EFFECTS OF SEMINAL PLASMA ON THE ENDOMETRIAL TRANSCRIPTOME AND EARLY FETAL DEVELOPMENT

By

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To my mother and father, for their endless love, support, encouragement, and sacrifices, they inspire me every day to be the best version of myself.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AKR1C4	Aldo-keto reductase family 1 member C4
APCs	Antigen presenting cells
BNC	Giant binucleate cells
CD	Cluster of differentiation
$CD4^+$	Helper T cells
$CD4^+CD25^+$	T regulatory cells
$CD8^+$	Cytotoxic T cells
cDNA	Complementary DNA
CIDR	Controlled internal drug release
CL	Corpus luteum
COX2/PTGS2	Prostaglandin-endoperoxide synthase 2
CSF2	Colony stimulating factor 2
CV	Coefficient variance
CXCL	CXC motif chemokine ligand
DCs	Dendritic cells
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
E2	Estradiol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunoassay
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FDR	False discovery rate

FGF	Fibroblast growth factor
FOXP3	Forehead box P3
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
IFNγ	Interferon gamma
IFNτ	Interferon tau
IL	Interleukin
IPA	Ingenuity pathway analysis
ISGs	Interferon stimulated genes
JAK	Janus kinase/signal transducer
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MIQE	Minimum information for publication of qPCR experiments
mRNA	mRNA
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
P4	Progesterone
PAGs	Pregnancy associated glycoproteins
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

PGE_2	Prostaglandin E_2
$PGF_2\alpha$	Prostaglandin F_2 alpha
PGR	Progesterone receptor
PI3K-AKT	Phosphatidylinositol-3 kinases-protein kinase B
RIN	RNA integrity number
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences
STAT3	Signal transducer and activator of transcription 3
TAI	Timed artificial insemination
Th	T helper
TGFβ	Transforming growth factor beta
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory cells
VEGFA	Vascular endothelial growth factor A

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In rodents, swine, and humans, seminal plasma facilitates sperm transport in the female reproductive tract and actively modulates the cellular and molecular maternal environment at the time of conception and early embryo development. While modulating the early endometrial environment using seminal plasma has been shown to alter embryonic and postnatal development in rodents, it is unclear if a similar phenomenon exists in cattle. I hypothesize that exposure to seminal plasma at conception alters the maternal tract environment and programs early embryo development in cattle. Here, seminal plasma was infused into the uterine lumen at the time of insemination with X-sorted semen to evaluate effects on endometrial transcriptome, embryonic development, and fetal growth. Embryos were recovered seven days after insemination and intrauterine infusion of either seminal plasma or saline. Treatment with seminal plasma did not affect the total number of fertilized objects or the proportion of embryo that were blastocyst, morula, degenerate, or arrested. Interestingly, blastocyst expression of *DNMT3A* and *PTGS2* were altered in cows infused with seminal plasma. In addition, seminal plasma altered the expression of 107 endometrial transcripts seven

days after insemination. A total of 19 upstream regulators of differentially expressed genes, seven gene networks and 22 canonical pathways related to 1) immune response and cell signaling, 2) cellular function, and 3) cell morphology and embryonic development were affected by seminal plasma infusion. The effect of seminal plasma on fetal growth was evaluated using ultrasound between d 32 and 81 after insemination in singleton, female pregnancies. Seminal plasma infusion reduced fetal crown-nose length, while fetal crown-rump length and abdominal diameter were not affected. The effect of seminal plasma infusion on placental and fetal morphometry were evaluated at d 96 of gestation. Exposure to seminal plasma at conception increased amniotic fluid volume and tended to reduce cotyledon diameter and fetal heart girth. In conclusion, I show that exposure to seminal plasma at conception alters the endometrial transcriptome seven days later, alters blastocyst gene expression and modifies fetal development. These data may help to optimize pregnancy outcomes and program postnatal development in cattle using seminal plasma.

CHAPTER 1
LITERATURE REVIEW

Introduction

Reproductive performance is the major hallmark of efficiency for livestock production. Fertility in lactating dairy cows has decreased in recent decades, causing a negative impact on reproductive, productive, and economic performance in dairy herds. Pregnancy loss in dairy cows; represents the major factor of poor reproductive performance, it is estimated that approximately 50% of pregnancy loss occurs within the first seven days post fertilization, with an additional 20% of pregnancy loss occurring between d 8 and d 28, and 5-20% of pregnancy loss occurring between d 28 and calving, resulting in a 30-35% chance of a live birth (Wiltbank et al., 2016). Pregnancy loss in cattle is attributed to genetic, physiological, endocrine, and environmental factors (Walsh et al., 2011).

Walsh et al. (2011) proposed different causes affecting fertility and recommended techniques to ameliorate infertility; 1) reduce postpartum negative energy balance and diseases, 2) improve expression of estrus and estrus detection followed by artificial insemination (AI) with high quality semen, 3) ovulation and fertilization of high quality oocytes, 4) improve early progesterone secretion from the corpus luteum (CL), 5) ensure an appropriate early endometrium environment to stimulate embryo development, and 6) promote a viable embryo that secretes interferon tau (IFNτ) to modulate uterine prostaglandin production and enhance maternal-fetal communication between d 15 and 20. Interestingly, the authors mentioned that use of high quality semen as a plausible way to overcome infertility. In the past two decades studies in different species demonstrated that male factors, specifically seminal plasma,

modulates the endometrial environment, influencing fetal programing, supporting embryo development and impacting the phenotype of subsequent offspring (Bromfield et al., 2014; Ortiz et al., 2019; Schjenken & Robertson, 2020). Therefore, the work presented in this thesis will focus on the influence of seminal plasma on the endometrial environment at the time of conception, preimplantation embryo development, and fetal development in an attempt to improve pregnancy outcomes in cattle.

Endometrial Environment Regulating Pregnancy

Female Reproductive Tract Environment During Early Embryo Development

The maternal environment at conception plays a critical role for early embryonic development, and pregnancy establishment and maintenance. A dynamic cross talk between the newly formed embryo and oviduct begins after fertilization. The oviduct facilitates gamete transport and provides the optimal physiological and biochemical environment to support and sustain early embryo development (Li & Winuthayanon, 2017). Following fertilization, the oviduct houses the preimplantation embryo for three to four days and is the initial interface of maternal-embryo communication, where the fertilized embryo receives signals from the oviduct to elicit molecular changes that subsequently shape the development of the embryo and epigenetic landscape of the future offspring (Pérez-Cerezales et al., 2018). In the bovine, the oviduct is comprised of ciliated and simple columnar secretory epithelial cells which contribute to the secretion of oviductal fluid that is rich in energy substrates, proteins, amino acids, lipids, and others factors to support the development of the early embryo (Mullins, 2000; Hugentobler et al., 2007; Maillo et al., 2016; Li & Winuthayanon, 2017).

Understanding the oviductal environment can provide insight into the regulatory mechanism affecting fertilization, and embryo survival and development. The presence

of the semi-allogenic embryo within the oviduct elicits a local immune response which is strictly regulated to provide a protective environment that allows the early embryo to develop and transit to the uterus (Maillo et al., 2016; Pérez-Cerezales et al., 2018). Despite this regulation, maternal immune dysfunction has been suggested as a major cause of infertility in cattle (Fair, 2015). Oviductal epithelial cells are the first maternal cells to encounter the preimplantation embryo, causing a maternal immune response that recruits and activates mostly macrophages and dendritic cells (DCs) (Ott, 2019). However, there is little information about maternal-embryo communication within the oviduct as it pertains to immune function, as studies suggest that maternal tissues do not recognize the embryo until trophoblast elongation (Marey et al., 2016).

Maillo et al. (2015), evaluated bovine oviductal cell transcriptome at the beginning of embryonic genome activation. In this experiment the presence of a single embryo failed to elicit *in vivo* changes to the oviductal transcriptome; however, when 50 *in vitro* produced embryos were transferred to the oviduct, a series of differentially expressed genes involved in immune function were detected in the oviduct, suggesting that a single embryo may elicit a subtle local response in the oviduct which is difficult to measure. Likewise, in another study using *in vitro* culture of bovine oviductal cells, a single embryo failed to induce changes in the transcriptome on day 8 or 13 of embryo culture. This is consistent with studies that suggest that maternal tissues cannot recognizes an embryo until trophoblast formation, due to the embryos inability to emit signals that drive cytokine and chemokine synthesis to activate immune cells (Yao et al., 2009; Schmaltz-Panneau et al., 2014; Marey et al., 2016). Conversely, most studies investigating the effect of the embryo on the oviductal transcriptome were achieved in

litter-bearing species where multiple embryos are present in the oviduct at the time of analysis (Lee et al., 2002; Almiñana et al., 2012).

Changes to the biochemical and physiological profiles of bovine oviductal cells are mediated by sex steroid hormones and their respective receptors (estrogen receptor 1, ESR1; estrogen receptor 2, ESR2; and progesterone receptor, PGR) (Ulbrich et al., 2003). Evidence suggests that the oviductal transcriptome is highly dependent of sequential exposure to estradiol (E2) and progesterone (P4) at proestrus and metestrus. Cows with greater exposure to E2 and P4 had increased abundance of transcripts related to tissue morphology, cellular changes, and secretion of growth factors that represent a potential impact to embryo survival and development (Gonella-Diaza et al., 2015; Binelli et al., 2018). Uterine receptivity to implantation is P4 dependent; however, implantation requires a loss of PGR and ESR1 from endometrial epithelial cells (Bazer et al., 2009).

Endometrial Environment During Preimplantation Embryo Development

On day five after fertilization, the morula stage embryo enters the uterus (Hansen & Tríbulo, 2019). Embryos continue cell division, forming the blastocyst stage embryo around day seven when blastocoel cavity formation occurs and differentiation of trophoblast cells and inner cell mass cells occurs that will give rise to the placenta and embryo proper, respectively (Valadao et al., 2018). Around day eight, embryos hatch from the zona pellucida, wherein the conceptus is first capable of direct contact with the maternal cells of the endometrium (Ott et al., 2014). After hatching, the trophoblast cells come into direct contact with endometrial cells and promote maternal-embryo communication. In order to survive, the conceptus must overcome several “hurdles”; 1) to ensure continued progesterone secretion from the CL, 2) avoid attack from maternal

immune cells, and 3) stimulate endometrial tissue remodeling to support placental formation (Ott, 2019). Following ovulation, E2 sharply decreases and the cells of the prior follicle transition from responding to follicle stimulating hormone and respond to luteinizing hormone. This transition period induces the remaining theca interna and granulosa cells of the ovulated follicle to begin CL formation and P4 secretion. A functional CL is formed within the first week of ovulation, leading to the dominance of P4, primarily associated with uterine receptivity (Bazer et al., 2009). From day 12 to 14, as the embryo grows and elongates, trophoblast cells begin to secrete significant amounts of IFNτ as a pregnancy recognition factor to prevent the luteolysis and ensure the maintenance of a functional CL for P4 synthesis (Forde & Lonergan, 2017).

During pregnancy establishment immune cells are active and present at the maternal-fetal interface to promote maternal tolerance; however, normal immune surveillance required to prevent uterine infection creates a hostile environment for the preimplantation embryo (Oliveira et al., 2012; Ott et al., 2019). As mentioned above the preimplantation embryo must avoid being attacked by maternal immune cells that recognize it as a foreign entity; how is it that the semi-allogenic embryo survives in this environment? It has been hypothesized that for survival the embryo requires continuous reciprocal maternal-fetal communication (Ott et al., 2014; Ott, 2019). Bovine endometrial immune cell populations are primarily composed of B cells, T cells, natural killer (NK) cells and monocytes during the estrous cycle and pregnancy. During maternal recognition of pregnancy, in response to the semi-allogenic embryo, the maternal immune system activates and promotes the expansion of NK cells, T cells, monocytes, macrophages and DCs (Oliveira & Hansen, 2009; Mansouri-Attia et al.,

2012; Vasudevan et al., 2017). Below is a descriptive summation for the role of the immune cells involved during pregnancy and their contribution to maternal recognition and tolerance towards the semi-allogeneic embryo in the bovine.

Endometrial Environment during Pregnancy Establishment

The maternal immune system actively supports a tolerogenic immune environment to permit trophoblast invasion, vasculature/tissue remodeling, embryo implantation/attachment, placentation, and ultimately, a successful pregnancy. Embryo survival during preimplantation development involves various mechanisms prior to establishment of pregnancy, including the suppression of luteolytic factors to maintain a functional CL by secreting IFNτ, maternal-fetal communication to modulate maternal immune tolerance of the semi-allogeneic embryo, and the onset of placentation (Spencer, 2015). After hatching from the zona pellucida (approximately day eight of development) the embryo develops into an ovoid and then tubular shape, and then continues to elongate. By day 12 of development, the embryo mediates maternal-fetal communication by secreting IFNτ for pregnancy recognition and maintenance of the CL. Around day 15-17 of gestation there is an increased expression of interferon stimulated genes (ISGs), including *ISG15*, *OASY1*, *MX1*, *RASD2*, and *MX2*. These ISGs act on uterine tissues in a paracrine manner to regulate uterine cellular functions and uterine vascular development that contribute to uterine receptivity of the embryo (Bazer et al., 2008, Bazer et al., 2009).

By day 19 the elongated conceptus is comprised of extraembryonic trophoblast cells lined with endoderm, at which time apposition and attachment of the conceptus to the luminal epithelial cells occurs and placentation can begin (Spencer et al., 2008; Spencer & Hansen, 2015; Valadao et al., 2018). Simultaneously, gastrulation occurs

and formation of the extraembryonic membranes, including the chorion, amnion and allantois is initiated (Spencer et al., 2008). Ruminants have an epitheliochorial type of placenta. During conceptus attachment the uterine epithelium is modified by invasion and fusion of trophoblast giant binucleate cells (BNC) that result in a semi-invasive type of placentation termed synepitheliochorial (Spencer, 2015). The BNC of the developing placenta release specific factors into the maternal circulation, including pregnancy associated glycoproteins (PAGs) (Klisch & Leiser, 2003). PAGs are inactive members of the aspartic proteinase family that have a yet unknown role in bovine pregnancy. Circulating PAGs are detectable from day 22 of pregnancy and can be employed as accurate measures of pregnancy establishment (Reese et al., 2018; Dalmaso de Melo et al., 2020; Filho et al., 2020). It has been suggested that PAGs may have a role in pregnancy maintenance and potentially placental function (Reese et al., 2019); however, more work is needed to identify PAG function. A comprehensive examination of the maternal-fetal interface, and the local and systemic immune system during the second and third trimester of gestation are lacking in the bovine.

T cells During Early Pregnancy

The maternal-fetal interface is modulated by conceptus signals that drive maternal immune responses (Ott et al., 2014). It has been proposed that this communication activates immune cells to secrete cytokines that stimulate placental growth, function, and embryo implantation (Fair, 2015). T cells are the major lymphocyte cell of the adaptive immune system that are thought to respond to paternal antigen present on the embryo and placenta. T cell populations, both cytotoxic ($CD8^+$) and helper ($CD4^+$), are present during early pregnancy recognition (Ott, 2019). Production of cytokines by T cells are essential to regulate immune responses, and specifically T

helper cells produce cytokines either in the Th1 or Th2 profile. The Th1 type immune response involves the secretion of pro-inflammatory interleukin (IL)-1β, IL-2, IL-15, IL-18, interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), whereas Th2 type immune responses involve anti-inflammatory IL-4, IL-5, IL-10, IL-13, and colony-stimulating factor 2 (CSF-2) (Sykes et al., 2012; Wilczyński, 2005; Fiorenza et al., 2021). During pregnancy, a cell-mediated immune response (Th1) of pro-inflammatory cytokines is reduced and an anti-inflammatory humoral response (Th2) is increased (Mansouri-Attia et al., 2012; Sykes et al., 2012; Ott, 2019;). A shift to a Th1 pro-inflammatory response during pregnancy can be detrimental to pregnancy success (Fiorenza et al., 2021), suggesting that uterine lymphocytes are key for the development of maternal immune tolerance and establishment of pregnancy.

In response to the semi-allogenic embryo another population of T cells, T regulatory (Treg) cells ($CD4^+CD25^+$), play an important role in pregnancy success. Treg cells effectively suppress inflammation in an antigen specific manner which is essential to modulate maternal immune tolerance toward the semi-allogeneic conceptus (Schjenken & Robertson, 2020). Treg cells express the transcription factor forehead box P3 (FOXP3) and are activated by antigen presenting cells (APCs) in a cytokine environment [IL-2, IL-5, and transforming growth factor β (TGFβ)] required to differentiate naïve $CD4^+$ to Treg cells (Robertson et al., 2009). In ruminants the role played by Treg cells during embryo implantation remains unclear, although, studies in mice show that depletion of Treg cells increases alloreactive T cell proliferation and prevents embryo implantation (Aluvihare et al., 2004; Heitmann et al., 2017). In addition, Treg cells have the capacity to secrete IL-4 to inhibit the activation of $CD8^+$

cells that are detrimental to the success of the semi-allogenic embryo (Mjösberg et al., 2007). It can be summarized that Treg cells are required to regulate the maternal immune response toward the semi-allogenic embryo in the bovine, but this requires further investigation.

Natural Killers During Early Pregnancy

Natural killer cells are highly sensitive to major histocompatibility complex (MHC) class I proteins, which is present on the surface of trophoblast cells. This suggests that NK cells are a continuous threat to the developing embryo that has the potential to express non-self-antigen via MHC class I. Various studies have aimed to investigate the dynamic regulation of NK cells and their control of maternal immune tolerance to facilitate placenta formation (Ott, 2019). In humans, NK cell killing is controlled by macrophages present in uterine tissues, whereby macrophages secrete TGFβ that inhibits the capacity of NK cells to specifically kill placental cells (Co et al., 2013; Vasudevan et al., 2017). In the bovine, endometrial NK ($NKp46^+$ marker) and $CD8^+$ T cells are present in the endometrium during early pregnancy, as are tolerogenic factors (CD274, LAG3, CTLA-4 and IL-10) known to control inflammation and induce T cell anergy and activation of tolerogenic macrophages. This finding suggests that, in the bovine, NK cell function may be suppressed by the maternal immune system to facilitate tolerance of the conceptus (Vasudevan et al., 2017).

