DEVELOPMENT AND CHARACTERIZATION OF A MOUSE MODEL OF HSV-2 INFECTION DURING PREGNANCY

DEVELOPMENT AND CHARACTERIZATION OF A MOUSE MODEL OF HSV-2 INFECTION DURING PREGNANCY

BY

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A Thesis
Submitted to the School of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree
Master of Science

McMaster University
Hamilton, Ontario, Canada

MASTER OF SCIENCE (2014) McMaster University
(Medical Sciences, Infection and Immunity) Hamilton, Ontario

TITLE: Development and characterization of a mouse model of HSV-2 infection during pregnancy

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NUMBER OF PAGES: xi, 111

Abstract

Problem: Primary HSV-2 infection during pregnancy is associated with adverse pregnancy outcomes. However the mechanisms underlying these outcomes remain largely unknown. In this study we developed and characterized a mouse model of primary HSV-2 infection during early pregnancy and examined its effects on pregnancy and fetal outcomes.

Methods of Study: C57BL/6 female mice positive for vaginal plugs were infected intravaginally (IVAG) with $10^3/10^4/10^5$ PFU/mouse of HSV-2 (333) or saline (control) on gestational day (GD) 5. For comparison, female mice in diestrus stage were infected with HSV-2 at the same doses. Survival, pathology scores and vaginal viral shedding were measured post-infection. Systemic viral dissemination was examined by real-time PCR. Vaginal tissue, implantation sites, placenta and fetuses were examined by histology. Maternal serum (GD 13) and amniotic fluid (GD 8) was collected for multiplex cytokine analysis.

Results: The minimum viral inoculation dose for infection in pregnant mice was 10^3 PFU of HSV-2, compared to 100-fold higher dose required to infect diestrus mice (10^5 PFU). There was a dose-dependent increase in implantation failure and number of resorptions with increasing dose of viral inoculum in pregnant mice at GD 8. In the 10^3 PFU group, although vaginal viral shedding was observed in all mice, 75% survived the infection, while all

the mice in 10^4 and 10^5 PFU groups succumbed to infection by GD 13-15. There was evidence of abnormal placental morphology and necrotic fetal tissues in HSV-2 infected, pregnant mice compared to controls. Presence of HSV-2 DNA was measured in the vaginal tract, uterus (mated non-pregnant mice), and implantations of infected mated mice. HSV-2 DNA was also present in the spleen of the GD 13 time point group.

Conclusions: These results indicate a 100-fold increase in susceptibility to HSV-2 infection during early pregnancy. At higher inoculation doses, IVAG HSV-2 infection spread systemically resulting in poor pregnancy outcomes and maternal mortality, especially in later gestation. At lower inoculation dose, the infection was localized in the reproductive tract and implantation sites, resulting in increased inflammation and adverse outcomes. This model will help to understand pathological mechanisms underlying adverse outcomes following primary HSV-2 infection in pregnancy.

Acknowledgments

"Nobody said it'd be easy, they just promised it would be worth it" – Harvey MacKay. Indeed, the road thus far has been filled with many exhausting, challenging, and rewarding experiences, but through all that, I have to learned to be a better student, teacher, scientist, and most important of all, a better person.

I would like to thank my supervisor, Dr. Charu Kaushic, who has provided me with the guidance, support, and many unique opportunities, both personally and professionally over the years.

To the past and present members of the Kaushic lab, thank you for your encouragement, friendships, and humour. Kristen, thank you for entertaining my questions and is always willing to help. Kristy, you have taught me a lot of the techniques that have proved to be invaluable in my project, you were a great teacher and colleague. Varun, thank for you for the life chats and the scientific insights.

I express my sincere gratitude to my committee members, Dr. Alison Holloway and Dr. Ali Ashkar; thank you for giving up your time and expertise to make my research project better.

JP & Sam, thank you for being there every step of the way. Your love and support meant more to me than words can express.

Most importantly, to my mom, who has sacrificed so much and worked so hard so that I can be where I am today. For that, I am eternally grateful.

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List of Abbreviations

AF	amniotic fluid
ANOVA	analysis of variance
AREB	animal research ethics board
Ct	crossing threshold
d.p.i	days post infection
ELISA	enzyme linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GD	gestational day
H_2O	water
H&E	hematoxylin and eosin
HRP	horseradish-peroxidase
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
IFN-γ	interferon gamma
IHC	immunohistochemistry
IL	interleukin
IVAG	intravaginal
KC	keratinocyte-derived chemokine
LIX	LPS induced CXC chemokines
LPS	lipopolysaccharide
MDG	millennium development goal
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NK	natural killer
NS	not significant
PAS	periodic acid-Schiff stain
PBS	phosphate buffered saline

PFU	plaque forming unit
p.i	post infection
PMN	polymorphonuclear
qPCR	quantitative real-time polymerase chain reaction
rpm	revolutions per minute
SEM	standard error of the mean
STI	sexually transmitted infection
TGF-β	transforming growth factor beta
Th	T helper lymphocyte
TNF-α	tumour necrosis factor-alpha
UN	United Nations

Declaration of Academic Achievement

All experiments were conceived and designed by Philip Nguyen and Charu Kaushic. Kathy Luinstra and Marek Smieja helped with the design of real-time PCR (qPCR) primers and protocols. John Mayberry histology facility staff optimized and performed all histology staining. Philip Nguyen performed all mouse and qPCR experiments. Philip Nguyen and Charu Kaushic performed data analysis. Philip Nguyen wrote this dissertation with contributions from Charu Kaushic.

CHAPTER 1: INTRODUCTION

1.1 Introduction

Pregnancy and childbirth represents both an exciting period in a woman's life and an important window of opportunity to improve maternal health and reduce fetal mortality and morbidity. In 2013, the United Nations (UN) released a special report as part of the United Nations Millennium Development Goal (MDG) 5, outlining the issue of maternal health (World Health Organization, 2012). The MDG 5 aims to reduce the maternal mortality rate by 75%, and ensures universal reproductive healthcare by 2015; however progress has been hampered by inequalities as outlined in the MDG progress report in 2012 (World Health Organization, 2012). One of the major contributions of perinatal mortality (approximately 70%), and long-term neurologic morbidity in the fetus is due to preterm labour (Goldenberg, Hauth, & Andrews, 2000; Velu et al., 2011). As a result, research aimed to tackle this issue is an important public health concern.

1.2 Pregnancy Complications

With so many physiological modifications and changes throughout gestation, there are inherent risks of complications associated with pregnancy. Some of these complications have both an immediate and/or long-lasting metabolic or psychological effect on the offspring. Previous

studies have shown increased maternal and fetal mortality and morbidity due to preeclampsia (a pregnancy condition characterized by hypertension and proteinuria), preterm labour, fetal inflammatory response syndrome (FIRS), intrauterine growth restriction (IUGR), and abortion (Arechavaleta-Velasco et al., 2008; Goldenberg et al., 2000; Gotsch et al., 2007; Romero et al., 2007; Sibai, Dekker, & Kupferminc, 2005). There are many contributing factors to these complications, for example, bacterial and/or viral intrauterine infections, genetics, environmental, and diet to name a few (Galtier-Dereure, Boegner, & Bringer, 2000; Kourtis, Read, & Jamieson, 2014; Sibai et al., 2005). A large proportion of preterm births are considered to be idiopathic, but up to 40% are associated with intrauterine infections (bacterial and/or viral) (Agrawal & Hirsch, 2012). The inflammatory environment in the placenta may affect the normal development of the fetal immune system, leading to abnormal responses later in life (G. Mor & Cardenas, 2010).

Previously, there were limited studies investigating the host-pathogen interaction at the maternal-fetal interface, and even more so in regard to fetal health outcomes. More recently, a series of papers have been published investigating selected prenatal pathogens (such as human cytomegalovirus, Parvovirus B19) and their effects on the clinical outcomes to fetus (Enders, Daiminger, Bader, Exler, & Enders, 2011; Iwasenko et al., 2011; Ross & Boppana, 2005; Syridou et al., 2008; Williams et al., 2013). These studies have shown to various extents that infections during first trimester with these

pathogens are associated with intrauterine fetal deaths, preterm births, and/or various other central nervous system sequelae. It is evident that investigation into the mechanisms by which these diverse pathogens initiate adverse outcomes in the fetus is a key area of research; not only to advance the understanding of host-pathogen interaction at the maternal-fetal interface, but also for its importance in improving maternal-fetal health.

1.2.1 Preterm births

Preterm labour is defined as labour that begins before 37 weeks of gestation (Agrawal & Hirsch, 2012). Preterm birth may be due to infection, multiple gestation, placental abruption, hormonal disruptions and other unknown factors. In 2010, more than 1 in 10 births (approximately 15 million babies) were born premature globally (before 37 weeks of gestation), of which, more than 1 million died as a result of prematurity (March of Dimes, 2012). Preterm births represent a major global health concern, especially when it is on the rise in most countries with available data (March of Dimes, 2012). In 2011-2012, preterm birth rate in Canada was approximately 1 in 12 births (8% of the population) (Canadian Institute for Health Information, 2013). More importantly, preterm birth is now the second leading cause of death in children under the age of 5, and it is the single most important cause of death in the first month of life (March of Dimes, 2012). The health implications of preterm birth extend beyond the neonatal period. Babies who

are born prematurely have an increased risk of developing autism, cerebral palsy, developmental delays, chronic lung disease, and vision and hearing impairments (G. Mor & Cardenas, 2010).

1.2.2 Fetal inflammatory response syndrome

Fetal inflammatory response syndrome (FIRS) is a condition characterized by systemic activation of the fetal innate immune system (Gotsch et al., 2007). The condition was originally classified as an elevated level of IL-6 in the fetal plasma > 11 pg/mL and involved multiple organ systems. (Gomez et al., 1998). Furthermore, funisitis and chorionic vasculitis (inflammation of the connective tissue of the umbilical cord) are the histopathologic hallmarks for FIRS. This is significant to note since two thirds of fetuses with FIRS have neutrophilia, which is defined as a neutrophil blood count above the 95th percentile for the gestational age (Romero et al., 2012). Fetuses with FIRS have been associated with higher risk of developing severe neonatal morbidity (e.g. respiratory distress syndrome, neonatal sepsis, pneumonia, bronchopulmonary dysplasia, or necrotizing enterocolitis) (Gomez et al., 1998). Due to the unique physical location of the fetus within the mother, it is important to understand the immunological response within non-pregnant and pregnant female reproductive tract (FRT).

1.3 Immunology of the Female Reproductive Tract

Sir Peter Medawar is generally considered to be the founding father of reproductive immunology. His 1953 essay to the Society for Experimental Biology helped put in focus the paradoxical relationship between the mother and the antigenically foreign fetus (Medawar, 1953). Later, Billingham and colleagues further postulated, in order for the semi-allograft fetus not to be rejected by the maternal immune system, the uterus, similar to the brain, eyes, and the hamster cheek pouch, is an immune-privileged site (Billingham, 1964). However, subsequent studies showed the uterus could, in fact, elicit immune responses to reject the allograft skin transplantation (Beer, Billingham, & Hoerr, 1971). Furthermore, both oral and intravaginal immunization can lead to the production of specific antibodies in the uterus and vaginal tract (Ogra & Ogra, 1973). Subsequently a number of studies in the last two decades have shown that the female reproductive mucosa is immunologically active, able to recognize and distinguish between pathogens (W. H. Kutteh, Hatch, Blackwell, & Mestecky, 1988; W. H. Kutteh & Mestecky, 1994; M. McDermott et al., 1984; Mestecky, 1987). These findings were further substantiated when Yeaman et al. (1997) showed that the uterine endometrium contains organized lymphoid aggregates that are located in the stratum basalis layer (Figure 1) (Nguyen, Kafka, Ferreira, Roth, & Kaushic, 2014). These structures have a B-cell core that is surrounded by $CD8^+$ T cells, with an outer halo of macrophages (Nguyen et al., 2014; Yeaman et al.,

1997). The size of these lymphoid aggregates vary with the stage of the menstrual cycle, it is significantly larger during the secretory phase when compared to the proliferative phase (Wira, Fahey, Rodriguez-Garcia, Shen, & Patel, 2014). Furthermore, immunohistochemical studies have shown that the endocervix contains the most IgG and IgA antibody-producing and antibody-containing cells compared to the ectocervix, vagina, and fallopian tubes (Russell & Mestecky, 2002). The FRT is unique in that the dominant antibody response in genital secretions is IgG instead of IgA, despite the presence of IgA. Both IgA1 and IgA2 are found in cervical secretions; interestingly, while cervical mucus contains approximately 70% of IgA in its polymeric form (pIgA), the vaginal secretions have approximately equal proportion of pIgA and monomeric IgA (mIgA) (W. Kutteh, Mestecky, & Wira, 1999).

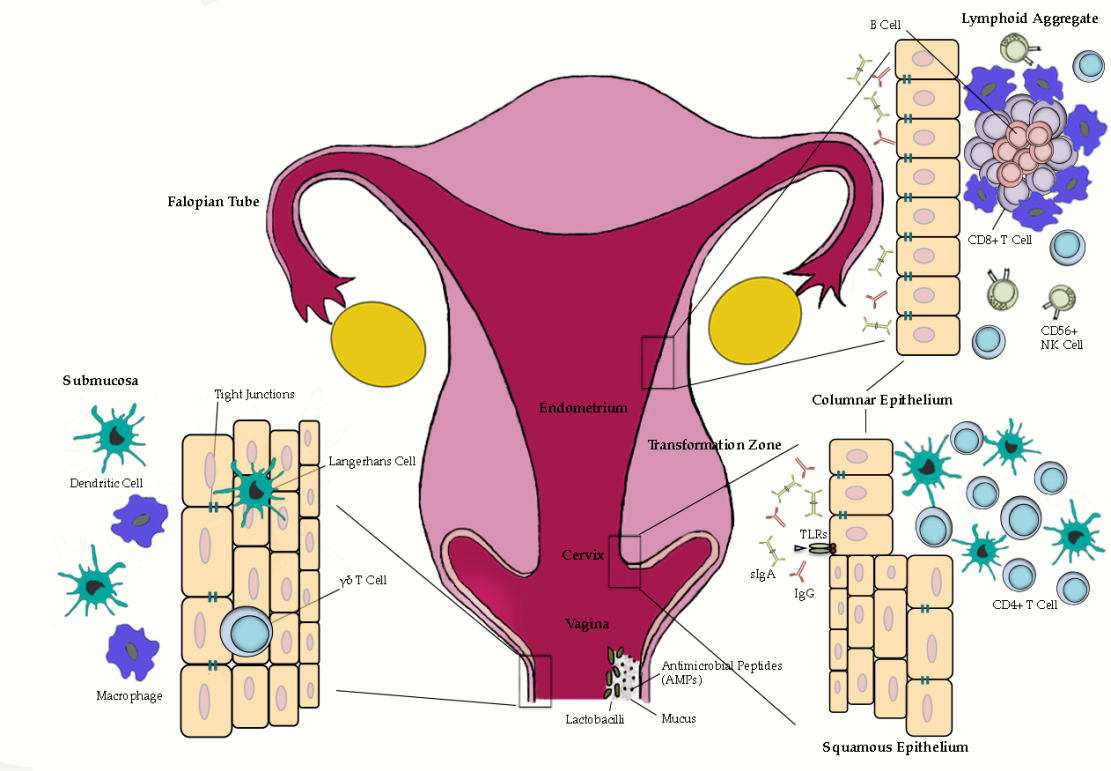


Figure 1 - Anatomical and immunological components of the female reproductive tract. The female reproductive tract consists of an upper (fallopian tubes, uterus and endocervix) and a lower (extocervix and vagina) tract. The vaginal epithelium has many innate immune-mediated protection mechanisms, such as tight junctions, AMPs and mucus, to neutralize, trap, and prevent the entry of potential pathogens. Commensal bacteria colonize the vaginal lumen, mainly Lactobacilli spp., which helps maintain a low pH environment and produce reactive oxygen species. Furthermore, innate immune cells, such as γδ T cells, DCs, and macrophages, are present beneath and between vaginal epithelial cells layer to survey the local environment for danger. Although traditional mucosal lymphoid structures are not found in the female reproductive tract, lymphoid aggregates in the endometrial tissue that are composed of B cells in the inner core and surrounded by $CD8^{+}CD4^{-}$ T cells and an outer layer of macrophages have been described. Scattered $CD56^{+}$ NK cells and $CD4^{+}$ T cells can be found between lymphoid aggregates. The immune cells and functions of the female reproductive tract are regulated by sex hormones that orchestrate cyclical changes with the menstrual cycle. AMP, anti-microbial peptide; DC, dendritic cell; NK, natural killer. (Nguyen et al., 2014)

1.4 Immunology of Pregnancy

Medawar offered some possible mechanisms through which the fetus escapes immune rejection: 1) the anatomical separation between mother and the fetus; 2) the antigenic immaturity of the fetus; and 3) the immunological inertness of the mother (Medawar, 1953). Much of this framework has been widely studied and reviewed comprehensively (Thaxton & Sharma, 2010; Trowsdale & Betz, 2006; Van Nieuwenhoven, Heineman, & Faas, 2003; Warning, McCracken, & Morris, 2011).

Previous studies have postulated pregnancy is an anti-inflammatory condition. The semi-allogeneic fetus protects itself by secreting T helper (Th2) cytokines (e.g. IL-4, IL-5, IL-6, IL-10, and IL-13) in order to down regulate harmful Th1 cytokines (e.g. IL-2, IFN-γ, and TNF) that may lead to abortions or other pregnancy complications (G. Mor, 2008; Wegmann, Lin, Guilbert, & Mosmann, 1993). However, various studies have provided evidence disputing the dogma that pregnancy is primarily a Th2 dominant state, but instead, the predominant immunological state corresponds with the different stages of pregnancy (Challis et al., 2009; G. Mor, 2008; G. Mor, Cardenas, Abrahams, & Guller, 2011; Gil Mor, 2006).

In order to have a successful implantation, the blastocyst and the maternal endometrium environment has to be tightly regulated during the so-called “implantation window” (Gil Mor, 2006). The implantation window ranges from gestational day (GD) 3-5 in mice and days 20-24 of the regular 28-day

menstrual cycle in humans (Gil Mor, 2006). The evidence of maternal T cells in the uterine endometrium with a strong Th1 profile as well as high expression of TNF-α mRNA during the implantation window suggests a moderate Th1 profile is essential for implantation (Hunt & Roby, 1994; Gil Mor, 2006). Furthermore, Ashkar et al. have shown that the production of a typical Th1 cytokine, interferon (IFN)-γ by uterine natural killer (uNK) cells, a special type of NK cell that is $CD56^{bright}$ $CD16^{-}$, largely responsible for uterine vasculature remodelling during gestation, is required for normal fetal implantation in mice (Ashkar & Croy, 1999, 2001; A. A. Ashkar, J. P. Di Santo, & B. A. Croy, 2000). It was shown that the level of IFN-γ production by peripheral lymphocytes in very early pregnancy was higher while IL-4 and IL-10 production was lower in normal pregnant women when compared to women who suffered from recurrent miscarriages (Liu et al., 2012).

As a result of these studies, it is evident that the balance between Th1 and Th2 cytokines during pregnancy is not as clearly demarcated as has been described previously in the literature. The inconsistencies seen in previous studies were due to the oversimplification of pregnancy as a single event (G. Mor & Cardenas, 2010). In fact, pregnancy can be separated into three distinct immunological stages – pro-inflammatory during the first to early-second trimester; anti-inflammatory environment during the second and much of the third trimester; this ultimately changes to an inflammatory

environment in order to promote the contraction of the uterus, expulsion of both the baby and the placenta (G. Mor & Cardenas, 2010).

During early implantation, Th1 cytokines (IL-6, IL-8, and TNF-α) can be secreted by endometrial cells, as well as immune cells that are recruited to the site of implantation to create an inflammatory environment (Zhou, 2012). The predominant population of uNK cells and monocytes are recruited through chemokine secretion by cytotrophoblasts, particularly macrophage inflammatory protein (MIP)-1α, these cells stay in the decidua throughout the pregnancy (Blencowe et al., 2012). As a result, the human decidua contains a large number of immune cells, such as macrophages (20-25%), NK cells (70%), dendritic cells (DCs) (1.7%), and T cells (3-10%) (T. Y. Tan et al., 2013). Within these different types of immune cells, some should be discussed with special interest, such as uterine DCs (uDCs), which have been recently associated as having a role in decidual tissue remodelling, just like the uNKs and macrophages (Erlebacher, 2013a). The anti-inflammatory environment during pregnancy is largely facilitated by non-lymphoid tissues, including the placenta and the decidua, which all produce a high amount of Th2 cytokines (e.g. IL-4, IL-10) (Ross & Boppana, 2005). Furthermore, some immune suppressive properties are conferred by the T regulatory (T_{reg}) population, which accounts for approximately 5% of the $CD4^+$ T cells (Erlebacher, 2013a). It is evident that some level of inflammation exists throughout pregnancy; however, this is kept under strict regulation. Loss of

uNKs or uDCs have been shown to result in severe impairment of implantation, and leads to embryo resorption (Braud et al., 1998; Enders et al., 2011); conversely, if too much inflammation is present (for example, during bacterial or viral infections), this could also lead to adverse pregnancy outcomes (Goldenberg et al., 2000; Srinivas et al., 2006).

