Placenta-on-a-chip: A microfluidic platform to study the drug transport across the human placental barrier

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Placenta-on-a-chip: A microfluidic platform to study the drug transport across the human placental barrier

by

Rajeendra Lakruwan Pemathilaka

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Mechanical Engineering

Program of Study Committee:
Nicole Hashemi, Major Professor
Reza Montazami
Juan Ren
Donald Sakaguchi
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

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DEDICATION

To my parents:

Mr. Sunil Pemathilaka

Mrs. Peduruwa Dewa Chandrani Kusumlatha

and

To my siblings:

Narmada and Hasindu

This humble work is a sign of my love to you!

In loving memory of my sister

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ABSTRACT

The human placenta is a gender-specific, interim endocrine organ that grows in the uterus during pregnancy, with the placenta attaching the evolving fetus to the uterine wall through the umbilical cord to supply oxygen and nutrients. The placenta is also responsible for facilitating removal of waste products and carbon dioxide from the fetus, and with protecting the fetus from infectious agents and foreign substances that can be introduced across the placental barrier. Until the revelation of thalidomide-induced birth defects in the late 1960s, the human placenta was assumed to be an impenetrable barrier shielding the fetus from xenobiotics transfused from the maternal bloodstream. Thereafter, the placenta became a very contentious subject within the scientific community and the pharmaceutical industry because of concerns related to uncertainty associated with usage of xenobiotics and prescribed drugs during pregnancy. Many in vivo, ex vivo, and in vitro models and platforms for conducting placental drug screening have been developed by researchers, but despite these numerous attempts, a lack of physiological functions exhibited by those models and platforms have restricted the modeling of realistic human-placenta responses for conducting accurate drug-transport studies. This dissertation aims to present a microfluidic platform that has been developed to mimic structural phenotypes and physiological characteristics of a human placenta that can be used to simulate near-transport studies of xenobiotics and pharmaceutical drugs across the human placenta.

The usage of pregnant animal subjects has been a previously-preferred method for in vivo experimentation, but ex vivo experimentation has mostly been conducted on perfusion models of human placentas derived from post-delivery. Because of differences between humans and animals with respect to physiological characteristics of placentation, current in vivo drug transport studies of the human placenta have mostly produced erratic outcomes and the ex vivo perfusion models
used lack representation of physiological characteristics over the course of pregnancy. Chapter 2 highlights how the placenta-on-a-chip, a micro-engineered device fabricated utilizing microfluidic technology, has revolutionized the processes of overcoming many issues previously experienced for both *in vivo* and *ex vivo* models.

Following fabrication and verification of the placenta-on-a-chip, it was initially used to study caffeine transport across the placental barrier *in vitro*. Caffeine, primarily found in natural sources such as coffee, tea, and cocoa, is one of the most widely-consumed psychoactive drugs in the world. Because of absence of enzymes responsible for metabolizing caffeine in the fetal liver, concerns have been raised that a high maternal caffeine intake might harm the fetus. Chapter 3 presents a study conducted to quantify fetal caffeine concentration and rate of caffeine transfer from a continuous maternal perfusion of 0.25 mg mL\(^{-1}\) over a span of 7.5 h. There is limited information about the safety of using naltrexone (NTX), a common form of medication, prescribed for treating opioid addiction during pregnancy, and concerns have been raised about the effects of this drug on the fetus and its brain. Chapter 4 is a near-transport study of NTX and its major metabolite, 6β-naltrexol, across the placental barrier to the fetus and its brain.

While the placenta-on-a-chip device and associated transport studies summarized in the following chapters suggest this device as a possible *in vitro* placental drug-testing platform, further studies are required to achieve a sufficiently accurate model for the pharmaceutical industry to use in performing placental drug transport studies.
CHAPTER 1. GENERAL INTRODUCTION

1.1. Motivation

The human placenta is a vital organ that performs as the liver, lungs, kidneys, gut, and endocrine glands of the fetus during pregnancy. Women are known to consume various xenobiotics and pharmaceutical drugs during pregnancy due to a long-term habit or medical condition. Most xenobiotics and pharmaceutical drugs cross the placental barrier unless they are either completely metabolized in the liver of the mother or the molecular size and lipid solubility of the compound restricts the placental passage. Fetal-exposure to drugs can result in fetal implications at the stage of fetal development and after birth. Despite the fact that most mothers consume some form of xenobiotic and/or pharmaceutical drug during pregnancy, very little is known about the amount that crosses the maternal-fetal interface.

In recent years, an extensive number of in vivo, ex vivo, and in vitro studies have been performed to study the drugs-transport across the human placenta. Outcomes from these studies have tended to provide inconsistent conclusions due to the difference between the models developed and the real-human placentas. Most animal-based in vivo experimentation has involved structural and physiological differences in the placenta, while ex vivo studies have replicated only a certain period of the placentation. With the development of in vitro studies, researchers have been encouraged to develop drug-testing platforms that can mimic the physiological and structural functions of human organs, including the human placenta. One in vitro model that has gained the attention of researchers is the use of organs-on-chips technology. This research provides a proof-of-concept of a drug-testing platform that utilizes organs-on-chips technology, which it then uses this to model the drug-transport across the human placenta.
1.2. Research Objectives

The objective of the studies presented in this dissertation is to develop an *in vitro* model of the human placenta utilizing microfluidics and organs-on-chips technology. The microfluidic device should present the structural phenotypes and physiological characteristics of a human placenta. Once this device is confirmed as providing a near-representation of the human placenta *in vitro*, it will be used to perform near-transport studies of xenobiotics and pharmaceutical drugs. Caffeine is used to simulate xenobiotic transport, while naltrexone and its primary metabolite, 6β-naltrexol, is used as the pharmaceutical drug transport.

1.3. Outline

The following chapters may be summarized thus:

Chapter 2: A literature review of the recent *in vivo*, *ex vivo*, and *in vitro* studies is presented, and how the placenta-on-a-chip revolutionizes placental drug transport studies is discussed;

Chapter 3: A study performed to quantify fetal caffeine concentrations and rate of transfers is presented;

Chapter 4: Fetal exposure to naltrexone and its major metabolite, 6β-naltrexol, is studied and a prototype for fetal-brain exposure during a similar exposure is presented;

Chapter 5: General conclusions of the research and recommendations for future work are described.

References


CHAPTER 2. DRUG TRANSPORT ACROSS THE HUMAN PLACENTA: REVIEW OF PLACENTA-ON-A-CHIP AND PREVIOUS APPROACHES

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Abstract

In the past few decades, the placenta became a very controversial topic that has had many researchers and pharmacists discussing the significance of the effects of pharmaceutical drug intake and how it is a possible leading cause towards birth defects. The creation of an in vitro microengineered model of the placenta can be used to replicate the interactions between the mother and fetus, specifically pharmaceutical drug intake reactions. As the field of nanotechnology significantly continues growing, nanotechnology will become more apparent in the study of medicine and other scientific disciplines, specifically microengineering applications. This review is based on past and current research that compares the feasibility and testing of the placenta-on-a-chip microengineered model to the previous and underdeveloped in vivo and ex vivo approaches. The testing of the practicality and effectiveness of the in vitro, in vivo, and ex vivo models requires the experimentation of prominent pharmaceutical drugs that most mothers consume during pregnancy. In this case, these drugs need to be studied and tested more often. However, there are challenges associated with the in vitro, in vivo, and ex vivo processes when developing a practical placental model, which are discussed in further detail.
2.1. Introduction

The human placenta is an extremely critical and complicated organ that performs a variety of functions. Those functions are comprised of a few of the following: ensuring the safety and security of fluid, nutrient, and oxygen circulation and flow; the removal of waste products and carbon dioxide; and the shielding from harmful diseases, infections, and xenobiotics from the mother to fetus. The performance of these actions supports the development and maturity of the fetus.\textsuperscript{[1-4]} The structure of the placenta is composed of a placental trophoblast and fetal capillary endothelium layer. The two cell layers separate the fetal circulation from the maternal blood, ensuring that no intermingling occurs between the two blood flows.\textsuperscript{[5]} Unfortunately, studying and monitoring the placenta is very difficult, considering the structure of the placenta goes through a considerable amount of alterations during pregnancy.\textsuperscript{[6]} Not to mention, as pregnancies increase within mothers, more dramatic alterations begin taking place in their placental barrier, making the testing and monitoring even more challenging.\textsuperscript{[6]} Although the placenta is very difficult to examine and study, the knowledge that humans are gaining from the placenta proves to be a vital source for humanity's understanding of the human placenta’s overall behavior and system. Therefore, the functionalities and structure of the placenta play a vital role in the testing, surveillance, and understanding of the placental barrier’s properties and purpose in the human body. Understanding the physiology behind the placenta creates a foundation for researchers to pave a model, for the scientific community and pharmaceutical industry, towards the testing and better understanding of the human placenta.

Until these past couple of decades, there was an idea circulating around researchers, pharmacists and the public that the placenta is an impenetrable barrier that does not impose any fatal or severe effects upon the mother or fetus.\textsuperscript{[7]} However, the discovery of thalidomide-induced birth defects in the late 1960s dismantled that argument.\textsuperscript{[7]} A few years after the discovery of the
thalidomide-induced birth defects, Boston University's Slone Epidemiology Center Birth Defects Study (BDS) began conducting an array of detailed interviews with a diverse selection of pregnant women, which still takes place annually.\textsuperscript{[8]} The intent of this program is to survey the medication that pregnant women are taking throughout the duration of their pregnancy. When the data is collected, researchers analyze the trend of medication intake by pregnant women and compare the intake towards the rise, causes and formation of birth defects. The collection of this data supports the proposed argument by researchers around the globe that pregnant women are taking medications more frequently and these medications need to be studied more thoroughly. The pharmaceutical industry is also faced with a considerable amount of complaints and concerns, surrounding their integrity and the safety of their drugs and adequacy.\textsuperscript{[9]} To bring statistics into the argument, statistics display the fact that approximately 45.3\% out of every pregnant woman uses prescription medicine, excluding vitamins.\textsuperscript{[10]} As the growth and skepticism of pharmaceutical and over-the-counter drugs become more popular for pregnant women, the testing of the feasibility and effectiveness of these drugs needs to be taken more seriously in order to prevent these increasing measures in birth defects. For example, the development and utilization of the \textit{in vivo} systems, and \textit{ex vivo} and \textit{in vitro} devices has helped scientists to study the effects and harms that these drugs are imposing on the human placenta.

A variety of \textit{in vivo} systems, and \textit{ex vivo} and \textit{in vitro} devices have been developed, for the most part, to mimic and replicate the metabolism process and active transport in the human placenta.\textsuperscript{[7, 11]} The development of the placenta-on-a-chip and other \textit{in vivo} and \textit{ex vivo} systems avoids the risk of the rupturing and disturbance of the fetus and mother.\textsuperscript{[12-14]} The use of these instruments creates a more viable and systematic approach towards the testing and understanding the effects that pharmaceutical and other medicated drugs inflict upon the placenta. However,
scientists have become increasingly more interested in the placenta-on-a-chip model for testing, especially in the past few years. While the \textit{in vivo} and \textit{ex vivo} systems do recapitulate a few of the behaviors and functions performed by the human placenta, they struggle with the resources and technology to carry out these tests effectively. Nonetheless, these approaches are being overshadowed by the growing desire and promise that the \textit{in vitro} model possess. With growing interest and development in the field of nanotechnology, the placenta is being infused into nanomedical related applications, particularly on-a-chip utilization.\cite{7, 15} The microengineered placenta-on-a-chip is the face of a new generation of technology that is becoming ever more so demanding in the study of the placenta. Although there are numerous amounts of challenges involved in the \textit{in vitro} models, the models’ abilities can be used to replicate the effects that pharmaceutical drugs are inflicting on the placental barrier. Most importantly, though, applying the placenta-on-a-chip to the study and understanding of the placenta will give rise towards the clarification on how pharmaceutical drugs and other diseases are leading causes of birth defects and mutations in the mother’s placental barrier.

2.2. Human Placenta

The placenta (Figure 2.1) serves as a vital organ in the production and growth of the fetus. The placenta performs as the kidneys, lungs, gut, and liver throughout the duration of pregnancy in order to keep the fetus alive.\cite{16-17} Based on the physiology of the placenta, the placental barrier is structurally defined by the placental trophoblast and fetal capillary endothelium layer, because they are the foundation and building blocks towards the development of other components of the placental barrier.\cite{5} The combination of the placental trophoblast and endothelium layer support the development of the chorionic villi. Throughout most of the pregnancy, the placenta’s chorionic villi serve as the main structural component of the placental barrier, which is composed primarily
Figure 2.1. Human placenta. (a) Fetus in developing stages attached to an umbilical cord and uterine wall. (b) Cross-sectional view of the placenta. (c) Detailed diagram of the cross-sectional view of villus that reveals the placental barrier’s intervillous space, villous endothelial cells, and syncytiotrophoblast. Reproduced with permission from [5]. Copyright © 2016 RSC.

of cytotrophoblasts. Cytotrophoblasts are the inner layer of the trophoblast and the stem cells for the syncytiotrophoblast. The main roles the syncytiotrophoblast plays into the development of the placenta is the synthesis of progesterone and estrogens, and the operation of the endocrine unit. Along with the synthesis and operation of estrogens, progesterone, and endocrine, the syncytiotrophoblast plays a significant role in the production of the placental growth hormone and peptide hormone. To take into consideration, the separation between the cytotrophoblast and the syncytiotrophoblast is largely dependent on the syncytiotrophoblast; the syncytiotrophoblast covers each villus after being compressed into a layer that separates the lucanue from the cytotrophoblast layer. Furthermore, the separation of the two layers creates intervillous spaces that ensure the growth of the fetus. In the course of the developments and processes carried out by the cytotrophoblasts and syncytiotrophoblast, their actions occur throughout the beginning portion of pregnancy, when the fetal organ development occurs in an environment with low oxygen pressure. Scientists need to acquire a great deal of knowledge regarding the physiology behind
the placental barrier in order to mimic the functionalities of the human placenta for pharmaceutical and other experimental purposes.

Understanding the solute across the placenta is critical when examining the placental barrier mechanisms. The migration of solute across the placental barrier, for the most part, begins in the later developments of the fetus when the structural components of the placental barrier are able to withstand the diffusion of fluids. In order for fetal growth to perform efficiently, the exchange of products and nutrients across the placenta is essential. Scientists still struggle to fully understand the inner mechanisms towards the exchange of fetal metabolism over the placental barrier. However, scientists have been able to discover and formulate a significant amount of information that gives researchers a general idea of the inner mechanisms of the placental barrier. In large part, the concentration gradient of the substance, extent, and thickness of the placental barrier are needed for the performance of the transfer of nutrients.\cite{18-19} The paracellular permeability and electrical potential difference are both major factors that come into play when studying the solute exchange across the placenta. Another significant mechanism towards the transferring of nutrients across the placenta are the functions that the transporter protein carries out: transporting glucose, hydrophobic molecules, and amino acids across the placenta. In fact, transporter proteins are located in the plasma membrane along with carriers, which are membrane proteins as well. The purpose of the carriers is to merge themselves with the solute and transport the solute to other parts of the membrane, similarly to the transporter proteins.\cite{20-21} The functionalities and system of exchange of fluids across the placental barrier is an important part of the placenta. In order for researchers to replicate the human placenta for experimental purposes, they must take the diffusion across the placental barrier and the complex physiology of the placenta into consideration when developing the most practical model possible.
2.3. In Vivo

The *in vivo* model for testing is an underdeveloped approach towards the study and testing of pharmaceuticals for the better understanding of the human placenta.\(^{[22]}\) One of the most common and extended methods of testing is the experimentation on animal subjects, especially for medical-related purposes. The use of pregnant animals is a favored *in vivo* model for testing and studying the functionalities and pharmaceutical effects of medicated drugs across the placenta.\(^{[23]}\) Rodents and mammals, like rats\(^{[24-26]}\), rabbits\(^{[27-28]}\), camels\(^{[29-31]}\), and pigs\(^{[32-34]}\) have all served as previous subjects for an *in vivo* model towards the replication of the functionalities and structure of the human placenta. Nonetheless, animal models vary dramatically from human physiology, making data comparisons difficult to match from an animal to a human placenta.\(^{[35]}\) Although scientists have utilized a variety of animal placentas for pharmaceuticals testing, the mouse and sheep placenta are the two *in vivo* subjects that will be discussed in further detail. The sheep and mouse placenta models are chosen for discussion for two particular reasons. One, the mouse and sheep model have proven to be popular approaches towards the evaluation and transability of using their placentas in place of human placentas for pharmaceutical testing, which is evident from the upcoming table and explained in the upcoming sections. Secondly and most importantly, though, the mouse model has a paracellular and extracellular route of exchange similarly with the human placenta,\(^{[36]}\) and the fetal-placental vascular structure in the sheep model shares more resemblances with the human placenta than the majority of other mammals.\(^{[14]}\) Similar physiological characteristics between the mouse, sheep and human placenta make for more effective assessments and support for the *in vivo*’s background in pharmaceutical and functional analysis evaluations.
2.3.1. Mouse and Sheep Model

Researchers of the past and present are still fixated on the idea of testing on animals, believing that genetically mutated animals, particularly the mouse (Figure 2.2b1-b3), can be used to replicate the human placenta (Figure 2.2a1-a3). The mouse is one of the most important, if not the best, subjects for *in vivo* testing of the placenta. The reason is that the mouse placental barrier has one of the closest structures to the human placenta due to the correspondents of both of their designs being a haemochorial type.\textsuperscript{[36]} The use of mouse trophoblast is also an alternative solution for the difficulty in obtaining primary human tissue, because researchers can efficiently use...
trophoblast stem cells to mimic the features of the human syncytiotrophoblasts.\textsuperscript{[38]} In addition to the resembling physiological traits between the two placentas, the mouse and human placenta both have a similar diffusion coefficient of water and allowance of inert hydrophilic solutes. Similar placental physiological exchange between the human and mouse placenta has played a vital role in the development and progression of the mouse model.\textsuperscript{[36]} Not to mention, for most scientists, time is a valuable factor when carrying out experiments and analysis, in which nominates mice a leading candidate for experimentation. The reason is that mice are particularly easy to breed and handle, due to their high reproductive rate and rapid development.\textsuperscript{[39]} Overall, though, scientists have acquired a considerable amount of information regarding the comparable biological and genetic characteristics between the mouse and human placenta in the past few decades, supporting the argument that the mouse model is an appropriate model for study.\textsuperscript{[36]}

While the mouse placenta does provide a comparative advantage over other rodents and mammals when testing the effects of medicated drugs on the placenta, scientists have spent a considerable amount of time evaluating the feasibility of the sheep placenta (Figure 2.2c1-c3) for future pharmaceutical testing. The reason is that researchers have achieved an extensive amount of progress modifying and assessing the sheep placenta for medical and analytical related purposes. For instance, the nutrient metabolism and respiration of the mammalian fetus are a couple of examples of the knowledge humans have gained from studying pregnant sheep.\textsuperscript{[40]} Scientists have also taken advantage of the sheep placenta when testing and exploring the maternal-fetal structure and interactions when developing an \textit{in vivo} model to examine placental nutrient transfer.\textsuperscript{[40]} To mention however, sheep, for the most part, have researchers surgically perform on their placenta when the fetus has reached a substantial weight and the maturity is quite high. In contrast though, the mouse model usually has the scientists genetically modify the mouse placenta
and operate the surgical procedures earlier in the fetal development.\textsuperscript{[41]} Nevertheless, the previous testing for nutrient metabolism and placental nutrient transfer on the sheep placenta, was only made possible due to the shared physiological traits between the sheep and human placenta. Since the sheep and human placenta share alike biological qualities, scientists have been able to conduct and translate medicated-drug related testing from the sheep to the human placenta. Corresponding physiological characteristics can be found in the villous tree of the sheep cotyledon, which shares a similar structure with the villous tree of the human placenta. Additionally, both the sheep and human placentas are structurally divided up into cotyledons and share alike fetal placental vascular structures.\textsuperscript{[42]} Nonetheless, scientists have acquired a significant volume of knowledge regarding the feasibility and structure of the sheep model for \textit{in vivo} pharmaceutical testing, making the following approach a candidate for study. To summarize, as researchers continue taking advantage of the mouse and sheep approaches, scientists will grasp a fuller understanding on how they can manipulate and apply these models towards the better understanding on how medicated-drugs affect the human placenta.