Macrophages and Dendritic Cells During in Early Pregnancy

For pregnancy recognition and pregnancy establishment, APCs contribute to the development of peripheral tolerance to the semi-allogeneic conceptus. During embryo attachment and placental development, macrophages and DCs are the most common APCs within the uterine environment (Nagamatsu & Schust, 2010). Furthermore, when

the semi-allogenic embryo interacts with endometrial epithelial cells, this interaction elicit secretion of cytokines and chemokines to recruit and activate macrophages and DCs (Nagamatsu & Schust, 2010). Endometrial macrophages control trophoblast invasion and induce immune tolerance by secreting TGFβ to modulate NK cell function. Additionally, macrophage activation causes secretion of cytokines and signaling molecules that contribute to uterine vascular remodeling, removal of apoptotic cells, and immunity against potential pathogens (Nagamatsu & Schust, 2010; Co et al., 2013; Schjenken & Robertson, 2020). In humans, decidual macrophages exhibit unique transcripts related to tissue remodeling, cell proliferation and metabolism (Gustafsson et al., 2008). Likewise, macrophages participates in vascular/tissue remodeling due to their ability of secretes vascular endothelial growth factor (VEGF) and promote trophoblast function by clearing apoptotic cells (Li et al., 2009).

An appropriate cytokine environment is required to induce DCs to mature; the presence of TGFβ, IL-10, CSF-2, and IL-4 give DCs the ability to signal and recruit Treg cells required for pregnancy success (Robertson et al., 2009). Macrophages and DCs are essential in regulating the balance between protective immunity and maternal immune tolerance toward the semi-allogeneic conceptus. This knowledge may help us better understand pregnancy progression, pregnancy success and their effect on postnatal phenotype of subsequent offspring.

Fetal Programing and the Developmental Origins of Health and Disease Hypothesis

Extensive studies in animals show how environmental factors experienced during gamete maturation, embryo development and fetal growth can affect subsequent offspring phenotype. This process is referred to as the Developmental Origins of Health

and Disease (DOHaD) hypothesis, stemming from the fetal programing hypothesis (Barker, 1998; Barker et al., 2007; Velazquez et al., 2019). This theory states that the origins of lifestyle-related disease is formed at the time of fertilization, embryonic, fetal, and neonatal development by the interaction between genes and environmental factors such as nutrition, ageing, diet or chemicals (Barker, 1995, 1998). This theory was established by the British epidemiologist David J. Barker and termed the “Barker hypothesis”, which suggests that deficient nutrition *in utero* programs the fetus to develop metabolic disorders and chronic diseases during later life (Barker, 1998). Barker’s studies demonstrate a correlation between maternal undernutrition during pregnancy, subsequent birth weight of offspring and the risk of cardiovascular diseases in adulthood (Barker 1995,1998; Arima & Fukuoka, 2020). Barker’s hypothesis awakened a vast interest in the area for fetal programing, and numerous studies now show that quality and quantity of maternal nutrition during gestation influence fetal development and impact long term cardiovascular and metabolic health of subsequent offspring (Fleming et al., 2018).

This revolutionary concept of fetal programing reframed studies to focus on the vulnerability of the uterine environment during conception and embryonic life to environmental factors. Studies in animal models demonstrated potential mechanisms for fetal programing that encompass physiological, cellular, molecular and epigenetic modifications caused by gestational exposures (Velazquez et al., 2019). Fetal programing mechanism likely involve modifications to the fetal epigenome that are impacted by the endometrial environment, maternal nutrient supply, and placenta metabolism, that may cause developmental adaptations in the fetus to alter cellular

function, organ development, endocrine function and metabolic activity that can have long-term consequence to offspring after birth (Fleming et al., 2018; Kwon & Kim, 2017; Limesand, 2019).

The placenta represents the major interface of maternal-fetal nutritional and oxygen exchange. It provides sources of hormonal endocrine signals and its optimal development likely plays a role in fetal programing by influencing fetal epigenome changes (Kwon & Kim, 2017). Studies in humans and animal models show that effects on offspring during the periconceptional period may be caused by maternal and paternal environmental exposures to altered nutritional status, age, and environmental pollutants (Morgan & Watkins, 2020). In recent years, a new focus of fetal programing has emerged to study the potential role of paternal contributions to embryo development, fetal growth, and subsequent programing of phenotype of offspring.

Effect of Mating on the Periconceptional Endometrial Environment

Previously, we discussed the endometrial environment of early pregnancy, whereas here we will discuss the role of mating on facilitating changes to the maternal reproductive tract environment. Depending on the species, the composition and volume of ejaculate produced by the male differs significantly. The main purpose of the ejaculate is to deliver spermatozoa into the female tract, whether directly into the uterus or the vagina. Copulation results in the deposition of semen containing billions of spermatozoa into the female reproductive tract facilitated by seminal plasma. In general, semen components can interact with cervical and uterine tissues that induce complex cellular and molecular changes in the female that influence conception, embryo development and potentially the future health of the offspring (Bromfield, 2014; Schjenken & Robertson, 2020). These complex cellular and molecular changes that

occur in the female tract after semen deposition include a transient post-mating inflammatory response which is partially induced by sperm (cellular fraction) and seminal plasma (acellular plasma fraction) (Schuberth et al., 2008). Semen itself is a foreign material to the female, containing paternal antigen and potential pathogens, and as such a maternal immune response to semen aims to eliminate foreign material while maintaining sperm viability required for fertilization (Schuberth et al., 2008).

Using the golden hamster, Yanagimachi and Chang (1963) described the influence of seminal plasma on mediating female post-mating inflammation. Female hamsters were mated either to an intact male or left unmated and leukocyte extravasation into the uterine lumen was evaluated at two or twelve hours after mating. Two hours after mating the authors observed an engorged uterus containing sperm but few leukocytes; however, twelve hours after mating a vast number of leukocytes that had engulfed sperm cells were present in the uterus. The authors posited at the time "One also wonders whether there are other functions of leucocytes in the uterus, besides elimination of bacteria and spermatozoa." (Yanagimachi & Chang, 1963). This question remains today and has sparked an investigational field into the role of the post-mating inflammatory response.

Studies in various species are investigating whether post-mating inflammation in female tissues is related to the immunological modulation necessary to support pregnancy and increase pregnancy success. Recent data in mice, using endometrial tissue from females mated to intact or vasectomized males (exposed to seminal plasma only), revealed a total of 110 differentially expressed genes in maternal tissues exposed to whole semen compared to seminal plasma alone (Schjenken et al., 2021). The IL-6,

IL-8 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways were increased in endometrial tissues of females mated to intact males compared to vasectomized males. Moreover, the expansion of Treg cells in the draining lymph nodes of the uterus was increased in females mated to intact males compared to those mated with vasectomized males. These data demonstrate that the whole ejaculate including sperm and seminal plasma is required to elicit post-mating inflammation and potentially promote maternal immune tolerance (Schjenken et al., 2021).

The Physiological Role of Seminal Plasma in Pregnancy

The Molecular Composition of Seminal Plasma

Seminal plasma is commonly known as a vehicle and survival medium to transport sperm cells to the site of fertilization, but recent evidence highlights the importance of seminal plasma in promoting pregnancy success in some mammals (Robertson, 2005; Robertson & Sharkey, 2016). In mammals, seminal plasma is secreted from the testes, epididymis, seminal vesicles, prostate, bulbourethral glands, and ampulla (Moura et al., 2006). Seminal plasma contributes around 90% of the total semen volume. It is a complex mix of sugars (energy substrate of sperm cells), antioxidants (to prevent sperm DNA damage), buffers, and an array of proteins of unknown function (Juyena & Stelletta, 2012; Bromfield, 2016; Ahmadi et al., 2022). Additionally, seminal plasma components can act as signaling agents to drive inflammation in the female reproductive tract, including estrogen, testosterone, prostaglandins, cytokines and chemokines, mostly secreted by the seminal vesicles (Robertson, 2005; Juyena & Stelletta, 2012; Ahmadi et al., 2022). Studies in different species have shown that the role for seminal plasma extends beyond sperm transport to

also modulate the cellular and molecular maternal environment at the time of conception, altering embryo development and programing the phenotype of the offspring (O'Leary et al., 2006; Bromfield et al., 2014; Ortiz et al., 2019; Morgan & Watkins, 2020; Schjenken & Robertson, 2020).

The components of seminal plasma described in this literature review are a compilation from different species including human, rodent, swine, and bovine. Seminal plasma is rich in proinflammatory cytokines such IL-1β, IL-6, IL-8, IL-10, IL-12, TNFα, IFNγ, and TGFβ (Robertson et al., 1996; Robertson, 2007). Many of these cytokines have been demonstrated to play crucial roles in embryo development and pregnancy outcomes and drive the maternal expression of other various cytokines in the endometrium including CSF-2. Maternal secretion of CSF-2 from uterine epithelium, acts in a paracrine manner to target the preimplantation embryo to support blastocyst formation and increase the number of viable blastomere by inhibiting apoptosis, assisting in glucose uptake, which likely facilitate the altered postnatal phenotype observed in subsequent offspring (Robertson et al., 2001; Robertson, 2005; Siqueira et al., 2017). In the bovine, CSF-2 promotes embryo survival and developmental competence, impacting number of blastomeres and regulating *de novo* methylation of genes that modify epigenetic reprograming (Loureiro et al., 2009; Siqueira et al., 2017).

Other embryokines secreted by maternal tissues due to semen exposure that affect embryo development include IL-6 and LIF which mediate embryonic cell development and maintenance by regulating the STAT-3 anti-apoptotic pathway. Supplementation of bovine embryos with IL-6 improves embryo competence and increases then number of inner cell mass (Wooldridge & Ealy, 2019; Seekford et al.,

2021). The growth factor LIF is part of the IL-6 family and plays an important role in uterine receptivity, embryo implantation, and placenta formation (Salleh & Giribabu, 2014). In mice, exposure of blastocyst to LIF significantly improved implantation and pregnancy rate (Mitchell et al., 2002). These observations suggest that exposure to embryokines either *in vivo* after semen exposure or *in vitro* using supplementation can alter embryo development and potentially modulate fetal programming.

In rodents and humans TGFβ has been identified as the major component of seminal plasma that triggers post-mating inflammation in female tissues and drives the development of Treg cells important in mediating maternal immune tolerance of paternal antigen (Tremellen et al., 1998; Robertson et al., 2009). The TGFβ signaling pathway is activated by phosphorylation and nuclear translocation of SMAD proteins that regulate transcription of cytokines and modulates cell function, apoptosis, immune regulation, and angiogenesis (Derynck & Zhang, 2003; Fleisch et al., 2006). Exposure to TGFβ is important in developing tolerogenic DCs and the induction of Treg cells by converting naïve $CD4^+CD25^-$ T cells into regulatory $CD4^+CD25^+$ Treg cells using the FOXP3 transcription factor (Mjösberg et al., 2007). In mice, TGFβ is primarily produced in the seminal vesicles in three major isoforms; TGFβ-1 (55% of seminal plasma TGFβ), TGFβ-2 (1% of seminal plasma TGFβ), and TGFβ-3 (44% of seminal plasma TGFβ). Nearly all seminal plasma TGFβ is secreted in a latent state and is activated in the female tract by plasmin, low pH, or interactions with trombospodin-1 or αvβ6 integrins (Tremellen et al., 1998; Robertson, 2007; Schjenken & Robertson, 2020).

Concentrations of TGFβ in seminal plasma differs between species; human seminal plasma contains 200 ng/mL of TGFβ-1, 5 ng/mL for TGFβ-2, and 170 ng/mL for

TGFβ-3; in mice seminal plasma contains 74 ng/mL of TGFβ-1; in swine seminal plasma contains 150 ng/mL of TGFβ-1; and in bovines seminal plasma contains 63 ng/mL of TGFβ-1 and 58 ng/mL of TGFβ-2 (Sharkey et al., 2012; Robertson et al., 2002; Rizo et al., 2019; Schjenken & Robertson, 2020). In mice and humans, seminal plasma TGFβ has been identified as be the principal signaling agent responsible for inducing secretion of CSF-2 from female tract tissues (Tremellen et al., 1998). Exposure of uterine epithelial cells to TGFβ *in vitro* increases expression of cytokines including CSF-2, IL-1α, IL-6, and TNFα, suggesting a role for seminal plasma TGFβ in post-mating inflammation, tissue remodeling and angiogenesis (Tremellen et al., 1998; Schjenken et al., 2015).

Prostaglandins in seminal plasma are key mediators of inducing and reducing inflammation in female tract tissues after mating. Prostaglandin E2 is the predominant prostaglandin detected in seminal plasma, and contributes to maternal immune tolerance by inducing DCs to differentiation into tolerogenic cells, and by inducing Th2 responses in immune cells known to be beneficial to pregnancy success by upregulating IL-10 and downregulating TNFα (Bromfield et al., 2018; Schjenken & Robertson, 2020).

Soluble CD38 produced by the seminal vesicles in mice and humans, is a potent immune regulatory factor that promotes recruitment of tolerogenic DCs and $FOXP3^{+}$ Treg cells required to facilitate maternal immune tolerance (Kim et al., 2015). Other factors produced by uterine epithelial cells in response to seminal plasma are involved in early embryo growth and implantation, including VEGFA, epidermal growth factor (EGF), and fibroblast growth factor (FGF) (Schjenken & Robertson, 2020).

Recent studies suggest that endogenous ligands of Toll-like receptor 4 (TLR4) found in seminal plasma are responsible for post-mating inflammation in female tissues (Schjenken et al., 2015). In mice, seminal plasma induced endometrial cytokine expression of *CFS3*, *CXCL1*, *CXCL2*, *IL1A*, *IL6*, *LIF* and *TNF* via the TLR4 pathway (Schjenken et al., 2015). While TRL4 signaling by seminal plasma may contribute to the post-mating inflammation in female tissues, further studies need to confirm this signaling mechanism in other species. For example, TLR4 has been identified in the endometrial cells of the cows, but there are currently no studies to demonstrate a functional role in post-mating inflammation in response to seminal plasma (Herath et al., 2006; Soboll et al., 2006).

The Role of Seminal Plasma in Maternal Immune Response

In rodents, pigs, and humans when seminal plasma interacts with cervical and/or uterine epithelial cells, it elicits a post-mating inflammatory response by inducing expression of cytokine and chemokines that attract and promote leukocyte activation, driving them into the uterine lumen (Robertson, 2007; Katila, 2012; Schjenken & Robertson, 2020). Among these cytokines and chemokines secreted by cervical and uterine epithelial cells are CFS-2, IL-6, macrophage chemotactic protein-1, and IL-8. This surge of proinflammatory cytokines causes recruitment of leukocytes into female tract tissues (neutrophils, macrophages, and dendritic cells) (Robertson et al., 1996; Robertson, 2005; Robertson et al., 1998; Robertson, 2007). Extravasation of leukocytes into female tract tissues after semen deposition acts to clear superfluous sperm and pathogens introduced during copulation (Schjenken & Robertson, 2020). Neutrophils that phagocytize paternal antigens are the first leukocytes to infiltrate female tract tissues in response to seminal plasma. Initial neutrophil recruitment into female tract

tissues is short-lived, and is resolved before embryo implantation (Robertson et al., 1996; Robertson, 2007; Song et al., 2016).

Monocytes and DCs from peripheral circulation infiltrate into the endometrium and cervix within 24 h of insemination (Katila, 2012). In pigs, an intrauterine infusion of seminal plasma at the onset of ovulation increased the abundance of leukocytes ($CD45^+$ leukocytes; $HB142^+$ macrophages; $MHCII^+$ macrophages/dendritic cells) causing inflammation in the endometrium 34 h after infusion which was sustained above control levels until nine days post infusion (O'Leary et al., 2004). Recruitment of macrophages and DCs, thought to contribute to tissue remodeling and embryo implantation, is the result of secretion of cytokines and chemokines by endometrial epithelial cells after exposure to seminal plasma (Ahmadi et al., 2022; Schjenken & Robertson, 2020). Macrophages have the potential to regulate angiogenesis, altering the local extracellular matrix, migration and proliferation of endothelial cells, and formation of capillaries (Ramirez-Pedraza & Fernández, 2019). Activation of endometrial macrophages by seminal plasma TGFβ causes secretion of VEGF that contributes to uterine vascular/tissue remodeling required for embryo implantation (Chow et al., 2003). In pigs, exposure to seminal plasma also enhances the recruitment of macrophages and DCs into ovarian tissues to increase the size of the CL and promote progesterone synthesis (O'Leary et al., 2006). These functions of seminal plasma modulating the maternal endometrial environment imply a role for seminal plasma in optimizing the endometrial environment during early pregnancy.

The Role of Seminal Plasma in Early Pregnancy

Implantation is a key event for pregnancy success where the fate of the embryo is dependent on the maternal endometrium. Seminal plasma promotes the activation

and expansion of Treg cells which play a critical role in pregnancy by protecting the semi-allogenic embryo from maternal immune attack (Guerin et al., 2011; Schjenken & Robertson, 2020). As mentioned above, seminal plasma contributes to the recruitment of macrophages and DCs into female tract tissues that can carry paternal antigen to the draining lymph nodes and induce the expansion of Treg cells required for maternal tolerance (Robertson, 2005; Bromfield, 2016; Schjenken & Robertson, 2020). Moreover in mice, exposure to seminal plasma results in hypertrophy of uterine draining lymph nodes, and an increased uterine $FOXP3^{+}$ Treg cell population (Johansson et al., 2004; Guerin et al., 2011). Importantly, Treg cells are key players for embryo implantation due to their immune regulatory, anti-inflammatory and vascular remodeling properties required for maternal tolerance toward the preimplantation embryo (Guerin et al., 2011). These data suggests that seminal plasma exposure at the time of mating enhances the recruitment of immune cells and regulates cytokine expression in uterine tissues that actively contribute to early pregnancy success.

