-drug BPS and UA Doppler examinations.

All before- and after- comparisons of the maternal vital sign data, BPS, S/D ratios, RIs, and PIs were performed using the paired Student’s t-Test or the non-parametric equivalent
Wilcoxon Ranked Sign Test when appropriate. Correlation analyses were performed using linear regression or multiple linear regression, where applicable. All data were analyzed using the SigmaStat for Windows, version 2.0, statistical software package (SPSS Science, Chicago, IL). All results are reported as mean ± standard deviation (SD).

Results

There were no differences between the study (n = 10) and control (n = 6) groups in terms of maternal age (31.7 ± 7.23 and 29.3 ± 4.63 years, respectively) and maternal weights (74.4 ± 20.9 and 68.6 ± 13.2 kg, respectively). Study and control group FBS were performed at 35.2 ± 4.19 and 28.4 ± 6.49 weeks gestation, respectively (p = 0.02).

The mean IM morphine dose administered to the study patients was 0.16 ± 0.02 mg/kg (range: 0.12 – 0.19 mg/kg). In the study group, the mean maternal plasma morphine concentration was 41.8 ± 14.6 ng/mL (range: 23.9 – 60.4 ng/mL) and the mean cord morphine concentration was 25.5 ± 11.6 ng/mL (range: 8.1 – 47.0 ng/mL). The mean F/M plasma morphine ratio was 0.61 ± 0.20 (range: 0.33 – 0.97).

The mean interval from the time that the patient received morphine to the time that the cord blood was drawn was 47.1 ± 14.1 minutes (range: 27.0 – 68.0 minutes). The mean time difference between FBS and aspiration of maternal blood was 4.0 ± 3.2 minutes (range: 1.0 – 11.0 minutes). The mean time that had elapsed between morphine administration to the study patient and the sampling of her blood during FBS was 51.1 ± 14.0 minutes (range: 30.0 – 71.0 minutes).

There were no statistically significant differences in maternal vital signs, temperature (°C), blood pressure, heart rate, and respiratory rate (Table 3-1) between the pre- and post-procedure values in either the study or control groups (p > 0.05).
Table 3-I. Maternal Vital Signs Before- and After-FBS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-FBS</th>
<th>Study (n=10)</th>
<th>Control (n=6)</th>
<th>Post-FBS</th>
<th>Study (n=10)</th>
<th>Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Systolic BP (mmHg)</td>
<td>Diastolic BP (mmHg)</td>
<td>Heart Rate (bpm)</td>
<td>Respiratory Rate (bpm)</td>
<td>Temperature (°C)</td>
<td></td>
</tr>
<tr>
<td>Pre-FBS</td>
<td>114.90 ± 14.63</td>
<td>74.10 ± 11.70</td>
<td>88.40 ± 10.10</td>
<td>18.50 ± 1.77</td>
<td>36.90 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>Study (n=10)</td>
<td>121.40 ± 20.24</td>
<td>77.00 ± 10.95</td>
<td>86.00 ± 12.65</td>
<td>20.50 ± 1.00</td>
<td>36.76 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>118.90 ± 18.02</td>
<td>74.40 ± 12.61</td>
<td>91.60 ± 9.73</td>
<td>20.70 ± 1.41</td>
<td>36.90 ± 0.39</td>
<td></td>
</tr>
<tr>
<td>Post-FBS</td>
<td>117.67 ± 18.78</td>
<td>80.67 ± 8.16</td>
<td>87.67 ± 9.99</td>
<td>20.33 ± 1.97</td>
<td>37.17 ± 0.69</td>
<td></td>
</tr>
</tbody>
</table>

FBS – fetal blood sampling; BP – blood pressure; bpm – beats per minute; bpm – breaths per minute; Results – mean ± SD; and All comparisons p > 0.05

Table 3-II. Measures of Fetal Health and Circulatory Status

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Patients (n = 10)</th>
<th>Control Patients (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-FBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPS</td>
<td>9.60 ± 0.84</td>
<td>8.00 ± 1.79</td>
</tr>
<tr>
<td>FHR</td>
<td>146.30 ± 14.02</td>
<td>140.33 ± 8.71</td>
</tr>
<tr>
<td>S/D Ratio</td>
<td>2.32 ± 0.43</td>
<td>3.44 ± 0.70</td>
</tr>
<tr>
<td>RI</td>
<td>0.56 ± 0.07</td>
<td>0.78 ± 0.21</td>
</tr>
<tr>
<td>PI</td>
<td>0.84 ± 0.13</td>
<td>1.06 ± 0.25</td>
</tr>
<tr>
<td>Post-FBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPS</td>
<td>6.40 ± 1.84*</td>
<td>8.67 ± 1.03</td>
</tr>
<tr>
<td>FHR</td>
<td>140.20 ± 13.74</td>
<td>145.83 ± 15.14</td>
</tr>
<tr>
<td>S/D Ratio</td>
<td>2.31 ± 0.34</td>
<td>2.92 ± 0.54</td>
</tr>
<tr>
<td>RI</td>
<td>0.57 ± 0.005</td>
<td>0.66 ± 0.10</td>
</tr>
<tr>
<td>PI</td>
<td>0.86 ± 0.10</td>
<td>1.03 ± 0.22</td>
</tr>
</tbody>
</table>

FBS – fetal blood sampling; BPS – biophysical profile score; FHR = fetal heart rate; S/D Ratio = systolic/diastolic ratio; RI = resistance index; PI = pulsatility index; Results = mean ± SD, *p = 0.001; and Doppler indices: n = 8/group.

Table 3-II shows the results of the BPS in both the study and control groups before and after FBS. There were no statistically significant differences between the FHR, S/D ratio, RI, or PI comparisons. There was no difference in the BPS in the control group before- and after-FBS (p = 0.50). In the study group, there was no difference in FM and FT before- and after-FBS. There was a statistically significant 3.2 point decrease in the overall BPS after fetal exposure to morphine (p = 0.001). FBM were absent and NSTs were nonreactive after FBS indicating that
the most sensitive variables to morphine exposure were FBM and the NST. Both variables scored 0 after morphine exposure in 80% and 60% of the cases, respectively (Table 3-III).

Table 3-III. Biophysical Profile Scores

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Patients</th>
<th>Control Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-FBS BPS</td>
<td>1 2 3 4 5 6 7 8 9 10</td>
<td>1 2 3 4 5 6 7 8 9 10</td>
</tr>
<tr>
<td>FBM</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>FT</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>FM</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>AF</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>NST</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>Composite Score</td>
<td>8 10 10 10 10 8 10 10 10</td>
<td>8 10 10 10 10 8 10 10 10</td>
</tr>
</tbody>
</table>

Post-FBS BPS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Patients</th>
<th>Control Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBM</td>
<td>0 0 0 2 2 0 0 0 0 0</td>
<td>0 0 0 2 2 0 0 0 0 0</td>
</tr>
<tr>
<td>FT</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>FM</td>
<td>2 2 2 2 2 2 2 2 0 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>AF</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
<td>2 2 2 2 2 2 2 2 2 2</td>
</tr>
<tr>
<td>NST</td>
<td>0 0 0 2 0 0 - 0 0 2</td>
<td>0 0 0 2 0 0 - 0 0 2</td>
</tr>
<tr>
<td>Composite Score</td>
<td>6 6 6 10 4 6 8 4 6 8</td>
<td>6 6 6 10 4 6 8 4 6 8</td>
</tr>
</tbody>
</table>

FBS – fetal blood sampling; BPS – biophysical profile score; FBM – fetal breathing movements; FT – fetal tone; FM – fetal movement; AF – amniotic fluid; NST – nonstress test; ‘-’ = NST not completed to ACOG standards148; sample size study (n=10) and control (n=6) groups; and Results – mean ± SD.

There was good correlation between the maternal and fetal plasma concentrations of morphine (r = 0.73, p = 0.02).

Figure 3-2 presents the least square linear regression of the maternal morphine administration-to-FBS time difference versus S/D ratio; there was a significant correlation exhibiting a positive trend (r = 0.74, p = 0.04). Similar results were determined for the RI and the PI (r = 0.82, p = 0.01 and r = 0.71, p = 0.05, respectively). There was no correlation between the maternal morphine administration-to-FBS time difference and the BPS or cord plasma morphine concentration.
Figure 3-2. Correlation Analysis of Time versus UA S/D Ratio Data after Fetal Exposure to Morphine
This graph (n = 8 patients) exhibits a moderate positive and significant correlation, \( r = 0.74, p = 0.04 \), between the time that morphine was administered to the mother and the time of FBS (minutes) and the UA S/D ratio. Each data point represents a single study patient and is the average S/D ratio of 5 umbilical artery waveform measures. The correlation coefficient was calculated by linear regression analysis.

Figure 3-3 shows the results of the linear regression of cord (fetal) plasma morphine concentration versus S/D ratio. There was a moderate correlation exhibiting a negative trend \( (r = 0.55, p = 0.16) \). Similar trends were determined for the RI and the PI \( (r = 0.45, p = 0.26 \) and \( r = 0.38, p = 0.35 \), respectively). There was no significant correlation between cord plasma concentration and the BPS or FHR.
This graph (n = 8 patients) exhibits a moderate negative correlation, $r = 0.55$, although not statistically significant, $p = 0.16$, between the cord plasma morphine concentration (ng/mL) and the UA S/D ratio. Each data point represents a single study patient and is the average S/D ratio of 5 umbilical artery waveform measures. The correlation coefficient was calculated by linear regression analysis.

Discussion

This study provides the first objective evidence that morphine transfer across the human placenta significantly affects some components of the BPS. Specifically, the most sensitive variables of the BPS to the effects of morphine are the FBM and the NST. There was no change in the measured Doppler indices, UA S/D ratio, RI, and PI after exposure to morphine. Linear regression showed a significant, positive correlation between the maternal morphine administration-to-FBS time difference and the Doppler indices. To our knowledge, these data are
the first *in vivo* human results showing that morphine functions as a vasoconstrictor of the placental vasculature.

Measures of fetal health and circulatory status that are commonly used in fetal medicine include the BPS and Doppler waveform analysis. The composite score of the five BPS variables is used in the evaluation of fetal CNS function. The Doppler flow velocity waveforms are used to assess the flow dynamics and vascular impedance in the fetoplacental unit. Changes in these scores can occur as a result of placental disease and/or therapeutic or illicit drug use by the mother. It has been shown that changes in these scores correlate strongly with adverse perinatal outcome.\(^{150,162,163}\) Abnormal results usually signify a hostile intrauterine environment and may precipitate intervention to deliver the fetus. It is therefore important to clearly define the expected effects of morphine on these variables such that post-procedure monitoring can be adjusted accordingly.

The decision to use morphine to sedate the fetus prior to FBS is a practice that depends on the obstetrician and institution, with no established evidence-based guidelines. Restricting fetal movements during FBS may be desirable in order to facilitate insertion of the needle into the target site and to minimize the chance of dislocation of the needle and/or shearing the vessel caused by unexpected fetal movement(s).\(^{117}\)

A dose of 10 to 15 mg of morphine, based on clinical practice, was administered intramuscularly to the study patients in order to facilitate fetal sedation. Because morphine was administered to the mother, who in effect served as an additional distribution compartment, two doses of morphine were used; maternal weight governed the dose of morphine given to the patient so as to achieve a morphine blood concentration that would effect fetal sedation.

Dimenhydrinate is an H\(_1\)-receptor antagonist that causes sedation and transfers across the placenta. It is unknown whether dimenhydrinate can produce adequate fetal sedation so as to restrict fetal movements for the duration of FBS. Because morphine causes nausea and vomiting,
each patient in the study group received morphine and dimenhydrinate as per standard clinical protocol. Since FM and FT were unaffected by this drug combination, it is unlikely that the sedation effects attributable to dimenhydrinate alone would restrict fetal movements enough to allow FBS.

The intramuscular dosing regimen used in this study yielded an average weight-adjusted morphine dose of 0.16 mg/kg. Higher doses of IV morphine, up to 0.20 mg/kg, have been used for fetal sedation\(^\text{117}\) and have not been reported to cause adverse maternal or fetal outcomes such as decreased rousability due to deep sedation or markedly decreasing the respiratory rate. The control group differed from the study group only in that the patients did not receive any IM morphine before FBS. An anterior placenta facilitates blood sampling from the UV because of easier access to the placental cord root. Since the sampling needle does not pass close to the fetus, fetal sedation to reduce movements of the fetus is less of an issue. The control group was used to rule out any possible effects of the invasive procedure per se on any of the measured parameters.

Although morphine was administered to these patients to determine its effect on the fetus as measured by the BPS and the Doppler indices, there is evidence to suggest that FBS using the IHV causes pain in the fetus. Elevated levels of cortisol and \(\beta\)-endorphins were measured in fetuses undergoing FBS via the IHV or when performing multiple cord root punctures.\(^\text{11,12}\) The authors concluded that the fetus mounts an hormonal stress response to invasive procedures, implying that the human fetus feels pain in utero. This may be another possible justification for morphine as a pretreatment for FBS.

There was no difference evident in the before- and after-FBS FM and FT variables of the BPS, indicating that fetal movements recovered by the time the post-procedure assessment was obtained. This may be attributed to two factors; first, the dose of morphine used and secondly,
the IM route of administration, which further attenuates the desired pharmacodynamic effect in
the fetus due to the distribution of the drug in the mother.

Despite the dose of morphine administered to the study patients, the BPS was
significantly decreased by an average of 3.2 points after morphine administration. Specifically,
the most sensitive variables of the BPS were the FBM and the NST. The NST is a function of
both FM and FHR accelerations. Although the morphine dose was insufficient to show a
decrease in FM, control of FHR accelerations are much more sensitive to drug exposure.
Therefore, it is conceivable that the NST changed from reactive (normal) to non-reactive
(abnormal) in fetuses exposed to morphine, until the drug was sufficiently metabolized and
eliminated, usually within 2 hours after the procedure was completed. After this time period
elapsed, the NST became reactive again.

We surmised a priori that the standard clinical dose of morphine administered to the
study participants was going to be too low to elicit an effect on vascular impedance. The results
indicate that there were no significant differences before and after morphine administration in the
UA S/D ratio, RI, and PI, thereby supporting our supposition.

There was a negative trend, although statistically not significant, in the correlation
between the fetal plasma morphine concentration and the S/D ratio, RI, and PI (Figure 3-3); as
the plasma morphine concentration was increased, each of the Doppler indices decreased.
However, when the maternal morphine administration-to-FBS time difference was correlated to
each of the Doppler indices (Figure 3-2) a statistically significant, positive trend was observed;
as the time from maternal morphine administration-to-FBS time difference increased, the S/D
ratio, RI, and PI also increased. If one postulates that time is related to morphine distribution into
the biophase and that it is the morphine concentration in the biophase that elicits a measurable
pharmacodynamic effect; then, as the length of time for morphine distribution increases, the
morphine concentration in the biophase also increases causing constriction of the placental vasculature and a corresponding increase in the Doppler indices.

It has been shown in both in vivo animal and in vitro tissue experiments that morphine acts as a vasoconstrictor of the placental and umbilical vasculature.¹²¹,¹²² As the morphine concentration increases in the placental biophase, there is a corresponding increase in placental vascular constriction. The resultant increased vascular impedance causes a decrease in the diastolic flow velocity. Because diastolic flow velocity is in the denominator of the Doppler equations, then as the diastolic flow velocity decreases with increasing morphine biophase levels, the S/D ratio, RI, and PI will increase. These are the first in vivo human data that support the animal and in vitro tissue experiments suggesting that morphine is a vasoconstrictor of the umbilical vasculature.

