ENDOMETRIAL RESPONSE TO SEMEN COMPONENTS IN THE BOVINE

By

JASON A. RIZO

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2019

To my mother, my father, and the Nicaraguan students who gave their life for a better country, who have given me the determination to be a better version of myself

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTB	Beta-actin
AI	Artificial insemination
ATCC	American type culture collection
BEND	Bovine endometrial epithelial cells
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BSE	Breeding soundness evaluation
BSP	Bovine Seminal Plasma protein
cDNA	Complementary deoxyribonucleic acid
COX	Cyclooxygenase
CSF	Colony-stimulating factor
DAPK	Death-associated protein kinase
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal–regulated kinases
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotropin-releasing hormone
HBSS	Hank's balanced salt solution

HCL	Hydrogen chloride
ICM	Inner cell mass
IFNγ	Interferon gamma
IFNτ	Interferon tau
IGF	Insulin-like growth factor
IL	Interleukin
ILR	Interleukin receptor
ILST	Interleukin signal transducer
IVF	*In vitro* fertilization
JAK	Janus kinase
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharides
MADD	MAP kinase-activating death domain
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIQE	Minimum information for publication of qPCR experiments
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NK cell	Natural killer cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
P13K	Phosphoinositide 3-kinase
PBS	Phosphate-buffered saline
PGE	Prostaglandin E

PGF2α	Prostaglandin F2 alpha
PGFS	Prostaglandin F synthase
PTGS	Prostaglandin-endoperoxide synthase
qPCR	Quantitative polymerase chain reaction
RIPK3	Receptor-interacting serine/threonine-protein kinase 3
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Standard error of the mean
SM	Semen
SP	Seminal plasma
SPSS	Statistical Package for the Social Sciences
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
Th cells	T helper lymphocytes
Treg cells	Regulatory T cells
uNK	Uterine natural killer cell
γδ T cells	Gamma-delta T cells

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

ENDOMETRIAL RESPONSE TO SEMEN COMPONENTS IN THE BOVINE

By

Jason A. Rizo

May 2019

Chair: John J. Bromfield
Major: Animal Molecular and Cellular Biology

Seminal plasma is the non-cellular fraction of semen derived from the male

accessory glands. Seminal plasma is important for sperm support; however, it can also

interact with the uterus to improve pregnancy outcomes. Seminal plasma, or seminal

plasma-derived transforming growth factor beta, induces synthesis of proinflammatory

cytokines following insemination in humans, mice and swine, which culminates in an

influx of leukocytes into the endometrium or cervix. This inflammatory response at

insemination influences the survival of the semi-allogenic conceptus during implantation

and the development of the offspring. Cows undergoing artificial insemination using

diluted/extended semen are not exposed to physiological components of the ejaculate

similar to natural mating. It is currently unknown whether components in semen or

seminal plasma directly elicit endometrial inflammation in cattle. We hypothesize that

seminal plasma or TGF-β would increase the expression of proinflammatory factors in

the bovine endometrium, influencing the uterine environment during early pregnancy.

Our data demonstrate that seminal plasma has a cytotoxic effect on endometrial cells

caused by a heat-labile substance. Using culture of bovine endometrial epithelial

(BEND) cells, seminal plasma increased expression of *IL6* and differentially regulated

expression of *IL1B* in an estrogen dependent manner. Exposure of endometrial cells to TGF-β increased expression of *TNF* but only in the absence of estrogen. In contrast, TGF-β increased expression of *IL6* only after estrogen exposure. Finally, intra-uterine infusion of seminal plasma in cows at estrus increased expression of endometrial *IL1B* but had no effect on the expression of other inflammatory markers 24 h after treatment. These data suggest that seminal plasma, and potentially TGF-β, influences endometrial expression of maternal cytokines; however, this response could be bull specific and may involve cytotoxic effects of semen. The identification of paternal factors contained in semen that have the potential to improve bovine reproduction and reduce economic losses associated with pregnancy failure could help optimize semen used for artificial insemination.

CHAPTER 1
LITERATURE REVIEW

Introduction

Reproductive success is a process mediated by the complex interaction of male and female factors. The maternal environment during the periconceptional period undergoes an inflammatory response promoted by immune and uterine cell secretions in response to semen (Katila, 2012). This inflammation at insemination influences the survival of the semi-allogenic conceptus during implantation and the development of the offspring. Disruptions to this process are detrimental for pregnancy outcomes (Robertson et al., 2018). In dairy cows, conception rates have declined by 20% from 1951 to 2001 (Lucy, 2001), which coincides with increased productivity that may affect negatively reproduction (Butler and Smith, 1989). Indeed, embryo losses in the first three weeks of pregnancy account for 35% of pregnancy failures in dairy cows (Santos et al., 2004; Diskin et al., 2006). A major challenge for dairy managers is to improve pregnancy rates and minimize embryonic losses.

In recent years, paternal genetics have been associated with improving pregnancy in the dairy cow, specifically preimplantation embryo development, conceptus elongation, placentation, and pregnancy recognition (Han and Peñagaricano, 2016; Abdollahi-Arpanahi et al., 2017; Ortega et al., 2018). The economic value associated with bull fertility corresponds to the number of inseminations required to achieve pregnancy; however, this number is increasing as a consequence of compromised fertility observed in the dairy industry (Lucy, 2001). Interestingly, factors present in the plasma fraction of semen in several species have immune-regulatory potential (Bromfield, 2016). The deposition of these factors, including hormones and

cytokines, into the female reproductive tract may modulate the maternal immune response to pregnancy (Bromfield et al., 2018). Considering these effects as evolutionary adaptions in reproductive strategies, males may have evolved to further their own reproductive success by 'dosing' females with factors present in semen that promote fertility outcomes (de Catanzaro and Pollock, 2016). The work presented here will attempt to appraise the capacity for seminal plasma to elicit acute inflammatory responses associated with the preparation of an optimal uterine environment in early pregnancy.

Changes in the Female Reproductive Tract Regulating Pregnancy

The success of implantation, placental development, fetal growth, and the long-term health of the offspring constitute the normal function of the female reproductive tract and is influenced by changes in the periconceptional uterine environment. The luminal content of the female tract is primarily regulated by immune and uterine cells which are the main sources of signaling molecules including cytokines (Hannan et al., 2014). The endometrium consists of different groups of cells that are subjected to dynamic remodeling in order to establish a microenvironment that promotes a positive maternal response to pregnancy (Okada et al., 2018). The main cell types are 1) the epithelial cells forming the uterine lining of the luminal compartment that have direct contact with the embryo and 2) fibroblast-like stromal cells, which form a layer below the uterine lining and are the main site of tissue remodeling by regulating secretion of proangiogenic cytokines (Forde and Lonergan, 2012). Considering this, the periconceptional uterine environment is a balance between factors that regulate cellular differentiation and apoptosis of the developing embryo before implantation and

15

remodeling events to support further development following embryo implantation or attachment.

In some mammals, female fertility is regulated in a cyclic manner by ovarian hormones with species-specific differences; therefore, uterine synthesis of cytokines fluctuates depending on the stage of the estrous cycle. The estrous cycle coordinates the expression of reproductive tract cytokines that affect the functional status of the oviduct and endometrium (Ozawa et al., 2016; Spencer et al., 2016a; Li and Winuthayanon, 2017; Tríbulo et al., 2018; Yu et al., 2018). Following ovulation, high concentrations of estrogen promote cellular differentiation and stimulate uterine motility associated with passive transport of sperm (Bulletti et al., 2000). Estrogen also regulates follicular growth and corpus luteum formation and function via estrogen receptor alpha (Li et al., 2017). The presence of estrogen increases the expression of its own receptor at estrus (Berisha et al., 2002). The presence of the embryo does not influence the expression of the estrogen receptor (Robinson et al., 1999). The pattern of endometrial expression of some cytokines varies during this estrogenic period, suggesting an interaction between estrogen receptor and the capacity for individual uterine cells to produce cytokines. In mice, the expression of cytokines including colony-stimulating factor 2 (*Csf2)*, tumor necrosis factor (*Tnf*), interleukin (*Il1b)*, and *Il6* varies depending on the stage of the estrous cycle (Orsi et al., 2006).

High levels of progesterone are present in the uterine lumen when the newly formed embryo implants or attaches. After fertilization, the bovine embryo reaches the endometrium on day 4 to 6 and continues to differentiate into morula and blastocyst stages around day 7. The success of these differentiation events coincides with

progesterone-mediated uterine quiescence and the synthesis of cytokines associated with the resolution of inflammation in the female reproductive tract, reducing expression of interferon-gamma (*IFNG*) and transforming growth factor (*TGF*), inhibiting cytotoxic immune cells (Albieri et al., 2005; Dorniak et al., 2011). Species-specific reproductive strategies may confer functional advantages at mating, especially in female reproductive tissues. For instance, semen deposition occurs in the vagina in humans and cattle, while in mice and swine semen is deposited directly into the uterus; this allows for local regulation of female tissues depending on the species. Sperm interaction with epithelial cells occurs mainly in the endometrium and oviduct, where selection by phagocytosis takes place (Suarez and Pacey, 2006).

Pre-Implantation Embryo Development

Following ovulation, fertilization of the oocyte by sperm results in the formation of a semi-allogeneic zygote containing paternal and maternal antigens. In the bovine, a series of cellular cleavage events occur in the oviduct resulting in the formation of the morula that will enter the uterus on day 5 after fertilization. The morula develops into a blastocyst, containing the inner cell mass, which give rise to the fetus and the trophectoderm that will later form the placenta. After the blastocyst hatches from the zona pellucida around day 7 to 9, it undergoes transformation into an ovoid or tubular shape around days 12 to 14 (Betteridge et al., 1980). This process is then followed by a rapid elongation of the conceptus required for secretion of interferon-tau (IFN-τ) which mediates maternal recognition of pregnancy (Roberts, 2007). Embryo attachment to the endometrium and subsequent placentation begins around day 19 of pregnancy.

The success of implantation is the result of appropriate embryo development to the blastocyst stage and at the same time a proper receptivity of the endometrium towards the embryo (Singh et al., 2011). This is a dynamic process controlled by a functional synchronization of the developmental stage of the embryo itself with cellular and molecular changes occurring locally in the endometrium. This process involves the coordination of effects from autocrine, paracrine and endocrine factors (Fujiwara et al., 2016), and the action of maternal immune cells to allow survival of the semi-allogeneic conceptus (Svensson-Arvelund et al., 2015).

A broad array of maternal molecules are released into the female reproductive tract to support embryo development. The biological function of a group of growth factors, hormones and cytokines involved in the regulation of preimplantation embryos has been described (Robertson et al., 2015), and specifically discussed below. Characterization of the maternal tract environment made *in vitro* fertilization (IVF) possible (Wang and Sauer, 2006), facilitating embryo development to the blastocyst stage under laboratory conditions; however, some pregnancy pathologies derived from IVF are still reported (Refaat et al., 2015). In humans, around 5% of women suffer from consecutive pregnancy losses, and nearly 75% of these pregnancies fail before embryo implantation (Comins Boo et al., 2016). Similarly in domestic species, approximately 20% to 50% of dairy cows experience embryo loss during the first week of pregnancy after oocyte fertilization (Wiltbank et al., 2016). The utility of IVF enables the development of the embryo to the blastocyst stage of development prior to transfer to embryo recipients; nevertheless, high rates of embryo mortality still occur following embryo transfer on day 7 in cows (Berg et al., 2010). The development of embryos from

the blastocyst stage until conceptus elongation has not yet been achieved *in vitro* (Martal et al., 1986), suggesting that the female reproductive tissues play a major role in embryonic development which may impact later stages of pregnancy.

Maternal Recognition of Pregnancy and Embryo Development after Implantation

The implantation of embryos requires trophoblast cells to interact with cells of the endometrium to establish the fetal-maternal interphase (Teh et al., 2016) and promote placental formation. Species-specific differences exist in the degree of trophoblast invasion into maternal tissues. Hemochorial placentation in humans and rodents requires high levels of invasion of placental tissues into the endometrium, regulated by the decidualization of endometrial stromal cells (Teklenburg et al., 2010). In contrast, the epitheliochorial placenta of ruminants is a non-invasive interaction between placental cells and the endometrium (Calle et al., 2019).

Prior to the establishment of the placenta, the survival of the embryo depends on maternal secretions, collectively referred to as histotroph (Gao et al., 2009). Several molecular markers expressed in the endometrium during the period of embryo implantation are associated with uterine receptivity, including cell adhesion molecules, cytokines, growth factors, uterine natural killer (uNK) cells, and other endometrial leukocytes (Achache and Revel, 2006). Combined, these factors promote angiogenesis and secretion of chemoattractant molecules to facilitate vascularization, tissue remodeling, trophoblast invasion, and placenta development (Koopman et al., 2003; Fu et al., 2017).

In ruminants, the major embryo-derived molecule modulating pregnancy recognition is IFN-τ secreted by trophectoderm cells of the elongated conceptus

(Spencer et al., 2008). Secretion of embryonic IFN-τ prevents endometrial secretion of

prostaglandin F2-alpha (PGF2-α), which has luteolytic effects on the corpus luteum

resulting in a return to estrus. Consequently, secretion of prostaglandin E2 (PGE2)

predominates (Schröpfer et al., 2008), resulting in maintained corpus luteum function

and elevated progesterone required for pregnancy maintenance. In the human, this

effect is similarly modulated by embryonic expression of human chorionic gonadotropin

(Schüler-Toprak et al., 2017). Bovine endometrial cells produce cyclooxygenases

(COX) and prostaglandin-endoperoxide synthases (PTGS) that modulate the synthesis

of prostaglandins (Okuda et al., 2002). Specifically, COX2 and PTGS2 play a role in the

protection of the corpus luteum from PGF2-α-induced luteolysis and in early embryo

survival by increasing progesterone concentration during the preimplantation period

(Spencer and Bazer, 2004; Zhang et al., 2007). The size of the conceptus is associated

with its capacity to produce IFN-τ (Hansen et al., 2017). Whether IFN-τ concentrations,

or larger embryos facilitate pregnancy success is difficult to test as flushing embryos to

discriminate by size and then transferring them into a new recipient presents several

technical challenges (Betteridge et al., 1980). In contrast, it has been demonstrated that

low concentrations of IFN-τ can induce maximal expression of IFN-responsive genes

(Matsuyama et al., 2012). The cellular response and molecular mechanisms of

pregnancy recognition and implantation are directly affected by the composition of the

histotroph under the influence of progesterone (Forde and Lonergan, 2012). Embryo

losses during this period can be the result of impaired embryo development, delayed

conceptus elongation, poor uterine receptivity and/or lack of signals necessary for

proper maternal immune response to pregnancy.

Maternal Immune Response to Pregnancy

Pregnancy involves a unique immune condition in the mother to permit the immune system to respond to pathogens, while allowing the survival of the semi-allogeneic embryo. Maternal immune modulation initiates early in pregnancy before embryo implantation and involves signals provided by the embryo, mother and potentially the father, which coordinate the response of the female reproductive tissues in a timely manner to ensure establishment and maintenance of pregnancy. Cytokines are critically involved in the communication between embryo and mother (Robertson et al., 2015) and their role will be discussed next.

Secretion of cytokines by endometrial immune and epithelial cells into the lumen of the uterus can have a direct influence on the survival of the preimplantation embryo (Robertson, 2005; Moldenhauer et al., 2010). The molecular events involved in cytokine signaling are complex and include several pathways, providing mechanisms to either support or possibly hinder embryos if conditions in utero do not favor reproduction; for example metabolic stress, infection, or environmental stressors (e.g. heat stress). Maternal cytokines support embryos and promote fetal growth (Sjöblom et al., 2005). Studies using gene knockout-mice have shown that disruption of cytokine signaling results in negative consequences for the developing embryo and subsequent pregnancy (Ingman and Jones, 2008). This evidence comes mainly from mouse models (Robertson et al., 2018); however, addition of cytokines such as CSF-2 to culture medium of bovine embryos improves embryo development and alters offspring phenotype at birth (Kannampuzha-Francis et al., 2015).

Several immune cells of the endometrium secrete cytokines including macrophages, dendritic cells, granulocytes, mast cells, and lymphocytes, which have

pivotal roles in endometrial inflammation during pregnancy (Fair, 2015). Human and mouse natural killer cells represents about 70% of the total endometrial leukocytes during pregnancy. The absence of these cells types during pregnancy results in the termination of pregnancy in humans and mice (Gaynor and Colucci, 2017). The initiation of pregnancy is marked by endometrial inflammation resulting from the physiological changes required to recognize the embryo when implantation occurs, which must be resolved for successful pregnancy maintenance (Mor and Cardenas, 2010; Katila, 2012; Robertson et al., 2018). The balance of pro-inflammatory and anti-inflammatory responses is mediated by thymus-derived T helper (Th) lymphocytes. Cytokines produced by Th1 cells promote inflammation whereas Th2 cells secrete cytokines with anti-inflammatory capacity. Th17 and T regulatory (Treg) lymphocytes also participate in the inflammatory transition required to balance the progression of pregnancy (Saito et al., 2010). The action of maternal immune cells during early embryonic development suggest that the maternal immune system is active within the endometrium and controlled in such a manner to facilitate and protect pregnancy.

Inflammation of the Pre-Implantation Uterus

Uterine inflammation contributes to the success of pregnancy. Following insemination, spermatozoa are removed from the maternal reproductive tract by granulocytes, including neutrophil-mediated phagocytosis (Katila, 2012). Uterine influx of immune cells and simultaneous expression of pro-inflammatory cytokines is associated with sperm and pathogen clearance, but may play a role in maternal recognition of paternal antigens expressed by the ensuing embryo. Neutrophils and dendritic cells are involved in triggering Th1-mediated inflammation of the female reproductive tract. Expression of pro-inflammatory cytokines and chemokines by these

cells further influences the activation, attraction and phenotypic behavior of additional immune cells in the uterus (Bennouna et al., 2003). This is important in the immune regulation of anti-inflammatory cytokines during implantation, when the embryo will express both maternal and paternal antigens. A myriad of cytokines that either promote or inhibit acute inflammation influence different aspects of pregnancy events. A balance of Th1, Th2, Th17, and Treg lymphocytes characterizes the maternal immune response of successful pregnancies (Saito et al., 2010; Robertson et al., 2018). Embryos express receptors for specific cytokines, and also produce cytokines, including growth factors, which impact embryo survival (Hardy and Spanos, 2002).

For embryo implantation to occur the endometrium undergoes a process of tissue remodeling, including angiogenesis mediated by cytokine secretion under the influence of estrogen and progesterone (Granot et al., 2012). The period of implantation results in the accumulation of endometrial macrophages (Korte et al., 1993). Hemochorial placentation provokes an injury to the endometrium, and therefore inflammation is required to resolve such damage. Specific to pregnancy, the maternal immune system must also support the growth of the semi-allogeneic embryo, which is foreign to the maternal immune system. In contrast, less invasive epitheliochorial placentation may involve a different degree of immune response (Tekin and Hansen, 2004); however, similar cytokines are produced in both scenarios (Robertson et al., 2015).

Pregnancy occurs under the influence of progesterone resulting in an anti-inflammatory endometrial phenotype (Mor and Cardenas, 2010). A balance in the expression of cytokines that modulate the maternal immune system is required for

embryo development, while an inappropriate balance of immune factors has been associated with pregnancy complications including embryo loss, restriction of fetal growth, and preeclampsia (Robertson et al., 2006, 2007).

Cytokines Influencing Early Embryonic Development

The function of cytokines go beyond regulation of immune cells during pregnancy, they also signal uterine cells and influence embryo survival. Embryos express receptors for cytokines (Robertson et al., 2015), and changes in the balance of cytokines in the oviduct and endometrium have the capacity to influence cellular differentiation and expression of genes in the embryo, consequently affecting embryo development (Sharkey et al., 1995; Guerin et al., 2011). A study using mice to monitor the serum profile of eighteen cytokines throughout the estrous cycle and pregnancy reported that most of the analyzed cytokines were present at all stages including interleukin (IL)-1α, IL-1β, IL-6, IL-10, IL-12, IL-17, CSF-2, TNF-α, and IFN-γ (Orsi et al., 2006). Similar expression patterns in the bovine uterus have been reported (Tríbulo et al., 2018). Concentrations of IFN-γ and IL-12 are decreased following coitus in mice, which is consistent with data suggesting that constant expression of these cytokines is detrimental for pregnancy (Lentsch et al., 1996; Mahdi, 2011). Conversely, high uterine expression of leukemia inhibitory factor (*Lif*) during early pregnancy, specifically during implantation, has been associated with embryo growth in mice (Bhatt et al., 1991). Supplementation of cytokines to embryo culture *in vitro*, including CSF-2, IL-6, and insulin-like growth factor 1 (IGF-1), results in embryotrophic effects, supporting embryo development. Conversely, exposure of embryos to TNF exerts embryotoxic effects (Hansen et al., 2014; Robertson et al., 2015). The synthesis of cytokines is normally regulated in response to different stressors (e.g. infection); therefore, the expression

profile of cytokines in the endometrium might imply a mechanism by which the embryo can react and adapt to the status of the mother. The role of well characterized embryotrophic and embryotoxic cytokines is discussed below.

Colony-stimulating factor 2 (CSF-2)

Colony-stimulating factor 2 (previously granulocyte-macrophage CSF) acts as a hematopoietic growth factor promoting cellular proliferation (Morstyn and Burgess, 1988), activating myeloid hematopoietic cells (Ruef and Coleman, 1990), and promoting competence of the preimplantation embryo (Hansen et al., 2014). CSF-2 is produced by lymphocytes, macrophages, mast cells, and cells in the oviduct and endometrium (De Moraes et al., 1999). In mice, maternal *Csf2* expression correlates with the period of oocyte fertilization along with embryo development and implantation around day 4 (Chegini et al., 1999); in fact, the concentration of CSF-2 in uterine fluid of mice at estrus is 173-fold higher than present in serum (Orsi et al., 2006). Bovine embryos cultured in the presence of CSF-2 (day 5 to 7) result in blastocysts containing an increased number of inner cell mass cells with higher survival rates compared to control embryos (Dobbs et al., 2013).

The signaling for CSF-2 is mediated by a cell surface receptor with two subunits, alpha and beta, and downstream cascades involve JAK/STAT, MAPK, and P13K pathways (Dhar-Mascareño et al., 2003). In embryos only the alpha subunit, which has low-binding affinity to CSF-2, is expressed, while the beta subunit of the receptor is not active in embryos of humans and other species (Sjöblom et al., 2002). However, signal transduction and phosphorylation of the STAT5 pathway occurs through the alpha subunit, activating transcription factors to promote cellular proliferation (Mui et al., 1995). This mechanism may allow to promote embryo survival in bovines (Dobbs et al.,

2013). Robertson et al. showed that *Csf2* is not expressed in the mouse embryo, suggesting that CSF-2 secreted from the oviductal and endometrial epithelium of the mother acts through paracrine mechanisms to control embryo development (Robertson et al., 2001). Knockout *Csf2* mice have smaller litters as a consequence of disrupted embryo development. While *Csf2* knockout-embryos contain less trophectoderm and inner cell mass cells compared to wild type embryos (Robertson et al., 2001). Knockout *Csf2* mice also have alterations in the expression of genes involved in placental function (Sferruzzi-Perri et al., 2009). Furthermore, the supplementation of CSF-2 to embryo culture improves clinical pregnancies in human and bovine IVF programs (Ziebe et al., 2013).

Calves resulting from embryos supplemented with CSF-2 have higher body weight at birth compared to non-CSF-2 treated embryos (Kannampuzha-Francis et al., 2015). *In vitro*, CSF-2 regulates bovine preimplantation embryos by inhibiting the expression of genes such as *DAPK1*, *MADD* and *RIPK3*, this results in decreased embryo apoptosis (Loureiro et al., 2011). The effects exerted by CSF-2 on the embryo may be sex-dependent. Male embryos cultured with CSF-2 are longer and consequently have higher IFN-τ secretion, while opposite effects are observed in female embryos (Dobbs et al., 2014). Similar evidence for dimorphic effects have been reported in mice where CSF-2 supports development of male embryos (Sjöblom et al., 2005). In parallel, CSF-2 increases bovine blastocyst development in female but not male embryos (Siqueira and Hansen, 2016). It has also been shown that supplementation with CSF-2 (day 0 to 7) promotes development of preimplantation embryos in pigs (Kwak et al., 2012). Taken together, the effects reported in mice, pigs, human, and cattle suggest a

role for the maternal cytokine CSF-2 to communicate with the embryo and promote its development during the preimplantation period.

Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is a cytokine capable of both pro- and anti-inflammatory responses. Signaling of IL-6 is mediated through a receptor complex formed with two units, IL-6 receptor (IL6R) and IL-6 signal transducer (IL6ST), which exert a cascade of molecular events involving JAK/STAT and MAPK/ERK signaling pathways (Mihara et al., 2012). Additional IL-6 family member cytokines, LIF and IL-11, also mediate responses through IL6ST, suggesting redundancy between IL-6 type cytokines related to early embryo development in mice and humans (Thouas et al., 2015). The signaling transduction and phosphorylation of the signal transducer and activator of transcription 3 (STAT3) pathway by IL-6 results in activation of transcription factors to prevent cellular apoptosis; this has been reported in mice where treatment of preimplantation embryos with IL-6 *in vitro*, reduces blastomeres apoptosis (Shen et al., 2012). Both the endometrium (Jasper et al., 2007) and embryo (Sharkey et al., 1995) secrete IL-6 starting at early stages of development. In addition, IL-6 is associated with maintaining the balance of Th17 and Treg lymphocytes required to prevent recurrent spontaneous abortions in human (Saito et al., 2010).

In different species, culture of embryos in the presence of LIF, IGF-1 and IL-6 improves the developmental potential of embryos (Robertson et al., 2015). The secretion of IL-6 by human endometrial epithelial cells is suggested to promote blastocyst formation and contribute to higher implantation rates. Specifically, viable embryos metabolize more IL-6 protein in culture compared to embryos that do not result in implantation (Dominguez et al., 2010). IL-6 increases blastocyst hatching and total

27

number of blastomeres while reducing apoptosis in the mouse. However, high concentration of IL-6 are detrimental to embryo development (Shen et al., 2012; Kelley and Gardner, 2017). Studies using *Il6* knockout-mice have shown that animals are still fertile but their reproductive success is compromised due to low implantation rates and high incidence of abortions (Robertson and Sharkey, 2001). The presence of cytokines like IL-6 not only influences proinflamatory responses in pregnant tissues but also prevent apoptosis and promotes embryo survival.

Tumor necrosis factor (TNF)

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine with pleiotropic capacity that is part of the TNF receptor (TNFR) superfamily. TNF signals via phosphorylation and translocation of the nuclear factor kappa B (NFκB) to promote transcription of pro-inflammatory cytokines including *IL6* and *IL1B* (Baker and Reddy, 1998; Tanabe et al., 2010). TNF is produced by different cell types but including macrophages, NK cells, T cells (Hunt et al., 1993), and epithelial cells of the reproductive tract. Leukocytes accumulate at the sites of inflammation during infections, and TNF mediates the activation of monocytes and neutrophils, indicating the capacity for TNF to act as a toxic effector in the defense against pathogens (Ming et al., 1987). Expression of uterine *Tnf* changes during the estrous cycle, and fluctuates during the periconception period in mice (Robertson and Moldenhauer, 2014). Mouse and human embryos express the TNF receptor, *TNFR1*, and can therefore respond to TNF signaling. Furthermore, embryos have the capacity to secrete TNF when conditions are not favorable, which occurs in cases such as diabetic pregnancies (Kirwan et al., 2002; Kawamura et al., 2007).

The development of the preimplantation embryo is compromised following exposure to TNF *in vitro*. Treatment of mouse blastocysts with TNF for 24 hours increased the number of dead blastomeres after treatment (Fabian et al., 2007). Soto et al. (2003) reported that exposure of bovine oocytes to TNF had no effect on subsequent embryo cleavage but did affect development to the blastocyst stage. Conversely, exposure of bovine embryos to TNF did not affect the proportion of resulting blastocysts, but did increase blastocyst apoptosis (Soto et al., 2003). The balance between the anti-inflammatory cytokine IL-10 and the pro-inflammatory TNF may influence the maternal immune response to pregnancy, by resolving inflammation in the environment of the preimplantation embryo. Studies using *Il10* knockout-mice have shown that IL-10 is not essential for pregnancy success (White et al., 2004); however, increased uterine expression of *Tnf* and *Il6* in response to lipopolysaccharides (LPS) is decreased following administration of exogenous IL-10. This process of IL-10 mediated regulation of inflammation reduces uterine inflammation and subsequent LPS mediated fetal loss (Robertson et al., 2006). Furthermore, TNF mediates the response of bovine stromal endometrial cells during periods of inflammation (Jacca et al., 2013).

Interleukin 1β (IL-1β)

Interleukin 1 beta (IL-1β) is a proinflammatory cytokine that acts as a paracrine and autocrine factor through its functional receptor IL1R1, which elicits signaling pathways via NFκB (Mantovani et al., 1998). IL-1β and TNF are involved in the downstream secretion of IL-8, which is a potent chemokine of leukocytes and is involved in luteal progesterone synthesis (Talbott et al., 2014). IL-1β has been shown to increase the expression of *PTGS2* and synthesis of PGE2 in the endometrium under the influence of estrogen (Bazer, 2013), along with promoting decidualization of stromal

cells in humans (White et al., 2007). The embryo and endometrium of humans, mice, cattle, and swine secrete IL-1β and express the *IL1R1* receptor. The addition of IL-1β to endometrial tissues *in vitro* increases the expression of *IL1B* receptor during early pregnancy in pigs (Geisert et al., 2012). Blocking IL-1β signaling with a receptor antagonist increases implantation failure in mice (Zheng et al., 1995). Jasper et al. showed that macrophages recruited during the post-coital inflammatory response in mice secrete LIF and IL-1β, which regulate the glycosylation of proteins that allow trophectoderm attachment to the endometrium (Jasper et al., 2011). It has been reported that human endometrial cells increase the secretion of IL-1β around the time of implantation (Srivastava et al., 2013); high concentrations of IL-1β in the media of IVF embryos have been associated with improving embryo implantation and pregnancy success in women (Sequeira et al., 2015). Furthermore, the bovine preimplantation embryo has the capacity of communicating with the maternal immune system through IL-1β signaling. It was reported that the concentration of IL-1β in the bovine endometrium increases locally in response to the presence of an embryo for 3 days, which suggests that embryos can regulate endometrial receptivity during the first days of pregnancy in cattle (Correia-Álvarez et al., 2015). In effect, supplementation of IL-1β to IVF bovine embryos improves development to blastocyst stage (Paula-Lopes et al., 1998).

The Influence of Cytokines during Pregnancy

The preimplantation embryo grows in an environment rich in cytokines secreted by immune cells, epithelial cells of the uterine lining, and the embryo itself. Some of these cytokines have a Th1 pro-inflammatory phenotype including IL-6, IL-1β, TNF, and TNF-related apoptosis-inducing ligand (TRAIL), while others have Th2 anti-inflammatory

effects such as IL-10, IL-13 and IL-17 (Robertson et al., 2015). The regulation and balance of the two types of cytokines is fundamental for implantation of a competent embryo and subsequent pregnancy success. However, special care must be considered when extrapolating from mouse and human models to domestic species such as the bovine since there are several physiological differences between species. For instance, embryo implantation occurs on day 4 in mice while the conceptus attaches to the endometrium around day 19 in bovines. Nevertheless, experiments performed under *in vitro* settings using recombinant proteins of human or mouse origin have proven beneficial to evaluate the role of specific cytokines in pregnancy events on several species including pigs (Geisert et al., 2012) and cattle (Dobbs et al., 2014). These data suggest that alterations in the balance of cytokines during the first days of pregnancy, including the period of semen exposure, oocyte fertilization, and development of the embryo prior to implantation, have the potential to establish molecular mechanisms that influence the developmental programming of the embryo. Of special interest to us is understanding the extent by which paternal factors contribute to the control of the maternal immune response to pregnancy.

Paternal Contribution to Fertility

The paternal contribution to pregnancy is mediated not only by transferring half of the genetic information to the embryo but also by influencing maternal tissues and the embryo. These changes to maternal tissues and the embryo are associated with molecular components contained in semen (Krawetz, 2005; Stuppia et al., 2015). In humans, the lifestyle of fathers has been associated with the health of their progeny, such as smoking, alcohol consumption, or obesity (Li et al., 2011; Kong et al., 2012; Sharma et al., 2013; Campbell et al., 2015). Factors in the ejaculate can be linked to

male infertility; for instance, semen with high concentrations of the cytokine IFN-γ are associated with infertility (Paradisi et al., 1996; Seshadri et al., 2009). In mice, paternal diet has been reported to impair the development and implantation of the embryo and the health of offspring, due to DNA damage in sperm (Karalis et al., 2009; Mitchell et al., 2011). In addition, poor paternal diets have been shown to affect the quality of the plasma fraction of semen, compromising offspring performance (Watkins et al., 2018). These reports suggest that paternal factors present in the ejaculate, other than spermatozoa, may affect reproductive outcomes. In the bovine, genes expressed in sperm cells are associated with fertilization success and early embryonic development (Cochran et al., 2013a). Single nucleotide polymorphism in genes *SERPINE2*, *TBC1D24* and *SLC18A2* have been associated with lower cleavage rates of *in vitro*-produced embryos (Cochran et al., 2013b).

Semen used for artificial insemination (AI) in cattle is diluted in extender to achieve an increased number of insemination doses from a single ejaculate. The extended semen product has a specific number of spermatozoa diluted in medium containing nutrients to support sperm cells until fertilization, factors to minimize sperm stress and facilitate cryopreservation (Layek et al., 2016). The use of extended semen at AI means that at breeding, cows are not exposed to similar semen components or volumes observed during natural mating. Two common sources of proteins used in semen extenders are milk and egg yolk. The source of protein used for extender does not alter conception rates following AI (Dejarnette and Marshall, 2005). However, the type of extender used to dilute semen has been shown to affect embryos produced by *in vitro* fertilization in the goat (Keskintepe et al., 1998) and cow (Cochran et al., 2013b),

with egg yolk-based extenders resulting in higher cleavage rates *in vitro* compared to milk based extenders (Cochran et al., 2013b).

In dairy cattle, approximately 35% of embryos die between day 0 to 7 after insemination with additional significant losses occurring up to day 35 (Santos et al., 2004; Wiltbank et al., 2016). Much of this early embryonic mortality is associated with maternal related factors including the metabolic requirements of milk production (Amann and DeJarnette, 2012). These early pregnancy losses may also be a consequence of dysfunctions related to semen quality (Walters et al., 2006; Ledoux et al., 2015). The identification of paternal factors contained in semen that have the potential to improve bovine reproduction and reduce economic losses associated with pregnancy failure is paramount to optimize the semen used for artificial insemination. Understanding the identity and role of these factors could aid in increasing pregnancy rates and improving offspring performance in domestic species.

A Role for Seminal Plasma in Pregnancy

Pregnancy is the result of a complex interaction between maternal and paternal factors to facilitate development of the semiallogeneic embryo. It has been proposed that the success of pregnancy requires adaptation of the maternal immune system. Specifically, some research suggests this modulation is mediated by the plasma fraction of semen, seminal plasma. Seminal plasma exposure in some species modulates maternal immune system recognition of the semi-allogeneic embryo, supports the development of the embryo, promotes formation of the placenta, and influences other pregnancy events leading to better fertility outcomes (Robertson et al., 2018).

Seminal plasma exposure elicits an inflammatory response in the female reproductive tract. This immune response is characterized by secretion of CSF-2 and

other proinflammatory cytokines by endometrial cells, leading to cellular inflammation (Moldenhauer et al., 2010). uNK cells secrete IFN-γ and IL-10 in response to seminal plasma (Vacca et al., 2010), while seminal plasma TGF-β and PGE2 induce the recruitment of macrophages and dendritic cells into uterine tissues under the influence of elevated estrogen (Blois et al., 2007; Rutella et al., 2011). Uterine macrophages promote further secretion of proinflammatory cytokines such as TNF, IL-1 and IFN-γ, stimulating uterine dendritic cells. Uterine dendritic cells process paternal antigens contained in semen and activate lymphocytes, including Treg lymphocytes, in the para-aortic lymph nodes draining the reproductive tissues (Aluvihare et al., 2004; Robertson et al., 2009; Samstein et al., 2013). T regulatory lymphocytes play a pivotal role in seminal plasma-mediated influence on the maternal immune response to pregnancy observed immediately after insemination (Robertson et al., 2018). The activation and proliferation of Treg lymphocytes allow the recognition and tolerance of the semi-allogeneic embryo by the maternal immune system (Kallikourdis et al., 2007). T regulatory lymphocytes accumulate in the uterus at the time of embryo implantation (Guerin et al., 2011), secreting TGF-β and IL-10 to inhibit Th1 and Th17 inflammation (Mjösberg et al., 2010; Vacca et al., 2010). The secretion of anti-inflammatory cytokines promote dendritic and uNK cell function to increase endometrial receptivity toward the implanting embryo under the influence of progesterone (Ghiringhelli et al., 2005; Du et al., 2014). Experiments in mice have shown that seminal plasma modulates the maternal immune responses to transplantation of allogenic paternal skin grafts and recognition of major histocompatibility complex (MHC) class I paternal antigens, facilitating the survival of allogeneic grafts only after exposure to whole semen that

matched the haplotype of the allogenic graft (Beer et al., 1975; Robertson et al., 1997; Robertson and Sharkey, 2001). These mechanisms may be critical in allowing implantation to occur.

Taken together, the mechanisms proposed by Robertson et al. (2018) suggest that the exposure of maternal tissues to factors contained in seminal plasma during fertilization may optimize pregnancy by modulating the cellular and molecular microenvironment of the female reproductive tract.

Molecular Constituents of Seminal Plasma

Seminal plasma is the non-cellular fraction of semen. Seminal plasma serves as a support medium for spermatozoa when the ejaculate is deposited in the female reproductive tract (Poiani, 2006). The composition of the plasma fraction of semen is unique from plasma contents in blood (Richter et al., 1999). Secretions from various male accessory glands and tissues form the content of seminal plasma including the epididymis, prostate, seminal vesicle glands, bulbourethral glands, and Sertolli cells (Druart et al., 2013; Harayama et al., 2017). The contribution of each gland to the final ejaculate volume and composition depends on their secretory capacity, which is different between species. For instance, the seminal vesicles of mice secrete more than 90% of the final ejaculate volume, while only contributing to approximately 50% of semen volume in bulls (Faulkner et al., 1968; Bromfield, 2016). The use of seminal plasma for sperm cryopreservation reduced sperm viability in swine and horses (Pursel and Johnson, 1976; Jasko et al., 1991) but such negative effects are not apparent in sheep and cattle (Leahy and de Graaf, 2012). In fact, bovine sperm can be exposed to seminal plasma for 1 to 4 hours before dilution with extenders for cryopreservation without experiencing cellular damage (Steinhauser et al., 2016). Proteins secreted from

the male accessory glands coat the cell membranes of sperm, affecting their viability

and function (Yanagimachi, 2005). The surgical removal of the seminal vesicle glands in

mice is detrimental for embryonic development (Bromfield et al., 2014).

Components of seminal plasma not only serve as nutritional support for sperm

but also belong to the repertoire of immuno-regulatory molecules that influence

pregnancy. Seminal plasma is a complex fluid containing minerals, sugars, amino acids,

enzymes, proteins, proteases, lipids, antioxidants, hormones, and cytokines (Juyena

and Stelletta, 2012). Minerals and sugars including zinc, calcium, potassium, sodium,

magnesium, fructose, sorbitol, and glycerylphosphorylcholine offer sources of energy for

sperm maturation prior to ejaculation and later for sperm survival and function in the

female reproductive tract (Poiani, 2006). The protein content of seminal plasma differs

among domestic species; however, more than 80% of proteins in bulls, boars, and

alpaca seminal plasma have molecular weights less than 25 kDa (Druart et al., 2013).

Furthermore, bull fertility has been associated with the protein content of seminal

plasma. Bulls with deficient protein content of seminal plasma have a higher incidence

of subfertility in artificial insemination programs (Moura et al., 2006; Harayama et al.,

2017). Interestingly, semen content of osteopontin (55 kDa) secreted by seminal vesicle

epithelial cells and lipocalin-type prostaglandin D synthase (26 kDa) synthetized by

Sertolli cells have been associated with fertility in bulls (Harayama et al., 2017).

Amongst the most abundant proteins in bull semen are the bovine seminal proteins

(BSP), including BSP-1, BSP-3, and BSP-5 (Druart et al., 2013). While the mechanism

is unclear, it appears that bull semen collection by electroejaculation has a different

seminal plasma protein composition compared to semen collected using an artificial vagina (Rego et al., 2015).

Cytokines are signaling molecules that affect the interaction between cells (Zhang and An, 2007). Seminal plasma contains numerous hormones and cytokines associated with maternal tissue immune signaling and male infertility. For instance, seminal plasma prostaglandins influence dendritic cell activation (Remes Lenicov et al., 2012) regulating the female reproductive immune system during pregnancy. In humans, seminal plasma PGE2 content has been reported to be more than 1000 times higher than in tissues under acute inflammation (Kelly, 1997; Bromfield, 2014). Depending on the species, seminal plasma contains several cytokines including TGF-β, nerve growth factor (NGF), IFN-γ, TNF, IL-1β, IL-6, IL-8, IL-10, IL-12, among other immune-regulatory cytokines (Poiani, 2006; Druart et al., 2013; Bromfield, 2014; Harayama et al., 2017). However, high levels of cytokines such as IL-6, IL-8, TNF, and IFN-γ in human seminal plasma are associated with male infertility (Shimoya et al., 1993; Naz and Kaplan, 1994; Seshadri et al., 2009; Eldamnhoury et al., 2018). Nonetheless, the composition of seminal plasma is variable between individuals but also between ejaculates of the same male over time (Sharkey et al., 2017).

Seminal Plasma Modulates the Maternal Response to Pregnancy

The molecular events mediated by seminal plasma that influence fertility have been well characterized using mouse models (Robertson et al., 2018), with some effects mirrored in humans (Robertson and Sharkey, 2016), and to a lesser extent in cattle and swine (O'Leary et al., 2004; Bromfield et al., 2018). Seminal plasma elicits functional responses in female reproductive tissues by exposing the maternal immune system to paternal antigens at insemination (Robertson et al., 2018). Seminal plasma

exposure elicits an inflammatory response in the maternal reproductive tract (Yanagimachi and Change, 1963). For instance, it has been reported that seminal plasma elicits inflammation in the human cervix (Sharkey et al., 2012b). The expression and secretion of proinflammatory cytokines such as *IL6*, *IL8*, *CSF2*, and *IL1A* increase in human cervical cells following *in vitro* treatment with 10% v/v seminal plasma (Sharkey et al., 2012a), while similar inflammation is induced after unprotected coitus (Sharkey et al., 2012b). In mice, CD45+ leukocytes and MHC class II-macrophages and dendritic cells are recruited into the endometrium following mating in response to seminal plasma (Robertson, 2005). A similar effect was reported in horses where the concentration of polymorphonuclear neutrophils was increased after seminal plasma exposure at insemination (Rota et al., 2012).

Exposure of human endometrial epithelial cells to seminal plasma increases the expression of proinflammatory cytokines such as *IL1B*, *IL16*, and *LIF* (Gutsche et al., 2003). Similarly, porcine seminal plasma increases endometrial expression of *CSF2*, *IL6*, *COX2* and monocyte chemoattractant protein-1 (*MCP-1*), and results in the recruitment of macrophages and dendritic cells into the uterus (O'Leary et al., 2004). The equine endometrium responds to seminal plasma by increasing expression of *IL6*, *IL1B*, *COX2*, and *TNF*; however, this response does not differ from the one elicited by saline or extenders (Palm et al., 2008). In the same project, it was reported that egg yolk-based semen extender caused the least inflammatory response compared to other commercial extenders used in equine semen cryopreservation (Palm et al., 2008). This result contrasts with data reported in bovine where egg yolk-based extenders promote development of *in vitro*-produced embryos (Cochran et al., 2013b). Furthermore, it has

been reported that seminal plasma influences the expression of proinflammatory and embryotrophic cytokines in the oviduct of mice (Bromfield et al., 2014).

Using Seminal Plasma to Improve Reproductive Outcomes

The success of assisted reproductive techniques confirms that seminal plasma is not required for pregnancy; pregnancy rates in cattle are similar when comparing artificial insemination to natural service (Lima et al., 2009). However, embryos produced by IVF have aberrant characteristics compared to *in vivo* derived embryos that have interacted with, and developed in the maternal reproductive tract (Rizos et al., 2002; Gad et al., 2012). For instance, pregnancy rates in cows using embryos produced *in vitro* are lower than for embryos produced *in vivo* (Lonergan et al., 2007). The addition of cytokines to IVF and embryo culture medium influences embryonic development. Bovine embryos exposed to CSF-2 for two days during IVF result in calves that grow faster after birth (Kannampuzha-Francis et al., 2015), and similar effects are observed in humans (Ziebe et al., 2013). Addition of IL-6 to embryo culture medium increases blastocyst cell number and reduces apoptosis in mice (Shen et al., 2012; Kelley and Gardner, 2017). Interestingly, the cytokines reported to promote development of embryos *in vitro* coincide with the cytokines whose expression or secretion are increased in response to seminal plasma (see above).

Clinical observations in humans, experimental data in mice, and research performed in domestic species show that uterine exposure to seminal plasma around the time of coitus or insemination improves early embryo development, implantation, conception rates, and progeny performance (Bromfield, 2016; Robertson et al., 2018). Bromfield et al. showed that pregnancies occurring in the absence of seminal plasma in mice, due to surgical removal of seminal vesicle glands in sires, is detrimental for the

development of the embryo (Bromfield et al., 2014). This may be in part due to decreased expression of embryotrophic cytokines such as *Csf2*, *Lif*, and *Il6* and increased pro-apoptotic *Trail* in the oviduct when seminal plasma is absent at mating (Bromfield et al., 2014). O'Leary et al. reported that circulating progesterone was increased in sows exposed to seminal plasma at insemination compared to controls, with higher progesterone concentrations observed up to nine days after insemination. In addition, seminal plasma exposure at insemination increased the number and viability of embryos collected from bred sows (O'Leary et al., 2004). In the horse, combining sperm cells with seminal plasma after cryopreservation increased pregnancy rates (Rota et al., 2012). Furthermore, clinical pregnancy rates are higher in couples that have unprotected intercourse around the time of embryo transfer compared to couples that abstain from intercourse (Tremellen et al., 2000).

The periconceptional period is a critical window of development than can influence the embryo survival and impact long-term health of offspring (Louis et al., 2008). In mice, male offspring sired in the absence of seminal plasma have increased fat deposition, increased blood pressure, and metabolic problems (Bromfield et al., 2014). It has been reported that the paternal contribution to offspring is also mediated through non-genetic mechanisms, specifically involving paternal diet. Paternal high-fat diets have been associated with metabolic problems related to insulin secretion and glucose tolerance in female rat offspring (Ng et al., 2010). In addition, paternal low protein diets affect seminal plasma composition, consequently impacting male offspring that develop increased adiposity, impaired fetal growth and skeletal development, metabolic dysfunction, and altered gut microbiota (Watkins et al., 2017, 2018). The

hypothesis that environmental factors can program male accessory glands and affect the secretion/profile of paternal factors in seminal plasma could have a significant impact on domestic animal industries and human health.

Seminal Plasma TGF-β

Seminal plasma derived TGF-β is responsible for inducing the post-coital inflammatory response in female reproductive tissues of mice and humans (Tremellen et al., 1998; Robertson, 2005). TGF-β is a pleiotropic cytokine that acts via phosphorylation and nuclear translocation of SMAD proteins to regulate transcription of pro-inflammatory cytokines and influence cell proliferation, angiogenesis, immune-modulation, and regulation of hormonal responses in the uterus (Derynck and Zhang, 2003; Fleisch et al., 2006). TGF-β is present in seminal plasma of various mammalian species as three major isoforms TGF-β1, TGF-β2, and TGF-β3 (Robertson et al., 2015). Average physiological concentrations of TGF-β1 in human semen are 200 ng/mL, 150 ng/mL in swine, 30 ng/mL in mice, and 0.4 ng/ml in sheep (Tremellen et al., 1998; Scott et al., 2006; O'Leary et al., 2011; Sharkey et al., 2012a). Recently, it was reported that seminal plasma in the bull contained an average concentration of 7.11 ng/mL of total TGF-β1 and 6.07 ng/mL of total TGF-β2 (Bromfield, 2016). TGF-β is synthetized in a latent state, which is activated by low pH or enzymatic activity in the female tract after deposition at insemination. In mice and humans, 70% to 80% of seminal plasma TGF-β is present in a latent form, whereas in pigs the majority of TGF-β is present in the active form (Robertson et al., 2015).