The Role of Seminal Plasma on Embryo Development

Early embryo development from the zygote to blastocyst stage is a critical time as conditions perceived during the peri-conceptual period can permanently alter the developmental program of the embryo and affect fetal development and the phenotype of offspring. The peri-conceptual environment is influenced by secretion of maternal cytokines into the luminal space of the female reproductive tract that bath the developing embryo. As such it is essential to have an optimal balance of embryotrophic (anti-apoptotic) and embryotoxic (apoptotic) factors being produced by the female tract tissues during the peri-conceptual window of development (Schjenken & Robertson, 2020). The early embryo expresses a number of cytokine receptors that can exert

action through one or more downstream pathways, including the phosphatidylinositol-3 kinases-protein kinase B (PI3K-AKT), Janus kinase/signal transducer and activator of transcription (JAK/STAT), and mitogen-activated protein kinase (MAPK) pathways (Robertson et al., 2015; Sang et al., 2020). Exposure of embryos to signaling molecules, and activation of these pathways can effect gene expression, metabolism, epigenome, and cell stress response in embryos, thus affecting embryo fate (Robertson et al., 2015; Schjenken & Robertson, 2020).

Cytokines and growth factors secreted by maternal tract tissues that support the development of the preimplantation embryo is often referred to as embryokines. Maternal expression of various embryokines can be influenced by exposure to seminal plasma, including *CSF1*, *CSF2*, *CSF3*, *IL6*, *VEGF*, and *LIF* (O'Leary et al., 2004; Bromfield, 2014; Bromfield et al., 2014). In mice, exposure to seminal plasma increases expression of oviductal *CSF2*, *LIF*, and *IL6* that contributed to increased embryo survival and improved developmental competence (Bromfield et al., 2014). Conversely, exposure to seminal plasma suppressed the expression of oviductal *TRAIL*, known to cause apoptosis in embryos (Bromfield et al., 2014). As a result, seminal plasma deprivation at the time of conception increased the number of arrested embryos recovered from mice and increased the incidence of embryo abnormalities (Bromfield et al., 2014). Intrauterine infusion of seminal plasma at the time of insemination in pigs increases the expression of endometrial *CSF2*, *IL6*, *PTGS2* and *MCP1*, and also increased in the total number of viable embryos recovered 34 h after treatment (O'Leary et al., 2004).

Recent work in bovines, demonstrated that seminal plasma increased endometrial epithelial cell expression of *CSF2*, *CXCL8*, *TGFB1*, *PTGS2* and *AKR1C4* (Ibrahim et al., 2019). In cattle, the transfer of in-vitro produced blastocysts into recipients mated to vasectomized bulls increased conceptus size at day 14 compared to recipients that were not mated (Mateo-Otero et al., 2020). This is the only evidence in cattle that has evaluated the effects of seminal plasma on embryo development, suggesting that the changes observed in embryo size are due to modulation of the female tract environment by seminal plasma. There is increasing evidence that indicate seminal plasma exposure increases endometrial or oviduct expression of embryokines that support preimplantation embryo development and survival.

The Role of Seminal Plasma in Offspring Development

In humans, rodents, bovine and other domestic species, the use of artificial insemination or embryo transfer demonstrates that seminal plasma is not required for pregnancy; however, there is emerging evidence that suggests an important role for seminal plasma in optimizing embryo development, fetal growth, and subsequent offspring performance (Bromfield et al., 2014; Watkins et al., 2018; Ortiz et al., 2019). Bromfield et al. (2014) demonstrated that deprivation of seminal plasma at the time of conception using seminal vesicle deficient males altered embryo development, placental formation, litter size and adult offspring metabolic health. Additionally, the authors examined the programing effects of seminal plasma exposure using embryo transfer to females primed with seminal plasma. Resultant offspring from the transfer of 2-cell embryos, but not blastocysts, into recipients that had never been exposed to seminal plasma experienced excess accumulation of adipose tissue in adulthood (Bromfield et al., 2014). Another study using mice, showed that the programing effects

of maternal seminal plasma exposure can be altered by paternal nutrition. Specifically, paternal low protein diet caused offspring to be heavier and present with glucose intolerance, altered gut bacterial profiles and disturbed hepatic function (Watkins et al., 2018). In cattle, a recent study evaluated the effects of seminal plasma exposure at the time of insemination on fertility and pregnancy outcomes (Ortiz et al., 2019). Results describe no effect of seminal plasma supplementation on fertility in agreeance with previous studies (Odhiambo et al., 2009); but interestingly exposure to seminal plasma at conception increased birth weight of heifer calves sired by X-sorted semen in primiparous cows (Ortiz et al., 2019). These observations suggest that paternal-maternal communication mediated by seminal plasma has the potential to modulate pregnancy establishment, progression, and impact phenotype of subsequent offspring.

The Role of Seminal plasma in Bovine Reproduction

The use of artificial insemination has become the primary assisted reproductive technology employed in the dairy industry, with over 66% of dairy cows bred by artificial insemination and 85% of registered dairy cows as the result of AI (Hall, 2019). Artificial insemination allows the producer to select from high genetic merit sires and increase the profitability of the farm; however, during semen processing for artificial insemination, seminal plasma is often diluted or removed from the semen. The success of artificial insemination indicates that seminal plasma is not required for establishment of pregnancy. But could seminal plasma have a physiological role in improving pregnancy outcomes in cattle?

The role of seminal plasma in eliciting an inflammatory response in the bovine is less well characterized than in other species. During natural mating in the bovine, semen is deposited in the fornix vagina. If seminal plasma reaches the uterine tissues

remains unclear, but is known that sperm are coated in seminal plasma proteins that are carried through the cervix and may act indirectly on tissues of the upper maternal tract (Gwathmey et al., 2006; Alghamdi et al., 2010). However, a leukocytosis response in the uterus has been shown to occur in young cattle 16 to 21 hours after semen deposition in the vagina (Howe & Black, 1963). More recently it has been shown that seminal plasma increases neutrophil binding of sperm, a function that may be associated with sperm selection or pathogen clearance after copulation (Alghamdi et al., 2009). Interestingly, endometrial tissues from cows mated with either intact bulls or vasectomized bulls revealed that only exposure to whole semen could alter the endometrial transcriptome compared to cows that were not mated. A total of 24 differentially expressed genes were identified in the endometrium of cows mated to intact bulls compared control cows, while mating with vasectomized bulls failed to elicit any change in the endometrial transcriptome (Recuero et al., 2020). Perhaps the changes observed in the endometrial environment in mice and pigs are reminiscent of intrauterine ejaculators where large volumes of seminal plasma can interact with endometrial tissues, whereas intravaginal ejaculators only a small volume (if any) interact directly with the endometrium and as such either whole semen or sperm cells coated with seminal plasma are required to elicit an endometrial response.

Odhiambo et al., (2009) investigated the role of seminal plasma in modulating the endometrial environment to facilitate pregnancy establishment. The authors observed that seminal plasma exposure at the time of AI did not improved pregnancy outcomes. Similarly Ortiz et al. (2019) evaluated the effects of intrauterine infusion of seminal plasma at the time of artificial insemination to assess fertility and postnatal outcomes of

calves. For this study seminal plasma was collected from 33 bulls, pooled, and loaded into straws to be applied intrauterine at the time of insemination. Results show that there was no difference in pregnancy rates between the seminal plasma infused (44.8%) or control group (44.7%). Interestingly, when primiparous cows were inseminated using X-sorted semen and infused with seminal plasma, the resultant heifer calves were heavier at birth when compared to the control group. Further studies are required to elucidate the physiological role of seminal plasma in optimizing the endometrial environment of the preimplantation embryo, fetal development, and the productive performance of offspring in cattle.

Thesis

The evidence presented above from mice, pigs, and humans, demonstrates that seminal plasma can positively influence maternal tissues to improve pregnancy outcomes; however, in the bovine the role of seminal plasma in pregnancy success remains unclear. A small body of evidence describes a role for seminal plasma in modulating maternal tissues and pregnancy in the bovine, by increasing endometrial cytokine expression and increasing heifer birth weight (Ibrahim et al., 2019; Ortiz et al., 2019). Therefore, I proposed to investigate the role of maternal exposure to seminal plasma on pregnancy outcomes in cattle. I hypothesized that exposure to seminal plasma at conception would alter the maternal tract environment, program early embryo development, and alter subsequent fetal growth in the cow. The aims of this thesis are to 1) evaluate the effect of intrauterine infusion of seminal plasma at insemination on embryo quality, 2) determine the impact of intrauterine infusion of seminal plasma on the endometrial transcriptome seven days later at the time the embryo would enter the

uterus, and 3) assess the effect of intrauterine infusion of seminal plasma at insemination on early fetal development. The experiments to address this hypothesis and associated aims are described in Chapter 2. These studies will contribute to our collective understanding for the role of seminal plasma in bovine reproduction and its potential to program the phenotype of subsequent offspring.

CHAPTER 2
EFFECTS OF SEMINAL PLASMA ON THE ENDOMETRIAL TRANSCRIPTOME AND EARLY FETAL DEVELOPMENT

Introduction

Seminal plasma, the fluid component of semen is a complex mixture of sugars, ions and biologically active signaling molecules. While seminal plasma is not required to establish or maintain a pregnancy, the question remains, why does the male invest in producing a complex biological fluid if its only role is to serve as a transport medium for sperm at the time of ejaculation? It has been proposed in rodents, swine and human that seminal plasma regulates the endometrial environment to optimize the maternal endometrium for pregnancy establishment and success (Robertson, 2007; Bromfield et al., 2018; Schjenken & Robertson, 2020). In rodents and swine, seminal plasma modulates the cellular and molecular environment of maternal tract tissues at the time of conception and improves early embryo development (O'Leary et al., 2004; Bromfield et al., 2014; Robertson & Sharkey, 2016).

Seminal plasma is commonly known to serve as a survival medium to transport sperm cells through the male and then female reproductive tract. Studies in several species show that seminal plasma elicits a localized inflammatory response in maternal tract tissues by inducing the expression of cytokines and chemokines in endometrial cells that promote cellular inflammation (O'Leary et al., 2004; Robertson, 2007; Katila, 2012; Robertson & Sharkey, 2016; Schjenken & Robertson, 2020). Seminal plasma increases the endometrial expression of several cytokines and embryokines including *CSF1*, *CSF2*, *CSF3*, *IL6*, *LIF*, *CXCL8*, *VEGF*, *MCP1*, *PTGS2* and *TNF* (Robertson et al., 1996; O'Leary et al., 2004; Robertson, 2007; Bromfield et al., 2014). These factors have the potential to influence a range of cellular events during embryo development

that can optimize pregnancy outcomes (O'Leary et al., 2004; Salmassi et al., 2005; Robertson, 2007; Loureiro et al., 2009; Katila, 2012; Robertson et al., 2015; Siqueira et al., 2017).

The success of assisted reproductive techniques such as artificial insemination, *in vitro* fertilization and embryo transfer affirms that seminal plasma is not required to achieve pregnancy in many species. In mice, deprivation of seminal plasma during mating causes a reduction in embryo competence, poor placental development, and subsequent development obese offspring with metabolic disorders (Bromfield et al., 2014). Moreover in pigs, an intrauterine infusion of seminal plasma at the time of insemination improved the development of viable embryos, and progesterone synthesis (O'Leary et al., 2004). Interestingly, in cattle an intrauterine infusion of seminal plasma at the time of insemination increased the birth weight of the resultant heifer calves (Ortiz et al., 2019). These improved pregnancy outcomes may be in part due to the ability of seminal plasma to mediate molecular changes in endometrial cells, increasing the expression of *CSF2*, *CXCL8*, *TGFB1*, *PTGS2* and *AKR1C4* that regulate endometrial function and embryo development (Salmassi et al., 2005; Loureiro et al., 2009; Robertson et al., 2011; Bromfield et al., 2018; Ibrahim et al., 2019; Rizo et al., 2019).

Experimentation in animals and epidemiological studies in humans show that maternal exposure to environmental factors during gamete maturation, embryo development and fetal growth can affect the phenotype of subsequent offspring (Bromfield et al., 2014; Watkins et al., 2018). This concept was first established by Dr. David J. Barker, who postulated that deficient maternal nutrition during pregnancy could program the fetus to develop metabolic disorders and chronic disease during adult life

(Barker, 1998). The revolutionary concept of fetal programing reframed studies to focus on the vulnerability of the uterine environment to environmental factors that could impact conception and early embryonic life. More recently, paternal influences on fetal programing have been investigated to evaluate the contribution of the sire to embryo development, fetal growth, and postnatal phenotype of offspring beyond genetic contributions (Bromfield et al., 2014; Velazquez et al., 2019; Morgan & Watkins, 2020; Schjenken & Robertson, 2020;).

The role of seminal plasma in promoting pregnancy outcomes in the bovine are unclear. The present experiments were performed to evaluate the effects of seminal plasma exposure at insemination on the endometrial environment, embryo development and fetal growth. I hypothesize that exposure to seminal plasma at conception will alter the maternal tract environment and programs early embryo development and subsequent fetal growth in the cow. These studies aim to provide a better understanding for the role of seminal plasma at insemination in optimizing pregnancy outcomes and modulating the health and productive performance of subsequent offspring.

Materials and Methods

Procedures involving cows were approved by the University of Florida Institutional Animal Care and Use Committee (protocol number 202111336) and the methods used to process samples followed applicable regulations and standard procedures. All reagents were obtained from Thermo Fisher Scientific (Pittsburgh, PA) unless otherwise stated. These experiments were conducted from April 2021 to April 2022, using the facilities at the University of Florida Dairy Research Unit.

Seminal Plasma Collection

Seminal plasma was collected on a single day from 44 Holstein bulls (age between 1.3 to 7.9 years) using an artificial vagina at Select Sires Inc (Plain City, OH). Ejaculates were processed according to Certified Semen Services (https://www.naab-css.org/uploads/userfiles/files/CSSMinReq-Jan2014201607-ENG.pdf). Whole ejaculates were pooled and combined with antibiotics (Spectinomycin, gentamicin, lincomycin and tylosin; Shin et al., 1988) before centrifugation (1,500 × *g* for 15 min) to remove cellular material. Subsequent seminal plasma was filtered using a 3 µm plated filter and immediately packaged into 0.5 mL straws and stored in liquid nitrogen. As a negative control, saline solution [0.9 % (wt/vol) NaCl] was packaged into 0.5 mL straws and stored in liquid nitrogen.

Study 1 – To Evaluate the Effect of Seminal Plasma at Insemination on Embryo Quality and Endometrial Transcriptome

Experimental Design

To evaluate the effect of seminal plasma at insemination on embryo quality and endometrial transcriptome, 17 multiparous, non-lactating Holsteins cows (aged 2 to 7 years old) were used. Cows were dried off with a final milking and an intramammary treatment of ceftiofur hydrochloride (Spectramast DC, Zoetis, Parsippany-Troy Hills, NJ) followed by a teat sealant of bismuth subnitrate (Orbeseal DC, Zoetis). Consequently, cows were moved to pasture, fed a total mixed ration and given free access to clean drinking water.

Cows were subjected to estrous synchronization with ovarian stimulation (see below) on the day of timed artificial insemination (TAI), donors were randomly assigned to receive one of two treatments, an intrauterine infusion of either pooled seminal

plasma or saline. A total of three replicates were performed. To ensure the absence of seminal plasma in the semen, all cows were bred using X-sorted semen from a single sire to avoid confounding effects of embryo sex (Hansen et al., 2016). Seven days after TAI, embryos were recovered by non-surgical flushing and the endometrium was sampled by cytobrush.

Estrous Synchronization, Artificial Insemination, and Intrauterine Infusion of Treatments

A modified double ovsynch protocol was used for estrous synchronization to avoid the use of an intravaginal CIDR. Simultaneous ovarian stimulation was performed using porcine pituitary-derived follicle stimulating hormone (Folltropin; Vetoquinol, Magny-Vernois, France). Estrous synchronization was initiated with a 25 mg injection of prostaglandin (PG) $F_{2\alpha}$ (dinoprost tromethamine; Lutalyse, Zoetis), followed by 100 µg of GnRH (gonadorelin; Cystorelin, Boerhringer Ingelheim, Ingelheim am Rhein, Germany) four days later. Five days after the initial GnRH, two injections of $PGF_{2\alpha}$ were administered 24 h apart, followed by a second GnRH injection two days later. Six days after the last GnRH a third injection of GnRH was administered. Two days after the final GnRH injection, ovarian stimulation began with an initial injection of 60 mg of Folltropin. Folltropin was administered every 12 h for 4 d in decreasing doses: 60 mg, 60 mg, 60 mg, 50 mg, 50 mg, 50 mg, 40 mg, and 40mg. At the time of the seventh Folltropin injection, all cows received a single 25 mg injection of $PGF_{2\alpha}$ followed by a second injection 12 h later with the last dose of Folltropin. Forty-eight h after the final $PGF_{2\alpha}$ and FSH injection, an estrus detection patch (Estrotect; Rockway Inc., Spring Valley, WI) was fixed to the tail head followed by a final injection of GnRH to induce ovulation. Insemination was performed 16 h after the last GnRH injection using two 0.25 mL

straws of X-sorted semen from sire Applause 029HO19052 (ABS Global, DeForest, WI). Cows were randomized using Microsoft Excel (Redmond, WA) and received 0.5 mL of either saline (*n* = 13) or pooled seminal plasma (*n* = 13) immediately following insemination. Saline straws were supplemented with 10 µL of antibiotic cocktail (#13504/9000; Minitube USA, Verona, WI) immediately prior to insemination to match the antibiotic concentration in seminal plasma straws. Cows were inseminated with an additional two 0.25 mL straws of X-sorted semen 10 h after initial insemination. The time at which insemination was completed and the status of the heat detection patch was recorded.

