SEMINAL PLASMA MODULATES EXPRESSION OF ENDOMETRIAL FACTORS IMPORTANT IN EARLY PREGNANCY SUCCESS

By

LAILA A. IBRAHIM

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To my parents, husband, family, and University of Benghazi

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AMH	Antimüllerian hormone
APC	Antigen-presenting cells
Aspp	Acidic seminal plasma protein
bFGF	Fibroblast growth factor
BOEC	Bovine oviduct epithelial cells
BUEC	Bovine uterine epithelial cell
BoLA	Bovine leukocyte antigen
BSE	Breeding soundness evaluations
BSP	Seminal plasma protein
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
COX	Cyclooxygenase
CSF	Colony-stimulating factor
CP	Cell pellet
DBPS	Dulbecco's phosphate-buffered saline
DC	Dendritic cells
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay

FBS	Fetal bovine serum
FOXP3	Forkhead box P3
HB-EGF	Heparin-binding epidermal growth factor
HBSS)	Hank's balanced salt solution
hCG	Human chorionic gonadotropin
ICM	Inner cell mass
IFNγ	Interferon gamma
IFNτ	Interferon tau
IGF-I	Insulin-like growth factor-I
IL	Interleukin
IUGR	Intrauterine growth restriction
IVF	In vitro fertilization
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharides
MCP1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
MMP	Metalloproteinases
mRNA	Messenger RNA
NK cell	Natural killer cell
NKT	Natural killer T cell
PBS	Phosphate-buffered saline
PGE	Prostaglandin E
PGF2α	Prostaglandin F2 alpha

PGFS	Prostaglandin F synthase
PTGS	Prostaglandin-endoperoxide synthase
PMN	Polymorphonuclear
PSP	Porcine sperm adhesions
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SA	Spiral arteries
SEM	Standard error of the mean
SM	Semen
SP	Seminal plasma
SPSS	Statistical Package for the Social Sciences
SVX	Seminal vesicle deficient
TGF	Transforming growth factor
TNF	Tumor necrosis Factor
TRAIL	Tumor necrosis factor related apoptosis-inducing ligand
Treg cells	Regulatory T cells
uNK	Uterine natural killer cell
VEGF	Vascular endothelial growth factor

Abstract of Thesis Presented to the Graduate School
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SEMINAL PLASMA MODULATES EXPRESSION OF ENDOMETRIAL FACTORS IMPORTANT IN EARLY PREGNANCY SUCCESS

By

Laila A. Ibrahim

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Are seminal plasma (SP) and the female reproductive tract friends or foes? Seminal plasma has conventionally been viewed as a transport and survival medium for mammalian sperm; however, its role is now proposed to extend beyond this process to target female tissues. Studies in rodents, swine, equine and humans show that seminal plasma induces molecular and cellular changes within the endometrium or cervix following insemination. Seminal plasma cytokines and growth factors that bind to cognate receptors on epithelial cells of the female reproductive tract result in production of embryokines and leukocyte-mediated events at the maternal-fetal interface. Potential seminal plasma induced alterations to the maternal environment have been hypothesized to facilitate embryo implantation, modulate maternal immunity toward the conceptus and potentially improve pregnancy success. It is unknown if a similar communication network exists in the bovine to modulate the uterine environment following insemination. We hypothesize that exposure of endometrial tissue to seminal plasma will modulate the expression of factors important in early pregnancy success. Here, responsiveness of bovine endometrial explants, semi-purified endometrial epithelial or stromal cells to semen components was evaluated. We observed an effect of seminal plasma exposure on endometrial explant expression of *CSF2*, *IL6*, *IL17A1*, *TGFB1*, *IFNE*, *PTGS*, and *PGFS*.