\textbf{2.3.2. Studies Concerning the Mouse and Sheep Model}

Since the mouse and sheep model are studied quite frequently among scientists, researchers have been able to conduct a variety of experiments across the mouse and sheep placentas. Table 2.1 presents an array of experiments pertaining towards the utilization and application of the mouse and sheep models towards the study and evaluation of pharmaceuticals and functional analysis on their placentas. More specifically, Table 2.1 categorizes each work by species, type of testing, details regarding experimentation, and conclusions from the analysis.
Table 2.1. Comparison and summary of mouse and sheep model medicated-drug and inner mechanism testing.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of testing</th>
<th>Details</th>
<th>Conclusions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Toxicity/Drug Testing</td>
<td>Test the effects that lithium, homocysteine, and alcohol in mouse placenta</td>
<td>The mouse placenta can prevent intrauterine growth retardation (IUGR) caused by lithium, homocysteine, and alcohol</td>
<td>[43]</td>
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<td></td>
<td></td>
<td>Evaluated the effects progesterone on Breast Cancer Resistance Protein (BCRP) in mouse placenta in vivo</td>
<td>Increased levels of progesterone did not have any noticeable effects on BCRP</td>
<td>[44]</td>
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<td>Tested for thalidomide-induced limb irregularities in humanized cytochrome P450 3A (CYP3A) mouse model in vivo</td>
<td>Limb abnormalities were recorded, human CYP3A was showed in mouse placenta, concluding that mouse model can predict toxicity in humans</td>
<td>[45]</td>
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<td></td>
<td></td>
<td>Analyze effects that antioxidant Tempol has on mouse model during fetal growth in vivo</td>
<td>Tempol improved the fetal growth in mouse placenta, and increase in the velocity of uterine artery blood flow</td>
<td>[46]</td>
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<tr>
<td></td>
<td>Functional Analysis</td>
<td>Use gestation in trophoblast of a mouse placenta to study subcellular localization and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions</td>
<td>GAPDH may have multiple purposes in trophoblast cells</td>
<td>[47]</td>
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<td></td>
<td></td>
<td>Evaluated venous morphological, functional, and arterial differences in the mouse fetoplacental vascular network</td>
<td>Fetoplacental veins and arteries differ between their branching scaling rules, and fetoplacental resistance is made up 26% of veins</td>
<td>[48]</td>
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<td></td>
<td></td>
<td>Evaluate Interleukin-(IL)11 in mouse placenta, and IL1β and TNFα their effects on placental villous explants</td>
<td>IL11 turned out to be regulated by pro-inflammatory cytokines IL1β, but not TNFα</td>
<td>[49]</td>
</tr>
<tr>
<td>Species</td>
<td>Type of testing</td>
<td>Details</td>
<td>Conclusions</td>
<td>Ref.</td>
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<td>Examined human placental mesenchymal stromal cells (PMSC) in a mouse model <em>in vivo</em></td>
<td>PMSC had positive effects on neovascularization in mouse model <em>in vivo</em></td>
<td></td>
<td>[50]</td>
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<td>Characterized lymphocyte antigen 6, locus E (LY6E) in mouse placenta</td>
<td>Syncytiotrophoblast differentiation is expressed from LY6E and may be found in progenitors</td>
<td></td>
<td>[51]</td>
<td></td>
</tr>
<tr>
<td>Studied parasite dynamics in mouse placental labyrinth layer using intravital microscopy</td>
<td>infected-Red Blood Cells (iRBCs) can be internalized by trophoblast cells, and quick removal of particles inside blood flow is a characteristic of placental blood flow</td>
<td></td>
<td>[52]</td>
<td></td>
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<tr>
<td>Evaluate <em>Cited2’s</em> effects on vascularization and trophoblast formation on mouse placenta <em>in vivo</em></td>
<td>Normal placental vascularization and development requires <em>Cited2</em> to promote embryo growth</td>
<td></td>
<td>[53]</td>
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<tr>
<td>Examined how transcriptional co-repressor TLE3 promotes development if giant trophoblast cells in mouse placenta</td>
<td>Growth and differentiation of trophoblast giant cells is regulated by TLE3, and TLE3 have placenta deficiencies</td>
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<td>[54]</td>
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<tr>
<td>Tested Argonaute 2 (AGO2) effects on differentiation mouse Embryonic stem cells <em>in vivo</em></td>
<td>Extra-embryonic endoderm genes need AGO2, and AGO2 plays a significant role in stem cell differentiation</td>
<td></td>
<td>[55]</td>
<td></td>
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<tr>
<td>Evaluated the Breast Cancer Resistance Protein (BCRP) in mouse placenta <em>in vivo</em></td>
<td>Mouse placenta expressed the fact that BCRP limits the effects of medicated-drugs during pregnancy</td>
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<td>[44]</td>
<td></td>
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<tr>
<td>Species</td>
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<td>Conclusions</td>
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<td></td>
<td></td>
<td>Studied the transfer of maternal cholesterol in mouse <em>in vivo</em></td>
<td>The transfer of maternal cholesterol was evident in the mouse, and the yolk and placenta are the sites for intermediate passage</td>
<td>[56]</td>
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<tr>
<td>Sheep</td>
<td>Toxicity/Drug Testing</td>
<td>Evaluated triclosan in sheep placenta</td>
<td>Triclosan is a potent inhibitor of sulfotransferase in the sheep placenta, and reduces the total placental estrogen secretion</td>
<td>[57]</td>
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<td></td>
<td></td>
<td>Examine the uptake of transdermal fentanyl in pregnant sheep</td>
<td>The fentanyl transfer rate was 77%, and recorded low pain scores after laparotomy and hysterotomy</td>
<td>[58]</td>
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<td>Analyze the rosiglitazone in the sheep plasma and the effects rosiglitazone has on pregnant sheep <em>in vivo</em></td>
<td>Study proves to be ideal for pharmacokinetic studies in sheep model, and successfully applied the determination of rosiglitazone in pregnant sheep</td>
<td>[22]</td>
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<td></td>
<td>Functional Analysis</td>
<td>Tested for the enhancement of the cytokine expression from maternal obesity (MO) in sheep placenta</td>
<td>MO increased the placental inflammatory responses of kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK)/c-Jun, thence enhancing cytokines</td>
<td>[59]</td>
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<tr>
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<td></td>
<td>Test the effects that human Amnion Epithelial cells (hAEC) have on sheep acute inflammatory response <em>in vivo</em></td>
<td>hAEC does not significantly alter the acute fetal inflammatory response in pregnant sheep</td>
<td>[60]</td>
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</tbody>
</table>
Table 2.1 portrays an array of studies towards the evaluation of medicated-drugs, toxins and the functional analysis of the mouse placental barrier in vivo. As illustrated, researchers have been able to test a few medicated drugs like Tempol \(^{[46]}\), progesterone \(^{[44]}\), thalidomide \(^{[45]}\) across the mouse placenta, and mimic a substantial quantity of the human placental features. More importantly, the collection of these data results on the mouse model proves to be vital when
comparing the results towards previous data from the effects of these medicated drugs have on the human placenta.\[39\] Fortunately, results in correlation with the effects that Tempol, progesterone, and thalidomide have on fetal growth and glucose metabolism showed evident similarities towards the natural reactive effects they impose on the human placental barrier. To acknowledge, however, Tempol, progesterone, and thalidomide are popular medications that most pregnant women consider throughout their pregnancy and require to be studied more often for behavioral interactions with the fetus. Nonetheless, if it was not for the mouse alternative tissue in place for human tissue, the imitation of Tempol, progesterone, and thalidomide across the placenta would not be as efficiently carried out as with other rodents and mammals.\[38\] In addition to medicated-drug testing on the mouse placenta, there is an immense amount of evaluating pertaining towards the functional analysis and inner structure of the mouse placenta, retaining towards the transability of using the mouse placenta in place of the human placenta for pharmaceutical testing. For example, scientists tested mesenchymal stromal cells \[50\], maternal cholesterol \[56\], proteins \[44\], and other characteristics across the mouse placenta in order to decide if the mouse placenta shares comparable biological characteristics with the human placenta. Based on data and conclusions of Table 2.1, researchers found that the mouse placenta shares similar protein and cellular features with the human placental barrier. Thus, the mouse placenta may be an appropriate model for medicated-drug testing in the near future, because of the shared qualities in physiological features and medicated-drug reactions with the human placenta.

While the mouse placenta has had a substantial amount of testing performed across the model’s placenta, the sheep approach has proven to have a solid record of accomplishment of assessing as well. Ever since researchers have been taking advantage of the sheep placenta, they have been able to evaluate the inner mechanisms of the sheep placental barrier, and the effects that
triclosan\(^{[57]}\), rosiglitazone\(^{[22]}\), and transdermal fentanyl\(^{[58]}\) have on fetal growth and glucose metabolism. Based from Table 2.1, there has been a substantial amount of accomplishments pertaining towards the evaluation of the functional analysis and inner mechanisms of the sheep placental barrier \textit{in vivo}. Similarly, with the mouse model, from fluid transfer to membrane and protein examinations, the sheep model has been a reliable method for the study and transability of the model’s functional analysis for the better understanding of the human placenta. Nonetheless, there have also been a few medicated tests performed across the sheep placental barrier. Those tests pertain towards the evaluation of triclosan, transdermal fentanyl, and rosiglitazone have on the sheep placenta. To make notice, triclosan, transdermal fentanyl, and rosiglitazone are common medications that women take during pregnancy and have a modest volume of information on their implications on fetal growth. Nevertheless, the medicated-drug testing conclusions from Table 2.1 revealed whether the sheep placenta is an alternative and suitable approach for medicated-drug testing in place of the human placenta. Fortunately, results in correlation with the effects that triclosan, transdermal fentanyl, rosiglitazone have on fetal growth and glucose metabolism demonstrated evident resemblances towards the normal reactive effects they impose on the human placental barrier based from the conclusions of their research in Table 2.1. Therefore, the sheep placenta may also be an appropriate model for pharmaceutical experimentation in the upcoming future, considering the human placenta’s similar qualities in medicated-drug reactions and physiological features. Moreover, given the fact that scientist have executed a countless amount of functional analysis tests and a few pharmaceutical evaluations on the sheep and mouse models, supports the argument that both models have the potential to be alternative approaches for studying the human placenta \textit{in vivo}. 
2.3.3. Mouse Model Limitations

Figure 2.3. Compares the physiology between the human and mouse placenta. (a) Section-views of the mouse and human placenta that reveal the similarities and differences between the generic physiologies of the two placentas. (b) Detailed views of the mouse and human placenta that compare and contrast the microscopic physiological characteristics of the two placentas. Reproduced with permission from [67]. Copyright © 2013, Elsevier.

Despite the mouse advantages as serving as an *in vivo* model for testing, the mouse placenta has only partially been able to prove the vitally of being an essential source for studying the effects of several medicated drugs across the placenta. The association towards the invalidity of the mouse placenta serving as a model for replicating the human placenta is because the physiology of the mouse placenta distinguishes itself quite substantially from the human placenta. The structure of the mouse placenta compares differently to the human placenta due to the mouse placenta consisting of labyrinthine and junctional zones (Figure 2.3). Another reason why the physiology behind the mouse and human placenta differentiate is because the mouse placenta consists of a three-layered trophoblast (haemotrichorial) while the human placenta consists of a single syncytiotrophoblast zone and layered trophoblast (haemomonochorial).[36, 39, 68] In addition to the
physiological trait variances between the two placentas, scientists are still struggling to replicate the alveolar growth and expansion in the mouse placenta, based on their own prototype alveologenesis. Not to mention, there is still no clear explanation why researchers cannot develop an intact basal membrane that can organize itself into the correct human placental tissue.\(^{[39]}\)

Contrasting physiology and the inability for scientists to duplicate the biological qualities between the mouse and human placenta are contributing factors towards comparison error and irrelevant data. In this case, the results from the tests will have limited valuable data for any success in creating a stable method for the testing of pharmaceutical drugs across the placenta. In order for the \textit{in vivo} model to become completely practical, the model must recapitulate the functionalities and physiology of a human placenta in almost every way possible, ensuring that the model can be vital towards the testing of pharmaceutical drugs.

Since scientists are struggling to find a solution that diminishes the contrasting physiological barriers between the mouse and human placenta, researchers have been unable to develop a concrete or practical procedure that can carry on any significant medicated-drug tests.\(^{[36]}\)

For example, even though scientists have evaluated Tempol, progesterone and thalidomide on the mouse placenta, researchers have been unable to perform a broad range of pharmaceuticals across the mouse placental barrier. For instance, the testing of diabetic or human immunodeficiency virus (HIV) medication — two commonly prescribed medicated-drugs to pregnant women — across the mouse placenta should be taking place more often by now, considering that scientists have been working on the \textit{in vivo} mouse model for decades.\(^{[69-70]}\)

Besides all the pessimism that surrounds the mouse model, researchers have successfully devoted the majority of their time trying to modify these mice to mimic human functionalities (Table 2.1), but should consider assessing the effects of pharmaceutical drugs on the mouse placenta more frequently.
2.3.4. Sheep Model Limitations

While there has been a substantial quantity of testing accomplished on the sheep placenta, the model is an underdeveloped approach towards the testing of medicated-drugs on the sheep placental barrier. One reason, particularly, is the fact that the physiological structure and glucose transfer between the sheep and human placenta distinguishes from each other quite substantially. For example, sheep have specialized territories of well vascularized, while the human placenta has a conceptus that invades into the uterine wall. Sheep also have a considerable amount of individual attachment sites composed of the maternal caruncle and fetal cotyledon; however, the human placenta has a large discoid placenta. In addition to the differences in physiological traits between the two placentas, glucose transporter proteins, GLUT-1 and GLUT-3, are developed and regulated in the sheep placenta but not in the human placenta. Not to mention, GLUT-1 is limited to the base of the syncytial layer of the sheep placenta, but localized in the vascular endothelial cells of the human placenta. This variation in placental structure and glucose transfer gives most scientists less motivation to carry out this method, because of the raised concerns in the invalidity of the sheep placenta serving as a model for replicating the human placenta.[42]

Let alone, although there has been success towards the testing of mineral traces, maternal-fetal structure and interactions, and transdermal fentanyl and triclosan in the sheep placenta approach, scientists have executed a modest number of pharmaceutical tests on the sheep placenta. Researchers have been spending the majority of their time testing the feasibility of the sheep placenta model in order to prove that their models will produce the same natural reactions from pharmaceutical intake that occurs in the human placenta. In order for the sheep model to become fully practical, the model first must be able to completely replicate the physiology and functionalities of the human placenta.[42] If not, then the tests will have no significance towards the comparison of data from the performance of pharmaceutical analyses on the sheep placenta to
the human placenta. Fortunately, scientists have been testing and ensuring that the sheep model imitates the similar physiological functionalities with the human placenta (Table 2.1), even though the structure between the two placentas differentiate from each other significantly. Nonetheless, researchers could consider the testing of more pharmaceuticals across the sheep placenta in the upcoming years to ensure that there is a balance between functional analysis and pharmaceutical experimentation on the sheep placenta. Furthermore, the \textit{in vivo} model of the sheep placenta is in the development stages of replicating the physiology of the human placental barrier, which has been limiting the potential testing of medicated-drugs on the model’s placenta.

2.3.5. Future of \textit{In Vivo} Model Development

Although the practicality of the \textit{in vivo} model for the testing of pharmaceutical drugs on the placenta has been tested in many unique and complicated ways, the use of animals for clinical and experimental trials is facing an extensive loss in support and disappreciation, due to the model’s invalidity of transability towards human study.\textsuperscript{[71-72]} The use of animals for the testing of pharmaceuticals is considered doubtful due to the difference in placentation between humans and other animals. Rodents and mammals also lack the surplus quantity of trophoblast in the villous structure within the myometrium and endometrium of the placenta. Moreover, financial and ethical constraints, from testing on animals, tend to play a critical role in the lack of resources and motivation for researchers to run trials and experiments on the placenta.\textsuperscript{[73-74]}

The leading reason why researchers are still analyzing this method for studying the human placenta is that drug responses can be predicted and tested most accurately \textit{in vivo}.\textsuperscript{[71]} Nevertheless, the replacement for the use of the \textit{in vivo} approach for testing and examining the human placenta will be largely due to the progress and improvement on the placenta-on-a-chip.\textsuperscript{[35]} The \textit{in vivo} method is the most primitive methods for studying the placenta, and as explained before, the \textit{in
\textit{vivo} model has not obtained a significant amount of progress in tested pharmaceuticals. In large fault, though, scientists are still in the development stages of the mouse, sheep, and other animal models, in which the models are still being improved upon in order for more pharmaceutical testing to take place.\cite{22} Nonetheless, although the \textit{in vivo} approach has seen a substantial amount of progress throughout the past couple of decades, the \textit{in vivo} method lacks current resources and knowledge to carry out an effective universal procedure for \textit{in vivo} model consisting of mice, sheep, and other animals. The testing of animal subjects may seem prominent for other medicated applications; however, because of the complexity of the placenta, the existence of a developed \textit{in vivo} method for testing the durability and validity of pharmaceutical drugs could potentially lose support in the near future. To ensure the continuation and support for the \textit{in vivo} approach, scientists could consider the testing of more pharmaceuticals on their animal models in the upcoming future.

\section*{2.4. Ex Vivo}

The utilization of the \textit{ex vivo} approach has been witnessing a decline in the scientists’ ability to develop a practical perfusion model that can test pharmaceuticals on real human placenta tissue. For clarification, \textit{ex vivo} means the testing on a live organism is done outside of the host, while \textit{in vivo} and \textit{in vitro} studies are conducted in the living host and cultured system, respectively.\cite{35} The \textit{ex vivo} model serves as possibly one of the most frequently tested, but complicated procedures when engaging in the testing of pharmaceutical drug intake of the placenta. However, most scientists prefer this model over the \textit{in vivo} method towards the study of recapitulating the features of the human placenta.\cite{35} The reason is that in the fetal and maternal umbilical plasma of a human, calculating the course of a drug is nearly impossible \textit{in vivo};
however, the \textit{ex vivo} has proven to be successful to calculate the course of a drug.\cite{75} Nevertheless, likewise with the \textit{in vivo} method for replicating the human placental barrier, the \textit{ex vivo} model of testing is found most frequently in mature placental subjects.\cite{76}

While the \textit{ex vivo} and \textit{in vivo} approaches share several similarities and differences, the perfusion model itself has a range of advantages towards the study of the human placenta. A few of the primary benefits of the human placental perfusion is that the model offers information about placental storage, metabolism, transplacental transfer and vascularization.\cite{35} Another benefit of the \textit{ex vivo} model is the fact that the approach does not cause severe damage to the maternal vasculature due to being torn from the uterine wall. The cause of interindividual fluctuation in the transplacental transfer is an additional benefit that has also been characterized by the \textit{ex vivo} perfusion model.\cite{77-78} The most essential factor when considering the applicability of the \textit{ex vivo} model is the representation of real human tissue, due to the fact, that real human tissue plays a vital role in the performance of perfusion experimental studies.\cite{77-79} The representation of real human tissue is the justification for why researchers have frequently been using and improving this method, in which their experiments will include the natural reactions that the placenta releases \textit{in vivo} during pharmaceutical drug intake.

\subsection*{2.4.1 \textbf{Ex vivo Perfusion Model}}

The following models are a human placental perfusion system (Figure 2.4a) and dual placenta perfusion model (Figure 2.4b1 and b2) from \textit{Malek et al.} \cite{80} and \textit{Conings et al.} \cite{81}, respectively, who adopted the first \textit{ex vivo} perfusion model developed by \textit{Panigel et al.} \cite{82} and later modified by \textit{Schneider et al.} \cite{83}, \textit{Myllynen et al.} \cite{84}, \textit{Mathiesen et al.} \cite{12,85}, \textit{Myren et al.} \cite{35}, and others research groups. Circulating (Figure 2.4) and non-recirculating perfusion are the two
most prominent types of ex vivo placental perfusion systems. Yet, the circulating model has proved to be the suitable approach when testing perfusion across the placental barrier.\cite{77-78} For example, the procedure carried out by Myllynen et al.\cite{79} for the experimentation of the ex vivo model to test antipyrine concentration was a major success in reconstructing the placental perfusion model. Since Myllynen et al. have had a substantial amount of success pertaining towards the utilization of their perfusion model, the construction of their perfusion model can be used as an example for the development of an ex vivo placental perfusion.\cite{79} Or, the following studies listed in the next
section can be investigated for further information on the construction of an *ex vivo* placental perfusion.

### 2.4.2. Studies Concerning Ex Vivo Perfusion Model

Considering the fact that the *ex vivo* perfusion model is studied quite frequently among scientists, researchers have been able to conduct a variety of experiments in *ex vivo*. Table 2.2 presents an array of experiments pertaining towards the utilization and application of the *ex vivo* perfusion model towards the examination and evaluation of pharmaceuticals, toxins and the inner mechanisms of the placental barrier in *ex vivo*. More specifically, Table 2.2 categorizes each work by type of testing, details regarding the study, and conclusions from analysis.

Table 2.2. Summary of *ex vivo* perfusion model pharmaceutical and functional analysis experiments.