1.5 Mouse Placental Development

The placenta represents both a mechanical and physiological barrier at the maternal-fetal interface, it is the first organ to form during mammalian embryogenesis and is the main site of gas and nutrient exchange between the mother and fetus (Rossant & Cross, 2001; Watson & Cross, 2005). The mature placenta consists of three layers: the labyrinth, the spongiotrophoblast, and the maternal decidua (Figure 2) (Watson & Cross, 2005). Apart from the maternal decidua, which usually is of epithelial or connective tissue origin of the ovary, oviduct or uterus, the fetal component of the placenta comes from the ectodermal epithelium, the trophoblast, or trophectoderm. The trophectoderm layer of the blastocyst is the first cell type to differentiate in the mammalian embryo, which makes up the trophoblast (Figure 2). Different lineages of the trophoblast provide the main structural and functional components needed to bring the fetal and maternal blood system into close contact (Rossant & Cross, 2001). The trophoblast, with its associated fetal blood vessels, undergoes extensive villous branching to

create a densely packed structure called the labyrinth (Rossant & Cross, 2001). Simultaneously, the chorionic trophoblast cells begin to differentiate into the various layers of labyrinth trophoblast cells. In mice, there are two layers of synctiotrophoblast cells (Figure 3) that are in direct contact with the endothelial cells of the fetal-derived blood vessels. While the labyrinth is developing, it is supported structurally by the spongiotrophoblast cells, which forms a compact layer of non-syncytial cells between the labyrinth and the outer giant cells (Rossant & Cross, 2001). The maternal blood eventually enters into the small spaces of the labyrinth where it bathes directly the fetal trophoblastic villi, ensuring the ease of material exchange between the two blood systems (Gabbe et al., 2012). Secondly, the synctiotrophoblasts form a highly polarized layer of microvilli at the apical surface in order to facilitate nutrient, and gas exchange between the mother and the fetus (Gabbe et al., 2012). Furthermore, the synctiotrophoblasts are multinucleated, terminally differentiated cells that are responsible for the production of various critical hormones during pregnancy (Gabbe et al., 2012; Rossant & Cross, 2001).

The normal morphology and immunological response within the placenta is critical to the health of the fetus. This has been demonstrated in the placenta of preeclamptic women, where there was an increase in the number of macrophages encircle spiral arteries accompanied by a few invaded trophoblasts (Redman, Sacks, & Sargent, 1999). As a result, the activated macrophages may have been a contributing factor to the damaged

spiral arteries in the decidua basalis and decidua vera (Redman et al., 1999; Reister et al., 1999). Furthermore, increased inflammatory cells, such as macrophages has been shown to be associated with necrosis, and hyperplastic blood vessels in pregnant women with primary antiphospholipid syndrome (Stone et al., 2006). Using a mouse model, Murphy and colleagues (2005) showed that fetal resorption in IL-$10^{-/-}$ mice was associated with a significant increase in uNK cell cytotoxic activation and invasion into the placenta. With this information in mind, the effects of infections and inflammation during pregnancy must be explored further.

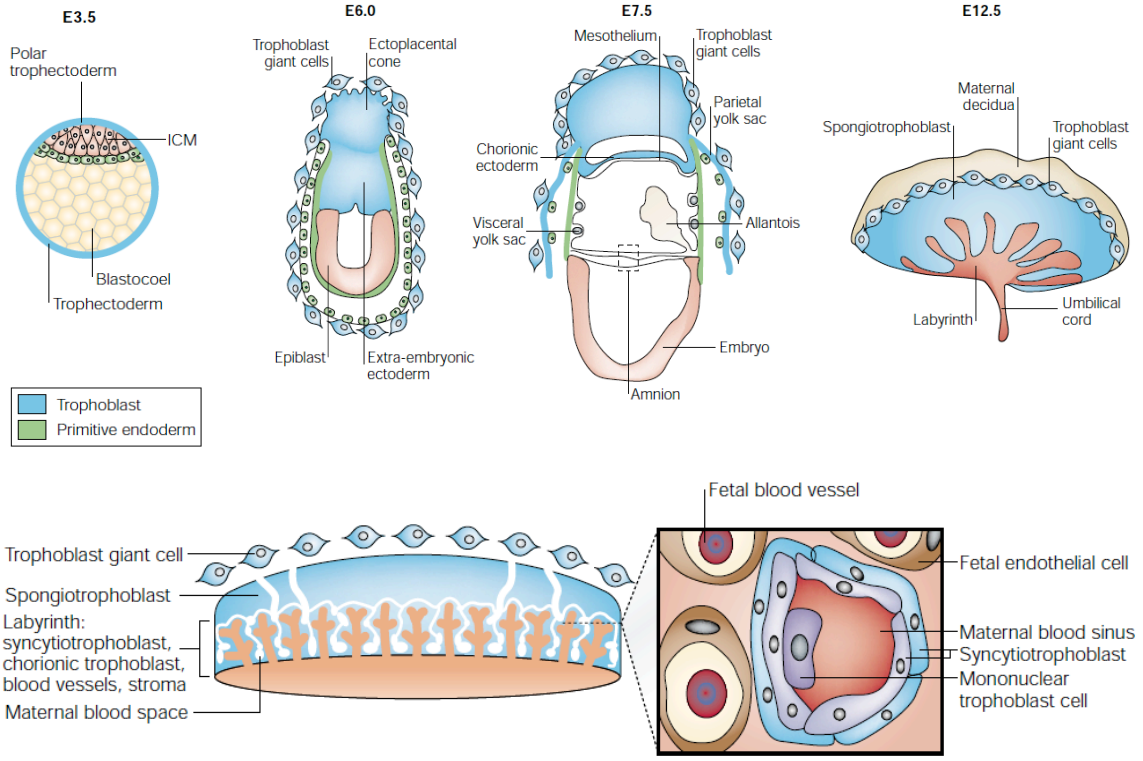


Figure 2 - Structure of the mouse placenta. Early development of the mouse embryo from embryonic day (E)3.5 – E12.5, is showing the origins of the extra-embryonic lineages and the components of the placenta. The inset details the fetal-maternal interface in the labyrinth. ICM, inner cell mass.

1.6 Infections in Pregnancy

In utero recognition of pathogens by transmission of pathogens from the mother to the fetus could theoretically occur via three different routes: 1) infected macrophage from the maternal blood could migrate to the placental trophoblast layers, 2) pathogen or infected cells could move from the maternal endothelial microvasculature to the endovascular extravillous cytotrophoblasts, and 3) ascending infection via the urogenital tract (Delorme-Axford, 2013). However, the exact mechanisms of pathogen transfer from mother to child remain poorly elucidated.

Various clinical studies have shown an association between pre-term births, FIRS, spontaneous abortions, and IUGR with intrauterine infections (Gil Mor, 2006). Thus understanding the role of the innate immune system at the fetal-maternal interface plays an important factor in the pregnancy outcome. One of the ways in which the innate immune system could differentiate between non-infectious self and infectious non-self is via the evolutionary conserved pathogen recognition receptors (PRRs) (Medzhitov & Janeway, 2002). These PRRs recognize and bind tightly to pathogen-associated molecular patterns (PAMPs), which are expressed on the surface of microorganisms, in order to elicit various immune responses in an attempt to control the pathogen, and/or activate the adaptive arm of the immune system (Janeway Jr & Medzhitov, 2002). There are many types of PRRs, including mannose-binding receptor and the scavenger receptor, however,

toll-like receptors (TLRs) have been implicated to play an important mechanistic role in infection associated inflammation, apoptosis, and preterm labour (Abrahams, 2008; Elovitz, Wang, Chien, Rychlik, & Phillippe, 2003; Koga et al., 2009). Previous studies have shown that trophoblast cells from term placenta express TLR 1-10 at the RNA level and TLR 2 and 4 at the protein level (Holmlund et al., 2002; Zarember & Godowski, 2002). Furthermore, different TLR activation triggers distinctive responses from the trophoblast. As an example, TLR-4 activation had been shown to promote cytokine production, while ligation of TLR-2 induces apoptosis in first trimester trophoblast cells (Gil Mor, 2006).

1.6.1 TORCH infections

The term TORCH was initially coined by Nahmias et al. to designate pathogens associated with known congenital and fetal diseases (Nahmias et al., 1971). This is especially important during pregnancy, since many maternal infections can potentially lead to vertical transmission and subsequent fetal morbidity or mortality. TORCH infections include toxoplasmosis, Parvovirus B19, varicella zoster virus (VZV), human immunodeficiency virus (HIV), enterovirus, *Listeria monocytogenes, Treponema pallidum*, rubella, cytomegalovirus, and herpes simplex virus type 1 and 2 (HSV-1, -2) (Kinney & Kumar, 1988). More recently, it has been proposed that syphilis should also be added to this panel of infections

(TORCHES) (Stegmann & Carey, 2002). Despite the fact that TORCHES infections have been shown to cause serious maternal and fetal health effects, routine screening for TORCHES infections is currently not recommended, since no clear benefits has been shown with the associated costs (Kaur, Gupta, Nair, Kakkar, & Mathur, 1999).

1.6.2 HSV-2 in pregnancy

Understanding the effect of *Herpes simplex 2* (HSV-2) during pregnancy is of critical importance due to the prevalence and potential detrimental effects of the infection on the pregnancy and/or fetus. A prospective study carried out in the United States by Brown et al. (1997) had shown that pregnant women represents the second largest group of adults with disseminated HSV; approximately 22% of pregnant women are HSV-2 positive, from this, about 2% of women acquire primary HSV-2 infection during gestation (Brown et al., 1997). Both primary infection and recurrent maternal infection could lead to transplacental infection and congenital disease, although the associated risk is lower in recurrent infections (Jaiyeoba, Amaya, Soper, & Kilby, 2012; Straface et al., 2012). Both HSV-2 primary infection and transplacental intrauterine infection (occurs in <5% of primary infections) have been associated with adverse pregnancy outcomes, including stillbirth, preterm labour (before 37 weeks of gestation), spontaneous abortion, and low birth weight (Arvaja et al., 1999; Brown et al., 1997). Recurrences of genital HSV infection increase in frequency during

pregnancy, although the clinical characteristics of recurrent genital HSV-infection are similar in pregnant women and non-pregnant women (Brown et al., 1985). Very little is known about the underlying mechanisms of preterm birth, and spontaneous abortions, especially in association with chronic viral infection, thus further research is warranted.

1.7 Herpes Simplex Virus 2 (HSV-2)

1.7.1 Epidemiology and pathogenesis

Herpes simplex virus 2, belonging to the *Herpesviridae* family, is a linear, double-stranded DNA virus that is largely responsible for genital ulcer disease, causing over $1 billion in direct and indirect medical costs in the US healthcare system alone (Blencowe et al., 2012; Goldenberg et al., 2000; March of Dimes, 2012). In 2003, it was estimated that 536 million people worldwide between the ages of 15-49 were infected with HSV-2 (Looker, Garnett, & Schmid, 2008). Globally, an additional 23.6 million new infections occur each year, underscoring the importance of public-health concerns for this disease (Looker et al., 2008).

Most primary HSV-2 infections are subclinical, thus these individuals are usually not aware that they are infected (Xu et al., 2002). As a result, asymptomatic viral shedding is responsible for the majority of transmitted HSV-2, since shedding occurs even in the absence of clinical reactivation of the disease (Kaushic, Jerse, & Beagley, 2014; Wald et al., 2000). It has been

estimated that HSV-2 could be transmitted fairly efficiently, with a median number of 40 sexual acts before transmission (Wald et al., 2006). When clinical symptoms are present, the patient experiences localized painful genital lesions and swollen regional lymph nodes. As the result of direct infection by the virus, the genital epithelium undergoes cellular lysis and focal necrosis. Primary infection involves the lytic cycle of HSV-2, in which the virus is able to replicate itself and progressively infect more cells (Knipe & Howley, 2007). However, HSV-2 could also travel to the dorsal root ganglia (DRG) via retrograde axonal transport to establish a latent infection, wherein the virus exists as circular DNA in the nucleus (Cunningham et al., 2006; Knipe & Howley, 2007). The virus population in DRGs serves as a reservoir for latent virus, which is responsible for periodic reactivation where the virus travels back to the epithelial layer causing viral shedding due to various emotional and physical triggers (Avgil & Ornoy, 2006; Johnston, Koelle, & Wald, 2011). As a result of this latency, HSV-2 is able to subvert the immune system, causing periodic flair-ups despite antiviral therapy and becomes one of the most prevalent STIs in the world.

HSV-2 infection has long-term health implications, such as higher risk of adverse pregnancy outcomes, neonatal herpes in pregnant women; central and peripheral nervous system infections, and increased risk of co-infections with other STIs and HIV-1 (Brown et al., 1997; Minton, 2013; Sauerbrei & Wutzler, 2007; Srinivas et al., 2006; Szucs, Berger, Fisman, & Harbarth,

2001). Women are more susceptible HSV-2 infection than men, the serological prevalence in Canada ranges from 7-28% in pregnant women who are between the ages of 15 to 44, underscoring the prevalence and importance of detection and treatment, especially in pregnant women (Mertz, Benedetti, Ashley, Selke, & Corey, 1992; Patrick et al., 2001).

Most HSV-2 infections are unrecognized and undiagnosed, which also contributes to the higher rate of transmission, especially for unprotected sexual activities; infected individuals, even with very mild symptoms, shed HSV-2 virus through their mucosa and genital fluids to infect their sexual partners (Gupta, Warren, & Wald, 2007). HSV-2 virion, composed of viral DNA enclosed in a capsid that is surrounded by tegument and encased in an outer envelope, has the capacity to infect both epithelial and neuronal cells (Connolly, Jackson, Jardetzky, & Longnecker, 2011; Paludan, Bowie, Horan, & Fitzgerald, 2011). Upon binding and fusion of the cell membrane with viral glycoproteins (gB and gC) via heparan sulphate, the tegument, capsid, and viral DNA are released into the cell (Connolly et al., 2011). The capsid and viral DNA are subsequently shuttled and released into the nucleus (Knipe & Howley, 2007). Once inside the nucleus, the HSV-2 virion utilizes host machinery to replicate and transcribe viral genome (Knipe & Howley, 2007). As a result of this, viral genes expressions are produced, and can be classified into α-, β-, and γ-genes (Knipe & Howley, 2007). The initial transcription of α-gene leads to the activation of β-gene, where the majority of

the enzymes and factors associated with viral DNA transcription are transcribed (Knipe & Howley, 2007). Most of the structural proteins are made by the transcription of the γ-gene (Knipe & Howley, 2007). Once the virion components and DNA are assembled as viral progeny, they are released from the infected cell either by exocytosis or lysis of the cell (Paludan et al., 2011). Free viral progenies are then able to infect other cells in the surrounding environment (Knipe & Howley, 2007).

1.7.2 Innate Immune responses

The immune response to HSV-2 infection involves both the innate and adaptive systems, in which the former controls early virus infection and replication, while the latter clears the infection. The innate immune response to HSV-2 is a non-specific response, in which recognition of the virus by the genital epithelial cells (GECs) results in the release of inflammatory cytokines, chemokines, and type I IFN (predominantly IFN-β) (Ferreira, Nazli, Mossman, & Kaushic, 2013; Koelle & Corey, 2008). To date, only a few studies have shown direct interaction between primary GECs and HSV-2, whereas others have used immortalized or transformed cell lines and HSV-1 (Huber et al., 2001; Li et al., 2006; MacDonald et al., 2007). Primary GECs have been shown to activate pro-inflammatory and type I IFN pathways in response to HSV-2 (Ferreira et al., 2013). Interestingly, Ferreira and colleagues (2013) have shown that viral host shutoff protein (VHS) present in wild type

replication-competent HSV-2 could suppress pro-inflammatory cytokines and chemokines, however, the upregulation of IFN-β was dependent only on viral replication.

Previous studies have shown that toll-like receptors (TLRs) 2, 3 and 9, which are receptors for hydrophophic pathogen-associated molecular patterns (PAMPs), double stranded RNA and CpG DNA, are involved in sensing HSV-2 (Paludan et al., 2011). As a result of IFN-β production, IFN-α is released due to the creation of a positive feedback loop, activating the IFN α/β receptor (IFN-α/βR) (Akira & Takeda, 2004). Furthermore, plasmacytoid dendritic cells (pDCs) have been implicated in TLR 9 sensing and subsequent release of IFN-α (Rasmussen et al., 2007). The release of type I IFN causes cells within the environment to enter an antiviral state by producing antiviral molecules, such as protein kinase R in order to block or reduce viral infection (Akira & Takeda, 2004; Paludan et al., 2011). An *in vitro* study by Nazli et al. (2009) had shown primary GECs were able to inhibit HSV-2 replication following treatment with TLR 3, TLR 5, and TLR 9 via biologically active IFN-β and nitric oxide (NO). Studies have also shown a TLR-mediated HSV-2 protection when mice are immunized with TLR 3 and TLR 9 ligands, poly (I:C) and CpG, respectively (Gill, Deacon, Lichty, Mossman, & Ashkar, 2006). However, a similar protective effect is not seen when TLR 2, TLR 4, and TLR 5 were used (Ashkar et al., 2004; Gill et al., 2006).

Upon activation, the cells within the genital tract microenvironment are capable of recruiting different immune cells, as well as becoming cytotoxic and releasing IFN-γ, perforin and granzyme (Lee & Ashkar, 2012). Of these, IFN-γ is very important in the attempt to control early HSV-2 infection. This is due to the fact that IFN-γ can induce the production of nitric oxide (NO) by other cells in the local environment, which subsequently is able to suppress the replication of HSV-2 (Bogdan, 2001). The importance of IFN-γ was further demonstrated when there was increased viral replication and pathogenesis, and decreased survival following genital infection with HSV-2 in IFN α/β receptor knockout ($IFNAR^{-/-}$) mice compared to wild-type mice (Conrady, Halford, & Carr, 2011; Gill, Chenoweth, Verdu, & Ashkar, 2011). Furthermore, immunization against HSV-2 failed to protect $IFNAR^{-/-}$ mice against subsequent viral challenge (Gill et al., 2006; Svensson, Bellner, Magnusson, & Eriksson, 2007). Type I IFNs also activate multiple effector cells as part of the innate immune response to HSV-2, such as natural killer (NK) cells (Kaushic et al., 2014). NK cells could mediate apoptosis of virally infected cells through the release of perforin and granzyme B and is an important early producer of IFN-γ (Martinez, Huang, & Yang, 2008). However the exact role of NK cells in HSV-2 remains controversial. Ashkar & Rosenthal had showed that $IL\text{-}15^{-/-}$ mice, which lack NK and NKT cells, are more susceptible to HSV-2 infection; while another study found that the clearance of HSV-2 from the

vaginal mucosa is independent of IFN-γ producing NK cells (Ashkar & Rosenthal, 2003; G. N. Milligan & Berstein, 1997).

1.7.3 Adaptive Immune responses

Both B- and T-cells, which are part of the adaptive immune response, are mainly responsible for clearing the infection and generating immunological memory against HSV-2 infection (Dobbs, Strasser, Chu, Chalk, & Milligan, 2005; Koelle et al., 1998; Morrison, Zhu, & Thebeau, 2001). Many studies have shown, via CD4 and CD8 T cell-depleted mouse models, that CD4+ T cells are mainly responsible for the protection against HSV-2 rather than CD8+ T cells (Gregg N Milligan, Bernstein, & Bourne, 1998; Parr & Parr, 2003). The protection by CD4 T cells is mediated mainly via IFN-γ, since it was shown that mice depleted of CD4 T cells also had a low level of IFN-γ in vaginal secretions (Harandi, Svennerholm, Holmgren, & Eriksson, 2001). Furthermore, IFN-γ deficient mice showed a lack of protection to HSV-2, even in the presence of CD4 T cells (Harandi et al., 2001). Previous studies have suggested that DCs and B-cells are able to act on CD4+ T-cells to release IFN-γ, which induces the secretion of chemokines such as CXCL9 and CXCL10, and in turn attracts the migration of IFN-γ producing CD8+ T-cells (Iijima et al., 2008; Nakanishi, Lu, Gerard, & Iwasaki, 2009). Moreover, B-cells have been observed to activate CD4+ T-cells to release IFN-γ, suggesting a function outside of antibody production (Iijima et al., 2008).