Furthermore, expression of endometrial epithelial *CSF2*, *IL8*, *TGFB1*, *PTGS* and *PGFS* were all increased after seminal plasma exposure, while endometrial stromal cells increased expression of *CSF2*, *IL6*, *IL8*, *IL17A1*, *TGFB1*, *PTGS* and *PGFS* following seminal plasma exposure. In the human and rodent, seminal plasma derived transforming growth factor beta (TGFβ) is one of the active molecules within the ejaculate to facilitate these maternal tract changes. Evaluated bull seminal plasma contains an average of 7.11 ± 1.55 ng/ml of total TGFβ-1, and 6.07 ± 1.16 ng/ml of total TGFβ-2. Collectively, our data suggest that endometrial changes induced by seminal plasma may help to improve reproductive outcomes in the cow. Endometrial changes induced by seminal plasma may help to improve reproductive outcomes in the cow by promoting embryo development, implantation and maternal immune modulation.

CHAPTER 1
LITERATURE REVIEW

Early Pregnancy – What Is Required for Success

Pregnancy is a complex, highly organized process which begins with the fertilization of the ovum, followed by attachment and implantation of the blastocyst, placentation, growth of the fetus and finally birth. The success of each event is essential to advance toward the next stage of pregnancy (Carson et al., 2000; Dey et al., 2004; Wang and Dey, 2006).

The most critical period of the reproductive process is early pregnancy, particularly the periconception and embryo implantation phase. In mammalian species, the majority of pregnancy loss occurs during the peri-implantation period (Norwitz et al., 2001; Cockburn and Rossant, 2010). In the human, most pregnancy loss occurs during embryo implantation; moreover, it has been reported that in Europe and the USA one in nine couples is affected by implantation disorders and pregnancy loss (Teklenburg et al., 2010). In livestock species, disruptions to the maternal environment and its capacity to support conceptus development are constraining factors of fertility with important economic implications (Kridli et al., 2016). In cattle, 70 to 80% of total embryonic loss occurs during the first three weeks after insemination, typically between days 7 and 16 of pregnancy (Diskin et al., 2006). It has been proposed that both the embryo and maternal tissues contribute to pregnancy loss, suggesting requirements for optimal embryo development and maternal preparedness.

Embryo development occurs in the oviduct following fertilization and continues in the uterus. A series of coordinated events must occur to ensure the survival of the embryo during this preimplantation period, orchestrated by the embryo itself and in response to secreted maternal factors. Embryo implantation occurs during a limited time period when the blastocyst participates in the first physical interaction with the maternal endometrium (Red-Horse et al.,

2004; Wang and Dey, 2006). To implant, the blastocyst needs to adhere to the endometrium and facilitate a bidirectional crosstalk with the uterus (Rogers et al., 1983; Rogers and Murphy, 1989; Paria et al., 1993; Ma et al., 2003). These interactions are necessary to facilitate placentation required to support the developing conceptus.

Unique to pregnancy, the conceptus is immunologically foreign to the maternal immune system due to the presence of foreign antigens derived from paternal chromosomes (Fernandez et al., 1999). Therefore, the conceptus is vulnerable to maternal immunological attack (Fowden et al., 2008). Maternal immune adaptation (or tolerance) toward the conceptus can influence the early embryo and either impair implantation or prevent placental morphogenesis. Failure to successfully establish T-regulatory cell-mediated immune tolerance toward the conceptus can result in poor fertility, pregnancy failure or impart long-term adverse consequences for the fetus (Thompson et al., 2006).

Other factors important in early pregnancy success include endometrial receptivity, endometrial tissue remodeling, and appropriate estrogen and progesterone (Teles et al., 2013; Robertson and Moldenhauer, 2014). In this thesis, we are interested in the modulation of the uterine environment during early pregnancy and the influence of semen in regulating this environment during early pregnancy.