In the mouse, TGF-β is responsible for the endometrial response to seminal plasma of epithelial cells, increasing the expression of proinflammatory cytokines including *Csf2*, *Il1a*, and *Tnf*. Additionally, intrauterine infusion of TGF-β elicits an influx

of leukocytes into the endometrium (Tremellen et al., 1998). Exogenous TGF-β exerts similar effects in human epithelial cells of the cervix and endometrium, increasing the expression of proinflammatory *CSF2*, *IL1B*, and *IL6*, which are associated with embryo development (Gutsche et al., 2003; Sharkey et al., 2012a). The maximal response of endometrial epithelial cells to TGF-β or seminal plasma occurs at the time of ovulation/mating in humans and mice, suggesting that responses may be mediated by estrogen (Robertson, 2005). It has been proposed that exposure of maternal tissues to TGF-β has the capacity to elicit an inflammatory response influencing the recognition of paternal antigens by activating Treg lymphocytes, neutrophils, and dendritic cells during pregnancy (Chen et al., 2003; Ghiringhelli et al., 2005; Fridlender et al., 2010).

A Role for Seminal Plasma in Bovine Reproduction

An influx of leukocytes into the bovine reproductive tract in response to semen was documented in young heifers (Howe and Black, 1963), which may be associated with the endometrial response to semen described in cattle over 50 years ago (Mattner, 1968). There are also reports of seminal plasma influencing fertility in cattle. Bovine seminal plasma enhances the interaction of sperm with neutrophils, and promotes the formation of DNA-based neutrophil extracellular traps (Alghamdi et al., 2009), which suggests a function in modulating sperm selection by the maternal immune system. Intramuscular administration of bovine seminal plasma increases plasma progesterone, likely due to the presence of nerve growth factor beta (NGF-β) (Tribulo et al., 2015) and pregnancy specific protein B (PSPB) (Stewart et al., 2018). These data suggest that bovine seminal plasma has luteotrophic benefits which may promote embryo development. Bovine seminal plasma also contains proteasomes, which are exosome-like vesicles (Ronquist et al., 2013), that in humans modulate spermatozoa cell function

42

(Samanta et al., 2018). This represents a potential mechanism by which factors contained in seminal plasma may modulate fertility in cattle.

Cows undergoing artificial insemination using diluted/extended semen are not exposed to physiological components and volumes of semen similar to natural mating. The infusion of seminal plasma or TGF-β1 at the time of artificial insemination does not affect pregnancy rates in beef or dairy cows (Odhiambo et al., 2009). However, Odhiambo et al., reported that seminal plasma infusion at AI increased pregnancy rates by 4%, but these studies were underpowered. Infusion of 40 ng/mL of TGF-β1 at AI tended to increase pregnancy rates in herds with low fertility (pregnancy rates below 50%), which beggars the questions for the capacity of seminal plasma or TGF-β to optimize pregnancy in the bovine.

Thesis

The physiological responses in mice and humans suggest that exposure of seminal plasma at conception influences embryo development and offspring health after birth (Robertson et al., 2015). Therefore, the capacity for semen components to improve pregnancy outcome in cattle should be evaluated, where the use of artificial insemination achieves pregnancy in absence of seminal plasma (Moore and Hasler, 2017). The overall objective of this thesis is to evaluate the capacity for seminal plasma or TGF-β to increase the expression of proinflammatory factors in the bovine endometrium. The basis for these investigations are reports from human and mice where seminal plasma or TGF-β elicit inflammatory responses in endometrial tissues associated with improving pregnancy outcomes and offspring performance (Robertson, 2005; Bromfield et al., 2014). The specific aims of this thesis are to 1) evaluate the responsiveness of endometrial cells to seminal plasma and to 2) characterize the

physiological response of the bovine endometrium to semen components. The experiments outlined in chapter 2 include two experimental models using *in vitro* culture of bovine endometrial cells with seminal plasma or TGF-β to evaluate cell viability and gene expression, and an *in vivo* model of intrauterine infusion of semen components to measure changes in endometrial gene expression. These experiments will advance our understating of the physiological role for semen components in bovine reproduction.

CHAPTER 2
ENDOMETRIAL RESPONSE TO SEMEN COMPONENTS IN THE BOVINE

Introduction

Seminal plasma is the non-cellular fraction of semen that contains factors that

facilitate the transport and function of spermatozoa when the ejaculate is deposited in

the female reproductive tract (Poiani, 2006). The components of seminal plasma also

interact with the cellular lining of the uterus to modulate the maternal response to

pregnancy (Robertson et al., 2018). The periconceptional period is a critical window of

development than can influence embryo survival and affect the long-term health of

offspring (Louis et al., 2008). Reports from humans and mice have shown that exposure

to seminal plasma at insemination elicits endometrial inflammation associated with

improving pregnancy outcomes and offspring performance (Bromfield et al., 2014;

Robertson et al., 2015). Seminal plasma derived TGF-β has been identified as the key

component of seminal plasma to elicit the post-coital inflammatory response in female

reproductive tissues of mice and humans (Tremellen et al., 1998; Robertson, 2005). In

several species, the endometrial inflammatory response is characterized by increased

expression of *CSF2*, *IL1B*, *IL6*, *TNF*, *PTGS2*, *LIF* and *IL17A* (Gutsche et al., 2003;

O'Leary et al., 2004; Robertson et al., 2015). These cytokines alter preimplantation

embryo development and influence maternal Treg lymphocytes, neutrophils, and

dendritic cells during pregnancy (Chen et al., 2003; Ghiringhelli et al., 2005; Fridlender

et al., 2010).

The success of assisted reproductive techniques confirms that seminal plasma is

not required for pregnancy. In fact, pregnancy rates using artificial insemination in cattle

are similar to natural service (Lima et al., 2009), and generally pregnancy rates

achieved by embryo transfer can be even higher than for artificial insemination (Pellegrino et al., 2016). However, cows undergoing artificial insemination using diluted/extended semen are not exposed to physiological components of the ejaculate. As such, the response of the endometrium to semen is not well studied in cattle, and the contribution of seminal plasma to bovine fertility is not yet well defined.

Intramuscular administration of bovine seminal plasma increases plasma progesterone, likely due to the presence of nerve growth factor beta (Tribulo et al., 2015), and pregnancy specific protein B (Stewart et al., 2018), which may further promote embryo development. Indeed, the intrauterine infusion of TGF-β1 at the time of artificial insemination increased pregnancy rates in cattle herds with low fertility (Odhiambo et al., 2009). This begs the question of the capacity for seminal plasma or TGF-β to optimize reproductive performance in the bovine. We hypothesize that seminal plasma or TGF-β increases the expression of proinflammatory factors in the bovine endometrium. In dairy cattle, the highest incidence of pregnancy loss occurs in the first week of pregnancy when approximately 40% of embryos fail to develop further than the blastocyst stage (Santos et al., 2004; Spencer et al., 2016b; Wiltbank et al., 2016). Modulating the maternal environment during this window of development may affect the survival of preimplantation embryos.

Here we utilized *in vitro* culture of bovine endometrial cells and intrauterine infusion to evaluate cell viability and expression of proinflammatory and embryotrophic factors in response to semen components, including TGF-β. The identification of paternal factors contained in semen that could improve bovine reproduction and reduce economic losses associated with pregnancy failure may help optimize semen use for

artificial insemination. These experiments advance our understating of the physiological role for semen components in bovine reproduction.

Materials and Methods

Reagents and Chemicals

All reagents were acquired from Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Seminal Plasma Collection

Whole semen was collected from healthy Angus bulls by electroejaculation during two separate breeding soundness evaluations (BSE) at the University of Florida North Florida Research and Extension Center. Whole semen from 21 bulls was collected on the first BSE and 12 bulls were collected during the second collection. Sample contamination was minimized as best could be achieved in field conditions. Whole semen was evaluated and further processed only if free of blood, urine and other visual abnormalities. Parameters recorded during the BSE included bull ID, collection date, ejaculated volume, scrotal circumference, sperm motility, morphology and concentration. On average, whole semen volume ranged from 4 to 15 mL, gross motility ranged from 10 to 90%, and scrotal circumference ranged from 31 to 49 cm. Following semen collection, samples were placed on ice and transported to the laboratory for processing under aseptic conditions. A 200 µL aliquot of whole semen was placed into a sterile micro-centrifuge tube and stored at -20°C. The remaining ejaculate was centrifuged at 12,000 x g for 20 min at 4°C to separate the plasma fraction from the cellular fraction. Cell free seminal plasma was placed in sterile tubes in aliquots of 500 to 1000 µL and stored at -20°C until use.

Preparation of Pooled Seminal Plasma

Ejaculates from 21 bulls were used to prepare a seminal plasma pool by combining 1 or 2 mL of seminal plasma from each bull into a 50 mL tube under aseptic conditions. A fraction of this seminal plasma pool was filter-sterilized using a 0.4 µm syringe filter before loading into 0.5 mL straws used for AI. Remaining pooled seminal plasma was directly stored or either filtered through a 0.22 µm filter, heat-treated at 90°C for 10 min, or both filtered and heat-treated. Pooled seminal plasma was stored in 500 µL aliquots at -20°C. A second pool of seminal plasma was generated as described above with ejaculates from 12 different bulls. The second pooled seminal plasma was stored in 100 µL aliquots at -20°C.

Reconstitution of TGF-β1 and TGF-β2

A total of 10 µg of recombinant human transforming growth factor beta 1 (rhTGF-β1) (Invitrogen, Frederick, MD) was reconstituted in 500 µL of sterile 4 mM HCl containing 1 mg/mL bovine serum albumin (BSA) fraction V in Dulbecco's phosphate-buffered saline (DPBS) to achieve a 20 µg/mL stock solution. Stock solutions were diluted 1:20 in sterile DPBS containing 25 µg/mL of gentamicin to achieve a working stock concentration of 1 µg/mL rhTGF-β1. Working stock solutions were stored in 40 µL aliquots at -80°C.

A total of 5 µg of recombinant human transforming growth factor beta 2 (rhTGF-β2) (Invitrogen, Frederick, MD) was reconstituted in 50 µL of molecular grade water to achieve a 0.1 mg/mL stock solution. Stock solutions were diluted 1:100 in sterile DPBS containing 1 mg/mL BSA to achieve a working stock concentration of 1 µg/mL rhTGF-β2. Working stock solutions were stored in 40 µL aliquots at -80°C.

Culture of Bovine Endometrial (BEND) Cells

BEND cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured according to the distributor's instructions. Briefly, BEND cells were cultured to 85% sub confluence in 75 cm^2 flasks (Greiner Bio-One, Frickenhausen, Germany) in a 1:1 mixture of Dulbecco's Minimum Essential Medium (DMEM) and Ham's F12 Nutrient Mixture Medium supplemented with 1.5 g/L sodium bicarbonate, 0.034 g/L D-valine, 10% fetal bovine serum (FBS), and 10% horse serum. Cultures were maintained at 38.5°C in a humidified 5% CO_2 environment. Complete culture medium was equilibrated to incubator conditions and changed every 48 h until cells reached sub confluence. Prior to treatment, cells were detached from flasks using 0.25% trypsin with 0.1%-EDTA, washed with warm DPBS by centrifugation and resuspended in equilibrated culture medium. Cells were plated at a final concentration of 10^5 cells/mL in 24 or 96-well culture plates (TPP, Trasadingen, Switzerland). Cultures were equilibrated for 24 h to facilitate cellular attachment before the addition of treatments (described below).

Isolation and Culture of Primary Bovine Endometrial Epithelial and Stromal Cells

Uteri from post pubertal, non-pregnant cows were collected at a local slaughterhouse and processed at room temperature (RT) within 4 h of collection. Whole reproductive tracts were collected; however, uteri were further dissected only if no signs of infection were observed. A total of 6 reproductive tracts at stage 3 (day 11 to 17 of the estrous cycle) were used for the assay. The stage of the estrous cycle was classified based on corpus luteum characteristics including internal and external appearance, diameter, surface vasculature, and presence of follicles as previously reported (Ireland et al., 1980).

After removal of surrounding fat and connective tissue, the external surfaces of the uteri were disinfected with 70% ethanol. The ipsilateral horn to the corpus luteum was opened with a transverse cut into the center of the horn, above the intercornual ligament and longitudinal to the ovary. The exposed endometrium was washed twice with DBPS supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin and 2.5 µg/mL amphotericin B to remove mucus and potential microorganisms. Endometrial tissue from intercaruncular regions was dissected into thin strips and placed into 50 mL pots containing Hank's balanced salt solution (HBSS) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin and 250 µg/mL amphotericin B. Endometrial tissue was further cut into 3 to 5 mm^2 pieces, transferred into a 50 mL centrifuge tube containing warm HBSS and maintained at 37°C for 10 min. Endometrial tissue was enzymatically digested in 40 mL of HBSS supplemented with 100 mg BSA, 125 CDU/mg collagenase II (Sigma-Aldrich, St. Louis, MO), 250 BAEE trypsin (Sigma-Aldrich), and 4% DNase I. Tissue was digested at 37°C in a shaking water bath for 1 h. The cell suspension was then filtered through a 40 µm filter into a new 50 mL centrifuge tube containing warm HBSS supplemented with 10% FBS to stop enzymatic activity. The filtered suspension was centrifuged at 500 x g for 7 min at RT. The supernatant was discarded and the endometrial cell pellet was resuspended in 5 mL of complete culture medium (RPMI 1640 medium supplemented with 10% FBS, 2 mM of L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 2.5 µg/mL amphotericin B) equilibrated to 38.5°C. Cells were transferred to 75 cm^2 flasks (Greiner Bio-One) containing 25 mL of equilibrated culture medium and maintained at 38.5°C in a humidified 5% CO_2 environment. Differential adhesion was utilized to semi-purify

endometrial epithelial and stromal cells. Following an 18 h culture period, epithelial cells in suspension were transferred to a new flask while stromal cells remained attached to the original culture flask. After a total of 66 h in culture, cells were detached from flasks using HyQTase, washed with warm DPBS and resuspended at a final concentration of 10^5 cells/mL. Purity of epithelial and stromal cells was assessed with flow cytometry analysis using the specific cell markers cytokeratin and vimentin, respectively, as previously reported (Ibrahim et al., 2018). Endometrial epithelial or stromal cells were plated in 24-well culture plates (TPP) in 500 μL of culture medium and equilibrated for 24 h before the addition of treatments.

All treatments were added to complete culture medium prior to the application to either BEND cells or primary endometrial cells. Treatments included medium alone as negative control, seminal plasma (0.001%, 0.01%, 0.1%, 1%, 5%, 10%, or 20% v/v), rhTGF-β1 (1 ng, 10 ng or 100 ng/mL) or rhTGF-β2 (0.1 ng, 1 ng or 10 ng/mL). Estradiol was used at 0 nM, 0.1 nM or 1 nM concentrations. Cells were exposed to treatments for 0, 1, 3, 6, 12 or 24 h. Experiments using BEND cells were repeated at least 4 times and each replicate represents cells between passage 1 to 11. Experiments using primary cells were replicated 6 times, with each replicate being representative of endometrial cells from an individual cow. Following treatment, cells were washed with warm DPBS and stored in 350 μL of RLT Lysis Buffer (Qiagen, Hilden, Germany) at -80°C to facilitate extraction of total RNA.

Evaluation of Endometrial Cell Viability

Cell viability was assessed by colorimetric analysis using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Viability of cells was determined by the capacity of living cells to reduce the membrane-permeable MTT

dye (Sigma-Aldrich) to formazan by mitochondrial-dependent metabolism. Endometrial cell viability was evaluated after 24 h treatment. Cells were plated at 10^5 cells/mL in 96-well culture plates (TPP) in a final volume of 200 µL of appropriate cell culture medium and equilibrated for 24 h prior to treatment. Treatments were added to complete culture medium and equilibrated to 38.5°C prior to exposure of cells in duplicate for 24 h. Assays were repeated 7 times. Each replicate included a blank with no cells to calculate background color. Following the 24 h treatment period, a total of 10 µL of 5 mg/mL MTT was added to each well. Cells were incubated for 2 h in MTT prior to washing in warm DPBS. Cellular formazan was solubilized by the addition of 100 µL of dimethyl sulfoxide (DMSO) per well and incubated for 15 min at RT in the dark. Optical density of each well was measured at 540 nm using a microplate reader (BioTek, Winooski, VT) and the blank corrected value for each treatment was calculated using the average of the duplicate wells. Data were normalized as fold change from the medium alone treated cells.

Synchronization, Blood Collection and Intra-Uterine Infusion of Cows

The study was conducted at the University of Florida, North Florida Research and Extension Center (NEFREC) in Marianna, Florida. All treatments, animal housing and procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

The endometrial response to intra-uterine infusion was characterized in 54 multiparous, non-lactating healthy Angus beef cows. Cows were randomly assigned to receive 1 mL of either saline, conventional extended semen from a single sire, pooled seminal plasma, or 1 mL of both conventional extended semen and seminal plasma. Each treatment was pre-loaded into 0.5 mL straws under aseptic conditions. Saline and

seminal plasma were filter-sterilized using a 0.4 µm syringe filter before loading into straws. Estrous synchronization was performed in all cows by administering GnRH (Factrel; 100 µg gonadorelin hydrochloride; Zoetis Animal Health, Parsippany, NJ), followed by PGF2α treatment (Lutalyse; 25 mg dinoprost tromethamine; Zoetis Animal Health) 7 days later. Transrectal ultrasound (Ibex ultrasound with 5 MHz multifrequency transducer: E. I. Medical Imaging, Loveland, CO) was performed to confirm the presence and location of an ovulatory follicle at the time of PGF2α treatment and again at the time of intra-uterine infusion. Only cows confirmed in heat were included in the study. Treatments were administered into the uterine body 48 h after PGF2α treatment by standard artificial insemination technique. Endometrial biopsies from both ipsilateral and contralateral uterine horns relative to the ovulatory follicle were collected 24 h after treatment infusion. Briefly, animals received local anesthesia by administering 4 mL of lidocaine to the epidural space between the second and third coccygeal vertebrate. Endometrial biopsy forceps (Jorgensen 195 Laboratories, Loveland, CO) were passed into the uterine horn, guided by rectal palpation. Once in place, the endometrium was gently pressed into the jaws of the biopsy forceps by rectal palpation and a small piece of endometrial tissue was clipped off by closing the instrument jaws. The process was repeated to facilitate the collection of endometrial tissue from both uterine horns. Endometrial tissue biopsies were rinsed in sterile DPBS and bisected into two pieces. Biopsies were stored in RNAlater (Invitrogen) for RNA extraction or fixed in 10% neutral buffered formalin for 24 h at 4°C for histological analysis. Blood was collected via coccygeal vein 7 days after insemination using 10 mL lithium heparin vacutainers (BD, Franklin Lakes, NJ), and stored at -20°C until plasma progesterone was quantified.

Quantification of Blood Plasma Progesterone Concentration by ELISA

Blood plasma progesterone concentrations were quantified by ELISA (DRG International Inc., Springfield NJ) following the manufacturer's instructions. The DRG progesterone ELISA kit is human specific and was previously validated in our laboratory for use with bovine blood plasma. The assay was validated by spike-in/recovery performance, using samples with known concentrations of bovine plasma progesterone and expected concentration recovery of progesterone supplied with the kit. Recovery of spike-in progesterone was above 89% with a coefficient of intra-assay variation at 6.5%.

Extraction and Purification of RNA from Endometrial Cells or Tissues

Total RNA was extracted from cultured cells using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Total RNA was extracted from endometrial biopsy tissues using TRIzol Reagent. Briefly, endometrial tissues were homogenized in TRIzol with the help of a disposable pellet pestle (Kimble, Rockwood, TN). Homogenates were suspended in a final volume of 1 mL TRIzol and centrifuged at 12,000 x g for 10 min at 4°C. Supernatants were transferred to a new centrifuge tube and a total of 200 µL of chloroform was added to homogenates and incubated at RT for 5 min. Samples were centrifuged at 12,000 x g for 15 min at 4°C. The RNA rich aqueous supernatant was transferred to a new tube and 500 µL 100% isopropanol was added to each sample, followed by a 10 min incubation at RT. Samples were centrifuged at 12,000 x g for 15 min at 4°C and supernatants were removed leaving the RNA pellet. The RNA pellet was washed twice with 1 mL of 75% ethanol and centrifuged at 12,000 x g for 15 min at 4°C. The RNA pellet was air-dried for 5 min at RT and resuspended in 50 µL of RNAase-free water. Extracted RNA was quantified using a NanoDrop ND1000 spectrophotometer, and the purity of each sample was

determined by the A260/A280 ratio. A total of 1 µg of RNA was subjected to reverse transcription using the Verso cDNA synthesis kit according to manufacturer's instructions. A genomic DNA wipe out step was included to remove genomic DNA. The recommended thermal cycling conditions for reverse transcription were 1 cycle of cDNA synthesis at 42°C for 30 min followed by inactivation at 95°C for 2 min. The cDNA was diluted 1:3 in molecular grade water and stored at -20°C.

Quantitative Real-Time RT-PCR

Primers were designed using the NCBI database and initial specificity was verified by BLAST to ensure no cross-reactivity with other loci. Primer length less than 200 bp and GC contents of each primer (50 to 60%) were selected to avoid primer dimer formation. Primer details are listed in Table 1. Amplification efficiency was evaluated for each primer by performing serial dilutions of cDNA to confirm linear amplification and confirmed by visualization of PCR product on a 1 % agarose gel containing diamond dye to confirm predicted PCR product size. All primers had to meet MIQE guidelines for use (Pearson correlation coefficient r > 0.98 and efficiency between 90%-110%).

Quantitative real-time RT-PCR was performed in 20 µL reactions using iTaq Universal SYBR green (Bio-Rad, Hercules CA) with 100 nM of each forward and reverse primer. A Bio-Rad CFX Connect light cycler (Bio-Rad) was employed to perform PCR using a two-step protocol with the following recommended thermal cycling conditions: initial denaturation/enzyme activation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec, and annealing/extension for 30 sec at 60°C. Samples were analyzed in duplicates and each PCR reaction was followed by melt curve analysis to ensure single product amplification. A no template negative control

was used in place of cDNA to determine non-specific amplification. Gene expression data from cultured cells were normalized independently to *GAPDH* mRNA expression. Gene expression data from endometrial biopsies were normalized independently to the geometric mean of *GAPDH* and *ACTB* mRNA expression. Data were further analyzed using the $2^{-\Delta Ct}$ method.

Statistical Analysis

Data were analyzed using SPSS software V24.0 (IBM Analytics, Armonk, NY). Data were log transformed where appropriate and normality was assessed with Shapiro-Wilk test before statistical analysis. Data were analyzed using the generalized linear mixed model with dose, treatment, time, and uterine horn as fixed factors. Replicate was included as random variable for the *in vivo* experiment. Pairwise comparisons were performed between each treatment dose against vehicle controls, or with estradiol concentrations within and among treatments. Data for gene expression were analyzed using the $2^{-\Delta Ct}$ method and cell viability data were analyzed using the values for fold-change normalized to control treatments for each experiment. Experiments were repeated at least four times. Data are presented as the mean ± standard error of the mean. Statistical significance was declared at a *P* value of ≤ 0.05.