HSV-2 infection induces both Ig(A) and Ig(G) in the genital tract of human and mice (Johnston et al., 2011; Parr & Parr, 2003). Even though IgG is the dominant isotype present in the human vaginal tract, their expression is not required for HSV-2 clearance, unlike IgA, where it was shown in a number of different studies to correlate with protection against an intravaginal challenge (Chan, Barra, Lee, & Ashkar, 2011; Roth, Ferreira, & Kaushic, 2013). These results support data from Parr and Parr, demonstrated IgG from HSV-2 immunized mice could neutralize HSV-2 in vitro, and adoptive transfer of serum IgG from immunized mice into the vaginal tract of naive mice could reduce the HSV-2 viral load (Parr & Parr, 2003). Furthermore, immunized B-cell-deficient mice had higher titers of HSV-2 in vaginal secretions and were more susceptible to viral re-challenge (Morrison et al., 2001; Parr & Parr, 2003). Conversely, other studies have shown T-cell mediated immunity was responsible for the protection and viral clearance in HSV-2-immunized mice, and this is done independently of anti-HSV-2 antibodies (Harandi et al., 2001; McDermott, Goldsmith, Rosenthal, & Brais, 1989; Gregg N Milligan et al., 1998).

1.8 Mouse models

1.8.1 Mouse Model of HSV-2 Infection

Animal models for HSV-2 infection reflect numerous aspects of human disease and have been extremely useful in examining factors that correlate

with clearance, pathogenesis and the development of protective immunity (M. R. McDermott et al., 1984; Parr et al., 1994; Parr & Parr, 2003). Of note is the mouse model of HSV-2; it has provided the most insights into susceptibility and protective immune responses post primary infection.

Previous studies have clearly shown that both susceptibility and immune responses in the female genital tract are regulated by sex hormones (Gallichan & Rosenthal, 1996; Kaushic, Ashkar, Reid, & Rosenthal, 2003; Kaushic, Zhou, Murdin, & Wira, 2000; White et al., 1997). Historically, all studies that used the mouse model of HSV-2 pre-treated mice with Depo-Provera (Depo; dihydroxyprogesterone acetate) prior to infection (Kaushic et al., 2003). In the absence of Depo treatment, infection is dependent on the stage of the estrous cycle (Gallichan & Rosenthal, 1996). Normal, untreated mice infected in estrous, when estrogen levels are high, are not susceptible to HSV-2, even at a dose of 10^7 plaque forming unit (PFU) (Kaushic et al., 2003). However, untreated mice inoculated in diestrus stage (when progesterone levels are high) showed significant viral shedding and external pathology at 10^6 PFU (Kaushic et al., 2003) In the same study, Kaushic and colleagues have shown that Depo-Provera and saline suspended progesterone increased susceptibility of HSV-2 infection by 100-fold and 10-fold respectively in treated compared to untreated mice (A. Gillgrass, Ashkar, Rosenthal, & Kaushic, 2003; Kaushic et al., 2003).

1.8.2 HSV-2 Mouse Models in Pregnancy and Gaps in the Literature

Even though the non-pregnant mouse model of HSV-2 has been extensively studied, to date there are only a few studies that have attempted to establish a pregnancy model of HSV-2 infection. More importantly, only one study assessed pregnancy outcomes as an experimental readout (Kinney & Kumar, 1988; Nahmias et al., 1971; Sanjuan & Zimberlin, 2001).

In the study by *Sanjuan et al.,* the authors infected intravaginally (IVAG) specific pathogen-free, pregnant BALB/c mice with HSV-2 (ATCC VR-734) using $5x10^5$ plaque forming unit (PFU) in 0.05 ml of cell culture medium at 10-14 days pregnant, and another group at 3-4 days pregnant (Sanjuan & Zimberlin, 2001). Fourteen out of 21 mice (66.66%) developed genital and neurologic signs of infection and died between 8 and 9 days post-infection (10/13 mice inoculated at 10-14 days pregnant and 4/8 mice inoculated at 3-4 days pregnant). HSV-2 infected mice had a higher rate of resorptions (69.23%) when compared to pregnant, uninfected mice. However, one drawback of this study was the high concentration of virus used in this study ($5x10^5$ PFU), which is 100 times higher than lethal dose in mice. In order to model human infection, there is a need to develop a clinically relevant mouse model, which characterizes the effects of various inoculation doses of the virus, in order to better understand the effects of primary HSV-2 infection during pregnancy.

While developing a mouse model has inherent advantages, such as cost, easy breeding, and a close modeling of human diseases, there are also many factors that must be taken into consideration when extrapolating data from one species to another. Despite the differences in placental anatomy and gestational length (approx. 21 days in mice versus 9 months in humans), human and mice both have hemochorial placentation, which is when maternal uterine blood vessels are infiltrated by trophoblast cells, causing rupture and release of blood into the intervillous space (Erlebacher, 2013b; Moffett & Loke, 2006) (Figure 3). However, trophoblast cells do not invade deeply into the decidual arterioles in mice, whereas in humans, the trophoblast temporarily replaces maternal endothelial cells all the way to the smooth muscle of the uterus (Figure 4) (Erlebacher, 2013b; Moffett & Loke, 2006). As a result, the limited trophoblastic invasion of the maternal arterioles, a characteristic of preeclampsia, and intrauterine growth restriction (IUGR) does not take place in mice (Erlebacher, 2013a). In mice, the presence of uterine NK cells in the media of the arteries indicates that they might have a direct physiological role in regulating the blood pressure and flow to the placenta (Erlebacher, 2013b; Moffett & Loke, 2006). Conversely in humans, uterine NK cells may have some direct effect on arterial function, but mainly act through indirect effects on trophoblast-cell invasion (Moffett & Loke, 2006).

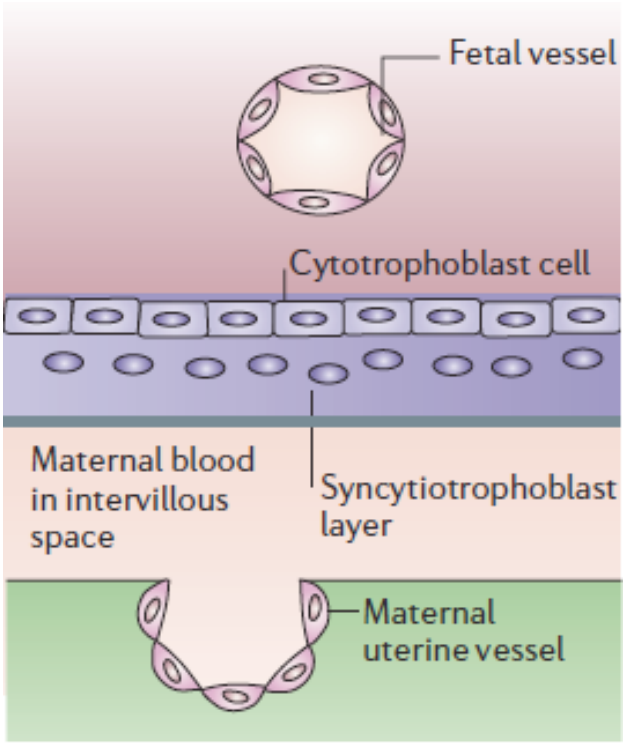


Figure 3 – Hemachorial placentation. Maternal uterine blood vessels are infiltrated by trophoblast cells causing rupture and release of blood into the intervillous space (Moffett & Loke, 2006).

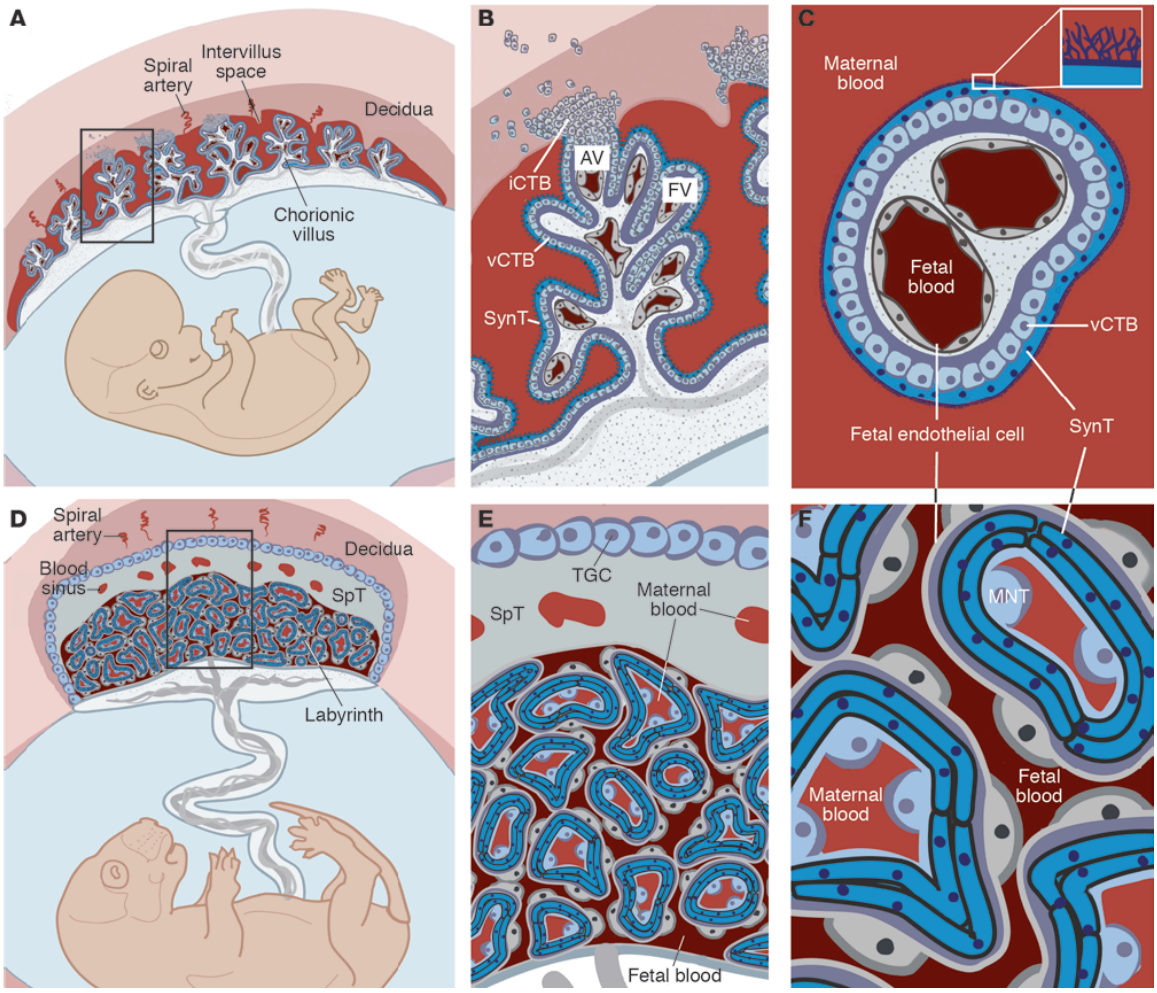


Figure 4 – Comparative anatomy of human and mouse placentas. In the hemochorial placentas of humans (A-C) and mice (D-F), the maternal vessels are invaded and colonize by invasive trophoblasts. (A) In humans, decidual spiral arterioles perfuse the chorionic villi that line the intervillous space. (B) In floating villi (FV), a continuous layer of multinucleated synctiotrophoblast (SynT) interfaces with maternal blood. (D) In mice, maternal blood from decidual spiral arterioles flows through blood sinuses in the spongiotrophoblast (SpT) layer to reach the labyrinth. (E) Trophoblast giant cells (TGCs), like invasive cytotrophoblasts (iCTBs), anchor the placenta to the uterus and invade the spiral arterioles. (Maltepe, Bakardjiev, & Fisher, 2010)

CHAPTER 2: RATIONALE

The mechanisms underlying adverse outcomes following primary HSV-2 infection during pregnancy remain largely uncharacterized. Recent studies have shown that pro-inflammatory responses to infections during the first stages of embryonic development have an impact on the peri-implantation and post-implantation developmental stages (Garbett, Hsiao, Kalman, Patterson, & Mirnics, 2012; Hsiao & Patterson, 2011; Kumar et al., 2014; Smith, Li, Garbett, Mirnics, & Patterson, 2007). Furthermore, an inflammatory environment could affect the vasculature remodelling, which occurs during early pregnancy, a process that is critical for the efficient diversion of uterine blood flow through the placenta. This suggests that primary infection during early gestation might pose a high risk for the abnormal development and adverse health outcomes for the fetus, which is in agreement with the current published studies.

The goal of this project was to develop and characterize a model of HSV-2 infection in pregnant mice during early gestation in order to elucidate its effects on pregnancy outcomes and examine underlying pathogenic mechanisms.

I hypothesize that **"*Primary HSV-2 infection during early gestation will lead to an inflammatory environment in utero, which ultimately will lead to poor pregnancy outcomes.*"**

To address this hypothesis, we proposed the following aims:

1) Develop a model of HSV-2 infection in early pregnancy

2) Determine the effects of HSV-2 infection on fetus and pregnancy

3) Examine the expression of proinflammatory cytokines and chemokines expression in maternal serum and amniotic fluid to determine if infection induces inflammatory environment

CHAPTER 3: MATERIALS AND METHODS

3.1 Mice

Inbred 6-8 week old virgin female C57BL/6 mice were obtained from Charles River laboratories (Constant, Quebec, Canada). All mice were housed and maintained in the Central Animal Facility at McMaster University in a 12-hour light/dark cycle. Mice were given low-fat mouse chow and water *ad libitum.* Mice were given 1 week to acclimatize prior to mating. Mice were mated by overnight cohabitation with syngeneic males. The morning that vaginal plug was observed is considered to be GD 0.5. All animal studies performed were approved by and were in compliance with the Animal Research Ethics Board (AREB) at McMaster University.

3.2 HSV-2 inoculation

Mice with a vaginal plug were anesthetised by intra-peritoneal injectable anaesthetic. Anaesthetic was prepared by mixing Ketamine® (Bimedia-MTC, Cambridge, Ontario) and Xylazine® (Bayer Inc., Toronto, Ontario) at the dosage of 150 mg per kg of body weight and 10 mg per kg of body weight respectively. The Ketamine®/Xylazine® combination was given at the dose of 0.1 ml/10 gm of body weight. Before infection, each and every mouse was gently swabbed IVAG with an approximately 0.5 cm tip of a sterile, dry cotton wool swab. Anaesthetized mice were infected IVAG with 10

µl (10^3, 10^4, or 10^5 PFU/mL) on GD 5 with wild type HSV-2 strain 333. After inoculation, mice were placed on their backs for approximately 45-60 minutes to allow for the inoculum to infect the vaginal tract. The infection in these mice was compared to mice at diestrus stage that were infected with HSV-2 (333) (10^3, 10^4, and 10^5 PFU), where kinetics of HSV-2 infection has previously been established (Kaushic et al., 2003). Normal mice with vaginal plugs inoculated with saline (0.9% NaCl) and vaginal plug positive mice not exposed to anything served as negative controls.

3.3 Viral titering and pathology monitoring

Vaginal washes were collected daily post infection by pipetting 2 x 30µl of PBS into and out of the vagina five to six times. Viral titers in vaginal washes were determined by viral plaque assay on Vero cell (ATCC, Manassas, USA) monolayers. Vero cells were grown in supplemented α-Medium Essential Medium (α-MEM) (Cat #12000063; GIBCO Laboratories, Burlington, Canada) supplemented with 5% fetal bovine serum (FBS; Cat# 16000-044; GIBCO, Burlington, Canada), 1% penicillin-streptomycin (Cat # 15140-122; Invitrogen, Burlington, Canada), L-glutamate (Cat # 56-85-9; BioShop Canada Inc., Burlington, Canada), and 1% HEPES (Cat #15630-080; Invitrogen, Burlington, Canada). For plaque assays, Vero cells were grown to confluence in 12-well plates (Becton Dickson, Oakville, Ontario). On the day of the experiment, lavages were removed from -70°C freezer and thawed on ice. Samples were diluted (10^{-2} to 10^{-7}) in α-MEM and added to monolayers.

Infected monolayers were incubated at 37°C for 2 hours and rocked every 15 minutes in order to facilitate viral absorption. Infected monolayers were overlaid with α-MEM in order to stop viral adsorption. Infection was allowed to occur for 48 hours at 37°C. Monolayers were subsequently fixed and stained with crystal violet, and viral plaques were counted under an inverted light microscope. Crystal violet was prepared by mixing 280 mL of 100% ethanol, 40 mL of 37% formaldehyde, 20 mL of glacial acetic acid, 120 mL of reverse osmosis filtered water and 4 gm of crystal violet powder (Sigma-Aldrich, Oakville, Canada). The number of PFU per millimeter was calculated by taking a plaque count for every sample and takes into account the dilution factors.

Genital pathology following HSV-2 infection was monitored daily and was scored on a 5-point scale: 0, no infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina; 3, severe swelling and redness of both vagina and surrounding tissue and hair loss in genital area; 4, genital ulceration with severe redness; 5, severe genital ulceration extending to surrounding tissue (Kaushic et al., 2003). Once mice reached pathology score of 4 and/or 5, they were sacrificed by cervical dislocation, abdominal cavity was opened, implantation sites, fetuses, placenta, vaginal tract, and spleen were collected and separated into 10% formalin for immunohistochemical fixing, or RNAlater® Solution (Cat #AM7020$1307013; Ambion, USA) for viral DNA extraction. Mice that do not reach genital

pathology score of 4 or 5 were sacrificed at the indicated time points, GD 8 or GD 13.

3.4 Viral DNA extraction

Standard protocols from DNeasy Blood & Tissue kit were followed from Qiagen Inc. Cat #69504 (Toronto, ON, Canada). Tissues (no more than 20 mg) were weighed and transferred to an Eppendorf tube. Each tissue was finely minced in 100 µl of Buffer ATL from Qiagen kit, and 20 µl of proteinase K was added and mixed by pulse-vortexing for a total of 15 seconds. The mixture was incubated overnight at 56°C. Following incubation, the tube was briefly centrifuged to remove drops from the inside of the lid and 200 µl of Buffer AL from Qiagen kit was added and mixed by pulse-vortexing for a total of 15 seconds. The mixture was incubated at 70°C for 10 minutes. Following incubation, the tube was briefly centrifuged and 200µl anhydrous ethyl alcohol was added to the sample, and mixed by pulse-vortex. After mixing, the solution was applied to a QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm for 1 minute. The tube containing the filtrate was discarded and a clean 2 ml collection tube from the kit was attached to the Mini spin column. 500 µl Buffer AW1 supplied from Qiagen kit was added to each QIAamp Mini spin column (part of Qiagen kit) and centrifuged at 8000 rpm for 1 minute. Again, the collection tubes were changed; 500 µl Buffer AW2 (part of Qiagen kit) was added to the spin column and centrifuged at full

speed 14 000 rpm for 3 minutes. Collection tubes were changed again and another spin at full speed was carried out for 1 minute in order to minimize the possible chance of AW2 carryover.

A clean 1.5 ml microcentrifuge tube was used instead of the collection tube and 200 µl of Buffer AE was added into the spin column. The Buffer AE (part of Qiagen kit) was allowed to incubate in the column for 3 minutes at room temperature in order to dissolve DNA into the solution. The Buffer AE-DNA mixture was then centrifuge at 8000 rpm for 1 minute. DNA purity and yield was determined via NanoVue Plus spectrophotometer (Cat #28-9569-62; GE Life Sciences, Mississauga, Canada) by pipetting 1 µl of the DNA sample onto the interface. Buffer AE served as the calibration for no DNA using the NanoVue Plus spectrophotometer. DNA products were stored in -20°C until qPCR experiment.