The results shown in Figure 3-3 can be explained using the aforementioned reasoning. When plasma morphine levels are high, the biophase levels of morphine will be low. Hence, less vasoconstriction occurs and there is correspondingly less vascular impedance. It follows then that the diastolic flow velocity is higher when vascular impedance is low and that the velocimetry scores will therefore be smaller. In other words, when the plasma morphine concentration is high, the biophase morphine concentration is low and, correspondingly, vascular impedance is low. This will have the effect of facilitating higher diastolic flow velocities and therefore, the numerical value of the velocimetry measures will be smaller eliciting a negative trend as seen in Figure 3-1.

The trend seen in Figure 3-3 is not statistically significant. This does not mean that these results are not clinically significant. The statistical insignificance may solely be a function of the sample size; the reader is reminded that sample size was calculated based on a ≥ 2 point change in the BPS. The data, however, are clinically significant because they show a trend moderately correlating plasma levels and, therefore, biophase levels of morphine to the S/D ratio, RI, and PI.
A clinical issue that arose as a result of this study involves the currently used morphine dosing protocol for these procedures. As in non-pregnant adult patients, a standard dose of 10 mg IM morphine may be administered to a patient prior to FBS. However, because the drug is administered to the mother, effectively adding an additional pharmacokinetic compartment, the clinician must factor in the weight of the mother in order to truly achieve a 'standard' dose. Consequently, we propose that morphine should be dosed on a mg/kg basis so as to achieve a truly standard dose for these procedures. The results indicate that morphine administered, as a 10 mg or 15 mg single dose, yielded a maternal plasma morphine concentration range of 0.12 to 0.19 mg/kg. It is not surprising therefore that the effect of morphine on both the mother and fetus was variable in these patients. Moreover, a considerable inter-individual variability in pharmacodynamic effect would still be expected, even if a truly standardized mg/kg dosing schedule was used. This is partially due to the wide inter-individual variability in the metabolic conversion of morphine to its active metabolite, morphine-6-glucuronide.\(^7\)

Although the average gestational age in the study and control groups was 35 weeks and 28 weeks, respectively, the gestational maturation difference between the two groups is not a confounding factor because each patient acted as her own control in the before- and after-treatment comparisons. The treatment in this clinical trial was the administration of morphine and FBS (study group) or the FBS procedure alone (control group).

In designing this clinical trial, three unavoidable limitations of the study design were encountered. First, the study subjects could not be randomized. Rather, obstetricians at the participating high-risk maternal fetal units maintained that patients would have to be assigned to the control or study group based on placental location, fetal position, and the extent of pre-procedure fetal movement. For example, despite an anterior placenta, if the fetus was positioned over the cord root insertion, an IHV sampling site was chosen necessitating the administration of morphine.
Secondly, blinding was not possible, since it would not be ethical for a placebo IM injection to be given to the mothers. Both design limitations are thought not to affect the interpretability of the results of the BPS and the Doppler measurements. That is, the BPS variables were either present or absent, scored 2 or 0, respectively. The S/D ratio, RI, and PI were obtained directly from the umbilical artery and automatically calculated from measurements made by the ultrasound scanner software programs. Both outcome measures in this study therefore did not rely on an equivocal interpretation of any recorded data to derive the final assessment scores.

Thirdly, in assessing the effect of morphine on restricting FM, the BPS is not administered specifically to detect a certain number of FM in the 30-minute assessment period. That is, the BPS simply evaluates the presence or absence of FM based on defined criteria; observing at least 3 FM over 30 minutes. However, to detect the actual effect of morphine on the number of fetal movements, the obstetrician should count all the FM during the 30 minute evaluation, before- and after-FBS. In this way, the effect of morphine on decreasing FM could be quantitated.

To conclude, morphine, even at relatively low doses\textsuperscript{117}, causes a significant decrease in the BPS. Specifically, the BPS decreases as a result of absent FBM and a non-reactive NST. There was no demonstrable effect on FM or FT; thus we conclude that the IM morphine doses administered to the mother in this study do not adequately immobilize the fetus. There was no significant correlation seen between fetal plasma concentrations of morphine and the BPS, FHR, S/D ratio, RI, and the PI. There was, however, a significant, positive trend between the maternal morphine administration-to-FBS time difference and the UA S/D ratio, RI, and PI. This correlation suggests that as time elapses, plasma morphine continues to distribute into the placental vasculature biophase causing an increase in vascular impedance. This has the effect of increasing the Doppler indices by decreasing the diastolic flow velocity. These results support
the limited *in vitro* tissue and *in vivo* animal data indicating that morphine is a vasoconstrictor of the placental vasculature.
Overall Summary and Conclusions
The placenta has a pivotal role in the development of the fetus. This fetal organ functions as an interface between the mother and child through which nutrients and drugs transfer to the fetus and metabolic waste products from the fetus pass into the maternal circulation for elimination. With the advent of maternal-fetal medicine, it has become clinical practice to use the mother as a vehicle for delivery of drugs, such as digoxin and morphine, directly to the fetus. The unborn child can be exposed to opioids throughout pregnancy both through maternal use and/or abuse of these drugs.

Although morphine has been administered to the mother specifically with the intent to elicit a response in the fetus, the drug has also been shown to function as a vasoconstrictor of placental blood vessels. This effect may affect fetal development by decreasing blood flow through the placenta and hence restricting blood flow to the fetus. The results of decreasing blood flow to the unborn child may include diminished growth (intrauterine growth retardation (IUGR)) due to inadequate nutrient supply and oxygenation and/or fetal demise due to inadequate removal of metabolic waste products.

To date, the effects of morphine on placental blood vessels in an intact placenta have not been determined. Moreover, albeit that morphine is administered therapeutically to pregnant women, an explanation for flaccid babies with diminished respirations born up to 12 hours after morphine administration to the mother was stopped, was unknown. A plausible explanation for these clinical observations was that the placenta stored morphine and subsequently released the drug into the fetal circulation, effectively prolonging fetal exposure and thus the pharmacodynamic effects attributable to morphine. The purpose of this thesis in part was to determine the rate of morphine transfer across, clearance by, and the storage potential of the term human placenta.

The in vitro placental perfusion model was used to effectively show that morphine transfers across the term human placenta, exhibiting a clearance index of $63 \pm 8\%$. That is, when
morphine transplacental transfer was compared against the transfer of a flow-dependent marker of diffusion (antipyrine), approximately 37% of the administered dose was not transferred across the placenta; an indication of morphine retention by the placenta. The placenta was shown to release morphine into the fetal circulation for at least one hour after administration of the drug into the maternal reservoir was stopped. To determine if this result was due to the adsorption of morphine to the perfusion system tubing, we conducted a morphine-binding study, which confirmed the results of other studies\textsuperscript{169} that morphine binding to the perfusion system tubing was negligible. The \textit{in vitro} study data, therefore, indicate that a fetus continues to be exposed to morphine, and consequently its pharmacodynamic effects (antinociception, sedation, and respiratory depression), after administration of morphine to the mother has been stopped.

There were no statistically significant differences in the perfusion pressures between the control and experimental periods. This result indicates that the steady-state morphine concentration used in the perfusion experiments did not cause a detectable effect on placental vascular constriction. However, the perfusion model employs a simple sphygmomanometer to measure perfusion pressure. This device may not be sensitive enough to measure a small change in perfusion pressure, if indeed such a morphine-attributable change occurred. The physiological consequence of such a small change in perfusion pressure is unknown and requires further study.

The morphine-naloxone perfusion experiments were designed to determine if the co-administration of naloxone would alter the transplacental transfer of morphine. Because naloxone reverses the peripheral vascular dilatation attributed to morphine, these experiments were done to determine if naloxone would reverse placental blood vessel vasoconstriction attributable to morphine. There were no differences measured in the rate of morphine transfer, the clearance of morphine, or any of the parameters measured throughout the perfusion experiments when the morphine-only and morphine-naloxone treatment groups were compared. These data indicate that naloxone did not have a detectable direct effect on altering vascular
resistance in the isolated placental cotyledon. Because of the simplistic method of measuring perfusion pressures as discussed in the aforementioned text, a small change in the perfusion pressure may not have been detected.

The *in vitro* placental perfusion studies have also shown that morphine readily transfers across the human placenta, facilitating rapid fetal exposure to morphine. That is, the data show that morphine appears in the fetal circulation within five minutes of its introduction into the maternal circulation. Therefore, fetal exposure to morphine is not delayed at the placenta, allowing obstetricians to quickly administer morphine to the fetus, through the mother, without having to wait for the drug to reach the target organ - the fetus - in emergency situations, when time is limited and pain control is necessary.

The medical literature contains little information regarding the effects of morphine on fetal response to morphine when administered therapeutically to the mother. This lack of information in turn creates an important need to determine how morphine-attributed fetal responses affect routinely used measures of fetal health and circulatory status in obstetrical medicine. Misinterpreting the results of these measures for fetal distress may lead to the unnecessary delivery of a premature child.

In order to ascertain the status of the unborn child, obstetricians rely on several measures of fetal health and circulatory status. First, the ultrasound-assisted BPS is used to assess fetal CNS function using five variables that are added together to provide a composite score. Secondly, the Doppler velocimetry examination of the fetus is used to assess the state of the fetal circulation using 3 calculated indices: the S/D ratio, the RI, and the PI. These measures are sensitive and specific for the fetus being examined. Therefore, when a drug is administered to a mother to effect a response in her baby, it is important to understand how and to what extent these fetal assessment measures may be affected. The purpose of the *in vivo* studies was to
determine if fetal exposure to morphine would affect the BPS and the Doppler indices; direct measures of fetal response to morphine administered to the mother.

The BPS and the Doppler indices are a measure of the pharmacodynamic effects of morphine on the fetus. Indeed, the composite BPS was decreased after exposure to morphine. This indicates that obstetricians must take into account that the drug, administered to the mother to produce fetal sedation and to ameliorate fetal pain, will also alter the results of the assessment tools used to assess the fetal response. The variables of the BPS that decreased in response to morphine (FBM and the NST) returned to pre-morphine values within 2 hours after FBS. Both FM and FT of the BPS were not affected by morphine, indicating that a dose of 10 to 15 mg of IM morphine is not enough to adequately restrict fetal movements for FBS. Therefore, a larger dose of morphine, must be administered to a patient prior to FBS, to produce adequate restriction of fetal movements for FBS.

The Doppler is a sensitive tool that is used to measure changes in blood flow velocity. Since blood flow velocity changes are directly proportional to changes in vascular impedance, a drug that increases vascular impedance would also facilitate a decrease in blood flow velocity. There was a positive correlation seen when the fetal biophase concentration of morphine was correlated with each of the Doppler indices. These data indicate that as morphine levels increase in the fetal biophase, vascular impedance also increases thereby decreasing blood flow to the fetus; a deleterious event that may lead to compromised fetal development such as, for example, IUGR. The impact of temporarily constricting placental blood vessels due to a single IM injection of morphine to the mother is unknown, although most probably will be unremarkable. The clinical importance of these results pertains to pregnant women consuming morphine for longer periods or abusing, for example, heroin which is metabolized to morphine in vivo leading to fetal exposure to morphine. Restricting blood flow through the placental blood vessels has been shown to cause IUGR. Obstetricians can now combine a history of maternal chronic
morphine use or maternal drug abuse with an elevated S/D ratio, RI, and PI and thus effectively diagnose and manage a compromised fetus due to placental blood flow restriction.

The *in vitro* studies did not show a morphine effect on vascular resistance. This may be due to the method used to measure perfusion pressures. Because the Doppler is a more sensitive measure of vascular impedance, the *in vivo* studies did show a concentration-dependent effect in the biophase on vascular impedance. A temporary increase in vascular impedance affecting blood flow velocity waveforms will probably produce unremarkable effects on the unborn child. However, in situations of chronic opioid use or narcotic abuse, consistently increased-for-gestation Doppler indices may be an indication of a deleterious fetal outcome.

In both the *in vitro* and *in vivo* studies, a F/M ratio was calculated for morphine transfer across the placenta. The F/M drug transfer ratio is an indication of the extent to which a drug passes from the maternal compartment to the fetal compartment. As indicated in the comparison graph (Appendix 13) between the *in vitro* and *in vivo* F/M ratios for morphine, the time-related *in vitro* ratios are lower than the *in vivo* ratios. This implies that the perfusion model cannot predict the extent to which morphine transfers across the placenta at a specific time interval from maternal administration of the drug.

There are three differences between the *in vitro* and *in vivo* studies that can affect F/M ratios. First, the placental perfusion model utilizes only one cotyledon with an approximate surface area of 5,038 cm² across which a drug transfers to the fetus. However, in the *in vivo* situation, a drug diffuses across the entire placenta: an approximate surface area of 215,000 cm². Thus, *in vivo* a 43-fold greater surface area is available for drug transfer. Consequently, at a given time point, the F/M ratio is thought to be greater *in vivo* compared to that of the *in vitro* model.

Secondly, in the *in vitro* perfusion model, the volume of blood flowing through one cotyledon is much less than the total blood volume perfusing the entire placenta *in vivo*. Because
morphine transfer is flow dependent, the substantially smaller total blood volume flowing through a single cotyledon in the *in vitro* perfusion model compared with the total blood volume that flows in apposition to the complete surface area of the placental membrane available for morphine transfer produces a lower F/M ratio.

Thirdly, the difference between the F/M ratios of the *in vitro* and *in vivo* studies is due to the difference in the fetal concentrations of morphine. That is, the F/M ratio is greater *in vivo* because the volume of the fetal reservoir (150 mL), *in vitro*, is proportionally much greater than the volume of distribution in the fetus (200 – 250 mL). If the average weight of a whole placenta is approximately 500 g and the average weight of a placental cotyledon is 12 g, then *in vivo*, 500 g of placental tissue transfers morphine into an average 225 mL fetal volume (2.22 g placental tissue/mL fetal volume). However, *in vitro*, 12 g of placental tissue transfers morphine into a 150 mL fetal reservoir volume (0.08 g placental tissue/mL fetal volume). Thus, the amount of morphine transferred to the fetal circulation *in vitro* is diluted in a proportionally greater volume thereby yielding lower F/M ratios at a given sampling time. The maternal volumes are not relevant because the morphine concentrations are approximately the same *in vivo* and *in vitro*.

Although several key questions have been answered by this work, there remain many unanswered questions regarding the use of opioids during pregnancy. Research is needed to explain the wide inter- and intraindividual variability associated with morphine use especially during pregnancy because of the potential impact that variable pharmacokinetics may have on the unborn child. Furthermore, as our knowledge increases regarding the pharmacological activity of opioid metabolites and if these metabolites were to be administered in place of the parent compounds, investigations into the transplacental transfer, clearance, and storage of opioid metabolites will be of clinical importance. Although morphine has been shown to cause vasoconstriction of placental blood vessels, the mechanism remains to be determined.
Newer opioid analgesics such as fentanyl, alfentanil and remifentanil are increasingly being administered to pregnant women to ameliorate labor pains because of their rapid onset of action, albeit shorter half-life. Future research endeavors should include studies on the transplacental pharmacokinetics of such drugs as alfentanil and remifentanil. In addition, the effects of these opioids and of fentanyl and sufentanil on the BPS and the Doppler indices have not been determined to date.

Future studies assessing Doppler flow velocity waveforms should include the scanning of both the UA and the middle cerebral artery (MCA) and then calculating the cerebroplacental ratio (for example, cerebral RI/umbilical RI ratio). The ratio identifies fetal central blood flow redistribution, to optimize perfusion to the brain, heart, and adrenals, attributable to abnormal placental blood flow to the fetus.