Results

The Effect of Seminal Plasma on BEND Cell Viability

The effect of seminal plasma on the viability of BEND cells was assessed by colorimetric analysis using the MTT assay after 24 h exposure with medium alone or 0.001%, 0.01%, 0.1%, 1%, 5%, 10%, or 20% (v/v) seminal plasma (Figure 2-1). A pool of seminal plasma from the first collection of 21 bulls was used. Viability of BEND cells was unaffected following exposure to low doses (0.001% to 0.1% v/v) of seminal

plasma regardless of seminal plasma treatment. However, exposure of BEND cells with 1% non-treated seminal plasma reduced cell viability by 86% compared to controls (P ≤ 0.05), while exposure to 5%, 10% or 20% non-treated seminal plasma reduced viability of BEND cells by at least 98% of controls (P < 0.05). Filtration of seminal plasma resulted in the same cytotoxic activity of non-treated seminal plasma at concentrations starting at 1% (P < 0.05). Heat-treatment of seminal plasma at 90°C for 10 min reduced the cytotoxic effects of seminal plasma, maintaining control cell viability at 1% and 5% heat-treated seminal plasma. The cytotoxic effect of seminal plasma was still evident after exposure to 10% or 20% heat-treated seminal plasma, compared to controls (P < 0.05). The combination of heat-treatment and filtration yielded the same degree of cytotoxicity as heat-treated seminal plasma, where cell viability cytotoxic effects were observed following exposure to 10% or 20% seminal plasma.

The Effect of Seminal Plasma on BEND Cell Gene Expression

The capacity for low concentration seminal plasma to modulate BEND cell expression of *CSF2*, *IL1B*, *TNF*, and *IL6* was evaluated following exposure to 0.001%, 0.01%, 0.1%, or 1% v/v seminal plasma or medium alone for 24 h (Figure 2-2). Seminal plasma was either non-treated (Figure 2-2A, E, I, M), filtered (Figure 2-2B, F, J, N), heat-treated (Figure 2-2C, G, K, O), or filtered and heat-treated (Figure 2-2D, H, L, P).

BEND cell expression of *CSF2* was unaffected following exposure to non-treated or filtered seminal plasma (Figure 2-2A-B). However, exposure to 0.1% or 1% (v/v) seminal plasma that was heat-treated or filtered and heat-treated reduced *CSF2* expression compared to control cells (Figure 2-2C-D; P ≤ 0.05). With the exception of exposure to 1% non-treated seminal plasma or filtered seminal plasma, the expression of BEND cell *IL1B* was reduced in response to seminal plasma exposure regardless of

treatment, compared to control cells (Figure 2-2E-H; P ≤ 0.05). Expression of BEND cell

TNF was increased following exposure to 0.001% v/v of heat-treated seminal plasma (P

< 0.05), while exposure to all other treatments had no effect on *TNF* expression (Figure

2-2I-L). BEND cell expression of *IL6* was increased following exposure to each

treatment of seminal plasma, compared to control cells (Figure 2-2M-P, P < 0.05).

Specifically, exposure to non-treated seminal plasma increased *IL6* expression at

0.01%, 0.1% and 1% (Figure 2-2M); exposure to filtered seminal plasma increased *IL6*

expression at 1% (Figure 2-2N); exposure to heat-treated seminal plasma increased *IL6*

expression at 0.001%, 0.01%, 0.1% and 1% (Figure 2-2O); and exposure to seminal

plasma that was both heat-treated and filtered increased *IL6* expression at 0.01%

(Figure 2-2P).

The Effect of Seminal Plasma on Viability of BEND Cells Supplemented with Estradiol

The capacity for estradiol to modulate BEND cell viability in response to seminal

plasma was assessed using the MTT assay (Figure 2-3). BEND cells were cultured in

the presence of 0 nM, 0.1 nM or 1 nM estradiol for 24 h before exposure to seminal

plasma. BEND cells were then treated with medium alone or 0.001%, 0.01%, 0.1%, 1%,

5%, 10%, or 20% (v/v) non-treated seminal plasma for 24 h in medium supplemented

with estradiol (Figure 2-3). A second pool of seminal plasma collected from 12 unique

bulls was used for this experiment. The viability of BEND cells was not affected

following exposure to seminal plasma at concentrations of 0.001%, 0.01%, 0.1% or 1%

(v/v) regardless of estradiol exposure. Following exposure to seminal plasma at 5%,

10% or 20% (v/v), BEND cell viability was decreased by a minimum of 95% compared

to control cells. Supplementation with estradiol did not affect seminal plasma induced

cytotoxicity of BEND cells.

The Effect of Seminal Plasma on Gene Expression of BEND Cells Supplemented with Estradiol

The capacity for estradiol to modulate BEND cell expression of *CSF2*, *IL1B*, *TNF*,

and *IL6* was evaluated following exposure to seminal plasma (Figure 2-4). BEND cells

were cultured in the presence of 0 nM (Figure 2-4A, D, G, J), 0.1 nM (Figure 2-4B, E, H,

K), or 1 nM (Figure 2-4C, F, I, L) estradiol for 24 h before exposure to seminal plasma.

BEND cells were then treated with medium alone or 0.001%, 0.01%, 0.1%, or 1% v/v

non-treated seminal plasma for 24 h in medium supplemented with estradiol (Figure 2-

4).

BEND cell expression of *CSF2* (Figure 2-4A, B, C) and *TNF* (Figure 2-4G, H, I)

was unaffected following exposure to seminal plasma regardless of estradiol treatment.

However, exposure to 1% v/v seminal plasma increased the expression of *IL1B* (Figure

2-4D, E, F; $P \leq 0.05$) and *IL6* (Figure 2-4J, K, L; $P \leq 0.05$) compared to control cells,

regardless of estradiol treatment. Interestingly, the magnitude of seminal plasma

induced *IL1B* expression was higher in cells exposed to 0.1 nM estradiol compared to

estradiol-free control cells (Figure 2-4E; $P \leq 0.05$).

The Effect of Transforming Growth Factor β on Viability of BEND Cells Supplemented with Estradiol

Seminal plasma-derived transforming growth factor β (TGF-β) modulates human,

mice and swine endometrial environment following insemination. The capacity for

rhTGF-β1 and rhTGF-β2 to affect BEND cell viability was evaluated using the MTT

assay (Figure 2-5). BEND cells were cultured in the presence of 0 nM, 0.1 nM or 1 nM

estradiol for 24 h before exposure to TGF-β. BEND cells were subsequently exposed to

medium alone, rhTGF-β1 (1 ng, 10 ng, or 100 ng/mL), or rhTGF-β2 (0.1 ng, 1 ng, or 10 ng/mL) for 24 h in medium supplemented with estradiol. There was no effect of either TGF-β or estradiol treatment on BEND cell viability ($P > 0.05$).

The Effect of Transforming Growth Factor β on Gene Expression of BEND Cells Supplemented with Estradiol

The capacity for estradiol to modulate BEND cell expression of *CSF2*, *IL1B*, *TNF*, and *IL6* was evaluated following exposure to rhTGF-β1 or rhTGF-β2 (Figure 2-6). BEND cells were cultured in the presence of 0 nM (Figure 2-6A, D, G, J), 0.1 nM (Figure 2-6B, E, H, K), or 1 nM (Figure 2-6C, F, I, L) estradiol for 24 h before exposure to TGF-β. BEND cells were then treated with medium alone, rhTGF-β1 (1 ng, 10 ng, or 100 ng/mL), or rhTGF-β2 (0.1 ng, 1 ng, or 10 ng/mL) for 24 h in medium supplemented with estradiol.

Expression of BEND cell *CSF2* was unaffected following exposure to rhTGF-β in the absence of estradiol (Figure 2-6A). Expression of *CSF2* was decreased following exposure to 1 or 10 ng/mL of rhTGF-β2 in combination with 0.1 nM estradiol compared to control cells cultured in 0.1 nM estradiol; however, exposure to 1 ng/mL of rhTGF-β2 co-treated with 0.1 nM estradiol resulted in lower *CSF2* expression compared to cells exposed to 1 ng/mL of rhTGF-β2 in the absence of estradiol (Figure 2-6A-B; $P ≤ 0.05$). Treatment with 1 nM estradiol also decreased *CSF2* expression following treatment with 10 ng/mL of rhTGF-β1, or either 1 ng/ml or 10 ng/mL of rhTGF-β2 compared to rhTGF-β2 exposure in the absence of estradiol (Figure 2-6A-C; $P ≤ 0.05$).

The expression of *IL1B* was unaffected following treatment with rhTGF-β in the absence of estradiol (Figure 2-6D), whereas cells treated with 0.1 nM estradiol the expression of *IL1B* was decreased after exposure to 1 ng/mL of rhTGF-β2 compared to

60

control cells (Figure 2-6E; P ≤ 0.05). Interestingly, estradiol treatment decreased the expression of *IL1B* regardless of exposure to rhTGF-β (Figure 2-6D-F; P ≤ 0.05).

Expression of *TNF* was increased following exposure to 10 ng/mL or 100 ng/mL rhTGF-β1 or 0.1 ng/mL rhTGF-β2 in the absence of estradiol, compared to control cells (Figure 2-6G; P ≤ 0.05). Interestingly exposure to 10 ng/mL rhTGF-β2 in combination with 0.1 nM estradiol reduced *TNF* expression compared to controls (Figure 2-6H; P ≤ 0.05). Collectively, exposure to estradiol ameliorated any TGF-β induced increase in *TNF* expression (Figure 2-6G-I; P ≤ 0.05).

Expression of *IL6* was increased in BEND cells exposed to 10 ng/mL or 100 ng/mL rhTGF-β1, or either 1 ng/mL or 10 ng/mL rhTGF-β2 that were supplemented with estradiol compared to control cells. There was no effect of TGF-β exposure on *IL6* expression in the absence of estradiol (Figure 2-6J-L; P ≤ 0.05).

Acute Effects of Transforming Growth Factor β on BEND Cell Cytokine Expression

To evaluate BEND cell response to acute TGF-β exposure cells were exposed to medium alone, 10 ng/mL rhTGF-β1, or 1 ng/mL rhTGF-β2 for a period of 0, 1, 3, 6, or 12 h (Figure 2-7). Expression of *IL1B* was decreased in response to either rhTGF-β1 or rhTGF-β2 following exposure for 1 or 3 h, compared to control cells at the same time points (Figure 2-7B; *P* ≤ 0.05). However, the expression of *IL1B* was increased after exposure to rhTGF-β1 or rhTGF-β2 for 3, 6 or 12 h compared to control cells at 0 h (Figure 2-7B; *P* ≤ 0.05). Expression of *IL6* was increased after exposure to rhTGF-β1 or rhTGF-β2 for 6 and 12 h compared to control cells at the same time points (Figure 2-7 D; *P* ≤ 0.05). In addition, *IL6* expression was increased in response to rhTGF-β1 or rhTGF-β2 exposure for 1, 3, 6 and 12 h compared to control cells at 0 h (Figure 2-7D; *P*

≤ 0.05). Expression of *CSF2* and *TNF* was unaffected following exposure to TGF-β (Figure 2-7A and C).

Effect of Seminal Plasma or Transforming Growth Factor β on Primary Endometrial Epithelial or Stromal Gene Expression

The capacity of seminal plasma or TGF-β to modulate semi-purified bovine endometrial epithelial or stromal cell expression of *CSF2*, *IL1B*, *TNF* and *IL6* was assessed by qPCR (Figure 2-8). Cells were exposed to non-treated seminal plasma (0.001%, 0.01%, 0.1%, or 1% v/v), rhTGF-β1 (1 ng, 10 ng or 100 ng/mL), rhTGF-β2 (0.1 ng, 1 ng or 10 ng/mL), or medium alone for 24 h. Stromal cells expression of *CSF2*, *TNF* and *IL6* was increased following exposure to 1% v/v seminal plasma compared to control cells (Figure 2-8A, C-D; $P ≤ 0.05$). Expression of selected genes in stromal cells was not affected following exposure to TGF-β. Exposure of cells to seminal plasma or TGF-β had no effect on expression of target genes in epithelial cells (Figure 2-8E-H).

Effect of Intrauterine Infusion of Semen Components on Endometrial Gene Expression and Circulating Progesterone

The endometrial response to semen components following intrauterine infusion was characterized in synchronized beef cows. Endometrial biopsies were collected 24 h after intrauterine infusion of saline, conventional semen, seminal plasma, or the combination of semen + seminal plasma (Figure 2-9). Seminal plasma infusion increased the expression of endometrial *IL1B* by 2.2-fold compared to saline infusion (Figure 2-9 J; $P ≤ 0.05$). Specifically, seminal plasma infusion increased *IL1B* expression in the contralateral horn compared to saline infusion. An effect of treatment was observed in the expression of *PTPRC* where semen + seminal plasma infusion reduced expression by 2.37-fold compared to saline infused control group (Figure 2-9G; $P ≤ 0.05$). This effect was strongest in the ipsilateral horn where *PTPRC* expression

was reduced following infusion of semen + seminal plasma compared to saline. Interestingly, the infusion of seminal plasma alone resulted in higher *PTPRC* expression compared to infusion of semen + seminal plasma in the contralateral horn. Expression of *PTGS2* was decreased following infusion of seminal plasma compared to saline in the ipsilateral horn (Figure 2-9E; $P \leq 0.05$). Basal expression of *CSF2* was 3.9-fold higher in the ipsilateral horn compared to the contralateral horn following saline infusion, while infusion of semen increased *CSF2* expression 3.8-fold compared to saline in the contralateral horn (Figure 2-9A; $P \leq 0.05$). Expression of *IL17A*, *AKR1C4*, and *TGFB* were higher in the ipsilateral horn compared to the contralateral horn following infusion of semen + seminal plasma (Figure 2-9C, H, L; $P < 0.05$). Basal expression of *AKR1C4* was also higher in the ipsilateral horn compared to the contralateral horn of the saline infused group (Figure 2-9K; $P \leq 0.05$).

Blood plasma progesterone concentration was measured by ELISA seven days following intrauterine infusion. Average circulating progesterone was above 3 ng/mL in each infusion group, however there was no effect of treatment ($P > 0.05$; Figure 2-10).

Table 2-1. List of qPCR primer sequences for target gene analysis

Gene	Primer	Sequence (5'–3')	GenBank accession no.	Product size (bp)
ACTB	5'	CAGAAGCACTCGTACGTGGG	NM_173979.3	200
	3'	TTGGCCTTAGGGTTCAGGG		
AKR1C4	5'	TGCAACCAGGTGGAATGTCA	NM_181027.2	122
	3'	ACCCATTCTTTTAGTCGTTGGGA		
CSF2	5'	TTCCTGTGGAACCCAGTTTATC	NM_174027.2	114
	3'	TTTGGCCTGCTTCACTTCT		
CXCL8	5'	GCAGGTATTTGTGAAGAGAGCTG	NM_173925.2	149
	3'	CACAGAACATGAGGCACTGAA		
GAPDH	5'	AGGTCGGAGTGAACGGATTC	NM_001034034.2	114
	3'	ATGGCGACGATGTCCACTTT		
IFNG	5'	CTCCGGCCTAACTCTCTCCT	NM_174086.1	174
	3'	GGCCCACCCTTAGCTACATC		
IL1A	5'	AGAGGATTCTCAGCTTCCTGTG	NM_174092.1	224
	3'	ATTTTTCTTGCTTTGTGGCAAT		
IL1B	5'	CTTCATTGCCCAGGTTTCTG	NM_174093.1	142
	3'	CAGGTGTTGGATGCAGCTCT		
IL6	5'	ATGACTTCTGCTTTCCCTACCC	XM_015468553.1	180
	3'	GCTGCTTTCACACTCATCATTC		
IL17A	5'	GCCCACCTACTGAGGACAAG	NM_001008412.2	134
	3'	ATTGCGGTGGAGAGTCCAAG		

Table 2-1. Continued.

Gene	Primer	Sequence (5'–3')	GenBank accession no.	Product size (bp)
PTGS2	5'	CGTGAAAGGCTGTCCCTTTA	XM_010813540.2	97
	3'	ATCTAGTCCAGAGTGGGAAGAG		
PTPRC	5'	AGACAAGGAAGCCTGTGCTC	XM_010813547.2	107
	3'	CACGGACGGTATACGCTGAA		
TGFB1	5'	AAGGGGTACCACGCCAATTT	NM_001166068.1	79
	3'	CCAGGACCTTGCTGTACTGTG		
TNF	5'	CACATACCCTGCCACAAGGC	NM_173966.3	261
	3'	CTGGGGACTGCTCTTCCCTCT		
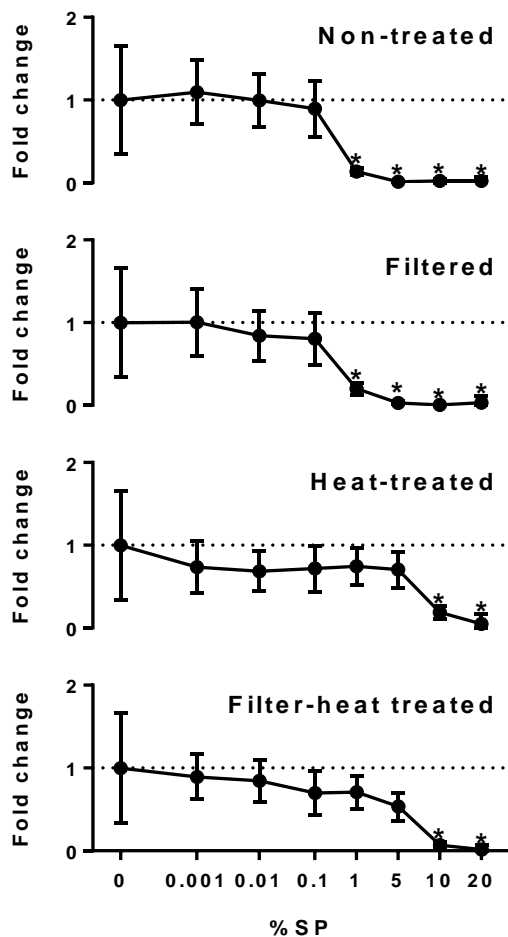
Figure 2-1. Effect of seminal plasma on BEND cell viability. Cell viability was assessed using the MTT assay after 24 h treatment with medium alone (0) or 0.001%, 0.01%, 0.1%, 1%, 5%, 10%, or 20% v/v seminal plasma (SP). Seminal plasma was either non-treated, filtered, heat-treated or both filtered and heat-treated. Data are presented as mean fold-change of medium alone treated cells ± SEM. Experiments were replicated in at least eight independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparison were made between medium alone and each seminal plasma concentration. *, represents $P \leq 0.05$ compared to medium alone.

Figure 2-2. Effect of seminal plasma on BEND cell gene expression. BEND cell expression of *CSF2* (A-D), *IL1B* (E-H), *TNF* (I-L), and *IL6* (M-P) was evaluated by qPCR after 24 h treatment with medium alone (0) or 0.001%, 0.01%, 0.1%, or 1% v/v seminal plasma (SP). Seminal plasma was either non-treated (A, E, I, M), filtered (B, F, J, N), heat-treated (C, G, K, O), or filtered and heat-treated (D, H, L, P). Data are presented as mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method ± SEM. Experiments were replicated in seven independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made between medium alone and each seminal plasma concentration. *, represents $P \leq 0.05$ compared to medium alone.
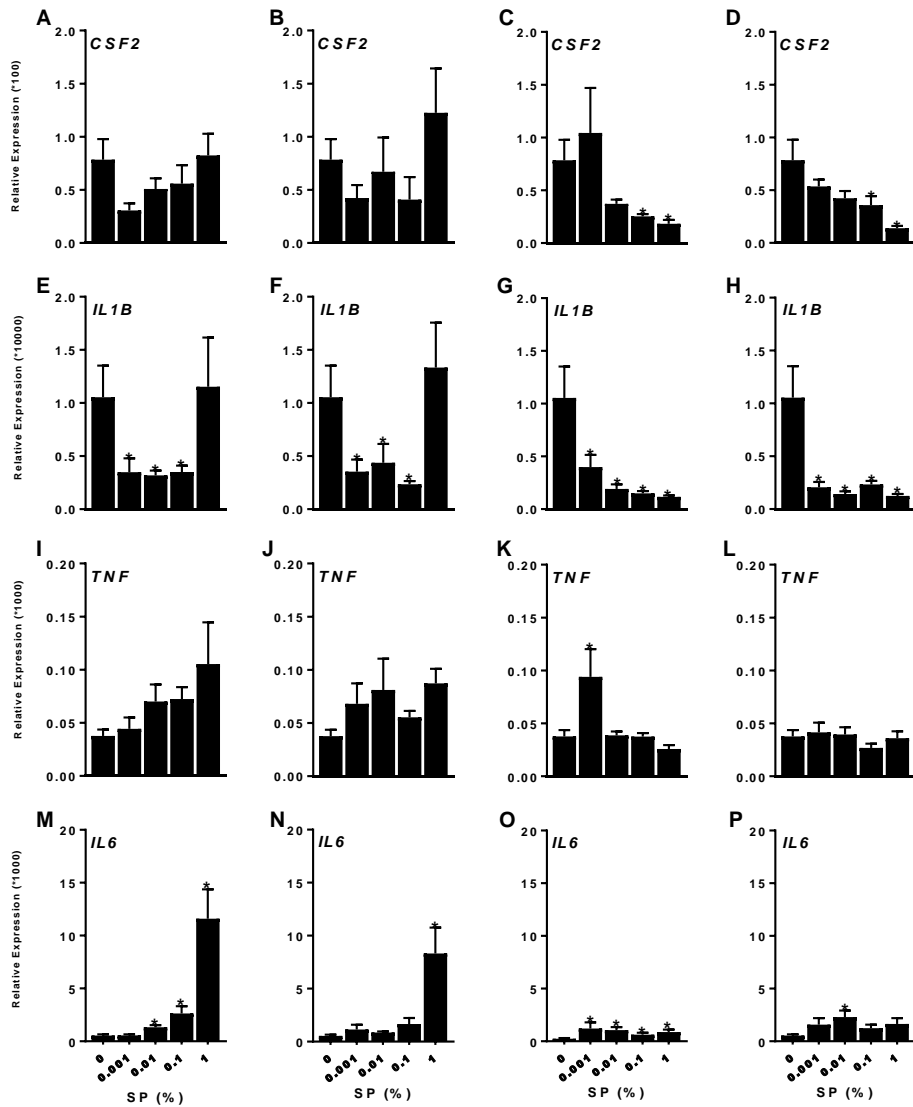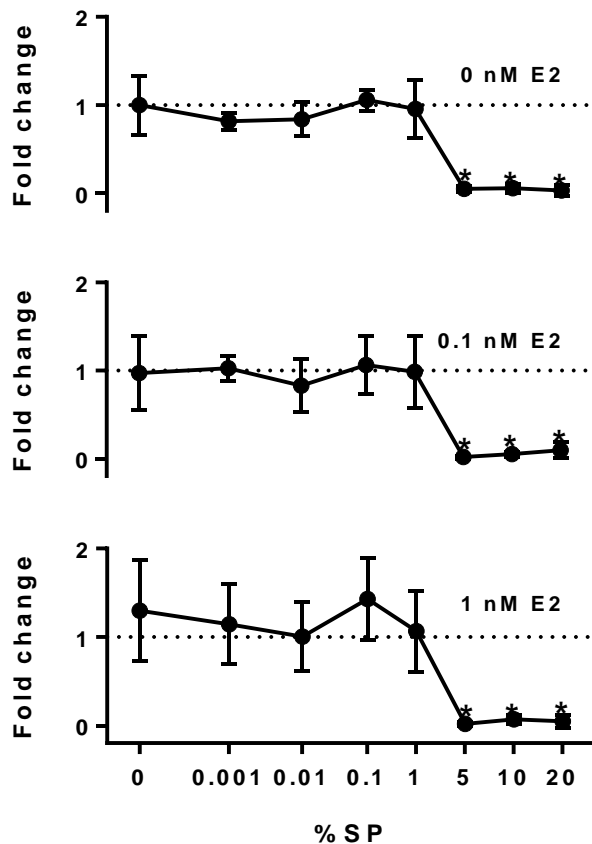
Figure 2-3. Effect of seminal plasma on viability of BEND cells supplemented with estradiol. Cell viability was assessed using the MTT assay. BEND cells were cultured in the presence of 0 nM, 0.1 nM or 1 nM estradiol for 24 h before addition of seminal plasma (SP) treatments. BEND cells were then treated with medium alone (0) or 0.001%, 0.01%, 0.1%, 1%, 5%, 10%, or 20% v/v SP for 24 h in medium supplemented with estradiol. Data are presented as mean fold-change of estradiol-free medium alone treated cells ± SEM. Experiments were replicated in four independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparison were made between medium alone and each seminal plasma concentration within estradiol concentrations.*, represents $P \leq 0.05$ compared to medium alone within estradiol concentration.