3.5 HSV-2 real-time polymerase chain reaction (qPCR)

In order to standardize the amount of DNA based on volume for all reactions, 5 µl of each DNA sample was added to 12.5 µl RT^2 SYBR Green ROX qPCR Master Mix (Cat #330521; Qiagen, Mississauga, Canada), 0.8 µM each forward and reverse primers (HSV DNA-pol; F: 5`-GGGGTGATCGGCGAGTAYTG-3`, R: 5`-ATCTGCTGGCCGTCGTARATG-3`; Integrated DNA Technologies, Coralville, IA), and 7.2 µl of water for a final volume of 25 µl. Samples underwent 50 cycles of PCR, and the fluorescent

dyes in each reaction were read automatically during PCR cycling in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). To quantify the amount of viral DNA in each sample, a standard curve was generated using known quantities of HSV-2 Polymerase Quantitated Viral DNA (Cat #08-922-000; Advanced Biotechnologies, Columbia, MD). Thermal conditions were as follows: 50°C for 3 minutes to degrade contaminating amplicons, 95°C for 10 minutes to activate the DNA polymerase, followed by 50 cycles of 95°C for 15 seconds and 57°C for 60 seconds.

3.6 Maternal serum and amniotic fluid collection

Whole blood was collected from each mouse on day 9 of gestation via orbital bleeding. Collected whole blood was allowed to clot at room temperature for 30 minutes in sterilized 1.5 mL Eppendorf tubes, after clotting, this was centrifuged at 8000 x g for 10 minutes. Serum was collected and stored at -20°C.

Amniotic fluid was collected and pooled from each implantation site for each mouse at time of sacrifice. Collected amniotic fluid was stored in -20°C until analysis.

3.7 Fetal homogenate supernatant collection

Individual fetuses were placed in 50 ml Falcon tubes, 500 µl of PBS was added. Each tissue was homogenized using a mechanical homogenizer

at increasing speed from 4500 to 30000 rpm until the tissue is a consistent homogenate (approximately 15-20 second intervals resting for 5 seconds between each interval for a total of 60 seconds. Each sample tube was placed in ice during the homogenization process. Care was taken to clean out the homogenizer tip between each tissue by removing any large tissues in the blade, running the homogenizer tip in 70% EtOH, followed by PBS, this protocol was repeated twice between each tissue. Homogenates were centrifuged at 8000 RPM for 5 minutes. Supernatants were collected and stored at -20°C.

3.8 Multiplex cytokines/chemokines analysis

The collected supernatants from amniotic fluid (GD 8), and maternal sera (GD 13) were used for measuring the induced amount of cytokines and chemokines. Serum samples were diluted 2-fold before analysis. Sera samples were sent to Eve Technologies (Calgary, AB, CA) for quantification using 32-plex array panel: eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF. Amniotic fluid samples were analyzed using a Milliplex Mouse Cytokine/Chemokine Magnetic Panel (IFN-γ, IL-1β, IL-6, IL-10, IL-17, MCP-1, and TNF-α) (Cat# MCYTOMAG-70K; Millipore, Toronto, ON).

3.9 Tissue staining

3.9.1 Tissue fixing and embedding

After each animal was sacrificed, implantation sites, vaginal tract, and/or uterus tissues were collected and fixed in 10% formalin for at least 7 days. Tissues were transferred to 70% ethanol for at least 7 days post fixation. Tissues were embedded in paraffin blocks and cut into approximately 3-5 µm sections and placed onto Aptex coated positive charge glass slides (Cat# 3800080; Leica Biosystems; Concord, ON) for future staining.

3.9.2 Hematoxylin and eosin staining

Paraffin blocks were cut in 4-5 µm serial sections and stained by John Mayberry histology staff using previously established protocols (Bancroft & Cook, 1984; Culling, Allison, & Barr, 1985). After paraffin embedded tissue was cut, it was allowed to air dry for 15 minutes before slides were placed in 60°C oven for 30 minutes. In order to clear sections of wax, slides were placed in Xylene I for 5 minutes before subsequently being transferred to Xylene II and III for 5 minutes each. Slides were dipped in and out of 100% alcohol I 10 times and left for 1 min, this step was repeated two more times to remove any Xylene residues. Tissues were rehydrated when transferred to 95%, then 70% alcohol. Each time the slides were dipped in and out 10 times and left for 1 min. Slides were placed in a slides rack and dipped in a tub filled with running tap water for 3 minutes, followed by distilled water.

Nuclei were stained with Modified Mayer's Hematoxylin (Cat# MHS16; Sigma-Aldrich, Oakville, ON) for 4 minutes, followed by dipping in the tub of water with running tap water. Slides were rinsed in Tris buffer (Cat# 161-0732; Bio-Rad; Mississauga, ON) pH 7.6 for 30 seconds, followed by another rinse in the tub with running tap water. Subsequently, slides were dipped in weak Acid Alcohol (15 ml of 1% Acid Alcohol in 190 ml distilled water) for 5-6 dips before being rinsed in the tub with running tap water. Slides were placed in Tris buffer pH 7.6 once again for 2 minutes to turn the Hematoxylin blue before being rinsed in a tub filled with running tap water. Slides were placed in buffered 0.5% Eosin pH 5.5 for 5 minutes, followed by 30 seconds dip in a tub filled with running tap water. Slides were differentiated and dehydrated by dipping into 70% alcohol 15 times before being transferred to 95% alcohol I (dip 15 times), 95% alcohol II (dip 10 times), 100% alcohol I (dip 10 times, leave for 1 min), 100% alcohol II (dip 10 times, leave for 1 min), and 100% alcohol III (dip 10 times, leave for 1 min). Slides were placed in Xylene I and were dipped in and out 10 times and left for 1 min. This step was repeated with Xylene II and Xylene III before slides were mount in resinous media such as Permount (Cat# SP15-500; Fisher Scientific; Ottawa, ON).

3.9.2 Periodic-acid Schiff staining

Paraffin blocks were cut in 4-5 μm serial sections and stained by John Mayberry histology staff using previously established protocols. After paraffin embedded tissue was cut, they were rinsed in distilled water. Subsequently,

slides were placed in 1% Periodic Acid (Cat#3951; Sigma-Aldrich; Oakville, ON) for 15 minutes before they were washed in a tub filled with running tap water and rinsed in distilled water. Slides were stained in Schiff's reagent (Cat# 3952016; Sigma-Aldrich; Oakville, ON) for 30 minutes before they were washed in a tub filled with warm running tap water for 10 minutes to develop full colour. Subsequently, slides were rinsed in distilled water and counterstained in Mayer's Hematoxylin (Cat# MHS1; Sigma-Aldrich; Oakville, ON) for 1-2 minutes. Slides were washed in a tub filled with cool running tap water to blue sections before they were dehydrate and mount in resinous medium such as Permount (Cat# SP15-500; Fisher Scientific; Ottawa, ON).

3.9.3 HSV-2 staining

Paraffin blocks were cut in 3 μm serial sections and stained by John Mayberry histology staff using previously established protocols. After paraffin embedded tissue was cut, slides were placed in 60°C oven for 45 minutes. Slides were then transferred to Xylene I, II, and III for 5 minutes each and subsequently transferred to 100% alcohol three times, each time the slides were dipped in and out for 10 seconds and left for 1 minute. Endogenous peroxidase was blocked with 5% H_2O_2 (190 ml methanol, 10 ml H_2O_2, 0.5 ml concentrated HCl) for 25 minutes at room temperature before they were transferred to 100% alcohol IV and dipped in and out for 10 seconds and left for 1 minute. Subsequently, slides were transferred to 95% alcohol, then 70% alcohol, dipping in and out for 10 seconds and left for 1 minute each time.

The slides were washed three times in distilled water for 1 minute each time. Antigen was retrieved by incubating the slides with Dako cytomation target retrieval solution 1X (Cat# S1699; Dako Canada Inc; Burlington, ON) and placed in the decloaker (BioCare Medical; Concord, CA) for 30 seconds at 125°C, followed by 90°C for 10 seconds before allowing slides to cool down for 15 minutes. Slides were washed for 1 minute in a tub filled with running tap water before being placed in TBS/TW (0.05M Tris buffered saline pH 7.6 with 0.1% Tween 20) for 5 minutes. The slides were placed in a humid chamber, 2 drops of 5% normal goat serum diluted in TBS/TW for 20 minutes at room temperature. HSV-2 antibody (Cat# B0116; Dako Canada Inc; Burlington, ON) was diluted 1:1000 in Ultra Clean Diluent (Cat# TA125UC; Fisher Scientific; Ottawa, ON) and incubated with the slides for 1 hour at room temperature. Subsequently, slides were rinsed in TBS/TW 3 x 5 minutes before Dako's Envision plus-rabbit (pre-diluted) (Cat# K4008; Dako Canada Inc; Burlington, ON) was added for 30 minutes at room temperature. The slides were rinsed in TBS/TW 3 x 5 minutes before being placed in 0.05 M acetate buffer pH 5.0 for 5 minutes. AEC chromogen/substrate solution was incubated with the tissues for 20 minutes before being washed under running tap water for 5 minutes. Slides were subsequently rinsed in distilled water and counterstained in Mayer's Hematoxylin (Cat# MHS1; Sigma-Aldrich; Oakville, ON) for 25 seconds. The slides were dipped in a tub filled with running tap

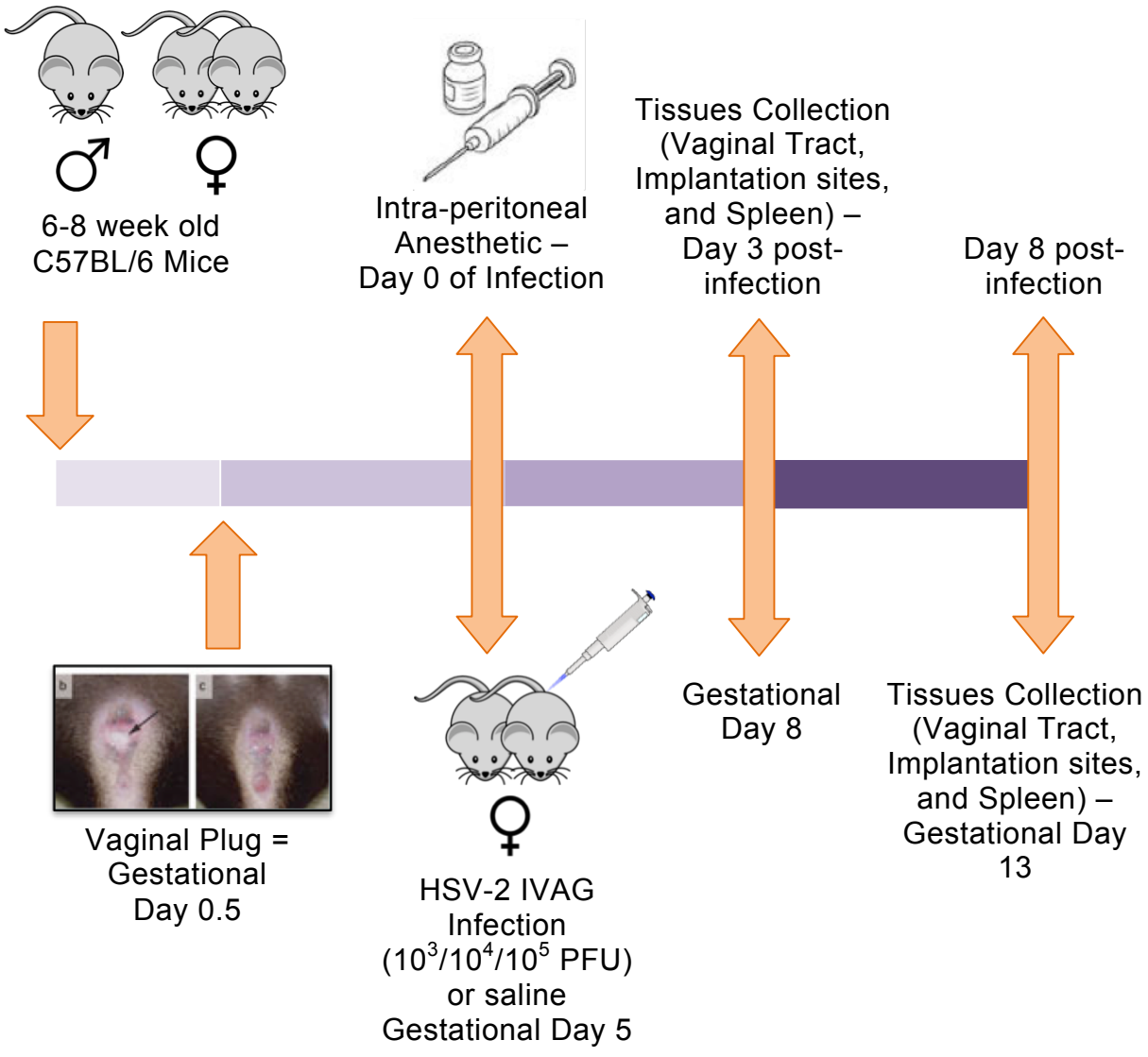
water for 1 minute before being placed in TBS for 1-2 minutes and mounted in glycerine gelatin.

3.10 Statistical Analysis

Statistical analysis and graphical representation was performed using GraphPad Prism 6.0d (GraphPad Software, San Diego, CA). Survival was analyzed by Kaplan-Meier test and other results were analyzed using one-way ANOVA.

3.11 Experimental Design

Intravaginal Infection



CHAPTER 4: RESULTS

4.1 Increased susceptibility of HSV-2 infection in mice positive for vaginal plugs

One male and two female C57BL/6 mice were allowed to copulate overnight. Since it is difficult to determine early pregnancy in a mouse, evidence of seminal fluid deposition in the vaginal opening (vaginal plug) was used as a proxy criterion for pregnancy. The day of vaginal plug discovery was considered to be gestational day (GD) 0.5. Mice were inoculated with saline (sham) or WT HSV-2 at three different doses (10^3, 10^4, 10^5 PFU) on GD 5. In addition to the sham infection control, a second control group was established, a group of mice served as pregnant, untreated control, where no anaesthetics or inoculation occurred. Vaginal plug positive mice were compared to diestrus mice, since these are non-pregnant mice where progesterone is the dominant hormone during this stage of cycle, similar to pregnancy and they have been shown to be naturally susceptible to HSV-2 infection (Kaushic et al., 2003) After inoculation, mice were examined daily for survival, genital pathology and vaginal washes were collected for viral titers (Materials and Methods 3.3).

In order to characterize HSV-2 infection during early pregnancy and its associated effects on pregnancy outcome, mated mice were sacrificed at two different time points, GD 8 and GD 13, which is 3 and 8 days post infection (d.p.i) respectively. While GD 13 (8 days post infection) results would be

important for survival and HSV-2 susceptibility analysis, however, other information such as the number of implantations might be lost or skewed at this point due to resorption sites being cleared by immune cells. Thus, by having an earlier time point at GD 8 (3 days post infection), the results should provide a better representation of the early effects of HSV-2 on pregnancy outcomes, such as the number of mice with implantations after mating and the number of implantations or resorptions per mouse.

Mice inoculated with WT HSV-2 had high viral shedding in the vaginal tract on Days 1-5 post infection (Figure 5). In the GD 8 group, 75% (n=6/8) of mice inoculated with 10^3 PFU showed viral shedding (Figure 5B), and 100% (n=6/6 and 7/7) of mice inoculated with 10^4 and 10^5 PFU, respectively, showed viral shedding throughout all three days post-infection (Figures 5C-D). In the GD 13 10^3 PFU group, 50% (n=4/8) of the mice showed local viral shedding on all 8 days post-infection (Figure 5F). However 100% of the mice with vaginal plugs from 10^4 and 10^5 PFU groups (n=10/10 and 9/9) had robust viral shedding (Figures 5G-H). The viral titer results from both GD 8 and GD 13 groups were markedly different than the diestrus group, where no shedding was seen in 10^3 PFU group, only 25% (n=1/4) of infected mice had viral shedding at 10^4 PFU, and 100% (n=5/5) at 10^5 PFU (Figures 5J-L).

Upon comparing the survival curve between GD 13 mice positive for vaginal plugs and diestrus mice infected with HSV-2, GD 13 mice succumbed to infection at a lower dose of inoculation (Figures 6A-B). Only results for GD

13 are shown here since at GD 8 (3 days post infection), infected mice do not exhibit advanced genital pathology and are still in the early stages of infection, thus mortality was not observed. At the lowest inoculation dose of HSV-2 (10^3 PFU), 25% of the mice positive for vaginal plugs succumbed to the infection (Figure 6A). It is important to note that mice inoculated with 10^4 and 10^5 PFU had 100% mortality rate (Figure 6A). Similar to what was seen in the viral titers, results from mated mice were distinctly different than the diestrus group. Mice inoculated with 10^3 PFU during diestrus stage had 0% mortality (Figure 6B). Furthermore, only 25% and 80% of mice succumbed to the infection when inoculated with 10^4 and 10^5 PFU respectively (Figure 6B).

Overall, these results provide evidence that mice positive for vaginal plugs are 100 times more susceptible to primary HSV-2 infection, when compared to diestrus non-pregnant mice.

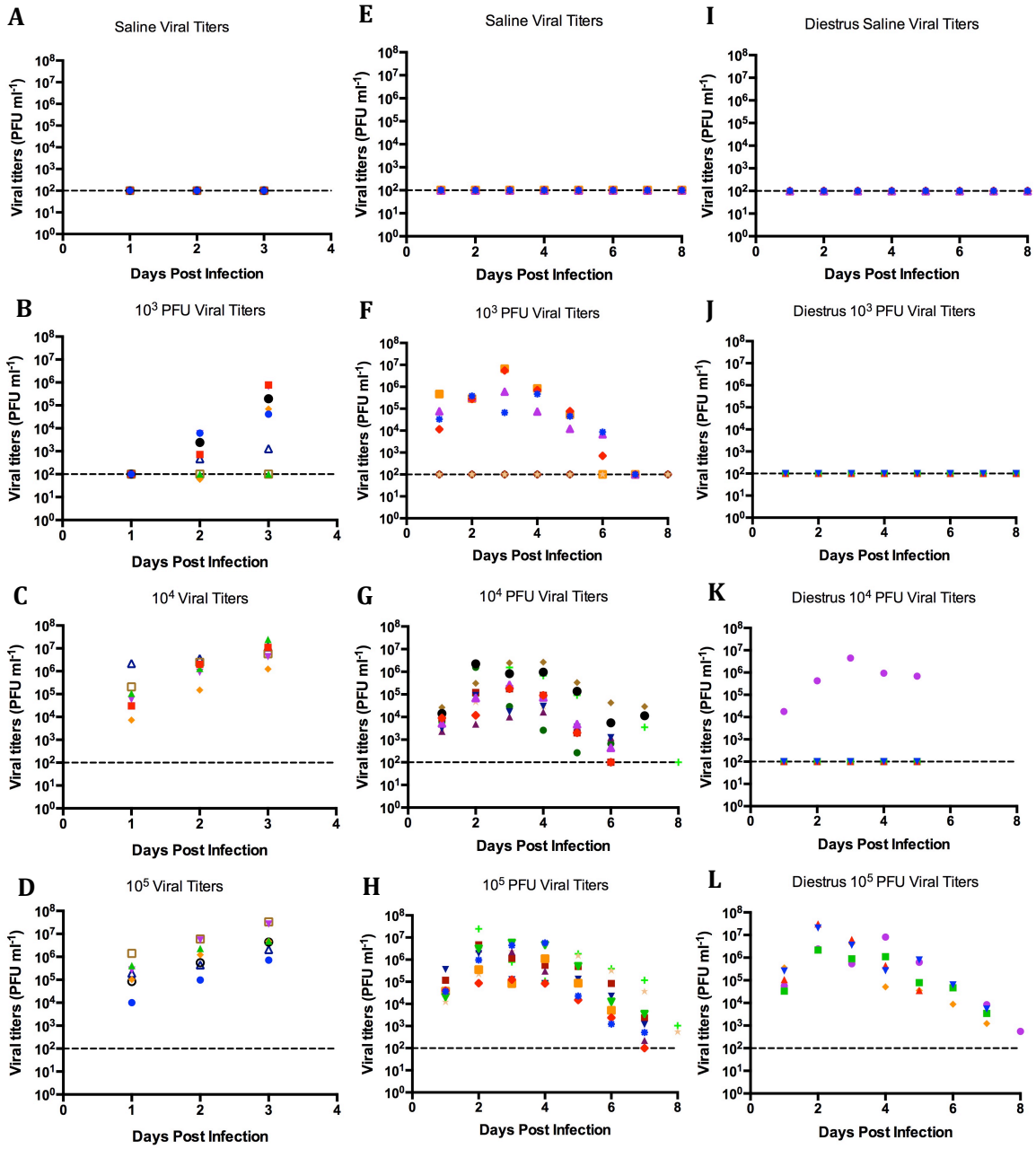


Figure 5 – Viral titers of diestrus and mated mice infected with HSV-2 (333). Mated mice inoculated with (A) saline (n=4), (B) 10^3 PFU (n=8), (C) 10^4 PFU (n=6), (D) 10^5 PFU (n=7) and sacrificed on gestational day (GD) 8. Mated mice inoculated with (D) saline (n=8), (E) 10^3 PFU (n=8), (F) 10^4 PFU (n=11), (G) 10^5 PFU (n=9) and sacrificed on gestational day (GD) 13. (H) saline diestrus (n=4), (I) 10^3 PFU diestrus (n=4), (J) 10^4 PFU diestrus (n=4), (K) 10^5 PFU diestrus (n=5). Viral plaque assays were done on all vaginal smears collected daily. Plaques were counted and viral titers were expressed as PFU ml^{-1}. Limit of detection of viral titers plaque assay was 100 PFU ml^{-1} (dashed line). Each symbol represents a single animal.