The Uterine Microenvironment during the Preimplantation Period as a Key Player in Pregnancy Establishment

The uterus is a dynamic organ which experiences molecular and functional changes during the estrous cycle, embryo implantation, pregnancy and parturition. The cellular and molecular environment of the uterus during the preimplantation period of early pregnancy is critical for embryonic development and subsequent implantation success. The main driving force for uterine transformation during the estrous cycle are ovarian steroid hormones; however, there

is also paracrine and autocrine release of cytokines from both immune and non-immune cells (epithelial and stromal) of the oviduct, cervix, and endometrium which contribute to these changes (Ruiz-Alonso et al., 2012). Maternal reproductive tract cytokines are secreted into the uterine lumen where the embryo develops, regulating embryo progression from a single cell zygote to the blastocyst stage prior to implantation (Robertson and Moldenhauer, 2014). Maternal cytokines are essential mediators of the cellular and molecular environment of the uterus, inducing inflammation, tissue remodeling and modulation of maternal immune tolerance toward the conceptus (Orsi and Tribe, 2008).

Embryo Development

Maternally derived cytokines referred to as embryokines, influence embryo development and can be categorized as embryotrophic (anti-apoptotic) or embryotoxic (apoptotic). In a healthy reproductive tract, cytokines including colony stimulating factor (CSF1), CSF2, leukemia inhibitory factor (LIF), insulin-like growth factor-I (IGFI) and heparin-binding epidermal growth factor (HB-EGF) act to stimulate blastocyst development, increasing cell number and the probability of implantation success (Robertson et al., 2018). Together these embryotrophic cytokines effect gene expression, metabolism and apoptosis pathways in the pre-implantation embryo (Loureiro et al., 2011). Consequently, by modulating embryo development these maternal cytokines can affect downstream events including implantation, placental development, fetal growth, and eventually the phenotype and health of progeny (Bromfield et al., 2014). Indeed, higher rates of bovine blastocysts development can be achieved when grown in an environment supplemented with growth factors and cytokines, including CSF2, LIF, IGFI, IGFII, fibroblast growth factor (bFGF), and transforming growth factor beta (TGFβ) (Neira et al., 2010).

One of the embryotrophic cytokines studied extensively is CSF2. Studies in several species including rodents, human, pigs and cattle indicate a crucial role for CSF2 in promoting optimal blastocyst development, implantation, placental development, and in programming metabolic health in offspring. In the mouse, embryo culture in the presence of CSF2 produces blastocysts with an increase in the total number of cells, particularly in the inner cell mass (ICM) (Sjoblom, 2002). Confirming a role for maternal CSF2 in vivo, a reduction in blastocyst development is observed in the *Csf2* null mouse. In humans, CSF2 increases development of healthy blastocysts in vitro (Sjöblom et al., 1999), while supplementation of CSF2 to embryo culture increases fertilization and implantation rates (Ziebe et al., 2013). Additionally, it has been observed that CSF2 enhances viability of in vitro developed embryos of pigs, sheep and cattle (Imakawa et al., 1993; Cui et al., 2004; Michael et al., 2006). In domestic species, CSF2 has been reported to upregulate blastocyst secretion of the pregnancy recognition molecule interferon-tau (IFNτ). While, supplementation of culture medium with CSF2 promotes post-transfer survival of preimplantation embryos (Loureiro et al., 2011).

The interleukin (IL)-6 family member, LIF, is another embryokine which improves embryo competence (Mathieu et al., 2012). The role of LIF as an embryokinvbc e has been associated with increased embryo implantation (Aghajanova, 2004). LIF is secreted by both the embryo and endometrium in several species including rodents, humans, and ruminants (Fry et al., 1992; Schofield and Kimber, 2004; Kimber, 2005; Vejlsted et al., 2005). LIF regulates various cellular functions including proliferation, survival, differentiation of a wide range of cell types and is considered important in embryo growth, implantation, and pregnancy (Schofield and Kimber, 2004; Vejlsted et al., 2005). LIF facilitates the progression of the embryo from morula to blastocyst, improves blastocyst hatching rate, and increases the number of blastomeres of the