<table>
<thead>
<tr>
<th>Type of testing</th>
<th>Details</th>
<th>Conclusions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicity/Drug Testing</td>
<td>Testing for antipyrine concentrations in <em>ex vivo</em></td>
<td>Results revealed that the antipyrine concentrations of both the maternal and fetal concentrations ended up equal, revealing the success of a circulating perfusion model and the model’s placental transfer</td>
<td>[79]</td>
</tr>
<tr>
<td></td>
<td>Examined the fetal drug compartment concentrations by exposing the placental-trophoblastic barrier with a variety of carboplatin concentrations</td>
<td>Revealed that fetal kidney cells are affected by the carboplatin and efficiently mimicked carboplatin in real human tissue.</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>Determined the extent and rate of maternal-fetal transplacental passage of bromocriptine (BCT) in <em>ex vivo</em></td>
<td>Concluded that only trace amounts of BCT could transport across the human placenta</td>
<td>[87]</td>
</tr>
<tr>
<td>Type of testing</td>
<td>Details</td>
<td>Conclusions</td>
<td>Ref.</td>
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<tr>
<td>Use the <em>ex vivo</em> for testing the placental transfer of maraviroc</td>
<td>Proved to be successful and relatable to the <em>in vivo</em> model, due to the accurate ratios in the maternal blood samples and umbilical cord</td>
<td></td>
<td>[88]</td>
</tr>
<tr>
<td>Test placental transfer of rosiglitazone in perfusion model</td>
<td>Discovered that rosiglitazone passes through the placenta at a low rate, and there is minimal fetal accumulation and transfer of rosiglitazone in the placenta</td>
<td></td>
<td>[89]</td>
</tr>
<tr>
<td>Measure the transfer of lamivudine across the human placenta in <em>ex vivo</em></td>
<td>By simple diffusion, lamivudine appears to passover the placenta</td>
<td></td>
<td>[90]</td>
</tr>
<tr>
<td>Measure the transfer of lamivudine across the human placenta with zidovudine</td>
<td>Appearance of lamivudine is not altered by the presence of zidovudine in the placenta</td>
<td></td>
<td>[90]</td>
</tr>
<tr>
<td>Tested placental transfer of enfuvirtide in perfusion model</td>
<td>Enfuvirtide placental transfer was not detected or observed, concluding that enfuvirtide can be used in human immunodeficiency virus (HIV)-infected pregnant women without causing harms to the fetus</td>
<td></td>
<td>[91]</td>
</tr>
<tr>
<td>Evaluate the maternal-fetal transfer of granisetron in <em>ex vivo</em> perfusion model</td>
<td>Granisetron has concentration dependent placental transfer, and at systemic granisetron concentrations there is no placental transfer detected</td>
<td></td>
<td>[92]</td>
</tr>
<tr>
<td>Test the human placental transfer of hydralazine in <em>ex vivo</em> perfusion model</td>
<td>Hydralazine crosses the <em>ex vivo</em> perfusion model readily, and that the absorption of hydralazine in the fetal compartment was in linear relationship with the maternal concentration</td>
<td></td>
<td>[93]</td>
</tr>
<tr>
<td>Examined the transportation of metformin across the placenta using <em>ex vivo</em></td>
<td>Metformin displayed a higher transport rate from the fetal-maternal side, and is mediated by a carrier that is likely apart of the organic cation transporter family</td>
<td></td>
<td>[94]</td>
</tr>
<tr>
<td>Type of testing</td>
<td>Details</td>
<td>Conclusions</td>
<td>Ref.</td>
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<tr>
<td>-----------------</td>
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</tr>
<tr>
<td>Tested the maternal-fetal transfer of saquinavir in perfusion model</td>
<td>Low rates of placental transfer of saquinavir were calculated, revealing that the use of this drug will possibly not be exposed significantly to the fetus</td>
<td>[95]</td>
<td></td>
</tr>
<tr>
<td>Analyzed the placental transfer of pioglitazone and sitagliptin in <em>ex vivo</em></td>
<td>Significant fetal accumulation of pioglitazone, clearance indexes, and extended half-life of pioglitazone and sitagliptin are not tolerable for treating diabetes during pregnancy</td>
<td>[96]</td>
<td></td>
</tr>
<tr>
<td>Evaluated the transplacental transfer of oseltamivir using <em>ex vivo</em> perfusion model</td>
<td>The transplacental transfer of oseltamivir phosphate was detected significantly, revealing that the results might have clinical importance</td>
<td>[97]</td>
<td></td>
</tr>
</tbody>
</table>

**Functional Analysis**

<table>
<thead>
<tr>
<th>Details</th>
<th>Conclusions</th>
<th>Ref.</th>
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</thead>
<tbody>
<tr>
<td>Characterize placental oxygenation levels in <em>ex vivo</em></td>
<td>Heterogeneity was displayed through intervillous space (IVS) oxygen mapping, and discovered that whole placental lobules displayed 3D placental oxygenation maps at great resolution</td>
<td>[98]</td>
</tr>
<tr>
<td>Analyzed the feto-placental vasculature of the placenta using perfusion model</td>
<td><em>Ex vivo</em> model can efficiently evaluate the dependency of Doppler ultrasound clinical measures for placental obstruction</td>
<td>[99]</td>
</tr>
<tr>
<td>Examined the transportation of immunoglobulin G (IgG) in <em>ex vivo</em></td>
<td>The human placenta develops a specific transport mechanism for IgG for the fetal and maternal direction</td>
<td>[100]</td>
</tr>
<tr>
<td>Examined the transportation IgG subclasses in <em>ex vivo</em></td>
<td>Transfer rates for all four classes varied. IgG₁ had the preferential transfer, while IgG₂ had the slowest transfer</td>
<td>[100]</td>
</tr>
<tr>
<td>Evaluated the differences of placental elasticity between normal and IUGR pregnancies using perfusion model</td>
<td>IUGR group displayed more histopathological changes and were stiffer than the control group, and that reduced placental elasticity could be the cause of these differences</td>
<td>[101]</td>
</tr>
</tbody>
</table>
Most of the following tests summarized in Table 2.2 use the circulating placental diffusion model for all experimental analysis, and carry out the perfusion assembly as mentioned in the previous section. However, there are perfusion composition differences between the experiments, which are not necessarily accounted for. Furthermore, previous ex vivo studies have proven to be beneficial when studying the causes and effects that certain pharmaceutical drugs have on the transplacental passage. Many of the pharmaceutical drugs relate towards anti-epileptic drugs and HIV protease inhibitors, which have been tested on the ex vivo model since the mid-1990s. As well as the testing and validating of anti-epileptic drugs and HIV protease inhibitors, the ex vivo model has been used to study and test the transplacental passage of exogenous and endogenous compounds and the examination of the transfer of amino acids in the placenta. Additionally, the replication of the effects of diseases like cancer and other drugs have been used as a model for assessing the effectiveness of the placental perfusion. Placental perfusion based on Table 2.2, and the ex vivo model in general, has proven to successfully replicate carboplatin in the human tissue, evaluate the maternal drug transfer across preterm birth, and analyze the functional components of the placental barrier on human placental tissue. In these cases, the ex vivo perfusion model has successfully analyzed for the reactive behavior released from the human placental tissue, and provided scientists accurate understandings on how glucose transfer and placental elasticity affect fetal growth. In addition, researchers have been able to use this

<table>
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<th>Type of testing</th>
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<th>Conclusions</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Examined the maternal transfer of Interleukin-6 in the ex vivo perfusion model</td>
<td>Minimal maternal to fetal transfer of Interleukin-6 was observed from the ex vivo perfusion model</td>
<td></td>
<td>[102]</td>
</tr>
</tbody>
</table>
knowledge to attain new understandings on how these drugs affect the human placenta, and what can be done to ensure that these drugs do not impose any harm on the mother and fetus. Thus, what can be concluded from the success of the \textit{ex vivo} model is that the placental perfusion process has not been a misfortune in the model’s development.

\subsection*{2.4.3. Future of Ex Vivo Model Development}

The \textit{ex vivo} model may present many significant contributions towards the study of the placenta and the effects that pharmaceutical drugs impose on the placental barrier; however, the \textit{ex vivo} approach does not have a standardized placental perfusion method, due to the difference in perfused compositions.\cite{75} Unfortunately, the \textit{ex vivo} model does not weigh placental transfer across the entire pregnancy, and cannot necessarily recapitulate the binding to serum proteins \textit{in vivo} conditions such as rilpivirine, a compound used to study placental transfer.\cite{104} The potential of the \textit{ex vivo} placental perfusion model has limitations, such as having a laborious placental perfusion set-up procedure and cannot handle the broad scale of chemical testing and screening.\cite{79} Not to mention, the \textit{ex vivo} model does not last long, because of the model’s short lasting performance and output of experimental results.\cite{73} Currently, the model can last approximately from 2-4 hr to 6-8 hr for short and long experiments, respectively.\cite{35} Comparatively, \textit{in vitro} studies do not include the usage of real human tissue, allowing experiments to last for days on end usage.

Although one could argue that the practicality of the \textit{ex vivo} model has proven to be more sustainable than the \textit{in vivo} approach, the perfusion model does not show the same compatibility as the placenta-on-a-chip. Likewise, with the \textit{in vivo} sheep and mouse model, the perfusion model does not necessarily have a concrete model that can test the effects that medicated drugs and other diseases can impose upon in the placental barrier. To argue rather, the perfusion model has gained
significantly more progress in medicated-drug testing than the \textit{in vivo} approach and has had more success with the model’s experiments dealing with the replication of antipyrine, placental transfer of maraviroc, and maternal drug transfer across preterm birth. Support for this claim can be found and exemplified in the variances in quantities of medicated-drug testing between Table 2.1 and Table 2.2. Nevertheless, the model has limitations and is arguably a difficult procedure to carry out effectively due to the short-lasting performance of the model, time composition, inability to handle a considerable amount of chemicals, and other constraints. In order for the \textit{ex vivo} model to not see a decline in the support in the placental perfusions continuation to run tests for pharmaceutical effects across the placenta tissue, scientists should consider developing a fully practical and universal model that diminishes the differences between perfused compositions and other limitations. In large fault though, the \textit{ex vivo} approach will likely see a decline in the support of the model’s experiments, due to the increasing interest and improvements in the \textit{in vitro} approach.

\textbf{2.5. Placenta-on-a-Chip}

\textbf{2.5.1. Microfluidic Devices}

The placenta-on-a-chip model (Figure 2.5) is the closest to the completion of a practical model that can replicate the physiology and functionality of the human placenta for the testing and transability of pharmaceutical intake from an \textit{in vitro} model to an \textit{in vivo} human placenta. Microfluidic devices serve as the foundation and system for the testing and analysis of pharmaceuticals in the \textit{in vitro} model. The development of the distinct physical setting of the microfluidic devices enables the ability for researchers to culture and manage the cellular and subcellular environments, and maintain a definite arrangement of the cells.\cite{106-107} Microfluidic
Figure 2.5. (a) An image of the inner structure of the microfluidic device that includes the poly(dimethylsiloxane) (PDMS) microchannels. (b) The physical setting of a common placenta-on-a-chip. The maternal (red) and fetal (blue) microchannels are separated by a vitrified collagen (VC) membrane in the center of the chip. Scale bar, 1 cm. Reproduced with permission from Miura et al. / CC BY

systems additionally allow researchers to gather the most significant amount of data in the shortest period of time, reduce sample utilization, and mimic the flow of fluids in parallel layers. A large portion of the microfluidic device’s abilities were developed and managed by a handful of research groups, Sadao et al., Matsunaga et al., Takinoue et al., Kiriya et al., Onone et al. and Kazayama et al., who have spent the past decade developing and modifying the microfluidic devices for drug, cellular, and tissue analytical simulation studies. Moreover, many of these advantages are influenced by the chip’s capability to structurally withstand the co-culturing of endothelial and trophoblast cells.

An important component of the microfluidic device, and the in vitro model in general, is that the placenta-on-a-chip requires the representation of endothelium and trophoblastic epithelium in order for the replication of the fetal and maternal interfaces of the placenta. When the endothelium and trophoblastic epithelium in the human placenta are represented accurately, the in vitro model can perform a variety of tests that replicate the physiology of the placental barrier, and the circulation of pharmaceutical intake and diseases between the mother and fetus. The growth factors, cytokines, hormones, nutrients, intercellular junctions, and extracellular matrices are all
additional examples of soluble and insoluble aspects that can be mimicked through the use of a microengineered co-culture model of a placenta-on-a-chip.\textsuperscript{[72]}

Data and tests have also proven well for the microfluidic devices, and have opened the doors to a realm of organs-on-chips\textsuperscript{[120]}: bone-on-a-chip\textsuperscript{[121]}, liver-on-a-chip\textsuperscript{[122]}, body-on-a-chip\textsuperscript{[123]}, kidney-on-a-chip\textsuperscript{[124]}, lung-on-a-chip\textsuperscript{[125]}, cancer-on-a-chip\textsuperscript{[126]}, heart-on-a-chip\textsuperscript{[127]}, and brain-on-a-chip\textsuperscript{[128]}. The following organs-on-chips reveal that the on-a-chip process is used quite frequently in the medical industry, and is becoming ever so demanding for the better understanding of the human body. For example, the in vitro models have proven to successfully develop treatments for diabetes, leukemia, and hyper-cholesterol.\textsuperscript{[74]} Nonetheless, most scientist can agree upon that the microengineering of the placenta-on-a-chip serves as the most practical model that requires the smallest budget compared to the ex vivo and in vivo, and procedure to test pharmaceutical intake on the in vitro model’s cultured cells and membranes. Overall, though, the 3D cell-culture model proves to be compatible and capable of withstanding the ability to support tissue differentiation, cell growth, and growth factors. In this case, there has been a growing interest in the placenta-on-a-chip’s ability for scientists to directly manipulate human endothelial and trophoblast cells.

![Microfluidic device schematic](image_url)

Figure 2.6. The following microfluidic device was tested for bacterial infection. (a) The following schematic depicts a diagram of a cross-sectional view of the microfluidic device; a porous membrane separates the maternal and fetal channels in the microfluidic device. (b) The maternal and fetal sides were composed of BeWo cells and HUVECs, respectively. (c) Illustrating how Bacteria commonly passes through the placental barrier to the fetus, causing nutrients and gasses to be exchanged.\textsuperscript{[129]} Copyright © 2018 American Chemical Society.
The microfluidic device (Figure 2.6) serves as the primary support for the placenta-on-a-chip and must be fabricated efficiently before cell culture, cell seeding in the chip, and analysis of the placental barrier can be performed.[5] Several research groups, including Miura et al.[105], Lee et al.[130], Blundell et al.[5, 131], Zhu et al.[129], Yin et al.[132], and Pemathilaka et al.[133], have carried out the previously stated procedures for their placenta-on-a-chip. In this case, the groups were able to successfully prepare and combine microfluidics and biology, produce confluent layers of endothelial and trophoblast cells within the chip’s channels, and perform experimental analyses on their placenta-on-a-chip. Therefore, their experimental methods will be elaborated in the next few sections.

Table 2.3. Summary and comparison of placenta-on-a-chip experiments.

<table>
<thead>
<tr>
<th>Type of Testing</th>
<th>Details</th>
<th>Cultured Cells</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microvilli formation and Vanilloid</td>
<td>Fluid shear stress (FSS) triggered microvilli formation in BeWo cells</td>
<td>BeWo cells (maternal channel)</td>
<td>[105]</td>
</tr>
<tr>
<td>family type-2 (TRPV6)</td>
<td>and human villous trophoblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exposing BeWo cells to FSS caused the development of a functional</td>
<td></td>
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<tr>
<td></td>
<td>microvilli with GLUT1</td>
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<td></td>
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<tr>
<td></td>
<td>TRPV6 played a significant role in FSS-induced Ca(^{2+}) influx</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Calcium signaling regulated apical localization of Ezrin</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Placenta-on-a-chip can effectively replicate a human placental</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>barrier microvilli surface in vitro</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Microfluidic device exhibits the polarized localization of multidrug</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>resistance-associated proteins and multidrug resistance proteins</td>
<td></td>
<td></td>
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<tr>
<td>Type of Testing</td>
<td>Details</td>
<td>Cultured Cells</td>
<td>Ref.</td>
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<tr>
<td><strong>Glucose</strong></td>
<td>Glucose was not affected by the JEG-3 cells and HUVECs interactions</td>
<td>JEG-3 cells (maternal channel)</td>
<td>[130]</td>
</tr>
<tr>
<td></td>
<td>Glucose transporters discovered within the cultured cell lines played a critical role in glucose transfer</td>
<td>HUVECs (fetal channel)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Confluent layers of trophoblast and endothelial cells were determined to be present throughout the device</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose crossed through the vitrified collagen membrane with little to no interference</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Placenta-on-a-chip recapitulates the characteristics on an <em>in vivo</em> human placenta when confronted with glucose transfer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Microchip has the potential to serve as a model for replicating the critical components of placental disorders and evolving into a platform for disease related-testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>Analyzed microvilli formation and intercellular junctions</td>
<td>BeWo b30 cells (maternal channel)</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td>Trophoblast channel-mediated glucose transport</td>
<td>HPVECs (fetal channel)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetal channel plays an underlying role in glucose transfer</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>The morphological and functional differentiation of BeWo cells reconstructed the syncytium of an <em>in vivo</em> human placenta</td>
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<tr>
<td></td>
<td>Model allows for precise management of trophoblast and endothelial cells and microvilli to analyze, visualize, and replicate the human placental structure <em>in vitro.</em></td>
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<td>Type of Testing</td>
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<td>Cultured Cells</td>
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<tr>
<td>Glyburide/Heparin</td>
<td>Microvilli generation on the apical side of the maternal channel</td>
<td>BeWo b30 cells (maternal channel)</td>
<td>[131]</td>
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<td></td>
<td>Heparin passage was efficiently deflected from crossing the fetal channel</td>
<td>HPVECs (fetal channel)</td>
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<td></td>
<td>BeWo cells were dominant in the administration of glyburide</td>
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<td>Syncytialization across the entire monolayer was not reachable</td>
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<td></td>
<td>Microfluidic device is capable of analyzing glyburide and heparin transport across the human placental barrier in vitro</td>
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<td>Model could potentially examine the alternations in placental tissue during pathogen, toxin, and disease-derived biomolecule exposure</td>
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<tr>
<td>Bacterial infection</td>
<td>BeWo cells formed the desired microvilli profile</td>
<td>BeWo cells (maternal channel)</td>
<td>[129]</td>
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<td>GLUT1 was expressed highly in the maternal channel</td>
<td>HUVECs (fetal channel)</td>
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<td>Bacterial infection stimulated the maternal channel’s inflammatory cytokines</td>
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<td>Bacterial infection encouraged the stimulation of maternal macrophages</td>
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<td>Placenta-on-a-chip can model the complicated inflammatory responses in human placenta in vitro</td>
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<td>Microfluidic device has the potential to incorporate</td>
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<td>Type of Testing</td>
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| Nanoparticles   | TiO₂-NPs accumulated and transferred across the maternal channel  
The integrity of cell junction, production of reactive oxygen species (ROS), and adhesion of maternal macrophages were explored  
Microvilli formation in trophoblastic cells was examined  
GLUT1 was expressed in the maternal channel  
No dramatic alternations in ROS production were detected  
Microchip can be used as a platform to investigate nanoparticles in human placenta *in vitro*  
Model possesses the capability to examine food safety and environmental toxins in a human placental barrier *in vitro* | BeWo cells (maternal channel)  
HUVECs (fetal channel) | [132] |
| Caffeine        | Cell detachment was reported to cause fluctuations in caffeine concentrations  
Semipermeable membrane integrity proved efficient overtime  
Caffeine rates warranted further investigation  
Caffeine absorption was identified across the edges of the maternal channel’s PDMS walls  
Placenta-on-a-chip can be used as a platform for investigating caffeine concentrations in human placenta *in vitro* | BeWo cells (maternal channel)  
HUVECs (fetal channel) | [133] |
Through the following studies listed in Table 2.3, Miura et al.\cite{105}, Lee et al.\cite{130}, Blundell et al.\cite{5, 131}, Zhu et al.\cite{129}, Yin et al.\cite{132}, and Pemathilaka et al.\cite{133} have successfully created a placenta-on-a-chip. The fabrication process for their microfluidic devices is a concrete and universal procedure method that most scientists use. The reason why is because the model allows researchers to compare their tests and results due to the consistency of their fabrication process. Since fabrication was performed adequately for all the studies, and similar culturing and seeding methods were used among the variety of experiments, analysis of their placental barriers produced satisfactory results pertaining towards the confluence of trophoblast and endothelial cells. In this case, their microfluidic devices were prepared for the study of glucose, glyburide, heparin, bacterial infection, nanoparticles and caffeine across their microengineered barriers. Moreover, the placenta-on-a-chip’s microengineered approach has a well-developed procedure that is being progressed to replicate the physiology and pharmaceutical intake of an in vivo placenta.

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<td>Microfluidic device provides a platform for xenobiotic testing</td>
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2.6. Experiments and Analysis from Placenta-on-a-Chip

2.6.1. Glucose

The replication of the physiology and transfer of fluids of the human placenta in the in vitro model is the most crucial step towards the testing of pharmaceuticals across the placenta-on-a-chip because the in vitro model must completely reproduce the natural reactions that the human placenta releases when confronting pharmaceutical intake. The analysis and study of the
Figure 2.7. Glucose perfused across Blundell et al. placenta-on-a-chip. (a) GLUT1 transporters and DAPI staining are expressed by the color red and blue, respectively. Scale bar, 10 µm. (b) Green dots represent the concentration gradient of glucose crossing from the maternal to the fetal compartment. (c) Glucose rates from the placenta-on-a-chip, Transwell co-culture, and ex vivo perfusion studies are displayed for comparison. (d) A graphical representation presents percent increase in fetal glucose concentration for a bare membrane, trophoblast monoculture and co-culture system. Reproduced with permission from [5]. Copyright © 2016, RSC.

Glucose transfer across the placenta-on-a-chip barrier is a significant and logical way of studying the strength, reactivity, and fluid flow of the model’s co-cultured placental barrier. Glucose serves as a vital factor during normal pregnancy when transporting overt and gestational diabetic medication on the placental barrier. Glucose is also predominantly found across the placental barrier.\textsuperscript{105, 130, 134} Two examples of the testing of glucose across the in vitro model’s placental barrier can be found in Lee et al.\textsuperscript{130} and Blundell et al.\textsuperscript{5} experiments, which carried a similar approach for the glucose perfusion.