Figure 2-4. Effect of seminal plasma and estradiol on BEND cell gene expression.
BEND cell expression of *CSF2* (A-C), *IL1B* (D-F), *TNF* (G-I), and *IL6* (J-L)
was measured by qPCR. BEND cells were cultured in the presence of 0 nM
(A, D, G, J), 0.1 nM (B, E, H, K) or 1 nM (C, F, I, L) estradiol for 24 h before
addition of seminal plasma (SP) treatments. BEND cells were then treated
with medium alone (0) or 0.001%, 0.01%, 0.1%, 1%, 5%, 10%, or 20% v/v SP
for 24 h in medium supplemented with estradiol. Data are presented as mean
relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method ± SEM.
Experiments were replicated in eight independent assays. Data were log
transformed and analyzed using the generalized linear mixed model and
pairwise comparisons were made between medium alone and each seminal
plasma concentration within estradiol treatment and between each estradiol-
free media and estradiol treatments within a seminal plasma treatment. *,
represents $P \leq 0.05$ compared to medium alone within an estradiol
concentration; $^{\Psi}$, represents $P \leq 0.05$ compared to corresponding SP
concentrations between estradiol concentrations.
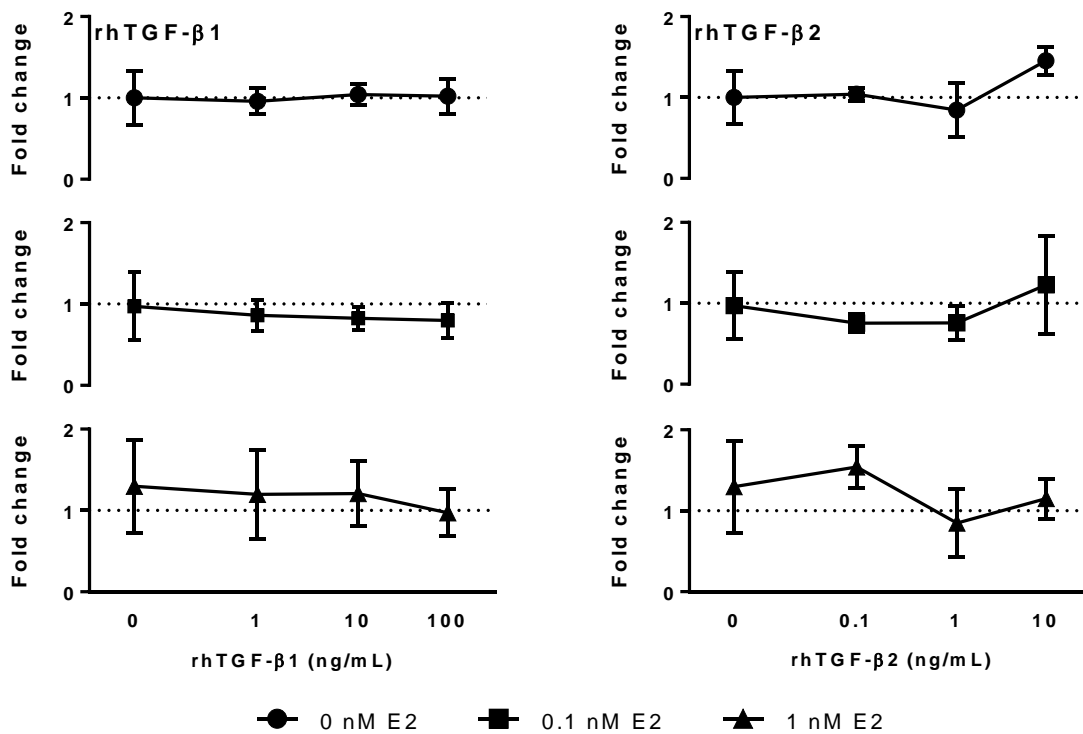
Figure 2-5. Effect of transforming growth factor β and estradiol on BEND cell viability. Cell viability was assessed using the MTT assay. BEND cells were cultured in the presence of 0 nM, 0.1 nM or 1 nM estradiol for 24 h before addition of TGF-β treatments. BEND cells were then treated with medium alone (0), rhTGF-β1 (1 ng, 10 ng or 100 ng/mL), or rhTGF-β2 (0.1 ng, 1 ng or 10 ng/mL) for 24 h in medium supplemented with estradiol. Data are presented as mean fold-change of estradiol-free medium alone treated cells ± SEM. Experiments were replicated in four independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparison were made between each treatment and vehicle control within an estradiol concentration. *, represents $P \leq 0.05$.

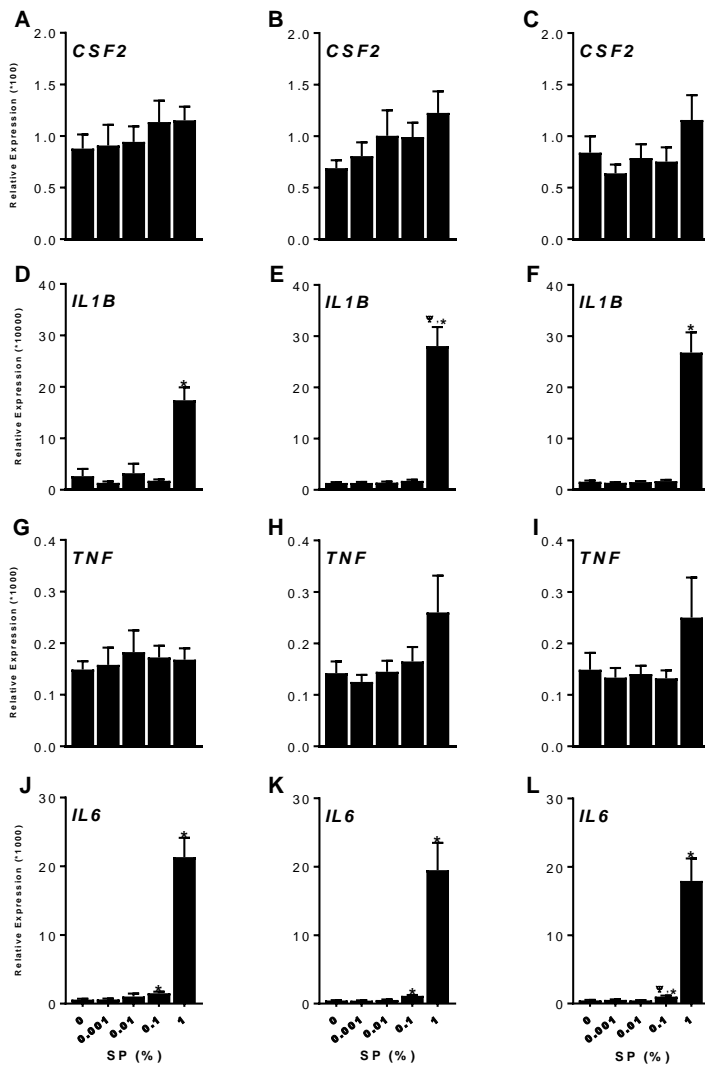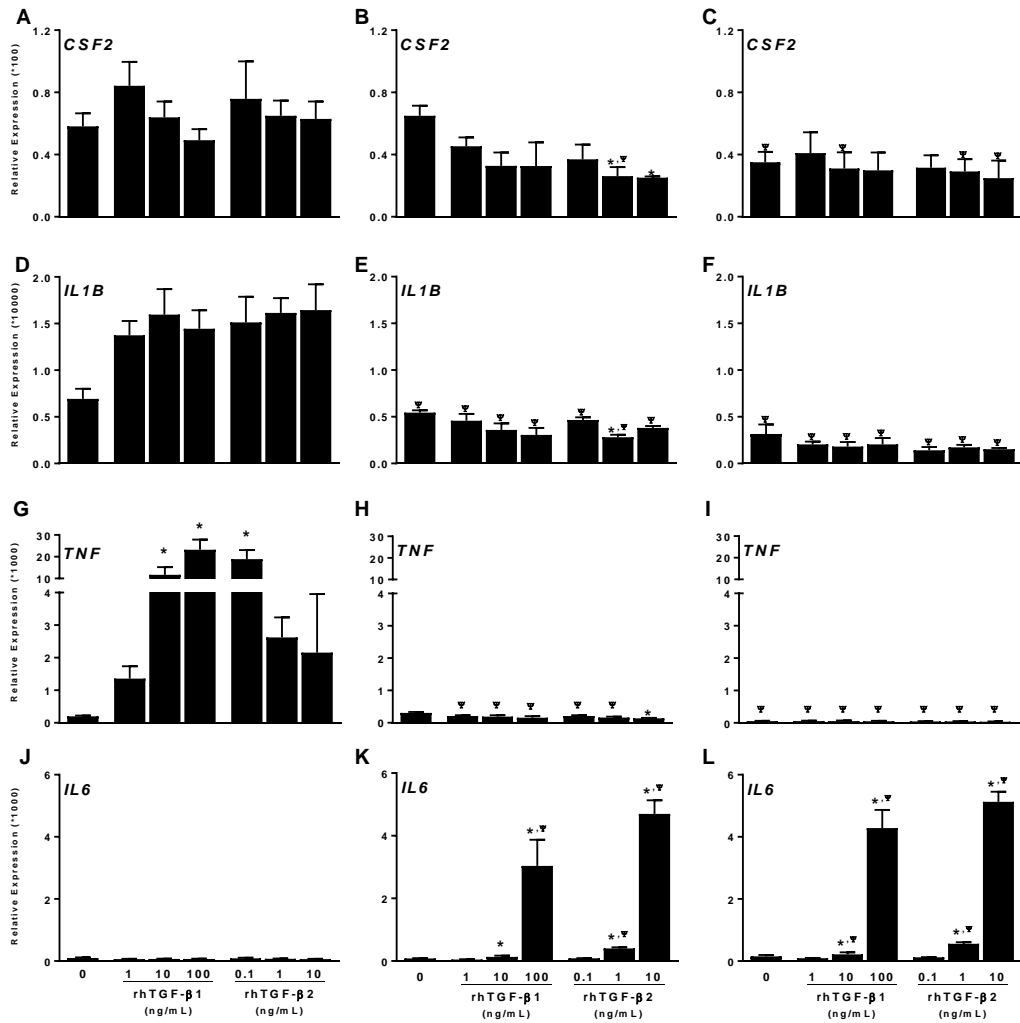Figure 2-6. Effect of transforming growth factor β and estradiol on BEND cell gene expression. BEND cell expression of *CSF2* (A-C), *IL1B* (D-F), *TNF* (G-I), and *IL6* (J-L) was measured by qPCR. BEND cells were cultured in the presence of 0 nM (A, D, G, J), 0.1 nM (B, E, H, K) or 1 nM (C, F, I, L) estradiol for 24 h before addition of TGF-β treatments. BEND cells were then treated with medium alone (0), rhTGF-β1 (1 ng, 10 ng or 100 ng/mL), or rhTGF-β2 (0.1 ng, 1 ng or 10 ng/mL) for 24 h in medium supplemented with estradiol. Data are presented as mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method ± SEM. Experiments were replicated in at least three independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparisons were made between appropriate medium alone and each treatment within an estradiol concentration and between each estradiol concentration within a given treatment. *, represents $P \le 0.05$ compared to appropriate medium alone within an estradiol concentration; $^{\Psi}$, represents $P \le 0.05$ treatment between estradiol concentrations.
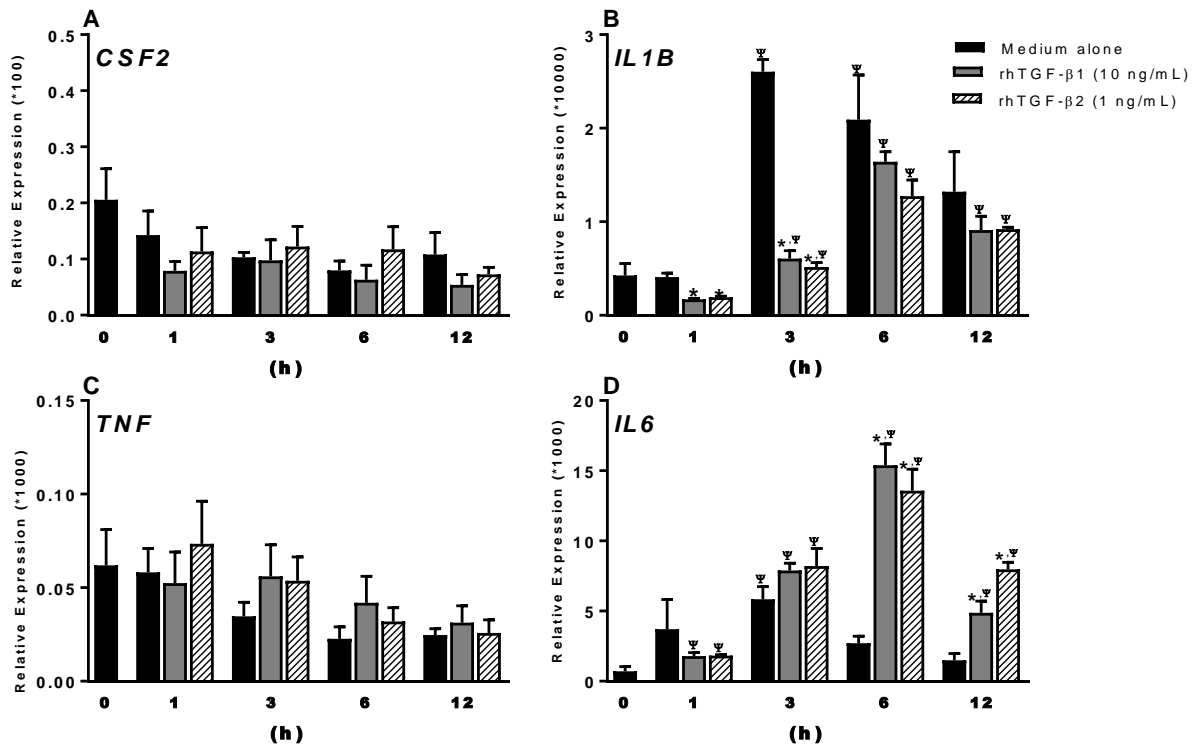
Figure 2-7. Acute effects of transforming growth factor β on BEND cell expression. BEND cell expression of *CSF2* (A), *IL1B* (B), *TNF* (C), and *IL6* (D) in response to medium alone, 10 ng/mL rhTGF-β1, or 1 ng/mL rhTGF-β2 for a period of 1 to 12 h. Data are presented as the mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method ± SEM. Experiments were replicated in four independent assays. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparison were made at each time point between medium alone and each treatment concentration and between 0 h and each treatment. *, represents $P \leq 0.05$ compared to medium alone within each time point; $^{\Psi}$, represents $P \leq 0.05$ compared to 0 h.
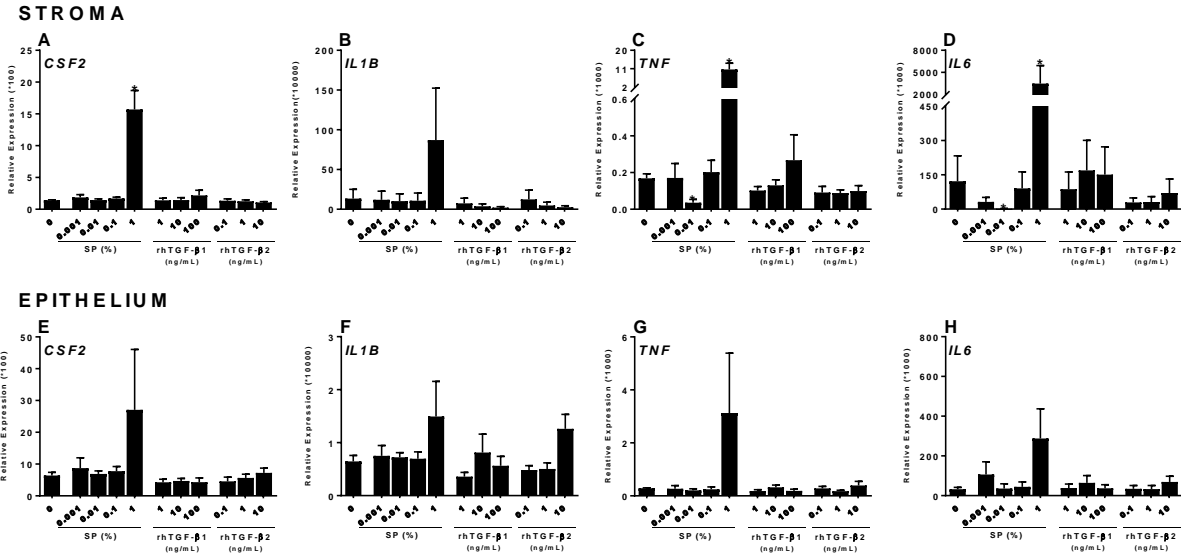
Figure 2-8. Effect of seminal plasma or transforming growth factor β on primary endometrial epithelial or stromal gene expression. Expression of *CSF2*, *IL1B*, *TNF*, and *IL6* in primary endometrial stroma (A-D) or epithelial (E-H) cells after 24 h treatment with medium alone (0), seminal plasma (SP; 0.001%, 0.01%, 0.1%, or 1% v/v), rhTGF-β1 (1 ng, 10 ng or 100 ng/mL), or rhTGF-β2 (0.1 ng, 1 ng or 10 ng/mL). Data are presented as the mean relative expression normalized to *GAPDH* using the $2^{-\Delta Ct}$ method ± SEM. Experiments were replicated in six independent assays with each replicate being representative of endometrial cells from an individual cow. Data were log transformed and analyzed using the generalized linear mixed model and pairwise comparison were made between medium alone and each treatment concentration. *, represents *P* ≤ 0.05 compared to medium alone.
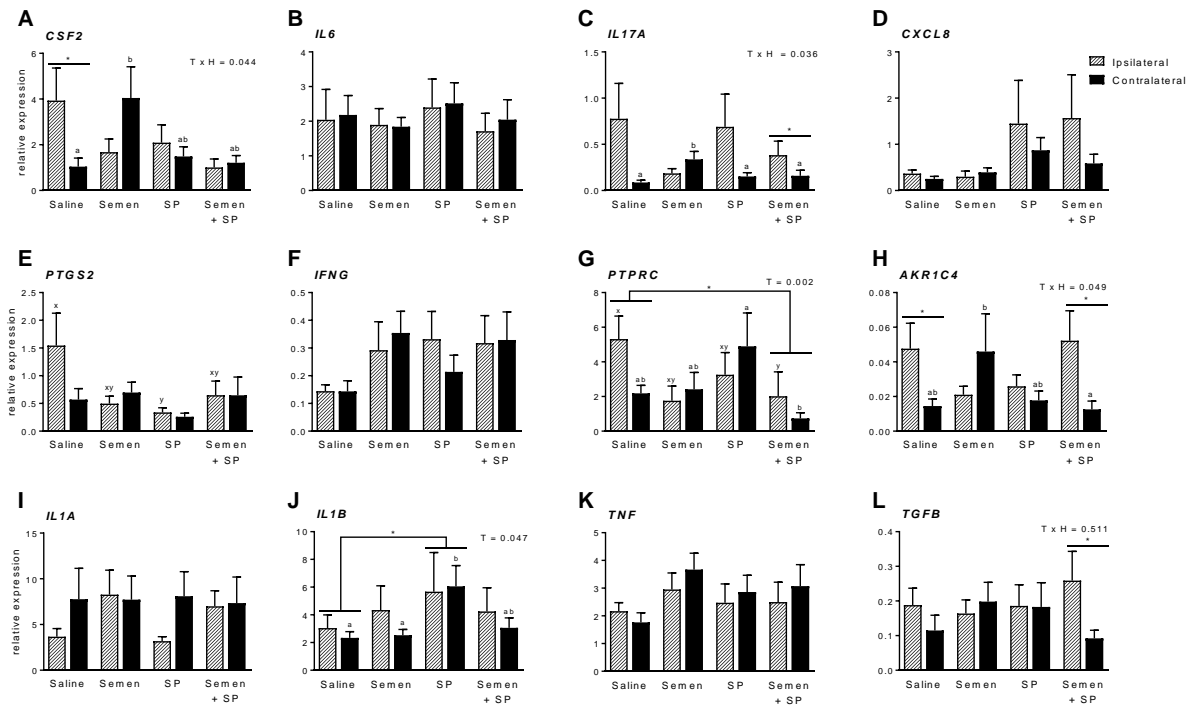
Figure 2-9. Effect of intrauterine infusion of semen components on endometrial gene expression. Expression of *CSF2*, *IL6*, *CXCL8*, *IL1A*, *IL1B*, *TNF*, *PTGS2*, *IFNG*, and *PTPRC* in endometrial biopsies collected 24 h following intrauterine infusion of conventional semen (n = 12), pooled seminal plasma (SP; n = 11), a combination of conventional semen and SP (n = 9), or saline (n = 11) as vehicle control. Biopsies were collected from uterine horns ipsilateral (hashed bars) and contralateral (solid bars) of the dominant follicle, identified by ultrasound the day before intrauterine infusion. Data are presented as the mean relative expression normalized to the geometric mean of housekeepers using the $2^{-\Delta Ct}$ method ± SEM. Data were log transformed and analyzed using repeated measures in the generalized linear mixed model with pairwise comparisons. The dam was used as the subject, treatment, horn, and treatment x horn were used as fixed effect. *, represents $P \leq 0.05$. Superscripts *a* and *b* refer to differences observed between treatments within the contralateral horn; superscripts *x* and *y* refer to differences observed between treatments within the ipsilateral horn. T, treatment effect; T_xH, treatment by horn interaction.
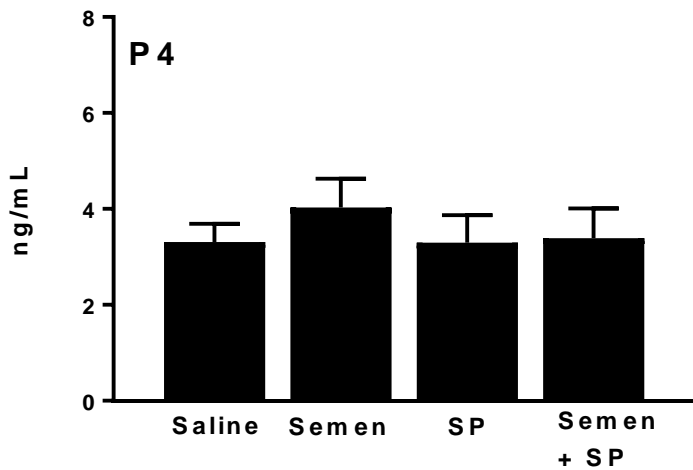
Figure 2-10. Effect of intrauterine infusion of semen components on day 7 blood plasma progesterone. Blood plasma progesterone was quantified by ELISA in cows 7 days after intrauterine infusion of saline (n = 11), conventional semen (12), pooled seminal plasma (SP; n = 11), or a combination of conventional semen and SP (n = 9). Data are presented as mean ng/mL of progesterone ± SEM. Data were analyzed using the generalized linear mixed model with pairwise comparisons. *, *P* value of ≤ 0.05.

CHAPTER 3
DISCUSSION AND CONCLUSIONS

Discussion

Reports from human and mice studies have shown that seminal plasma elicits endometrial inflammation associated with improving pregnancy outcomes and offspring performance. Specifically, seminal plasma derived TGF-β induces this inflammatory response in female reproductive tissues at the time of insemination (Tremellen et al., 2000; Robertson, 2005; Bromfield et al., 2014). Currently, there is little evidence to suggest that seminal plasma or components in semen, directly elicit endometrial inflammation in cattle (Bromfield, 2016). Furthermore, cows undergoing artificial insemination using diluted/extended semen are not exposed to physiological components and volumes of semen comparable to natural mating. The present study evaluated the responsiveness of bovine endometrial cells to seminal plasma or TGF-β using *in vivo* and *in vitro* exposures. We hypothesized that seminal plasma or TGF-β would increase the expression of proinflammatory factors in the bovine endometrium. Our data demonstrates that seminal plasma has a cytotoxic effect on endometrial cells above concentrations of 1% v/v that is heat-labile. In addition, exposure to seminal plasma increased *IL6* expression and differentially regulated expression of *IL1B* in an estrogen dependent manner. Exposure of endometrial cells to TGF-β increased expression of *TNF* but only in the absence of estrogen. Paradoxically, TGF-β increased expression of *IL6* in an estrogen dependent manner. Finally, intra-uterine infusion of seminal plasma at estrus increased expression of endometrial *IL1B* but had little effect on the expression of other inflammatory markers.