Although the focus of this dissertation was on the effects of therapeutic morphine doses and plasma concentrations seen in clinical medicine, future studies are needed utilizing bolus doses of morphine consistent with the much higher doses used by pregnant addicts. These much higher concentrations of morphine should be investigated to determine the effects on the placenta and on the BPS and Doppler flow velocity waveform analyses.

In summary, the purpose of this doctoral thesis was to contribute to the overall understanding of the transplacental pharmacokinetics of morphine and to elucidate if fetal exposure to morphine would alter the two measures of fetal health and circulatory status. This research has shown that not only does morphine transfer freely across the term human placenta, but that the drug is released from the placenta prolonging fetal exposure after maternal administration ceases. Furthermore, this work has shown that obstetricians must take into account the pharmacodynamic effects of morphine on the fetus when interpreting the results of the BPS and the Doppler indices. This will have the effect of avoiding unnecessary hospital
admissions and/or administering inappropriate interventions based on the misinterpretation of the commonly used measures of fetal health and wellbeing.
Appendices
Correlation of Morphine Sulfate in Blood Plasma and Saliva in Pediatric Patients
Objective: This study sought to determine whether saliva concentrations of morphine correlate with plasma levels of morphine in pediatric patients receiving morphine analgesia for severe pain, and to evaluate whether the measurement of morphine concentrations in saliva could be a useful, noninvasive, clinical tool to diagnose systemic exposure to morphine.

Patients and Methods: Fifteen pediatric patients were enrolled; for the control group, 18 adult volunteers were recruited. Patients received continuous morphine drips to ameliorate pain caused by a sickle cell vaso-occlusive crisis (range: 10 - 40 μg/kg/hr). Control subjects were randomized into those receiving acetaminophen with either 8 mg (n = 13) or 30 mg (n = 5) of codeine. All participants were fasting at least 4 hours before sample collection. Blood and saliva samples were collected simultaneously. All samples were analyzed by radioimmunoassay for morphine.

Results: There was no correlation between saliva and plasma morphine concentrations in either the patients receiving intravenous morphine (r = 0.04, p = 0.89) or in the controls receiving codeine (r = 0.43, p = 0.08). There was no observed difference in the mean counts per minute (CPM) for saliva samples in the pH range 3.96 to 8.06.

Conclusions: Saliva concentrations of morphine cannot be used to predict the plasma concentration of morphine in children or adults. However, the concentration of morphine in saliva may be used as a qualitative indicator of systemic exposure to morphine in a subject.
Introduction

The therapeutic and toxic effects of certain drugs correlate better with their plasma concentrations than with dosage of the drug. For certain drugs, measurements of concentrations are required clinically to enable titration of the drug to specific therapeutic levels and to avoid toxicity. This is particularly useful for drugs with a narrow therapeutic index during the course of long-term therapy. Traditionally, drug monitoring was conducted using blood samples, but numerous studies in children and adults have shown that saliva can replace blood for monitoring therapy with theophylline, anticonvulsants (phenytoin, carbamazepine, and phenobarbital), digoxin, salicylic acid, acetaminophen, and other drugs.

Drug passage into saliva follows the general principles of drug movement across biological membranes and is influenced by the physicochemical characteristics of both the drug molecule (molecular size, degree of ionization, lipid solubility) and the membrane (lipid bilayer). Saliva is a low-protein secretion and, accordingly, only the unbound fraction of the drug in plasma, considered the pharmacologically active fraction, diffuses into saliva.

Distinct advantages exist with the use of saliva rather than blood for measuring the concentration of many drugs. In pediatric and neonatal patients, saliva sampling is convenient, less invasive, painless; spares blood; avoids problems of catheter access; encourages patient cooperation; and decreases the anxiety associated with venipuncture. Minimal interference in the child's medical management, convalescence, or daily routines may permit single or multiple saliva samples to be obtained in hospital for 24-hour drug monitoring. Because saliva collection does not require specialized training, it may be done in the home by the family after a short training session. This aids in the evaluation of drug compliance in outpatients. A recently identified advantage of saliva sampling compared with blood drawing is the reduced risk of contracting blood-borne infections through needle stick injuries. Saliva may be a
useful medium for determining drug levels because, in that saliva is a blood ultrafiltrate, the unbound drug in plasma is in equilibrium with saliva.\textsuperscript{16}

The required volume of a saliva sample may be obtained through stimulating salivation by chewing paraffin wax, polystyrol tube with cotton wool, elastic bands, or sour gum\textsuperscript{12,14,17} or applying citric acid on the tongue in infants and younger or uncooperative patients.\textsuperscript{3,18,19} Stimulated saliva has several advantages compared with resting saliva: the required sample volume is more easily obtained, the pH gradient between plasma and saliva is smaller, the variability in the saliva to plasma (S:P) ratios of some drugs is narrowed, and fewer specimens are too viscous or discolored to permit drug analysis.\textsuperscript{10,11,20} The rate of salivary flow may have variable effects on the saliva concentration of different drugs: rapid flow may or may not narrow the variability of the S:P ratios.\textsuperscript{10}

Morphine is an opioid analgesic that exerts its primary effect on the central nervous system and organs containing smooth muscle. Morphine acts as an agonist at specific, saturable opioid \( \mu \) receptors in these regions. Common uses include acute and chronic relief of severe pain as in cases of vasoocclusive crisis in patients with sickle cell disease and cancer-related pain. In addition, it is used in patients requiring post-operative analgesia. Morphine is also used in pediatric patients with terminal illnesses such as malignancies and human immunodeficiency virus infections, and to relieve respiratory distress associated with end-stage cardiopulmonary disease.

Because saliva sampling is more convenient, simple, and comfortable than blood sampling, we wished to verify the usefulness of using this body fluid to study morphine pharmacokinetics and toxicokinetics. This study sought to determine whether saliva concentrations of morphine correlate with plasma morphine concentrations in pediatric patients receiving morphine analgesia for severe pain and in adult volunteers receiving codeine.
Patients and Methods

The study conformed to the guidelines for use of human subjects in research and received approval from the Hospital for Sick Children's Research Ethics Board. Before enrolling in the study, each patient (or his or her legal guardian) and volunteer provided written informed consent.

Fifteen hospitalized patients with sickle cell disease who were experiencing severe pain attributable to vasoocclusive crisis that was being controlled by intravenous morphine sulfate (Morphine HP®, Sabex Inc., Boucherville, Canada) were enrolled in the study. Only patients receiving continuous intravenous morphine infusions participated in the study. Eligible patients were at or near steady state morphine concentrations before commencing each study. Patients given orally administered medicines such as uncoated or chewable tablets had their mouths thoroughly rinsed with plain water before the saliva specimens were obtained to reduce the risk of specimen contamination.21 No opioids other than morphine were administered during the course of the study.

For the control group, healthy, adult volunteers without salivary gland pathology who were not taking any analgesic medications were randomly assigned to receive acetaminophen with codeine (either Tylenol #1 [8 mg codeine/tablet, n=10] or Tylenol #3 [30 mg codeine/tablet, n=5], McNeil Consumer Products Company, Guelph, Canada). Each patient and volunteer was instructed to fast, except for water, for at least 4 hours before predrug baseline blood and saliva samples were obtained. The Tylenol 1 group then received two tablets every 4 hours times three dosing intervals. The Tylenol 3 group received two tablets every 4 hours times two dosing intervals. Patients and volunteers fasted, except for water, for at least 4 hours before postdrug blood and saliva samples were obtained.

Citric acid crystals, 5 to 10 mg, were placed on the tongue to stimulate salivation. A mucous extractor or a syringe was used to extract 1 to 2 mL of saliva from the younger children's
oral cavities. Older children expectorated saliva directly into labeled specimen containers. Saliva samples were frozen at temperatures of -20°C or lower until analysis. Simultaneous blood and saliva samples were obtained from the patient and control groups. In all control volunteers, blood (3 mL) was obtained for pharmacokinetic assessment. In the patient group, study bloods were drawn at the same time as specimens for routine blood work to avoid additional needle sticks. Lithium heparin vacutainers were used for blood sample collection. Polypropylene tubes were used for storage of plasma. Blood was withdrawn into the vacutainer. The sample was immediately centrifuged and the plasma harvested and transferred into a labeled polypropylene tube. Plasma samples were frozen at a temperature of -20°C or lower until the time of analysis.

Each plasma and saliva sample was centrifuged to settle any debris and assayed for morphine using a solid phase serum morphine radioimmunoassay (RIA) (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, USA). Plasma samples (25 μL) were analyzed in duplicate. Data provided by the Coat-A-Count® RIA kit manufacturer indicated a 0.06% cross-reactivity with codeine. The RIA is highly specific for morphine (limit of detection 0.8 ng/mL morphine).

To test the analytical effect of saliva pH on the RIA antibody reaction and the resultant morphine concentration, six 0.5 mL saliva samples were spiked with the same concentration of morphine sulfate, and the pH was adjusted with concentrated HCl to pH 1.33, 3.96, 5.41, 6.21, 6.88, and 8.06 at 25°C. The pH levels were read in a Beckman pH 34 meter (Beckman Instruments Inc., Fullerton, USA). The samples were analyzed for morphine by a Coat-A-Count RIA kit. The within-run coefficient of variation was 2.2%.

For statistics, the correlation between plasma and saliva morphine concentrations for each patient and control was calculated using a regression analysis. The counts in pH-adjusted saliva
were compared by a paired Student’s t-test. All statistical analyses were conducted using SigmaStat for Windows, version 1.0 (Jandel Scientific Software, San Rafael, USA).

Results

The mean age of the patients (± SD) enrolled in the study was 9.3 ± 5.4 years (range: 0.78 - 17 years). The mean age of the adult control population was 28.8 ± 5.4 years (range: 21 - 41 years). Table A1-1 shows the distribution of the number of male and female subjects enrolled in the study.

Table A1-1. Saliva Study Demographic Data

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
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<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Volunteers</td>
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<td></td>
<td></td>
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<tr>
<td>Tylenol #1</td>
<td>5</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Tylenol #3</td>
<td>3</td>
<td>2</td>
<td>5</td>
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</tbody>
</table>

Fifteen patients were on continuous intravenous morphine infusion to treat severe pain associated with sickle cell crisis (dose: 10 - 40 μg/kg/hr = 1 - 4 mg/hr). There was no correlation between saliva and plasma morphine concentrations (r = 0.04, p = 0.89) (Figure A1-1).

The mean (± SD) S:P ratio was 2.28 ± 2.84 (range: 0.35 - 9.58). Eighteen volunteers were randomly allocated into one of two groups given either Tylenol #1 or Tylenol #3. O-demethylation of the codeine produced detectable morphine levels in both plasma and saliva. The mean saliva and plasma morphine levels after codeine administration were 6.42 ± 5.48 ng/mL and 5.06 ± 2.52 ng/mL, respectively. Here too, there was poor correlation between saliva and plasma morphine concentrations after codeine administration (r = 0.43, p = 0.08) (Figure A1-2). The mean S:P ratio was 1.31 ± 1.04 (range: 0.10 - 3.04).
Figure A1-1.
Correlation between saliva and plasma morphine concentrations in pediatric patients with sickle cell disease (n=15). The regression equation was obtained by least-squares regression analysis.

\[ y = -0.015x + 38.0, \ r = 0.04, \ p = 0.89 \]

Figure A1-2.
Relationship between saliva and plasma morphine concentrations in 18 healthy, adult volunteers (control group) after Tylenol administration. The regression was calculated by least-squares regression analysis.

\[ y = 0.198x + 3.79, \ r = 0.43, \ p = 0.08 \]
Because citric acid decreased the saliva pH ± SD to an average pH of 4.39 ± 1.36, we examined the analytic effect of different pH values on the morphine concentration in saliva measured by RIA. Across a pH range of 3.96 to 8.06 (Table A1-2), there was no difference in assay performance. An observed difference was detected only at pH 1.33.

Table A1-II. pH Effect on Saliva Morphine Concentrations

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean Counts/Minute</th>
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<td>1.33</td>
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<tr>
<td>5.41</td>
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<td>6.21</td>
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<td>6.88</td>
<td>11563</td>
</tr>
<tr>
<td>8.06</td>
<td>11208</td>
</tr>
</tbody>
</table>

Discussion

Our data clearly demonstrates that determination of morphine levels from saliva cannot be used as a quantitative tool to predict serum concentrations of the drug. However, we wished to verify whether this test could be used qualitatively to identify subjects systemically exposed to the opioid.

Morphine has been shown in numerous studies to be the biotransformation product of codeine by O-demethylation. Tylenol #1 and #3 both contain codeine that is converted to morphine and detectable by RIA. Because only 10% of codeine is O-demethylated into morphine, two control groups were used to ensure that sufficient codeine was given to the subjects to produce detectable levels of morphine in plasma and saliva. The morphine RIA kit is sensitive (0.8 ng/mL) and specific for morphine and has a coefficient of variation of less than 10% for concentrations between 2.5 and 250 ng/mL. Our results indicated that in adults 8 mg of codeine per tablet (Tylenol #1) is sufficient to produce detectable morphine concentrations in both media.
The use of an adult control group was necessary to verify that the patient saliva morphine concentrations reflected true drug levels and not endogenous opiate-like substances cross-reacting with the $^{125}$I-labeled morphine in the RIA kit. Hence, baseline (predrug administration) specimens in the control group were analyzed to determine the level of background noise: false-positive drug levels attributable to the RIA's potential cross-reactivity with endogenous morphine-like substances such as endorphins and enkephalins.

Our results showed a trace amount of morphine in the precodeine saliva and plasma of the controls. The RIA has approximately a 20% error in the detection of morphine at concentrations below the lowest kit calibrator concentration (2.5 ng/mL). Therefore, results showing concentrations below 2.5 ng/mL were reported as trace amounts of morphine detected in the samples. However, the mean morphine levels in both saliva and plasma were approximately two-fold higher if codeine was administered to the control volunteers, indicating that the detected saliva morphine concentrations reflected morphine levels derived from codeine. These results also indicated that the detected patient saliva morphine concentrations were not results generated by the cross reactivity of the RIA with endogenous substances but rather definitive morphine levels.

To determine whether the trace amounts of morphine before codeine administration in the adults were attributable to a dietary source of morphine-like substances, such as those found in poppy seeds, we recruited several of the volunteers to provide a saliva sample before and after consuming one bagel with poppy seeds. Bagels were purchased locally to ensure emulation of frequently consumed poppy seeds by the control subjects. To make sure that the bagels given to the control volunteers did in fact contain morphine, we scraped off approximately 25% of the poppy seeds from one bagel (0.24 g dry weight) and measured the morphine concentration. A very high morphine level (beyond the 250 ng/mL upper limit of the standard curve) was detected in the poppy seed homogenate, which indicated that the bagels consumed by our control subjects
contained morphine. No morphine was found in the saliva of these volunteers, which enabled us to rule out the hypothesis that a dietary source of morphine might have been responsible for the trace amounts of morphine detected in the precodeine controls.