ICM (Paria and Dey, 1990; Martal et al., 1998). The process of embryo implantation does not occur in the absence of LIF, as witnessed in the *Lif* null mouse (Bhatt et al., 1991; Pampfer et al., 1991; Stewart et al., 1992; Yang et al., 1995). In sheep, the addition of human LIF to embryo culture medium improves the development of ovine embryos and pregnancy rates after transfer to recipient ewes (Fry et al., 1992). Similarly, in cows, it has been reported that an improvement of pregnancy rate is achieved by the intrauterine administration of human LIF at the time of embryo transfer (Roh et al., 2016). Al Naib and colleagues have suggested that LIF regulates the expression of *NC4* in bovine blastocysts, a binding site of non-classical major histocompatibility complex (MHC) class I, potentially modulating maternal immune recognition of the embryo (Al Naib et al., 2011). LIF is also known to regulate the expression of the trophoblast-specific MHC class I protein that is specifically expressed at the time of uterine invasion and plays a role in facilitating immune tolerance of the conceptus (Bamberger et al., 2000). In the rodent, LIF induces stromal decidualization by increasing the production of cytokines and prostaglandins, and induces trophoblast proliferation (Salleh and Giribabu, 2014).

Preimplantation bovine embryos expresses the receptor for IGF1 throughout early development (Palma et al., 1997). Many studies have described a positive effect of IGF1 supplementation during in vitro culture on development to the blastocyst stage (Palma et al., 1997; Moreira et al., 2002; Sirisathien and Brackett, 2003; Sirisathien et al., 2003). The supplementation of the embryokine IGFI to culture medium has been shown to improve pregnancy and calving per embryo transfer rates when recipients are exposed to heat stress, suggesting a protective role against exogenous stress (Block et al., 2011). An increase in blastocyst cell number was observed when IGFI is added to bovine embryo culture medium by reducing apoptosis (Byrne et al., 2002; Sirisathien and Brackett, 2003). In the human, IGFI also

reduces early preimplantation embryo apoptosis (Kawamura et al., 2005; Neira et al., 2007). Neira and colleagues found that IGFI accelerated embryonic development, particularly the progression from the expanded blastocyst to the hatched blastocyst stage.

In addition to embryotrophic cytokines, growth factors including TGFα and TGFβ play crucial roles during embryo development (Martal et al., 1998). TGFβ1 is known have a mitogenic effect on the blastocyst and the expression of maternal *TGFB1* in cattle and sheep supports the hypothesis that this factor has a role in embryo development and implantation (Watson et al., 1992; Neira et al., 2010). Supplementation of TGFβ to culture medium increases the proportion of embryos developing to the blastocyst stage (Marquant-Le Guienne et al., 1989), and the combination of bFGF and TGFβ act synergistically to promote the development of bovine embryos (Larson et al., 1992). Endometrial *TGFB2* expression is downregulated during the ovine and bovine implantation period but appears to increase during placentation (Mansouri-Attia et al., 2012). In many species, several roles have been proposed for TGFβ-2 during placentation including; as a chemoattractant for macrophage recruitment to the placentation foci, a regulator of trophoblast invasion and regulation of macrophage inflammatory status (Wahl et al., 1987; Graham and Lala, 1991).

Embryotoxic cytokines, including tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL) and interferon gamma (IFNγ) induce apoptosis and exert potent inhibitory effects on embryo development (Pitti et al., 1996; Gao et al., 2015). Both, IFNγ and TNFα also significantly reduced sperm motility in vitro. In contrast, another study showed that TNFα in vitro did not affect human sperm motility, hamster ova penetration, mouse in vitro fertilisation (IVF) and embryo development (Wincek et al., 1991). The balance between maternal embryotrophic (anti-apoptotic) and embryotoxic (apoptotic) cytokines appears to be a

physiological quality control mechanism to decide whether a given reproductive cycle will result in embryo survival and progression to pregnancy or could result in disorders such as recurrent pregnancy loss, pre-term labor or fetal growth restriction.