Embryo Collection, Embryo Morphological Evaluation, and Endometrial Sampling

In vivo produced embryos were recovered seven days after TAI. On the day of collection, cows received epidural anesthesia (Lidocaine Injectable; 80 mg lidocaine hydrochloride, Aspen Veterinary Resources, Liberty, Missouri) between the second and third coccygeal vertebrae. External genitalia were cleaned with 1% (w/v) chlorhexidine solution (2% chlorhexidine gluconate, Aspen Veterinary Resources, Liberty, MO), followed by 70% (v/v) ethanol, and dried with surgical gauze. Embryos were recovered using a Foley catheter (Vortech silicone catheter 23” long 18FR30CC balloon, Agtech Inc, Manhattan, Kansas) employing standard non-surgical uterine horn flushing using commercial embryo collection medium (BioLife™ Advantage Embryo Collection Medium, Agtech). Collected uterine flushings were filtered and evaluated for the number and morphological stage of any embryos recovered using a standard stereomicroscope (Bó & Mapletoft, 2013). Collected objects were recorded as morula, blastocyst (including early blastocyst, blastocyst, and expanded blastocyst), arrested (any embryo

structure prior to morula stage), degenerate (dead or non-viable embryos), or unfertilized oocytes. Blastocyst stage embryos from each donor were pooled, snap frozen in liquid nitrogen and stored at -80°C.

Immediately after uterine flushing an endometrial cytological brush sample was collected. A cytobrush tool (Aries Surgical, Davis, CA) with an attached cytobrush (Cooper Surgical, Trumbull, CT) was passed through the cervix guided by rectal palpation. Once in place the cytobrush was exposed and placed in contact with the endometrium of the uterine body and rotated three times to sample the endometrium (Cardoso et al., 2017). The cytobrush was retracted into the cytobrush tool to prevent contamination by cervical or vaginal cells and immediately snap frozen in liquid nitrogen and stored at -80°C. Following endometrial sampling all cows received two injections of $PGF_{2\alpha}$ (25 mg) 12 h apart to terminate any ensuing pregnancies and regress corpus lutea.

Study 2 - Evaluate the Effect of Seminal Plasma at Insemination on Early Fetal Development

Experimental Design

To evaluate the effect of seminal plasma exposure at insemination on early fetal development, 24 non-lactating Holsteins cows (aged 2 to 7 years old) were dried off as previously stated and moved to pasture, fed a total mixed ration and given free access to clean drinking water. Cows were subjected to estrous synchronization (see below), and on the day of TAI cows were randomly assigned to receive one of two treatments, an intrauterine infusion of either seminal plasma or saline. All cows were bred using X-sorted semen from a single sire to ensure the absence of seminal plasma and to avoid

any potential confounding effect of fetal sex (Hansen et al., 2016). Only female singleton pregnancies were included in the analysis.

Estrous Synchronization, Artificial Insemination, and Intrauterine Infusion of Treatments

Cows were synchronized using a double ovsynch protocol as previously described by Wiltbank et al. (2015). Day 0 was established as the day of TAI and intrauterine infusion of treatments. Cows were randomly assigned using Microsoft Excel to receive a 0.5 mL intrauterine infusion of either saline (*n* = 12) or pooled seminal plasma (*n* = 12), as above. All inseminations consisted of two 0.25 mL straws of commercial X-sorted semen from sire Halifax 029HO19215 (ABS Global).

Blood Collection, Pregnancy Diagnosis, and Evaluation of Fetal Growth

Blood was collected from the coccygeal vein into heparin vacutainers (Greiner Bio-One, Monroe, NC) on d 21, 28, and then weekly from d 32 until d 81 post TAI. On d 21, peripheral blood mononuclear cells (PBMC) were isolated using an ammonium chloride red cell lysis buffer and centrifugation at 500 × *g* for 10 min. Pelleted PBMC were snap frozen and stored at -80°C.

Pregnancy was confirmed by transrectal ultrasound (Evo I, E.I. Medical Imaging, Loveland, CO) on d 32 by visualization of an embryo with an intact amniotic vesicle, fetal heartbeat, and the presence of a CL. Weekly transrectal ultrasound was performed to evaluate fetal crown/rump length, crown/nose length, and abdominal diameter. Fetal parameters were quantified utilizing Image J software (National Institutes of Health, Bethesda, MD) after initial calibration using ultrasound scale landmarks.

Quantification of Peripheral Progesterone Concentration

Plasma progesterone was measured utilizing a commercially available ELISA according to the manufacturer's instructions (DRG International, Springfield Township, NJ). Validity of the assay for bovine plasma was previously reported following spike-in/recovery assays (Dickson et al., 2020). The limit of detection and CV for the assay as performed here were 0.14 ng/mL and 4.2%, respectively.

Conceptus Evaluation at d 96 of Pregnancy

Cows with female singleton pregnancies (*n* = 9) were slaughtered at d 96 of gestation by captive bolt and exsanguination. Whole reproductive tracts were recovered, placed on ice, and processed within ~1 h to evaluate fetal and placental morphometric characteristics. The conceptus was manually detached from the maternal tissues to retrieve the conceptus intact. The excised conceptus was weighed and measured to obtain conceptus weight, chorion length and width, and amniotic vesicle length and width. Amniotic fluid was collected, and total amniotic fluid was measured using a volumetric cylinder. The placenta was extended to determine the total number of cotyledons, and ten cotyledons in close proximity to the fetus were randomly selected to measure individual cotyledon diameter and weight. Fetal body weight, crown/rump length, crown/nose length, hearth girth, and umbilical cord diameter were measured. Fetal heart, paired lungs, paired kidneys, brain, liver, and spleen were weighed for each fetus. Samples of fetal liver and the semitendinosus muscle were stored in RNAlater or snap frozen in liquid nitrogen for later analysis.

General Procedures

RNA Extraction

Blastocysts were lysed in RLT buffer using QIAshredder columns (Qiagen, Hilden, Germany) and total RNA was extracted using the RNeasy Micro Kit according to the manufacturer's instructions (Qiagen). Total RNA from cytobrush samples and PBMC were extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Extracted RNA was quantified using a NanoDrop ND2000 spectrophotometer (Thermo Fisher Scientific) and stored at -80°C.

Gene Expression by Semi-Quantitative Real-Time RT-PCR

For blastocysts and PBMC, reverse transcription was performed using the Verso cDNA synthesis kit according to the manufacturer's instructions (Thermo Fisher Scientific). Blastocyst cDNA was subjected to a target-specific preamplification using SsoAdvanced PreAmp Supermix (Bio-Rad, Hercules, USA). A total of 12 preamplification cycles were performed for each sample using a pool of 50 nM of each required primer (Table 2-1). Preamplification consisted of a 50 µL reaction with an initial polymerase activation and DNA denaturation step at 95°C for 3 min, followed by 12 cycles consisting of denaturation at 95°C for 15 sec followed by annealing/extension at 58°C for 4 min. Primers for all PCR were designed utilizing the NCBI data base (Table 2-1). Amplification efficiency was evaluated for each primer pair by performing serial dilution of cDNA and all primer pairs met MIQE guidelines of $r^2 > 0.98$ and primer efficiency between 90% and 110% (Bustin et al., 2009).

Real time RT-PCR using preamplified blastocyst cDNA and PBMC cDNA was performed in 20 µL reactions using 500 nM of each specific primer (except for *OAS1Y*; 300 nM, and *MX2*; 250nM) and iTaq Universal SYBR Green master mix (Bio-Rad). A

CFX Connect light cycler (Bio-Rad) was employed using either a three-step or two-step protocol. Blastocyst samples were evaluated using a three-step protocol with an initial denaturation step at 95°C for 30 sec followed by 40 cycles at 95°C for 5 sec, annealing temperature specific for each primer for 10 sec (*IGF2R*, *SLC2A1*, *IFNT2*, *DNMT3A*, *HSP1A1*, *GAPDH*, *RPL19* and *SDHA* at 60°C and *PTGS2* at 62°C) and extension at 72°C for 30 sec. Samples from PBMC were subjected to a two-step protocol with an initial denaturation step at 95°C for 30 sec, followed 40 cycles of denaturation at 95°C for 5 sec followed by an annealing/extension step for 30 sec at 60°C (*ISG15, MX1, MX2, OAS1Y, GAPDH* and *ACTB*). Each sample was assayed in triplicate and each primer pair was evaluated in a no template negative control where cDNA was replaced by water.

Relative gene expression data was calculated using the $2^{-\Delta Ct}$ method with normalization to the geometric mean of the housekeepers (*GAPDH*, *RPL19* and *SDHA* for blastocyst; *ACTB* and *GAPDH* for PBMC).

RNAseq, Read Mapping and Differential Gene Expression Analysis

Total RNA from cytobrush samples was subjected to RNA sequencing performed by Novogene (Sacramento, CA, USA). Total RNA was quantified and analyzed for RNA integrity utilizing an RNA Nano 6000 Assay Kit and Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Samples with an RNA integrity number (RIN) ≥ 5.3 were utilized for library preparation. For library preparation, samples underwent first and secondary cDNA synthesis followed by PCR and quality assessment using a Bioanalyzer 2100 system. Preparation of cDNA templates was performed utilizing a

TruSeq PE Cluster Kit v3-cBot-HA (Ilumina, CA, USA), before sequencing on an Illumina NovaSeq 6000 platform to generate 150 bp paired-end reads.

Original raw data from high-throughput sequencing was transformed into sequenced reads by CASAVA stored in FASTQ(fq) format, which cleans data to contain reads without adapters and low quality reads. Paired-end, clean reads were aligned to the reference genome (ARS-UCD1.2) that was built using Hisat2 v2.0.5, then mapped-reads were assemble and quantified as full-length transcripts by StringTie (v1.3.3B) (Pertea et al., 2015). To determine differential expression between treatment groups (seminal plasma compared to saline), pairwise comparison was performed using the DESeq2 R package (1.20.0). Resulting *P*-values were adjusted utilizing the Benjamini and Hochberg's approach for controlling multiple comparisons and false discovery rate (FDR). Genes with an adjusted *P*-value ≤ 0.1 were declared as being differentially expressed in samples from seminal plasma infused cows compared to the saline control group.

Downstream Analysis of Differentially Expressed Genes

Ingenuity Pathway Analysis (IPA) (Qiagen) was employed to identify the potential canonical pathways, upstream regulators, and molecular networks of differentially expressed genes altered by seminal plasma infusion at insemination. Only differentially expressed genes with an FDR ≤ 0.1 were used for analysis. Canonical pathways were identified with a $-\log_{10}(p\text{-value}) > 1.3$ and a calculated z-score where ≤ -2 predicted inhibition or ≥ 2 predicted activation. Upstream regulators of differentially expressed genes were identified based on the predicted activation state that is determined by z-score. Molecular networks affected by differentially expressed genes

were determined by the network score; a network score > 2 provides a 99% confidence that the network in question is not being identified by chance.

Statistical Analysis

Data of absolute number and proportion of embryos recovered were analyzed using SAS v. 9.4 (SAS Institute, Cary, NC). Absolute number of embryos recovered was analyzed using a General Linear Model (GLM) procedure, including the fixed effect of treatment (saline *vs.* seminal plasma) and cohort, and cow ID was included as a random effect. The proportional data pertaining to embryo collection was analyzed by the Generalized Linear Mixed Model (GLIMMIX) procedure, including the fixed effect of treatment (saline *vs.* seminal plasma) and cohort, and cow ID was included as a random effect. Data for relative gene expression (embryo quality and PBMC), fetal growth measurements, peripheral progesterone concentration, and conceptus morphometry were analyzed using SPSS v. 27 (IBM Corporation, Armonk, NY). Normal distribution of data was evaluated using the Shapiro-Wilk test and data were transformed if appropriate before analysis (see figure legends for specific examples). Gene expression data was analyzed using a general linear model including treatment (saline *vs.* seminal plasma) as a fixed effect. Data for weekly fetal growth parameters, weekly peripheral progesterone concentrations, cotyledon diameter and weight from day 96, was analyzed by repeated measures ANOVA using a general linear model including the fixed effects of treatment (saline *vs.* seminal plasma), day and the interaction between treatment and day (day was not used as a fixed effect when analyzing cotyledon data from d 96). For conceptus measurements, fetal morphometry

and fetal organ weight on d 96 of gestation, data were analyzed using a general linear model including the fixed effects of treatment (saline *vs.* seminal plasma).

For transcriptome analysis, differentially expressed genes were identified in cows infused with seminal plasma compared to saline using an FDR ≤ 0.1. A heat map of differentially expressed genes was assembled using average linkage clustering and Pearson correlation distance in Heatmapper (Babicki et al., 2016).

Results

Effect of Intrauterine Infusion of Seminal Plasma on Embryo Number and Morphology

In vivo produced embryos were recovered 7 d after TAI and intrauterine infusion of either saline (*n* = 13) or seminal plasma (*n* = 13). A total of four cows did not respond to estrous synchronization or ovarian stimulation or could not be non-surgically flushed because of anatomical reasons and were excluded from the analysis (saline; *n* = 10, seminal plasma; *n* = 12). A total of 162 fertilized objects and an additional 4 unfertilized objects were recovered after flushing 22 cows. Intrauterine infusion of seminal plasma had no effect on the absolute number of recovered objects, recovered fertilized objects, recovered morula, recovered blastocysts, recovered transferable embryos (morula plus blastocysts), recovered arrested embryos, or recovered degenerate embryos (Table 2-2). When expressed as a proportion of fertilized structures recovered, intrauterine infusion of seminal plasma had no effect on the proportion of morula, proportion of blastocysts, proportion of transferable embryos (morula plus blastocysts), proportion of arrested embryos or proportion of degenerate embryos, compared to saline infusion (Fig. 2-1).

Effect of Intrauterine Infusion of Seminal Plasma on Embryo Quality

To evaluate the potential of seminal plasma to modulate embryo quality, genes associated with DNA methylation (*DNMT3A*), stress response (*HSPA1A*), maternal recognition of pregnancy (*IFNT2*), growth factor signaling (*IGF2R*), prostaglandin synthesis (*PTGS2*), and glucose transport (*SLC2A1*) were assessed in blastocyst stage embryos using real time RT-PCR (Tesfaye et al., 2004; Sagirkaya et al., 2006). Intrauterine infusion of seminal plasma increased ($P = 0.042$) expression of blastocyst *DNMT3A* by 32.4% compared to saline infusion (Fig. 2-2A). Expression of blastocyst *PTGS2* was reduced ($P = 0.040$) by 22.0% following intrauterine infusion of seminal plasma compared to saline infusion (Fig. 2-2E). Expression of blastocyst *HSPA1A*, *IFNT2*, *IGF2R,* and *SLC2A1* were not affected by intrauterine infusion of seminal plasma (Fig. 2-2).

Intrauterine Infusion of Seminal Plasma Alters the Endometrial Transcriptome

Endometrial cytobrush samples were collected 7 d after TAI and intrauterine infusion of treatment to evaluate the endometrial transcriptome by RNA sequencing. Only samples with a RIN ≥ 5.3 were included in the analysis (saline; $n = 7$, seminal plasma; $n = 5$; Table 2-3). After sequencing a total of 604,715,296 high quality reads were aligned to the reference genome to provide an average of 44,123,050 mapped reads per sample (87.5% reads mapped per sample).

A total of 107 differentially expressed genes were identified in the endometrium after intrauterine infusion of seminal plasma compared to saline infusion (FDR < 0.1; Table 2-4). Forty-six endometrial genes were upregulated, and 61 genes were downregulated after intrauterine infusion of seminal plasma compared to saline infusion (Fig. 2-3). The five genes with the greatest increase in expression after intrauterine

infusion of seminal plasma included *SCT*, *RADIL*, *APLP1*, *PCP4*, and *ILVBL*, while the five genes with the greatest decrease in expression included *CCNB2*, *PLAC8*, *CDKN2A*, *SCNN1B*, and *GRAP* (Table 2-5). A heat map shows the hierarchical clustering of the 107 differentially expressed genes and highlights the expression pattern within a treatment group (Fig. 2-3).

Ingenuity Pathway Analysis was employed to investigate the enrichment and functionality of endometrial genes differentially expressed after intrauterine infusion of seminal plasma. Intrauterine infusion of seminal plasma affected 22 canonical pathways (Fig. 2-4; Table 2-6). Among these canonical pathways the top 5 according to *P*-value include 1) leukocyte extravasation signaling, 2) granulocyte adhesion and diapedesis, 3) 3-phosphoinositide biosynthesis, 4) IL-13 signaling pathway, and 5) superpathway of inositol phosphate compounds. No canonical pathway was identified to have a z-score that predicted activation or inhibition. A total of 19 predicted upstream regulators of differentially expressed genes were identified after intrauterine infusion of seminal plasma (Table 2-7). Three predicted upstream regulators had a z-score ≥ 2 and are predicted to be activated after intrauterine infusion of seminal plasma, including CITED2 (transcription regulator), SIRT1 (transcription regulator), and miR-124-3p (microRNA). Conversely, 16 predicted upstream regulators had a z-score ≤ -2 and are predicted to be inhibited after intrauterine infusion of seminal plasma, including beta-estradiol (steroid hormone), IFNγ (cytokine), and STAT1 (transcription regulator).