Although correction for saliva pH has been advocated for calculating the S:P ratio for acidic and basic drugs, most reports in the pediatric literature indicate that there are no changes in the S:P ratio compared with saliva pH for neutral or very weakly acidic and basic drugs that are largely unionized at normal plasma pH.20,22,34 However, drugs highly ionized at plasma pH 7.35 - 7.40, such as chlorpropamide, tolbutamide, propranolol, and meperidine, produced as much as a two-fold change in the saliva drug concentration.20

Because morphine is a weakly basic drug (pKa = 7.9 - 8.05),35,36 it is ionized at normal physiologic plasma pH. Although the assay reagent has a buffering capacity, we wanted to make sure that the buffering capacity of the reagent was sufficient to compensate across a wide pH range. We were able to eliminate the potential impact of a wide pH range on the ability of the assay to measure morphine in saliva. Our results indicated (Table A1-2) that across a range of salivary pH values, 3.96 - 8.06, there was no effect on the analytic result of salivary morphine concentrations. Thus, the antibody reaction of the RIA kit produced reliable results in this pH range; but it is possible that a pathologic pH change, such as is seen in metabolic acidosis or alkalosis, could cause the amount of morphine present in the saliva to change with changing pH and thereby alter the S:P ratio for morphine. Although it was beyond the scope of this study, it will be important to investigate the effects of salivary pH on the transfer characteristics of morphine from plasma to saliva due to the influence of pH on the ionization of morphine.

In summary, our results indicated that there is no correlation between saliva and plasma morphine concentrations in children and adults. However, because morphine was detected in the saliva, saliva may have potential use as a qualitative test to indicate the presence or absence of morphine in a patient.
References


The major limitation to studying human placental function is that bioethical considerations preclude studies during the course of human gestation. Albeit that *in vitro* and *in vivo* animal models are available, their applicability to the understanding of human placental function is questionable because of the large interspecies differences in anatomy and physiology.

*In vitro* human placental perfusion techniques have been developed to facilitate the collection of both quantitative and qualitative information regarding almost all aspects of human placental function noninvasively. Unlike subcellular preparations or tissue homogenates, data obtained from intact tissue approximate the *in vivo* situation more closely because the cells maintain structural integrity and cell-to-cell organization. Another advantage of the *in vitro* placental perfusion technique is that confounding metabolic and/or physiologic influences of maternal and/or fetal origin are eliminated. Even after undergoing the trauma associated with delivery, the human placenta has been shown to be a resilient tissue retaining its functional capacity and structural integrity during *in vitro* perfusion for up to 12 hours.

Perfusing an isolated single human placental cotyledon has the advantage of structural integrity and cost effectiveness in that perfusing an entire placenta would require large volumes of maternal and fetal perfusing solutions. The major advantages of using a single placental cotyledon are that the tissue dose not require extensive manipulation, the tissue is better supported in the perfusion chamber, and ultimately, the tissue sustains less mechanical trauma at harvesting.

The perfusion system described in this appendix is generally based on the method and equipment developed by Miller et al.¹ and modified by Derewlany et al (Figure A2-1).²
Fig. A2-1. Placental Perfusion System

Diagrammatic representation of the placental perfusion system design for perfusing a human placental cotyledon in vitro.


Materials

1. Maternal Ismatec pump (Ismatec Instruments, Zurich, Switzerland)
2. Fetal polystaltic SA 8031 pump (Buchler Instruments, (VWR Canlab) Mississauga, Canada)
3. Maternal reservoir – 500 mL flask
4. Fetal reservoir – chromatography Ampholine® column (type 8101) glass water-jacket (LKB, Sweden)
5. Model 86 hot water bath (Precision Scientific, Chicago, USA)
6. Plexiglass perfusion chamber
7. Buchi rotary evaporator - Brinkmann CH 9230 Rotavapor R (Laboratorium Technik AG, Switzerland)
8. ABL 330 acid-base analysis laboratory (Radiometer A/S, Copenhagen, Denmark)
9. Model 759 sphygmomanometer (Sunbeam Corp. (Canada) Ltd., Toronto, Canada)

10. Surgical instruments: fine and coarse scissors, 5.5” straight needle holder, 5.5” straight Kelly clamps x 2, fine and coarse straight-edge splinter forceps, German Surgicals 3.75” curved iris Castroviejo scissors (Irex, Toronto, Canada)

11. Three-way stop cocks x 13

12. Three-hole rubber stopper x 1

13. #5 French catheter

14. #8 French feeding tube

15. Blunt-ended catheter

16. Narrow bore tubing (PE 280)

17. 19 gauge metal bulbous-tip cannula

18. 15 gauge metal bulbous-tip cannula

19. 1 cc and 3 cc syringes

20. 20 mL syringe

21. Oxygen – D tank (Proxair, Mississauga, Canada) with a pressure gauge, flow meter regulator

   Model No. ME1-540-FG (Medigas, Mississauga, Canada)

22. Carbon dioxide – D tank (Proxair, Mississauga, Canada) with a pressure gauge, flow meter regulator

   Model No. ME1-540-FG (Medigas, Mississauga, Canada)

23. Nitrogen – C tank (Proxair, Mississauga, Canada) with a pressure gauge, flow meter regulator

   Model No. M1 940 PG (Western Enterprises, Westlake, USA)

24. 4-0 black braided silk sutures with a 17 mm T-31 needle (Daves & Geck, Wayne, USA)

25. Catheter introducer (Becton-Dickinson, Rutherford, USA)

26. Gas dispersion tube

27. T-connector x 3

28. Y-connector x 1
Perfusion System – Materials and Hardware Setup

Fetal Circuit

The fetal reservoir is a chromatography column glass water-jacket, fitted with a three-hole rubber stopper on the top and a three-way stopcock at the base (Figure A2-2).

A gas dispersion tube is inserted into the reservoir, through one of the three stopper holes, and functions to both gas and mix the fetal perfusate. The fetal venous line is connected to a tube that is inserted in the second of the three stopper holes; it returns perfusate to the reservoir for recirculation. Sampling of fetal perfusate from the reservoir is done through an umbilical catheter (#5 French) which is inserted in the third hole of the rubber stopper located at the top of the fetal reservoir.

Tubing extending from the base of the reservoir leads to the fetal pump (Figure A2-3).
Fig. A2-3. Fetal Perfusion Circuit

From the pump, the fetal circuit tubing leads first to a flowmeter and then to a sampling port (three-way stopcock) from which fetal arterial (FA) samples are drawn. Distal to the sampling port, a sphygmomanometer is connected to the fetal circuit tubing by a T-connector. From the sphygmomanometer, the tubing leads to a connector located on the outside of the fetal perfusion chamber. The FA cannula is connected to the inside of the chamber; it is a #5 French umbilical vessel catheter with a short 19 gauge metal bulbous-tip cannula that inserts into the terminal end of the catheter. The fetal vein (FV) cannula is also connected to the inside of the chamber; it is a #8 French feeding tube with a short 15 gauge metal bulbous-tip cannula that inserts into the terminal end of the tube. Tubing connected to outside of the perfusion chamber at the venous port leads to the FV sampling port (three-way stopcock). From the FV sampling port, tubing extends back to the fetal reservoir enabling the perfusate to be re-circulated.
Maternal Circuit

The maternal reservoir is composed of a 500 mL flask attached to the steam duct of a rotary evaporator (Figure A2-4).

Fig. A2-4. Maternal Reservoir

Rotation of the flask aids in mixing and oxygenation of the maternal perfusate. A gas dispersion tube is not used to oxygenate the maternal perfusate because of the extent of perfusate frothing, which occurs because of protein and red blood cell accumulation from the placenta. Oxygen is introduced into the maternal reservoir through narrow bore tubing that minimizes frothing and also adequately oxygenates the perfusate even when the tubing is not completely inserted into the maternal perfusate.

There are three lines inserted into the maternal reservoir. One line delivers oxygen into the maternal reservoir while the other two lines deliver perfusate to and from the placenta. Perfusate samples from the reservoir are withdrawn through a #5 French umbilical catheter that inserts into the maternal reservoir.
The maternal pump is a peristaltic pump that delivers perfusate to the placenta and pumps maternal venous return from the placenta (Figure A2-5).

![Maternal Perfusion Circuit Diagram](image)

**Fig. A2-5. Maternal Perfusion Circuit**

The bubble trap within the maternal circuit consists of a 20 mL syringe barrel fitted with a rubber stopper at the top (inflow). A small piece of gauze is put inside the bubble trap to filter any tissue debris that could clog the maternal arterial cannulas and/or the cannulated maternal artery (MA) itself. The MA sampling port (three-way stopcock) branches off the main line tubing by way of a T-connector. Before reaching the perfusion chamber, the arterial line branches at a Y-connector thereby creating two arterial inflows that enter the maternal side of the perfusion chamber. The MA cannulas are two #5 French umbilical catheters. As maternal venous return collects on the
3. The top section of the perfusion chamber has three ports. Two of the ports support MA cannulas, which are attached to ports on the inside of the chamber. Tubing from the arterial branch of the maternal circuit is attached to ports on the outside of the chamber. The third port supports tubing that is attached on the inside of the chamber for suctioning off maternal venous return. Tubing of the maternal venous circulation is connected to this port on the outside of the chamber. The top section of the perfusion chamber is securely clamped to the middle section enabling the maternal venous return to collect around the cotyledon without leaking out of the perfusion chamber.
4. The final component of the perfusion chamber is a lid that is used to cover the opening at the top of the chamber setup. The lid prevents heat dissipation and helps maintain the tissue at physiologic temperature.

**Perfusion System Setup**

Before each perfusion experiment was carried out, all supporting items such as surgical instruments, sutures, surgical gloves, syringes, gauzes, and perfusates were set out. The area of the lab bench where the perfusion experiment was going to be performed was covered with absorbent pads. The tubing of the maternal and fetal circuits were assembled to create an intact perfusion circuit. The glassware (beakers, graduated cylinders) used during the perfusion experiments were placed appropriately to facilitate easy reach and all syringes, glass tubes, microcentrifuge tubes, ultracentrifuge tubes, and sample storage tubes used to collect, store, and/or process samples were assembled and labeled. The solutions used in the perfusion experiments (maternal perfusate, fetal perfusate, phosphate buffer) were placed in a warm water bath and brought to 37 °C before each experiment.

Once the solutions were at 37 °C, the perfusion circuits were primed with the appropriate solutions and were kept re-circulating until cannulation of the placenta was started. The fetal perfusate (Table A2-1) was equilibrated with a 95% O₂/5% CO₂ gas mixture. The maternal perfusate (Table A2-1) was not gased until after the placenta was ready for cannulation.

Placentas used in all studies were obtained from Mt. Sinai Hospital, Labor and Delivery, in Toronto, Canada. Placentas were delivered either by cesarean section or vaginally. Only those placentas obtained from uncomplicated deliveries were used in the placental perfusion studies. Specific details regarding patient screening may be found in chapter 2, Methods and Materials.

During delivery, the time of umbilical cord clamping was noted. After the clinicians obtained a sample of cord blood for clinical purposes, the entire placenta (usually including at least a 20 cm margin of fetal membranes and approximately a 75 cm length of umbilical cord)
was transferred the laboratory in a portable ice-chest containing ice-cold, heparinized saline. A 5.5” straight Kelly clamp was used to clamp the umbilical cord in order to prevent placental exsanguination; blood maintained within the placental vasculature helped to prevent the vessels from collapsing.

After the placenta was brought to the laboratory, it was placed in a shallow plastic tray for examination to determine the suitability of the tissue for perfusion. The fetal and maternal sides of the placenta were examined for evidence of tissue damage sustained during delivery.

Cannulation of the vessels was only attempted if an area could be identified where both the fetal and maternal sides of the placenta appeared to be intact. The fetal side of the placenta was almost always intact. The maternal side of the placenta was gently blotted with a gauze sponge to remove as much blood as possible. Damage to the maternal side of the placenta was often difficult to assess due to numerous blood clots. Moreover, assessment of the tissue was complicated by the indentations created by the placental septa, which often resemble tissue damage even when the tissue is intact.

Table A2-I. Constitution of the Perfusion Medium

<table>
<thead>
<tr>
<th>M199/HBSS plus:</th>
<th>Maternal Perfusate</th>
<th>Fetal Perfusate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>For 10 L</td>
</tr>
<tr>
<td>Heparin</td>
<td>2000 U/L</td>
<td>20,000 U/L</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100 mg/L</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.77 mM</td>
<td>5.0 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>30 mM</td>
<td>25.2 g</td>
</tr>
<tr>
<td>Dextran</td>
<td>7.5 g/L</td>
<td>75.0 g</td>
</tr>
</tbody>
</table>

U = units
Once an intact area of a placenta was identified, a single chorionic artery and vein pair (vessels on the fetal side of the placenta) were selected for cannulation (Figure A2-7); ideal vessels would supply a single, usually peripheral, cotyledon and should not be extensively branched. Any anastomoses distal to the site of cannula insertion were tied off to avoid perfusing multiple cotyledons. Sutures used in these experiments were 4-0 black braided silk with a 17 mm T-31 needle. The arterial and venous vessels were tied off proximally (Figure A2-8). The fetal artery was cannulated first. A small incision was made in the artery distal to the proximal tie, the cannula was inserted and tied securely to the vessel. The fetal vein was cannulated in a similar fashion. The bulbous tip of the cannulas enabled the tissue and/or cannula to be manipulated without fear of the catheters dislodging. During the cannulation procedure, the fetal pump speed was turned down to an extremely low flow rate (< 0.1 mL/min), which was sufficient to maintain

Fig. A2-7. Isolated Fetal Artery-Vein Pair
fluid within the arterial cannula. This procedure maintained patency of the cannula without creating excessive pressure within the fetal vessels prior to cannulating the fetal vein. The fetal flow rate was not increased until cannulation of the maternal side of the placenta was complete.

After completing the cannulations on the fetal side of the placenta, the 95% O$_2$/5% CO$_2$ gas mixture (which until now was oxygenating the fetal circuit) was transferred to oxygenate the maternal circuit. The fetal circuit was now gased with a 95% N$_2$/5% CO$_2$ mixture to de-oxygenate the fetal perfusate, thus emulating the relative maternal and fetal oxygen tensions observed in vivo (Figure A2-1). By the time the maternal cannulations were complete, the maternal perfusate was well oxygenated and the oxygen tension of the fetal perfusate dropped to $<100$ mmHg. Oxygenation of the placental tissue, which was initiated as soon as possible after delivery, was maintained throughout the cannulation procedure.

Before mounting the placenta in the perfusion chamber, the tissue was trimmed leaving a substantial margin of placental tissue and fetal membranes surrounding the cotyledon to be
perfused. The placenta was then mounted to the bottom section of the perfusion chamber; the fetal side facing down (Figure A2-9).

![Fig. A2-9. Placental Cotyledon Mounted in a Perfusion Chamber](image)

The lower section of the perfusion chamber was filled with phosphate-buffered saline (pH 7.35) (Table A2-2), warmed to 37 °C. The phosphate buffered saline solution was used as a substitute for amniotic fluid. This fluid supported the placenta in the chamber and also helped to maintain the tissue at a physiologic temperature. The tissue was clamped in place.

Table A2-II. Constitution of Phosphate Buffered Saline

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Quantity needed for 10 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>152 mM</td>
<td>89.1 g</td>
</tr>
<tr>
<td>Na₂HPO₄ • 7H₂O</td>
<td>1.0 mM</td>
<td>2.65 g</td>
</tr>
</tbody>
</table>
using the middle section of the perfusion chamber. At this point, the maternal side of the placenta was carefully examined for any fetal perfusate appearing on the maternal surface; an indication of a gross leak between the maternal and fetal circulations. If a leak was observed, the perfusion experiment was terminated at this point. If there was no observable leak, then the top portion of the perfusion chamber was firmly clamped in place on top of the middle section. The maternal pump speed was turned down to approximately 4 mL/min to avoid significant loss of maternal perfusate during the cannulation procedure. The maternal side of the placenta does not have any blood vessels that can be cannulated. Therefore, the maternal catheters were inserted into the intervillous space by gently piercing the decidual plate with two blunt-ended umbilical catheters (Figure A2-10). Once the maternal cannulas were in place, the maternal pump speed was increased to a flow rate of 15 mL/min. At the same time, the fetal pump speed was increased to a flow rate of 3 mL/min.