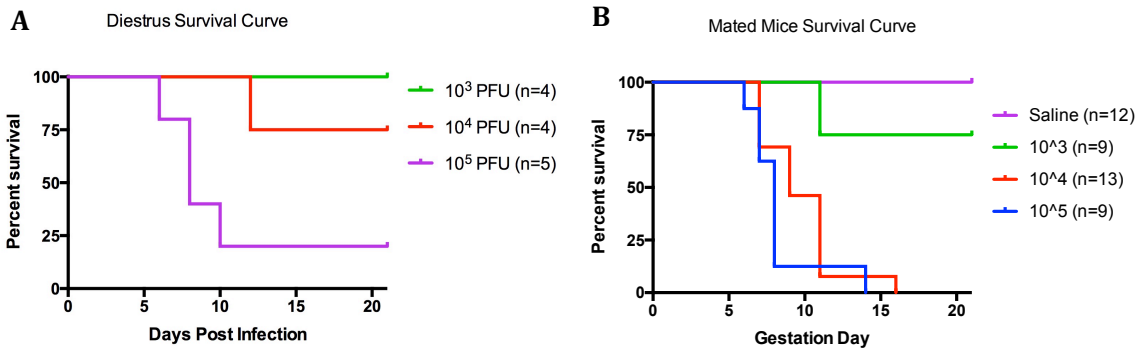


Figure 6 – Mated mice (A) are more susceptible to HSV-2 infection than diestrus mice (B). At day 5 of gestation, mice with vaginal plugs were infected IVAG with 10^3/10^4 or 10^5 PFU of HSV-2 (333), they were monitored daily for survival. Mice with vaginal plugs in 10^4 and 10^5 PFU groups had 100% mortality within 16 days post infection compared to 75% and 20% respectively in the diestrus group.

4.2 Increased pathology score in mated mice infected with HSV-2

In order to compare genital pathology with the survival and viral shedding seen in mated mice compared to diestrus mice, external genital pathology was monitored daily. External genital pathology indicated the extent of infection in the mice and based on the severity of the pathology, mice were sacrificed accordingly (pathology scores >4). The pathology was scored based on a 5-point scale as indicated in Materials and Methods 3.3.

Results from all experiments indicated that mice positive for vaginal plugs had higher pathology scores in the GD 13 group at all three inoculation doses when compared to their diestrus controls (Figure 7). The difference in pathology score between GD 13 and diestrus mice could be seen at the lowest inoculation dose of 10^3 PFU, where 1/10 mice had a pathology score of 2 or higher, compared to 0/4 in diestrus group (Figures 7D, G). This difference is more prominent at 10^4 and 10^5 PFU, where 10/10 (100%) of vaginal plug positive mice in GD 13 had a pathology score of 2 or higher compared to 1/4 (25%) and 4/5 (80%) in the diestrus group respectively (Figures 7E-F, H-I). Mice positive for vaginal plugs sacrificed at GD 8 (day 3 post infection) did not have high pathology score due to the short time point after infection (Figures 7A-C).

Overall, pathology scores showed a good correlation with results obtained from survival and viral titers. Mice with positive vaginal plugs had higher pathology scores when compared to diestrus controls.

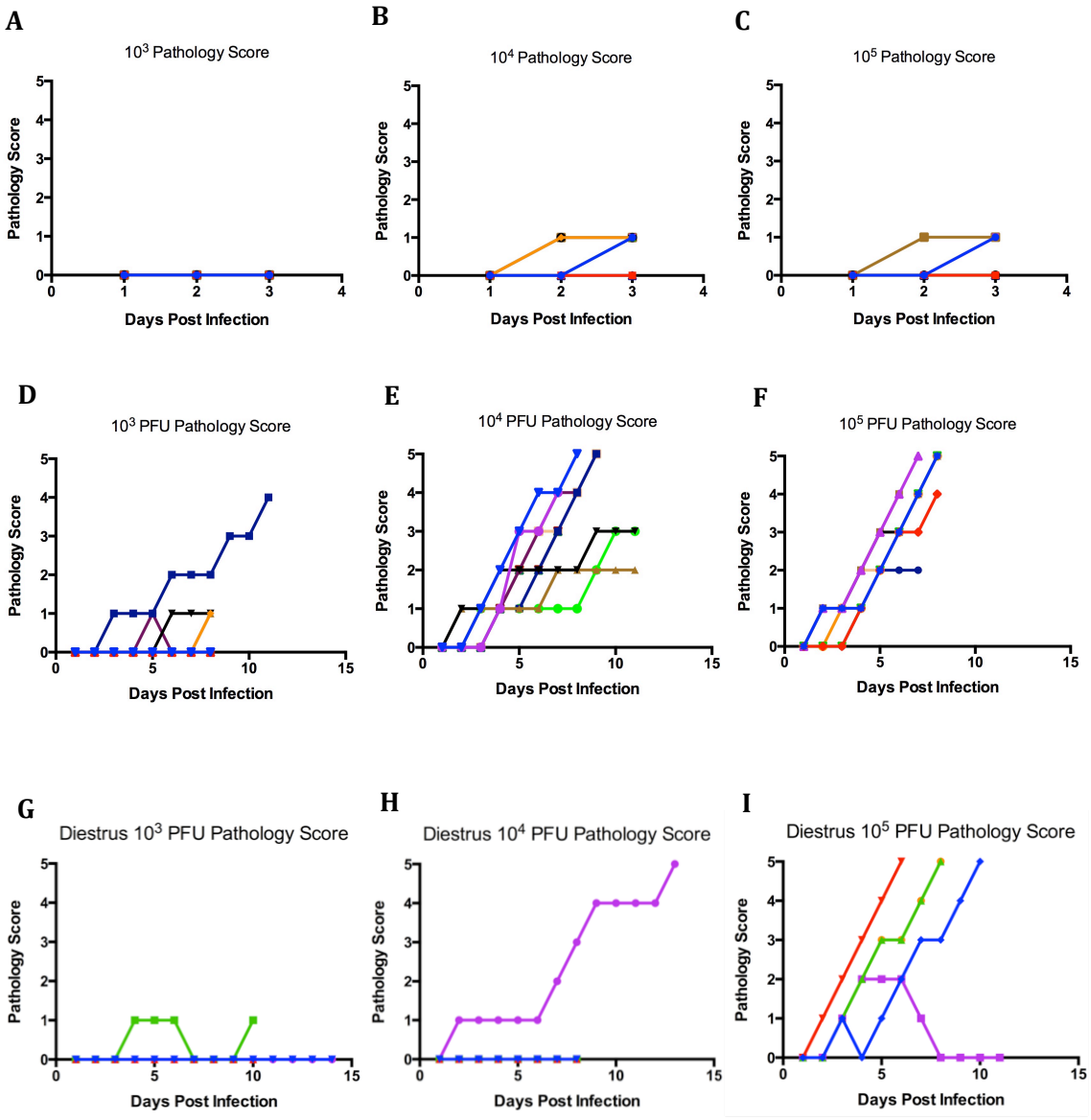


Figure 7 – Genital Pathology Score. Mice with vaginal plugs have higher pathology (GD 13) at a lower HSV-2 infection dose compared with diestrus mice. Vaginal pathology monitored daily post infection both GD 8 (A-C) and GD 13 (D-F) groups. (A) Mice with plugs 10^3 PFU (n=8), (B) mice with plugs 10^4 PFU (n=6), and (C) mice with plugs 10^5 PFU (n=7). (D) Mice with plugs 10^3 PFU (n=10), (E) mice with plugs 10^4 PFU (n=10), and (F) mice with plugs 10^5 PFU (n=10), (G) 10^3 PFU diestrus (n=4), (H) 10^4 PFU diestrus (n=4), (I) 10^5 PFU diestrus (n=5). Each symbol represents a single mouse.

4.3 Pregnancy Outcomes

One of the aims of this study was to determine the effects of primary HSV-2 infection on the fetus and pregnancy outcomes. As a result, mice positive for vaginal plugs were sacrificed by cervical dislocation at either GD 8 (3 d.p.i) or GD 13 (8 d.p.i) and the number of implantation sites and resorptions were counted for each mouse.

The overall results are summarized in Table 1 and 2 (Appendix). There were no differences in the percentage of mice with plugs that had implantation sites at time of sacrifice on GD 8 and GD 13 (Figure 8). However, normal mice with vaginal plugs not exposed to saline or HSV-2 had a higher percentage of mice with visible implantation sites at time of sacrifice when compared to other groups, including saline (Figure 8). The percentage of resorption (ratio of resorptions/total fetuses) increased in a dose dependent manner at GD 8 (Figure 9A). However there was no clear discernable trend present for the percentage of resorption in the GD 13 group (Figure 9B). Unfortunately, the standard error of the mean was too large to draw any significant conclusions at both sacrifice time points. Thus a larger sample size is needed to obtain meaningful analysis for the percentage of resorptions in HSV-2 vaginal plugged infected mice.

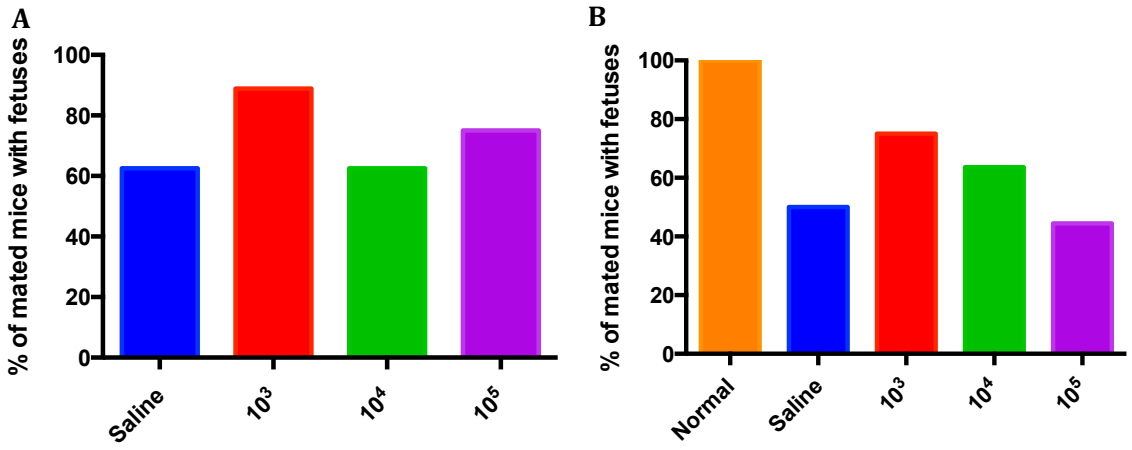


Figure 8 – Percentage of mice with plugs that had fetuses at time of sacrifice on (A) gestational day 8, and (B) gestational day 13.

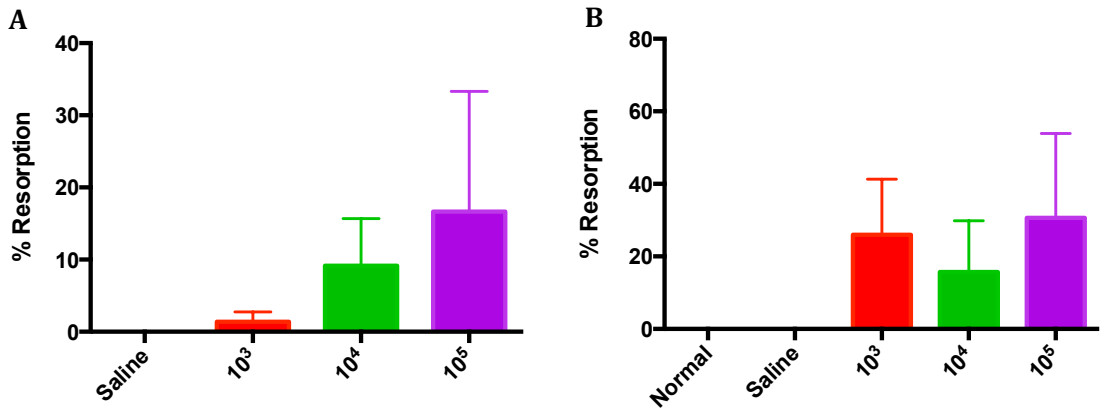


Figure 9 – Percentage of resorption (number of resorptions/total number of fetuses) for (A) gestational day 8, and (B) gestational day 13. Data are presented as mean ± SEM).

4.4 Assessing Tissue Histopathology in Vaginal Plug Positive, HSV-2 Infected Mice

Results from survival, viral titers and genital pathology scores confirmed that mice with a vaginal plug are more susceptible to HSV-2 compared to non-pregnant mice in diestrus. Furthermore, adverse pregnancy outcomes were observed in infected mice. However, in order to examine the local tissue environment for inflammation more closely, histology staining was performed. Vaginal tract, uterus (in mated mice with absence of implantation sites), and implantation sites at GD 8 and GD 13 were fixed and tissue staining was done as described in Materials and Methods 3.9. H&E staining was used in this study to reveal morphologic changes in the tissues and aid in the identification of polymorphonuclear cells, when the nucleus is stained with haematoxylin, giving it a blue-purple color (Fischer, Jacobson, Rose, & Zeller, 2008). PAS reaction was used to detect glycoproteins in the granules of NK cells present in the examined tissues (Yadi et al., 2008). HSV-2 specific immunohistochemistry (IHC) was done to identify extent and location of infection in the tissues.

Polymorphonuclear cell infiltration was observed in the vaginal tissues of pregnant mice at the lowest inoculation dose of 10^3 PFU at both GD 8 and GD 13 time points (Figure 10). Furthermore, HSV-2 specific staining was observed consistently in the vagina in both GD 8 & 13 groups (Figure 13). The local infection observed through HSV-2 specific IHC correlated well with

polymorphonuclear cells infiltration in the vaginal tract of mated mice. H&E staining revealed that there were necrotic tissues and structural changes present in the decidua, and junctional zone in implantation sites of pregnant mice at 10^4 and 10^5 PFU when compared to the sham infected mice at GD 13 (Figure 11). Evidence of necrotic fetal tissues was observed at higher infection doses (10^4 PFU and 10^5 PFU); this was not evident in the tissue histopathology of saline and 10^3 PFU groups. It is likely that HSV-2 infection led to histopathological changes in placental tissue indicated by necrotic tissue, suggesting that this likely led to fetal distress and mortality.

PAS staining showed that there was evidence of abnormal localization of NK cells in placentas at both GD 8 and GD 13 (Figure 12). Furthermore, blood vessel (spiral arteries) in the labyrinth of the placenta of mice infected at 10^4 and 10^5 PFU were more constricted when compared to the sham infected at GD 13 (Figure 12E-F). The abnormal localization of NK cells within different layers of the placenta could affect spiral arteries remodeling, which likely contributed to fetal distress and deaths.

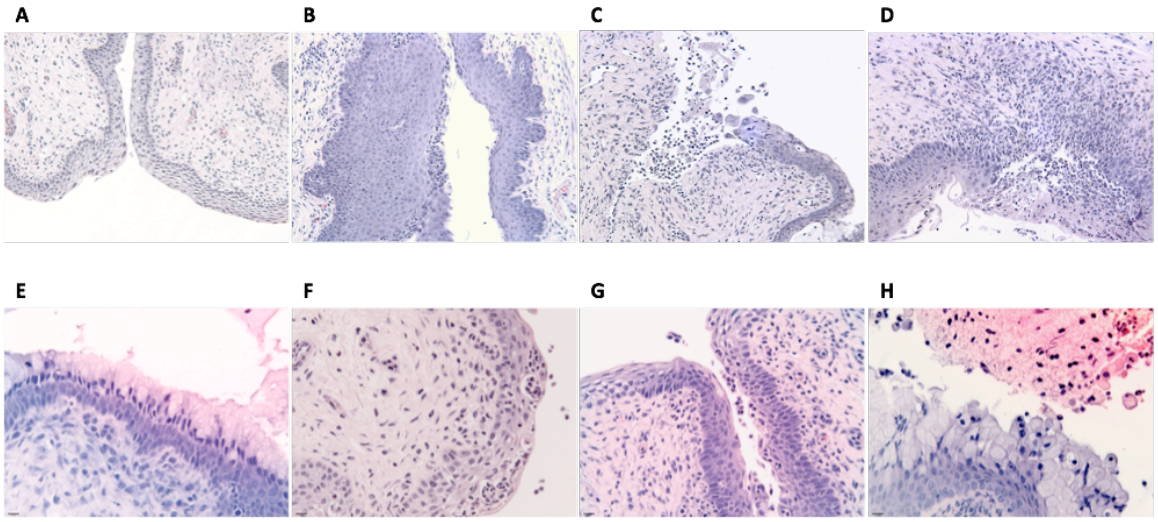


Figure 10 – Histopathology and localization of leukocytic infiltration in the vaginal tract of mated mice inoculated with saline (A) and HSV-2 (333) at $10^3/10^4/10^5$ PFU (B-D) at GD 8 (Magnification 20x). HSV-2 infected mice showed extensive leukocytic infiltration in the tissue and in the lumen (B-D) when compared to pregnant mice that were inoculated with saline (A). Similar results are seen at GD 13 (E) saline, $10^3/10^4/10^5$ PFU (F-H) (Magnification 40x). Vaginal tissues were sectioned and stained with H&E (A-H). Representative tissue sections are shown.

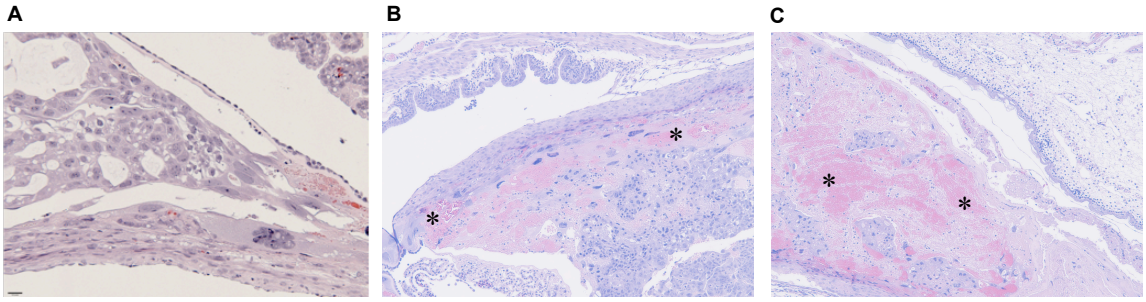


Figure 11 – Placental Histopathology of mated mice inoculated with saline (A) and HSV-2 (333) at $10^4/10^5$ PFU (B-C), respectively. Whole implantation sites were sectioned and stained with H&E (A-C). Representative tissue sections are shown. HSV-2 infected mice showed extensive pathological morphology changes and necrotic tissues (*) in the placenta and fetus (B-C) when compared to pregnant mice that were inoculated with saline (A). (Magnification 10x).