Ingenuity Pathway Analysis identified seven gene networks altered after intrauterine infusion of seminal plasma (Table 2-8). The five highest ranked gene networks identified include 1) cancer, cell-to-cell signaling and interaction, molecular

transport (Fig. 2-5A), 2) cellular movement, hematological system development and function, immune cell trafficking (Fig. 2-5B), 3) cancer, organismal injury and abnormalities, reproductive system disease (Fig. 2-5C), 4) cell death and survival, cell signaling, organismal injury and abnormalities, 5) metabolic disease, neurological disease, organismal injury, and abnormalities.

Effect of Intrauterine Infusion of Seminal Plasma on Expression of Interferon Stimulated Genes in Peripheral Blood Mononuclear Cells

To determine the effect of intrauterine infusion of seminal plasma on expression of interferon stimulated genes in PBMC on d 21, expression of *ISG15*, *MX1*, *MX2*, and *OAS1Y* was evaluated only in cows that were confirmed pregnant on d 32 by transrectal ultrasound (Fig. 2-6). Expression of selected *ISGs* at d 21 in PBMC was evident in all cows, except one cow in the seminal plasma group where *ISG15* was not detectable. Intrauterine infusion of seminal plasma (n = 5) did not significantly alter the expression of *ISG15*, *MX1, MX2*, and *OAS1Y* when compared to saline infusion (n = 4).

Effect of Intrauterine Infusion of Seminal Plasma on Circulating Progesterone

Peripheral progesterone concentration was quantified on d 28 and then weekly from d 32 until d 81 only in cows confirmed to be pregnant from d 32 to d 81. There was no effect of intrauterine infusion of seminal plasma (P = 0.17) on circulating progesterone; however, there was an effect (P < 0.001) of day on circulating progesterone regardless of the treatment (Fig. 2-7). There was no effect of the interaction between treatment and day on circulating progesterone. The highest average progesterone concentration during the study period was observed on d 28 in the seminal plasma group at 18.5 ng/mL, with progesterone in the saline group at 14.3

ng/mL. The lowest average progesterone concentration was observed on d 74 in the seminal plasma group at 7 ng/mL, with progesterone in the saline group at 7.6 ng/mL.

Effect of Intrauterine Infusion of Seminal Plasma on Fetal Growth

On d 32 after TAI, pregnancy diagnosis was completed by transrectal ultrasound. Pregnancy rate was not affected by treatment, with 58% (7 of 12 cows) pregnant in the saline group and 50% (6 of 12 cows) pregnant in the seminal plasma group. Fetal growth was evaluated in singleton pregnancies with female calves only, excluding male pregnancies, twin pregnancies, and pregnancy losses (saline, $n = 4$; seminal plasma, $n = 5$).

Fetal crown-rump length was evaluated from d 32 until d 53 (Fig. 2-8A). Fetal crown-rump length increased ($P < 0.001$) over time from 13.9 mm to 43.2 mm but was not affected by treatment ($P = 0.467$) or the interaction between treatment and time ($P = 0.882$). Fetal crown-nose length was evaluated from d 53 until d 81 (Fig. 2-8B). Fetal crown-nose length was reduced ($P = 0.02$) after intrauterine infusion of seminal plasma due to a tendency ($P \leq 0.08$) for reduced length on d 60 and d 74. Further, fetal crown-nose length increased ($P < 0.001$) over time from 19.6 mm to 47.2 mm, with no effect of the interaction between treatment and time. Fetal abdominal diameter was evaluated from d 53 until d 81 (Fig. 2-8C). Fetal abdominal diameter increased ($P < 0.001$) over time from 15.8 mm to 34.4 mm but was not affected by treatment ($P = 0.936$) or the interaction between treatment and time ($P = 0.359$).

Effect of Intrauterine Infusion of Seminal Plasma on Conceptus Development

A total of nine female singleton pregnancies were collected at d 96 of gestation (saline; $n = 4$, seminal plasma; $n = 5$). Conceptus, extraembryonic, and fetal morphometric measurements are displayed in Table 2-9. Conceptus weight, conceptus

length, amniotic vesicle width, and amniotic vesicle length prior to dissection was not affected by treatment. Interestingly, intrauterine infusion of seminal plasma increased (*P* = 0.050) amniotic fluid by 125 mL compared to saline controls. The total number of cotyledons and average cotyledon weight was not affected by treatment, while intrauterine infusion of seminal plasma tended to reduce (*P* = 0.086) average cotyledon diameter by 9.1% compared to saline controls. Fetal body weight, eviscerated fetal weight, umbilical cord diameter, crown-rump length, and crown-nose length were not affected by treatment; however, intrauterine infusion of seminal plasma tended (*P* = 0.080) to reduce fetal heart girth by 0.3 cm compared to saline controls.

Fetal organs weights evaluated at d 96 of gestation are displayed in Table 2-10. Intrauterine infusion of seminal plasma had no effect on the weight of the fetal heart, fetal lungs, fetal kidneys, fetal spleen, fetal brain or combined fetal heart and lungs.

Table 2-1. Primer sequences used for real time RT-PCR.

Gene	Primer	Sequence (5'-3')	Accession no.
ACTB	Fwd	TTGGCCTTAGGGTTCAGGG	NM_173979.3
	Rev	CAGAAGCACTCGTACGTGGG	
DNMT3A	Fwd	CCATGTACCGCAAGGCTATCTA	XM_024998.68.1
	Rev	CCTGTCATGGCACATTGGAA	
GAPDH	Fwd	AGGTCGGAGTGAACGGATTC	NM_001034034.2
	Rev	ATGGCGACGATGTCCACTTT	
HSPA1A	Fwd	GACAAGTGCCAGGAGGTGATTT	NM_203322.3
	Rev	CAGTCTGCTGATGATGGGGTTA	
IFNT2	Fwd	TCCATGAGATGCTCCAGCAGT	NM_001015511.4
	Rev	TGTTGGAGCCCAGTGCAGA	
IGF2R	Fwd	CAGGTCTTGCAACTGGTGTATGA	NM_174352.2
	Rev	TTGTCCAGGGAGATCAGCATG	
ISG15	Fwd	AGAGAGCCTGGCACCAGAAC	NM_174366.1
	Rev	TTCTGGGCGATGAACTGCTT	
MX1	Fwd	AGACGAGTGGAAAGGCAAAGTC	NM_173940.2
	Rev	GATGGCAATCTGGGCTTCAC	
MX2	Fwd	TCAGAGACGCCTCAGTCGAA	NM_173941.2
	Rev	ACGTTTGCTGGTTTCCATGAA	
OAS1Y	Fwd	TAGGCCTGGAACATCAGGTC	NM_001040606.1
	Rev	TTTGGTCTGGCTGGATTACC	
PTGS2	Fwd	CGTGAAAGGCTGTCCCTTTA	NM_001105323.1
	Rev	ATCTAGTCCAGAGTGGGAAGAG	
RPL19	Fwd	ATGCCAACTCCCGCCAGCAGAT	NM_001040516.2
	Rev	TGTTTTTCCGGCATCGAGCCCG	
SDHA	Fwd	GGAACACTGACCTGGTGGAG	NM_174178.2
	Rev	CGTCAACCCTCTCCTTGAAGT	
SCLC2A1	Fwd	AGCGTCATCTTCATCCCAGC	NM_174602.2

Table 2-2. Effect of intrauterine infusion of seminal plasma on the number of *in vivo* recovered embryos.

	Saline (*n* = 10)	Seminal Plasma (*n* = 12)	*P*-value
Total objects recovered	6.5 ± 1.3 (66)	8.1 ± 1.2 (100)	0.330
Fertilized objects recovered[1]	6.2 ± 1.2 (63)	8.1 ± 1.1 (99)	0.240
Morula	0.9 ± 0.5 (9)	0.6 ± 0.5 (7)	0.633
Blastocyst	3.1 ± 1.0 (32)	4.5 ± 1.0 (55)	0.344
Transferable[2]	4.0 ± 1.3 (41)	5.2 ± 1.2 (62)	0.547
Arrested[3]	0.7 ± 0.5 (8)	0.5 ± 0.5 (6)	0.769
Degenerate	1.4 ± 0.5 (14)	2.6 ± 0.5 (31)	0.103

Donors were subjected to estrus synchronization and ovarian stimulation, on d 0 cows were inseminated and received an intrauterine infusion of either saline or seminal plasma. Embryos were recovered 7 d after insemination. Cows that did not respond to estrus synchronization, ovarian stimulation or were not flushed were excluded from the analysis. Data are presented as least square means ± SEM.; effect of treatment was determined by general linear model, using treatment (saline *vs.* seminal plasma) and cohort as fixed effect, and cow ID as a random effect. Total observations of all cows are presented in parentheses.

[1] Excludes unfertilized objects.

[2] Includes morula and blastocyst stage embryos.

[3] Includes objects arrested prior to the morula stage of development.

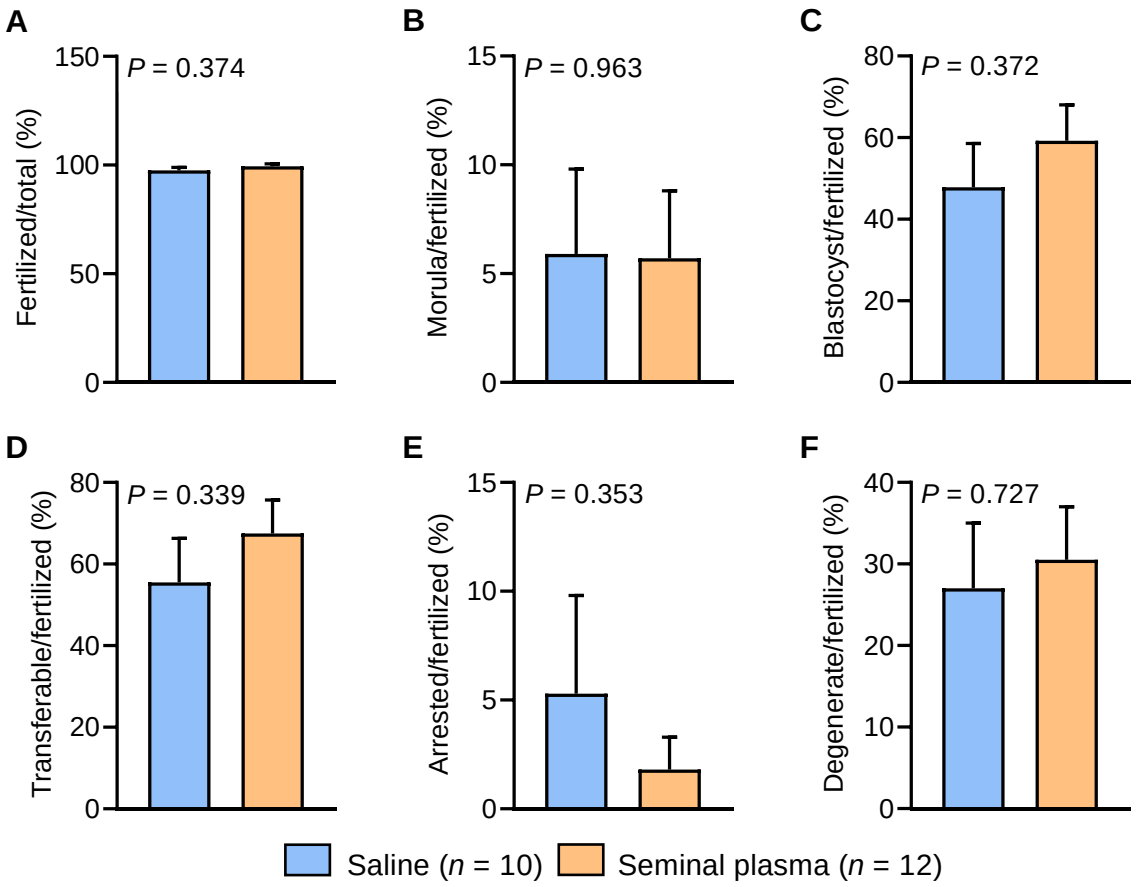

Figure 2-1. Effect of intrauterine infusion of seminal plasma on the proportion of *in vivo* recovered embryos. Donors were subjected to estrus synchronization and ovarian stimulation, on d 0 cows were inseminated and received an intrauterine infusion of either saline or seminal plasma. Cows that did not respond to estrus synchronization, ovarian stimulation or were not flushed were excluded from the analysis. (A) The proportion of fertilized objects that were recovered, (B) the proportion of fertilized objects that were morula, (C) the proportion of fertilized objects that were blastocyst, (D) the proportion of fertilized objects that were transferable embryos that included morula and blastocyst, (E) the proportion of fertilized objects that were arrested, and (F) the proportion of fertilized objects that were degenerate. Data are presented as least square means ± SEM.; effect of treatment was determined by General linear mixed model, using treatment (saline *vs.* seminal plasma) and cohort as fixed effect, and cow ID as a random effect.

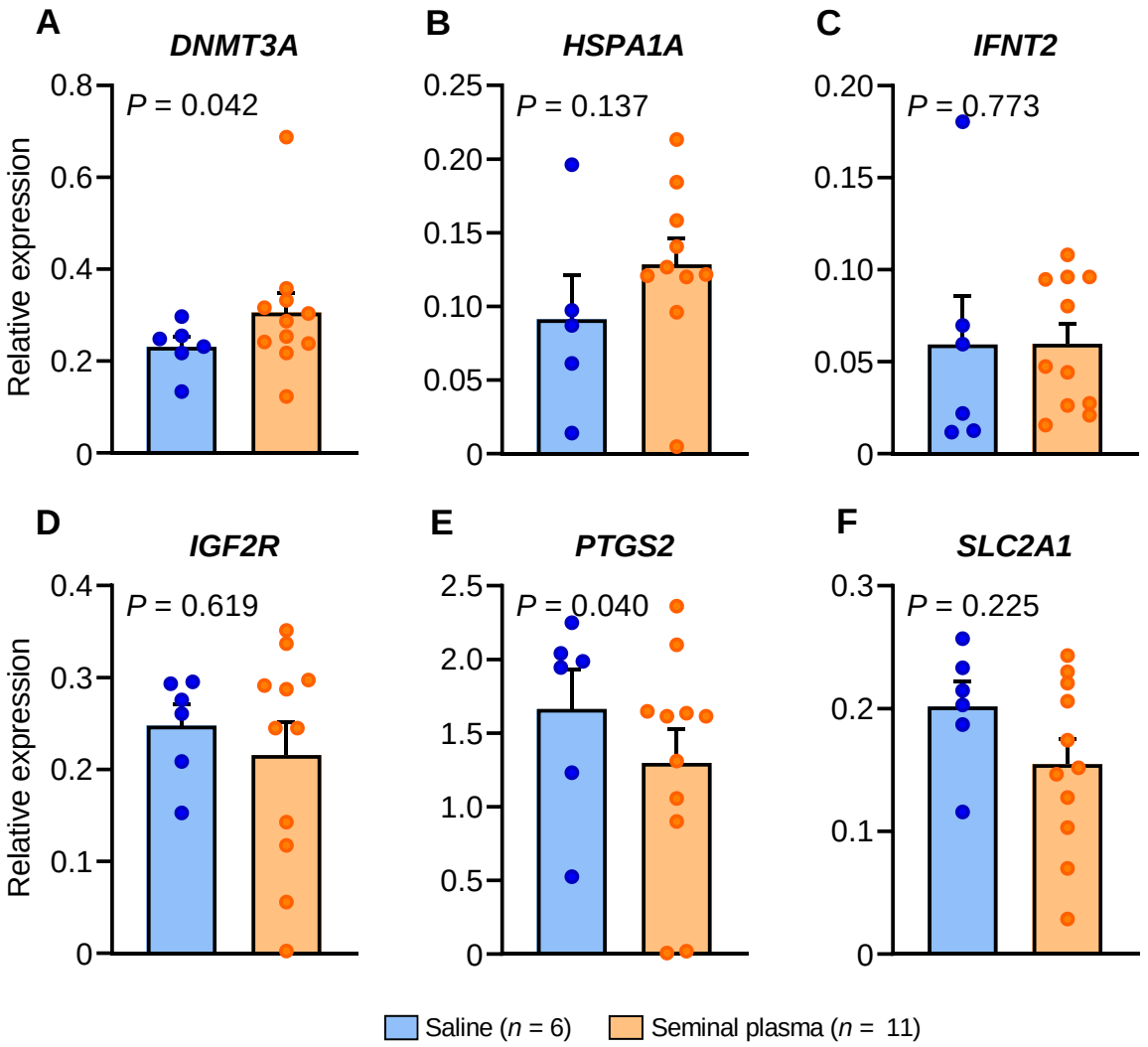

Figure 2-2. Effect of intrauterine infusion of seminal plasma on molecular markers of blastocyst quality. Donors were subjected to estrus synchronization and ovarian stimulation, on d 0 cows were inseminated and received an intrauterine infusion of either saline or seminal plasma. Gene expression for *DNMT3A* (A), *HSPA1A* (B), *IFNT2* (C), *IGF2R* (D), *PTGS2* (E), and *SLC2A1* (F) was assessed in blastocyst stage embryos collected 7 d after insemination. Data are presented as expression relative to the geometric mean of the housekeeper genes *GAPDH*, *RLP19* and *SDHA*. Data for *DNMT3A* and *IFNT2* was log transformed following a Shapiro-Wilk test before analysis. A sample from both treatment groups were excluded outliers for *HSPA1A*. Gene expression was analyzed using a general linear model using treatment (saline *vs.* seminal plasma) as a fixed effect. Each dot represents a sample of pooled blastocysts from a single donor and the bars depicts the mean + SEM.

Table 2-3. Summary of read mapping for endometrial samples collected seven days after intrauterine infusion of seminal plasma.