Lee et al. began the testing and analyses of glucose transfer across their placenta-on-a-chip, by introducing glucose concentrations to the fetal and maternal sides of the chip. EGM\textsuperscript{TM}-2 containing a lower glucose concentration (1.1 g mL\textsuperscript{−1}) was introduced to the endothelial channel, while DMEM supplemented with a higher concentration (4.5 g mL\textsuperscript{−1}) of glucose was added in the trophoblast channel. The introduction of these glucose concentrations replicates glucose gradients across the placental barrier, so the in vitro membranes release a more recognizable reaction that
mimics the behavior of an in vivo placenta. Next, 30 µL h⁻¹ of a continuous flow of the glucose-supplemented culture media was maintained in the channels for 68 h. Lastly, the outlets at the end of the lower and upper channels were used to collect the perfused media. An evaluation of the lower maternal compartment’s reduction in glucose concentration was also evaluated to determine if gradient-induced passive diffusion or glucose transporters were responsible for fluctuations in glucose concentrations across the chip. Analysis from the placental cells revealed that glucose transporters were a significant factor towards the differences in maternal and fetal glucose concentrations, as well as gradient-induced passive diffusion.\cite{130}

**Blundell et al.** began the placenta-on-a-chip glucose study by perfusing the maternal channel with F-12K medium, supplemented with 10 mM of glucose to increase the glucose concentration levels generated by F-12K medium. Nonetheless, on the fetal side, the media contained 5.5 mM of glucose during perfusion. During the perfusion process, outflow from both microchannels was collected over a 2 h period. The collected flow was analyzed by a glucose meter, that measured glucose concentrations.\cite{5}

The results carried out in the testing and analysis of glucose transfer rates turned out to be successful and revealed metabolic consumption. *Lee et al.* results indicated that the final transfer rates of the co-culture model yielded from 17.3%-39.1%, which is approximately in the same range of the in vivo transfer rates.\cite{130} Similar rates can also be compared to *Blundell et al.* placenta-on-a-chip glucose transfer rates. *Blundell et al.* microchip produced a glucose rate of 34.8%. The following glucose transfer rate was found to be in the same approximately of an ex vivo glucose study, that produced glucose rates from 26.5%-38.3% (Figure 2.7c).\cite{5} Based on observation, the yielded glucose rates from both *Lee et al.* and *Blundell et al.* shared similar rates with an in vivo placenta and an ex vivo study, respectively, indicating the proficiency of both glucose experiments.
Successful and related results of the following analyses reveal the *in vitro* model’s ability to mimic the structure and placental transfer of glucose in an *in vivo* human placenta. More importantly, the model’s ability to replicate glucose transfer rates provides evidence that the model is prepared for the perfusion of medicated-drugs.\[5, 130\]

2.6.2. Glyburide

The progress of the *in vitro* model for the replication of the physiology and glucose transfer across the *in vivo* placental barrier has played a significant part in the replication and comparison of medicated-drug reactions between the placenta-on-a-chip and *in vivo* human placenta. For example, the testing of glyburide across the placenta-on-a-chip proved to be successful in replicating the common occurring effects that the *in vivo* placenta release during glyburide intake. Glyburide is a common medication that is prescribed to pregnant women to combat and contain diabetes. However, the traceability of glyburide is nearly undetectable in the fetal circulation as reported by the success of the *ex vivo* clinical and perfusion models.\[131, 135-136\]

Nonetheless, with the development of the placenta-on-a-chip, Blundell et al. have efficiently imitated the usual effects that the human placenta releases when glyburide is diffused across the placental barrier (Figure 2.8). The study began when Blundell et al. introduced glyburide into their placenta-on-a-chip. The continuous perfusion of BODIPY-conjugated glyburide diluted in serum-free DMEM/F-12K to a final concentration of $1\times10^{-6}$ M was perfused through for the maternal channel, while the EGM-2 medium was injected into the fetal channel to examine glyburide transport. After a 30 min equilibrium phase, the outflow was collected from the fetal compartment in 30 min intervals and then analyzed with a microplate reader. Next, to validate the presence of breast cancer resistance proteins (BCRP), Ko143 ($150\times10^{-9}$ M in DMSO), an inhibitor of BCRP, was included in the drug-containing medium and perfused in the maternal channel.\[131\]
After testing was completed, Blundell et al. began interpreting their data. Low concentrations were measured throughout the first 3 h due to the large absorptions from the PDMS slabs, and trophoblast cells. Unexpectedly the concentrations began to rise in the last 3 h of the experiment, resulting in glyburide rates of 5.6% which were relatively close to the ex vivo model’s glyburide rates from 0.62% to 3.9%. These sudden rises were most likely caused by BCRP-mediated active transport of BODIPY-glyburide, in which the placental barrier was absorbing BODIPY-glyburide back into the maternal circulation. Nevertheless, the similar results between the in vitro and ex vivo model reveal that the simple in vitro chip’s design can duplicate the active drug transport of an in vivo human placenta, like the complicated and underdeveloped ex vivo model was able to accomplish.⁹¹ Observations were also taken from the constant fetal
concentrations of glyburide. The fetal concentrations turned out to be lower than the maternal concentrations. In addition, microscopic imaging was carried out to determine why there was differences in the fetal and maternal concentrations. Based on microscopic imaging, observations conclude that BeWo cells were predominantly in control of glyburide transport. This observation indicates the fact that BeWo cells in the in vitro model were one of the most significant aspects towards the transporting of glyburide across the chip’s barrier, which happens in most cases in an in vivo placenta.\[131\]

Blundell et al. microengineered placental barrier was also analyzed to determine the presence of BCRP, major efflux transporter in the placental tissue. The BCRP plays a critical role in preventing medicated-drugs from entering the fetal compartment of the placenta, making the presence of BCRP essential for the in vitro model to mimic the diffusion of drugs across the placental barrier completely. Based on fluorescent images, BCRP turned out to be distributed throughout the trophoblast channel of the in vitro model, which is parallel to the in vivo human placenta. Nevertheless, including Ko143 in the perfusion of BODIPY-conjugated glyburide reduced the transport function BCRP and prevented the BeWo cell from transferring glyburide back into the maternal channel. This discovery supports the presence of BeWo cells when glyburide is moved across the placental barrier, supporting the previous argument. Therefore, the placenta-on-a-chip’s progress, pertaining towards the model’s ability to reproduce the natural reactions that are released from an in vivo placenta when confronted with glyburide, reveals one of the chip’s highlighted accomplishments.\[131\]

2.6.3. Heparin

Not only was there success pertaining towards the replication of glyburide on the in vitro model, but the replication of the natural effects of heparin in the placenta-on-a-chip was carried
out successfully as well. Heparin is a commonly used medication to prevent vein thrombosis and arterial thromboembolism. Not to mention, heparin is a commonly used anticoagulant medication to assess the microengineered placental interface’s barrier integrity. Blundell et al. assessed heparin transport across the placental membrane and measured the intensity of fluorescence outflow from the microchambers of the placenta-on-a-chip. The analysis of heparin transport was also carried out in order to determine if the in vitro model can efficiently duplicate the maternal-fetal interface and restrictive barrier of an in vivo placenta. Using heparin as a medicated drug subject additionally allowed researchers to verify that their in vitro model fully imitates the common reactions from an in vivo placenta in order to acknowledge the model is entirely practical.[131]

At the beginning of the study, Blundell et al. diluted green fluorescent fluorescein-heparin conjugate to a concentration of 0.05 mg mL$^{-1}$ in DMEM/F12K medium. Subsequently, the diluted fluorescein-heparin was injected into the maternal compartment of the placenta-on-a-chip. Following 5 h of perfusion, the outflow was then collected from both channels. The fluorescence levels were then quantified with a microplate reader. The following procedure was carried out for both the co-culture and bare membrane (acellular) conditions in the microchip. Briefly, the acellular system included a bare semipermeable membrane without the support of trophoblast and endothelial cells; however, the co-culture system was supported with BeWo cells and HPVECs, and consisted of lower fluorescence intensity.[131]

Results from the acellular devices indicated high fluorescence levels and a concentration of 11.3% of the maternal compartments’ initial absorption. High fluorescence levels and low concentrations prove that the acellular device efficiency replicated the natural reactions released from an in vivo placenta from heparin intake. The co-culture system resulted in a fluorescent final-
fetal-concentration of 0.2% of the maternal concentration initial value. This lower concentration that of the acellular device can be explained by the presence of BeWo cells and HPVECs. Nonetheless, the 11.3% and 0.2% minuscule concentration percentages reveal that Blundell et al. placental barriers can effectively mimic the case that heparin reported to be not transported across the placenta barrier due to its larger molecular size. In this case, the placenta-on-a-chip can successfully replicate the natural occurring reactions from heparin intake, in which the in vivo placenta’s common response from heparin is to show aggressive rejective behavior. Testing heparin in the in vitro model provided support for the fact that the placenta-on-a-chip possesses the ability to test for medicated-drug intake reactions. More importantly, though, the study’s success establishes that the microchip’s similar fabrication processes with the glucose and glyburide studies prove that the placenta-on-a-chip is closing in on becoming the practical model for revolutionizing pharmaceutical drug testing.\[131\]

2.6.4. Bacterial Infection

Zhu et al. chose to model placental inflammatory responses to bacterial infections through their microfluidic device (Figure 2.9). Through the establishment of the microfluidic device, they were able to construct a placenta-on-a-chip that enabled them to analyze tissue and cellular responses from bacterial infections. Bacterial infections are a common cause of placental inflammation that can cause damage to fetal organs and transplacental infection in the growing fetus. In return, these damages can cause inflammatory response syndrome, sepsis and other underlying diseases within the human placenta. In these cases, these responses can lead to short-term and long-term limitations during fetal development stages. Therefore, scientists need to be incorporating bacteria into the placenta-on-a-chip if they want to account for these limitations and fully replicate the physiology of the human placenta in vitro.\[129\] Bacterial transformation and
infection within Zhu et al. chip began with the cultivation of gram-negative *Escherichia coli* (*E. coli*) labeled with a green fluorescence protein (GFP) from LB broth. *Escherichia coli* is a dominant bacterium found within the intestines of humans and other animals, which happens to be harmful in most cases. Cultivation took place for 12 h at 37°C and 5% CO₂. Subsequently, the *E. coli* were resuspended in DMEM/F-12K medium and immunized with trophoblast medium in the upper microchannel of the microfluidic device. After 6 h of immunization, the cells were prepared for biological analysis.¹²⁹

![Figure 2.9](image)

Figure 2.9. Bacterial infections were introduced into Zhu et al. placenta-on-a-chip. (a1,a2) Maternal macrophages behavior in the maternal-fetal interface during bacterial infection (GFP expressing bacteria). (b1,b2) Images depict proliferated GFP-expressing bacteria in the microchip. (c1-c6) Fluorescence microscopy shows the behavior of BeWo and THP-1 cells in *E. coli* (d) Inflammatory genes in BeWo cells were stimulated with *E. coli* and were represented by a RT-PCR quantitative analysis. Scale bars, 100 µm. Reproduced with permission from [¹²⁹]. Copyright © 2018, American Chemical Society.

Results and data from biological investigation concluded that the immunization with the *E. coli* and trophoblast cells resulted in high amounts of inflammatory cytokines, which includes Interleukin-1α (IL-1α), IL-1β, and IL-8. In other words, there were increased levels of inflammatory from the immune responses (IL-1α), cell proliferation (IL-1β), and the host defense
mechanisms (IL-8). These increased levels of inflammatory responses resulted in a significant amount of death in the trophoblast and endothelial cells. The conclusion from these results reveals that the placenta-on-a-chip was able to mimic the inflammatory reactions on an in vivo placenta when confronted with E. coli, based on the similarly increased levels of inflammatory responses. Nevertheless, conclusions from Zhu et al. study also indicated that the BeWo cells activated the circulating macrophages when inflammatory began to develop along the maternal side of the chip. These activation interactions are common in an in vivo human placenta, since the macrophages responsibilities include the protection of the maternal and fetal organs from bacterial inflammatory infections. Thus, these results show the placenta-on-a-chip’s capabilities to replicate the bacterial interactions and behaviors between the macrophages and trophoblast and BeWo cells in vitro.[129]

2.6.5. Nanoparticles

The placenta-on-a-chip has been applied for, fluid analysis, medicated-drug testing, physiological examinations; however, Yin et al. used their in vitro model for nanoparticle (NP) analysis (Figure 2.10). The study included a common NP (TiO₂-NPs) that are environmentally exposed to the majority of pregnant women. Unfortunately, studies lack on this subject of research, so the adverse effects NPs on the placental barrier are lesser known. However, through the fabrication of Yin et al. placenta-on-a-chip, they were able to extrapolate data to explore the effects TiO₂-NPs imposed on their micro-engineered placenta while bringing awareness to this overlooked NP.[132]

Yin et al. began this experiment by exposing their fabricated placenta-on-a-chip with NPs. The process began by injecting DMEM/F12-K medium with TiO₂-NPs at a concentration of 50 or 200 μg mL⁻¹ into the maternal channel at a rate of 20 μL h⁻¹ for 24 h. Next, monocyte adhesion
Figure 2.10. Nanoparticle exposure across placenta-on-a-chip: (a1-a3) Schematic shows TiO$_2$-NPs being perfused in the maternal channel and transferring into the matrix in the middle channel after 24 h. (b1-b3) ROS levels were analyzed in the BeWo cells with NPs exposure. Scale bars, 50 µm. (c1,c2) The diagram represents perfused differentiated THP-1 cells in the maternal channel. (d1-d3) After NPs exposure, the trophoblast cells were analyzed for THP-1 adhesion. Scale bars, 100 µm. Reproduced with permission from [132]. Copyright © 2019, Elsevier.

analysis was performed. This included differentiating monocyte THP-1 cells into macrophages, and then perfusing them into the channels at a flow rate of 40 µL h$^{-1}$ for 30 min. The channels were then washed with PBS 3 times before imaging was taken place. Once imaging was acquired from a confocal microscopy, Yin et al. began ROS detection in their cells. The inspection was conducted using a ROS Assay kit [132].

The findings Yin et al. gathered begins with their ROS fluorescence image discoveries. The production of ROS, a natural occurring metabolism by-product in trophoblast, was used to study when detecting for NPs exposure. On the maternal side of the chip, when lower NPs concentrations were introduced, ROS generation was almost nondetectable. Once levels were increased, ROS generation activated substantially, which is observable based on the decrease of cells. Additionally, once the NPs transferred across into the fetal channel of the microchip, endothelial cells began to die off. These increased distributions were in theory, enhanced by the close-fitting cellular junctions within the placental barrier. Therefore, tight junctions are possibly more sensitive to
environmental stress, NPs. Nevertheless, since the placental barrier includes more than just endothelial and trophoblast cells, Yin et al. decided to analyze the effects the NPs had on macrophages.\footnote{132}

During the procedural examinations, monocyte adhesion was included. Based on the discussion of Zhu et al. study on bacterial infections, monocytes are prominent for cellular and tissue defense from outside pathogens.\footnote{132} Once data was collected from the NPs channel exposure, macrophages were introduced into the maternal side of the chip. Observations discovered that the macrophages adhered to the trophoblastic cell layer. This behavioral reaction revealed the significant immune system changes towards NPs. In other words, macrophages were able to detect the NPs and determine the harmfulness of their nature. Moreover, the results of the study concluded the destructive nature of NPs, and the damaging effects they impose on the placental barrier. In this case, these destructive behaviors can have a long-term impact on the placenta, and even worse, the fetus.\footnote{132}

Therefore, in conclusion of the study, there are two take ways. One, the fabrication of the placenta-on-a-chip was carried out similarly with the previous elaborated studies, providing consistency between analyses. More importantly, though, the placenta-on-a-chip was used to study an overlooked pathogen- a pathogen that scientist have not been paying close attention to when searching for mutational defects in the fetus and placental barrier. So, in this case, the study provides one of the first documentation on the effects NPs imposes on the placental barrier, based on their placenta-on-a-chip. In this case, this study will improve placenta-on-a-chips progress for testing medicated-drug testing, considering nanoparticles play a factor in placental mutations and fetal birth defects.\footnote{132}
2.6.6. Caffeine

While placenta-on-a-chip has undergone fluid flow, medicated-drug, bacterial infection, and nanoparticle testing, Pemathilaka et al.\cite{133} provides one of the first studies of caffeine transport across the placenta using a placenta-on-a-chip model. Caffeine happens to be one of the most widely consumed stimulants in the world and consisting as a main ingredient in coffee, tea, chocolate, soda, etc. In this case, the over-consumption of caffeine can have damaging effects on the fetus. So, in this study, Pemathilaka et al. fabricated a placenta-on-a-chip to assess the overlooked behaviors the xenobiotic compound is imposing on their chip. This study is further discussed in Chapter 2.

While the placenta-on-a-chip is one of the most recently developed approaches toward medical drug treatment, the model has acquired a significant amount of progress regarding fluid, medicated-drug, bacterial infection, nanoparticle, and xenobiotic testing. With limited testing and analysis, the success to fail ratio for the placenta-on-a-chip is substantially high, considering that the model was developed relatively recently. A substantial amount of progress has been gained from the broad spectrum of testing in the in vitro model’s placental barrier: mimicking glucose transfer, glyburide and heparin drug reactions, bacterial infections, and nanoparticle and caffeine expose responses. The variety of studies, especially the testing of fluid transporters and bacteria and xenobiotic compounds, improves the overall functionality of the in vitro approach, reproducing the physiological characteristics and common reactions that an in vivo placenta releases when confronted with medicated-drugs. Most importantly, the consistency between the experiments and their fabrication processes for their microengineered in vitro models has played a vital role towards the chip’s practicality, when comparing data between different fluid, pharmaceutical, bacterial, and xenobiotic intake tests. Therefore, the testing of glucose, glyburide, heparin, bacterial infections, nanoparticles and caffeine have proven to be significant for the better
understanding of their interactions with the human placental barrier. As researchers continue improving the fabrication process, and conducting successful medical treatment tests on the in vitro chip, the placenta-on-a-chip could potentially become one of the most demanded products in the pharmaceutical and medical industry.

2.7. Challenges of Placenta-on-a-Chip

Figure 2.11. Pregnant mother surrounded by recently developed placenta-on-a-chips. (a) Miura et al. used their placenta-on-a-chip to determine whether fluid shear activates microvilli formation and TRPV6. Scale bar, 1 cm. Reproduced with permission from Miura et al.[105]/CC BY. (b) Blundell et al. microchip was analyzed for glucose transport studies. Reproduced with permission from [5]. Copyright © 2016, RSC. (c) Blundell et al. microchip was used for heparin and glyburide transport studies. Reproduced with permission from [131]. Copyright © 2018, John Wiley and Sons. (d) Zhu et al. studied bacterial infections within their microfluidic device. Reproduced with permission from[129]. Copyright © 2018, American Chemical Society. (e) Yin et al. analyzed nanoparticle exposure into their placenta-on-a-chip. Reproduced with permission from[132]. Copyright © 2019, Elsevier. (f) Pemathilaka et al. perfused caffeine across their microengineered barrier, and examined the channels respective concentrations. [133] Reproduced with permission from Pemathilaka et al.[133]/CC BY
Although the placenta-on-a-chip (Figure 2.11) has made a substantial amount of progress towards the testing of medicated-drugs across the microengineered placental barrier, the model was developed fairly recently compared to the *in vivo* and *ex vivo* models. One of the most significant problems when defending the placenta-on-a-chip’s accomplishments is the fact that the model has only had a few efficient pharmaceutical intake tests performed on the chip’s microengineered placental barrier. This is largely because scientists have been devoting the majority of their time in the past few years modifying the fabrication process for the placenta-on-a-chip. Ironically, though, while scientists have been investing a considerable amount of time improving the chip’s structural and functional components, researchers lack the replication of prominent physiological characteristics of the placental barrier. For example, the placenta-on-a-chip lacks the testing of fatty and amino acids, which are essential characteristics when predicting the capability and reactivity of the chip, and mimicking the physiology of the human placenta.\[^5\] However, unlike the *in vivo* and *ex vivo* model, the *in vitro* approach has seen a significant amount of testing in the model’s early development stages compared to the previous models.

Not only are there issues pertaining the model’s experience with pharmaceutical testing, but also faces challenges towards the chip’s aptitude to perform complicated tests, and control and distribute PDMS. For example, when the functionality and complexity of the 3D co-cultured model of the cell increases, analysis of the chip becomes more difficult to perform high-resolution screening of the tissue and to visually locate the tissue.\[^{137}\] In addition, when analyzing for biological responses the chip’s small cell volumes, when compared to an *in vivo* placenta, give rise towards detection sensitivity issues for drug absorption and cellular interactions. Therefore, the mapping and interpretation of the chip’s clinical endpoints make the data difficult for pharmacists and scientists to translate to patients and themselves.\[^{72}\] There are also limitations dealing with
PDMS. PDMS has shown to reduce the pharmacological and drug absorption reactions, because of the PDMS consuming hydrophobic molecules. PDMS also lacks the ability for vast-scale manufacturing, because the PDMS fabrication process is intended for laboratory prototype only. In this situation, the adoption of this technology into the pharmaceutical and medical industry will make the process difficult for large-scale industries to obtain PDMS. Furthermore, the complexity and limitations with the placenta-on-a-chip and PDMS are both major disadvantages when considering the operation and control of the chip’s abilities and performance.