The relevance of these various factors in early embryo development is important to our understanding of reproductive physiology and potential mediators of pregnancy success. However, little is known about what regulates the maternal secretion of these embryokines in a temporal fashion to coordinate embryo development with endometrial receptivity.

Uterine Cellular Environment

Leukocytes residing (or recruited) to the endometrium are an effective source of cytokines and chemokines to alter the local environment (Tafuri et al., 1995). Resident uterine leukocytes consist of uterine natural killer (uNK) cells, natural killer (NK) cells, antigen presenting cells (APC) (including macrophages and dendritic cells (DC)), granulocytes (including neutrophils and eosinophils), mast cells and lymphocytes (T and B cells).

During implantation uNK cells of the innate immunity compartment, represent the major immune cell population at the early feto-maternal interface (Hanna et al., 2006). Uterine NK cells work together with uterine macrophages to regulate maternal uterine vasculature remodeling and production of cytokines to facilitate placentation (Hatta et al., 2011). Uterine macrophages and uNK cells, mediated by IFNγ, initiate the remolding of spiral arteries prior to placentation (Ashkar and Croy, 2001). Uterine NK cells are also known to promote angiogenesis at the feto-maternal interface. In vivo, carbon monoxide treatment induces the in situ expansion of uNK cells and vascular endothelial growth factor (VEGF) secretion, further promoting vascular remodeling (Greenwood et al., 2000; Bilinski et al., 2008). Mice lacking uNK cells have impaired spiral artery modification and placenta development, resulting in intrauterine growth restriction (IUGR) and smaller progeny (Greenwood et al., 2000). Interestingly, uNK cells are

rare in bovine endometrial tissue of cycling animals but increase during the early stages of pregnancy (Oliveira et al., 2013).

Macrophages are recruited to the pregnant endometrium in a range of mammalian species including the mouse, human, sheep and cattle (Mincheva-Nilsson et al., 1994; Tekin and Hansen, 2004; Fest et al., 2007; Oliveira and Hansen, 2009; Oliveira et al., 2010). Endometrial macrophages generally have an immunosuppressive M2 phenotype and secrete anti-inflammatory TGFβ and IL10 (Heikkinen et al., 2003; McIntire and Hunt, 2005). Uterine macrophages are hypothesized to clear cellular debris, regulate endometrial apoptosis, and modulate placental lactogen concentrations at the fetal-maternal interface (Straszewski-Chavez et al., 2005; Kzhyshkowska et al., 2008). An additional proposed role for these cells, which is related to ruminant species, is regulating the activation of anti-conceptus immune reactions in response to IFNτ stimulation and antigenicity of the conceptus (Doyle et al., 2009; Oliveira et al., 2010). Macrophage numbers are reduced by more than half in *Lif* knockout mice, indicating the important role for endometrial LIF in macrophage recruitment (Schofield and Kimber, 2004). Care and colleagues reported that specific depletion of $CD11b^{+}$ macrophages prior to mating caused implantation failure, not because of a uterine defect, but due to the fact that macrophage depletion altered the luteal microvasculature that is essential to support progesterone production during pregnancy (Care et al., 2013).

Similar to macrophages an important function of dendritic cells (DCs) is the presentation of antigen to T cells and the preparation of the uterus for implantation. Specifically, DCs are thought to support decidual transformation important in implantation in the rodent (Laskarin et al., 2007; Plaks et al., 2008). A large population of immature endometrial DCs are present during early pregnancy in the bovine endometrium (Mansouri-Attia et al., 2012). Immature DC's have

been associated with the initiation and maintenance of peripheral tolerance and their presence has been positively associated with the establishment of healthy pregnancies in women (Rieger et al., 2004; Tirado-González et al., 2010). The expansion of these populations in the maternal endometrium of ruminants is likely to be induced by IFNτ. However, CSF2 is also an important regulator of DC maturity (Moldenhauer et al., 2010). Du and colleagues have shown that DCs are mediators of the communication between trophoblast and decidual cells in the human (Du et al., 2014). Indeed, the depletion of DCs in mice causes substantial fetal loss due to implantation failure and impaired decidual proliferation related to uterine receptivity (Plaks et al., 2008; Negishi et al., 2012)..