Pregnancy rates using artificial insemination in cattle are similar to natural service (Lima et al., 2009), and generally pregnancy rates achieved by embryo transfer are higher than for artificial insemination (Pellegrino et al., 2016). However, these artificial reproductive techniques differ from the natural physiology of conception in the bovine where semen is deposited in the vagina during natural service. During artificial insemination, diluted/extended semen is infused into the uterine body and both the composition and volume are substantially different from semen ejaculated by bulls. In rodents, swine and humans, seminal plasma increases expression of proinflammatory cytokines in the female reproductive tract (Robertson, 2005). It has been proposed that expression of these cytokines at the time of conception promote various changes important in pregnancy success, including tissue remodeling, embryo development and maternal immune adaption. Cytokines including IL-6, IL-8, and TGF-β promote angiogenesis, which establishes vascular changes in uterine tissues required to develop a functional/competent placenta that will support fetal growth until birth (Neufeld and Kessler, 2006; Forde and Lonergan, 2012; Fu et al., 2017). Exposure to seminal plasma in mice promotes the secretion of embryotrophic factors in the oviduct, improving embryo development (Bromfield et al., 2014). While seminal plasma induced inflammation enables maternal immune sensitization to paternal antigens, it also facilitates the establishment of maternal tolerance toward the semi-allogenic conceptus (Bromfield et al., 2018). It is unclear if any of the effects we have characterized here mediate changes to pregnancy success or downstream physiological processes.

We report that bovine seminal plasma is cytotoxic to endometrial cells, and that heat treatment alleviates this effect to some degree. This heat-labile effect implies that

seminal plasma induced cytotoxicity is protein mediated; however; concentrations of 10% v/v seminal plasma remained cytotoxic even after heat-treatment. It is unclear how seminal plasma cytotoxicity at higher concentrations is mediated, but may include alteration to culture medium pH or osmolality as additional seminal plasma is added to a closed system. Seminal plasma cytotoxicity has been associated with low fertility bulls (Nongbua et al., 2018), while bull fertility has been associated with the protein content of seminal plasma; protein deficient bulls have a higher incidence of subfertility in artificial insemination programs (Moura et al., 2006; Harayama et al., 2017). Interestingly, we observed that the concentration of seminal plasma required to achieving a cytotoxic effect varied between two pools of seminal plasma collected from unique populations of bulls. Here, we used pooled seminal plasma from two unique populations of Angus bulls collected by electroejaculation. The seminal plasma pool from the first collection was toxic for cells at doses of 1% v/v or higher; no viable BEND cells were observed in the wells. However, the seminal plasma pool prepared from the second cohort of Angus bulls was only cytotoxic for doses of 5% v/v or higher. Independent of the cohort, heat-treatment of seminal plasma alleviated cytotoxicity, but did not completely quench this activity. Several studies suggest that bovine seminal plasma is not a protective medium for sperm long after ejaculation, since seminal plasma compromises sperm quality (reviewed by Leahy and de Graaf, 2012). Curiously, epididymal fluid is not toxic to sperm while ejaculated seminal plasma kills sperm (Shannon, 1965; Way et al., 2000). This suggests that the cytotoxic factors in seminal plasma may be produced in specific accessory glands and this may explain why bulls vary in the toxicity of the seminal plasma. We speculate that the variation observed between bull cohorts may be either

associated to the fertility of bulls or be consequence of the environment affecting semen quality. In fact, it has been reported that season changes the protein content and composition of seminal plasma in rams, boars, and horses (Trudeau and Sanford, 1986; Janett et al., 2003; Cardozo et al., 2006). This coincides with reports in humans where the composition of seminal plasma is variable between individuals but also between ejaculates of the same male over time (Sharkey et al., 2017). It is possible that electroejaculation used for semen collection might have altered any effect of seminal plasma in our model, as seminal plasma composition varies between collection methods (Rego et al., 2015). Further studies using semen collected from electroejaculation and artificial vagina to evaluate endometrial responses should be performed.

A large proportion of pregnancy loss in dairy cattle occurs in the first week of pregnancy when approximately 40% of embryos fail to develop further than the blastocyst stage (Santos et al., 2004; Wiltbank et al., 2016). Early embryonic loss is likely a consequence of compromised oocyte fertilization, embryo development, oviduct/uterine support, and ovarian insufficiency (Sartori et al., 2002; Spencer et al., 2016b; Wiltbank et al., 2016). In dairy cows, this period of early pregnancy may be affected by postpartum complications including metabolic stress and uterine infection (Lucy, 2001). The utility of IVF enables the development of the embryo to the blastocyst stage of development prior to transfer to embryo recipients, bypassing this period of embryonic loss. It has been reported that proinflammatory cytokines secreted by the endometrium have embryotrophic properties, emphasizing the importance of the maternal environment for embryo development. In mice, pigs, humans, and cattle the

maternal cytokine CSF-2 communicates with the embryo and promotes development during the preimplantation period (Robertson et al., 2001; Sjöblom et al., 2005; Kwak et al., 2012; Siqueira and Hansen, 2016). Our data suggests that heat treatment of seminal plasma reduces *CSF2* expression in BEND cells, while intra-uterine infusion of semen or seminal plasma did not affect endometrial *CSF2* expression. However, *CSF2* expression was increased in semi-purified endometrial stromal cells. This is in agreement with Ibrahim et al. (2018) that demonstrated a seminal plasma mediated increase in *CSF2* expression of endometrial explants, stromal cells and epithelial cells. Tribulo et al. reported that endometrial *CSF2* expression is modulated throughout the estrous cycle, resulting in maximal endometrial expression at day 5 (Tríbulo et al., 2018). It is possible that the time selected to measure endometrial gene expression following intra-uterine infusion (24 hours) do not coincide with the time of maximal *CSF2* expression and that BEND cells are not an ideal model system to evaluate endometrial response to seminal plasma. More studies are required to evaluate the capacity for seminal plasma to modulate *CSF2* in a temporal fashion or perhaps in the oviduct, which would benefit embryo development.

Expression of *IL6* was increased in BEND cells following seminal plasma exposure. Interestingly, seminal plasma mediated *IL6* expression was still apparent after heat treatment, but to a lower relative expression. These results confirm that seminal plasma increases the expression of *IL6* and that this response may be mediated by the protein fraction of seminal plasma. In humans, Cross (1996) reported that the sperm acrosome reaction is inhibited by exposure to seminal plasma, and that this inhibitory action is not quenched after seminal plasma heat treatment at 90°C for 10

minutes (Cross, 1996). A similar effect has been observed in bulls, where seminal

plasma modulation of neutrophil phagocytosis of sperm was not affected after heat

treatment of seminal plasma at 56°C for 30 minutes (Strzemienski, 1989).

Odhiambo et al. reported a tendency for improving pregnancy rates in beef cows

with subfertility (pregnancy rate < 50%) after intrauterine infusion of TGF-β1 at

insemination but not seminal plasma (Odhiambo et al., 2009). Here we report that

treatment of BEND cells with TGF-β1 or TGF-β2 increased *IL6* and *TNF* expression.

Expression of BEND cell *TNF* was increased in response to TGF-β in the absence of

estrogen. In contrast, expression of *IL6* was only increased in BEND cells in the

presence of estrogen after 24 h. However, primary epithelial cells did not respond to

either TGF-β1 or TGF-β2. Conversely, we did not see an increase in endometrial *IL6*

expression following intrauterine infusion of semen or seminal plasma. Our findings are

similar to reports in humans where seminal plasma increased *IL6* expression without

altering *TNF* in endometrial cells (Gutsche et al., 2003), and in pigs where seminal

plasma or seminal plasma derived heparin-binding protein increased *IL6* expression in

cervical cells (Madej et al., 2012). Interestingly, treatment of human endometrial cells

with *TNF* for 24 hours increased *IL6* expression (Bersinger et al., 2011). However, we

observed that seminal plasma increased *IL6* without affecting *TNF* expression, and that

TGF-β has this same effect under the influence of estrogen. Therefore, it could be

speculated that seminal plasma or TGF-β promote a uterine environment beneficial for

early embryonic development by either inhibiting overproduction of *TNF*, independently

promoting IL-6 secretion or both. This potential mechanism is supported by reports in

humans where endometrial IL-6 is associated with promoting blastocyst development

and increasing implantation rates (Dominguez et al., 2010). While IL-6 supplementation to embryo culture medium promotes blastocyst development in the bovine (Wooldridge and Ealy, 2019).

Seminal plasma derived TGF-β elicits inflammation in the murine endometrium and human cervix (Tremellen et al., 1998; Robertson, 2005). However, expression of endometrial *IL6* could mediate TGF-β signaling and influence endometrial inflammation via IL-17A secretion. In humans, endometrial IL-6 is associated with reducing the incidence of recurrent spontaneous abortions by maintaining the balance between Treg and Th17 which are IL-17A dependent (Saito et al., 2010). In mice, seminal plasma induced secretion of IL-17A and mediated gamma-delta (γδ) T cells function (Song et al., 2016). We did not observe an effect of semen or seminal plasma on *IL17A* expression *in vivo*; however, Ibrahim et al. (2018) reported that bovine seminal plasma increased the expression of *IL17A* and *TGFB1* in endometrial cells (Ibrahim et al., 2018).

Using seminal plasma from the first cohort of bulls we observed a decreased expression of *IL1B* in BEND cells, however seminal plasma derived from the second cohort of bulls increased *IL1B* expression, which was further increased in the presence of estrogen. Excitingly, we observed that the expression of *IL1B* increases following intrauterine infusion of seminal plasma compared to infusion of saline. These results are similar to findings in human were seminal plasma increases *IL1B* and *IL6* expression in endometrial epithelial cells (Gutsche et al., 2003). Endometrial IL-1β increases the expression of *PTGS2* and synthesis of PGE2 in the endometrium under the influence of estrogen (Bazer, 2013), along with promoting decidualization of stromal cells in humans

82

(White et al., 2007). Blocking IL-1β signaling with a receptor antagonist, increases

implantation failure in mice (Zheng et al., 1995). Jasper et al. showed that macrophages

recruited during the post-coital inflammatory response in mice secrete LIF and IL-1β,

which regulate the glycosylation of proteins that allow trophectoderm attachment to the

endometrium (Jasper et al., 2011). Furthermore, endometrial IL-1β is elevated locally at

the site of embryo-uterine interaction (Correia-Álvarez et al., 2015).

The use of artificial insemination has been linked to lower progesterone

concentration in the early luteal phase (Spencer et al., 2016a), which is detrimental for

embryo development (Lonergan, 2011). O'Leary et al. reported that infusion of seminal

plasma at insemination increases plasma progesterone and embryo development in

swine (O'Leary et al., 2004). In cattle, seminal plasma derived NGF is luteotrophic and

promotes increased progesterone synthesis (Tribulo et al., 2015), increasing

pregnancy-specific protein B (PSPB) in circulation (Stewart et al., 2018). Following

intrauterine infusion of semen or seminal plasma, we did not observe any effect on

endometrial *PTSG2* or *AKR1C4* expression, or day 7 plasma progesterone. This is in

conflict with previous reports that have shown seminal plasma induced endometrial

expression of *PTGS2* and *AKR1C4 in vitro* (Ibrahim et al., 2018).

Seminal plasma elicits an inflammatory response in the maternal reproductive

tract (Yanagimachi and Change, 1963). An influx of leukocytes into the bovine

reproductive tract in response to semen was documented in young heifers (Howe and

Black, 1963) which may be associated with the endometrial response to semen

described in cattle over 50 years ago (Mattner, 1968). We evaluated cellular

inflammation of the endometrium following intrauterine infusion by measuring

expression of *PTPRC*, a marker for CD45 cells. Interestingly, intrauterine exposure of semen or seminal plasma decreased *PTPRC* expression, while the expression of other inflammatory genes remained unchanged. These results suggest that there was not a marked inflammation in the endometrium in response to conventional, diluted semen or seminal plasma. However, it is possible that the timing of evaluation failed to capture the window of response. It is also likely that evaluating the interaction between semen components and cervix might be more informative as in humans where semen is deposited at coitus. It will be important to evaluate the volume of seminal plasma that reaches the endometrium following coitus in future experiments, or perhaps utilize vasectomized bulls for seminal plasma exposure prior to artificial insemination. Experiments evaluating the effect of semen exposure on the cervix or endometrium in a temporal manner will be required to fully characterize cellular inflammation in the bovine.

Conclusion

The reproductive performance of dairy cows has been declining in recent history, with the biggest pregnancy losses taking place during the first weeks after insemination. Interestingly, seminal plasma or TGF-β infusion at insemination have a tendency to increase pregnancy rates in herds with low fertility. We speculate that there is a reproductive advantage for maternal exposure to seminal plasma in cattle that regulates maternal immune responses to pregnancy, triggering acute endometrial inflammation, and synthesis of factors to support embryo development. Our data suggest that seminal plasma, and potentially TGF-β, influence endometrial expression of maternal cytokines; however, this response is bull specific and may involve cytotoxic effects of semen. The identification of paternal factors contained in semen that have the potential to improve

84

bovine reproduction and reduce economic losses associated with pregnancy failure could help optimize semen used for artificial insemination. Additional experiments will be required to determine the potential of seminal plasma or TGF-β exposure at conception to improve fertility in the cow.

LIST OF REFERENCES

Abdollahi-Arpanahi, R., G. Morota, and F. Peñagaricano. 2017. Predicting bull fertility using genomic data and biological information. J. Dairy Sci. 100:9656–9666. doi:10.3168/jds.2017-13288.

Achache, H., and A. Revel. 2006. Endometrial receptivity markers, the journey to successful embryo implantation. Hum. Reprod. Update 12:731–746. doi:10.1093/humupd/dml004.

Albieri, A., M.S. Hoshida, S.M. Gagioti, E.C. Leanza, I. Abrahamsohn, A. Croy, A.A. Ashkar, and E. Bevilacqua. 2005. Interferon-gamma alters the phagocytic activity of the mouse trophoblast. Reprod. Biol. Endocrinol. 3:1–11. doi:10.1186/1477-7827-3-34.

Alghamdi, A.S., B.J. Lovaas, S.L. Bird, G.C. Lamb, A.K. Rendahl, P.C. Taube, and D.N. Foster. 2009. Species-specific interaction of seminal plasma on sperm-neutrophil binding. Anim. Reprod. Sci. 114:331–344. doi:10.1016/j.anireprosci.2008.10.015.

Aluvihare, V.R., M. Kallikourdis, and A.G. Betz. 2004. Regulatory T cells mediate maternal tolerance to the fetus. Nat. Immunol. 5:266–271. doi:10.1038/ni1037.

Amann, R.P., and J.M. DeJarnette. 2012. Impact of genomic selection of AI dairy sires on their likely utilization and methods to estimate fertility: A paradigm shift. Theriogenology 77:795–817. doi:10.1016/j.theriogenology.2011.09.002.

Baker, S.J., and E.P. Reddy. 1998. Modulation of life and death by the TNF receptor superfamily. Oncogene 17:3261–3270. doi:10.1038/sj.onc.1202568.

Bazer, F.W. 2013. Pregnancy recognition signaling mechanisms in ruminants and pigs. J. Anim. Sci. Biotechnol. 4:1. doi:10.1186/2049-1891-4-23.

Beer, A.E., R.E. Billingham, and J.R. Scott. 1975. Immunogenetic aspects of implantation, placentation and feto-placental growth rates. Biol. Reprod. 12:176–189. doi:10.1095/biolreprod12.1.176.

Bennouna, S., S.K. Bliss, T.J. Curiel, and E.Y. Denkers. 2003. Cross-Talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. J. Immunol. 171:6052–6058. doi:10.4049/jimmunol.171.11.6052.

Berg, D.K., J. van Leeuwen, S. Beaumont, M. Berg, and P.L. Pfeffer. 2010. Embryo loss in cattle between Days 7 and 16 of pregnancy. Theriogenology 73:250–260. doi:10.1016/j.theriogenology.2009.09.005.

Berisha, B., M.W. Pfaffl, and D. Schams. 2002. Expression of estrogen and progesterone receptors in the bovine ovary during estrous cycle and pregnancy. Endocrine 17:207–214. doi:10.1385/ENDO:17:3:207.

Bersinger, N.A., A.R. Günthert, B. McKinnon, S. Johann, and M.D. Mueller. 2011. Dose-response effect of interleukin (IL)-1β, tumour necrosis factor (TNF)-α, and interferon-γ on the in vitro production of epithelial neutrophil activating peptide-78 (ENA-78), IL-8, and IL-6 by human endometrial stromal cells. Arch. Gynecol. Obstet. 283:1291–1296. doi:10.1007/s00404-010-1520-3.

Betteridge, K.J., M.D. Eaglesome, G.C.B. Randall, and D. Mitchell. 1980. Collection, description and transfer of embryos from cattle 10-16 days after oestrus. J. Reprod. Fertil. 59:205–216. doi:10.1530/jrf.0.0590205.

Bhatt, H., L.J. Brunet, and C.L. Stewart. 1991. Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. Proc. Natl. Acad. Sci. 88:11408–11412. doi:10.1073/pnas.88.24.11408.

Blois, S.M., U. Kammerer, C.A. Soto, M.C. Tometten, V. Shaikly, G. Barrientos, R. Jurd, D. Rukavina, A.W. Thomson, B.F. Klapp, N. Fernández, and P.C. Arck. 2007. Dendritic cells: key to fetal tolerance? Biol. Reprod. 77:590–598. doi:10.1095/biolreprod.107.060632.

Bromfield, J.J. 2016. A role for seminal plasma in modulating pregnancy outcomes in domestic species. Reproduction 152:R223–R232. doi:10.1530/REP-16-0313.

Bromfield, J.J. 2014. Seminal fluid and reproduction: Much more than previously thought. J. Assist. Reprod. Genet. 31:627–636. doi:10.1007/s10815-014-0243-y.

Bromfield, J.J., J.A. Rizo, and L.A. Ibrahim. 2018. Paternal priming of maternal tissues to optimise pregnancy success. Reprod. Fertil. Dev. 30. doi:10.1071/RD17345.

Bromfield, J.J., J.E. Schjenken, P.Y. Chin, A.S. Care, M.J. Jasper, and S.A. Robertson. 2014. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. Proc. Natl. Acad. Sci. 111:2200–2205. doi:10.1073/pnas.1305609111.

Bulletti, C., D. De Ziegler, V. Polli, L. Diotallevi, E. Del Ferro, and C. Flamigni. 2000. Uterine contractility during the menstrual cycle. Hum. Reprod. 15:81–89. doi:10.1093/humrep/15.suppl_1.81.

Butler, W.R., and R.D. Smith. 1989. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J. Dairy Sci. 72:767–783. doi:10.3168/jds.S0022-0302(89)79169-4.

Calle, A., S. López-Martín, M. Monguió-Tortajada, F.E. Borràs, M. Yáñez-Mó, and M.Á. Ramírez. 2019. Bovine endometrial MSC: mesenchymal to epithelial transition during luteolysis and tropism to implantation niche for immunomodulation. Stem Cell Res. Ther. 10:23. doi:10.1186/s13287-018-1129-1.

Campbell, J.M., M. Lane, J.A. Owens, and H.W. Bakos. 2015. Paternal obesity negatively affects male fertility and assisted reproduction outcomes: A systematic review and meta-analysis. Reprod. Biomed. Online 31:593–604. doi:10.1016/j.rbmo.2015.07.012.

Cardozo, J.A., M. Fernández-Juan, F. Forcada, A. Abecia, T. Muiño-Blanco, and J.A. Cebrián-Pérez. 2006. Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. Theriogenology 66:841–850. doi:10.1016/j.theriogenology.2006.01.058.

de Catanzaro, D., and T. Pollock. 2016. Absorption and distribution of estradiol from male seminal emissions during mating. J. Endocrinol. 231:245–257. doi:10.1530/JOE-16-0247.

Chegini, N., X.M. Tang, and Q. Dou. 1999. The expression, activity and regulation of granulocyte macrophage- colony stimulating factor in human endometrial epithelial and stromal cells. Mol. Hum. Reprod. 5:459–466. doi:10.1093/molehr/5.5.459.

Chen, W., W. Jin, N. Hardegen, K. Lei, L. Li, N. Marinos, G. McGrady, and S.M. Wahl. 2003. Conversion of peripheral CD4+ CD25− naive T cells to CD4+ CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. J. Exp. Med. 198:1875–1886. doi:10.1084/jem.20030152.

Cochran, S.D., J.B. Cole, D.J. Null, and P.J. Hansen. 2013a. Discovery of single nucleotide polymorphisms in candidate genes associated with fertility and production traits in Holstein cattle. BMC Genet. 14:1. doi:10.1186/1471-2156-14-49.

Cochran, S.D., J.B. Cole, D.J. Null, and P.J. Hansen. 2013b. Single nucleotide polymorphisms in candidate genes associated with fertilizing ability of sperm and subsequent embryonic development in cattle. Biol. Reprod. 89:1–7. doi:10.1095/biolreprod.113.111260.

Comins Boo, A., A.G. Segovia, N.N. del Prado, L. de la Fuente, J. Alonso, and S.S. Ramon. 2016. Evidence-based update: immunological evaluation of recurrent implantation failure. Reprod. Immunol. Open Access 01. doi:10.21767/2476-1974.100024.

Correia-Álvarez, E., E. Gómez, D. Martín, S. Carrocera, S. Pérez, J. Otero, N. Peynot, C. Giraud-Delville, J.N. Caamaño, O. Sandra, V. Duranthon, and M. Muñoz. 2015. Expression and localization of interleukin 1 beta and interleukin 1 receptor (type I) in the bovine endometrium and embryo. J. Reprod. Immunol. 110:1–13. doi:10.1016/j.jri.2015.03.006.

Cross, N.L. 1996. Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. Biol Reprod 54:138–145. doi:10.1137/1.9780898718713.ch12.

Dejarnette, J.M., and C.E. Marshall. 2005. Straw-thawing method interacts with sire and extender to influence sperm motility and conception rates of dairy cows. J. Dairy Sci. 88:3868–3875. doi:10.3168/jds.S0022-0302(05)73072-1.

Derynck, R., and Y.E. Zhang. 2003. Smad-dependent and smad-independent pathways in TGF-B family signaling. doi:10.1038/nature02006.

Dhar-Mascareño, M., J. Chen, R.H. Zhang, J.M. Cárcamo, and D.W. Golde. 2003. Granulocyte-macrophage colony-stimulating factor signals for increased glucose transport via phosphatidylinositol 3-kinase- and hydrogen peroxide-dependent mechanisms. J. Biol. Chem. 278:11107–11114. doi:10.1074/jbc.M212541200.

Diskin, M.G., J.J. Murphy, and J.M. Sreenan. 2006. Embryo survival in dairy cows managed under pastoral conditions. Anim. Reprod. Sci. 96:297–311. doi:10.1016/j.anireprosci.2006.08.008.

Dobbs, K.B., D. Gagné, E. Fournier, I. Dufort, C. Robert, J. Block, M.-A. Sirard, L. Bonilla, A.D. Ealy, B. Loureiro, and P.J. Hansen. 2014. Sexual dimorphism in developmental programming of the bovine preimplantation embryo caused by colony-stimulating factor 2. Biol. Reprod. 91:1–12. doi:10.1095/biolreprod.114.121087.

Dobbs, K.B., F.A. Khan, M. Sakatani, J.I. Moss, M. Ozawa, A.D. Ealy, and P.J. Hansen. 2013. Regulation of pluripotency of inner cell mass and growth and differentiation of trophectoderm of the bovine embryo by colony stimulating factor 2. Biol. Reprod. 89:1–10. doi:10.1095/biolreprod.113.113183.

Dominguez, F., B. Gadea, A. Mercader, F.J. Esteban, A. Pellicer, and C. Simón. 2010. Embryologic outcome and secretome profile of implanted blastocysts obtained after coculture in human endometrial epithelial cells versus the sequential system. Fertil. Steril. 93. doi:10.1016/j.fertnstert.2008.10.019.

Dorniak, P., F.W. Bazer, and T.E. Spencer. 2011. Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. Biol. Reprod. 84:1119–1127. doi:10.1095/biolreprod.110.089979.