Fig. A2-10. Placement of the Maternal Artery Cannulas in a Placental Cotyledon
The ideal placement of the maternal cannula would be in the intervillous space of the same cotyledon as the one being perfused by the vessels cannulated on the fetal side of the placenta. Placement of the maternal cannulas can be aided by an observable slight blanching of the tissue where the blood has been cleared from the fetal vessels. This tissue blanching effectively defines a target area for maternal cannula insertion. However, in the majority of cases, there was no such visual guide and the cannulas were usually inserted in the area directly opposite the site of fetal vessel cannulation. To verify that the cannulas were in the correct place, a FV sample was drawn and the oxygen tension measured. Of the blood gas measurements, the pO$_2$ of the fetal artery and vein were critical indicators of the degree of overlap between the fetal and maternal circulations. A fetal veno-arterial pO$_2$ difference of approximately 100 mmHg was the best indicator that the maternal and fetal cannulations pertained to the same cotyledon and that oxygen was being transferred from the maternal circulation (high O$_2$ tension) to the fetal circulation (low O$_2$ tension). If necessary, the maternal cannulas were removed, repositioned, and the positioning reevaluated by way of measuring a fetal veno-arterial pO$_2$ difference. To ensure viability of the tissue, all cannulations were completed as quickly as possible with both circulations being established within 45 minutes of delivery.

During maternal cannula placement confirmation, the placenta was perfused with both the maternal and fetal circuits open (no perfusate recirculation). During this time (5 to 6 minutes), much of the blood within the perfused tissue was washed out. After this initial wash-out period, the two circuits were closed and the perfusates were re-circulated. The fetal venous line was reconnected to the fetal reservoir for recirculation and the pool of maternal venous effluent that accumulated on the surface of the decidua (generally < 5 mL) was aspirated (via the maternal pump) and returned to the maternal reservoir for recirculation.

Specific details regarding the morphine and morphine-naloxone perfusion experiments may be found in chapter 2, Materials and Methods.
References


<table>
<thead>
<tr>
<th>Compound</th>
<th>mg/L</th>
<th>Compound</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-Alanine</td>
<td>50.0</td>
<td>Thiamine HCl</td>
<td>0.01</td>
</tr>
<tr>
<td>L-Arginine • HCl</td>
<td>70.0</td>
<td>Thymine</td>
<td>0.30</td>
</tr>
<tr>
<td>DL-Aspartic acid</td>
<td>60.0</td>
<td>Vitamin A acetate</td>
<td>0.14</td>
</tr>
<tr>
<td>L-Cysteine HCL • H2O</td>
<td>0.11</td>
<td>Xanthine • Na</td>
<td>0.34</td>
</tr>
<tr>
<td>L-Cystine • 2HCl</td>
<td>26.0</td>
<td>Calcium chloride • 2H2O</td>
<td>185.0</td>
</tr>
<tr>
<td>DL-Glutamic acid</td>
<td>133.6</td>
<td>Ferric nitrate • 9H2O</td>
<td>0.72</td>
</tr>
<tr>
<td>Glycine</td>
<td>50.0</td>
<td>Magnesium sulfate (anhydrous)</td>
<td>97.7</td>
</tr>
<tr>
<td>L-Histidine • HCL • H2O</td>
<td>21.9</td>
<td>Potassium chloride</td>
<td>400.0</td>
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<tr>
<td>L-Hydroxyproline</td>
<td>10.0</td>
<td>Potassium phosphate</td>
<td>60.0</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>40.0</td>
<td>Sodium acetate (anhydrous)</td>
<td>50.0</td>
</tr>
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<td>DL-Leucine</td>
<td>120.0</td>
<td>Sodium chloride</td>
<td>8000.0</td>
</tr>
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<td>L-Lysine • HCl</td>
<td>70.0</td>
<td>Sodium phosphate dibasic (anhydrous)</td>
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<td>DL-Methionine</td>
<td>30.0</td>
<td>D-Glucose</td>
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<td>DL-Phenylalanine</td>
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<td>DL-Serine</td>
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<tr>
<td>DL-Threonine</td>
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<tr>
<td>DL-Tryptophan</td>
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<tr>
<td>L-Tyrosine • 2Na</td>
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<td>DL-Valine</td>
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<tr>
<td>Adenine sulfate</td>
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<td>Adenosine triphosphate • 2Na</td>
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<td>Adenylic acid</td>
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<tr>
<td>α-Tocopherol phosphate • 2Na</td>
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<tr>
<td>Ascorbic acid</td>
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</tr>
<tr>
<td>Biotin</td>
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</tr>
<tr>
<td>Calciferol</td>
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</tr>
<tr>
<td>Cholesterol</td>
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<td>Choline chloride</td>
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<td>Deoxyribose</td>
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<tr>
<td>Folic Acid</td>
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</tr>
<tr>
<td>Glutathione (reduced)</td>
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<tr>
<td>Guanine • HCl</td>
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<td></td>
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</tr>
<tr>
<td>Niacinamide</td>
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<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
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<td></td>
</tr>
<tr>
<td>PABA</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Pantothenic acid • Ca</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyoxyethylene sorbitan</td>
<td>20.0</td>
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<td>Monooleate</td>
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<td></td>
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<tr>
<td>Pyridoxal HCl</td>
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<tr>
<td>Pyridoxine HCl</td>
<td>0.03</td>
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<tr>
<td>Riboflavin</td>
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<td></td>
</tr>
<tr>
<td>Ribose</td>
<td>0.50</td>
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<td></td>
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</table>
Samples were analyzed using the Coat-A-Count serum morphine RIA kit (Diagnostic Products Corporation, Los Angeles, USA). The assay is a solid-phase $^{125}$I RIA designed for the quantitative measurement of morphine in serum and heparinized plasma. $^{125}$I-labelled morphine competes for one hour with morphine in the patient sample for antibody sites. The antibody is immobilized to the wall of a polypropylene tube. Decanting the supernatent suffices to terminate binding competition and to isolate the antibody-bound fraction of the radiolabeled morphine, which is then counted in gamma counter. Comparison of the counts to a calibration curve yields a measure of the morphine present in the patient sample. The level of radioactivity is inversely proportional to the concentration of morphine present in the sample. Ready-to-use human serum-based liquid standards (0, 2.5, 10, 25, 75, and 250 ng/mL) are included with each kit.

The detection limit of the assay is 0.8 ng/mL. Severe icterus (bilirubin up to 20 mg/dL) had no effect on the assay. The antiserum is highly specific for free, unconjugated morphine exhibiting very low crossreactivity to other naturally occurring compounds that may be present in a patient’s sample. At 5000 ng/mL, the assay yielded detectable results for M6G and M3G; the percent reactivity was 0.17% and 0.04%, respectively.

Specimens collected into Vacutainer tubes with EDTA produced false positive morphine concentrations; samples collected in EDTA are unsuitable for use. Samples collected with lithium heparin are suitable for analysis.

Materials Supplied with each RIA Kit

1. Morphine Ab-coated tubes.

2. $^{125}$I serum morphine with gentamicin and sodium azide preservatives, in ready-to-use liquid form.

3. Serum morphine calibrators (standards); six ready-to-use liquid morphine calibrators in morphine-free human serum with gentamicin and sodium azide preservatives.
4. Serum morphine controls; 2 ready-to-use liquid controls containing different levels of morphine in a processed human serum matrix with gentamicin and sodium azide preservatives.

**Materials Required but not Provided**

1. Gamma counter – compatible with standard 12x75 mm tubes
2. Vortex mixer
3. Centrifuge
4. Distilled or deionized water or phosphate buffered saline (PBS)
5. Plain 12x75 mm glass tubes for use as Total and NSB tubes
6. Micropipets
   i. 25 μL disposable tip, air-displacement pipet for serum standards, controls, and plasma samples
   ii. 1000 μL (1 mL) repeating precision pipettor for $^{125}\text{I}$ serum morphine dispensing
7. Decanting rack

**Assay Procedure**

1. Thaw plasma samples at room temperature. Once samples are liquid, keep samples cold.
2. Keep $^{125}\text{I}$ serum morphine and kit calibrators at room temperature.
3. Prepare all assay tubes in duplicate; glass tubes should be labeled Total (total counts) and NSB (nonspecific binding) – morphine Ab tubes (orange polypropylene reaction tubes) should be labeled and used for the standards, controls, maternal and fetal perfusate 0 ng/mL controls, and each maternal and fetal sample.
4. Vortex, then centrifuge all samples.
5. Pipet 25 μL of maternal and fetal perfusate 0 ng/mL controls, each of the standards, and the maternal and fetal morphine samples into the morphine Ab tubes. Pipet directly to the bottom of each reaction tube.
6. Pipet 1 mL of $^{125}$I-labeled morphine using the repeating precision pipettor into the Total tubes and each of the tubes in step 5.

   Note: Steps 5 and 6 should be completed in approximately ≤ 30 minutes.

7. Vortex all reaction tubes.

8. Incubate for one hour at room temperature.

9. Remove $^{125}$I tracer (discard into Seal-Lite) except for the reaction tube labeled Total.

10. Add 1 mL of PBS per reaction tube using the repeating precision pipettor.

11. Vortex all reaction tubes.

12. Remove PBS (discard into Seal-Lite) wash from each reaction tube.

13. Air dry for approximately 2 hours. There should be no PBS droplets remaining in the reaction tubes after the drying process is complete. Removing all visible moisture will significantly enhance precision.

14. Count for 1 minute in a gamma counter.

15. Calculation of results (ng morphine/mL).

   **Computer-assisted method:**

   i. To calculate the morphine concentration from a logit-log calibration curve, first calculate for each duplicate set of tubes the mean NSB-corrected CPM:

      \[
      \text{Net CPM} = \text{Mean CPM} - \text{Mean NSB CPM}
      \]

   ii. Determine the binding of each duplicate set of tubes as a percent of maximum binding (MB), with the NSB-corrected counts of the maternal perfusate blank taken as 100\%: Percent Bound = (Net CPM/Net MB CPM) x 100

   iii. Data reduction results are obtained from a computer-assisted linear regression analysis based on 7 calibration curve points: 1.3, 2.5, 5, 10, 20, 40, and 250 ng/mL. The resulting regression equation, \( y = mx + b \), is solved for \( x \), \( x = (y - b)/m \), which is the desired [morphine] in units of ng/mL. (Note: \( y = \text{CPM} \)
iv. Input each unknown sample, \( y \), into the \( x = (y-b)/m \) equation, where \( b \) and \( m \) are known; \( b \) and \( m \) were calculated in the regression analysis of the standard curve, to obtain the corresponding morphine level in units of ng morphine/mL.

16. Calculation of morphine clearance (mL/min).

The clearance of morphine is calculated according to the method established by Challier\(^1\) as follows:

\[
\text{Clearance (mL/min)} = \left[ \frac{(FV - FA)}{(MA - FA)} \right] \times Q_f \quad \text{where in the open system,}
\]

\( FA = 0 \) ng morphine/mL perfusate. Therefore, the equation simplifies to:

\[
\text{Clearance (mL/min)} = \left( \frac{FV}{MA} \right) \times Q_f \quad \text{where } FV = \text{morphine concentration in the fetal vein; } FA = \text{morphine concentration in the fetal artery; } MA = \text{morphine concentration in the maternal artery; and } Q_f = \text{fetal flow rate (mL/min).}
\]

Note: \( FV/MA = \text{transfer fraction (extraction ratio). The clearance standard deviation is calculated according to the equation:}

\[
\text{Cl}_{SD} = \text{SQ ROOT } \left( \frac{(n_1-1)SD_1^2 + (n_2-1)SD_2^2}{n_1 + n_2} \right) - 2, \quad \text{where } n_1 \text{ and } n_2 \text{ equal the number of measurements per perfusion clearance experiment.}
\]

Antipyrine clearance is calculated using the same equation.

17. Calculation of the clearance index.

\[
\text{Cl}_{\text{index}} = \frac{\text{Cl}_{\text{morphine}}}{\text{Cl}_{\text{antipyrine}}} \times 100 = \% \text{ Cl}_{\text{index}}
\]

Reference

Samples were analyzed using a Tandem®-R HCG immunoradiometric assay (Hybritech Inc., San Diego, USA) for quantitative and qualitative measurement of hCG in human serum. Tandem-R HCG is a solid-phase, two-site immunoradiometric assay. Samples containing hCG are reacted with monoclonal Ab-coated plastic beads (solid phase), directed toward a unique antigenic site on the hCG molecule, and with a radiolabeled monoclonal Ab directed against a distinctly different antigenic site on the same hCG molecule. Once the solid phase hCG-labeled Ab complex is formed, the beads are washed to remove unbound, labeled Ab. A gamma counter is used to measure the radioactivity bound to the solid-phase. The level of radioactivity is directly proportional to the concentration of hCG present in the test sample.

Even the highest routinely reported physiological concentrations of human luteinizing hormone (hLH), follicle stimulating hormone (hFSH), and thyroid stimulating hormone (hTSH) do not interfere with the hCG Ab. The kit utilizes two different monoclonal Abs directed against two different antigenic sites to detect hCG. Both Abs must bind to hCG for detection and measurement. This two-site Ab-binding method increases the specificity for hCG because the interferences of biologically inactive cross-reactants and hCG molecular fragments, including the beta subunit, are eliminated. There is no detectable (< 0.01%) crossreactivity with hFSH and hTSH and 0.24% crossreactivity with hLH. Hemoglobin and bilirubin (up to concentrations of 320 mg/dL, respectively), triglycerides (up to concentrations of 1015 mg/dL), and total protein (at concentrations between 3 and 15 g/dL) do not affect the results of the Tandem-R HCG assay. The minimum detectable concentration of hCG is 1.5 mIU/mL.

Serum or perfusate, but not plasma, may be used for the assay. Turbid samples or samples with particulate matter should be centrifuged prior to assay.
Materials Supplied with each Kit

1. Anti-hCG tracer Ab; ready-to-use liquid $^{125}$I-labelled mouse monoclonal IgG (anti-hCG) in horse/mouse protein matrix containing a blue dye of approximately 10 μCi/vial and 0.1% sodium azide as preservative.

2. Anti-hCG coated beads; mouse monoclonal IgG (anti-hCG) coated plastic beads in a buffer containing 0.1% sodium azide.

3. Zero diluent/calibrator (0 mIU/mL); ready-to-use liquid human serum with undetectable hCG and 0.1% sodium azide.

4. Calibrators (standards); ready-to-use liquid human serum containing 5, 10, 25, 100, 200, and 400 mIU/mL of hCG and 0.1% sodium azide.