Dependent on local stimuli, neutrophils, eosinophils and mast cells produce several cytokines and chemokines, including IFNγ, CSF2 and TNFα, (Cocchiara et al., 1995; Yeaman et al., 1998). Neutrophils support angiogenesis and secrete VEGF to support implantation and placentation (Valent et al., 1998; Smith et al., 2006). As described for other cells of the innate immune system, uterine mast cells represent a different population of mast cells found in other tissues (Woidacki et al., 2013). Schmerse and colleuges observed that uterine mast cells are abundant in the uterus of pregnant mice, localized in close proximity to blood vessels in a similar pattern to uNK cells (Woidacki et al., 2013; Schmerse et al., 2014). Studies in rats show a positive influence of uterine mast cells on promoting angiogenesis at the feto-maternal interface, supporting tissue remodeling during implantation, placentation, and fetal growth (Varayoud et al., 2004; Bosquiazzo et al., 2007; Woidacki et al., 2013). Mice deficient of uterine mast cells are fertile; however, breeding colonies often display irregular birth rates due to high death rates (Lyon and Glenister, 1982). Depletion of uterine mast cells in murine allogeneic pregnancies reduces implantation rates (Woidacki et al., 2013; Zenclussen and Hämmerling, 2015).

The majority of uterine lymphocyte are uNK cells; however, there are other lymphocyte populations comprised of T cells and B cells. No information is available about the participation of B cells at the early stages of blastocyst development; however, it is likely that B cells play a role in later gestation. B cells are present in the decidua in low numbers (Fettke et al., 2014; Muzzio et al., 2014). In mice, failure of IL10 producing B cells to expand is related with pregnancy loss (Jensen et al., 2013). In vitro, human B cells are able to hinder TNFα production by effector T cells (Rolle et al., 2013), while in mice, early transfer of IL10 producing B cells can save pregnancies from immunological abortion (Jensen et al., 2013). Early studies focused on the capacity of T helper cells to produce either Th1 or Th2 type cytokines. A number of studies conclude that Th2 immune function is critical to the establishment and maintenance of pregnancy, while Th1 phenotypes are associated with reproductive failure and pregnancy pathology (Morelli et al., 2015; Bonney, 2016). More recently, a specialized subset of T cells (characterized by the presence of the cell surface marker CD25 and the transcription factor FOXP3) have been identified which regulate antigen specific immunosuppression, termed T regulatory (Treg) cells (Sakaguchi et al., 2001; Saito et al., 2013). Treg cells are critical for the maternal tolerance of paternally expressed antigens expressed on the conceptus, required for pregnancy success. In mice, Treg cells accumulate in the uterus shortly following insemination. Populations of Treg cells, including $CD4^+$, $CD8^+$ and $FOXP3^+$ lymphocytes are present in both pregnant and cycling cattle (Fair, 2016). Although the population size of these cells does not change during the estrous cycle, the gene expression profile of these cells is temporally changed; Th1 immune factors *IFNA, LIF, IL1B, IL8,* and *IL12A* are down regulated during the luteal phase, whereas Th2 factors *LIF* and *IL10* are upregulated. Depletion of Treg cells results in reduced implantation in both allogeneic and syngeneic matings of mice (Aluvihare et al., 2004;

Shima et al., 2010; Teles et al., 2013). However, when Treg cells are depleted after conception, pregnancy is not compromised to the same degree as pre-conception reduction, suggesting that Treg cells have an essential role during early implantation and placental development (Samstein et al., 2012).