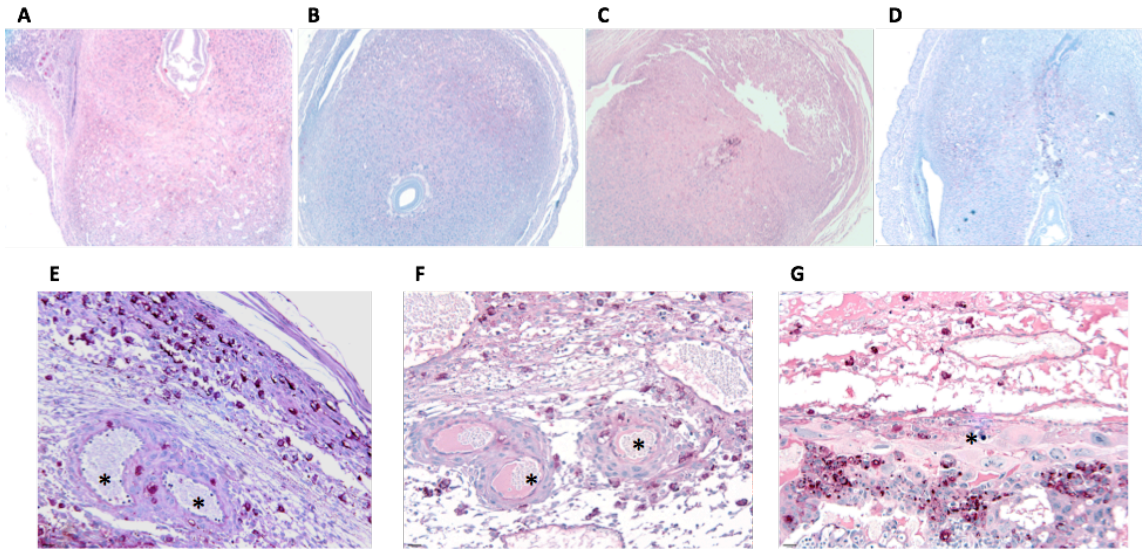


Figure 12 - Localization of NK cells in whole implantation of pregnant mice (GD 8) inoculated with saline (A) and HSV-2 (333) at $10^3/10^4/10^5$ PFU (B-D), respectively (Magnification 10x). GD 13 whole implantations from pregnant mice inoculated with saline (E) and HSV-2 (333) at $10^4/10^5$ PFU (F-G) (Magnification 20x). Whole implantation sites were sectioned and stained with PAS (A-G). Representative tissue sections are shown. Differences in blood vessel morphology (*) were noted in saline (E) when compared to HSV-2 $10^4/10^5$ PFU groups (F-G). HSV-2 infected mice showed an abnormal localization of NK cells (C-D, F-G) when compared to pregnant mice that were inoculated with saline (A, E).

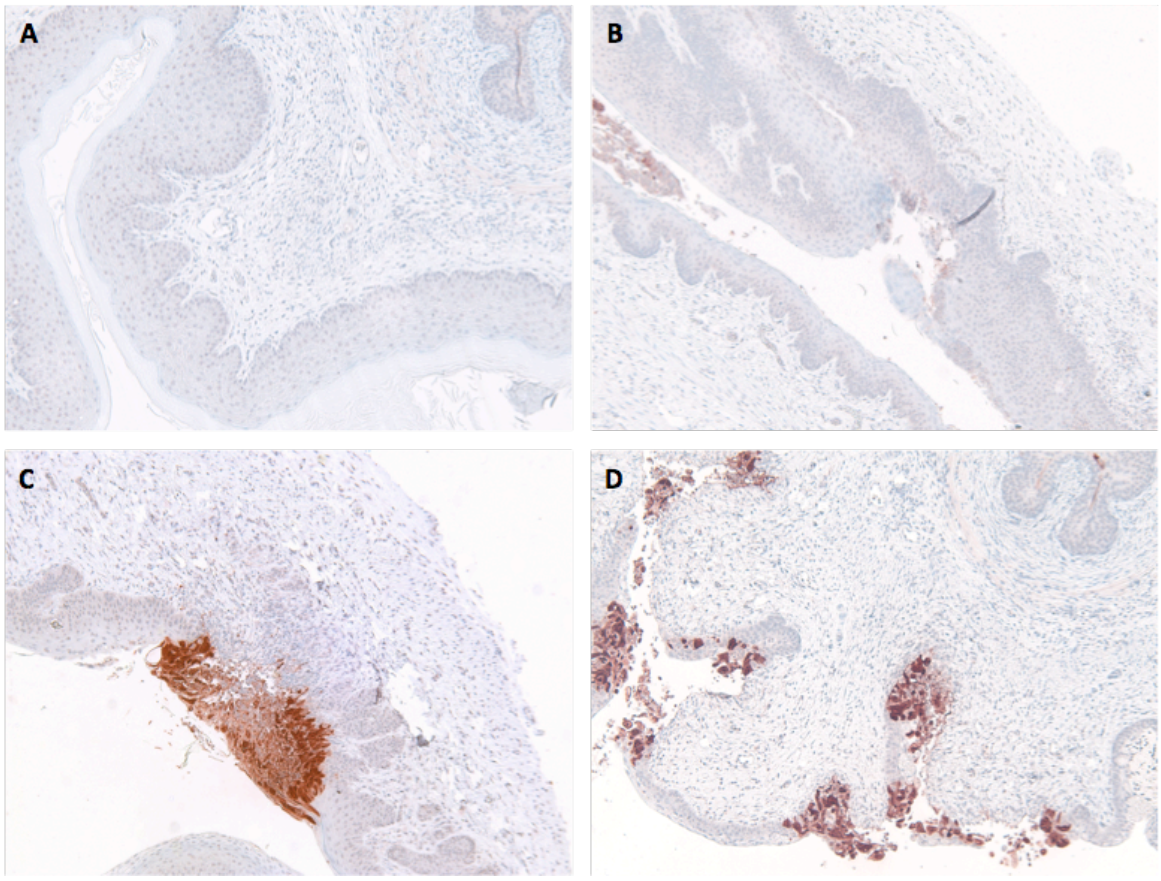


Figure 13 - Localization of HSV-2 in vaginal tract of mated mice (GD 8 group) that were inoculated with saline (A) and HSV-2 (333) at $10^3/10^4/10^5$ PFU (B-D), respectively. Vaginal tracts were sectioned and stained for HSV-2 (A-D). Representative tissue sections are shown. (Magnification 10x).

4.5 Real-time PCR assessment of HSV-2 viral DNA in the vaginal tract, spleen, placenta and/or whole implantations

Results from experiments described above showed that HSV-2 shedding was detected in the vaginal tract of mated mice; as well the presence of HSV-2 DNA was detected by histological staining. However, in order to quantitate the infection in tissues and determine the extent of HSV-2 dissemination in various tissues (spleen, vagina, and implantation sites), a sensitive analysis based on HSV-2 qPCR was carried out. The HSV-2 qPCR was adapted from previous assays and optimized to detect HSV-2 DNA (Roth et al., 2013; Thean Yen Tan et al., 2013). Using HSV-2 (G strain) quantitated viral DNA targeting DNA polymerase gene of HSV-2 as a reference, a standard curve was created, from which the number of viral copies per microliter was calculated for each sample. Each tissue sample was tested in triplicates in order to ensure consistency within the real-time PCR assay.

Viral DNA was detected in the vaginal tract in 7 out of 8 animals (mean = 4.97×10^6 copies/µl) and in implantations of 3 out of 8 animals (mean = 5.39×10^3 copies/µl) of mated mice inoculated with 10^3 PFU of HSV-2 in the GD 8 group (Figure 14B). It should be noted that there were no non-pregnant mice in the GD 8 10^3 PFU group; therefore data for uterine viral DNA was not available for collection. In the 10^4 PFU group, the mean HSV-2 DNA in the vaginal tract was 6.5×10^4 copies/µl (5 out of 8 animals) compared to 6.44 copies/µl in implantation sites (3 out of 5 animals and 1.99×10^3 copies/µl in

resorptions (only 1 animal examined) (Figure 14C). Mice in the 10^5 PFU treated group had an average of 3.48 x 10^5 copies/µl of HSV-2 (7 out of 7 animals) in the vaginal tract and 2.5 x 10^2 copies/µl in implantation sites (4 out of 6 animals) (Figure 14D). HSV-2 DNA was also detected in the uterus of mated, non-pregnant mice. However, no HSV-2 DNA was detected in the spleen of mated mice, despite the higher inoculation dosage.

Upon examining the GD 13 group, similar pattern of dissemination was seen in the vaginal tract of 10^3 PFU (4 out of 8 animals), 10^4 PFU (7 out of 8 animals), and 10^5 PFU (8 out of 9 animals) and uterus of mated, non-pregnant mice 10^3 PFU (0 out of 2 animals), 10^4 PFU (2 out of 8 animals), and 10^5 PFU (8 out of 9 animals) when compared to the GD 8 mated mice. Although the mean number of HSV-2 DNA was lower in the tissues of GD 13 group, there was evidence that HSV-2 disseminated into the spleen at all inoculation doses 10^3 PFU (n= 4 out of 8 animals), 10^4 PFU (n= 1 out of 8 animals), and 10^5 PFU (n= 1 out of 9 animals) (Figure 15D). Unlike the resorption tissue in 10^4 PFU of GD 8, a resorption tissue in 10^5 PFU in GD 13 group did not have any measurable HSV-2 DNA (Figures 14C, 15D). However, a fetus sample from the 10^4 PFU GD 13 group showed low level of HSV-2 DNA (mean = 3.78 copies/µl) (Figure 15D).

The current results have shown for the first time that WT HSV-2 can ascend up the vaginal tract into the uterus, and disseminate into the placenta of mated mice. The highest HSV-2 DNA was usually detected in the vaginal

tract of inoculated mated mice at both earlier (GD 8) and late (GD 13) time points. Furthermore, HSV-2 DNA was frequently detected in the uterus of mated non-pregnant mice inoculated with 10^4 and 10^5 PFU of both GD 8 and GD 13 groups. Similarly, HSV-2 DNA was frequently detected in the implantations of GD 8 and the decidua of GD 13 group. The fact that HSV-2 DNA was observed infrequently in the placental-fetal tissues suggests that the virus could cross the cervical mucus plug, which could be directly or indirectly responsible for some of the observed fetal histopathology.

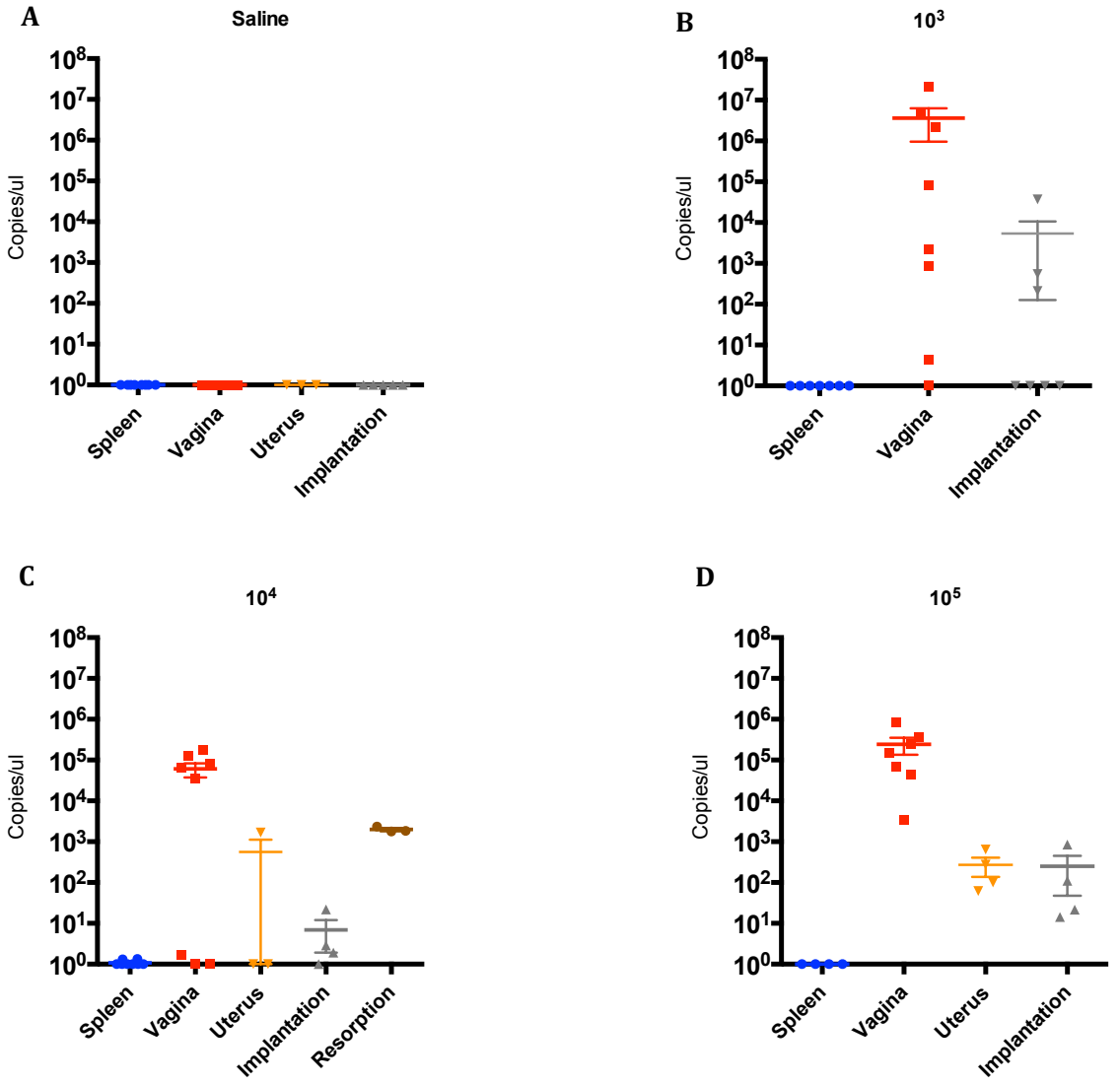


Figure 14 – Absolute HSV-2 real-time PCR quantification (copies/ul) from tissues of vaginal plug positive mice sacrificed at gestational day 8. Each tissue sample was tested in triplicates.

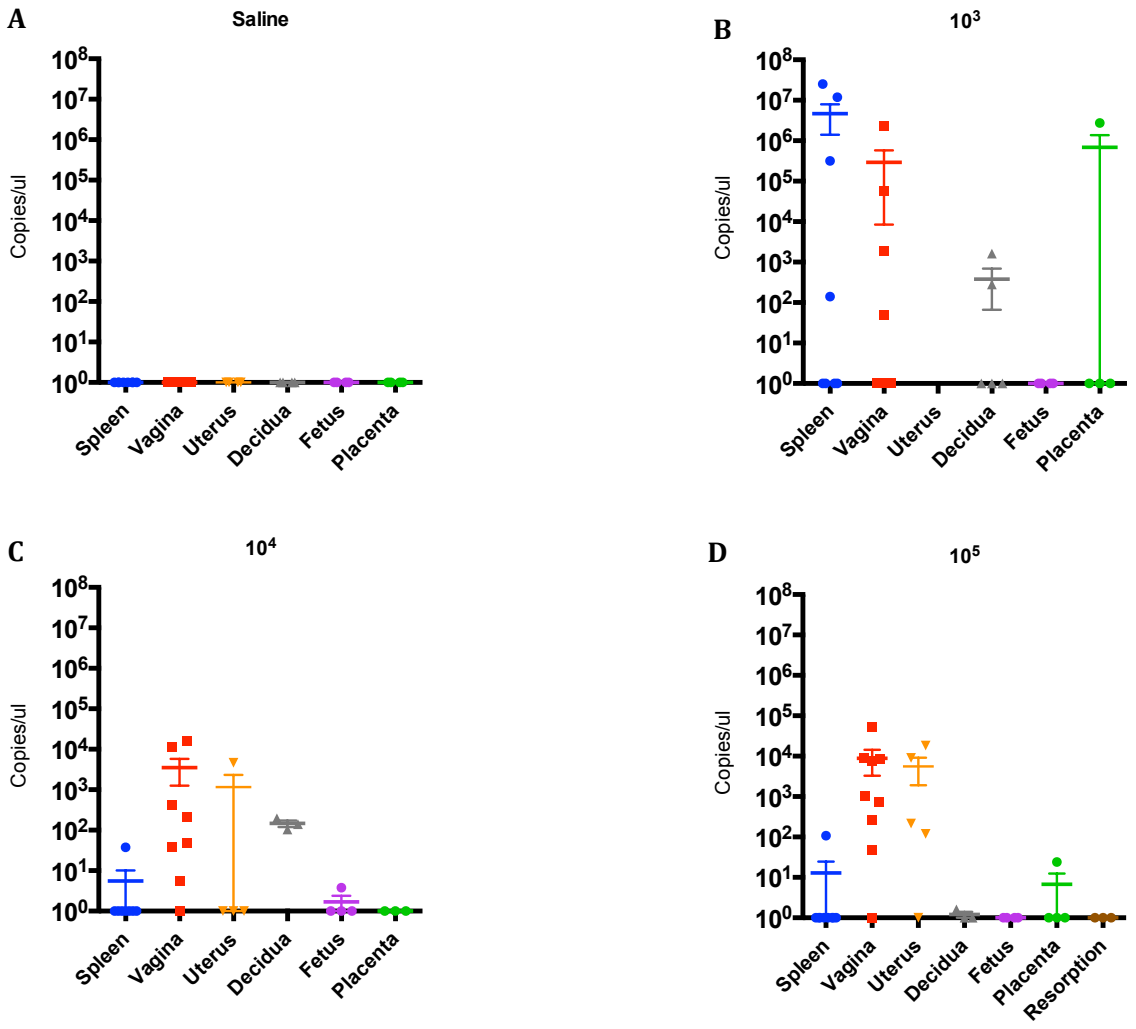


Figure 15 - Absolute HSV-2 real-time PCR quantification (copies/ul) from tissues of vaginal plugged mice sacrificed at gestational day 13. Each tissue sample was tested in triplicates.

4.5 Characterizing maternal serum in HSV-2 infected mice

Fetal inflammatory response syndrome (FIRS) in human is clinically described as a fetal plasma concentration of IL-6 > 11 pg/mL and has been associated with pregnancy complications and pre-term labour (Gotsch et al., 2007). An increase in pro-inflammatory cytokines, such as IL-1, IL-6, TNF, IFN, and a decrease in IL-10 is a hallmark of FIRS; as a result, fetuses with FIRS have a higher rate of severe neonatal morbidity, pneumonia, or neonatal sepsis (Gotsch et al., 2007).

Various animal studies have shown that the increased pathogenesis in pregnant mice is due to the elevated viral infections (e.g. influenza A, B, human cytomegalovirus (CMV)), and elevated or skewed inflammatory cytokines and chemokines (Hamilton et al., 2012; Kang, Song, Lee, Kim, & Seo, 2011; H. M. Kim, Kang, Song, Kim, & Seo, 2012; J. C. Kim et al., 2014). With this background in mind, one of the aims of the present study was to examine the cytokines and chemokines profile in HSV-2 infected pregnant mice. Thus a 32-plex cytokine array (Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α, and VEGF) was carried out using maternal sera collected on GD 9 of the GD 13 experimental group.

Based on the 32-plex cytokine array results, 8 cytokines were selected for in-depth analysis based on their relevance to the model and trends or

differences in the maternal sera between control and infected mice: Eotaxin, IL-1α, IL-1β, IL-6, IP-10, KC, RANTES, and TNF-α (Figure 16). One-way ANOVA was used to analyze the results, however, there were no statistical differences between the experimental groups. Upon closer examination, there was a clear trend showing a dose dependent increases in IL-6; however, not enough samples were collected for 10^5 PFU to draw significant conclusion from the differences between each group of mice (Figure 16). IL-1 b also showed dose-dependent increase in infected groups; however, levels were high in saline control group as well. Moreover, there was higher level of TNF-α in the 10^5 PFU group when compared to other experimental groups (Figure 16). Surprisingly, there was no significant measurable difference in IFN-γ, a common proinflammatory cytokines that is associated with viral infections, such as HSV-2.