More challenging, though, the placenta-on-a-chip lacks the maintenance for human cells and accountability of placental alterations during pregnancy. Scientists can agree that the maintenance of confluent trophoblast cells is challenging. In order to maintain and control confluent trophoblast cells, patience and precise cellular cultivation are needed for the operation and control of the model to function properly. Maintaining and preventing cellular contamination is also difficult when considering that the contamination of the cells will lead towards false results and insignificant data. Additionally, there needs to be new developments within the chip in order to account for alterations in an in vivo placental barrier throughout pregnancy. The human placental barrier goes through many alterations in the course of pregnancy. Thus, the model must account for these alterations in order for the chip to entirely mimic pharmaceutical intake of an in vivo placenta during the course of an entire pregnancy. In this case, if the model is unable to account for placental developments during pregnancy, the chip will oppose the model’s own goal of being fully practical. Nonetheless, if scientists continue improving upon the chip’s performance and sustainability of more challenging analysis, then the model’s advantages will overshadow the placenta-on-a-chip’s low track record of tests, complexity of transability and evaluation, maintenance of PDMS and cells, and other disadvantages.
The continuation of support and funding for the placenta-on-a-chip will assist scientists to progress improvements and developments on the chip’s overall functionality. As researchers continue innovating upon the *in vitro* approach, scientists will be able to conduct an array of tests across the microengineered barrier, other than pharmaceutical testing. For example, the experimentation and analysis of diseases and bacteria could be tested more frequently when the model reaches a sturdier compatible barrier. Scientists will also be competent to possibly use the *in vitro* model to gain new knowledge on the development stage of the fetus. For instance, there will be gains in the understandings on how slower or faster, or altered or damaged developments in the placenta impact the fetus’s growth. Nevertheless, the practicality of a universal model was discussed in detail, which dealt with the fact that the model must have a concrete procedure and method that can fully imitate the physiological characteristics of an *in vivo* human placenta, and be carried out in the same manner for all medical related testing in order for the comparison of data to take place. The development of a practical model that can run medicated intake tests is important towards the better understanding of the effects that pharmaceuticals impose on the fetus and placental barrier. In this case, the placenta-on-a-chip as explained throughout most of the paper, was discussed in detail on how this practical model will revolutionize pharmaceutical testing for a better understanding on how these medicated-drugs affect the human placenta when compared to the *in vivo* and *ex vivo* approaches. In conclusion, the application and progression of the placenta-on-a-chip’s testing of medicated-drugs and improved understanding of the human placenta will help scientists decrease birth defects from medicated-drugs, and develop a better understanding on how these drugs affect the human placenta.
References


CHAPTER 3. PLACENTA-ON-A-CHIP: IN VITRO STUDY OF CAFFEINE TRANSPORT ACROSS PLACENTAL BARRIER USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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Abstract

Due to the particular structure and functionality of the placenta, most current human placenta drug testing methods are limited to animal models, conventional cell testing, and cohort/controlled testing. Previous studies have produced inconsistent results due to physiological differences between humans and animals and limited availability of human and/or animal models for controlled testing. To overcome these challenges, a placenta-on-a-chip system is developed for studying the exchange of substances to and from the placenta. Caffeine transport across the placental barrier is studied because caffeine is a xenobiotic widely consumed on a daily basis. Since a fetus does not carry the enzymes that inactivate caffeine, when it crosses a placental barrier, high caffeine intake may harm the fetus, so it is important to quantify the rate of caffeine transport across the placenta. In this study, a caffeine concentration of 0.25 mg mL$^{-1}$ is introduced into the
maternal channel, and the resulting changes are observed over a span of 7.5 h. A steady caffeine concentration of 0.1513 mg mL\(^{-1}\) is reached on the maternal side after 6.5 h, and a 0.0033 mg mL\(^{-1}\) concentration on the fetal side is achieved after 5 h.

3.1. Introduction

Caffeine is one of the most popular and widely consumed stimulants across the globe.\(^{[1]}\) Coffee, tea, and cocoa are the primary natural sources of caffeine. Both health authorities and regulatory bodies have raised concerns about consumption of caffeine-enhanced food and beverages because of the increased availability of caffeine-enhanced food products containing synthetic caffeine.\(^{[2]}\) Because caffeine is found not only in food and beverages, but also in prescription and over-the-counter medications, many pregnant women are very likely to consume caffeine in some form, so they may risk exposing underdeveloped fetuses to this behaviorally active substance. There are concerns that overly heavy caffeine consumption may harm the fetus, since pregnant women require a half-life 1.5 to 3.5 times greater to metabolize caffeine than non-pregnant women, possibly causing caffeine to remain in body tissues for longer periods of time. Caffeine has been found on locations within the fetal compartment, suggesting caffeine transport across the placental barrier, and since a fetus would have difficulty in metabolizing caffeine due to lower levels of enzyme production in its developing liver, there is a real possibility that caffeine exposure could damage the fetus.\(^{[1, 3]}\) Because of undetermined effects from maternal caffeine intake on the fetus and the increased number of caffeine products available for prenatal consumption, health authorities such as the World Health Organization (WHO) and the Food and Drug Administration (FDA) have developed recommendations restricting caffeine intake during pregnancy and declaring caffeine consumption during pregnancy a global healthcare problem.\(^{[3-5]}\)
Over the last decade, organ-on-a-chip technology has grown to become one of the most popular alternatives for drug testing and toxicology in vitro. Its aim is to create a 3D microenvironment reminiscent of specific human organs as a means for replicating their functionality. The placenta has been one of the most difficult organs to replicate using organ-on-a-chip because it is a temporary organ that develops only during pregnancy and changes its structure and functionality over the course of the gestational period. To study this vital organ, in vivo, ex vivo, and in vitro tests have been conducted. In vivo studies conducted on rats and baboons have yielded inconsistent results. It is difficult to transfer the results from an animal testing model to a human model because placenta development is different in different mammals. For example, in humans, glucose transport through the placental barrier is mediated by GLUT1 glucose transporters, while in mice it is mediated by GLUT3 transporters. This difference shows why experiments using mice can yield lower glucose transfer rates than those found in similar experiments using human placentas. Previous studies have reported that in vitro microphysiological placenta systems are capable of exhibiting similar levels of glucose transport as those of actual placenta. Moreover, in another study, the transport of two xenobiotic substances, heparin and glyburide, was studied using a bioengineered placental model, demonstrating the capability of a placenta-on-a-chip model to successfully mimic at least some of the physiological functions of the human placenta. It has also been shown that these in vitro models are capable of mimicking placental inflammatory behavior from a placenta attacked with a bacterial infection.

Even though humans are closely related to many other mammals, there is no yolk sac placentation in humans, and an allantoic stalk rather than an allantoic sac is present in a human placenta. This makes it challenging to compare the results of experiments conducted on
placentas of other mammals to results of studies conducted on human placentas. Ex vivo studies in humans have also been conducted by obtaining placentas immediately after childbirth or cesarean sections.\textsuperscript{[19]} These human studies only provide opportunities to study placentas and gain insight from women in their final term of pregnancy. Difficulty in obtaining consent from women for such participation in such studies and gaining access to placentas before they become no longer viable makes these studies difficult to conduct. Moreover, ethical issues may limit experimental research of this type, and observational studies may be difficult to perform because the women could be from a group self-selected for testing a particular drug.\textsuperscript{[20]}

During pregnancy, both endogenous substances and xenobiotic substances consumed by a pregnant woman can pass through the placental barrier, possibly causing severe damage to a fetus either before or after birth. For example, an exogenous compound with the chemical nomenclature 1,3,7-Trimethylpurine-2,6-dione, commonly known as caffeine, is quite often consumed worldwide by pregnant women on a daily basis by way of ingesting coffee, tea, energy drinks, chocolate, etc., because it acts as a stimulant for the central nervous system (CNS).\textsuperscript{[20]} Unfortunately, it has been found that such increased intake of caffeine by pregnant women can result in birth weight (BW) reduction in newborn children, or in reduced neonate size for its gestational age (SGA).\textsuperscript{[20-22]} A meta-analysis of 32 studies suggests that caffeine intake is associated with an increased risk for reduction in BW, and another meta-analysis of 26 studies appeared to show a 43 g weight reduction in newborn children whose mothers appeared to be heavy caffeine consumers.\textsuperscript{[21-22]} Despite these findings, it has never been recorded that caffeine directly causes BW reduction, also known as intrauterine growth retardation (IUGR).\textsuperscript{[23-29]} While both meta-analyses exhibited some correlation with caffeine intake and BW, inconsistencies found in these studies suggest that there remains a need to identify a proper method for measuring exact
caffeine levels crossing the placental barrier when a prospective mother consumes a certain amount of caffeine. Note that the effects on a fetus due to excessive intake of caffeine by pregnant women are beyond the scope of this study because it primarily focuses on the amount of caffeine infiltrating a placental barrier.

Caffeine is easily absorbed by the placental barrier and crosses the barrier freely. Since the primary enzyme responsible for caffeine metabolism, cytochrome P450 1A2, is absent both from the placenta and the fetus, the rate of metabolism of caffeine depends totally on the metabolism capacity of pregnant women. One study states that the half-life of caffeine has a range of 6–16 h in pregnant women compared to that for non-pregnant women, i.e., 2–8 h. According to guidelines provided by the WHO and FDA, the intake of caffeine during pregnancy should not exceed 300 mg per day, making it necessary to measure a pregnant woman's concentration of caffeine perfused into the fetus in relation to her total caffeine intake. Our placenta-on-a-chip device is designed to represent the trophoblastic epithelium and endothelium of the maternal interface and the fetal interface in a human placenta, respectively. The chip was designed to carry two cell lines to represent both the maternal and the fetal sides. A porous membrane was placed between the two channels to serve as a barrier between the two bloodstreams. This membrane acts as an extracellular matrix (ECM) to provide support for surrounding cells used in our design. Human umbilical vein endothelial cells (HUVECs) and trophoblasts cells (BeWo) were respectively chosen to represent the endothelium in the fetal interface and the epithelium in the maternal interface. This work will enable us to establish a platform for studying the pharmacokinetics of different xenobiotic drugs across the placental barrier, and also enable us to examine the safety of drugs administered to pregnant women.
3.2. Results and Discussion

3.2.1. Cell Growth and Characterization on Membrane

The HUVECs and BeWo cocultured microfluidic device provided a relevant environment for representing the propagation of a human placenta. The human placenta in vivo consists of three main parts: the epithelium, the endothelium, and the placental barrier. As intended, we were able to replicate an in vivo–like microsystem with HUVECs representing the endothelium, BeWo cells representing the epithelium, and a semipermeable membrane representing the placental barrier. CellTracker results (Figure 3.1a–d) showed a proliferation of cells over time and cell characterization was used to further study the formation of a placental barrier–like interface used to replicate and mimic placenta-related physiology. During medium perfusion, cells were able to cover the entire area of both sides of the membrane within 24–30 h from the cell seeding. Cell adhesion on the porous membrane is an important step in properly representing each cell line, and ECM macromolecules play an important role in proper growth and normal function of primary cells. The most important cell-adhesion control variable was the cell adhesion time. Various time periods were tested to identify the optimal time for cells to reach solid attachment. Another important parameter affecting cell viability on the channel was the flow rate. Since high flow rates produce high levels of shear stress on the channel walls, and can thereby force attached cells to detach from the membrane and flush out of the device, we tested different flow rates to seek the best results while also satisfying the previously discussed conditions. In the upper channel where BeWo cells were introduced, cells began forming a 3D structure and thereby affected long-term cell growth in the upper channel. As cell coverage increased, the space remaining for the medium to cross the channel had decreased, causing medium flow to exert pressure on the cells.
When fabricating a placental-barrier-like semipermeable membrane, it is important to verify the formation of tight cell–cell junctions. E-cadherin is considered to be an important molecule when seeking to maintain cell–cell adhesion in the epithelial cell layer because it is restricted to regions of adherence junctions.\textsuperscript{31} We used E-cadherin present on trophoblast cells to validate the formation of tight junctions and strong cell–cell adhesion in the epithelium. After 3
days, BeWo cells were stained with anti-E-cadherin and scrutinized for red fluorescent protein (RFP). As shown in Figure 3.1e, BeWo cell–cell boundaries tested positive when stained for E-cadherin, verifying existence of tight junctions across the epithelial cell layer. Tight junctions in the endothelial cell layer ensure tissue integrity and play a vital role in maintenance and control of endothelial cell contacts.\textsuperscript{[32]} VE-cadherin was used to investigate cell–cell interactions and the formation of tight junctions on HUVECs that represent the endothelium. Similarly, after 3 days of medium perfusion, HUVECs were marked with anti-VE-cadherin and analyzed for green fluorescent protein (GFP). As shown in Figure 3.1f, VE-cadherin was detected on cell–cell partitions, verifying the occurrence of tight junctions in the endothelial cell layer. The E-cadherin and VE-cadherin-labeled cell–cell boundaries implied the formation of tight junctions and verified that both the epithelial and endothelial cell layers consisted of a confluent monolayer of cells on the membrane.

Placental barrier permeability was evaluated using 3000 MW fluorescein–dextran anionic probes. When dextran was introduced to the maternal side, fluorescence intensities on both the maternal side and the fetal side were recorded, and the data represented as a fraction, with maternal intensity the numerator and fetal intensity the denominator, as shown in Figure 3.1g. We observed that, while maternal fluorescence increased over time due to the dilution of the dextran-mixed medium by the remaining medium in the channels and by the tubing, fetal fluorescence intensity remained at a lower level. Even though a few molecules were diffused from the maternal side to the fetal side across the membrane, overall fetal intensity remained insignificant over time, verifying the integrity of the placental-barrier-like semipermeable membrane.
3.2.2. Quantitative Analysis of Caffeine Transport

3.2.2.1. Concentration of Caffeine Transported through Placental Barrier

Before calculating caffeine concentrations, we plotted the data obtained from the area under the curve for each chromatogram with respect to time, as shown in Figure 3.2, and the fetal side (Figure 3.2a) of the control (samples collected from a chip consisting of a bare membrane with perfusing EGM and F-12K) showed more fluctuation in terms of the number of counts (representing the area) with a positive gradient with respect to time up to \( t = 6.5 \) h. Between \( t = 6.5 \) h and \( t = 7.5 \) h, concentrations (represented by the number of counts) sought to reach a steady-state while achieving a peak-level of caffeine diffusion through the placental barrier. Conversely, the actual data (from chips with cells and medium) show less data variability, with a positive gradient, but data remained in a lower range than in the controlled tests. The actual data also exhibited reaching a peak diffusion between \( t = 5 \) h and \( t = 7.5 \) h. On the maternal side (Figure 3.2b), control data always remained lower than actual data, but it exhibited greater fluctuation than the actual data while the system was moving toward its optimum diffusion stage, and this trend was also observed on the fetal side. The data show attainment of steady-state between 6 and 7.5 h for actual data.

Using data obtained from both the maternal and fetal calibration curves, individual quadratic curves were fitted and equations with a 95% confidence level found for them. Equations 3.1 and 3.2 represent curves fitted for EGM (fetal) and F-12K (maternal), respectively.

\[
A_f = -8.06e^8C_f^2 + 5.80e^8C_f + 3.46e^5 \tag{3.1}
\]

\[
A_m = -2.04e^8C_m^2 + 3.97e^8C_m + 4.64e^6 \tag{3.2}
\]
Where A and C represent the area under the curve from the liquid chromatography mass spectrometry (LCMS) method and the calculated caffeine concentration, respectively. Roots were obtained from each equation for both maternal and fetal caffeine transport. We neglected one root under the condition: \( C \leq 0.25 \text{ mg mL}^{-1} \) because the highest caffeine concentration introduced was 0.25 mg mL\(^{-1}\) on the maternal side.

Figure 3.2. Area under the curve for each chromatogram from LCMS, which were generated for each sample collected from both the maternal and fetal outlet after every 30 min. (a) Chromatogram area output for EGM (fetal side). (b) Chromatogram area output for F-12K (maternal side). Actual tests have both cells in the chip and the control has just the bare membrane with media perfusing through the channels. \( n = 3 \) independent experiments. Data are presented as mean (±SD).

Figure 3.3 a,b shows calculated concentrations with respect to time that followed the same trend as in Figure 3.2. Examination of caffeine concentrations for actual tests on the fetal side (Figure 3.3a) reveals a more conclusive result than that for concentrations represented by areas under the curve (Figure 3.2a). In this study, we investigated both the steady-state concentration and with the amount of time required to reach this condition. Knowing steady-state concentrations on the maternal and fetal sides will assist in verifying the safest dose of caffeine to be taken by a mother when a certain concentration is described in terms of the safe concentration level in the
fetus. Since this system was used only as a proof-of-concept to verify the caffeine transport across the placenta in vitro, only one caffeine concentration (0.25 mg mL\(^{-1}\)) within the safe amount of caffeine according to FDA was tested. After 5 h, the caffeine concentration began to reach a steady-state of 0.0032 mg mL\(^{-1}\), and between 5 and 7.5 h, it maintained an average of 0.0033 mg mL\(^{-1}\) in steady-state. Fetal caffeine concentration in controlled tests reached its peak at \(t = 6.5\) h, and between 6.5 and 7.5 h it achieved its steady-state at an average of 0.0179 mg mL\(^{-1}\). Similarly, analyzing the caffeine on the maternal side (Figure 3.3b) shows that steady-state for the actual tests was achieved between 6 and 7.5 h at a value of 0.1513 mg mL\(^{-1}\) (average). During the controlled experiment, it was noted that steady-state was achieved after 7 h at a caffeine concentration of 0.1307 mg mL\(^{-1}\). After 7.5 h, we observed cells detaching from the membrane. At this point, we concluded that the system could no longer provide a confluent layer of cells and would therefore not adequately represent a system to be used for actual experiments. Such failure could be attributed either to effects of caffeine on the cells or flow phenomenon inside the channels. Further studies are required to identify or confirm reasons for underlying cell detachment. Steady-state was defined at the point in time or time range where caffeine concentrations seemed to maintain a steady value with respect to time while caffeine continued to be introduced into the maternal side at a constant flow rate.

A study on a physiologically based human model of a pregnant woman concluded that, after introducing caffeine, the concentration increased until it reached a steady-state value.\(^{[33]}\) In that study, multiple doses were introduced, and each time the peak concentration was increased until it reached a steady-state condition. We introduced about 0.0938 mg of caffeine to the maternal side within 7.5 h through medium perfusion, and while we can relate our tests to a similar study using multiple doses over time, the doses were continuously given, possibly explaining why
Figure 3.3. Caffeine concentrations calculated for both the maternal and fetal sides. (a) EGM (fetal side). (b) F-12K (maternal side). Actual tests have both HUVECs and BeWo cells on the chip and the control has solely the bare membrane with media perfusing through the channels. (c) Calibration curve for caffeine concentrations in EGM (concentrations ranging from 0.00001 to 0.25 mg mL$^{-1}$). (d) Calibration curve for caffeine concentrations in F-12K (concentrations ranging from 0.05 to 0.3 mg mL$^{-1}$). n = 3 independent experiments. Data were presented as mean (±SD).

Figure 3.2 a,b has multiple peaks but only reached a single steady-state condition. Since we continuously perfused caffeine diluted medium for 7.5 h, continuous perfusion resulted in multiple peaks with only a single steady-state region. In addition, concentrations reported in controlled experiments were significant in the absence of caffeine on either the maternal side or the fetal side because caffeine that perfused to the maternal side should come from either the maternal side or the fetal side; we believe this is due to caffeine absorption to the edges on poly(dimethylsiloxane)
(PDMS) side walls in the maternal channel. This was not significantly observed in actual experiments due to the cell coverage in channels.

While the collected volume might have only a minimal error, even this small error could affect the final calculated value. For example, while the expected volume within a 30 min perfusion period is 25 µL (with a flow rate of 50 µL h⁻¹), only a small error in volume could dilute the medium with an incorrect volume of methanol. This error was minimized by measuring the volume of the sample collected during each 30 min period diluted with the correct amount of methanol. In Figure 3.2a, the number of counts measured at later time points (i.e., t = 6 h) at the fetal side showed the same standard deviation order value as the average. This was observed at early time points (i.e., t = 2 h) on the maternal side, as shown in Figure 3.2 b. Fluctuations of caffeine concentrations on the both maternal and fetal sides at later time points could be attributed to cell detachment, but earlier fluctuation of the maternal concentration could be a result of different medium dilutions with methanol, as mentioned earlier. Further studies are needed to find specific reasons for these errors. In Figure 3.3b, it was noted that the highest calculated caffeine concentration (0.2591 mg mL⁻¹) was detected at t = 1.5 h. While this value is greater than the caffeine concentration introduced to the maternal side (0.25 mg mL⁻¹), during the calculation process the fitted curves (in Figure 3.3 c,d) were made with a 95% confidence level and that error could affect the caffeine concentrations calculated on both maternal and fetal sides.

### 3.2.2.2. Rate Transfer of Caffeine

The rate of caffeine transfer was calculated for both maternal and fetal sides using the following equation (Equation 3.3):
\[
\% \text{RT} = \frac{\Delta C_f}{\Delta C_m} \times 100
\]

(3.3)

Where \(\Delta C_f\) and \(\Delta C_m\) represent the change in caffeine concentrations in the fetal and maternal channels, respectively, during perfusion. Initial and final caffeine concentrations from both the maternal and fetal sides were used when calculating the values for \(\Delta C_f\) and \(\Delta C_m\). To calculate the initial maternal and fetal caffeine concentrations, the values at a previous time point were used for both the actual and controlled experiments (Figure 3.4). Calculated rates were used to measure the change in rate of caffeine transfer with respect to the rate of caffeine transfer calculated at the previous time point (i.e., if the rate of caffeine transfer was calculated at \(t = 5 \text{ h}\), the values at \(t = 4.5 \text{ h}\) were used as the initial concentrations).