Uterine Receptivity and Embryo Implantation

There are important anatomical and physiological differences in the reproductive strategies of domestic species compared to that of the human or rodent models. For example, embryo implantation occurs much later, and placentation is less invasive in ruminants than in the mouse or human. Ruminants have different types of placentation and sequential embryonic development and implantation. The growth trajectory of the bovine placenta differs from the ovine and rodent, and is, in fact, more similar to the human (Wooding and Flint, 1994; Hernandez-Medrano et al., 2015). Unlike small ruminants, bovine embryo development occurs in a similar temporal fashion as the human (Copping et al., 2016). The differences in reproductive strategies between species are important to consider when drawing conclusions from the various models.

Generally, after fertilization, implantation is initiated when the trophoblast cells reach the surface epithelium of the endometrium to attach and invade, leading to the formation of the placenta. The success of implantation is dependent on the coordinated balance between the invading trophoblast and a receptive maternal endometrium. In addition to endometrial and endothelial cells, infiltrating immune cells represent a major cellular component of the maternal implantation site (Mor et al., 2017). At this time the blastocyst becomes a component of the host immune system during pregnancy (Guilbert et al., 1993). A key event of mammalian implantation is the cross talk between the embryo and the receptive endometrium that allows the invasion of the blastocyst and the rapid growth of the placenta while supporting the

transformation of uterine tissue to support the placenta. This transformation is facilitated by maternal immune cell populations, a variety of genes that include cytokines, homeobox transcription factors, and developmental genes working together with ovarian steroids to confer uterine receptivity (Zhang et al., 2013; Zenclussen and Hämmerling, 2015).

The human endometrium increases expression of a number of factors during the period of receptivity, such as IL1, EGF, HBEGF, CSF1, LIF and PTGS2 (Aghajanova, 2004). During the window of implantation, the embryo itself actively participates in establishing contact with the endometrium by expressing LIF-specific receptors (Sharkey et al., 1995; Sherwin et al., 2002). The embryo increases LIF expression, whereas the endometrium increases expression of the LIF receptor genes IL6ST and LIFR (Chen et al., 1999). When the embryo invades the epithelium and reaches the endometrial stroma, it begins to synthesize several cytokines, such as IL1, TNF, and TGFβ, inducing further secretion of LIF by stromal cells (Selick et al., 1994).

Ruminant preimplantation embryos produce the type 1 interferon, IFNτ, which is the main signaling factor in maternal recognition of pregnancy to avoid regression of the corpus luteum and maintain ovarian progesterone secretion (Hansen et al., 1999; Kim et al., 2003; Spencer, 2004; Gierek et al., 2006; Bazer, 2013). IFNτ is secreted by the trophectoderm of the elongating conceptus (Roberts et al., 2003; Robinson et al., 2006). IFNτ acts on the endometrium to induce the expression of genes that promote corpus luteum survival, conceptus growth and development, and induce uterine receptivity (Hansen et al., 2010, Johnson et al., 2000; Mansouri-Attia et al., 2012). Progesterone and conceptus secreted molecules, including prostaglandins and cortisol also regulates the uterine environment during the implantation period (Klein et al., 2006; Dorniak et al., 2012; Spencer et al., 2013)

Maternal Immune Tolerance toward the Conceptus

The maternal immune system plays a critical role in mammalian embryo implantation. Activation of an appropriate maternal immune response and tolerance towards paternal antigens expressed by the semi-allogenic embryo is required to allow the growth and development of the foreign tissue graft of the conceptus. More than 60 years ago, Sir Peter Medawar postulated theories to explain how maternal immune tolerance toward the allogeneic conceptus might occur, including physical separation between maternal and fetal tissues, fetal antigenic immaturity, and maternal immune suppression (Medawar, 1953). Since then, data supporting the incomplete validity of these explanations has been revealed (Jiang et al., 2014).

It is probable that MHC class I play a role in embryo-maternal interaction and modulation of the maternal immune response to pregnancy. MHC class I genes, named bovine leukocyte antigen (BoLA) in cattle, are involved in immunological differentiation of self and non-self-antigens by presenting antigenic peptides to T lymphocytes (Davies et al., 2006; Robertson et al., 2013). MHC-I mRNA expression by bovine embryos can be controlled by many cytokines including IFNγ, IL4, and LIF (O'Gorman et al., 2010; Al Naib et al., 2011).