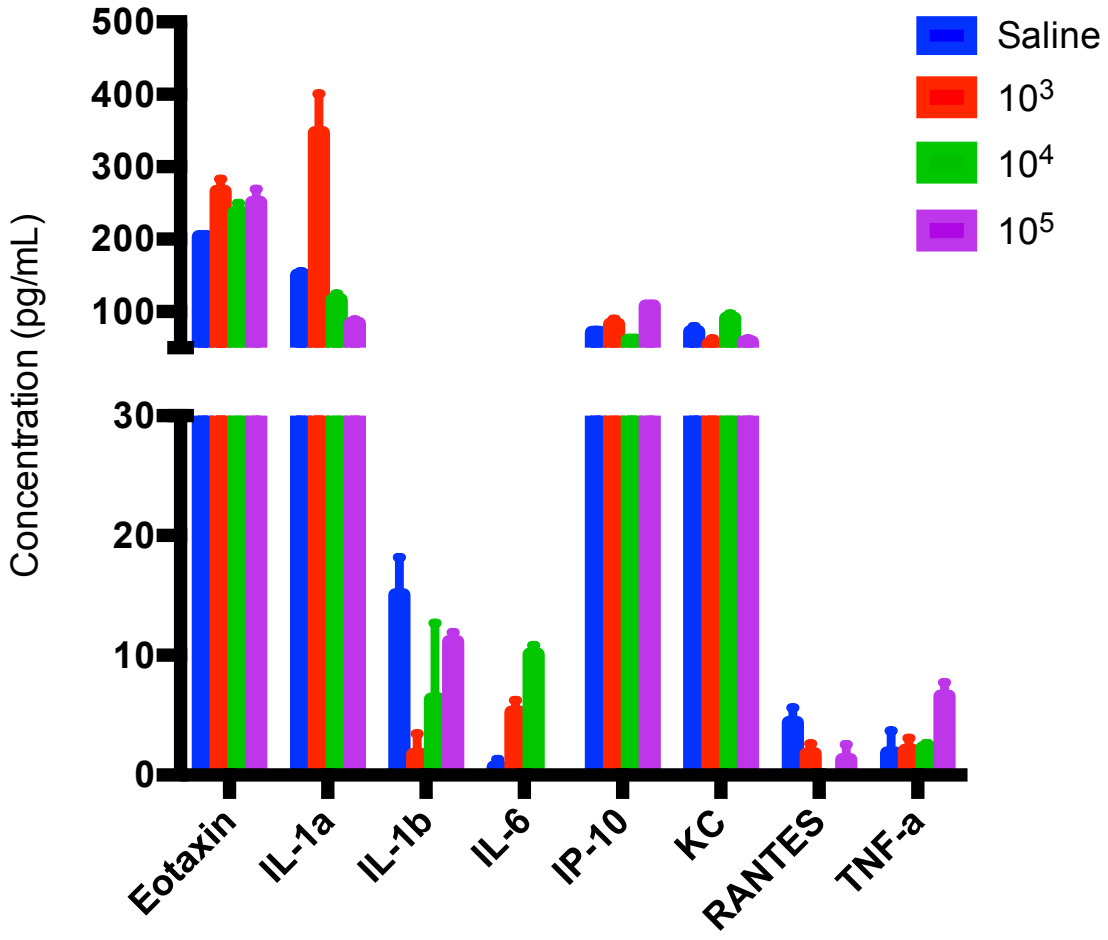


Figure 16 – Detection of cytokines and chemokines in maternal sera. Sera of vaginal plug positive HSV-2 infected and sham infected mice in the GD 13 group (saline n= 3, 10^3 n= 5, 10^4 n= 1, 10^5 n= 3) were collected on GD 9 (4 d.p.i). Each sample was run in triplicates. One-way ANOVA was used for statistical analysis, no significant differences were observed. Data are represented as mean ± standard error of the mean (SEM).

4.6 Characterizing amniotic fluid in HSV-2 infected mice

The association of microbial infection in the amniotic fluid (AF) and adverse pregnancy outcomes, such as preterm births with or without ruptured membranes, and cervical insufficiency has been well established in the literature (Gomez et al., 2005; Greig, 1998; Oh et al., 2010; Romero et al., 2007; Romero et al., 2001; Romero & Mazor, 1988; Romero et al., 1988). However, only more recently has the issue of viral infections in the amniotic cavity has been investigated. Previous studies have attempted to examine the presence of virus in the amniotic cavity using PCR-based methods, however, the results have been varied, some showing that there was no viral DNA present in the AF of 277 patients (12.3 to 32 weeks of gestation) and some studies detecting viral DNA in 44/686 samples collected during mid-trimester (16 to 29 weeks of gestation) (McLean, Chehab, & Goldberg, 1995; Oh et al., 2010). Furthermore, viral DNA was detected in the AF of up to 15% of asymptomatic low-risk pregnancies in a prospective observational study (Baschat, Towbin, Bowles, Harman, & Weiner, 2003). In the study by Baschat (2003) and colleagues, the most common viral DNA isolates found were adenovirus, CMV, and enterovirus. However, as Agrawal & Hirsch (2012) pointed out in their recent review, the lack of a global marker for viral genomes, paralleling bacterial 16S ribosomal RNA, limits the available molecular methods to screen for specific, known viruses.

In association with the presence of viral DNA in the amniotic fluid, the presence of IL-6 in the AF has been considered to be a marker of intraamniotic inflammation (Romero, Avila, Santhanam, & Sehgal, 1990; Yoon et al., 1995). Similarly, patients with preterm births were shown to have higher levels of matrix metalloproteinase-8, IL-6, TNF-α, and angiogenin in their AF when compared to those delivered at term (Romero, Chaiworapongsa, & Espinoza, 2003). As a result, proinflammatory cytokines and chemokines were measured in AF of GD 8 group using a 7 cytokines/chemokines Milliplex Magnetic Panel (IFN-γ, IL-1β, IL-6, IL-10, IL-17, MCP-1, and TNF-α). Samples were run in duplicates (Normal n= 2, saline n= 2, 10^3 n= 3, 10^4 n= 2, 10^5 n= 1). Unfortunately there was no clear evidence of a dose dependent increase in proinflammatory cytokines in the AF of mated mice infected with HSV-2 (Figure 17). However despite the low sample size, some trends were noted. There was a marked increased in the expression of IFN-γ at the high inoculation dose of 10^5 PFU when compared to other experimental groups (Figure 17E). There appeared to be a reverse correlation between IL-6 and IL-10 levels of expression in the amniotic of GD 8 implantations (Figures 17 B-C).

In conclusion, given the low n numbers and the variability observed in these experiments, it is difficult to interpret the available data. Plausible reasons for this have been discussed in chapter 5.

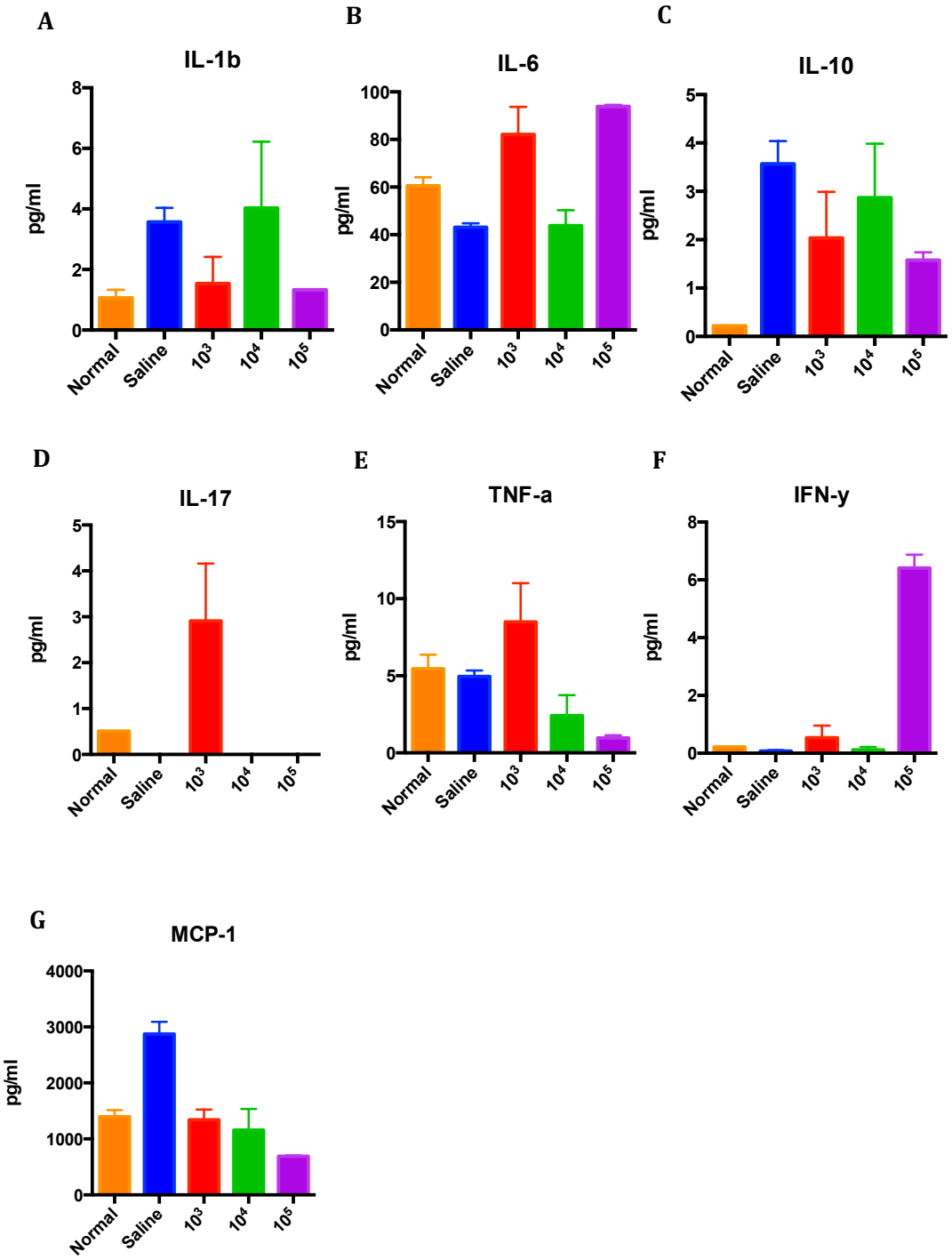


Figure 17 - Detection of cytokines and chemokines in amniotic fluid (AF). Amniotic fluids of GD 8 implantation sites (Normal n= 2, saline n= 2, 10^3 n= 3, 10^4 n= 2, 10^5 n= 1) were collected. Each sample was run in duplicates. Data are represented as mean ± standard error of the mean (SEM).

CHAPTER 5: DISCUSSION

5.1 Summary

Viral infections have long been associated with various adverse pregnancy outcomes (Avgil & Ornoy, 2006; Gervasi et al., 2012; Kourtis et al., 2014; McIntosh & Isaacs, 1992; Robb, Benirschke, & Barmeyer, 1986; Robb, Benirschke, Mannino, & Voland, 1986). During the 2009 influenza A (H1N1) pandemic, pregnant women were shown to be more susceptible to severe influenza illness, accompanied by an increased risk of adverse pregnancy outcomes and maternal death (Haberg et al., 2013; Pierce, Kurinczuk, Spark, Brocklehurst, & Knight, 2011; Siston et al., 2010). Similarly, HSV-2 and other sexually transmitted infections during pregnancy have been associated with gestational complications, such as spontaneous abortions, intrauterine growth restriction, still births, and preterm births (Padmavathy et al., 2013). In a study conducted in the US by Brown et al. (1997), it was shown that up to 22% of pregnant women are seropositive for HSV-2, and 2% of them acquired HSV-2 during gestation. However, very little is known regarding the underlying mechanisms of preterm birth, and spontaneous abortions, especially in association with HSV-2 infection. Unfortunately, there remains a lack of a suitable animal model in order to address the mechanism of pathogenesis. As a result, in an attempt to fill the gap in the current research, the goal of this project was to develop and characterize a primary HSV-2 infection model in pregnant mice. This model could be used in the future to examine various

pregnancy outcomes (e.g. preterm births) and underlying pathogenesis and immunological mechanisms that would affect pregnancy outcome, in response to intravaginal HSV-2 infection.

The overall hypothesis of the current study was that primary HSV-2 infection during early gestation would lead to an inflammatory environment in utero, which ultimately will lead to poor pregnancy outcomes. To address this hypothesis we formulated three goals for this study: 1) define HSV-2 infection in early pregnancy, 2) determine the effects of HSV-2 infection on fetus and pregnancy, and 3) examine proinflammatory cytokines and chemokines expression in maternal serum and amniotic fluid to determine if infection induces inflammatory environment. To accomplish these goals, we infected mated mice intravaginally with three different doses (10^3, 10^4, and 10^5 PFU) of wild-type HSV-2 on gestational day 5 and separated them into two different end-point groups, gestational day 8 (3 days post infection) and gestational day 13 (8 days post infection). Gestational day 5 represents infection during early gestation; since GD 4.5-5 has been shown to be the window for implantation in mice (Croy, Yamada, DeMayo, & Adamson, 2013). Gestational day 8 (3 days post infection and GD 13 (8 days post infection) were chosen as the two sacrifice time points since HSV-2 infection has been shown to typically peak at approximately 3 days post intra-vaginal infection in non-pregnant mouse models. Furthermore, at GD 13, implantation sites should be well developed enough that it is easier to separate different tissues, such as

the decidua, placenta, and fetus from each other. The mice were monitored, and vaginal washes were collected daily. Spleen, vaginal tract, uterus/decidua, and implantations were collected for histology and real-time PCR at two time points of sacrifice.

The cumulative results from the current study show that we have successfully developed and characterized a mouse model of HSV-2 infection early in pregnancy. Mated mice were shown to be more susceptible to primary HSV-2 infection when compared to diestrus (progesterone dominant) non-pregnant mice. Using a sensitive real-time PCR assay, HSV-2 DNA was detected in the upper reproductive tract and implantation sites of mated mice at GD 8. At later time point viral DNA was detected in the vagina, decidua, and placenta of mated mice. Furthermore, at the later time point of infection (GD 13), HSV-2 DNA was found to have disseminated into the spleen. This is the first report that HSV-2 viral DNA can be detected in upper tract, decidua, placenta and fetus during pregnancy. Additionally, infections in mated mice corresponded with local inflammation, demonstrated via leukocytic cell infiltration and HSV-2 specific staining in the vaginal tract. We also observed a trend for increased resorptions following HVS-2 infection of mated mice that was dose-dependent on the virus. Interestingly, despite small n numbers our results indicated that infections in these mated mice could be responsible for the inversed relationship between protein levels of IL-6 and IL-10 seen in amniotic fluid of GD 8 implantations, which could affect pregnancy outcomes.

Pathological changes in the infected mated mice, especially with fetal and placental necrotic tissues, were used as an indirect measurement of pregnancy outcomes.

Results from previous studies have shown an association of viral infections, such as CMV, influenza, HSV-2, MHV-68, and pregnancy complications (Finger‐Jardim et al., 2014; Goldenberg et al., 2000; Hamilton et al., 2012; Iwasenko et al., 2011; J. C. Kim et al., 2014; McIntosh & Isaacs, 1992; Racicot et al., 2013). However, there were various limitations in these study designs, ranging from non-clinically relevant route of infection (e.g. intraperitoneal), to high viral inoculation dosage, and non-human herpes virus, that prevented these studies from recapitulating a primary WT HSV-2 infection during pregnancy in humans.

Taking previous studies limitations into consideration, the current experimental design uses WT HSV-2 that is delivered via a biologically relevant route, similar to what would happen during sexual intercourse transmission (i.e. intravaginally). Additionally, by using three different doses of inoculation (10^3, 10^4, and 10^5 PFU), details of how HSV-2 inoculation dosage affects pregnancy outcomes, dissemination of infection and different immunological parameters could be elucidated. Since HSV-2 seropositive human adults do not die from the infection, unlike mice, varying doses of inoculation in pregnant mice could help to determine the concentration of virus that will result in an infection, but would not cause mortality in these

mice. This study is the first of its kind to demonstrate WT HSV-2 could disseminate into the upper reproductive tract and into the placenta of mated mice. As a result, this model could be used to better understand how primary HSV-2 could affect the pregnancy outcomes, fetal health, and the underlying mechanisms for these observations.

Previous studies have shown that pregnant mice, and mice treated with progesterone are more susceptible to intravaginal HSV-2 infection (Baker & Plotkin, 1978; A. Gillgrass et al., 2003; A. E. Gillgrass, Fernandez, Rosenthal, & Kaushic, 2005; A. E. Gillgrass, Tang, Towarnicki, Rosenthal, & Kaushic, 2005; Kaushic et al., 2003; Young & Gomez, 1979). The present study has shown that like progesterone-treated mice, mated mice are more susceptible to intravaginal HSV-2. Furthermore, the current study also showed the affects of increasing inoculation viral doses (10^3, 10^4, and 10^5 PFU) in mated mice compared to non-pregnant diestrus mice. The difference could be seen at the lowest inoculation dose of 10^3 PFU, where mated mice had 25% mortality compared to 0% mortality in diestrus mice. At higher inoculation doses (10^4 and 10^5 PFU), all of the mated mice succumbed to the infection, whereas the diestrus mice had 25% and 80% mortality respectively. The viral shedding in each experimental group corresponded with the genital pathology scores and survival, with mated mice showing highest pathology and viral shedding. Given that mated mice likely have high progesterone levels, as could have been predicted, out results show that mated mice were more susceptible to

HSV-2 infection when compared to diestrus mice. More interesting was the dose-dependent difference in susceptibility between each inoculation dose in relation to the pathobiology and pregnancy outcomes. Since data showing the relationship between HSV-2 inoculation dose and adverse pregnancy outcomes has been missing in the literature, these results serve as a strong foundation for examining the affects of a non-lethal infection in pregnant mice.

Once the infection model was established, pregnancy outcomes and related immunological read-outs were examined. There was no significant decrease in the percentage of infected mated mice that have fetuses at time of sacrifice for both GD 8 and GD 13 time points, when compared the sham infected group. However, upon examination of the GD 13 placentas from the fetuses of infected mated mice using immunohistochemical staining, there were sections of necrotic tissues and constricted spiral arteries remodeling based on H&E and PAS staining. NK cells are usually localized in the decidua basalis, close to the placenta, as visualized via PAS staining (Moffett-King, 2002). However, placentas of infected mated mice showed localization of NK cells in the band of decidua, next to spiral arteries in the 10^5 PFU GD 13 group. This suggests that HSV-2 infection could affect the localization of NK cells within implantation sites and may prompt the NK cells to change their phenotypes to activated NK cells, resulting in adverse pregnancy outcomes.

These types of morphological and histopathological changes were not observed in the sham-infected group. Previous studies have shown abnormal

placental weight and spiral arteries remodeling has a negative impact on fetal morbidity and mortality (Ashkar & Croy, 2001; A. A. Ashkar et al., 2000; Croy, Chantakru, Esadeg, Ashkar, & Wei, 2002). Despite the fact that the number of mated mice that had fetuses did not decrease in the HSV-2 infected group compared to the sham infected control; the pathological changes in different tissues of the implantation sites suggests that the infection has an affect on the fetal and pregnancy outcomes. Whether this effect is mediated through increase in proinflammatory cytokines/chemokines, directly through viral infection of the tissues, increased maternal stress due to infection, or a combination of factors is unclear, and requires further analysis.

Post HSV-2 infection, one would expect to see an increase in proinflammatory cytokines in maternal serum. An overt inflammatory response would correspond with the observed genital pathology in the mother and may contribute to the resorptions and abnormal placental histology. However, results from the 32-plex cytokines array showed no significant changes in cytokines or chemokines expression between experimental groups. Although the results did not correspond to the initial hypothesis of increased expression of proinflammatory cytokines and chemokines in the sera of infected mated mice, other studies such as by Kim et al. (2014) failed to find increased inflammatory cytokines when maternal serum in post-influenza B infection was analyzed. However, Kim et al. (2014), and various other studies have shown an increase in proinflammatory cytokines, such as

IL-6 and TNF-α and decrease IL-10 in tissues (e.g. lungs, cervix, and/or placenta) (Cardenas et al., 2010; Hamilton et al., 2012; Kfutwah et al., 2009; J. C. Kim et al., 2014). This would support the results obtained from the amniotic fluid in our GD 8 group, where IL-6 and IL-10 were shown to have an inversed correlation to each other. Instead of an overt inflammatory cytokine profile in the maternal serum, this data suggests the presence of a more local inflammatory effect, at the level of the tissues. This is significant since Cardenas et al. (2010) had shown that the local inflammatory response in the placenta and decidua could transiently delay the development of the fetus, such as differentiation of the eye, tails, and limbs. The detrimental effects of severe pathogenesis and pregnancy outcomes are present even in the absence of a systemic infection suggests that mated mice are more susceptible to viral infections when compared to non-pregnant mice.

Using a sensitive real-time PCR approach, viral HSV-2 DNA was detected in the vaginal tract, uterus (mated non-pregnant mice), implantations, and resorptions (when applicable) of GD 8 mated mice. Similarly, HSV-2 DNA was found in the spleen, vaginal tract, uterus (when applicable), decidua, fetus, and placenta of mated mice at GD13. The presence of viral DNA in tissues, especially of implantations has been shown to cause adverse effects in pregnancy outcomes (Iwasenko et al., 2011; McIver et al., 2005; Romero et al., 2007; Syridou et al., 2008). However, to date, no other animal studies have shown the presence of HSV-2 DNA in

implantations of infected mice. This suggests that in the current model, HSV-2 could cross the cervical plug, which serves as an immunological and physical barrier, and has previously been thought to be responsible for blocking pathogens from ascending up the reproductive tract during pregnancy (Racicot et al., 2013).