Treatment	RIN[1]	Raw reads	Clean reads	Mapped reads	Mapped reads (%)
Saline	6.7	50,213,384	49,012,928	40,281,199	82.18%
Saline	6.4	51,302,938	50,332,498	46,567,928	92.52%
Saline	6	64,476,260	63,148,902	57,489,883	91.04%
Saline	6.8	52,706,750	51,715,580	47,975,030	92.77%
Saline	6	51,142,132	50,123,426	45,196,981	90.17%
Saline	5.8	50,515,526	49,579,672	43,584,298	87.91%
Saline	5.6	45,060,900	44,195,560	39,613,272	89.63%
Seminal plasma	6.2	45,162,738	44,378,308	38,226,479	86.14%
Seminal plasma	5.5	55,377,724	54,050,838	45,009,494	83.27%
Seminal plasma	5.4	48,054,240	47,324,826	39,259,061	82.96%
Seminal plasma	6.1	52,583,948	51,535,146	42,143,551	81.78%
Seminal plasma	5.3	50,288,468	49,317,612	44,129,423	89.48%

[1] RNA integrity number.

Table 2-4. Differentially expressed genes of the endometrium after intrauterine infusion of seminal plasma.

Gene	Description	Log_2FC^1	Adj *P*-value
ANO1	Anoctamin 1	-1.819	0.025
AOX1	Bos taurus aldehyde oxidase 1	-1.218	0.063
APLP1	Bos taurus amyloid beta precursor like protein 1	1.575	0.039
ARHGEF3	Rho guanine nucleotide exchange factor 3	-1.157	0.065
ATP2B2	Bos taurus atpase plasma membrane Ca2+ transporting 2	-1.276	0.051
BARX2	BARX homeobox 2	0.632	0.084
BOLA-DRB3	Major histocompatibility complex, class II, DRB3	-0.980	0.069
BRF1	BRF1, RNA polymerase III transcription initiation factor subunit	0.751	0.065
C18H16orf74	Novel gene	0.906	0.099
C1QTNF5	Bos taurus C1q and TNF related 5	-1.771	0.065
C3H1orf87	Chromosome 3 c1orf87 homolog	-0.898	0.060
C7orf61	Chromosome 25 c7orf61 homolog	-0.980	0.099
CASKIN1	CASK interacting protein 1	1.333	0.099
CCNB2	Bos taurus cyclin B2	-2.517	0.063
CCRL2	Bos taurus C-C motif chemokine receptor like 2	-1.735	0.069
CD44	Bos taurus CD44 molecule	-1.157	0.065
CDK16	Bos taurus cyclin dependent kinase 16	0.647	0.069
CDKN2A	Cyclin-dependent kinase inhibitor 2A	-2.199	0.069
CEP83	Centrosomal protein 83	-0.830	0.099
CLDN10	Bos taurus claudin 10, transcript variant 2	-1.473	0.088
CORO1A	Bos taurus coronin 1A	-0.835	0.069
CXCL10	C-X-C motif chemokine ligand 10	-1.765	0.051
DECR2	Bos taurus 2,4-dienoyl-coa reductase 2	0.986	0.084
DGKD	Bos taurus diacylglycerol kinase delta	0.499	0.099
DOK1	Bos taurus docking protein 1	-1.118	0.025
DUSP8	Dual specificity phosphatase 8	0.990	0.061
EEF1AKMT1	EEF1A lysine methyltransferase 1	0.509	0.061
ENDOD1	Bos taurus endonuclease domain containing 1	-0.938	0.064
FAM71E1	Family with sequence similarity 71 member E1	-1.885	0.060
FBXL2	Bos taurus F-box and leucine rich repeat protein 2	1.211	0.084
FBXW5	Bos taurus F-box and WD repeat domain containing 5	0.684	0.069

Table 2-4. Continued

FGD3	Bos taurus FYVE, rhogef and PH domain containing 3	-0.918	0.088
FMC1	Bos taurus formation of mitochondrial complex V assembly factor 1 homolog	0.699	0.051
FOLR1	Folate receptor 1	-1.005	0.088
GALNT6	Bos taurus polypeptide N-acetylgalactosaminyltransferase 6	-0.851	0.051
GIMAP7	Bos taurus gtpase, IMAP family member 7	-1.195	0.088
GRAP	GRB2-related adaptor protein	-2.045	0.020
IFITM3	Interferon induced transmembrane protein 3	-1.052	0.025
ILVBL	Bos taurus ilvb acetolactate synthase like	1.476	0.025
KDELR3	Bos taurus KDEL endoplasmic reticulum protein retention receptor 3	-0.894	0.051
LAMA3	Laminin subunit alpha 3	-1.261	0.020
LCP1	Lymphocyte cytosolic protein 1	-0.801	0.063
LITAF	Bos taurus lipopolysaccharide induced TNF factor	-0.646	0.060
LOC615733	Novel gene	-1.324	0.099
LRRN2	Leucine rich repeat neuronal 2	1.056	0.094
LTF	Lactotransferrin	-1.352	0.069
LYN	Bos taurus LYN proto-oncogene, Src family tyrosine kinase	-0.603	0.088
LYPD3	Bos taurus LY6/PLAUR domain containing 3	-1.315	0.099
METTL27	Methyltransferase like 27	0.705	0.099
MIB2	Mindbomb E3 ubiquitin protein ligase 2	0.792	0.088
MIIP	Bos taurus migration and invasion inhibitory protein	0.777	0.042
MKRN2OS	MKRN2 opposite strand	-1.864	0.045
MTMR9	Bos taurus myotubularin related protein 9-like	-0.841	0.061
NEK6	Bos taurus NIMA related kinase 6	-0.671	0.099
NLRC5	NLR family CARD domain containing 5	-0.928	0.099
OSGIN1	Bos taurus oxidative stress induced growth inhibitor 1	0.988	0.088
PALM3	Paralemmin 3	1.019	0.082
PALMD	Palmdelphin	-1.366	0.084
PCP4	Purkinje cell protein 4	1.498	0.060
PI4K2B	Bos taurus phosphatidylinositol 4-kinase type 2 beta	-0.717	0.060
PKD1L3	Polycystin 1 like 3, transient receptor potential channel interacting	1.053	0.037

Table 2-4. Continued

PLAC8	Placenta-specific 8	-2.430	0.099
POP1	Bos taurus POP1 homolog, ribonuclease P/MRP subunit	1.063	0.061
PPFIA4	PTPRF interacting protein alpha 4	1.220	0.060
PPWD1	Bos taurus peptidylprolyl isomerase domain and WD repeat containing 1	0.592	0.088
PTGES	Bos taurus prostaglandin E synthase	-1.371	0.060
RAC2	Bos taurus Rac family small gtpase 2	-0.725	0.022
RADIL	Rap associating with DIL domain	1.601	0.084
RASSF5	Bos taurus Ras association domain family member 5	-0.603	0.088
RFLNA	Bos taurus refilin A	-1.137	0.099
ROGDI	Rogdi homolog	0.825	0.084
RPAIN	RPA interacting protein	1.052	0.088
RRP15	Ribosomal RNA processing 15 homolog	-0.868	0.069
RTN3	Bos taurus reticulon 3	0.587	0.084
S100A11	S100 calcium binding protein A11	-0.815	0.084
SAMD4A	Sterile alpha motif domain containing 4A	-1.398	0.025
SCNN1B	Sodium channel epithelial 1 beta subunit	-2.048	0.025
SCT	Novel gene	1.888	0.060
SDC1	Syndecan 1	-0.849	0.099
SELPLG	Bos taurus selectin P ligand, transcript variant 1	-1.135	0.060
SFTA2	Surfactant associated 2	1.055	0.025
SLC41A3	Solute carrier family 41 member 3	0.854	0.063
SLX4	SLX4 structure-specific endonuclease subunit	0.851	0.069
SMC4	Bos taurus structural maintenance of chromosomes 4	-0.719	0.029
SNRNP48	Small nuclear ribonucleoprotein U11/U12 subunit 48	1.119	0.099
SOX17	SRY-box 17	0.771	0.099
SPDEF	Bos taurus SAM pointed domain containing ETS transcription factor	-1.451	0.084
SPTB	Spectrin beta, erythrocytic	0.943	0.088
SQOR	Bos taurus sulfide quinone oxidoreductase	-0.764	0.088
TMEM150C	Bos taurus transmembrane protein 150C	-1.643	0.061
TMEM158	Bos taurus transmembrane protein 158	1.319	0.098

Table 2-4. Continued

TTC28	Tetratricopeptide repeat domain 28	1.185	0.069
TUBA4A	Tubulin alpha 4a	-0.923	0.098
USP40	Ubiquitin specific peptidase 40	0.580	0.084
WARS	Tryptophanyl-trna synthetase	-0.721	0.069
ZNF467	Zinc finger protein 467	-1.317	0.082
ZNF821	Bos taurus zinc finger protein 821	1.009	0.088
novel.1307	Novel gene	0.850	0.025
novel.1335	Novel gene	0.869	0.060
novel.1350	Novel gene	1.332	0.029
novel.2132	Novel gene	-0.851	0.029
novel.2512	Novel gene	0.677	0.080
novel.2598	Novel gene	0.780	0.097
novel.2693	Novel gene	-0.729	0.088
ENSBTAG00000033166	Novel gene	-0.845	0.060
ENSBTAG00000050877	Novel gene	-1.748	0.061
ENSBTAG00000051239	Novel gene	0.784	0.065

[1] Log_2 Fold Change

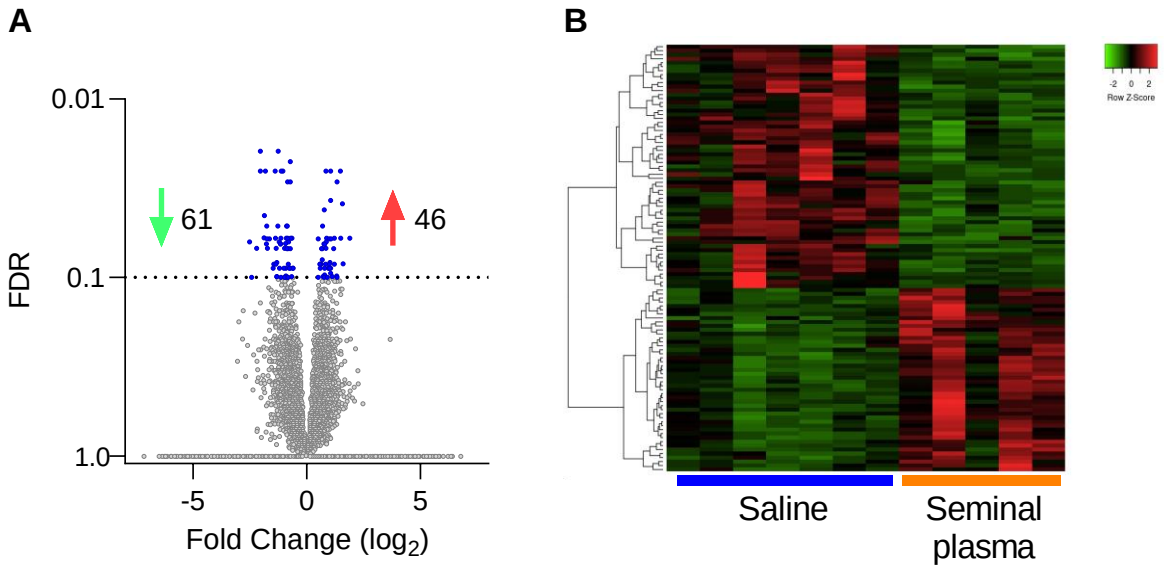

Figure 2-3. Differentially expressed genes of the endometrium after intrauterine infusion of seminal plasma. Differentially expressed genes were identified in the endometrium 7 d after infusion of seminal plasma (n = 5) compared to saline (n = 7) using an FDR ≤ 0.1. A total of a 107 differentially expressed genes were identified. A) Volcano plot displays the distribution of differentially expressed genes alter by seminal plasma in the endometrium compared with saline. Each dot represents a single transcript, blue dots represent differentially expressed genes with an FDR > 0.1 and grey dots represent genes not affected by treatment. B) Heat map of differentially expressed genes of the endometrium after intrauterine infusion of seminal plasma. The heat map uses average linkage clustering and Pearson correlation distance. Differentially expressed genes are represented by a single row and each column represents an individual sample.

Table 2-5. Top five upregulated and downregulated differentially expressed genes of the endometrium after intrauterine infusion of seminal plasma.

Gene ID	Symbol	Description	Log_2 FC[1]	Adj *P*-value
ENSBTAG00000054832	*SCT*	Protein coding	1.888	0.060
ENSBTAG00000030690	*RADIL*	Rap associating with DIL domain	1.601	0.084
ENSBTAG00000001151	*APLP1*	Bos taurus myeloid beta precursor like protein 1	1.575	0.039
ENSBTAG00000051421	*PCP4*	Purkinje cell protein 4	1.498	0.060
ENSBTAG00000015186	*ILVBL*	Bos taurus ilvB acetolactate synthase like	1.476	0.025
ENSBTAG00000005269	*CCNB2*	Bos taurus cyclin B2	-2.517	0.063
ENSBTAG00000009849	*PLAC8*	Placenta-specific 8	-2.430	0.099
ENSBTAG00000034220	*CDKN2A*	Cyclin-dependent kinase inhibitor 2A	-2.199	0.069
ENSBTAG00000012290	*SCNN1B*	Sodium channel epithelial 1 beta subunit	-2.048	0.025
ENSBTAG00000003707	*GRAP*	GRB2-related adaptor protein	-2.045	0.020

[1] Log_2 Fold Change

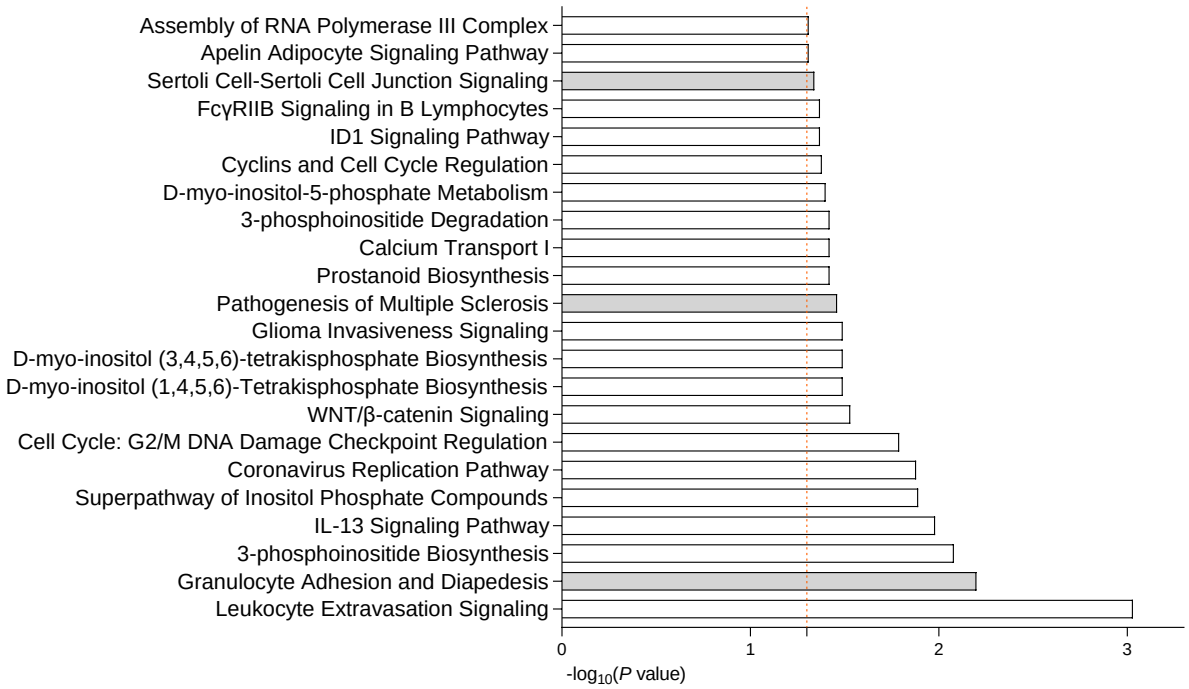

Figure 2-4. Canonical pathways affected in the endometrium after intrauterine infusion of seminal plasma. Ingenuity Pathway Analysis identified a total of 22 canonical pathways altered in the endometrium 7 d after infusion of seminal plasma (n = 5) compared to saline (n = 7). Canonical pathways are considered significant when -$\log_{10}$(P-value) ≥ 1.3. White bars represent canonical pathways that did not reach the z-score threshold to predict activation/inhibition status and grey bars represent pathways where the z-score could not be calculated. A detailed list of canonical pathways can be found in Table 2-6.

Table 2-6. Canonical pathways affected in the endometrium after intrauterine infusion of seminal plasma.