As shown in Figure 3.4, the transfer rates for actual tests (with cells) reflected less fluctuation when compared to the rate calculated for controlled tests (without cells), when more caffeine was introduced into the system. In actual experiments, the transfer rates calculated from \(t = 1 \text{ h}\) to \(t = 3 \text{ h}\) show a gradual decrease, followed by a sudden increase and another gradual decrease in caffeine transfer rate observed between \(t = 3 \text{ h}\) and \(t = 5 \text{ h}\). Similar patterns were seen in transfer rates for the controlled tests from \(t = 1.5 \text{ h}\) to \(t = 4 \text{ h}\). The frequent fluctuations were attributed to the constant perfusion of caffeine at 50 \(\mu\text{L h}^{-1}\) to the maternal side, although further investigation is needed to find the exact reasons for such fluctuations. It has been previously reported that the transfer rate of caffeine across a placental barrier depends also on its physiochemical properties such as its size (molecular weight), ionization yield, lipophilicity (Log P), and protein binding.\(^{[34]}\) High-permeability coefficients are observed for small polar molecules because such compounds pass readily through lipid membranes.\(^{[35]}\)
Figure 3.4. The rate of caffeine transfer calculated at every 30 min for both the actual (with cells) and control (without cells) tests. The rates were calculated cumulatively using the values at previous time point as the initial-maternal and initial-fetal concentrations (i.e., if the rate of transfer is calculated at $t = 6$ h, values at $t = 5.5$ h were used as the initial-maternal and initial-fetal concentrations). $n = 3$ independent experiments. Data were presented as mean ($\pm$SD).

Assertions about the amount of caffeine safe for consumption during pregnancy vary depending on the study referenced, and the FDA, taking into account for all the variations for this value has stated that any amount less than 300 mg per day is safe for pregnant women.$^{[29]}$ In our study, we used a concentration of 0.25 mg mL$^{-1}$ of caffeine, less than the FDA-specified amount (300 mg per day = 0.67 mg mL$^{-1}$), for perfusion analysis.
3.3 Conclusions

In this study, we successfully fabricated a placenta-on-a-chip device using PDMS soft lithography techniques. After confirming that we had a confluent layer of cells, we used it to conduct caffeine transport analysis. A caffeine calibration curve was initially established to quantify the caffeine in collected media from both maternal and fetal channels. Using an integrated equation, caffeine concentrations in each media were calculated for each sample over a 7.5 h time span, producing a result showing that caffeine concentration on the fetal side increases until it reaches a steady-state condition. In actual tests (with cells), caffeine concentration on the fetal side reached a steady-state of 0.0033 mg mL\(^{-1}\), while in controlled tests it reached the steady-state of 0.0179 mg mL\(^{-1}\) in the interval between 6.5 and 7.5 h. On the maternal side, while initial concentrations fluctuated, they reached steady-state within 7.5 h. The steady-state value was 0.1513 mg mL\(^{-1}\) between 6.5 and 7.5 h for the actual tests and 0.1307 mg mL\(^{-1}\) after 7 h for the controlled tests. This result clearly warrants further investigation on perfusing different caffeine concentrations to the maternal interface and the way they affect transfer rates.

3.4 Experimental Section

3.4.1. Cell Culture

HUVECs (Lonza) were chosen to represent the cells at the fetal interface. The cells were cultured with endothelial basal medium (EBM, R&D Systems), supplemented with Endothelial cell growth supplement (R&D Systems) containing fetal bovine serum (FBS). BeWo (ATCC) was selected from a variety of trophoblast cell lines based on its adhesive properties, functionality, and phenotype.\(^{[36]}\) BeWo was used to represent the cells at the maternal interface. The cells were cultured in Kaighn's Modification of Ham's F-12K medium (Thermofisher), supplemented with
10% FBS (Thermofisher). Both cell lines were maintained in an incubator at 37 °C with 5% CO₂ in air until they were 80–90% confluent.

### 3.4.2. Design and Fabrication of the Chip

The placenta-on-a-chip device (Figure 3.5) consisted of two microchannels (height: 100 microns; width: 400 microns) fabricated on two PDMS layers. An SU-8 mold for the chip was created using standard soft lithography techniques. The silicon wafer mold was placed in a 15 cm diameter petri dish, and then a 10:1 w/w mixture of PDMS base and curing agent solution (Dow Corning) were introduced into the mold. Once the PDMS had solidified at room temperature, it was cut and peeled away from the mold to separate it into upper and lower layers. To provide fluid access for each individual channel, inlet/outlet holes (1 mm diameter) were created using a biopsy punch. A 0.4 micron pore-sized polyester track etched (PETE) membrane from the membrane inserts (Corning) was used to represent the barrier between fetal and maternal bloodstreams. The membrane covered the mid-section of the lower channel before both layers were treated with plasma for 1 min, and the two PDMS layers were then aligned, attached, and left overnight to perfectly cure the bond. 1/16 ft diameter PEEK tubes (IDEX Health and Science) were then inserted into the inlets and outlets, attached to 0.062 × 0.125 in laboratory tubing (DOW Corning), then left overnight before use. After the layers were permanently bonded, the chip was UV-sterilized for 20 min. Entactin–collagen IV–laminin (E–C–L, Millipore) solution was prepared from a diluted solution of E–C–L with a sterile serum-free medium for each cell line up to a final concentration of 10 µg mL⁻¹. Both sides of the membrane were initially coated with E–C–L solution, after which the chips were refrigerated overnight at 4 °C. Prior to cell seeding, the channels were washed twice with phosphate-buffered saline (PBS) to remove excess E–C–L.
3.4.3. Microfluidic Cell Seeding and Culturing on the Chip

Once the HUVECs and BeWo cells reached 80–90% confluence, the cells were prepared for infusion. The density of the dissociated cells was adjusted to $5 \times 10^6$ cells mL$^{-1}$. The HUVECs
were suspended in EGM medium, seeded into the lower channel, and incubated in an inverted position at 37 °C with 5% CO₂ in air for 1 h to ensure reliable attachment to the membrane. Similarly, the BeWo cells suspended in F-12K medium were introduced into the upper channel and incubated at the original position under similar conditions for 1 h. Once cell attachment was confirmed, the inlet of each channel was connected to 3 mL syringes (Becton, Dickinson and Company) filled with the respective growth media for each cell type seeded into the channels, after which the syringes were connected to a syringe pump driven at a constant volumetric flow rate of 50 µL h⁻¹.

3.4.4. Observing Live Cells

The HUVECs and BeWo cells were stained with CellTracker green and CellTracker orange fluorescent probes (Life Technologies), respectively. Dissociated cells were incubated with staining diluted serum-free medium (final working concentration of 0.5–25 × 10⁻⁶ M) at 37 °C with 5% CO₂ for 45 min.

3.4.5. Investigating the Barrier Permeability

Three thousand megawatt fluorescein–dextran anionic probes (Invitrogen, ThermoFisher) were used to measure the barrier permeability function based on its transport between maternal and fetal channels. Fluorescein–dextran was first diluted in PBS to 100 mg mL⁻¹ then brought to a final concentration of 0.1 mg mL⁻¹ in F-12K medium. F-12K supplement for the maternal channel was replaced with dextran-mixed F-12K and perfused for 4 h. Flow from both maternal and fetal channels was collected each hour and the fluorescence intensity of the collected samples was analyzed using a microplate reader (BioTek Synergy 2).
3.4.6. Cell Characterization for Analyzing Intercellular Junctions

After confirming proliferation of cells on membranes inside the channels for a minimum of 3 days, the channels were rinsed twice with 0.1 M PO₄ buffer, after which the cells were fixed in 4% paraformaldehyde and incubated at room temperature for 20 min. The channels were then washed thrice with PBS at 7 min increments. The channels were subsequently incubated at room temperature in a blocking solution created using 5% normal donkey serum as the normal blocking serum (NBS, Jackson Immuno Research Labs), 0.4% bovine serum albumin (BSA), and 0.2% Triton X-100 for 60 min. Following incubation, primary antibodies (E-cadherin and VE-cadherin [Cell Signaling Technologies] for BeWo and HUVECs, respectively) were diluted in previously prepared blocking serum and incubated in each channel overnight at 4 °C. After being washed in PBS 4 times, the channels were incubated for 90 min with secondary antibodies and DAPI solution diluted in the same blocking solution. The channels were then rinsed with PBS 4 times with 8 min intervals between each rinse. After carefully separating the membrane from the chip, it was mounted to a coverslip and imaged with an inverted microscope (Zeiss Axio Observer Z1).

3.4.7. Analysis of Caffeine Transport

An LC/MS analytical method was used to determine the caffeine concentrations, using an Agilent Technologies 1100 Series advanced high-performance liquid chromatography (HPLC) tandem mass spectrometer equipped with a Poroshell 120 EC-C18 2.7 μm 4.6 mm × 50 mm (Agilent) column (W.M. Keck Metabolomics Laboratory, Iowa State University), to detect caffeine levels.[39] This instrument is composed of a UV–vis capable diode array detector and an Agilent Technologies Mass Selective Trap SL detector equipped with an electrospray ion source.
To prepare the collected samples for runs, each sample was diluted at a ratio of 1:3 in methanol and vortexed for several seconds, after which the samples were centrifuged at 16 × g for 5 min and 100 µL from each sample was transferred to separate vials. The mobile phase was a mixture of water (80% with 0.1% acetic acid) and acetonitrile (20% with 0.1% acetic acid). The mass analyzer operated with an ESI source in positive ion mode, and the flow rate and injection volume were 0.75 mL min⁻¹ and 5 µL, respectively. The quantification for caffeine was determined by measuring the intensity of protonated molecular ions of caffeine at m/z 195.

### 3.4.8. Caffeine Transport across the Placental Barrier

Calibration curves were initially developed so that the correlation could be used to calculate the amount of caffeine transported from the maternal side to the fetal side. Different caffeine (Sigma Aldrich) concentrations ranging from 0.00001 to 0.25 mg mL⁻¹ in EGM and from 0.05 to 0.3 mg mL⁻¹ in F-12K were used to create calibration curves using the data collected via the LCMS method. Assuming that the area under the curve for the caffeine spike from chromatograms was proportional to the concentration of the caffeine in each medium, the two different graphs shown in Figure 3.3 c,d were used to represent the correlation between concentration of caffeine and area under the curve. A 0.25 mg mL⁻¹ caffeine solution in F-12K medium was then introduced into the maternal side. Following a 1 h perfusion period, samples were collected after every 30 min from both the maternal and fetal outlets, and each sample was analyzed using the LCMS method to identify the exact amount of caffeine transported across the placental barrier.
3.4.9. Quantification of Caffeine Concentrations and Transfer Rates

Using Figure 3.3 c,d, the caffeine concentrations for both the maternal and fetal sides were quantified and used to study the percentage increase of caffeine concentration in the fetal compartment over a period of medium perfusion. Equation 3.3 was used to calculate the rate of caffeine transfer.

References


CHAPTER 4. MATERNALLY ADMINISTERED NALTREXONE AND ITS MAJOR ACTIVE METABOLITE 6-BETA-NALTREXOL TRANSPORT ACROSS THE PLACENTAL BARRIER IN VITRO

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Abstract

Opioid use disorder (OUD) has become a growing concern in the U.S. and has been a dominant presence among pregnant women, resulting in an unprecedented amount of prescription medications, particularly naltrexone (NTX), prescribed for pregnant women. Because of unknown potential harm that NTX can impose on the fetus and its premature brain, the needs for safety and regulation of NTX are still undetermined, so we fabricated a microfluidic device to mimic structural phenotypes and physiological characteristic of in vivo placental barrier to evaluate near-transport simulations of NTX and its primary metabolite, 6β-naltrexol, across the human placental-barrier-like membrane. Following transport analysis, epithelial and endothelial cell layers were evaluated for possible gene-expressions released by an in vivo human placenta during NTX and 6β-naltrexol placental exposure. Mean NTX and 6β-naltrexol concentrations in co-culture devices exhibited ~4.21-fold and ~4.67-fold lower concentrations, respectively, compared to concentrations measured in acellular devices. When a 100 ng/mL of NTX and 6β-naltrexol (1:1) were administered to the maternal channel, the mean concentration for co-culture models exhibited
~2.5 % of NTX and ~2.2% of 6β-naltrexol of the initial maternal concentration. To prototype and simulate fetal-brain exposure, perfusate from a fetal channel was directed to cultured N27 cells that were then evaluated for gene-expression. Evidence of cell apoptosis was evaluated with staining for live/dead cells. Similar to procedures for an epithelial or an endothelial cell layer, gene-expression levels were assessed in N27 cells following 8-hour exposure, and the fabricated microfluidic device provided a most satisfactory platform for exploring NTX and 6β-naltrexol virtual transport across a simulated placental barrier environment.

4.1. Introduction

Opioids are composed of natural, artificial, and semisynthetic mediators that can play an essential role in relieving chronic and severe pain,[1] and the discomfort released by opioid intake has caused a demand upsurge for prescription opioids in the U.S., with more than 650 prescription opioids prescribed every day, particularly for back pain, injuries, and disorders in human body movement.[1-2] However, this is an alarming increase in opioid intake, with OUD usage more than quadrupling during the past decade.[3] For example, approximately 115 people fatally overdose from opioids every day, contributing to more than 66% of all drug overdoses,[4-5] unfortunately including much OUD use by pregnant women.[6] Over the past decade, published reports have shown that opioid mediators, especially heroin, are being used extensively among pregnant women in the U.S. (5.6 per 1000 live births).[3] In OUD cases, fetuses have faced incidents of neonatal abstinence syndrome (NAS), postnatal growth deficiency, neonatal mortality, neurobehavioral complications, and other similar impairments,[6] with studies reporting that over 85% of OUD instances among pregnant women were unintended, with an estimated 3.39 per 1000 births facing NAS.[3,7-8] Given this alarming increase in OUD in pregnant women, prescription medications for treating opioid dependence have become more prominent.[3]
NTX, that exhibits little to no addictive qualities,[9-10] is a common form of medicine for treating opioid addiction. Its opioid antagonist functions by blocking $\mu$-, $\kappa$-, and $\delta$-opioid receptors, thereby reducing opioid craving and drug-seeking behaviors.[3, 9] The medication operates by metabolizing cystolic dihydrodiol dehydrogenase into $6\beta$-naltrexol through the liver. $6\beta$-Naltrexol is a primary active metabolite of NTX that contributes 43% of its pharmacological response.[9, 11] While NTX is commonly used for OUD, two other opioid antagonists, methadone and buprenorphine, have been extensively used,[6] with both endorsed by the World Health Organization (WHO) as effective means for treating OUD, accompanied by office-based treatment, brief NAS development, and minimal drug exchanges.[3, 12-13] The same two opioid antagonists have been successful in treatment of cigarette and tobacco addiction[14] and cocaine[15] usage. Although buprenorphine and methadone have been successful in treating opioid-dependent patients, policy constraints have prevented some patient access to these medications,[6, 9] while NTX, both affordable and accessible,[16] has been successful in treating alcoholism[11, 17] and heroin dependence[18], and has also been effective in treating pruritus of uremia[19] and cholestasis[20], and in therapy related to obesity.[21] However, with limited data on safety and effectiveness of NTX during pregnancy, the NTX’s validity as an efficient means for treating OUD in pregnant women is still uncertain.[3] Currently, the Food and Drug Administration (FDA) is still undetermined as to whether NTX crosses the placenta, and it also cautions against use of NTX during lactation.[16, 22] In the latter case, the FDA labels NTX under the lactation subsection for prescription drug labeling, since medical practitioners know only of potential harm and side-effects NTX can impose during breastfeeding,[16] so an increase of in vivo and placebo-controlled NTX testing has occurred during the past decade.[3]
In vivo testing has been the most common form of testing for analyzing NTX concentration levels, with subjects that include mice\textsuperscript{[23-24]}, rats \textsuperscript{[25]}, and rhesus monkeys.\textsuperscript{[26]} The downside of using these models for testing is that while their anatomies resemble those of human anatomy, their placental tissues lack the analogous cellular and tissue interactions.\textsuperscript{[27]} However, the in vivo studies have successfully analyzed NTX with respect to adverse levels of morphine in mice,\textsuperscript{[23]} spinal cord transition states between different doses of NTX in rats,\textsuperscript{[25]} and have also monitored behavioral changes in rhesus monkeys from NTX and 6β-naltrexol consumption.\textsuperscript{[26]} In addition to in vivo as a form of NTX testing, placebo-controlled testing has also been utilized to some extent.\textsuperscript{[17, 20, 28]} For the most part, these studies have recorded only psychological, and antipruritic reactions from NTX and did not directly evaluate opioid exposure in the placental tissue.\textsuperscript{[17, 20, 28]} Fortunately, with the success of the organs-on-chips, specifically the placenta-on-a-chip, NTX and 6β-naltrexol can be examined for its effects in a human placental barrier in vitro.

The placenta-on-a-chip allows us to co-culture trophoblast and endothelial cells, two of the most prominent cell lines in the human placenta (Figure 4.1 (a)-(c)), and to monitor NTX and 6β-naltrexol exposure on the micro-engineered barrier. Culturing the following cell lines allows us to replicate the physiology of an in vivo human placenta, providing us with similar natural and reoccurring reactions from NTX and 6β-naltrexol. Previous studies have proven the validity of using placenta-on-a-chip for assessing and replicating different drug transport analysis while mimicking physiological function of human placenta in vitro.\textsuperscript{[20-35]} Through our most recent study, we analyzed caffeine transport across our fabricated microchip, and proved the model to be compatible with withstanding xenobiotic exposure.\textsuperscript{[35]} Therefore, since placenta-on-a-chip has yet to be tested with opioid contact, our study provides the first appraisal examining NTX and 6β-naltrexol on an organ-on-a-chip.
Figure 4.1. Structure of the human placenta and design of the placenta-on-a-chip device. (a) The placenta is an interim organ that develops only during pregnancy and connects the fetus to the uterine wall via an umbilical cord. (b) Cross-sectional schematic of the human placenta containing important structures called chorionic villi that occupy and demolish uterine decidua and absorb nutritive materials to support fetus maturation; their development is due to the rapid proliferation of trophoblasts. (c) Structure of the placental villi. Syncytiotrophoblast and endothelial cells in maternal and fetal interfaces, respectively, are separated by a basal lamina (a layer of extracellular matrix), at the end of gestational period. During the first trimester, the maternal interface consists of syncytiotrophoblast and cytotrophoblast cells. (d) – (f) Exploded, assembled, and cross-sectional views of the fabricated placenta-on-a-chip before microfluidic cell culture. This device, fabricated to resemble the human placental barrier in vitro, consists of two microchannel-etched PDMS layers separated by a thin semipermeable membrane. (g) Cross-section of a placenta-on-a-chip after microfluidic cell culture, with the endothelium in the fetal interface and the epithelium in the maternal interface are respectively represented by HUVEC and BeWo cell layers.

This study’s intended purpose was to evaluate opioid transport in our placenta-on-a-chip model, and this stage of testing is made possible through the system and foundation of the placenta-on-a-chip, microfluidic device. Microfluidic devices provide us with the ability to culture and manage cellular and subcellular environments within our microchip, maintain fixed compositions of our cell lines, and replicate fluid movement in parallel layers. Our microfluidic device ((Figure 4.1 (d)-(f-f)) was fabricated using standard lithography techniques. To represent the human placenta’s endothelium and trophoblastic epithelium, human umbilical vein endothelial
cells (HUVECs) and trophoblasts cells (BeWo) were cultured accordingly. Once these cells had exhibited behavioral bonding and placental structure in vitro after cell seeding and culturing in their respective microchannels, we began exposing our microfluidic device with NTX and 6β-naltrexol. Additionally, by transferring outflow from the fetal channel to cultured N27 embryonic-dopamine cells (N27 cells), we presented a proof-of-concept for modeling NTX and 6β-naltrexol transport to a fetus brain across the placental barrier. We also used a quantitative polymerase chain reaction (qPCR) method to analyze gene-expression changes that ensue from post-exposure to NTX and 6β-naltrexol. This work provides a platform for both scientific and pharmaceutical communities to examine and extrapolate information regarding safety and potential side-effects caused by prescription opioid medication during pregnancy. More specifically, the knowledge gained should encourage other researchers to test and validate the short and long-term influences NTX imposes on the mother’s placental barrier and the fetus’s premature brain.

**4.2. Experimental Section**

**4.2.1. Cell Culture**

HUVECs and BeWo were chosen to present the cells at the fetal and maternal interfaces, respectively, as previously described.[35] BeWo cells (ATCC) were cultured in Kaighn’s Modification of Ham’s F-12K medium (Thermofisher), supplemented with growth factors and 10% fetal bovine serum (FBS, Gibco). HUVECs (Lonza) were cultured in endothelial growth medium (EGM, R&D Systems). N27 embryonic-dopamine cell line was generously provided by Dr. Anumantha Kanthasamy at Iowa State University. N27 cells were cultured in RPMI 1640 medium (Gibco), supplemented with 10% FBS, 2mM L-glutamine (Gibco), and 100 U/mL penicillin and streptomycin (Gibco). Cell lines were incubated at 37 °C with 5% CO₂ in air until they were 80-90% confluent. After at least three passages, N27 cells were dissociated with
trypsin/EDTA (1X) (Cascade Biologics) and plated into individual wells of a 6-well plate at a density of 25 ×10³ cells/well.