5.2 Advantages and disadvantages compared to related studies

There has been an increase in the number of studies examining the incidence rate, as well as the associated morbidity and mortality risk associated with infections during pregnancy (Agrawal & Hirsch, 2012; Arechavaleta-Velasco, Koi, Strauss, & Parry, 2002; Arvaja et al., 1999; Brown et al., 1997; Cardenas et al., 2010; Challis et al., 2009; Christiansen, Nielsen, & Kolte, 2006; Corey & Wald, 2011; Kourtis et al., 2014) . However, there remains a lack of understanding regarding the underlying mechanisms responsible for the detrimental effects seen during pregnancy due to infections. This could partly be contributed to the scarcity of appropriate animal models. The present study showed pregnant mice are more susceptible to sexually transmitted HSV-2 viral infection and are at risk of adverse pregnancy outcomes. Furthermore, this is the first study to show primary IVAG HSV-2 infection could disseminate into the upper reproductive tract and placenta of fetuses. In another study designed to examine the effects of viral infection during pregnancy, Cardenas et al. (2010) infected

pregnant mice intraperitoneally (i.p.) with 10^6 PFU of murine γ-herpes virus 68 (MHV-68) at GD 8.5. In their results, they showed high viral titers in the decidua, and placenta, but not in the fetus. However, even without the presence of virus in the fetus, the authors were able to show lower fetal weight and slower development at 3 days post-infection, corresponding to high levels of IFN-γ and TNF-α in the fetus, thus suggesting that the presence of an active inflammatory response in the placenta and decidua can have a direct effect on fetal development (Cardenas et al., 2010). Additionally, there were evidence of edema in the decidua; necrosis and inflammation foci were also observed in the placental labyrinth of infected mice (Cardenas et al., 2010). In our study, while we saw increased resorption with corresponding with the inoculation dose, these differences were not significant. We also did not observe IFN and TNF increase in GD 13 maternal serum or GD 8 AF. The pathohistological changes in the placenta of the infected mice reported in this study are similar to the results found in our study (Figures 10 & 11). Some of the limitations of this study included the systemic route of infection (intraperitoneal), as well as the high dosage of infection. By injecting intraperitoneally with such a high dose of virus, this ensured almost all the mice will be infected, however, the resulting data might be confounded by such a large number of viruses present, skewing the rate of infection as well as the associated adverse pregnancy outcomes. Furthermore, by using murine γ-herpes virus 68 (MHV-68), even though it shares significant genomic

colinearity with Epstein Bar Virus and Kaposi's sarcoma-associated herpesvirus, it is difficult to make conclusions or extrapolations regarding the effects of HSV-1 or HSV-2 in during pregnancy.

In a follow up study done by Racicot et al. (2013), the authors using the same mouse model and infection route (Cardenas et al., 2010), showed for the first time that viral infection during pregnancy makes the mother more susceptible to ascending bacterial infections. The authors suggested the increase in susceptibility could be associated with the high sex hormones level (estrogen and progesterone), which induced modifications in the cervix such as decreased toll-like receptors (TLRs) and antimicrobial peptides (Racicot et al., 2013). Despite showing an affect of viral infection, specifically MHV-68 on pregnancy, this study has some of the same limitations as that found in Cardenas et al. (2010), a systemic route (i.e. intraperitoneal) and high dose of infection. The current study used a more anatomically relevant and physiologically appropriate route for HSV transmission, which is via the vaginal tract.

To date, there has only been one other study that examined pregnancy outcomes post IVAG HSV-2 infection. Sanjuan and Zimberlin (2001) infected mice IVAG with wild-type HSV-2 at an earlier GD 3-4 and late GD 10-14 time points. Sanjuan and colleagues (2001) concluded that the average mortality of IVAG HSV-2 infected pregnant mice was not significantly different from that in non-pregnant mice. The authors used a very high inoculation dose of $5x10^5$

PFU of HSV-2 that resulted in high mortality across all groups (14 out of 21 mice (66.66%); 10/13 mice inoculated at GD 10-14; 4/8 mice inoculated at GD 3-4). This is different from the current study results where HSV-2 infected mice had higher mortality rate when compared to diestrus non-pregnant mice, even at the lowest inoculation dose of 10^3 PFU. A possible explanation for this discrepancy could be due to the fact that the authors failed to infect the non-pregnant mice in the appropriate stage of estrous cycle. Instead of staging the mice and infect them during diestrus, the authors argued that swabbing the vaginal tract with a dry cotton wool is crucial for HSV-2 IVAG infection in non-pregnant mice, even more so than the serum levels of the progesterone. Thus contradicting previous studies showing that susceptibility to vaginal HSV-2 infection is dependent on the stage of the estrous cycle (Gallichan & Rosenthal, 1996). The authors also observed that infection at earlier time point had higher abortion rate when compared to infection at later time point, suggesting that infection at earlier time point during pregnancy could result in deaths of embryos 4 to 5 days p.i. However, there was no evidence of dissemination of HSV-2 in the liver, spleen, bone marrow or kidneys despite a high HSV-2 inoculation dose of $5x10^5$ PFU. This could be due the lack of a quantitative method used in this study; the authors relied on immunohistochemistry and electron microscopy to detect the presence of virus in various tissues. This method is not very sensitive since detection of infection is dependent on where the sections were taken. Similar to the

thickening of the endothelium surrounding the spiral arteries in the present study (Figures 11E-G), Sanjuan and Zimberlin (2001) showed coagulative necrosis at abortion sites, which is usually caused when blood supply to tissues is diminished.

In other related viral infection models in pregnant mouse, Pazos et al. (2012) and Kim et al. (2014) examined the effects of influenza during pregnancy. In both studies, pregnant mice had higher mortality rate and delayed viral clearance in the lungs. Furthermore, the fetuses from the infected mothers failed to achieve the same weight compared to the controls and had fewer implantations. However, what is interesting is both studies differed on the effects of sex hormones on pregnancy outcomes. Pazos and colleagues (2012) showed that mice treated with estrogen pellets had a delayed viral clearance in the lungs compared to placebo treated mice. In comparison, Kim et al. (2014) suggests that pregnant infected mice had significantly lower level of estrogen and progesterone when compared to pregnant uninfected mice, suggesting that sex hormones may confer a protective effect against viral infections. Further research is needed to determine the role of progesterone and estrogen has in infections during pregnancy.

5.3 Limitations

Despite the fact that we were successful in developing the mouse model and showed for the first time the presence of HSV-2 DNA being disseminated into the upper reproductive tract and the implantations, there were a few limitations in our studies. The first was that we had found no difference in number of mated, uninfected mice with successful implantations compared to HSV-infected mice at both GD 8 and GD 13 time points. Given the timing of infection (GD 5, which corresponds with implantation) we expected a significant decrease in number of mice with successful implantations in mated mice infected with HSV-2 compared to control uninfected group. This lack of difference in number of mated mice with implantations was even more pronounced when comparing between the saline and normal group. It is clear that there is a decrease in the number of mated mice with implantations that were infected with HSV-2 compared to normal un-inoculated mice. However, it was difficult to ascertain and draw conclusions if HSV-2 infection had any effects on pregnancy outcomes, especially when the saline inoculated mice had a similar decrease in the number of mated mice with implantations as that of HSV-2 infected ones. Although the underlying cause of this is observation is unclear, however, a possible explanation for this could be due to the intraperitoneal anesthetic and the act of inoculation in the vaginal tract, which may cause stress in these mated mice. Numerous experimental models have shown maternal

stress could adversely affect pregnancy outcomes, including fetal programming (Couret, Jamin, Kuntz-Simon, Prunier, & Merlot, 2009; Kay, Tarcic, Poltyrev, & Weinstock, 1998; Knackstedt, Hamelmann, & Arck, 2005). A preliminary study should be conducted in order to find the confounding variable, if it is the inoculation versus anesthesia. In order to do this, one group of mated mice would receive only the anesthesia without the inoculation, and the other group will continue to have both. If there were a decrease in the number of mated mice with implantation sites in the group that received only anesthesia, it would suggest the anesthesia is the reason for lower number of mated mice with implantation sites. If this were the case, switching to gas anesthesia could be a good alternative to determine if the injectable anesthesia was responsible for the decrease in the number of mated mice with implantations.

The second limitation of our study was that we found no significant difference in the percentage of fetal resorption at GD 13; however, GD 8 group showed an inoculation dose dependent increase in the number of resorptions. A possible explanation as to why there was no significant difference in the percentage of fetal resorption at GD 13 might be due to the more advanced gestational period. At GD 13, resorption sites might be no longer visible if the implantation site was resorbed earlier in gestation and has been cleared by GD 13. Two approaches should be carried out order to address this issue. In order to draw meaningful conclusions from the

difference in percentage of resorption between each experimental group at two time points, additional samples should be added to prevent a type two-analysis error. Furthermore, since it is difficult to visualize implantation sites early in gestation, injection of dye (Chicago Sky Blue 6B) just prior to euthanasia can be used to aid in implantation site localization (Croy et al., 2013). As implantation is associated with neoangiogenesis and vascular leakage, dye spots will indicate the implantation sites even after resorption.

In our study, pathological changes such as spiral arteries constriction and a demolished implantation morphological structure were found in the implantation sites of GD 13 mated mice. Furthermore, abnormal localization of NK cells was present in the band of the decidua surrounding the constricted spiral arteries of these implantation sites. NK cells were stained in the placenta using PAS staining, however, more information regarding specific cell sub-types could be gained by using FlowCytometry or specific staining for different NK cells surface markers. An example of this would be comparing uterine NK cells ($CD56^{bright}/CD16^{-}$) and killer NK cells (Sca-1/CD69) (Fogel, Sun, Geurs, Carayannopoulos, & French, 2013).

Another limitation was the relatively low number of mated mice with implantations in the GD 8 and GD 13 experiments. If the difference between each experimental groups and time points were relatively small, a larger sample size would be required to demonstrate the difference between each group. In order to address this issue, a power calculation could be made to

determine the necessary number of mice needed to be included in the statistical sample. This would increase the sample size for resorption percentage and the number of tissues collected for different assays. By increasing the number of mated mice, it is possible to reveal the presence of any type II errors present, which could be masked by a small population size.

The current study was not designed to examine fetal health and development, thus there are some aspects that were not included in these experiments were weighing of the fetuses, organs, and especially the placentas as an assessment of fetal health and development. A study by Heinonen *et al.,* found that small for gestational age infants had smaller placenta than the control group, suggesting that fetal growth depends on the actual weight of the placenta (2001) . High placental weight to birth weight ratio could lead to the development of hypertension, diabetes, and possibly stroke later in life (Barker et al., 1993; Lao & Wong, 1999).

Additionally, the 32-plex-cytokine array testing the maternal sera of GD 13 mated mice (collected on GD 9) showed no increase in proinflammatory cytokines/chemokines or a corresponding decrease in Th_2 cytokines. The lack of significant difference could be due to the small number of sample size in this array. However by increasing the number of samples, the results would show a clearer difference in the level of cytokines/chemokines expression, especially in those cytokines already showing a clear trend. As mentioned previously, other studies have also shown no significant increase in

proinflammatory cytokines/chemokines in maternal sera of infected mice but rather in different tissues (Cardenas et al., 2010; Hamilton et al., 2012; Kfutwah et al., 2009; J. C. Kim et al., 2014). Therefore instead of screening maternal sera for proinflammatory cytokines and chemokines, similar cytokines panel could be done on different tissues, such as decidua, fetus, placenta, and spleen of the infected mated mice. Similarly, the number of amniotic samples need to be increased for future studies since the preliminary cytokines/chemokines results based on a few samples showed promising results, however it remains inconclusive.

5.4 Implications

The novel data from the current study showed a dose dependent effect on susceptibility and pregnancy outcomes in a primary HSV-2 infection pregnant mouse model. Furthermore, it provided evidence for the first time that HSV-2 DNA could disseminate up the reproductive tract and into implantation sites of pregnant mice. To date, few studies have shown pregnancy outcomes in an HSV-2 pregnant mouse model. While clinical observations have linked various viral/bacterial infections to preterm births and spontaneous abortions, little information exists about the underlying mechanisms of these adverse pregnancy outcomes. Thus making the connection between primary HSV-2 infection and adverse pregnancy and developmental outcomes has not been concretely established. Using the

mated mouse model established in this study, future investigations regarding the pathogenic mechanisms of HSV-2 that may be responsible for the adverse pregnancy outcomes could be explored. Ultimately such studies could be correlated to clinical outcomes and could help physicians make informed evidence-based prenatal care clinical decisions and screening for STIs, especially in the population with high-risk of HSV-2 acquisition.

5.5 Future Directions

This study has laid the foundation for future studies in primary HSV-2 infection during pregnancy in a mouse model. In order to move this study forward, further characterization and enumeration of specific immune cells, such as uterine NK cells is required, using either immunohistochemical staining and/or FlowCytometry techniques. This would be of interest since it has been previously shown that the lack of uterine NK cells ($CD56^{bright}/CD16^{-}$) and IFN-γ signalling leads to a failure in initiating pregnancy-induced modification of decidual arteries and display hypocellularity or necrosis of decidua in mice (Ali A Ashkar, James P Di Santo, & B Anne Croy, 2000; Croy et al., 2002). Furthermore, future experimental designs should include comparison of fetal and placental weight, and have pre-term births as one of the primary objectives of investigation. This is important since high placental weight to birth weight ratio could lead to the development of hypertension, diabetes, and possibly stroke later in life (Barker et al., 1993; Lao & Wong,

1999). Moreover, pre-term births have been associated with higher risks of autism, cerebral palsy, developmental delays, chronic lung disease, and vision and hearing impairments development (G. Mor & Cardenas, 2010).

Additionally, increasing the sample size for both experimental time points (GD 8 and GD 13) would allow for better comparison and differences in the percentage of resorption and failure to implant could be delineated between experimental groups. Furthermore, with an increased sample size, a multiplex cytokine inflammatory array similar to that of GD 13 group could be conducted for implantation sites in the GD 8 (day 3 p.i.) group. With this information, further comparison and the kinetics profile of different chemokines and cytokines post-HSV-2 infection could be explored, especially with that of the GD 13 group.

CHAPTER 6: CONCLUSION

Over 536 million people between the ages of 15 and 49 are living with HSV-2, making it one of the most prevalent sexually transmitted diseases (STIs) worldwide (Looker et al., 2008). Although the pathogenesis and underlying mechanisms for immune responses and protection against HSV-2 infection have been well characterized in the non-pregnant mouse model, the effects of primary HSV-2 infection during pregnancy remains unclear. This is especially concerning since previous clinical studies have shown that genital HSV infection acquired during pregnancy could be associated with preterm labour, intrauterine growth restriction, and spontaneous abortions (Brown et al., 1997; Brown et al., 1985; Whitley et al., 1991).

The results from this study supported the hypothesis that HSV-2 infection during early pregnancy could lead to adverse pregnancy outcomes. It was demonstrated that mated mice were more susceptible to HSV-2 infection when compared to mice in progesterone dominant diestrus stage of the cycle. The dose-dependent increase in susceptibility and infection correlated with increasing number of resorptions in the GD 8 group. Furthermore, there were evidence of necrosis in the placenta and thickening of the endothelial layer of the spiral arteries, suggesting abnormal remodelling of the tissue during implantation. The current study has shown for the first time that HSV-2 could disseminate into the upper reproductive tract and

placentas, suggesting that either the presence of virus and/or proinflammatory cytokines and chemokines are responsible for adverse pregnancy events. As a result, this study is highly relevant in both basic science and clinical study contexts, and provides a strong foundation for future studies exploring the mechanisms and potential novel maternal and fetal screenings and health management strategies in this special demographic.

CHAPTER 7: REFERENCES

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APPENDICES

Appendix A – Summary of Mated Mice (Gestational Day 8)

PBS								
Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Intact Fetuses Rate	Resorption Rate
1	Yes	Yes	6	0	6	62.5%	100%	0.00%
2	Yes	Yes	9	0	9			
3	Yes	Yes	9	0	9			
4	Yes	No	N/A	N/A	N/A			
5	Yes	Yes	13	0	13			
6	Yes	Yes	7	0	7			
7	Yes	No	N/A	N/A	N/A			
8	Yes	No	N/A	N/A	N/A			
	Total = 8	Total = 5/8	44	0	44			
		Averages	8.8	0	8.8			

10^3								
Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Intact Fetuses Rate	Resorption Rate
1	Yes	Yes	11	0	11	100.00%	98.72%	1.28%
2	Yes	Yes	8	1	9			
3	Yes	Yes	10	0	10			
4	Yes	Yes	11	0	11			
5	Yes	Yes	10	0	10			
6	Yes	Yes	12	0	12			
7	Yes	Yes	10	0	10			
8	Yes	Yes	5	0	5			
	Total = 8	Total = 8/8	77	1	78			
		Averages	9.625	0.125	9.75			

10^4								
Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Intact Fetuses Rate	Resorption Rate
1	Yes	No	N/A	N/A	N/A	83.33%	95%	5%
2	Yes	Yes	2	1	3			
3	Yes	Yes	7	1	8			
4	Yes	Yes	8	0	8			
5	Yes	Yes	10	0	10			
6	Yes	Yes	11	0	11			
	Total = 6	Total = 5/6	38	2	40			
		Averages	7.6	0.4	8			

10^5								
Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Intact Fetuses Rate	Resorption Rate
1	Yes	Yes	8	0	8	85.71%	97.78%	2.22%
2	Yes	No	N/A	N/A	N/A			
3	Yes	Yes	0	1	1			
4	Yes	Yes	9	0	9			
5	Yes	Yes	6	0	6			
6	Yes	Yes	10	0	10			
7	Yes	Yes	11	0	11			
	Total = 7	Total = 6/7	44	1	45			
		Averages	7.333333333	0.166666667	7.5			

Appendix B – Summary of Mated Mice (Gestational Day 13)

Normal

Mouse #	Presence of Plugs	Successful Implantation	Number of Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Developmentally Normal Fetuses	Resorption Rate
1	Yes	Yes	9	0	9			
2	Yes	Yes	8	0	8			
3	Yes	Yes	10	0	10	100%	100%	0%
4	Yes	Yes	9	0	9			
5	Yes	Yes	7	0	7			
	Total = 7/7		43	0	43			
		Averages	8.6	0	8.6			

PBS

Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Developmentally Normal Fetuses	Resorption Rate
1	Yes	No	N/A	N/A	N/A			
2	Yes	Yes	11	0	11			
3	Yes	Yes	9	0	9			
4	Yes	Yes	10	0	10	50.00%	100%	0%
5	Yes	No	N/A	N/A	N/A			
6	Yes	No	N/A	N/A	N/A			
7	Yes	No	N/A	N/A	N/A			
8	Yes	Yes	10	0	10			
	Total = 8	Total = 4/8	40	0	40			
		Averages	10	0	10			

10^{-3}

Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Developmentally Normal Fetuses	Resorption Rate
1	Yes	No	N/A	N/A	N/A			
2	Yes	Yes	6	1	7			
3	Yes	No	N/A	N/A	N/A			
4	Yes	Yes	10	0	10			
5	Yes	Yes	3	1	4			
6	Yes	Yes	10	2	12	75.00%	86.36%	13.6%
7	Yes	Yes	0	2	2			
8	Yes	Yes	9	0	9			
	Total = 8	Total = 6/8	38	6	44			
		Averages	6.333333333	1	7.333333333			

10^{-4}

Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Developmentally Normal Fetuses	Resorption Rate
1	Yes	No	N/A	N/A	N/A			
2	Yes	No	N/A	N/A	N/A			
3	Yes	Yes	10	0	10			
4	Yes	Yes	8	0	8			
5	Yes	No	N/A	N/A	N/A			
6	Yes	Yes	9	0	9	63.64%	96.49%	3.51%
7	Yes	Yes	9	1	10			
8	Yes	Yes	1	1	2			
9	Yes	Yes	8	0	8			
10	Yes	No	N/A	N/A	N/A			
11	Yes	Yes	10	0	10			
	Total = 11	Total = 7/11	55	2	57			
		Averages	7.857142857	0.285714286	8.142857143			

10^{-5}

Mouse #	Presence of Plugs	Successful Implantation	Number of Intact Fetuses	Number of Resorptions	Total Fetuses	% of mice with successful implantations	Developmentally Normal Fetuses	Resorption Rate
1	Yes	No	N/A	N/A	N/A			
2	Yes	No	N/A	N/A	N/A			
3	Yes	Yes	9	1	10			
4	Yes	No	N/A	N/A	N/A			
5	Yes	Yes	7	1	8	44.44%	72.97%	27.03%
6	Yes	No	N/A	N/A	N/A			
7	Yes	No	N/A	N/A	N/A			
8	Yes	Yes	11	0	11			
9	Yes	Yes	0	8	8			
	Total = 9	Total = 4/9	27	10	37			
		Averages	6.75	2.5	9.25			

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