5. Positive reference; ready-to-use liquid human serum containing 25 mIU/mL of hCG and 0.1% sodium azide.

6. Wash concentrate; ready-to-use liquid buffer with 0.3% sodium azide.

Materials Required but not Provided

1. Gamma counter – compatible with standard 12x75 mm tubes

2. Vortex mixer

3. Centrifuge

4. Sarstedt 55.535 (3.5 mL) tubes (Sarstedt, Newton, USA)

5. Micropipets

   i. 100 μL disposable tip, air-displacement pipet for serum standards, controls, and plasma samples

   ii. 100 μL and 2 mL repeating precision pipettor for $^{125}$I tracer and wash solution dispensing, respectively

6. Decanting rack

7. 37°C water bath
8. Forceps

Assay Procedure

1. Thaw plasma samples at room temperature. Once samples are liquid, keep them cold.
2. Keep $^{125}$I tracer and kit calibrators at room temperature.
3. Prepare all assay tubes in duplicate; hCG reaction tubes should be labeled and used for the standards, quality controls, maternal and fetal perfusate 0 ng/mL controls, and each maternal and fetal sample.
4. Label Eppendorf tubes with maternal sample times (preparation for 1:8 dilution of maternal samples due to high hCG concentrations in the placental perfusion samples).
5. Dilute the kit standards with maternal perfusate to produce 50, 12.5, and 6.25 mIU/mL standards.
6. Dilute the maternal samples 1:8 with maternal perfusate.
7. Place the 1:8 diluted maternal samples in the tray with the fetal samples for analysis.
8. Add one monoclonal Ab-coated bead to each reaction tube. (Before putting the bead into the reaction tube, gently blot dry; do not allow bead to dry)
9. Pipet 100 μL of the standards, quality controls, and sample pre reaction tube.
10. Pipet 100 μL of the radio-labeled tracer using the repeating precision pipettor.
11. Agitate all reaction tubes (while in the test tube rack) for approximately 1-2 minutes and cover the tubes.
12. Incubate samples for 60 minutes at 37°C in the water bath.
13. Wash samples twice using the kit Wash Solution as follows:
   i. Dispense 2 mL of Wash Solution/reaction tube using the repeating precision pipettor.
   ii. Aspirate the Wash Solution using an Erlenmeyer flask suction unit.
14. Cap all reaction tubes.
15. Count for 1 minute in a gamma counter.
16. Calculation of results (mIU hCG/mL).

Computer-assisted method:

i. Data reduction results are obtained from a computer-assisted linear regression analysis based on 6 calibration curve points: 0, 6.25, 12.5, 25, 50, and 100 mIU hCG/mL. The resulting regression equation, \( y = mx + b \), is solved for \( x \), \( x = (y - b)/m \), which is the desired hCG concentration in units of mIU/mL. (Note: \( y \) = mean CPM; \( CPM1 + CPM2/2 = \) mean CPM) Each maternal sample mean CPM result is multiplied by 8 to account for the 1:8 dilution done in step 6.

ii. Input each unknown sample, \( y \), into the \( x = (y-b)/m \) equation, where \( b \) and \( m \) are known; \( b \) and \( m \) were calculated in the regression analysis of the calibration curve, to obtain the corresponding hCG level in units of mIU hCG/mL.
A modified spectrophotometric analysis as described by Brodie et al.¹ was used to determine antipyrine concentrations in the perfusate samples. Antipyrine is nitrosated in a dilute acid medium with sodium nitrite. Absorbance of the resulting 4-nitrosoantipyrine is measured at 350 nm. The absorbance measured is proportional to the antipyrine concentration in the sample.

At the time of sample collection, each sample of perfusate was deproteinized with the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA), mixing, and centrifuging in a microcentrifuge for 5 minutes at 13000 x gravity.

**Required Materials**

1. NaNO₂ (Sigma Chemical Company, St. Louis, USA)
2. Antipyrine reaction mixture (can be purchased from Sigma or prepared in-lab)
3. Distilled water
4. Vortex mixer
5. Small cuvettes (10x4x45 mm) No. 67.742 (Sarstedt, Newton, USA)
6. Paraffin paper to cover each cuvette during inversion for mixing.
7. Micropipets
   i. 0.9 mL repeating precision pipettor for the reaction mixture
   ii. 0.3 mL disposable tip, air displacement pipet for 10% TCA and samples
   iii. 0.15 mL disposable tip, air displacement pipet for 10% TCA and the standards
   iv. 30 µL disposable tip, air displacement pipet for NaNO₂ mixture
8. Spectrophotometer

**Assay Procedure**

1. Order, in duplicate, small sized cuvettes (No. 67.742 [10x4x45 mm]) for sample analysis:
   i. Blank - 0.3 mL 10% TCA to the cuvette.
   ii. Standards - 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, and 2.0 mM
iii. Samples

2. Thaw samples at room temperature, then keep the samples cold by placing on ice.

3. Prepare the NaNO₂ mixture as follows: add 200 mg NaNO₂ to 100 mL distilled water.

4. Prepare the antipyrine reaction mixture as follows:
   i. 7% (w/v) TCA
   ii. 0.3% (v/v) H₂SO₄
   iii. 0.3 N NaOH

   Reaction mixture = i + ii + iii (Note: Dispense into a bottle and store at room temperature)

5. Add 0.9 mL of the antipyrine reaction mixture to all cuvettes.

6. Add 0.3 mL of 10% TCA to the blank cuvette.

7. Add 0.15 mL of 10% TCA to each of the cuvettes that will have standards.

8. Vortex, then add 0.15 mL of each standard into the cuvettes prepared in step 5.

9. Vortex, then add 0.3 mL of sample (diluted 1:2 in 10% TCA) to the sample cuvettes prepared in step 3.

10. Add 30 μL of the NaNO₂ mixture to each cuvette to initiate the reaction process.

11. Cover each cuvette with Paraffin paper before inverting twice to mix.

12. Incubate at room temperature for 20 minutes.

13. Read sample on a Shimadzu UV-VIS recording spectrophotometer UV-160A (Shimadzu Corporation, Toyo, Japan) at 350 nm versus a blank containing 0.3 mL 10% TCA instead of sample as a reference.

14. Antipyrine concentration calculations:

   Computer-assisted method:

   i. Data reduction results are obtained from a computer-assisted linear regression analysis based on 6 calibration curve points: 0.03, 0.06, 0.125, 0.25, and 0.5 mM. The resulting regression equation, y = mx + b, is solved for x, x = (y – b)/m,
which is the desired antipyrine concentration in units of mM. (Note: \( y = \) absorbance at 350 nm in a 1 cm lightpath)

ii. Input each unknown sample, \( y \), into the \( x = (y-b)/m \) equation, where \( b \) and \( m \) are known; \( b \) and \( m \) were calculated in the regression analysis of the calibration curve, to obtain the corresponding antipyrine level in units of mM.

iii. Each sample concentration obtained in step 14ii is multiplied by a dilution factor of 2 to account for the equal volume dilution of sample with 10% TCA at the time of collection.

iv. Conversion of mM to ng/mL: \(((\text{mM}/1000) \times 188.2)) \times 1000000\)

**Reference**

The concentrations of lactate produced by the placenta were determined by measuring the reduction of nicotinamide adenine dinucleotide (NAD) by lactate dehydrogenase (LD). LD catalyzes the reversible conversion of lactate to pyruvate, reaction I.

\[
\text{Reaction I}
\]

\[
\text{LD} \quad \text{Lactate + NAD} \rightleftharpoons \text{Pyruvate + NADH}
\]

In this presence of excess NAD, all lactate present in the sample will be converted to pyruvate.

The concentration of lactate is calculated by quantifying the increase in absorbance at 340 nm as NAD is reduced to NADH.

At the time of sample collection, each sample of perfusate was deproteinized with the addition of an equal volume of 10% (w/v) trichloroacetic acid (TCA), mixing, and centrifuging in a microcentrifuge for 5 minutes at 13000 x gravity.

**Required Materials**

1. Sigma Metabolite Control (Sigma Chemical Company, St. Louis, USA); an assayed, lyophilized preparation with 2,3-diphosphoglyceric acid (2,3-DPG), L(+)lactic acid, phenylalanine, pyruvic acid, buffer salts, and 0.025% sodium azide preservative in bovine albumin base.

2. Sigma Glycine Buffer (Sigma Chemical Company, St. Louis, USA); 0.2 mM glycine in 0.17 mM hydrazine at pH 9.2 with 1% chloroform preservative.

3. Sigma β-NAD (Sigma Chemical Company, St. Louis, USA); 99% pure, 1.7 mg/mL β-NAD (disodium salt Grade III).

4. Sigma LDH (Sigma Chemical Company, St. Louis, USA); 20 units/mL, crystalline suspension in 2.1 M (NH₄)₂SO₄ solution at pH 6 and < 0.03% pyruvate kinase activity.
5. Vortex mixer

6. Distilled water

7. Large cuvettes (10x10x45 mm) No. 67.741 (Sarstedt, Newton, USA).

8. Paraffin paper to cover each cuvette during inversion for mixing.

9. Micropipets
   i. 2.4 mL repeating precision pipettor for the reaction mixture
   ii. 0.1 mL disposable tip, air displacement pipet for Blank, Quality Control, Standard, and samples

10. Spectrophotometer

Assay Procedure

1. Order, in duplicate, large sized cuvettes (No. 67.741 [10x10x45 mm]) for sample analysis:
   i. Blank – add 0.1 mL 10% TCA to the cuvette.
   ii. Quality Control (undiluted, 2.3 mmol/L [range 2.0 – 2.7 mmol/L])
   iii. Standard
   iv. Samples

2. Thaw samples at room temperature, then keep the samples cold by placing on ice.

3. Calculate the required amount of the reaction mixture as follows: (Note: 3 mL of buffer/sample is required); Reaction mixture = i + ii + iii.
   i. Dilute glycine buffer 1:3 in distilled water. In this mixture, do not use the glycine buffer preservative; this appears at the bottom of the bottle as oil droplets.
   ii. Dissolve 1.7 mg/mL NAD in the diluted buffer.
   iii. Add 3.45 μL of ~20u/mL LDH per mL of buffer.

Example: Amount of Reaction Mixture Required for 14 Samples, Blank, QC, and Standards
a. Need 3 mL x 14 samples = 42 mL diluted buffer = 14 mL glycine buffer + 28 mL distilled water.

b. Need 1.7 mg x 42 mL = 71.4 mg NAD.

c. Need 3.45 μL x 42 mL = 145 μL LDH.

4. Obtain ice and completely immerse a 600 mL beaker.

5. Take the Sigma Metabolite Control vial, reconstitute with 5 mL distilled water, and gently swirl. Let the reconstituted mixture stand for 5 minutes, then place on ice.

6. Dilute the Sigma Standard (4.44 mmol/L) 1:5 with distilled water and place on ice.

7. Prepare the reaction mixture in the 600 mL beaker, while keeping the beaker immersed in ice, as per calculations in step 3.

8. Aliquot 2.4 mL reaction mixture into each cuvette.

9. Add 0.1 mL aliquot of the Quality Control, Standard, and sample into the cuvettes.

10. Cover each cuvette with Paraffin paper before inverting twice to mix.

11. Incubate at room temperature for 60 minutes.

12. Read sample on a Shimadzu UV-VIS recording spectrophotometer UV-160A (Shimadzu Corporation, Tokyo, Japan) at 340 nm versus a blank containing 0.1 mL 10% TCA instead of sample as a reference.

13. Lactate concentration calculations:

Concentration = \( \frac{A}{6.22} \times \frac{2.5}{0.1} \times 2 \)

where \( A \) = absorbance of sample at 340 nm

\[ 2.5 = \text{reaction volume (mL)} \]

\[ 6.22 = \text{absorptivity of 1 mM of NADH at 340 nm in a 1 cm lightpath} \]

\[ 2.0 = \text{dilution factor for samples in 10% TCA (1:2 dilution)} \]

\[ 0.1 = \text{sample volume (mL)} \]

Concentration = lactate concentration of sample (mM)
The YSI Model 23A Glucose Analyzer (Yellowstone Instrument Company, Inc., Yellowstone, USA) is a device used for the quantitative measurement of glucose. Glucose determination using the YSI 23A glucose analyzer operates on the principle that glucose and oxygen are converted to gluconic acid and hydrogen peroxide, reaction I.

**Reaction I**

Glucose oxidase

\[ \text{D-glucose} + \text{oxygen} \rightarrow \text{gluconic acid} + \text{H}_2\text{O}_2 \]

In the YSI Model 23A, the glucose probe oxidizes a constant portion of the hydrogen peroxide at the platinum anode, reaction II.

**Reaction II**

\[ \text{H}_2\text{O}_2 \rightarrow 2\text{H}^+ + \text{O}_2 + 2e^- \]

The current produced is directly proportional to the glucose level in the sample.

The circuit is completed at a silver cathode where oxygen is reduced to water, reaction III.

**Reaction III**

\[ 4\text{H}^+ + \text{O}_2 \rightarrow 2\text{H}_2\text{O} - 4e^- \]

The tip of the 23A's glucose probe is covered by a three-layer membrane, which functions both to protect the electrodes and to define a diffusion path to the electrodes. The outer layer, a polycarbonate material with a pore size of 0.03 \( \mu \text{m} \), is large enough to allow diffusion of glucose, oxygen, hydrogen peroxide, water, and salt yet small enough to block the diffusion of cells and enzymes. Glucose oxidase is immobilized in a thin intermediate layer made of glutaraldehyde resin. The inner layer, a cellulose acetate material with a very small pore size, prevents the diffusion of glucose, ascorbic acid, and most other potentially interfering substances from the electrodes while allowing hydrogen peroxide, oxygen, water, and salts to diffuse.
Glucose at the probe diffuses through the membrane's outer layer and comes into contact with glucose oxidase facilitating reaction I to occur. The resultant hydrogen peroxide in turn diffuses through the membrane's inner layer to contact the platinum anode where reactions II and III now occur, yielding a current proportional to the quantity of hydrogen peroxide diffused.

The platinum anode is polarized at +0.7 volts with respect to the silver cathode, which facilitates the oxidation of hydrogen peroxide and other reducing agents normally found in blood, for example, ascorbic acid and glutathione. The inner layer of the membrane effectively filters these interfering substances to minimize their effect on the glucose measurement.

A temperature probe is incorporated into the system because both the enzyme reaction rate and the membrane's permeability are temperature dependent. The measurement chamber contents are mixed by an air-driven silicone diaphragm, which ensures adequate mixing of the buffer and sample and an abundant supply of oxygen to support the reaction.

To ensure measurement accuracy, calibrate the analyzer prior to each analysis using the 200 and 500 mg/dL standards.

Heparinized samples may be frozen at -10°C or colder until analysis. Avoid drawing air and/or cellular debris with the SyringePET. Therefore, vortex, then centrifuge each sample prior to injecting into the injection port of the analyzer.

Required Materials
1. YSI Model 23A Glucose Analyzer (Yellow Springs Instrument Company, Inc., Yellow Springs, USA)
2. Buffer Supply solution
3. 200 and 500 mg/dL glucose standards
4. SyringePET (syringe-pipet combination) precision 25 µL glass syringe with Teflon-tip plunger (Yellow Springs Instrument Company, Inc., Yellow Springs, USA)
5. Reagents for buffer supply, glucose standards, and potassium ferrocyanide; refer below to steps 2i, 3i, 4i, and 5i, respectively

6. Distilled water

7. Vortex mixer

Assay Procedure

1. Thaw samples at room temperature, then keep the samples cold by placing on ice.

2. Prepare the buffer as follows:
   i. Buffer powder compounds:
      
      14.3% K$_2$H$_2$EDTA \( \cdot \) 2H$_2$O
      
      7.1% Na benzoate
      
      13.6% NaH$_2$PO$_4$ \( \cdot \) H$_2$O
      
      53.4% Na$_2$HPO$_4$
      
      0.059% gentamicin sulfate; equivalent to 2.25 mg free base

      Balance NaCl

      Add 3.67 g of powder to 450 \( \pm \) 25 mL distilled water. Shake until dissolved. Makes 450 mL of isotonic buffer at pH 7.3.