### 4.2.2. Design and Fabrication of the Chip

The placenta-on-a-chip consists of two polydimethylsiloxane (PDMS) slabs, each with a microchannel constrained by the following dimensions: 100 µm (height) and 400 µm (width). The microfluidic device was fabricated using a silicon wafer SU-8 mold produced through standard soft lithography techniques. A mixture of PDMS base and curing agent solution (Dow Corning) at 10:1 (w/w) was introduced into a SU-8 mold placed in a petri dish (15 cm in diameter), as previously described.[35] The PDMS was cut and removed from the mold as upper and lower layers after the PDMS had solidified at room temperature. A biopsy punch was then used to produce inlet/outlet holes (1 mm in diameter) on the upper PDMS layer. A polyethylene terephthalate (PETE) membrane (0.4 µm pore size) as the barrier between two channels, taken from the membrane inserts (Corning®), was placed over the mid-section of the lower channel, after which both PDMS layers were plasma-treated, aligned, bonded together, and left overnight to completely cure the bond. The chip was UV-sterilized for 20 minutes following attachment of inlet/outlet tubing to provide fluid access to each channel, as described previously.[35]

### 4.2.3. Microfluidic Cell Culture

Following UV-sterilization, an Entactin-collagen IV-laminin (E-C-L, Millipore) solution (10 µg/mL in sterile serum-free medium) was coated across both faces of the PETE membrane through the channel inlets, and the chip then was refrigerated overnight at 4°C. On the following day, phosphate-buffed saline (PBS) was perfused twice to remove and wash excess E-C-L from the microfluidic device’s channels, after which the microchip was prepared for microfluidic cell
culture. Before infusion, both cell lines were dissociated with trypsin/EDTA (1X) and cells were resuspended in full growth medium (EGM or F-12K medium). Initially, the HUVECs were introduced into the lower channel at a density of ~5 × 10^6 cells/mL, then incubated at an inverted position at 37°C with 5% CO₂ in air for 1 hour to ensure adherence of cells to the membrane. Similarly, the BeWo cells were seeded into the upper channel at a density of ~5 × 10^6 cells/mL, and after the cells had exhibited adhesion to the membrane, 3 mL syringes (Becton, Dickinson, and Company) filled with EGM and F-12K medium were connected to the inlets of the lower and upper channels, respectively. The syringes were then connected to a syringe pump operated at a flow rate of 50 μL/hour.

4.2.4. Live Cell Staining

The BeWo cells and HUVECs were initially stained with CellTracker orange and green (Life Technologies), respectively. Both cell lines were incubated at 37°C with 5% CO₂ for 45 minutes with staining in a diluted serum-free medium before dissociation, conforming to the manufacturer’s recommended protocol.

4.2.5. Immunohistochemistry

Investigation of the epithelial and endothelial integrity was carried out after confirming proliferation of cells in the microchannels. Initially, cultures in the fetal and maternal channels were fixed in 4% paraformaldehyde at room temperature for 20 minutes then rinsed with PBS, after which the microchannels were incubated with a blocking buffer (0.4% bovine serum albumin (BSA), 0.2% Triton X-100, and 5% normal donkey serum (Jackson Immuno Research Labs)) for 60 minutes at room temperature. After incubation, microchannels were incubated overnight at 4°C with anti-vascular endothelial-cadherin (VE-cadherin) and anti-epithelial cadherin (E-cadherin)
(Cell Signaling Technologies) for HUVECs and BeWo cells, respectively (primary antibodies were diluted with blocking buffer). On the following day, the microchannels were rinsed with PBS followed by incubation with secondary antibodies and DAPI solution (diluted with blocking buffer) in the dark for 90 minutes. Finally, both microchannels were rinsed with PBS and the chip’s membrane was carefully separated from the microchip, mounted to a coverslip, and examined for immunostaining using an inverted fluorescence microscope (Zeiss Axio Observer Z1).

4.2.6. Staining to Visualize Microvilli Formation

After 72 hours of proliferation of cells in the channels, BeWo cells were stained for filamentous actin (F-actin) to visualize formation of microvilli. Following a PBS rinse, the maternal channel was fixed with 4% paraformaldehyde for 15 minutes, after which the cultures in the channel were rinsed with PBS and permeabilized with 0.5 % Triton X-100 for 10 minutes at room temperature. After another PBS rinse, cultures were stained with CF® 488A-conjugated phalloidin (Biotium) (5 µL of a 200 U/mL (in cell culture grade water) stock solution in 200 µL of PBS). Following a counterstain of cell nuclei with DAPI, cells were incubated for 20 minutes at room temperature, washed with PBS, then imaged with a Zeiss Axio Observer Z1 Microscope.

4.2.7. Investigating the Barrier Permeability

The barrier permeability function of the transport between the fetal and maternal microchannels was analyzed using 3000 MW fluorescein-dextran anionic probes (Invitrogen, ThermoFisher), as described previously. The fluorescein-dextran was initially diluted in PBS to 100 mg/mL, in conformance with the manufacturers’ recommended protocol, then the fluorescein-dextran solution was placed in F-12K medium and brought to a final concentration of 0.1 mg/mL. The maternal channel’s F-12K supplement was then replaced and perfused with
dextran-mixed F-12K for 10 hours. At subsequent 2-hour intervals, the perfusate from both the fetal and maternal channels was collected and its fluorescence intensity quantified utilizing a microplate reader (BioTek Synergy 2).

4.2.8. NTX and 6β-Naltrexol Transport Across the Placental Barrier

NTX and 6β-naltrexol were generously provided by Dr. Wolfgang Sadee and Dr. John Oberdick of Ohio State University. NTX and 6β-naltrexol at 1:1 (w/w) were first diluted to 1 mg/mL (in cell culture grade water) then diluted to a final concentration of 100 ng/mL (in F-12K medium). The medium supplement for the maternal channel was then substituted for the NTX/6β-naltrexol-mixed F-12K medium and perfused for 1 hour to remove the NTX/6β-naltrexol-free F-12K medium from the maternal channel. After 1 hour of equilibrium, perfusate was collected every 30 minutes from the fetal channel and analyzed by liquid chromatography/mass spectrometry (LC-MS), and the permeability between the fetal and maternal channels in the microfluidic device during NTX/6β-naltrexol perfusion was analyzed using dextran anionic probes. Fluorescein-dextran was diluted in NTX/6β-naltrexol-mixed F-12K medium (to a final concentration of 0.1 mg/mL) and perfused through the maternal channel, after which the outflow was sequentially collected at 2-hour intervals and its fluorescence intensity was evaluated using a microplate reader, as described above.

4.2.9. LC-MS for Detection of NTX and 6β-Naltrexol

An Agilent Technologies 6540 Ultra-High-Definition (UHD) Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC-MS system equipped with an Agilent Technologies Eclipse C18 1.8 μm 2.1 mm × 100 mm column (W. M. Keck Metabolomics Laboratory, Iowa State University) was used to perform analysis of NTX and 6β-naltrexol in collected outflow samples. The Agilent
system was equipped with a thermostatically-controlled dual-needle injector coupled to an Agilent Technologies 1290 Infinity Binary Ultra High-Pressure Liquid Chromatography (UHPLC) system. The samples were initially transferred into 300 µL conical volume inserts (Thermo Scientific) in 2 mL Surestop vials (Thermo Scientific), loaded onto the Autosampler tray and kept at 4°C during the Q-TOF measurement. A gradient of 1.2 mM ammonia formate (pH 3.5) in High Performance Liquid Chromatography (HPLC) grade water (buffer A) and 1.2 mM ammonia formate (pH 3.5) in a mixture of HPLC grade acetonitrile and methanol (1:2) (buffer B) was used for chromatographic separation. After a 0.2-minute holding time in 100% buffer A, the gradient had transitioned to 33.3% and 48% buffer B in 5.2 to 6.2 minutes, respectively. The composition was switched to 100% buffer B at 8 minutes, followed by a 1-minute hold before returning it to initial conditions by a 4-minute post-run. Electrospray ionization in positive mode was used to detect NTX and 6β-naltrexol as [M + H]^+ ions (flow rate: 0.4 mL/minute, injection volume: and 10 µL). NTX and 6β-naltrexol were identified at m/z 342.17 and 344.18, respectively. Calibration standards were prepared as a diluted series of combined NTX and 6β-naltrexol at 1:1 (w/w) (first diluted in cell culture grade water then to a final concentration in F-12K medium) at the following concentrations: 0.1, 0.5, 1, 5, 12.5, and 25 ng/mL. Agilent Mass Hunter Quantitative Analysis B.07.00 software was utilized to quantify the detection of NTX and 6β-naltrexol. The calculated concentrations of NTX and 6β-naltrexol were adjusted to their final values based on relative volume of samples collected from the outflow of the fetal channel.^[47-48]

4.2.10. Midbrain-Derived Embryonic N27 Cell Exposure to NTX and 6β-Naltrexol

After 5 days of culture, the N27 cells in the 6-well plates were prepared for exposure to NTX and 6β-naltrexol. Following the NTX/6β-naltrexol-mixed F-12K perfusion, outflow of the fetal channel was directed to N27 cell-cultured individual wells of a 6-well plate. The treatment
was performed for 8 hours after 1 hour of equilibrium phase, as described above. Three different experiment groups including two control conditions were used for data analysis under the following conditions: N27 cells cultured in RPMI 1640 medium and had no exposure to NTX and 6β-naltrexol, N27 cells (in RPMI 1640 medium) exposed to the outflow from the fetal channel without NTX and 6β-naltrexol in system, and N27 cells (in RPMI 1640 medium) treated with NTX and 6β-naltrexol through the outflow of the microfluidic channel, for 8 hours.

4.2.11. Quantitative Polymerase Chain Reaction (qPCR)

After exposure to NTX and 6β-naltrexol, HUVECs and BeWo cells from the microfluidic device or N27 cells from 6-well plates were quantified using the qPCR method. After treatment, control and experimental samples were trypsinized, pelleted, and frozen at -80°C, then integrated into single-control and single-experimental sets before homogenization in TRIzol reagent (Invitrogen, ThermoFisher). Following homogenization, RNA isolation and reverse transcription were performed using the Absolutely RNA Miniprep kit (Stratagene) and cDNA synthesis system (Applied Biosystems), respectively. A Qiagen RT² SYBR Green master mix with validated qPCR human primers (for HUVECs and BeWo cells) or mouse primers (for N27 cells) from Qiagen (Frederick) were used to determine relative magnitudes of gene-expression levels using qPCR. Human 18S rRNA or mouse 18S rRNA, the housekeeping genes, were used to normalize each sample, and melting curves and dissociation curves were constructed to verify the gathering of nonspecific amplicons-free peaks, as described in manufacturer’s recommended guidelines. The ΔΔCt method developed to utilize threshold cycle (Ct) values from housekeeping gene and respective gene was used to calculate and report the results as a fold change in gene-expression.⁴⁹⁻⁵₀
4.2.12. Live/Dead Cell Assay

After exposure to NTX and 6β-naltrexol, assessment of both live and dead cells on the N27 cell line was performed with a combination of 10 µM CellTracker green and 4 µM Propidium Iodide (PI, Invitrogen, ThermoFisher) solutions. Each solution was diluted in serum-free medium, following the manufacturers’ recommended protocol. Live and dead cells were detected with green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively, using an Inverted Microscope (Zeiss Axio Observer Z1). The numbers of live and dead cells were determined using ImageJ software.

4.2.13. Statistical Analysis

All experiments were repeated at least three times, with results reported as mean ± S.E.M. Data analysis was performed using a two-tailed Student’s t-test or one-way analysis of variance (ANOVA) using MATLAB (The MathWorks Inc.) or OriginPro (OriginLab Corp.), respectively.

4.3. Results and Discussion

4.3.1. Fabrication and Verification of Placental-Barrier-Like Semipermeable Membrane

The co-cultured microfluidic device consisted of three layers (Figure 4.1 (g)) that replicate a human placenta in vitro, similar to that from a previous study.[35] The top layer, or maternal channel, was composed of BeWo cells that represent the trophoblastic epithelium of the human placenta. Beneath the top layer is a thin semipermeable membrane that imitates the human placental barrier, and adhered to the bottom of the porous membrane is the fetal channel, comprised of HUVECs, to recapitulate the endothelium of a human placenta. The combination of the three layers provides a microfluidic platform for reconstructing the human placental barrier in vitro to study NTX and 6β-naltrexol transport across the maternal-fetal interface. To validate that the
Figure 4.2. Following 24-30 hours of media perfusion under dynamic flow conditions, epithelial and endothelial cell layers were stained with CellTracker live cell staining. (a) BeWo cells were stained with CellTracker orange and scrutinized for red fluorescent protein (RFP). (b) HUVECs were stained with CellTracker green and analyzed for green fluorescent protein (GFP). (c) Fluorescent microscopic image showing epithelial cells (BeWo) stained with anti-E-cadherin and nuclei labeled with DAPI. E-cadherin is a crucial transmembrane protein to maintain cell-cell junctions in epithelial adherens junctions (AJs). (d) Fluorescent microscopic image displaying endothelial cells (HUVECs) stained with anti-VE-cadherin and nuclei labeled with DAPI. VE-cadherin is an essential endothelial-specific protein responsible for controlling endothelial permeability and maintaining cell-cell junction stabilization in AJs. (e) Formation of microvilli on the apical surface of the trophoblast was assessed by testing for the presence of filamentous
actin (F-actin) protein. (f) Placental barrier permeability was investigated using fluorescein-dextran transport across the epithelial-endothelial barrier. \( n = 3 \) independent experiments. Data represented as mean (± S.E.M.). Scale bars, 50 µm. Two-tailed Student’s t-test, ****, \( p < 0.0001 \).

Microarchitecture of the microfluidic device’s soluble microenvironments was represented accurately, cell characterization was performed within the chip. For example, to ensure that the entire surface of the maternal and fetal sides of the porous membrane in channels was covered with BeWo cells and HUVECs, both cell lines were seeded on their respective sides of the cellular matrix and maintained under dynamic flow conditions. After 24-30 hours of microfluidic cell culture, the cells labeled with CellTracker live cells staining (Figure 4.2 (a) and (b)) reflected a proliferation over time of cell populations across the porous membrane in the corresponding channels. The dynamic flow environment in both channels precisely resembled the blood circulation within the maternal and fetal interfaces of the human placenta.

After cell proliferation across both microchannels within their corresponding sides of the ECM-coated membrane had been confirmed, after 48 hours of microfluidic cell culture under dynamic flow conditions, we verified the epithelial and endothelial integrity of the microengineered barrier. For investigation of epithelial integrity, because epithelial cell-cell junctions formed by cell surface protein have a prominent role in retaining epithelial integrity, E-cadherin antibody was used to detect efficient cell-cell junctions in the epithelium.\[^{51}\] E-cadherin, a transmembrane protein essential for maintaining cell-cell junctions in epithelial adherens junctions (AJs), reflects the stability and durability of cell-cell junctions in epithelium. As shown in Figure 4.2 (c), strong complexes of E-cadherin detected on the BeWo cells suggested existence of the epithelium cell-cell junctions throughout the membrane in the maternal compartment. Additionally, in a mature epithelium, high cadherin densities are formed within AJs,\[^{52}\] and the uniform E-cadherin found within the epithelial cell layer suggests the presence of AJs in the maternal cell layer of the microfluidic device. VE-cadherin antibody was next used to examine the
endothelial integrity with respect to adopting AJs in endothelial cells. Interendothelial adhesive junctions play a crucial role in maintaining structural integrity and controlling permeability in endothelium,[53] and, importantly, VE-cadherin is considered an essential component of AJs to maintain endothelial permeability and cell-cell junction stabilization. [54] VE-cadherin was detected across the HUVECs, validating the existence of a distinct network of AJs on the endothelial cell layer. In addition, the immunofluorescence micrograph in Figure 4.2 (d) shows that this cell-specific cadherin protein was homogeneously distributed throughout the endothelium, so the complexes displayed from the anti-E-cadherin in epithelium and the anti-VE-cadherin in endothelium confirmed the formation of cell-cell junctions in AJs and the epithelial and endothelial integrity of the placental barrier in vitro.

Following the investigation of cell-cell interactions, the maternal channel was evaluated for microvilli formation, because the microvillus plasma membrane is involved in facilitating hormonal and immunological interaction between mother and fetus.[55] The highly-structured microvilli based in the intervillous space and surrounded by plasma membrane, are considered to be major placental structural components that facilitate materno-fetal transport of nutrients and metabolites through facilitated/simple diffusion, active-transport, phagocytosis, and pinocytosis.[56-57] As pregnancy proceeds, microvilli are also responsible for placental surface increase by their increase in number and density,[58] so the microvilli in the maternal surface of the syncytial trophoblast cell layer are considered another key characteristic of the human placenta in vivo when constructing a placental-barrier-like semipermeable membrane. The microvilli herein were evaluated by the presence of F-actin protein, essential for cell stability and morphogenesis. As shown in Figure 4.2 (e), a dense layer of microvilli was observed on the fluorescence microscopy images, and variability this dense layer was detected along the apical surface of the
trophoblast cell layer, possibly attributed to differing fluid shear stresses caused by trophoblast cells forming a 3D structure inside the maternal channel, as previously described.\textsuperscript{35} Previous studies for visualizing microvilli under static conditions have resulted in sparse microvillar surfaces.\textsuperscript{29, 31, 33} In conjunction with such previous studies, microvilli visualized herein dynamic flow conditions have demonstrated elongated microvilli formation in our study.

Once the surface of the trophoblast cell layer was evaluated for formation of microvilli in the maternal channel, placental barrier permeability was analyzed to investigate integrity of the placental barrier. The placental barrier function was assessed using 3000 MW fluorescein-dextran anionic probes as a permeability assay. Over a 10-hour period, perfusate from both channels was collected from acellular and co-culture devices and analyzed using calculated mean fluorescence intensities (MFIs). As shown in Figure 4.2 (f), MFIs of the fetal perfusate from acellular devices confirmed a significant passage of fluorescein-dextran ($p < 0.0001$) compared to those collected from the fetal compartment of co-culture devices. Results in Figure 4.2 (f) also revealed that in co-culture devices, while a few molecules passed through the semipermeable membrane, over a 10-hour period, the overall number of MFIs in the fetal compartment of co-culture devices were negligible compared to those in the perfusates collected from the maternal compartments of both acellular and co-culture devices.

The structural phenotypes of the \textit{in vitro} placental barrier were examined to determine the capability of co-culturing epithelial and endothelial cell layers, to verify the proliferation of the cells in each layer, to validate the epithelial and endothelial integrity, and to analyze barrier permeability. Formation of microvilli in the maternal surface was used as an important physiological characteristic in validating the placental barrier \textit{in vitro}. Considering the ability of replicating structural phenotypes and physiological characteristics of an \textit{in vivo} placental-barrier,
our placental-barrier-like membrane fabricated on a microfluidic device provides an ideal platform for modeling near-transport simulation of nutrients and metabolites of a human placental barrier.

### 4.3.2. Analysis of NTX and 6β-Naltrexol Transport Across the Placental Barrier

In this study, we investigated the possibility of mimicking NTX and its primary metabolite, 6β-naltrexol transport, across our fabricated *in vitro* placental barrier. NTX is capable of blocking μ-, κ-, and δ-opioid receptors, but its clinical effects are mainly administered by blocking the μ-opioid receptor.\[^{59}\] NTX to 6β-naltrexol plasma levels were used at dilutions of 1: 10 and 1:1 for oral dosing and long-acting injection, respectively.\[^{60}\] Previous studies have demonstrated that blood NTX of ~8 ng/mL has achieved and putrefied to a level of 1.1 ng/ mL within 24 hours after digesting one 50 mg tablet,\[^{61}\] and ~2-10 ng/mL was identified as a clinically-relevant NTX plasma concentration during sustained NTX exposure from NTX implants.\[^{62-63}\] While it is an oral NTX accomplice with weak compliance, e.g., for pregnant women with opioid dependence, long-acting injection of NTX provides a potential for using it as a probable medication.\[^{64-65}\]

Since this study was carried out to demonstrate the possibility of utilizing our placental barrier in a microfluidic device to study the transport of NTX and 6β-naltrexol across human placenta *in vitro*, we introduced a final concentration of 100 ng/mL NTX and 6β-naltrexol (1:1) to the maternal channel. A concentration of 100 ng/mL in this proof-of-concept was also used to receive a detectable level of NTX and 6β-naltrexol concentrations via LC-MS in perfusate collected from the fetal channel.