Canonical Pathway	-Log_{10} (*P*-Value)	z-score[1]	Differentially expressed genes in the pathway
Leukocyte Extravasation Signaling	3.03	N/A[2]	*CD44, CLDN10, RAC2, RASSF5, SELPLG*
Granulocyte Adhesion and Diapedesis	2.20	N/A	*CLDN10, CXCL10, SDC1, SELPLG*
3-phosphoinositide Biosynthesis	2.08	0	*DUSP8, MTMR9, PI4K2B, PPFIA4*
IL-13 Signaling Pathway	1.98	N/A	*ANO1, LYN, SPDEF*
Superpathway of Inositol Phosphate Compounds	1.89	0	*DUSP8, MTMR9, PI4K2B, PPFIA4*
Coronavirus Replication Pathway	1.88	N/A	*IFITM3, TUBA4A*
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.79	N/A	*CCNB2, CDKN2A*
WNT/β-catenin Signaling	1.53	N/A	*CD44, CDKN2A, SOX17*
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	1.49	N/A	*DUSP8, MTMR9, PPFIA4*
D-myo-inositol (3,4,5,6)-Tetrakisphosphate Biosynthesis	1.49	N/A	*DUSP8, MTMR9, PPFIA4*
Glioma Invasiveness Signaling	1.49	N/A	*CD44, RAC2*
Pathogenesis of Multiple Sclerosis	1.46	N/A	*CXCL10*
Prostanoid Biosynthesis	1.42	N/A	*PTGES*
Calcium Transport I	1.42	N/A	*ATP2B2*
3-phosphoinositide Degradation	1.42	N/A	*DUSP8, MTMR9, PPFIA4*
D-myo-inositol-5-phosphate Metabolism	1.40	N/A	*DUSP8, MTMR9, PPFIA4*
Cyclins and Cell Cycle Regulation	1.38	N/A	*CCNB2, CDKN2A*
ID1 Signaling Pathway	1.37	N/A	*CDKN2A, LYN, PLAC8*
FcγRIIB Signaling in B Lymphocytes	1.37	N/A	*DOK1, LYN*
Sertoli Cell-Sertoli Cell Junction Signaling	1.34	N/A	*CLDN10, SPTB, TUBA4A*
Apelin Adipocyte Signaling Pathway	1.31	N/A	*PTGES, RAC2*
Assembly of RNA Polymerase III Complex	1.31	N/A	*BRF1*

[1] z-score: indicates the predicted activation or inhibition status, were a z-score ≥2 means activation or ≤-2 means inhibition.
[2] N/A means that Ingenuity Pathway Analysis could not calculated a z-score to predict activation/inhibition.

Table 2-7. Predicted upstream regulators affected in the endometrium after intrauterine infusion of seminal plasma.

Upstream regulator	Biotype	Predicted activation state[1]	z-score[2]	*P*-value	Target molecules differentially expressed in dataset
CITED2	Transcription regulator	Activated	2.00	0.017	*ARHGEF3, CDKN2A, CXCL10, ENDOD1, PLAC8, WARS1*
SIRT1	Transcription regulator	Activated	2.39	0.010	*ANO1, CCNB2, CDKN2A, CORO1A, IFITM3, NLRC5*
miR-124-3p	MicroRNA	Activated	2.40	0.000	*ENDOD1, LITAF, NEK6, RASSF5*
Beta-estradiol	Chemical endogenous	Inhibited	-2.95	0.013	*ANO1, ATP2B2, CCNB2, CD44, CXCL10, FOLR1, IFITM3, KDELR3, LAMA3, LITAF, LTF, PTGES, RAC2, RASSF5, S100A11, SDC1, SPDEF, TMEM150C*
IFNG	Cytokine	Inhibited	-2.89	0.000	*CCRL2, CD44, CDKN2A, CORO1A, CXCL10, DOK1, ENDOD1, IFITM3, LYN, NLRC5, PTGES, RAC2, SCNN1B, SDC1, TMEM158, TTC28, WARS1*
STAT1	Transcription regulator	Inhibited	-2.59	0.002	*CCRL2, CD44, CXCL10, IFITM3, NLRC5, SELPLG, WARS1*
IL6	Cytokine	Inhibited	-2.46	0.020	*CCNB2, CD44, CDKN2A, CXCL10, IFITM3, LTF, PTGES, WARS1*
Lipopolysaccharide	Chemical drug	Inhibited	-2.43	0.002	*ARHGEF3, CCNB2, CCRL2, CD44, CORO1A, CXCL10, DUSP8, GIMAP7, LITAF, LTF, LYN, NEK6, NLRC5 PLAC8, PTGES, SCNN1B, SDC1, SELPLG, SOX17, WARS1*
AGT	Growth factor	Inhibited	-2.29	0.013	*ANO1, ATP2B2, CCNB2, CD44, CDKN2A, CXCL10, DOK1, PLAC8, SDC1*

Table 2-7. Continued.

TGFB1	Growth factor	Inhibited	-2.239	0.016	*CCNB2, CCRL2, CD44, CDKN2A, CXCL10, DOK1, KDELR3, LITAF, PI4K2B, PTGES, S100A11, SDC1, SELPLG, SMC4, TTC28*
RAF1	Kinase	Inhibited	-2.186	0.002	*CD44, CDKN2A, LAMA3, SDC1, TUBA4A*
E. coli B4 lipopolysaccharide	Chemical toxicant	Inhibited	-2.17	0.007	*CXCL10, FOLR1, IFITM3, LCP1, PTGES*
Poly rI:rC-RNA	Biologic drug	Inhibited	-2.159	0.009	*BRF1, CCNB2, CD44, CXCL10, IFITM3, LTF, LYN, NLRC5*
Tretinoin	Chemical endogenous mammalian	Inhibited	-2.156	0.276	*CCRL2, CD44, CDKN2A, CLDN10, CXCL10, LTF, LYN, PTGES, SOX17*
SMARCA4	Transcription regulator	Inhibited	-2.155	0.001	*ANO1, CCRL2, CD44, CDKN2A, FOLR1, IFITM3, LAMA3, RAC2, SOX17*
TLR4	Transcription regulator	Inhibited	-2.148	0.026	*CCRL2, CD44, CXCL10, IFITM3, PTGES*
IRF3	Transmembrane receptor	Inhibited	-2.148	0.002	*CCRL2, CXCL10, IFITM3, NLRC5, PLAC8*
IL2	Cytokine	Inhibited	-2.047	0.181	*ARHGEF3, CD44, CXCL10, PLAC8, SELPLG*

[1] Predicted activation status based on the z-score value.
[2] z-score: indicates the predicted activation or inhibition status, where a z-score ≥ 2 suggests activation or ≤ -2 suggests inhibition.

Table 2-8. Genes networks predicted to be affected in the endometrium after intrauterine infusion of seminal plasma.

Gene network[1]	Score[2]	Molecules in Network
Cancer, Cell-To-Cell signaling and interaction, Molecular transport	40	*ANO1, APLP1, ARHGEF3, CDK16, DECR2, ENDOD1, LAMA3, LITAF, LYPD3, MTMR9, OSGIN1, PPFIA4, SAMD4A, SCT, SDC1, SOX17, SPDEF, WARS1*
Cellular movement, Hematological system development and function, Immune cell trafficking	32	*CCNB2, CD44, CORO1A, DGKD, LCP1, LYN, NLRC5, PCP4, PI4K2B, PTGES, RAC2, RRP15, S100A11, SELPLG, SNRNP48, SPTB*
Cancer, Organismal injury and abnormalities, Reproductive system disease	27	*BRF1, CCRL2, CDKN2A, CG, CXCL10, FBXL2, FBXW5, KDELR3, LTF, MIB2, NEK6, RADIL, SCNN1B, SLX4, TMEM158*
Cell death and survival, Cell signaling, Organismal injury and abnormalities	27	*AOX1, ATM, CBX3, CEP83, CFLAR, DCK, FAM71E1, GLIPR2, GRAP, GRB2, HNF4A, HUNK, JRKL, LAX1, METTL27, NFE2L2, PALMD, PPWD1, PRRG4, PTPN11, RFLNA/ZNF664-RFLNA, ROGDI, RPAIN, RTN3, SFTA2, TBRG1, THEMIS, TNF, TOX, USP40, XPO1, ZNF420, ZNF821*
Metabolic disease, Neurological disease, Organismal injury, and abnormalities	25	*ABCB9, APP, ATP2B2, BARX2, C18orf21, Ca2 ATPase, CLDN10, CLDN16, COL13A1, COL4A6, DECR2, ESR1, FCHO1, GALNT6, GBA2, GIMAP7, ILVBL, PALM3, PCP4, PIGO, PLAC8, PLPP2, POP1, RASSF6, SLC41A3, SLC45A2, SQOR, SYK, TASP1, TP73*
Cell death and survival, Cellular growth and proliferation, Organ development	18	*AKT3, ATAD2, CASKIN1, CDC42, EEF1A2, EEF1AKMT1, FGD3, FMC1, GALNT10, LRRN2, mir-26, MKRN2OS, NRIP3, PKD1L3, PLAGL1, PPARG, PRKRIP1, QSOX1, RARRES2, RASSF5, SEPTIN4, SLC44A1, STEAP1, STS, TMEM150C, TMEM245, TRAF2, TRIM25, WWP2, ZNF467*
Cancer, Endocrine system disorders, Organismal injury, and abnormalities	13	*C1QTNF5, DOK1, LAX1, MIIP, SMC4, SRMS, TTC28, TUBA4A*

[1] Gene networks identified by Ingenuity Pathway Analysis based on differentially expressed genes.
[2] Ingenuity Pathway Analysis calculated a network score utilizing differentially expressed genes. When a network score is > 2 there is 99% confidence that the network and genes are not being identified by chance.

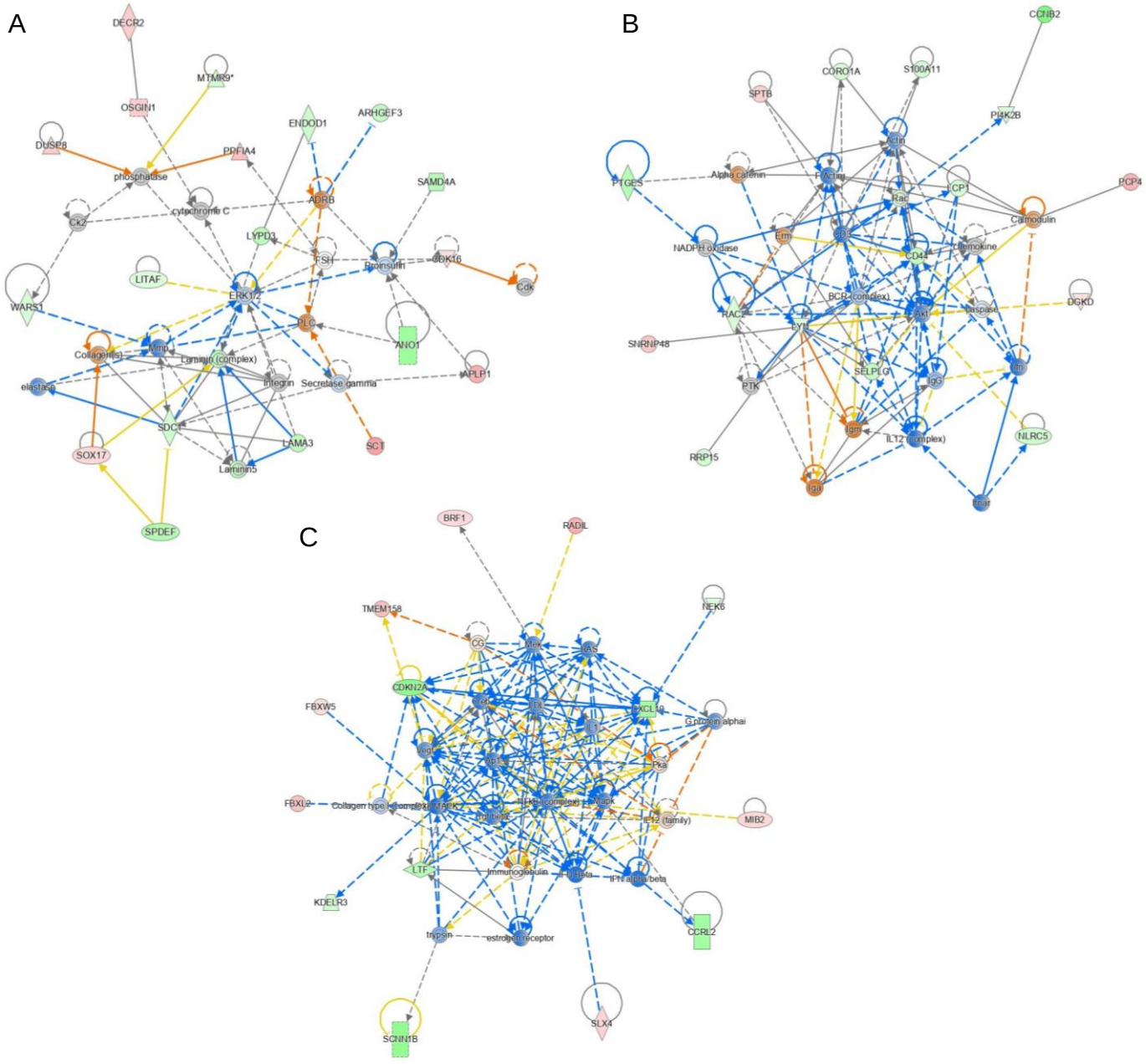

Figure 2-5. Gene networks predicted to be affected in the endometrium after intrauterine infusion of seminal plasma. (A) Cancer, cell-to-cell signaling and interaction, molecular transport; (2) cellular movement, hematological system development and function, Immune cell trafficking; (3) cancer, organismal injury and abnormalities, reproductive system disease. Red symbols indicate upregulated in our dataset, green symbols indicate downregulated in our dataset, orange symbols indicate a predicted activation and blue symbols indicate predicted inhibition.

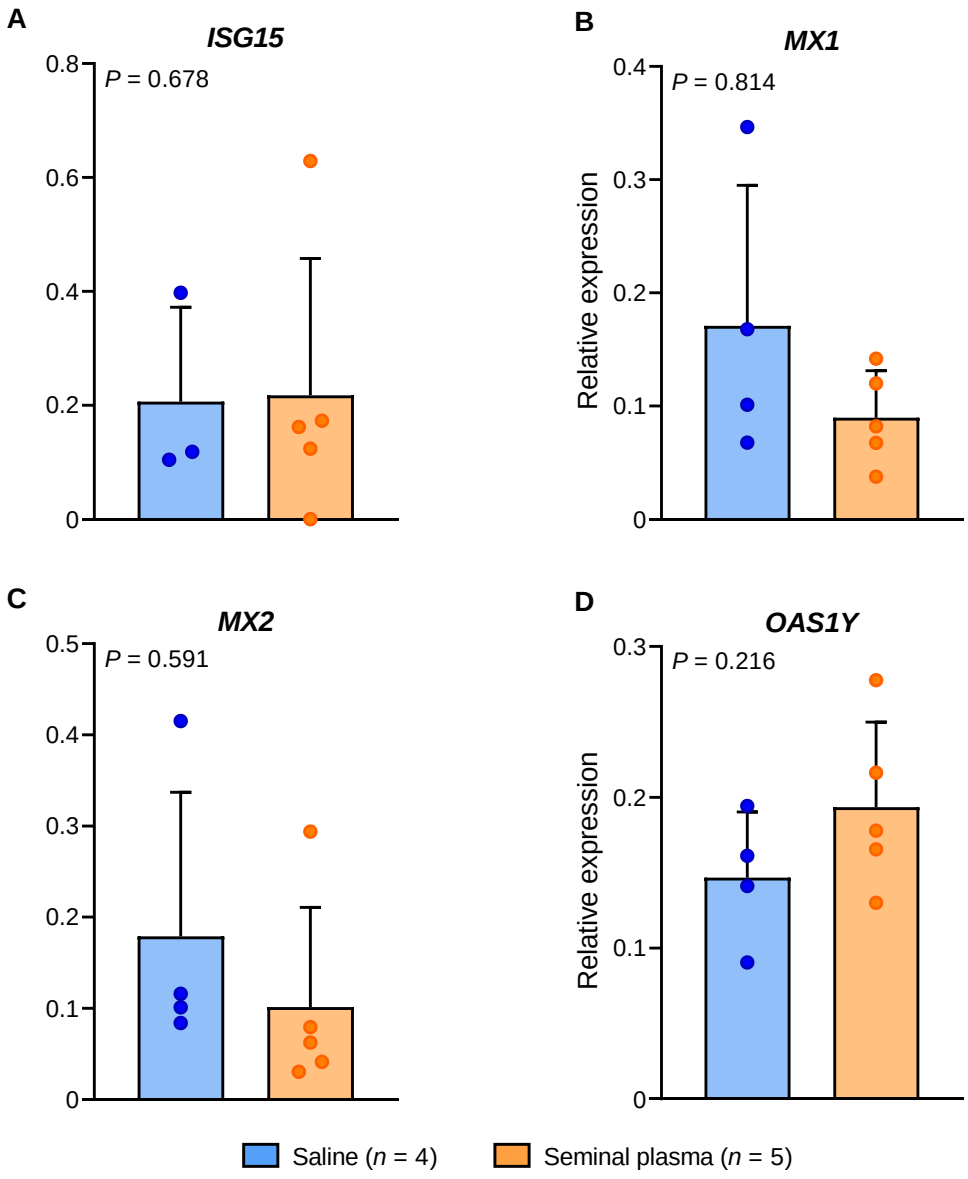

Figure 2-6. Effect of intrauterine infusion of seminal plasma on expression of interferon stimulated genes in peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated on d 21 after TAI and intrauterine infusion of either saline or seminal plasma. Expression of *ISG15* (A), *MX1* (B), *MX2* (C) and *OAS1Y* (D) were evaluated by real time RT-PCR. Data are presented as expression relative to the geometric mean of *ACTB* and *GAPDH*. Gene expression was analyzed using a general linear model including treatment (saline *vs.* seminal plasma) as a fixed effect. Each dot represents a single cow, and the bars represent the mean ± SEM. Expression of *ISG15* for one sample from the saline group was excluded as an outlier.