Druart, X., J.P. Rickard, S. Mactier, P.L. Kohnke, C.M. Kershaw-Young, R. Bathgate, Z. Gibb, B. Crossett, G. Tsikis, V. Labas, G. Harichaux, C.G. Grupen, and S.P. de Graaf. 2013. Proteomic characterization and cross species comparison of mammalian seminal plasma. J. Proteomics 91:13–22. doi:10.1016/j.jprot.2013.05.029.

Du, M., P.-F. Guo, H. Piao, S. Wang, C. Sun, L.-P. Jin, Y. Tao, Y.-H. Li, D. Zhang, R. Zhu, Q. Fu, and D.J. Li. 2014. Embryonic trophoblasts induce decidual regulatory T cell differentiation and maternal-fetal tolerance through thymic stromal lymphopoietin instructing dendritic cells. J. Immunol. 192:1502–1511. doi:10.4049/jimmunol.1203425.

Eldamnhoury, E.M., G.A. Elatrash, H.M. Rashwan, and A.I. El-Sakka. 2018. Association between leukocytospermia and semen interleukin-6 and tumor necrosis factor-alpha in infertile men. Andrology 6:775–780. doi:10.1111/andr.12513.

Fabian, D., S. Juhás, G. Il'ková, and J. Koppel. 2007. Dose- and time-dependent effects of TNFα and actinomycin D on cell death incidence and embryo growth in mouse blastocysts. Zygote 15:241–249. doi:10.1017/S0967199407004200.

Fair, T. 2015. The contribution of the maternal immune system to the establishment of pregnancy in cattle. Front. Immunol. 6:1–8. doi:10.3389/fimmu.2015.00007.

Faulkner, L.C., M.L. Hopwood, and J.N. Wiltbank. 1968. Seminal vesiculectomy in bulls. J. Reprod. Fertil. 16:179–182.

Fleisch, M.C., C.A. Maxwell, and M.H. Barcellos-Hoff. 2006. The pleiotropic roles of transforming growth factor beta in homeostasis and carcinogenesis of endocrine organs. Endocr. Relat. Cancer 13:379–400. doi:10.1677/erc.1.01112.

Forde, N., and P. Lonergan. 2012. Transcriptomic analysis of the bovine endometrium: what is required to establish uterine receptivity to implantation in cattle ? J. Reprod. Dev. 58:189–195. doi:10.1262/jrd.2011-021.

Fridlender, Z.G., J. Sun, S. Kim, V. Kapoor, G. Cheng, G.S. Worthen, and S.M. Albelda. 2010. Polarization of tumor-associated neutrophil (TAN) phenotype by TGF-β: "N1" versus "N2" TAN. Cancer 16:183–194. doi:10.1016/j.ccr.2009.06.017.Polarization.

Fu, B., Y. Zhou, X. Ni, X. Tong, X. Xu, Z. Dong, R. Sun, Z. Tian, and H. Wei. 2017. Natural killer cells promote fetal development through the secretion of growth-promoting factors. Immunity 47:1100–1113.e6. doi:10.1016/j.immuni.2017.11.018.

Fujiwara, H., Y. Araki, K. Imakawa, S. Saito, T. Daikoku, M. Shigeta, H. Kanzaki, and T. Mori. 2016. Dual positive regulation of embryo implantation by endocrine and immune systems – step-by-step maternal recognition of the developing embryo. Am. J. Reprod. Immunol. 75:281–289. doi:10.1111/aji.12478.

Gad, A., M. Hoelker, U. Besenfelder, V. Havlicek, U. Cinar, F. Rings, E. Held, I. Dufort, M.-A. Sirard, K. Schellander, and D. Tesfaye. 2012. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. Biol. Reprod. 87:1–13. doi:10.1095/biolreprod.112.099697.

Gao, H., G. Wu, T.E. Spencer, G.A. Johnson, X. Li, and F.W. Bazer. 2009. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biol. Reprod. 80:86–93. doi:10.1095/biolreprod.108.071597.

Gaynor, L.M., and F. Colucci. 2017. Uterine natural killer cells: Functional distinctions and influence on pregnancy in humans and mice. Front. Immunol. 8. doi:10.3389/fimmu.2017.00467.

Geisert, R., M. Lucy, and D. Mathew. 2012. Pregnancy in mammals : role of interleukin 1β. Cell Tissue Res. 349:825–838. doi:10.1007/s00441-012-1356-1.Interaction.

Ghiringhelli, F., P.E. Puig, S. Roux, A. Parcellier, E. Schmitt, E. Solary, G. Kroemer, F. Martin, B. Chauffert, and L. Zitvogel. 2005. Tumor cells convert immature myeloid dendritic cells into TGF-β–secreting cells inducing CD4+ CD25+ regulatory T cell proliferation. J. Exp. Med. 202:919–929. doi:10.1084/jem.20050463.

Granot, I., Y. Gnainsky, and N. Dekel. 2012. Endometrial inflammation and effect on implantation improvement and pregnancy outcome. Reproduction 144:661–668. doi:10.1530/REP-12-0217.

Guerin, L.R., L.M. Moldenhauer, J.R. Prins, J.J. Bromfield, J.D. Hayball, and S.A. Robertson. 2011. Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. Biol. Reprod. 85:397–408. doi:10.1095/biolreprod.110.088591.

Gutsche, S., M. von Wolff, T. Strowitzki, and C.J. Thaler. 2003. Seminal plasma induces mRNA expression of IL-1β, IL-6 and LIF in endometrial epithelial cells in vitro. Mol. Hum. Reprod. 9:785–791. doi:10.1093/molehr/gag095.

Han, Y., and F. Peñagaricano. 2016. Unravelling the genomic architecture of bull fertility in Holstein cattle. BMC Genet. 17:1–11. doi:10.1186/s12863-016-0454-6.

Hannan, N.J., K. Bambang, T.J. Kaitu'u-Lino, J.C. Konje, and S. Tong. 2014. A bioplex analysis of cytokines and chemokines in first trimester maternal plasma to screen for predictors of miscarriage. PLoS One 9:2–8. doi:10.1371/journal.pone.0093320.

Hansen, P.J., K.B. Dobbs, and A.C. Denicol. 2014. Programming of the preimplantation embryo by the embryokine colony stimulating factor 2. Anim. Reprod. Sci. 149:59–66. doi:10.1016/j.anireprosci.2014.05.017.

Hansen, T.R., L.D.P. Sinedino, and T.E. Spencer. 2017. Paracrine and endocrine actions of interferon tau (IFNT). Reproduction 154:F45–F59. doi:10.1530/REP-17-0315.

Harayama, H., K. Minami, K. Kishida, and T. Noda. 2017. Protein biomarkers for male artificial insemination subfertility in bovine spermatozoa. Reprod. Med. Biol. 16:89–98. doi:10.1002/rmb2.12021.

Hardy, K., and S. Spanos. 2002. Growth factor expression and function in the human and mouse preimplantation embryo. J. Endocrinol. 172:221–236. doi:10.1677/joe.0.1720221.

Howe, G.R., and D.L. Black. 1963. Spermatozoan transport and leucocytic responses in the reproductive tract of calves. J. Reprod. Fertil. 6:305–311.

Hunt, J.S., H.L. Chen, X.-L. Hu, and J.W. Pollard. 1993. Normal distribution of tumor necrosis factor-alpha messenger ribonucleic acid and protein in the uteri, placentas, and embryos of osteopetrotic (op/op) mice lacking colony-stimulating factor-1. Biol. Reprod. 49:441–452. doi:10.1095/biolreprod49.3.441.

Ibrahim, L.A., J.A. Rizo, P.L.P. Fontes, G.C. Lamb, and J.J. Bromfield. 2018. Seminal plasma modulates expression of endometrial inflammatory meditators in the bovine. Biol. Reprod. 0:1–12. doi:10.1093/biolre/ioy226.

Ingman, W. V., and R.L. Jones. 2008. Cytokine knockouts in reproduction: the use of gene ablation to dissect roles of cytokines in reproductive biology. Hum. Reprod. Update 14:179–192. doi:10.1093/humupd/dmm042.

Ireland, J.J., R.L. Murphee, and P.B. Coulson. 1980. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. J. Dairy Sci. 63:155–160. doi:10.3168/jds.S0022-0302(80)82901-8.

Jacca, S., V. Franceschi, A. Colagiorgi, M. Sheldon, and G. Donofrio. 2013. Bovine endometrial stromal cells support tumor necrosis factor alpha-induced bovine herpesvirus type 4 enhanced replication. Biol. Reprod. 88. doi:10.1095/biolreprod.112.106740.

Janett, F., R. Thun, S. Bettschen, D. Burger, and M. Hassig. 2003. Seasonal changes of semen quality and freezability in Franches-Montagnes stallions. Anim. Reprod. Sci. 77:213–221. doi:10.1016/S0378-4320(03)00039-3.

Jasko, D.J., D.M. Moran, M.E. Farlin, and E.L. Squires. 1991. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 35:1059–1067. doi:10.1016/0093-691X(91)90354-G.

Jasper, M.J., A.S. Care, B. Sullivan, W. V. Ingman, J.D. Aplin, and S.A. Robertson. 2011. Macrophage-derived LIF and IL1B regulate alpha(1,2) fucosyltransferase 2 (Fut2) expression in mouse uterine epithelial cells during early pregnancy. Biol. Reprod. 84:179–188. doi:10.1095/biolreprod.110.085399.

Jasper, M.J., K.P. Tremellen, and S.A. Robertson. 2007. Reduced expression of IL-6 and IL-1α mRNAs in secretory phase endometrium of women with recurrent miscarriage. J. Reprod. Immunol. 73:74–84. doi:10.1016/j.jri.2006.06.003.

Juyena, N.S., and C. Stelletta. 2012. Seminal plasma: An essential attribute to spermatozoa. J. Androl. 33:536–551. doi:10.2164/jandrol.110.012583.

Kallikourdis, M., K.G. Andersen, K.A. Welch, and A.G. Betz. 2007. Alloantigen-enhanced accumulation of CCR5 "effector" regulatory T cells in the gravid uterus. Pnas 104:594–599. doi:10.1073/pnas.0604268104.

Kannampuzha-Francis, J., A.C. Denicol, B. Loureiro, K. Kaniyamattam, M.S. Ortega, and P.J. Hansen. 2015. Exposure to colony stimulating factor 2 during preimplantation development increases postnatal growth in cattle. Mol. Reprod. Dev. 82:892–897. doi:10.1002/mrd.22533.

Karalis, K.P., P. Giannogonas, E. Kodela, Y. Koutmani, M. Zoumakis, and T. Teli. 2009. Mechanisms of obesity and related pathology: Linking immune responses to metabolic stress. FEBS J. 276:5747–5754. doi:10.1111/j.1742-4658.2009.07304.x.

Katila, T. 2012. Post-mating inflammatory responses of the uterus. Reprod. Domest. Anim. 47:31–41. doi:10.1111/j.1439-0531.2012.02120.x.

Kawamura, K., N. Kawamura, J. Kumagai, J. Fukuda, and T. Tanaka. 2007. Tumor necrosis factor regulation of apoptosis in mouse preimplantation embryos and its antagonism by transforming growth factor alpha/phosphatidylionsitol 3-kinase signaling system. Biol. Reprod. 76:611–618. doi:10.1095/biolreprod.106.058008.

Kelley, R.L., and D.K. Gardner. 2017. Addition of interleukin-6 to mouse embryo culture increases blastocyst cell number and influences the inner cell mass to trophectoderm ratio. Clin. Exp. Reprod. Med. 44:119–125. doi:10.5653/cerm.2017.44.3.119.

Kelly, R.W. 1997. Prostaglandins in primate semen: Biasing the immune system to benefit spermatozoa and virus? Prostaglandins Leukot. Essent. Fat. Acids 57:113–118. doi:10.1016/S0952-3278(97)90000-4.

Keskintepe, L., A.A. Simplicio, and B.G. Brackett. 1998. Caprine blastocyst development after in vitro fertilization with spermatozoa frozen in different exterders. Theriogenology 1265–1274.

Kirwan, J., S. Hauguel-De Mouzon, J. Lepercq, J.-C. Challier, L. Hunson-Presley, J.E. Friedman, S. Kalhan, and P.M. Catalano. 2002. Pregnancy after laparoscopic sacral colpopexy: A case report. Int. Urogynecol. J. 23:651–653. doi:10.1007/s00192-011-1606-x.

Kong, A., M.L. Frigge, G. Masson, S. Besenbacher, P. Sulem, G. Magnusson, S.A. Gudjonsson, A. Sigurdsson, A. Jonasdottir, A. Jonasdottir, W.S.W. Wong, G. Sigurdsson, G.B. Walters, S. Steinberg, H. Helgason, G. Thorleifsson, D.F. Gudbjartsson, A. Helgason, O.T. Magnusson, U. Thorsteinsdottir, and K. Stefansson. 2012. Rate of de novo mutations and the importance of fathers age to disease risk. Nature 488:471–475. doi:10.1038/nature11396.

Koopman, L.A., H.D. Kopcow, B. Rybalov, J.E. Boyson, J.S. Orange, F. Schatz, R. Masch, C.J. Lockwood, A.D. Schachter, P.J. Park, and J.L. Strominger. 2003. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. J. Exp. Med. 198:1201–1212. doi:10.1084/jem.20030305.

Korte, K., K. Marzusch, and J. Dietl. 1993. The production of prostanoids and the identification of macrophages in human decidua vera tissue. Gynecol. Obstet. 149–150.

Krawetz, S.A. 2005. Paternal contribution: New insights and future challenges. Nat. Rev. Genet. 6:633–642. doi:10.1038/nrg1654.

Kwak, S.S., S.H. Jeung, D. Biswas, Y.B. Jeon, and S.H. Hyun. 2012. Effects of porcine granulocyte-macrophage colony-stimulating factor on porcine in vitro-fertilized embryos. Theriogenology 77:1186–1197. doi:10.1016/j.theriogenology.2011.10.025.

Layek, S.S., T.K. Mohanty, A. Kumaresan, and J.E. Parks. 2016. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. Anim. Reprod. Sci. 172:1–9. doi:10.1016/j.anireprosci.2016.04.013.

Leahy, T., and S.P. de Graaf. 2012. Seminal plasma and its effect on ruminant spermatozoa during processing. Reprod. Domest. Anim. 47:207–213. doi:10.1111/j.1439-0531.2012.02077.x.

Ledoux, D., C. Ponsart, B. Grimard, J. Gatien, M.C. Deloche, S. Fritz, R. Lefebvre, and P. Humblot. 2015. Sire effect on early and late embryonic death in French Holstein cattle. Animal 9:766–774. doi:10.1017/S1751731114003140.

Lentsch, A.B., M.J. Edwards, D.E. Sims, K. Nakagawa, S.R. Wellhausen, and F.N. Miller. 1996. Interleukin-10 inhibits interleukin-2-induced tumor necrosis factor production but does not reduce toxicity in C3H/HeN mice. J. Leukoc. Biol. 60:51–57. doi:10.1002/jlb.60.1.51.

Li, S., S.R.S. O'Neill, Y. Zhang, M.J. Holtzman, K.I. Takemaru, K.S. Korach, and W. Winuthayanon. 2017. Estrogen receptor a is required for oviductal transport of embryos. FASEB J. 31:1595–1607. doi:10.1096/fj.201601128R.

Li, S., and W. Winuthayanon. 2017. Oviduct: roles in fertilization and early embryo development. J. Endocrinol. 232:R1–R26. doi:10.1530/JOE-16-0302.

Li, Y., H. Lin, Y. Li, and J. Cao. 2011. Association between socio-psycho-behavioral factors and male semen quality: systematic review and meta-analyses. Fertil. Steril. 95:116–123. doi:10.1016/j.fertnstert.2010.06.031.

Lima, F.S., C.A. Risco, M.J. Thatcher, M.E. Benzaquen, L.F. Archbald, J.E.P. Santos, and W.W. Thatcher. 2009. Comparison of reproductive performance in lactating dairy cows bred by natural service or timed artificial insemination. J. Dairy Sci. 92:5456–5466. doi:10.3168/jds.2009-2197.

Lonergan, P. 2011. Influence of progesterone on oocyte quality and embryo development in cows. Theriogenology 76:1594–1601. doi:10.1016/j.theriogenology.2011.06.012.

Lonergan, P., A. Woods, T. Fair, F. Carter, D. Rizos, F. Ward, K. Quinn, and A. Evans. 2007. Effect of embryo source and recipient progesterone environment on embryo development in cattle. Reprod. Fertil. Dev. 19:861–868. doi:10.1071/RD07089.

Louis, G.M.B., M.A. Cooney, C.D. Lynch, and A. Handal. 2008. Periconception window: advising the pregnancy planning coupe. Fertil. Steril. 89:1–6. doi:10.1016/j.fertnstert.2007.12.043.Periconception.

Loureiro, B., L.J. Oliveira, M.G. Favoreto, and P.J. Hansen. 2011. Colony-stimulating factor 2 inhibits induction of apoptosis in the bovine preimplantation embryo. Am. J. Reprod. Immunol. 65:578–588. doi:10.1111/j.1600-0897.2010.00953.x.

Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: where will it end? J. Dairy Sci. 84:1277–1293. doi:10.3168/jds.S0022-0302(01)70158-0.

Madej, M., M. Norrby, M.T. Madsen, A. Johannisson, C. Hansen, and A. Madej. 2012. The effect of boar seminal plasma on the release of prostaglandins and interleukin-6 by porcine endometrial and cervical cells and bovine endometrial cells. Reprod. Domest. Anim. 47:113–124. doi:10.1111/j.1439-0531.2011.01809.x.

Mahdi, B.M. 2011. Role of some cytokines on reproduction. Middle East Fertil. Soc. J. 16:220–223. doi:10.1016/j.mefs.2011.03.002.

Mantovani, A., M. Muzio, P. Ghezzi, C. Colotta, and M. Introna. 1998. Regulation of inhibitory pathways of the interlekin-1 system 91:399–404.

Martal, J., J.E. Fléchon, M. Guillomot, and M. Charlier. 1986. Experimental studies on the elongation of the ewe blastocyst. Reprod. Nutr. Dev. 26:1017–1024. doi:10.1051/rnd:19860609.

Matsuyama, S., T. Kojima, S. Kato, and K. Kimura. 2012. Relationship between quantity of IFNT estimated by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic mortality after AI or ET. Reprod. Biol. Endocrinol. 10:21. doi:10.1186/1477-7827-10-21.

Mattner, P.E. 1968. The distribution of spermatozoa and leucocytes in the female genital tract in goats and cattle. J Reprod Fertil 17:253–261. doi:10.1530/JRF.0.0170253.

Mihara, M., M. Hashizume, H. Yoshida, M. Suzuki, and M. Shiina. 2012. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. Clin. Sci. 122:143–159. doi:10.1042/CS20110340.

Ming, W.J.I., L. Bersani, and A. Mantovani. 1987. Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J. Immunol.

Mitchell, M., H.W. Bakos, and M. Lane. 2011. Paternal diet-induced obesity impairs embryo development and implantation in the mouse. Fertil. Steril. 95:1349–1353. doi:10.1016/j.fertnstert.2010.09.038.

Mjösberg, J., G. Berg, M.C. Jenmalm, and J. Ernerudh. 2010. FOXP3+ regulatory T cells and T helper 1, T helper 2, and T helper 17 cells in human early pregnancy decidua. Biol. Reprod. 82:698–705. doi:10.1095/biolreprod.109.081208.

Moldenhauer, L.M., S.N. Keenihan, J.D. Hayball, and S.A. Robertson. 2010. GM-CSF is an essential regulator of T cell activation competence in uterine dendritic cells during early pregnancy in mice. J. Immunol. 185:7085–7096. doi:10.4049/jimmunol.1001374.

Moore, S.G., and J.F. Hasler. 2017. A 100-Year Review: reproductive technologies in dairy science. J. Dairy Sci. 100:10314–10331. doi:10.3168/jds.2017-13138.

Mor, G., and I. Cardenas. 2010. The immune system in pregnancy: a unique complexity. Am. J. Reprod. Immunol. 63:425–433. doi:10.1111/j.1600-0897.2010.00836.x.The.

De Moraes, A.A.S., F.F. Paula-Lopes, N. Chegini, and P.J. Hansen. 1999. Localization of granulocyte-macrophage colony-stimulating factor in the bovine reproductive tract. J. Reprod. Immunol. 42:135–145. doi:10.1016/S0165-0378(98)00075-8.

Morstyn, G., and A.W. Burgess. 1988. Hemopoietic growth factors: a review. J. Clin. Pharmacol. 48:486–501.

Moura, A.A., H. Koc, D.A. Chapman, and G.J. Killian. 2006. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: A proteomic approach. J. Androl. 27:201–211. doi:10.2164/jandrol.05089.

Mui, A.L., H. Wakao, A.M. O'Farrell, N. Harada, and A. Miyajima. 1995. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. EMBO J. 14:1166–75. doi:10.1139/w99-143.

Naz, R.K., and P. Kaplan. 1994. Increased levels of interleukin-6 in seminal plasma of infertile men. J. Androl. 15:220–227. doi:10.1002/j.1939-4640.1994.tb00436.x.

Neufeld, G., and O. Kessler. 2006. Pro-angiogenic cytokines and their role in tumor angiogenesis. Cancer Metastasis Rev. 25:373–385. doi:10.1007/s10555-006-9011-5.

Ng, S.F., R.C.Y. Lin, D.R. Laybutt, R. Barres, J.A. Owens, and M.J. Morris. 2010. Chronic high-fat diet in fathers programs β 2-cell dysfunction in female rat offspring. Nature 467:963–966. doi:10.1038/nature09491.

Nongbua, T., Y. Guo, A. Edman, P. Humblot, and J.M. Morrell. 2018. Effect of bovine seminal plasma on bovine endometrial epithelial cells in culture. Reprod. Domest. Anim. 53:85–92. doi:10.1111/rda.13069.

O'Leary, S., D.T. Armstrong, and S.A. Robertson. 2011. Transforming growth factor-β (TGF) in porcine seminal plasma. Reprod. Fertil. Dev. 23:748–758. doi:10.1071/RD11001.

O'Leary, S., M.J. Jasper, G.M. Warnes, D.T. Armstrong, and S.A. Robertson. 2004. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. Reproduction 128:237–247. doi:10.1530/rep.1.00160.

Odhiambo, J.F., D.H. Poole, L. Hughes, J.M. DeJarnette, E.K. Inskeep, and R.A. Dailey. 2009. Pregnancy outcome in dairy and beef cattle after artificial insemination and treatment with seminal plasma or transforming growth factor beta-1. Theriogenology 72:566–571. doi:10.1016/j.theriogenology.2009.04.013.

Okada, H., T. Tsuzuki, and H. Murata. 2018. Decidualization of the human endometrium. Reprod. Med. Biol. 17:220–227. doi:10.1002/rmb2.12088.

Okuda, K., Y. Miyamoto, and D.J. Skarzynski. 2002. Regulation of endometrial prostaglandin F2αsynthesis during luteolysis and early pregnancy in cattle. Domest. Anim. Endocrinol. 23:255–264. doi:10.1016/S0739-7240(02)00161-3.

Orsi, N.M., N. Gopichandran, U. V. Ekbote, and J.J. Walker. 2006. Murine serum cytokines throughout the estrous cycle, pregnancy and post partum period. Anim. Reprod. Sci. 96:54–65. doi:10.1016/j.anireprosci.2005.11.010.

Ortega, M.S., J.G.N. Moraes, D.J. Patterson, M.F. Smith, S.K. Behura, S. Poock, and T.E. Spencer. 2018. Influences of sire conception rate on pregnancy establishment in dairy cattle. Biol. Reprod. 99:1244–1254. doi:10.1093/biolre/ioy141.

Ozawa, M., M. Sakatani, K.B. Dobbs, J. Kannampuzha-Francis, and P.J. Hansen. 2016. Regulation of gene expression in the bovine blastocyst by colony stimulating factor 2. BMC Res. Notes 9:1–9. doi:10.1186/s13104-016-2038-y.

Palm, F., I. Walter, S. Budik, J. Kolodziejek, N. Nowotny, and C. Aurich. 2008. Influence of different semen extenders and seminal plasma on PMN migration and on expression of IL-1β, IL-6, TNF-α and COX-2 mRNA in the equine endometrium. Theriogenology 70:843–851. doi:10.1016/j.theriogenology.2008.04.054.