Initially, as a control condition, we administered NTX and 6β-naltrexol through an acellular device with no epithelial and endothelial cell layers. As shown in Figure 4.3 (a), fetal naltrexone concentrations calculated with compiling calibration standards (Figure 4.3 (b)) exhibited multiple fluctuation phases over a span of 8 hours. NTX concentrations exhibited sudden
Figure 4.3. (a) Naltrexone (NTX) concentrations in the fetal channel were quantified via liquid chromatography/mass spectrometry (LC-MS). (b) Calibration standards were plotted to perform NTX analysis. (c) 6β-Naltrexol concentrations in the fetal channel were assessed via LC-MS. (d) Diagram showing calibration standards prepared for quantification of 6β-naltrexol. Co-culture devices have both epithelial and endothelial cells. Acellular devices contain only the bare membrane with perfusion of media as a control condition. n = 3 independent experiments. Data represented as mean (± S.E.M.).

drops at 4, 6.5, and 8 hours, and between t = 1 to t = 3.5 hours, t = 4 to t = 6 hours, and t = 6.5 to t = 7.5 hours, with similar patterns of increase observed during the study. This could be attributed to active transport of NTX back and forth between maternal-fetal interfaces. Overall, fetal concentration in acellular devices began to rise from an initial value (0.815 ± 0.044 ng/mL) and reached a maximum concentration of 16.882 ± 0.888 ng/mL at 7.5 hours. Conversely, fetal naltrexone concentrations in the perfusate collected from co-culture devices remained nearly stable without major significant fluctuations compared to concentrations in perfusates collected from
acellular devices. A slight drop in concentration was observed at 4 hours, and from t = 1 to t = 3.5 hours and t = 4 to t = 8 hours, fetal NTX concentration maintained a mean of 1.491 ± 0.323 and 3.176 ± 0.307 ng/mL, respectively. Additionally, before and after the slight drop, fetal NTX levels achieved a concentration of 2.712 ± 1.470 and 4.489 ± 0.834 ng/mL at 3.5 and 7.5 hours, respectively. Overall, the co-culture model maintained a ~4.21 times lower mean NTX concentration compared to that of the acellular model.

Similarly, fetal 6β-naltrexol concentrations were analyzed in perfusates collected in acellular and co-culture devices. As shown in Figure 4.3 (c), final 6β-naltrexol concentrations were quantified after interpolating with generated concentration standards (Figure 4.3 (d)). Correspondingly to fetal NTX concentrations in acellular devices, fetal 6β-naltrexol concentrations in acellular devices exhibited alike trend over a span of 8 hours. The concentrations of 6β-naltrexol in acellular devices exhibited rapid decrease at 4 and 6.5 hours, and, in contrast to NTX, this trend was also observed in fetal 6β-naltrexol concentrations in co-culture devices. For those hours, concentrations of 6.133 ± 3.009 and 12.497 ± 0.459 ng/mL for acellular devices and 1.474 ± 0.715 and 2.542 ± 0.624 ng/mL for co-culture devices were detected, respectively. In comparison, mean fetal 6β-naltrexol concentrations from t = 1 to t = 3.5 hours, t = 4 to t = 6 hours, and t = 6.5 to t = 8 hours were observed as 6.707 ± 1.329, 12.998 ± 2.147, and 14.336 ± 1.487 ng/mL for acellular devices and 1.267 ± 0.275, 2.259 ± 0.427, and 3.612 ± 0.428 ng/mL for co-culture devices, respectively. The mean fetal 6β-naltrexol concentrations analyzed for perfusates collected from acellular devices exhibited a ~4.67-fold higher level compared to those in co-culture devices, for an interval of 8 hours.

Interestingly, in comparison with the initially-administered maternal NTX and 6β-naltrexol concentration (100 ng/mL), the mean fetal NTX and 6β-naltrexol concentrations evinced ~2.5%
and ~2.2% levels in devices in the presence of HUVECs and BeWo cells, respectively. Conversely, in the absence of HUVECs and BeWo cells, NTX and 6β-naltrexol concentrations were ~10.5% and ~10.3% levels with respect to the initial maternal concentrations. Uncommonly, the mean NTX and 6β-naltrexol concentrations evaluated for co-culture model began to rise after 6 hours and continued to increase until the end of the experiments, reaching higher mean concentrations compared to those from t = 1 to t = 6 hours (NTX: 1.936 ± 0.272; 6β-naltrexol: 1.718 ± 0.257). After 6 hours, concentrations sustained mean concentrations of 4.058 ± 0.288 and 3.612 ± 0.428 ng/mL for NTX and 6β-naltrexol, respectively. We suspect that this may be due to a disturbance of barrier integrity of the epithelial and endothelial cell layers in our microfluidic device. If there was such a possible disruption in HUVEC and BeWo cell layers, a rise in concentration levels would be expected for both NTX and 6β-naltrexol because structural and physiological functions were not precisely represented during this scenario. Initially, to verify whether there was a disruption in epithelial and endothelial cell layers, after 8 hours, BeWo cells and HUVECs in microchannels were stained with CellTracker to visualize live cells, and as a control condition, the maternal channel in co-culture devices was perfused with F-12K medium with the absence of NTX and 6β-naltrexol. As shown in Figure 4.4 (a), for control conditions, the epithelial cell layer was not disrupted as seen for the co-culture devices with exposure to NTX and 6β-naltrexol ((Figure 4.4 (b)), and similar observations were identified for the endothelial layer. HUVECs stained for live cells in the control ((Figure 4.4 (c)) seems to have a matured cell layer compared to the raptured cell layer in the co-culture devices with perfusing NTX and 6β-naltrexol ((Figure 4.4 (d)).

Barrier permeability function was also evaluated to identify the time frame in which the disruption of cell layers occurs on the fabricated placental membrane. Fluorescein-dextran was mixed with NTX/6β-naltrexol diluted F-12K medium and perfused over a span of 8 hours.
Figure 4.4. Following NTX and 6β-naltrexol exposure, epithelial and endothelial cell layers were stained with CellTracker live-cell staining. Control and experimental conditions represent co-culture devices perfused with and without NTX/6β-naltrexol in the maternal flow, respectively. BeWo cells and HUVECs were stained with CellTracker orange and green, respectively. (a) BeWo cell layer under control conditions. (b) BeWo cell layer under experimental conditions. (c) HUVEC cell layer under control conditions. (d) HUVEC cell layer under experimental conditions. (e) Transport analysis of fluorescein-dextran across acellular and co-culture devices to evaluate barrier permeability during the NTX/6β-naltrexol transport study. \( n = 3 \) independent experiments. Data represented as mean (± S.E.M.). Scale bars, 50 µm. Two-tailed Student’s t-test, *, \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \); ****, \( p < 0.0001 \).
In contrast to live-cell analysis during NTX/6β-naltrexol exposure, control and experimental studies were performed in acellular and co-culture devices, respectively. No significant differences were observed, and virtually-constant levels of MFIs were displayed in perfusates collected from co-culture devices during the first six hours. The MFIs evaluated from \( t = 6 \) to \( t = 8 \) hours in co-culture devices implied a statistical-significant increase \( (p < 0.001) \) compared to MFI at \( t = 6 \) hours. In comparison with MFI levels measured over 6 hours in co-culture devices, the levels showed \(~4.5\) times greater values in perfusates evaluated from \( t = 6 \) to \( t = 8 \) hours. Interestingly, no significant differences were observed within MFIs of perfusates collected from acellular devices at 2-hour intervals for 8 hours. In addition, the MFIs in co-culture devices always remained lower than those evaluated in perfusates collected from acellular devices. This not only verified that the disruption visualized in microscopic imaging of HUVEC and BeWo cell layers occurred between \( t = 6 \) to \( t = 8 \) hours, but also confirmed that, after 6 hours of NTX/6β-naltrexol exposure, the \textit{in vitro} placental barrier became profoundly permeable to fluorescein-dextran. This placental barrier alteration existing after 6 hours of NTX and 6β-naltrexol perfusion also supported the possibility of becoming eminently permeable to aforesaid drugs. Such a rupture of the \textit{in vitro} placental barrier could be attributed to cell apoptosis due to effects of NTX and 6β-naltrexol on epithelial and endothelial cell layers.

\subsection*{4.3.3. Genetic Analysis of the Placental Barrier Following Post-NTX/6β-Naltrexol Exposure}

In this phase of the study, we evaluated possible genetic changes that the placental barrier undergoes following post-NTX and -6β-naltrexol exposure. Following an 8-hour perfusion of NTX/6β-naltrexol through the maternal channel, HUVECs and BeWo cells were dissociated and quantified using the qPCR method. A previous study reported that low-dose naltrexone (LDN) exhibited a reduced plasma concentration of interleukin (IL)-6 and tumor necrosis factor (TNF)-
α.\(^{[66]}\) TNF-α is considered a proinflammatory cytokine that worsens disease,\(^{[67]}\) and IL-1α is another proinflammatory cytokine that binds IL-1 receptor.\(^{[68]}\) Conversely, IL-6 not only performs as a proinflammatory cytokine, but also an anti-inflammatory myokine and a cytokine involved in responding to inflammation and infection.\(^{[69-70]}\) IL-8 is another cytokine responsible for activating neutrophils in inflammatory regions.\(^{[71]}\) These properties were evaluated with IL-1α, IL-6, and TNF-α for the HUVEC layer and IL-1α, IL-6, and IL-8 for the BeWo cell layer.

![Graph](image)

**Figure 4.5.** Gene-expression analysis of epithelial and endothelial cells following NTX and 6β-naltrexol exposure, via qPCR method. Control conditions consisted of cells in a co-culture device with perfusing NTX- and 6β-naltrexol-free media through the maternal channel. Expression levels were reported as a fold change in gene-expressions analogous to that of human 18S rRNA, the housekeeping gene. (a) BeWo cells from maternal side were assessed for IL-1α, IL-6, and TNF-α genes. (b) HUVECs from the fetal side were evaluated for IL-1α, IL-6, and IL-8 genes. n = 3 independent experiments. Data represented as mean (± S.E.M.). Two-tailed Student’s t-test, *, p < 0.01.

As shown in Figure 4.5 (a), proinflammatory cytokines, IL-1α and IL-6, implied lower fold changes in gene-expression levels when exposed to NTX and 6β-naltrexol compared to levels reported under control conditions. Interestingly, only IL-1α exhibited a significant difference (p < 0.01) even though IL-6 also exhibited a decrease in fold change. TNF-α gene-expression revealed an increase in fold change levels, but no significant difference was detected. Intriguingly, when exposed to NTX and 6β-naltrexol, IL-1α and IL-6 fold change levels in HUVECs (Figure 4.5 (b)) exhibited completely different tendencies compared to fold change levels achieved in BeWo cells.
When evaluated for IL-1α and IL-6 in HUVECs, fold change displayed higher levels with no significant increase than those measured under control conditions. This change in fold change patterns could be attributed to epithelial and endothelial cell apoptosis during NTX/6β-naltrexol exposure. IL-8, a cytokine responsible for activating neutrophils in inflammatory regions, and evaluated in the endothelial cell layer, exhibited a decrease in fold change compare to that under control conditions. Since no significant difference was observed, IL-8 presented the possibility of achieving inflammatory-free conditions in fetal interfaces during an NTX/6β-naltrexol exposure. Due to the lack of availability of gene-expression analysis performed on the maternal and fetal interfaces during an NTX/6β-naltrexol exposure, further investigation is recommended to validate these results before comparing them to genetic analysis of an in vivo human placental barrier.

### 4.3.4. N27 Embryonic-Dopamine Cell Line Exposed to NTX and 6β-Naltrexol

In the next phase of the study, we proposed a possible concept for simulating effects on fetus brain cells from maternally-administered NTX and 6β-naltrexol to the placental barrier, because a previous study on pregnant mice reported that 6β-naltrexol enters the fetal brain at greater levels after promptly crossing the placental barrier. In the previous phase of the study, we evaluated and quantified that both 6β-naltrexol, the primary metabolite of NTX, and the parent drug are capable of entering the fetal compartment. Even though this claim requires further study for validation, we speculated that, it is also possible for NTX to reach the fetus brain because NTX was found in the fetal compartment within the course of this study.

Initially, N27 cells, plated and cultured for 5 days, were exposed to NTX and 6β-naltrexol by simply directing the outflow of the fetal channel, and two control condition measurements were also used to compare the results obtained from N27 cells exposed to NTX and 6β-naltrexol. For
Figure 4.6. The outflow from the fetal channel was directed toward N27 cells cultured in 6-well plates and a live/dead assay was performed on the cells after 8 hours of continuous exposure to the following conditions: (a) Cells were maintained in RPMI 1640 medium with no exposure to outflow from the fetal channel. (b) Cells (in RPMI 1640 medium) were exposed to the fetal-channel outflow from the co-culture devices that perfused NTX- and 6β-naltrexol-free medium through the maternal channel (c) Cells (in RPMI 1640 medium) were exposed to NTX and 6β-naltrexol through the fetal-channel outflow from co-culture devices. Live and dead cells indicated in green and red, respectively. (d) N27 cells under same conditions as above quantified to examine cell viability. Data were calculated from 3-4 images. (e) Gene-expression analysis on N27 cells subjected to conditions (a), (b), and (c). The mouse 18S rRNA, the housekeeping gene, was referenced to report gene-expression levels as a fold change. n = 3 independent experiments. Data represented as mean (± S.E.M.). Scale bars, 50 µm. One-way ANOVA, *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

the first control, N27 cells were maintained in RPMI-1640 medium without introducing outside conditions. The second control condition was to expose N27 cells to outflow from a fetal channel of co-culture devices operated without perfusing NTX or 6β-naltrexol, mainly to verify whether any significant differences can be observed due to the EGM from the fetal compartment because perfusate from the fetal channel contains EGM. Following an 8-hour transfer of perfusate to N27 cells cultured in 6-well plates, cells were stained with a live/dead cell assay. As indicated in Figure 4.6 (a) and (b), fluorescent images displayed only minor cell death under both control conditions,
while live/dead cell assays identified a large amount of cell death on the cell layer (Figure 4.6 (c)) exposed to the fetal channel from co-culture devices perfusing NTX and 6β-naltrexol. To quantify cell viability, fluorescent images were analyzed by counting live and dead cells. Cell viability (Figure 4.6 (d)) exhibited no significant difference ($p > 0.05$) for both control studies, verifying minimal effects against cell apoptosis after N27 cells exposed to EGM. In addition, an experimental study with N27 cells exposed to NTX and 6β-naltrexol showed a significant decrease in cell viability compared to cell viability found in both control studies ($p < 0.001$).

We next evaluated N27 cells for genetic changes following post-exposure to NTX and 6β-naltrexol. The most important segment of this study phase is to determine possible, genetically-defined aftereffects the fetal brain cells exhibit following a post-NTX and -6β-naltrexol exposure through a placental barrier \textit{in vitro}. It has been reported that IL-6 and IL-1β expression levels have produced increases in plasma levels of fetal brains,\textsuperscript{72} and acute inflammatory insult to a developing brain from IL-6 gene-expression,\textsuperscript{73} and the possibility of TNF-α reducing embryonic development of the brain have also been reported.\textsuperscript{74} Sphingosine kinase (sphk)1 enzyme is associated with increasing survival and proliferation of cells,\textsuperscript{75} and sphk1 exhibits standard physiological functions in developing brain cells.\textsuperscript{76} As indicated in Figure 4.6 (e), N27 cells exposed to NTX and 6β-naltrexol exhibited significantly higher fold change levels in IL-6 compared to those under both control conditions, while the control conditions exhibited a significant difference ($p < 0.05$) in fold change levels for IL-6 gene-expression. Fold change levels revealed lower expression levels of IL-1β in N27 cells exposed to EGM and NTX/6β-naltrexol compared to levels in N27 cells maintained in RPMI-1640, but no significant differences were observed. Interestingly, sphk1 gene-expressed fold change levels measured in N27 cells in RPMI-1640 and N27 cells exposed to NTX and 6β-naltrexol remained virtually-constant while the levels
for cells exposed to EGM showed a significantly higher fold change values compared to cells in RMI-1640 and exposed to NTX and 6β-naltrexol (for both, $p < 0.0001$). This could be attributed to rapid cell growth in N27 cells when exposed to EGM, because extra growth factors in EGM could be promoting cell growth. While cells exposed to NTX and 6β-naltrexol were also exposed to EGM, we suspect that NTX and 6β-naltrexol exposure resulted in more effects than EGM exposure. Conversely, TNF-α gene-expression exhibited significantly lower fold change values in N27 cells exposed to EGM than in N27 cells exposed to NTX and its primary metabolite, and EGM. Further studies are warranted to validate these results achieved from genetic analysis of N27 cells following post-exposure to NTX and 6β-naltrexol. This preliminary study was conducted to demonstrate the possibility of utilizing placenta-on-a-chip not only for investigating placental drug transport, but also for post-transport studies to different organs in a fetus, because drugs expected to be transported to other organs in a fetus when crossed the placental barrier.

### 4.4. Conclusions

In our study, we fabricated a human placental barrier in vitro, allowing us to investigate NTX and 6β-naltrexol transport across our micro-engineered barrier. Our multi-layered placenta-on-a-chip design consisted of a maternal and fetal channel and a semipermeable membrane, with the maternal and fetal channels containing co-cultured trophoblast and endothelial cell lines, respectively. Both cell lines adhered to the opposite surfaces of the semipermeable membrane, confirming the placental barrier’s practical behavior. This multilayered placenta-on-a-chip allowed us to replicate the maternal-fetal interfaces of the human placenta and flow of the dynamic environment in the maternal and fetal bloodstreams in vitro. Initially, in vitro placental barrier was evaluated for structural phenotypes and physiological characteristics of a human placenta. Following the barrier verification, 100 ng/mL of NTX and 6β-naltrexol was introduced to the
maternal channel and perfused for 8 hours, and mean fetal NTX and 6β-naltrexol concentrations over this interval were recorded as 2.503 ± 0.255 and 2.223 ± 0.2515 ng/mL, respectively, for coculture devices. The epithelial cell layer after NTX and 6β-naltrexol exposure was evaluated for IL-1α, IL-6, and TNFα and endothelial cell layer was examined for IL-1α, IL-6, and IL-8 genes. During the next phase of the study, perfusate for the fetal channel was directed to investigate embryonic brain cells exposed to NTX and 6β-naltrexol. Following cell viability evaluation, cells were observed for IL-6, IL-1β, sphk1, and TNFα gene-expressions. With enhanced detection through LC-MS, this proof-of-concept can be used to analyze the transport of ~2-10 ng/mL (clinically-relevant plasma concentration for NTX) of NTX and 6β-naltrexol and its effects on a fetus and its premature brain.

References


CHAPTER 5. GENERAL CONCLUSIONS

The chapters in this dissertation provide proof-of-concept with respect to investigating transport of caffeine, naltrexone (NTX), and 6β-naltrexol, the major metabolite of NTX, across the placental barrier in vitro. In each study, a single concentration of caffeine, NTX, or 6β-naltrexol was used to quantify the fetal concentrations. To further investigate caffeine transport and identify a safe dosage of caffeine consumption during pregnancy, multiple maternal caffeine concentrations should be investigated. Similarly, multiple NTX and 6β-naltrexol concentrations should also be analyzed to more precisely determine how different dosages of NTX consumed by a mother could affect the fetus. Additionally, even though gene-expression analysis was performed on both endothelial and epithelial cell layers to identify genetic changes occurring post-NTX/6β-naltrexol exposure, further long-term studies must be performed to confirm whether those results validate the reported findings. Although cancer-derived trophoblast cells (BeWo) and human umbilical vein endothelial cells (HUVECs) were used to represent the epithelium in the maternal interface and the endothelium in the fetal interface, respectively, there have been concerns about the accuracy of this microfluidic device’s maternal-fetal interface. To improve the physiological characteristics of the maternal-fetal interface in vitro, the current co-culture cell model should be replaced with a co-culture of primary villous trophoblast cells and human primary placental villous endothelial cells (HPVECs).

In addition to studies presented in this dissertation, further drug-transport analysis and structural phenotype examination can be performed using with either the current placenta-on-a-chip microfluidic platform or a slightly-modified system. Over the course of a pregnancy, the placenta goes through numerous structural changes that can affect the transport of oxygen, nutrients, xenobiotics, and pharmaceutical drugs, one change being an increase in villous surface
area, and by the third trimester the cytotrophoblast cell layer disappears and decreases the thickness of the placental membrane.\textsuperscript{[2]} These structural changes allow the placental barrier to become more permeable to nutrients and drugs and enhance the passive diffusion transport mechanism. Conversely, during non-ideal situations such as infective conditions, the thickness of the placental membrane will increase and become less permeable by both drugs and nutrients due to a reduced passive diffusion transport mechanism.

In addition to effects of such structural changes on the placental membrane, passive diffusion across the placenta is also dependent on a drug’s physiochemical properties, such as molecular weight, lipid solubility, degree of ionization, and protein binding,\textsuperscript{[2]} and these properties can be effectively used when modeling drug-transport studies with the placenta-on-a-chip device. Along with passive transport analysis, since drugs are often transported by active transport and facilitated diffusion mechanisms, if facilitated diffusion studies are simulated in the placenta-on-a-chip device, this will not only support verification of the respective transport mechanisms, but also will verify the potential of the \textit{in vitro} model to mimic transporter physiology in the human placenta, because facilitated diffusion requires a carrier substance for transfer to occur. There have been previous investigations of glucose transport across the placenta, and further studies should be performed to simulate metabolic transfer of, for example, amino acid, fatty acid, electrolytes, vitamins, and water. Taken together, if oxygen supply to the fetus and carbon dioxide removal from the fetus across the placenta are modeled using the placenta-on-a-chip device, this suggests a possibility that similar investigation of non-ideal conditions will eliminate the need for some current underdeveloped \textit{in vivo} and \textit{ex vivo} drug transport studies.
References