3. Prepare the 200 mg/dL glucose standard as follows:
   i. Glucose standard compounds:
      
      2.0 g anhydrous \( \alpha \)-D-glucose adjusted to equivalence with National Bureau of Standards (NBS) standard
      
      2.0 g Na benzoate
      
      1.0 g dipotassium EDTA

      Dilute to 1 L with distilled and let stand for 12 hours before use.

4. Prepare the 500 mg/dL glucose standard as follows:
   i. Glucose standard compounds:
5.0 g anhydrous α-D-glucose adjusted to equivalence with NBS standard
2.0 g Na benzoate
1.0 g dipotassium EDTA

Dilute to 1 L with distilled and let stand for 12 hours before use.

5. Prepare the potassium ferrocyanide as follows:
   i. Potassium ferrocyanide compounds:
      1.0 g A.C.S. certified potassium ferrocyanide
      100.0 mL distilled water
      Mix potassium ferrocyanide with distilled water.

6. Fill the YSI Model 23A buffer supply with the buffer prepared in step 2i.

7. Vortex, then centrifuge each sample.

8. Use the Syringe pet to draw samples (approximate required sample volume = 25 μL).

9. Inject sample into the injection port.

10. Rinse the Syringe pet twice with distilled water between samples.

11. Record the sample value and convert to desired units as follows:
   i. mmol/L = (mg/dL)/18, where 18 mg/dL = 1 mmol/L
   ii. mg/dL = (mmol/L) × 18, where 18 mg/dL = 1 mmol/L
Blood gases taken from the maternal and fetal arterial and venous circuit sampling ports are used to calculate oxygen transfer from the maternal artery (area of high oxygen tension) to the fetal venous circulation (area of low oxygen tension). Maternal artery blood gas oxygen tensions (pO$_2$) are usually measured between 400 and 500 mmHg; values that are high compared with in vivo arterial pO$_2$ levels of 100 mmHg. However, the placenta is actually perfused in vitro under hypoxic conditions because oxygen delivery to the placenta during perfusion is much lower than in the in vivo situation. That is, in vivo, uterine blood flow to the placenta is approximately 1 mL/min/g of placenta (500 mL/min for an average placental weight of 500 g) and the oxygen content of blood is 20 mL O$_2$/100 mL blood or 0.2 mL O$_2$/mL blood. Therefore, O$_2$ delivery in vivo is estimated to be 200 mL O$_2$/min/kg. The oxygen delivery during in vitro placental perfusion is approximately 10 times lower than in vivo. These low rates of oxygen delivery in perfusion experiments are not due to ischemia, since the in vitro maternal flow rates approximate those seen in vivo, but are rather due to the lower solubility of oxygen in the perfusion medium compared with whole blood (0.2 mL O$_2$/mL blood versus 0.024 mL O$_2$/mL perfusate). The presence of an oxygen carrier in the perfusion medium would improve oxygen delivery to the perfused placental cotyledon; however, the use of whole blood is not practical because the increased viscosity of blood would lead to increased fetal-to-maternal perfusate leaks. In addition, a ready source of fresh cross-typed and matched whole blood (or AB negative whole blood) would be required. Although hospital blood banks often have a supply of outdated blood, the oxygen saturation of out-dated blood has a decreased pO$_{250}$ making it inappropriate for use as an oxygen carrier in perfusion experiments. Despite the apparent low rate of oxygen delivery to the placenta, compared with in vivo estimates, the placenta perfused in vitro maintains its physiological viability as shown by the tissue's ability to synthesize hCG.
Oxygen consumption has been shown to be dependent on the rate of oxygen delivery to the placental tissue; the more oxygen that is delivered to the tissue, the more oxygen is utilized. In addition, Challier et al. also showed that placental oxygen consumption will continue to increase even when the oxygen supply to the tissue is increased to 200 mL/min/kg. These data support the conclusion that it is the delivery of oxygen to the tissue that is the rate-limiting step for placental oxygen consumption. Albeit that the rate of oxygen transfer to the fetal circulation also increases with increased oxygen delivery to the placenta, a significantly higher proportion of the oxygen delivered to the perfused lobule is consumed by the placenta than is transferred to the fetal circulation.

Although the values for oxygen consumption measured in vitro are lower than the estimates of in vivo rates of consumption, the efficiency of oxygen utilization in vitro when using perfusate is high compared to that calculated from data obtained from perfusions using whole blood, where O₂ delivery is an order of magnitude higher. Hence, although the placenta perfused in vitro with perfusate does not quantitatively mimic the in vivo situation with respect to oxygen delivery, the tissue does appear to maintain its requirements for energy metabolism through a compensatory increase in efficiency of oxygen utilization.

The clearance of oxygen from the maternal circulation has been shown to be inversely proportional to the size of the perfused lobule, suggesting that more efficient oxygen transfer is achieved by perfusing smaller lobules.¹

Materials
1. 1 cc Tuberculin syringes
2. ABL 330 acid-base analysis laboratory (Radiometer A/S, Copenhagen, Denmark)

Blood Gas Determination Procedure
1. Aspirate 0.20 mL of perfusate from the desired port into a 1 cc tuberculin syringe.
2. Inject 0.15 mL of the sample into the APL 330 acid-base laboratory.
3. Record the pO₂ result and sample time.

**Formulas for Calculation of Placental Oxygenation**

The oxygen content of the perfusate samples, delivery of oxygen to the maternal circulation, rate of transplacental transfer, and oxygen consumption by the placenta are calculated using the following equations.³

**I. Oxygen Loss from the Maternal Circulation**

The loss of oxygen from the maternal circulation in the absence of placental tissue must be measured before calculating any of the parameters relating to placental oxygen consumption. This is done to correct for insensible oxygen loss from the perfusion medium, which does not contain an oxygen carrier molecule such as hemoglobin. Perfusion in the maternal reservoir is oxygenated to a partial pressure ranging between 350 and 500 mmHg, circulated through the maternal perfusion tubing circuit, and returned back to the maternal reservoir. Perfusate is sampled from the maternal artery (MA) and maternal vein (MV) and the pO₂ of the perfusate is measured using an automated blood gas analysis system (ABL 330 Acid-Base Analysis Laboratory, Radiometer A/S, Copenhagen, Denmark). The amount of oxygen that is lost from the perfusion system is given by the measured arteriovenous difference in pO₂ and is expressed as a function of the oxygen tension in the MA.

Maternal arteriovenous differences (MA – MV) in pO₂ must be corrected to account for this non-specific O₂ loss from the maternal circulation according to the following equation:

\[ pO₂ = (MA - MV)_{\text{corrected}}, \text{ where the correction} = \text{non-specific O}_2 \text{ loss} = 0.779(MA pO₂) - 203.135 \]

The equation for nonspecific O₂ loss was obtained by collecting MA and MV perfusate samples from a perfusion experiment conducted without a placenta. A linear regression analysis of time (x-axis) versus MA pO₂ (y-axis) yielded the equation 0.779(MA pO₂) – 203.135.

**II. Oxygen Content of Perfusate Samples**
\[ O_2 \text{ Content} = \frac{0.0239}{(BP - 47)} \times pO_2 \text{ mL } O_2/\text{mL perfusate}, \text{ where} \]

0.0239 – solubility of oxygen expressed as mL O2/mL fluid at 37°C and 1
atmosphere dry gas pressure;

BP – barometric pressure in mmHg;

47 – saturated vapor pressure of water at 37°C in mmHg;

pO2 – pO2 of the sample in mmHg.

III. Maternal Oxygen Delivery

\[ O_2 \text{ Delivery} = MA \times \frac{Q_m}{WT} \text{ mL } O_2/\text{min/kg, where} \]

MA – O2 content of the maternal arterial perfusate sample in mL O2/mL
perfusate;

Qm – flow rate (mL/min) of the perfusate on the maternal side of the placenta;

WT – weight of the perfused lobule expressed in kilograms.

IV. Rate of Transplacental Oxygen Transfer

\[ O_2 \text{ Transfer} = \frac{Q_f}{WT} \times (FV - FA) \text{ mL } O_2/\text{min/kg, where} \]

Qf – flow rate (mL/min) of the perfusate on the fetal side of the placenta;

WT – weight of the perfused lobule expressed in kilograms;

FV – O2 content of the fetal venous perfusate sample in mL O2/mL perfusate;

FA – O2 content of the fetal arterial perfusate sample in mL O2/mL perfusate.

V. Placental Oxygen Consumption

\[ O_2 \text{ Consumption} = [(MA - MV)_{\text{corrected}} \times \frac{Q_m}{WT}] - O_2 \text{ Transferred}, \text{ where} \]

\( O_2 \text{ leaving maternal circulation} \quad O_2 \text{ appearing in fetal circulation} \]

\[ O_2 \text{ Consumption} – \text{ expressed as mL } O_2 \text{ consumed/min/kg;} \]

\( (MA - MV)_{\text{corrected}} – \text{ maternal perfusate arteriovenous difference in } O_2 \text{ content} \)

corrected for oxygen loss in the absence of tissue expressed as mL O2/mL
perfusate;
Q_m – flow rate (mL/min) of the perfusate on the maternal side of the placenta;

WT – weight of the perfused placental lobule expressed in kilograms; O_2

Transferred – oxygen transferred to the fetal side of the placenta as calculated in

IV above expressed as mL O_2/min/kg.

References


CLINICAL INFORMATION FORM

Title of Research Project
THE CORRELATION BETWEEN MATERNAL AND FETAL MORPHINE SULFATE PLASMA CONCENTRATIONS AND THE ULTRASOUND BIOPHYSICAL PROFILE AND UMBILICAL ARTERY DOPPLER VELOCIMETRY SCORES; DRUG EFFECTS ON FETAL RESPONSE

Investigators: Mr. Ernest A. Kopecky - Ph.D. (Candidate)
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Women's College Hospital
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for Sick Children

Dr. Kofi Amankwah - Professor of Obstetrics and Gynecology
University of Toronto
Department of Obstetric and Gynecology
Women's College Hospital

Purpose of the Research:

The purpose of this research is to determine how much of a drug given to a mother will cross to her baby and what effects the medication will have on the baby. The drug is called morphine. It is a pain reliever and sedative given to mothers before a cordocentesis to decrease the baby’s movements during the procedure. No one has investigated exactly how much drug crosses to the baby and what effect the medication will have on the child. We have used this drug for many years without harmful effect. The effects we are looking for relate to how the baby behaves after the mother receives morphine. Secondly, we are also proposing to determine the relationship between the mother’s and baby’s morphine blood concentrations and the way the blood flows through the umbilical artery.
Description of the Research:

Based on the clinical judgment of your obstetrician, you will be scheduled for a cordocentesis. This procedure involves the insertion of a needle into your abdomen until the umbilical cord is entered. A local anaesthetic will be used to anaesthetize your abdomen. A small amount of blood will be withdrawn and sent to the hospital's laboratory for analysis.

At the time that you give consent to participate in the study, you will be randomly assigned to either the experimental group or the control group. If you are in the experimental group, you will be given a morphine injection into your arm before the procedure starts. This will help your baby sleep through the procedure. If you are in the control group, everything is conducted the same way as in the experimental group except that you will not receive the morphine injection. Currently, there are no specific guidelines stipulating that morphine must be used as part of the cordocentesis procedure. The decision to give you morphine is made by your obstetrician based on his/her experience and the hospital’s policy.

We will ask you to arrive at the special procedures room 2 hours before the cordocentesis. Once registered, a nurse will take your temperature, blood pressure, pulse, and respirations. These vital signs will serve as baseline values. Then, you will have a short ultrasound examination prior to the procedure, much like the examination performed in your obstetrician’s office, to observe your baby’s movements and the amount of amniotic fluid (water) around the baby. The entire procedure is done by ultrasound guidance.

Once the cord is localized and if you have been randomized to the experimental group, a nurse will give you an injection of morphine into your arm in order to sedate your child. This is done to decrease your baby’s movements making it easier to enter the umbilical cord with the needle while avoiding your baby. This part of the procedure is the same whether or not you are in the study. Once the obstetrician has entered the umbilical cord, he/she will withdraw enough cord blood for clinical analysis. Any excess cord blood will be used to measure the exact level of morphine in the cord after the morphine injection. The actual method of taking blood from your babies’ cord is the same whether or not you are enrolled in the study. Almost at the same time, a nurse will draw some of your own blood that will enable us to measure the exact morphine level in your body after the morphine injection that you received earlier.

Finally, the same vital signs and ultrasound examination performed before the cordocentesis will be done after the procedure. We will then compare the before-procedure and after-procedure examination results to see exactly what effect, if any, the procedure or drug have had on your baby’s responses. Once this is completed, your participation in the study is complete. You will be observed for a while as part of the standard ward care for every mother undergoing a cordocentesis.

Potential Benefits:

There are no direct benefits to your child. The results of this study will enable obstetricians to decide whether morphine should be given to other pregnant women.
Potential Harms (Injury, Discomfort, Inconvenience):

The risks associated with the cordocentesis have already been explained before obtaining your consent for the clinical procedure. The most common risks associated with morphine include sedation, decreased rate and depth of respiration, nausea, vomiting, and itchy skin. Morphine is expected to sedate your baby. No harmful effects to your baby are known nor expected with the clinical use of morphine; this is in fact what we are studying. There are no additional risks or harms involved in the study. You may feel a pin-prick when the nurse draws some of your blood (2mL) from your arm while the obstetrician draws blood from the cord.

Confidentiality:

Confidentiality will be respected and no information that discloses your identity or the identity of your child will be released or published without consent. For your information, the research consent form will be inserted into your clinic and hospital patient health record.

Participation:

Participation in research must be voluntary. If you choose not to participate, you and your family will continue to have access to quality care at this hospital. If you choose to participate in this study, you can withdraw from the study at any time. Again, you and your family will continue to have access to quality care at this medical institution.
CONSENT FORM

Title of Research Project

THE CORRELATION BETWEEN MATERNAL AND FETAL MORPHINE Sulfate Plasma Concentrations AND the Ultrasound Biophysical Profile AND Umbilical Artery Doppler Velocimetry Scores; Drug Effects on Fetal Response

Investigators: Mr. Ernest A. Kopecky - Ph.D. (Candidate)
Department of Pediatrics
Division of Clinical Pharmacology and Toxicology
The Hospital for Sick Children

Dr. Kofi Amankwah - Professor of Obstetrics and Gynecology
University of Toronto
Department of Obstetric and Gynecology
Women's College Hospital

I acknowledge that the research procedures described above have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in this study, including the right not to participate and the right to withdraw without compromising the quality of medical care at the Women's College Hospital. As well, the potential harms and discomforts have been explained to me and I also understand the benefits (if any) of participating in the research study. I know that I may ask now, or in the future, any questions I have about the study or the research procedures. In addition, I have read and also received a copy of the Clinical Information Form. I have been assured that records relating to me/my child and me/my child's care will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I __________________________ hereby consent to participate in this research study.

Print Name

_______________________________
Signature

Name of Person who obtained Consent ________________ Signature ________________ Date ________________

The persons who may be contacted about the research are Ernest A. Kopecky who may be reached at telephone number (416) 813 8216 or pager (416) 589-5196 OR Dr. K. Amankwah at (416) 323-7326.
Although exposure during pregnancy to therapeutic medications and illicit drugs has been reported for many years, the clinical state of the baby could not be accurately assessed \textit{in utero} until the 1970s. It was the advent of real time B-mode ultrasonographic imaging in the late 1970s that made it possible to observe and objectively assess fetal health and circulatory status. As a result, the fetal biophysical profile score (BPS) was developed, enabling physicians to assess the health and wellbeing of the baby prior to birth.