Paradisi, R., M. Capelli, M. Mandini, E. Bellavia, and C. Flamigni. 1996. Increased levels of interferon-gamma in seminal plasma of infertile men. Andrologia 28:157–161. doi:10.1111/j.1439-0272.1996.tb02775.x.

Paula-Lopes, F.F., A.A.S. de Moraes, J.L. Edwards, J.E. Justice, and P.J. Hansen. 1998. Regulation of preimplantation development of bovine embryos by interleukin-1β. Biol. Reprod. 59:1406–1412. doi:10.1095/biolreprod59.6.1406.

Pellegrino, C.A.G., F. Morotti, R.M. Untura, J.H.F. Pontes, M.F.O. Pellegrino, J.P. Campolina, M.M. Seneda, F.A. Barbosa, and M. Henry. 2016. Use of sexed sorted semen for fixed-time artificial insemination or fixed-time embryo transfer of in vitro–produced embryos in cattle. Theriogenology 86:888–893. doi:10.1016/j.theriogenology.2016.03.010.

Poiani, A. 2006. Complexity of seminal fluid: a review. Behav. Ecol. Sociobiol. 60:289–310. doi:10.1007/s00265-006-0178-0.

Pursel, V.G., and L.A. Johnson. 1976. Frozen boar spermatozoa: methods of thawing pellets. J. Anim. Sci. 42:927–931. doi:10.2527/jas1976.424927x.

Refaat, B., E. Dalton, and W.L. Ledger. 2015. Ectopic pregnancy secondary to in vitro fertilisation-embryo transfer: pathogenic mechanisms and management strategies. Reprod. Biol. Endocrinol. 13:1–18. doi:10.1186/s12958-015-0025-0.

Rego, J.P.A., A.A. Moura, A.S. Nouwens, M.R. McGowan, and G.B. Boe-Hansen. 2015. Seminal plasma protein profiles of ejaculates obtained by internal artificial vagina and electroejaculation in Brahman bulls. Anim. Reprod. Sci. 160:126–137. doi:10.1016/j.anireprosci.2015.07.015.

Remes Lenicov, F., C. Rodriguez Rodrigues, J. Sabatte, M. Cabrini, C. Jancic, M. Ostrowski, A. Merlotti, H. Gonzalez, A. Alonso, R.A. Pasqualini, C. Davio, J. Geffner, and A. Ceballos. 2012. Semen promotes the differentiation of tolerogenic dendritic cells. J. Immunol. 189:4777–4786. doi:10.4049/jimmunol.1202089.

Richter, R., P. Schulz-Knappe, M. Schrader, L. Ständker, M. Jürgens, H. Tammen, and W.G. Forssmann. 1999. Composition of the peptide fraction in human blood plasma: database of circulating human peptides. J. Chromatogr. B Biomed. Sci. Appl. 726:25–35. doi:10.1016/S0378-4347(99)00012-2.

Rizos, D., F. Ward, P. Duffy, M.P. Boland, and P. Lonergan. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev. 61:234–248. doi:10.1002/mrd.1153.

Roberts, M. 2007. Interferon-tau, a type 1 interferon involved in maternal recognition of pregnancy. J. Polym. Sci. Polym. Chem. Ed. 23:291–301. doi:10.1016/j.cytogfr.2007.06.010.

Robertson, S.A. 2005. Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res. 322:43–52. doi:10.1007/s00441-005-1127-3.

Robertson, S.A., A.S. Care, and L.M. Moldenhauer. 2018. Regulatory T cells in embryo implantation and the immune response to pregnancy T. J Clin Invest 128:4224–4235. doi:10.1172/JCI122182.

Robertson, S.A., A.S. Care, and R.J. Skinner. 2007. Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in Mice. Biol. Reprod. 76:738–748. doi:10.1095/biolreprod.106.056143.

Robertson, S.A., P. Chin, J.E. Schjenken, and G. Jeremy. 2015. Cell Signaling during Mammalian Early Embryo Development.

Robertson, S.A., L.R. Guerin, J.J. Bromfield, K.M. Branson, A.C. Ahlström, and A.S. Care. 2009. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol. Reprod. 80:1036–1045. doi:10.1095/biolreprod.108.074658.

Robertson, S.A., V.J. Mau, S.N. Hudson, and K.P. Tremellen. 1997. Cytokine-leukocyte networks and the establishment of pregnancy. Am. J. Reprod. Immunol. 37:438–442. doi:10.1111/j.1600-0897.1997.tb00257.x.

Robertson, S.A., and L.M. Moldenhauer. 2014. Immunological determinants of implantation success. Int. J. Appl. Eng. Res. 11:5250–5255. doi:10.1387/ijdb.140096sr.

Robertson, S.A., and D.J. Sharkey. 2001. The role of semen in induction of maternal immune tolerance to pregnancy. Semin. Immunol. 13:243–254. doi:10.1006/smim.2000.0320.

Robertson, S.A., and D.J. Sharkey. 2016. Seminal fluid and fertility in women. Fertil. Steril. 106:511–519. doi:10.1016/j.fertnstert.2016.07.1101.

Robertson, S.A., C. Sjoblom, M.J. Jasper, R.J. Norman, and R.F. Seamark. 2001. Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. Biol Reprod 64:1206–1215. doi:10.1095/biolreprod64.4.1206.

Robertson, S.A., R.J. Skinner, and A.S. Care. 2006. Essential role for iL-10 in resistance to lipopolysaccharide-induced preterm labor in mice. J. Immunol. 177:4888–4896. doi:10.4049/jimmunol.177.7.4888.

Robinson, R.S., G.E. Mann, G.E. Lamming, and D.C. Wathes. 1999. The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. J. Endocrinol. 160:21–33. doi:10.1677/joe.0.1600021.

Ronquist, K.G., B. Ek, J. Morrell, A. Stavreus-Evers, B. Ström Holst, P. Humblot, G. Ronquist, and A. Larsson. 2013. Prostasomes from four different species are able to produce extracellular adenosine triphosphate (ATP). Biochim. Biophys. Acta - Gen. Subj. 1830:4604–4610. doi:10.1016/j.bbagen.2013.05.019.

Rota, A., D. Panzani, C. Sabatini, and F. Camillo. 2012. Donkey jack (Equus asinus) semen cryopreservation: studies of seminal parameters, post breeding inflammatory response, and fertility in donkey jennies. Theriogenology 78:1846–1854. doi:10.1016/j.theriogenology.2012.07.015.

Ruef, C., and D. Coleman. 1990. Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness. Oxford Univ. Press. 12:41–62.

Rutella, S., S. Danese, G. Leone, and W. Dc. 2011. Tolerogenic dendritic cells : cytokine modulation comes of age tolerogenic dendritic cells : cytokine modulation comes of age. Hematology 108:1435–1440. doi:10.1182/blood-2006-03-006403.

Saito, S., A. Nakashima, T. Shima, and M. Ito. 2010. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am. J. Reprod. Immunol. 63:601–610. doi:10.1111/j.1600-0897.2010.00852.x.

Samanta, L., R. Parida, T.R. Dias, and A. Agarwal. 2018. The enigmatic seminal plasma: a proteomics insight from ejaculation to fertilization. Reprod. Biol. Endocrinol. 16:1–11. doi:10.1186/s12958-018-0358-6.

Samstein, R.M., S.Z. Josefowicz, A. Arvey, P.M. Treuting, and A.Y. Rudensky. 2013. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict 150:29–38. doi:10.1016/j.cell.2012.05.031.Extrathymic.

Santos, J.E.P., W.W. Thatcher, R.C. Chebel, R.L.A. Cerri, and K.N. Galvão. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim. Reprod. Sci. 82–83:513–535. doi:10.1016/j.anireprosci.2004.04.015.

Sartori, R., G.J.M. Rosa, and M.C. Wiltbank. 2002. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J. Dairy Sci. 85:2813–2822. doi:10.3168/jds.S0022-0302(02)74368-3.

Schröpfer, R., B. Klenner-Fringes, R. Schropfer, and B. Klenner-Fringes. 2008. Minimizing interspecific competition by different foraging strategies in two North African desert rodents. Acta Theriol. (Warsz). 36:109–117. doi:10.1016/j.semcdb.2007.10.007.

Schüler-Toprak, S., O. Treeck, and O. Ortmann. 2017. Human chorionic gonadotropin and breast cancer. Int. J. Mol. Sci. 18. doi:10.3390/ijms18071587.

Scott, J.L., N. Ketheesan, and P.M. Summers. 2006. Society for Reproductive Biology. Pages 1–100.

Sequeira, K., A. Espejel-Núñez, E. Vega-Hernández, A. Molina-Hernández, and P. Grether-González. 2015. An increase in IL-1β concentrations in embryo culture-conditioned media obtained by in vitro fertilization on day 3 is related to successful implantation. J. Assist. Reprod. Genet. 32:1623–1627. doi:10.1007/s10815-015-0573-4.

Seshadri, S., M. Bates, G. Vince, and D.I.L. Jones. 2009. The role of cytokine expression in different subgroups of subfertile men. Am. J. Reprod. Immunol. 62:275–282. doi:10.1111/j.1600-0897.2009.00736.x.

Sferruzzi-Perri, A., A.M. Macopherson, C.T. Roberts, and S.A. Robertson. 2009. All disease comes from the heart: the pivotal role of the emotions in classical chinese medicine. J. Chinese Med. 221:26–35. doi:10.1095/biolreprod.108.073312.

Shannon, P. 1965. Presence of a heat-labile toxic protein in bovine seminal plasma. J Dairy Sci 48:1362–5. doi:10.3168/jds.S0022-0302(65)88464-8.

Sharkey, A.M., K. Dellow, M. Blayney, M. Macnamee, S. Charnock-Jones, S.K. Smith, B. M, M. M, C.-J. S, and S. SK. 1995. Stage-specific expression of cytokine and receptor messenger ribonucleic acids in human preimplantation embryos. Biol. Reprod. 53:974–981.

Sharkey, D.J., A.M. Macpherson, K.P. Tremellen, D.G. Mottershead, R.B. Gilchrist, and S.A. Robertson. 2012a. TGF mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. J. Immunol. 189:1024–1035. doi:10.4049/jimmunol.1200005.

Sharkey, D.J., K.P. Tremellen, N.E. Briggs, G.A. Dekker, and S.A. Robertson. 2017. Seminal plasma pro-inflammatory cytokines interferon-3 (IFNG) and C-X-C motif chemokine ligand 8 (CXCL8) fluctuate over time within men. Hum. Reprod. 32:1373–1381. doi:10.1093/humrep/dex106.

Sharkey, D.J., K.P. Tremellen, M.J. Jasper, K. Gemzell-Danielsson, and S.A. Robertson. 2012b. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after Coitus. J. Immunol. 188:2445–2454. doi:10.4049/jimmunol.1102736.

Sharma, R., K.R. Biedenharn, J.M. Fedor, and A. Agarwal. 2013. Lifestyle factors and reproductive health: taking control of your fertility. Reprod. Biol. Endocrinol. 11:1. doi:10.1186/1477-7827-11-66.

Shen, X., X. Cui, S. Lee, and N. Kim. 2012. Interleukin-6 enhances porcine parthenote development in vitro, through the IL-6 / Stat3 signaling pathway. J. Reprod. Dev. 58:1–8.

Shimoya, K., N. Matsuzaki, T. Tsutsui, T. Taniguchi, F. Saji, and O. Tanizawa. 1993. Detection of interleukin-8 (IL-8) in seminal plasma and elevated IL-8 in seminal plasma of infertile patients with leukospermia. Fertil Steril 59:885–888. doi:10.1016/S0015-0282(16)55876-2.

Singh, M., P. Chaudhry, and E. Asselin. 2011. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. J. Endocrinol. 210:5–14. doi:10.1530/JOE-10-0461.

Siqueira, L.G.B., and P.J. Hansen. 2016. Sex differences in response of the bovine embryo to colony-stimulating factor 2. Reproduction 152:645–654. doi:10.1530/REP-16-0336.

Sjöblom, C., C.T. Roberts, M. Wikland, and S.A. Robertson. 2005. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. Endocrinology 146:2142–2153. doi:10.1210/en.2004-1260.

Sjöblom, C., M. Wikland, and S.A. Robertson. 2002. Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. Biol. Reprod. 67:1817–1823. doi:10.1095/biolreprod.101.001503.

Song, Z.H., Z.Y. Li, D.D. Li, W.N. Fang, H.Y. Liu, D.D. Yang, C.Y. Meng, Y. Yang, and J.P. Peng. 2016. Seminal plasma induces inflammation in the uterus through the γδT/IL-17 pathway. Sci. Rep. 6:1–8. doi:10.1038/srep25118.

Soto, P., R.P. Natzke, and P.J. Hansen. 2003. Actions of tumor necrosis factor-α on oocyte maturation and embryonic development in cattle. Am. J. Reprod. Immunol. 50:380–388. doi:10.1034/j.1600-0897.2003.00101.x.

Spencer, T.E., and F.W. Bazer. 2004. Conceptus signals for establishment and maintenance of pregnancy. Reprod. Biol. Endocrinol. 15:9–10. doi:10.1186/1477-7827-2-49.

Spencer, T.E., N. Forde, and P. Lonergan. 2016a. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. J. Dairy Sci. 99:5941–5950. doi:10.3168/jds.2015-10070.

Spencer, T.E., O. Sandra, and E. Wolf. 2008. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction 135:165–179. doi:10.1530/REP-07-0327.

Spencer, T.E., O. Sandra, E. Wolf, J.G.N. Moraes, S.K. Behura, T.W. Geary, P.J. Hansen, H.L. Neibergs, J.E.P. Santos, W.W. Thatcher, R.C. Chebel, R.L.A. Cerri, K.N. Galvão, M.C. Wiltbank, G.M. Baez, A. Garcia-Guerra, M.Z. Toledo, P.L.J. Monteiro, L.F. Melo, J.C. Ochoa, and R. Sartori. 2016b. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim. Reprod. Sci. 135:165–179. doi:10.1530/REP-07-0327.

Srivastava, A., J. Sengupta, A. Kriplani, K.K. Roy, and D. Ghosh. 2013. Profiles of cytokines secreted by isolated human endometrial cells under the influence of chorionic gonadotropin during the window of embryo implantation. Reprod. Biol. Endocrinol. 11:1–15. doi:10.1186/1477-7827-11-116.

Steinhauser, C.B., J.K. Graham, R.W. Lenz, and G.E. Seidel. 2016. Removing seminal plasma improves bovine sperm sex-sorting. Andrology 4:1131–1137. doi:10.1111/andr.12245.

Stewart, J.L., V.R.G. Mercadante, N.W. Dias, I.F. Canisso, P. Yau, B. Imai, and F.S. Lima. 2018. Nerve growth factor-beta, purified from bull seminal plasma, enhances corpus luteum formation and conceptus development in Bos taurus cows. Theriogenology 106:30–38. doi:10.1016/j.theriogenology.2017.10.007.

Strzemienski, P.J. 1989. Effect of bovine seminal plasma on neutrophil phagocytosis of bull spermatozoa. J. Reprod. Fertil. 87:519–528. doi:10.1530/jrf.0.0870519.

Stuppia, L., M. Franzago, P. Ballerini, V. Gatta, and I. Antonucci. 2015. Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health. Clin. Epigenetics 7:1–15. doi:10.1186/s13148-015-0155-4.

Suarez, S.S., and A.A. Pacey. 2006. Sperm transport in the female reproductive tract. Hum. Reprod. Update 12:23–37. doi:10.1093/humupd/dmi047.

Svensson-Arvelund, J., R.B. Mehta, R. Lindau, E. Mirrasekhian, H. Rodriguez-Martinez, G. Berg, G.E. Lash, M.C. Jenmalm, and J. Ernerudh. 2015. The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages. J. Immunol. 194:1534–1544. doi:10.4049/jimmunol.1401536.

Talbott, H., A. Delaney, P. Zhang, Y. Yu, R.A. Cushman, A.S. Cupp, X. Hou, and J.S. Davis. 2014. Effects of IL8 and immune cells on the regulation of luteal progesterone secretion. Reproduction 148:21–31. doi:10.1530/REP-13-0602.

Tanabe, K., R. Matsushima-Nishiwaki, S. Yamaguchi, H. Iida, S. Dohi, and O. Kozawa. 2010. Mechanisms of tumor necrosis factor-α-induced interleukin-6 synthesis in glioma cells. J. Neuroinflammation 7:3–10. doi:10.1186/1742-2094-7-16.

Teh, W.T., J. McBain, and P. Rogers. 2016. What is the contribution of embryo-endometrial asynchrony to implantation failure? J. Assist. Reprod. Genet. 33:1419–1430. doi:10.1007/s10815-016-0773-6.

Tekin, S., and P.J. Hansen. 2004. Regulation of numbers of macrophages in the endometrium of the sheep by systemic effects of pregnancy, local presence of the conceptus, and progesterone. Am. J. Reprod. Immunol. 51:56–62. doi:10.1046/j.8755-8920.2003.00125.x.

Teklenburg, G., M. Salker, M. Molokhia, S. Lavery, G. Trew, T. Aojanepong, H.J. Mardon, A.U. Lokugamage, R. Rai, C. Landles, B.A.J. Roelen, S. Quenby, E.W. Kuijk, A. Kavelaars, C.J. Heijnen, L. Regan, J.J. Brosens, and N.S. Macklon. 2010. Natural selection of human embryos: decidualizing endometrial stromal cells serve as sensors of embryo quality upon implantation. PLoS One 5:2–7. doi:10.1371/journal.pone.0010258.

Thouas, G.A., F. Dominguez, M.P. Green, F. Vilella, C. Simon, and D.K. Gardner. 2015. Soluble ligands and their receptors in human embryo development and implantation. Endocr. Rev. 36:92–130. doi:10.1210/er.2014-1046.

Tremellen, K., D. Valbuena, J. Landeras, A. Ballesteros, J. Martinez, S. Mendoza, R. Norman, S. Robertson, and C. Simón. 2000. The effect of intercourse on pregnancy rates during assisted human reproduction mechanisms: the introduction of infection and the initiation. Hum. Reprod. 15:2653–2658.

Tremellen, K.P., R.F. Seamark, and S.A. Robertson. 1998. Seminal transforming growth factor β1, stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. Biol. Reprod. 58:1217–1225. doi:10.1095/biolreprod58.5.1217.

Tribulo, P., O. Bogle, R.J. Mapletoft, and G.P. Adams. 2015. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. Theriogenology 83:1394–1401. doi:10.1016/j.theriogenology.2014.12.014.

Tríbulo, P., L.G.B. Siqueira, L.J. Oliveira, T. Scheffler, and P.J. Hansen. 2018. Identification of potential embryokines in the bovine reproductive tract. J. Dairy Sci. 101:690–704. doi:10.3168/jds.2017-13221.

Trudeau, V., and L.M. Sanford. 1986. Effect of season and social environment on testis size and semen quality of the adult Landrace boar. J. Anim. Sci. 63:1211–1219. doi:10.2527/jas1986.6341211x.

Vacca, P., C. Cantoni, M. Vitale, C. Prato, F. Canegallo, D. Fenoglio, N. Ragni, L. Moretta, and M.C. Mingari. 2010. Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. Proc. 107:11918–11923. doi:10.1073/pnas.1001749107.

Walters, A.H., R.G. Saacke, R.E. Pearson, and F.C. Gwazdauskas. 2006. Assessment of pronuclear formation following in vitro fertilization with bovine spermatozoa obtained after thermal insulation of the testis. Theriogenology 65:1016–1028. doi:10.1016/j.theriogenology.2005.07.005.

Wang, J., and M. V. Sauer. 2006. In vitro fertilization (IVF): a review of 3 decades of clinical innovation and technological advancement. Ther. Clin. Risk Manag. 2:355–364. doi:10.2147/tcrm.2006.2.4.355.

Watkins, A.J., I. Dias, H. Tsuro, D. Allen, R.D. Emes, J. Moreton, R. Wilson, R.J.M. Ingram, and K.D. Sinclair. 2018. Paternal diet programs offspring health through sperm- and seminal plasma-specific pathways in mice. Proc. Natl. Acad. Sci. 115:10064–10069. doi:10.1073/pnas.1806333115.

Watkins, A.J., S. Sirovica, B. Stokes, M. Isaacs, O. Addison, and R.A. Martin. 2017. Paternal low protein diet programs preimplantation embryo gene expression, fetal growth and skeletal development in mice. Biochim. Biophys. Acta - Mol. Basis Dis. 1863:1371–1381. doi:10.1016/j.bbadis.2017.02.009.

Way, A.L., L. Griel, and G.J. Killian. 2000. Effects of accessory sex gland fluid on viability, capacity, and the acrosome reaction of cauda epididymal bull spermatozoa. J. Androl. 21.

White, C.A., E. Dimitriadis, A.M. Sharkey, C.J. Stoikos, and L.A. Salamonsen. 2007. Interleukin 1 beta is induced by interleukin 11 during decidualization of human endometrial stromal cells, but is not released in a bioactive form. J. Reprod. Immunol. 73:28–38. doi:10.1016/j.jri.2006.05.003.

White, C.A., M. Johansson, C.T. Roberts, A.J. Ramsay, and S.A. Robertson. 2004. Effect of interleukin-10 null mutation on maternal immune response and reproductive outcome in mice. Biol. Reprod. 70:123–131. doi:10.1095/biolreprod.103.018754.

Wiltbank, M.C., G.M. Baez, A. Garcia-Guerra, M.Z. Toledo, P.L.J. Monteiro, L.F. Melo, J.C. Ochoa, J.E.P. Santos, and R. Sartori. 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 86:239–253. doi:10.1016/j.theriogenology.2016.04.037.

Wooldridge, L.K., and A.D. Ealy. 2019. Interleukin-6 increases inner cell mass numbers in bovine embryos. BMC Dev. Biol. 1–11.

Yanagimachi, R. 2005. Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. Reprod. Biomed. Online 10:247–288. doi:10.1016/S1472-6483(10)60947-9.

Yanagimachi, R., and M. Chang. 1963. Infiltration of leucocytes into the uterine lumen of the golden hamster during the oestrous cycle and following mating. J Reprod Fertil. 5:389–96.

Yu, C., X. Zhang, L. Wang, Y. Liu, N. Li, M. Li, L. Chen, Y. Liu, and Y. Yao. 2018. Interleukin-6 regulates expression of Fos and Jun genes to affect the development of mouse preimplantation embryos. J. Obstet. Gynaecol. Res. 44:253–262. doi:10.1111/jog.13498.

Zhang, J.-M., and J. An. 2007. Cytokines, inflammation and pain. Int Anesth. Clin. 69:482–489. doi:10.1097/AIA.0b013e318034194e.Cytokines.

Zhang, Q., V. Collins, K. Chakrabarty, J.C. Rose, and W.X. Wu. 2007. Regulation of the prostaglandin enzymatic system by estradiol and progesterone in nonpregnant sheep cervix. Reproduction 133:1027–1034. doi:10.1530/REP-06-0328.

Zheng, H., D. Fletcher, and W. Kozak. 1995. Resistance to fever induction and impaired acute-phase response in interleukin-1B deficient mice. Current 3:197–219.

Ziebe, S., A. Loft, B.B. Povlsen, K. Erb, I. Agerholm, M. Aasted, A. Gabrielsen, C. Hnida, D.P. Zobel, B. Munding, S.H. Bendz, and S.A. Robertson. 2013. A randomized clinical trial to evaluate the effect of granulocyte- macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. Fertil. Steril. 99:1600–1609.e2. doi:10.1016/j.fertnstert.2012.12.043.

BIOGRAPHICAL SKETCH

Jason Rizo was born in Matagalpa, Nicaragua. Jason was raised in the small town of San Dionisio, where he was exposed to beef cattle and crop production. In January 2012, he moved to Tegucigalpa, Honduras to start his undergraduate studies at Zamorano Agricultural University; he graduated in December 2015 with a Bachelor of Agricultural Sciences. During his time in Zamorano, Jason did an internship with Dr. Jud Heinrichs at The Pennsylvania State University, working with heifer management and organic trace minerals supplementation in dairy cattle. In 2017, Jason moved to Gainesville, Florida and started his master's studies with Dr. John Bromfield in the Animal Molecular and Cellular Biology Program at the University of Florida. After graduating with a Master of Science degree in May 2019, Jason will move to Columbia, Missouri to continue his studies towards Doctor of Philosophy at University of Missouri in the laboratory of Dr. Sofia Ortega and Dr. Thomas Spencer.