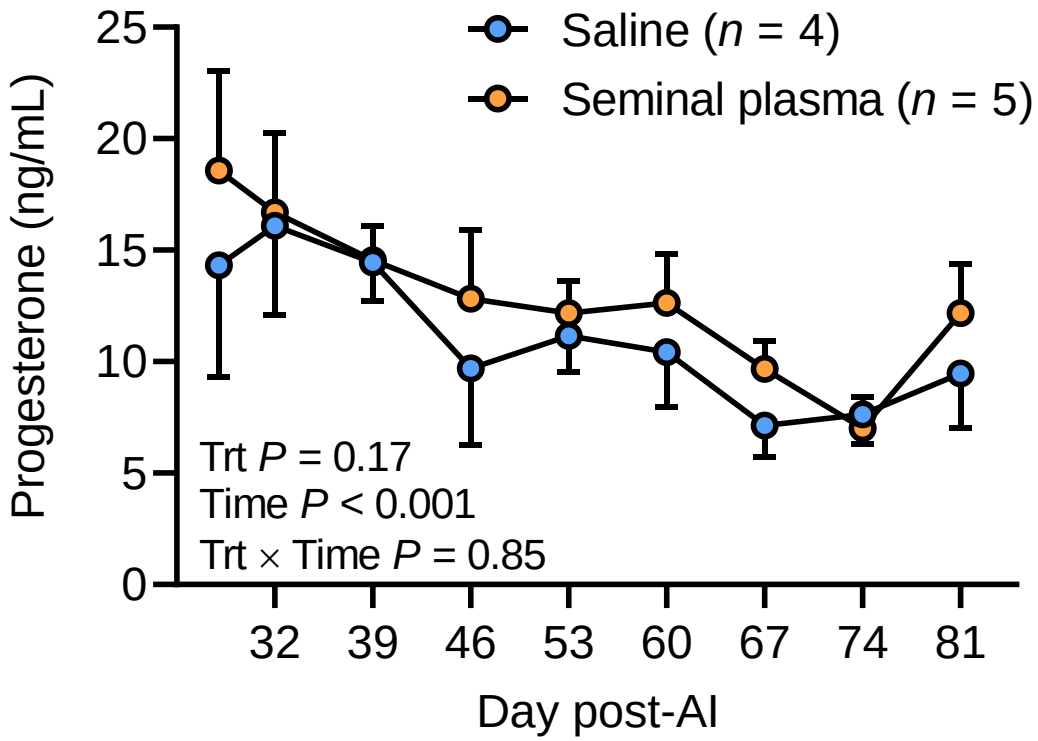

Figure 2-7. Effect of intrauterine infusion of seminal plasma on circulating progesterone. Plasma was collected on d 28, 32, 39, 46, 53, 60, 67, 74 and 81 after TAI and intrauterine infusion of either saline or seminal plasma. Peripheral progesterone concentration was analyzed by repeated measures ANOVA using a general linear model including the fixed effects of treatment (saline *vs.* seminal plasma), day and the interaction between treatment and day. Data are presented as least squares means ± SEM.

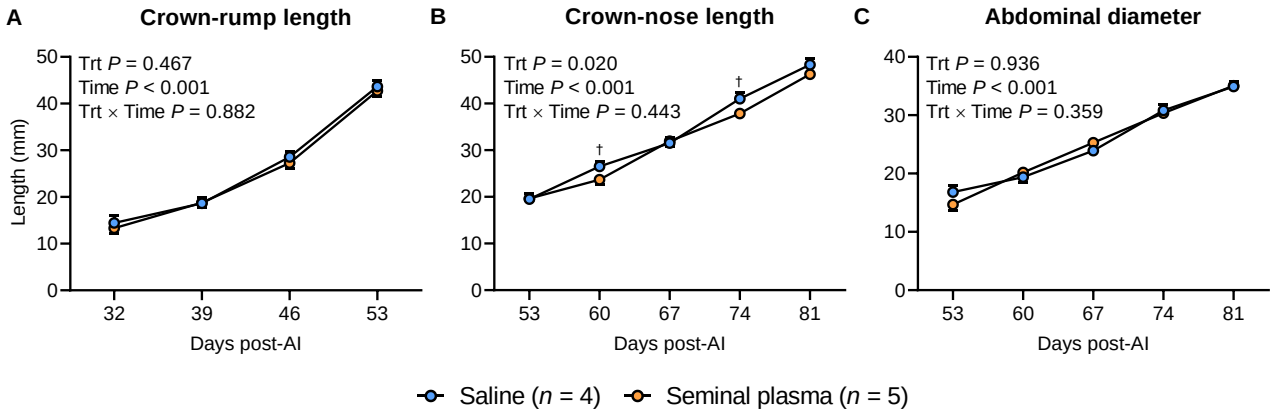

Figure 2-8. Effect of intrauterine infusion of seminal plasma on fetal growth. Cows were subjected to transrectal ultrasound weekly from d 32 until d 81 after TAI and intrauterine infusion of either saline or seminal plasma. Fetal crown/rump length (A), crown/nose length (B) and abdominal diameter (C) were evaluated in singleton female pregnancies only. Fetal growth parameters were analyzed by repeated measures ANOVA using a general linear model including the fixed effects of treatment (saline *vs.* seminal plasma), day and the interaction between treatment and day. Data are presented as least squares means ± SEM. † represents $P \leq 0.08$ when comparing treatments within a single time point.

Table 2-9. Effect of intrauterine infusion of seminal plasma on conceptus parameters at d 96 of gestation.

	Saline (*n* = 4)	Seminal Plasma (*n* = 5)	*P*-value
Conceptus weight (kg)	1.6 ± 0.1	1.8 ± 0.1	0.133
Conceptus length (cm)	109 ± 3	125 ± 9	0.157
Amniotic vesicle width (cm)	19.0 ± 0.4	19.1 ± 0.6	0.897
Amniotic vesicle length (cm)	31.1 ± 1.2	33.4 ± 1.1	0.229
Amniotic fluid (mL)	856.3 ± 23.6	981.3 ± 45.5	0.050
Number of cotyledons[1]	62.3 ± 4.9	69.8 ± 6.3	0.397
Average cotyledon weight (g)[2]	3.9 ± 0.5	3.5 ± 0.3	0.448
Average cotyledon diameter (mm)[3]	49.2 ± 1.7	44.7 ± 1.5	0.086
Fetal body weight (g)	273.8 ± 9.2	281.0 ± 8.7	0.588
Eviscerated fetal weight (g)	208.8 ± 5.5	221.0 ± 8.3	0.285
Umbilical cord diameter (mm)	9.7 ± 0.3	8.3 ± 0.6	0.131
Crown-rump length (cm)	17.8 ± 0.5	18.1 ± 0.3	0.498
Crown-nose length (cm)	6.5 ± 0.0	6.4 ± 0.2	0.529
Heart girth (cm)	14.6 ± 0.2	14.3 ± 0.1	0.080

Only cows confirmed pregnant with a female singleton pregnancy were included in the analysis. For fetal morphometry and conceptus measurements on d 96 of gestation, data was analyzed using a general linear model including the fixed effect of treatment (saline *vs.* seminal plasma). Data are presented as the least square mean ± SEM.

[1] Absolute number of cotyledons of each placenta.

[2] Average cotyledon weight of 10 cotyledons in close proximity of the fetus.

[3] Average diameter of 10 cotyledons in close proximity of the fetus that were also evaluated for cotyledon weight.

Table 2-10. Effect of intrauterine infusion of seminal plasma on fetal organ weight at day 96 of gestation.

Fetal Organ	Saline (n = 4)	Seminal Plasma (n = 5)	P-value
Heart (g)	2.0 ± 0.1	1.8 ± 0.1	0.287
Paired lungs (g)	11.2 ± 0.6	10.5 ± 0.8	0.511
Combined heart and lungs (g)	13.3 ± 0.6	12.4 ± 0.8	0.400
Paired kidney (g)	2.6 ± 0.3	2.7 ± 0.1	0.684
Liver (g)	13.9 ± 0.3	12.8 ± 0.8	0.283
Spleen (g)	0.6 ± 0.0	0.6 ± 0.0	0.250
Brain (g)	7.5 ± 0.2	7.8 ± 0.5	0.714

Data was analyzed using a general linear model including the fixed effect of treatment (saline *vs.* seminal plasma). Data are presented as least square means ± SEM.

CHAPTER 3
DISCUSSION AND CONCLUSIONS

Discussion

The fluid fraction of the ejaculate, seminal plasma, is not required for pregnancy establishment as shown by the success of artificial insemination and embryo transfer in cattle. In rodents, swine and humans, exposure to seminal plasma at the time of insemination has been shown to improve pregnancy outcomes by increasing establishment of pregnancy, improving embryo development and even programing postnatal outcomes of subsequent offspring. Evidence suggests that seminal plasma supplementation at insemination increases birth weight of heifer calves in cattle; however, it is not clear if seminal plasma exposure improves bovine pregnancy outcomes in a similar fashion to other species. The data described in this thesis were collected to address the hypothesis that seminal plasma exposure at the time of insemination modifies the endometrial environment and improves embryo quality and fetal growth. Results presented here demonstrate that exposure to seminal plasma at insemination 1) alters the endometrial transcriptome 7 d after insemination, 2) altered blastocyst expression of *DNMT3A* and *PTGS2*, and 3) altered fetal development during the first trimester of gestation. Collectively these studies suggest that seminal plasma exposure at insemination has the potential to modulate pregnancy outcomes in cattle.

Artificial insemination has been employed worldwide in cattle for more than 100 years due to its effectiveness in disseminating superior genetic traits (Hall & Ph, 2019). Improvements in methods for cryopreservation and semen processing have provided producers access to high fertility bulls based on phenotypic assessments of sperm motility and morphology (Butler et al., 2020). The seminal plasma fraction of semen

used in artificial insemination is often diluted in semen extenders or removed completely during processing, and contrary to natural mating when semen is deposited into the vagina, diluted semen is placed directly into the uterine body during artificial insemination. Interestingly, two independent studies have demonstrated that seminal plasma supplementation at the time of insemination does not improve pregnancy rate or pregnancy loss in cattle (Odhiambo et al., 2009; Ortiz et al., 2019). While it has been demonstrated that cattle have a leucocytic response in the uterus after semen deposition into the vagina, it is unclear if this response is beneficial to pregnancy success, or if it can be recapitulated when semen or seminal plasma is deposited directly into the uterus (Howe & Black, 1963). This beggars the question if pregnancy outcomes following artificial insemination truly reflect the normal physiological state when undiluted semen is deposited into the vagina during copulation.

Studies in mice, humans and swine demonstrate that seminal plasma is the active component of the ejaculate to elicit an inflammatory response in maternal tract tissues and subsequently impact the developing preimplantation embryo (Robertson, 2007). Here, I demonstrate that seminal plasma exposure at insemination did not increase the yield or developmental progression of embryos collected after ovarian stimulation; however, in blastocyst of cows exposed to seminal plasma at insemination the expression of *DNMT3A* was increased, while expression of *PTGS2* was decreased (Tesfaye et al., 2004; Sagirkaya et al., 2006). In mice, DNMT3A is responsible for *de novo* DNA methylation and is important for developmental progression of the embryo and establishment of the epigenome (Uysal et al., 2021). The cyclooxygenase enzyme PTGS2 (also known as COX-2) is involved in prostaglandin synthesis and has been

shown to be required for preimplantation embryo survival in sheep (O'Neil et al., 2020). Additional analysis and further experimentation is required to understand the molecular mechanism by which maternal exposure to seminal plasma modifies gene expression in the embryo; however, it is intriguing to consider that altered DNA methylation of the embryo (due to increased *DNMT3A* abundance) could impact fetal/placental development and even postnatal phenotype.

Of great interest is the fact that intrauterine infusion of seminal plasma at insemination could induced changes to the endometrial transcriptome seven days later at a time when the embryo is leaving the oviduct for the developmental environment of the uterus. Many of the identified canonical pathways and gene networks regulated by seminal plasma here are involved with 1) cellular immune responses, specifically cell-to-cell signaling and immune cell trafficking, 2) cellular growth, proliferation, and development, and 3) cell morphology and embryonic development. This suggest that an intrauterine infusion of seminal plasma has a long-term effect on the endometrium that could participate in the regulation of an immune response that promotes cell signaling and immune cell trafficking to the uterine environment - similar to what is observed in other species after seminal plasma or semen exposure. Moreover, pathways involved with cellular proliferation and development of the endometrium may impact endometrial tissue repair and remodeling processes required for embryo attachment and subsequent placentation. Finally, seminal plasma modulation of pathways involved in embryonic development; it may suggest that seminal plasma infusion could be altering the manner in which the endometrium supports the developing embryo and may be responsible for the altered gene expression observed in blastocysts collected after

seminal plasma infusion. A total of 19 upstream regulators of differentially expressed genes were identified seven days after intrauterine infusion of seminal plasma. Three upstream regulators [CITED2 (transcription factor), SIRT1 (transcription factor) and miR-124-3p (microRNA)] were predicted to be activated, while 16 upstream regulators were predicted to be inhibited after intrauterine infusion of seminal plasma. Of the inhibited upstream regulators TLR-4 and IL-2 were similar to molecules inhibited in the pig endometrium following seminal plasma infusion. It has been surmised that uncontrolled immune activation by TLR or IL-2 signaling during early pregnancy can result in pregnancy failure (Sykes et al., 2012; Firmal et al., 2020; Martinez et al., 2020). Together these data provide evidence that seminal plasma exerts an effect on the endometrial transcriptome at least seven days after initial exposure that could prepare the endometrium for embryo implantation and impact embryo development in cows.

In pigs, intrauterine infusion of seminal plasma increases the weight of the CL and enhances progesterone synthesis on d 5 and 9 of pregnancy (O'Leary et al., 2006). Here, exposure to seminal plasma did not affect peripheral progesterone circulation at d 28 of pregnancy, however we did not measure progesterone in peripheral blood at earlier time points when seminal plasma may be impacting CL function in the cow. In addition, embryonic factors like interferon tau may be contributing to CL function in the cow. As a proxy measure of interferon tau production by trophectoderm cells of the embryo, I measured interferon stimulated genes in peripheral blood mononuclear cells at d 21 of pregnancy (Spencer, 2014; Ding et al., 2022). Here, seminal plasma exposure did not impact the expression of the interferon stimulated genes *ISG15,*

OAS1Y, MX1 or *MX2* in peripheral blood, suggesting seminal plasma exposure does not alter interferon secretion by the early embryo.

Intrauterine infusion of seminal plasma at insemination increases birth weight of heifer calves born to primiparous dams bred with X-sorted semen. (Ortiz et al., 2019); however, it is not clear when the effect of seminal plasma on fetal growth begins in cattle. As such I evaluated the effects of an intrauterine infusion of seminal plasma on early fetal growth using ultrasound and on fetal/placental development at the end of the first trimester of pregnancy. Seminal plasma reduced fetal crown-nose length between d 53 and d 81 of gestation, however this effect was no longer observed when morphometric measurements of the fetus were assessed at d 96 of gestation. Bromfield et al. (2014) demonstrated that the absence of seminal plasma at insemination caused placental hypertrophy and reduced fetal:placental weight ratio in mice at the end of gestation, suggesting an effect of seminal plasma on placenta development. In the present study evaluated extraembryonic features on d 96 of gestation and observed that seminal plasma tended to reduce the average cotyledon diameter while increasing amniotic fluid by volume by 125 mL. I will need to further characterize the chemical constituents of the amniotic fluid and attempt to evaluate the effect of seminal plasma exposure on placental function and efficiency to ascertain the role of seminal plasma in modulating placental development and function. While seminal plasma exposure moderately reduced fetal heart girth, there was no apparent effect on fetal weight or weight of fetal organs including the heart, brain, lungs, kidney, liver, or spleen. However, to understand how seminal plasma mediates changes to birth weight I will need to further assess fetal/placental development later in gestation in addition to evaluating

global DNA methylation of fetal liver and muscle tissues. The ultimate goal is to assess the validity of using intrauterine seminal plasma supplementation at the time of conception to modulate pregnancy outcomes and postnatal phenotype of cattle.

Conclusion

The effects of intrauterine infusion of seminal plasma at insemination in cows seem to be long-term, altering blastocyst quality, endometrial transcriptome, fetal development and ultimately birth weight. Here, I present a novel study that adds to our understanding of how seminal plasma exposure may regulate pregnancy outcomes by modulating the endometrial environment that drives the developmental progression of the early embryo. As such, I observed altered gene expression in blastocysts developed in the presence of seminal plasma that may be associated with the changes in fetal growth observed during early gestation. Future work will clarify the effect of seminal plasma on placental function and DNA methylation in fetal tissues. The current results expand our understanding for a contribution of the male at conception beyond genetics, and further support the role of paternal factors in pregnancy outcomes observed in other species. These data may help to develop practical applications by which pregnancy outcomes, fetal development and postnatal success could be improved in cattle to advance production beyond the use of genetics by using fetal programing as a tool.

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BIOGRAPHICAL SKETCH

Gabriela was born in Morazan, El Salvador. In 2012, she moved to Honduras to pursue her undergraduate studies at Zamorano University, where she earned a Bachelor of Agricultural Sciences degree in 2015. During her time at Zamorano, she was exposed to dairy and beef cattle management. Following graduation in 2016, Gabriela worked at North Florida Holsteins (Bell, Florida) where she spent a year working in different departments including the calf yard, maternity barn, hospital barn, heifer management, herd health and reproduction management. During her time at North Florida Holsteins, she was exposed to veterinarians form the University of Florida and they awakened her desire to conduct research. She returned to El Salvador to work in a small dual-purpose farm. In 2018, she came back to North Florida Holsteins to train in reproductive biotechnologies such as embryo transfer, conventional embryo flushing, and ultrasonography. This experience reinforced her experience in herd health and reproductive management. She is profoundly grateful to Mr. Donald Bennik, John Karanja, Dr. Kelsey Arrellano and Dr. Rafael Bisinotto, for mentoring and training her during her time at North Florida Holsteins. In 2019, she started an internship in the laboratory of Dr. John J. Bromfield to work on a large-scale research project testing a novel drug for prevention of metritis. In the same year, after COVID hit, she persevered and won a scholarship to begin her master's degree with Dr. Bromfield in the Department of Animal Sciences at the University of Florida. She focused her studies on the role of seminal plasma in regulation of the endometrial environment and early and conceptus development. After graduating with a Master of Science degree in August 2022, Gabriela will stay in Gainesville to work towards her Doctor of Philosophy degree with Dr. Bromfield.