Experimental and observed clinical data compiled for the fetal BPS confirm that the fetus expresses health through a variety of biophysical activities. The occurrence and pattern of these activities undergo a predictable and consistent change in the presence of fetal compromise; the end pattern reflecting both the severity and chronicity of the insult.

An initial BPS is attained to determine the parameters listed in Table A10-1.

Table A10-I. Objectives of the Fetal Biophysical Profile\textsuperscript{1}

1. Fetal number and position.
2. Placental position and anatomy.
3. Fetal morphometric data: biparietal diameter, head circumference, femur length, abdominal circumference.
4. Presence/absence of structural or functional developmental anomalies

Requirements for the BPS

1. High-resolution ultrasound with pulsed wave and color flow Doppler unit (Acuson EXP [Acuson, Mountainview, USA] or ATL Ultramark 9 [ATL, Bothell, USA])
2. Curved linear array 3.5 MHz transducer
3. Conducting gel
Procedure

A minimum procedure time of 30 minutes must be allotted in order to rule out the influence of fetal sleep-wake cycles on the variables being assessed. Because fetal BPS variables are subject to modulation by sleep state and because the absence of an activity can occur in either non-rapid eye movement sleep or asphyxia, the observation period for the examination must be a minimum of 30 minutes (approximately 1 fetal sleep cycle) before a variable can be considered abnormal.\(^1\) The presence of a variable, according to defined criteria,\(^2\) is scored 2 while the absence of a variable is scored 0. The overall BPS is a composite score encompassing the following five variables: fetal breathing movements (FBM), fetal tone (FT), gross fetal movements (FM), amniotic fluid volume (AF), and the non-stress test (NST).

1. Lay patient in the supine position, dim the procedure room lights, and apply the conducting gel to the gravid abdomen.

2. The examiner should scan the uterine cavity and assess the following variables according to the following defined\(^2\) criteria:

   i. FBM – At least 1 episode of FBM of at least 30 seconds duration within the 30 minute observation period

   ii. FT – At least 1 episode of active extension-flexion of fetal limbs or trunk

   iii. FM – At least 3 discrete body/limb movements in 30 minutes

   iv. AF – At least one pocket of AF measuring 2 cm by 2 cm, without cord

   v. NST – At least 2 episodes of fetal heart rate accelerations of \(\geq\) 15 seconds duration associated with fetal movement

3. Calculate the overall BPS for a maximum score of 8/8 (10/10 with NST).
References


A major development in perinatal medicine has been ability to study fetal blood flow noninvasively in the two placental circulations. Current duplex Doppler systems are capable B-mode imaging and allow a Doppler sample volume or range gate to be set to record from a desired vessel are currently available. In addition, Doppler ultrasound units can color code frequency shifts, which enable blood flow to be presented as color Doppler images. Flow velocity waveform color Doppler systems operate by superimposing the Doppler information on a real-time B-mode ultrasound image. The B-mode ultrasound image is produced by plotting the intensity of the ultrasound reflection at all tissue interfaces in the field of study.\(^1\)

A change in the frequency of a reflected wave relative to a transmitted wave is caused when an ultrasound wave is back-scattered from a target (red blood cell) that is moving relative to the source of the transmitted wave. This is known as the Doppler effect. When the frequency of the returning echoes differs from the transmitted frequency, the frequency difference is the Doppler frequency shift. The Doppler frequency shift for red blood cells moving with a velocity \(V\), can be mathematically described by the following equation:

\[
F_D = F_1 - F_0 = \frac{2F_0 \cdot V \cdot \cos \theta}{c},
\]

where \(F_0\) is the transmitted frequency, \(F_1\) is the returning frequency, \(c\) is the velocity of sound in tissue (1540 m/s) and \(\theta\) is the angle between the ultrasound beam and the direction of blood flow.\(^1\) It becomes clear that if a drug or disease affects vascular impedance of the blood vessel(s) being studied, this will alter blood flow velocity, and correspondingly, the Doppler waveform. Consequently, the detected changes in blood flow velocity will be incorporated into the calculated measures of circulatory status: systolic/diastolic (S/D) ratio, pulsatility index (PI), and resistance index (RI).

Blood flow velocity waveforms are produced by Doppler ultrasound studies of blood flow. The shape of a flow velocity waveform is characteristic of the scanned vessel. Therefore,
the flow velocity waveform of each of the following vessels is inherently unique and can be readily differentiated upon examination: umbilical artery, umbilical vein, uterine artery, middle cerebral arteries, and the aorta. Furthermore, each waveform has a characteristic gestation-related appearance because of the increasing diastolic flow velocity attributable to a decrease in the vascular impedance with increasing gestation (Figure A11-1).

Fig. A11-1. Umbilical Artery Doppler Velocimetry Waveforms

Clinical evidence has established that there is an association between adverse fetal outcome and an abnormal umbilical artery Doppler study. An abnormal umbilical artery Doppler study alerts one to the possibility of fetal compromise associated with placental pathology.

Requirements for the BPS

1. High-resolution ultrasound with pulsed wave and color flow Doppler unit (Acuson EXP [Acuson, Mountainview, USA] or ATL Ultramark 9 [ATL, Bothell, USA])
2. Curved linear array 3.5 MHz transducer

3. Conducting gel

Procedure

1. Lay patient in the supine position, dim the procedure room lights, and apply the conducting gel to the gravid abdomen.

2. Localize the desired vessel (umbilical artery), generate a blood flow velocity waveform tracing with at least 5 complete waveforms, and freeze the waveform image.

3. Calculate each of the following indices as an average of 5 waveforms:
   i. \( \text{S/D ratio} = \frac{\text{maximum velocity}}{\text{minimum velocity}} \)
   ii. \( \text{PI} = \frac{\text{maximum velocity} - \text{minimum velocity}}{\text{average velocity}} \)
   iii. \( \text{RI} = \frac{\text{maximum velocity} - \text{minimum velocity}}{\text{maximum velocity}} \)

References

Ultrasoundographically guided transabdominal percutaneous access to the fetal umbilical circulation has become a widely used diagnostic tool, permitting direct visualization of the fetus and yielding diagnostic information about fetal health.

Despite its usefulness, direct access to the fetal circulation is associated with risk. The procedure is technically difficult, time-consuming, painful for the mother and fetus, and unable to be done transplacentally. Moreover, potential complications of FBS include fetal bradycardia, fetal bleeding, chorio-amnionitis, preterm labor, premature rupture of membranes, cord thrombosis, placental abruption, acute maternal complications such as pain, contraction, and anxiety, and fetal death. The complication rate associated with cordocentesis is between 0.5–5% and up to 10% when procedure-related complications are included.

FBS can be done using the umbilical vein (UV) at the cord root insertion or the fetal intrahepatic vein (IHV). Less commonly, the needle can be inserted into the fetal heart (cardiocentesis) as a reliable way of obtaining fetal blood at 13 to 17 weeks’ gestation or in dire clinical situations.

Table A12-I lists the distribution of indications for FBS. Conversely, there are many

<table>
<thead>
<tr>
<th>Clinical Indication</th>
<th>Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid karyotyping</td>
<td>38</td>
</tr>
<tr>
<td>RBC alloimmunization</td>
<td>23</td>
</tr>
<tr>
<td>Infection</td>
<td>10</td>
</tr>
<tr>
<td>Nonimmune hydrops</td>
<td>7</td>
</tr>
<tr>
<td>Twin-twin transfusion syndrome</td>
<td>5</td>
</tr>
<tr>
<td>Neonatal alloimmune thrombocytopenia</td>
<td>5</td>
</tr>
<tr>
<td>Immune thrombocytopenia</td>
<td>2</td>
</tr>
<tr>
<td>Immune deficiency</td>
<td>2</td>
</tr>
<tr>
<td>Coagulopathy</td>
<td>1</td>
</tr>
<tr>
<td>Hemoglobinopathy</td>
<td>1</td>
</tr>
<tr>
<td>Miscellaneous*</td>
<td>1</td>
</tr>
</tbody>
</table>

*Includes fetal drug concentrations, thyroid function, and metabolic disorders.
contraindications for FBS. Safety of the mother and fetus must be the primary concern; the contraindication is therefore relative where urgent diagnosis may supersede most of the common contraindications listed in Table A12-II.

Table A12-II. Contraindications for FBS

<table>
<thead>
<tr>
<th>Contraindication</th>
<th>Course of Action/Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atypical umbilical cord insertion</td>
<td>Choose an alternate needling site</td>
</tr>
<tr>
<td>Single umbilical artery in IUGR</td>
<td>Serious post-cordocentesis bradycardia</td>
</tr>
<tr>
<td>Inadequate visualization</td>
<td>Defer procedure</td>
</tr>
<tr>
<td>Maternal or fetal activity</td>
<td>Maternal or fetal sedation; defer procedure</td>
</tr>
<tr>
<td>Uterine hyperactivity</td>
<td>Determine FHR decelerations before procedure</td>
</tr>
<tr>
<td>Unstable maternal conditions (preeclampsia)</td>
<td>Stabilize maternal condition before procedure</td>
</tr>
<tr>
<td>Unstable fetal conditions (hypovolemia from feto-maternal hemorrhage,</td>
<td>Effects of cordocentesis on fetal vascular regulation are a clinically</td>
</tr>
<tr>
<td>circulatory embarrassment from severe hydrothoraces with hydrops)</td>
<td>significant threat; only a single procedure may be tolerable</td>
</tr>
</tbody>
</table>

Prerequisites for FBS

1. High-risk obstetrical team
   i. Multidisciplinary team: obstetrics, anesthesiology, neonatology
   ii. Technical expertise: obstetricians, nurses, and ultrasonographers with direct FBS-procedure training and experience

2. Appropriate facility (tertiary center with a fetal assessment unit)
   i. FBS-procedure room
   ii. Adjacent delivery operating room
   iii. Obstetric anesthesiology service
   iv. Pre- and post-procedure monitoring facility
   v. Neonatal intensive care unit
   vi. Full-service laboratory capabilities

3. Equipment
   i. Bedside testing: cardiotocograph, blood pressure
ii. Needle guide
iii. 20 and 22 gauge, 6 to 9 cm needles
iv. 1 mL heparinized fetal blood sample syringes
v. 10 mL syringes
vi. 2% lidocaine without CO₂ (Xylocaine®)

vii. 10 mL (reconstituted in 0.9 % normal saline) vecuronium (Norcuron®)
viii. Skin marker
ix. 0.9% normal saline flush solution
x. High-resolution ultrasound with pulsed wave and color flow Doppler unit (Acuson EXP [Acuson, Mountainview, USA] or ATL Ultramark 9 [ATL, Bothell, USA])
xii. Image storage and hard copy capabilities

4. Preparation

i. Pre-procedure orders

a. Admit patient to obstetrics
b. Weigh patient upon admission
c. DAT, then NPO x 6 hours prior to procedure
d. NST x 30 minutes before procedure (if > 24 weeks gestation)
e. CBC
f. Type and crossmatch x 2 Units PRBC
g. IV normal saline TKVO @ 100 mL/hr
h. Cefazolin (Ancef®) 1 g IV, 30 minutes before procedure
i. Morphine (Duramorph®) 10 – 15 mg IM, 30 minutes before procedure
j. Diazepam (Valium®) 10 mg PO, 60 minutes before procedure, if indicated
k. Send the following medications to the procedure room with the patient:

1. 100 Units/10 mL heparin flush solution x 1 vial
2. Midazolam (Versed\textsuperscript{®}) (1 mg/mL) 5 mL vial x 1
3. Fentanyl (Sublimaze\textsuperscript{®}) (50 \mu g/mL) 5 mL vial x 1
4. Dimenhydrinate (Gravol\textsuperscript{®}) (50 mg/mL) 1 mL vial x 1
5. Vecuronium (Norcuron\textsuperscript{®}) (10 mg/10 mL) 10 mL vial x 1

ii. Skin preparation (Hibatane\textsuperscript{™}) and sterile drapes

iii. Sterile procedure tray

iv. Neonatal resuscitation tray

v. Post-procedure orders

   a. DAT 60 minutes after procedure
   b. Routine vital signs
   c. NST x 2 hours
   d. Cefazolin (Ancef\textsuperscript{®}) 1 g IV q6h x 1 – 2 dose(s) prn
   e. If NST reactive, D/C IV and D/C patient

5. Detailed parental counseling

6. Written informed consent

Procedure

1. Localize, using ultrasound guidance, the needling site: UV cord root insertion, free cord, IHV

2. Anesthetize the needle insertion site using lidocaine.

3. Technique: surgeons

   i. Two-surgeon method

      a. Surgeon 1: continuous ultrasound referencing of the needle tip, vessel puncture, and needle stabilization
b. Surgeon 2: obtaining blood samples and in cases of fetal transfusions, transfusing the fetus

4. Technique: ultrasound orientations

i. Through the ultrasound plane (free-hand approach) (Figure A12-1).

The transducer and needle are positioned as close to the vertical axis as possible, directly above the target. Slight angulation of the transducer toward the needle enables intersection of the ultrasound plane and the needle tip.

![Figure A12-1. Fetal Blood Sampling: Free-Hand Technique](image)


ii. Within the ultrasound plane (needle guide approach) (Figure A12-2).

The transducer and needle are positioned in the same plane, usually with complementary angles away from the vertical axis, in opposite directions.
5. Vessel identity is established by infusion of a 0.2-0.3 mL bolus of normal saline; turbulence in the vein is usually unmistakable while turbulence in the artery may be more difficult to visualize.

6. 0.2-0.3 mg/kg of estimated fetal weight vecuronium, if required.

7. Blood sampling
   i. Blood samples are collected into heparinized 1 mL syringes. The order of sampling should be noted.
   ii. Separate samples into test-specific tubes: CBC, chemistry, Kleihauer

8. Needle removal – maintain angle of insertion and withdraw gently but firmly to avoid rebounding back into the cord.

9. Post-procedure monitoring
   i. 10 minute direct Doppler-assisted ultrasound to R/O puncture site bleeding
ii. 5–10 minutes post-procedure, draw blood for maternal Kleihauer to R/O large-scale transplacental hemorrhage, if suspected

10. Sample validation – Kleihauer test results will confirm blood samples are of pure fetal origin.

To correctly interpret laboratory results, the blood sample origin (maternal or fetal) must be confirmed. Table A12-3 presents a comparison of the differing factors within maternal and fetal blood.

Table A12-III. Differentiation between Maternal and Fetal Blood

<table>
<thead>
<tr>
<th>Factor</th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell antigens</td>
<td>Lewis a or b</td>
<td>Absent</td>
</tr>
<tr>
<td>Cell size</td>
<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td>Kleihauer-Betke acid elution test*</td>
<td>Ghosts</td>
<td>Intact</td>
</tr>
<tr>
<td>Acid elution test (APT)*</td>
<td>Brown/green</td>
<td>Red</td>
</tr>
<tr>
<td>Hemoglobin type</td>
<td>A₁ A₂</td>
<td>F</td>
</tr>
<tr>
<td>Turbulence from saline infusion</td>
<td>Deep Spreading</td>
<td>Superficial Confined</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Human chorionic gonadotropin (hCG)</td>
<td>High</td>
<td>Absent</td>
</tr>
</tbody>
</table>

*Tests become less distinct with increasing gestation

References


Figure A13-1. Comparison of F/M Ratios Obtained from Placental Perfusion Experiments versus Cordocenteses

F/M ratios obtained from cordocenteses represent in vivo data while in vitro data were obtained from the placental perfusion experiments. Each in vivo data point represents a single patient's F/M ratio (n = 8). Each in vitro data point represents the mean ± SD value of 4 morphine-only placental perfusions.


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