In humans, the maternal immune response to pregnancy has long been described as a Th1/Th2 switch with an imbalance toward a Th2 type immune response (Wegmann, 1988; Raghupathy, 1997). Uterine macrophages, dendritic cells and Treg cells stimulate the adaptive immune system. Consequently, antigen-specific immune responses can be initiated and switched from the inflammatory (Th1) to the anti-inflammatory (Th2) type. This switch is vital for future related requirements for inflammatory signaling during the establishment of pregnancy and implantation (Lin et al., 1993; Chaouat et al., 2007; Gellersen et al., 2007). Furthermore, it has been reported that uterine inflammation is essential for successful human embryo implantation and pregnancy maintenance. For example, an increase in inflammatory Th1 cytokines such as

IL18, IL12, INFγ, and decreased Th2 cytokines such as LIF in the blood and endometrium is observed in patients with recurrent pregnancy loss (Comba et al., 2015). Furthermore, Th2 cells have tissue-remodeling roles and communicate with epithelial cells and stromal cells to affect endometrial receptivity for blastocyst implantation (Chan et al., 2015). It is unclear how maternal tolerance to the conceptus is developed in a timely manner to allow embryo implantation to occur in species such as the human or mouse where implantation occurs within a week of fertilization.

Hormonal Regulation of Early Pregnancy

Steroid Hormones

The expression of uterine cytokines is influenced by ovarian steroid hormones (Caballero-Campo et al., 2002). The balance between estrogen and progesterone is a major driver of the changes in cytokine, chemokine and growth factor expression by uterine endometrial cells (Robertson et al., 1992a; Miller et al., 1996). The combination of ovarian hormones and endometrial factors direct the preparation of the uterus for implantation in mice and rats (Harper and Walpole, 1967; Roblero et al., 1987; Vinijsanun and Martin, 1990; Aldo et al., 2014). It is generally accepted that progesterone is required for implantation in nearly all the animals studied (Finn and Martin, 1972; Dey et al., 2004; Tranguch et al., 2006; Wang and Dey, 2006).

Proliferation and differentiation of uterine stromal and epithelial cells throughout the estrous cycle and during pregnancy is mainly under the control of the ovarian steroid hormones (Bigsby and Cunha, 1986; Kurita et al., 1998). In the rodent and human, estrogen regulates the synthesis of several important pro-inflammatory cytokines in uterine epithelial cells including CSF1, CSF2, TNFα, IFNγ and IFNε (Fox et al., 1991; Robertson et al., 1992b; Fung et al., 2013). Interestingly, progesterone hinders epithelial cell production of CSF2, IL1 and numerous other chemokines. The suppression of pro-inflammatory cytokines by progesterone during the

luteal phase influences endometrial receptivity and immune adaptation for possible embryo implantation and pregnancy. Moreover, ovarian steroids are reported to play a role in regulating *LIF*, *LIFR*, and *IL6ST* expression in the uterus throughout the implantation window. In mice, endometrial LIF secretion can be altered by estrogen exposure (Kimber, 2005). Exogenous estrogen and progesterone administration to ovariectomised mice is able to increase *IL6ST* expression in uterine glands (Ni et al., 2002). In hamsters, LIF secretion is induced by estrogen while the expression of *LIFR* and *IL6ST* is induced by progesterone (Ding et al., 2008). An in vitro study using human endometrial stromal cells shows that estrogen and progesterone are able to upregulate *LIFR* expression (Shuya et al., 2011). In mice, TGFβ responsiveness was lower in epithelial cells from diestrous mice than from estrous mice, signifying that estrogen might reduce expression of TGFβ receptors, binding proteins, or other components of the signaling web leading to initiation of CSF2 production (Tremellen et al., 1998). Estradiol is able to potentiate mast cells degranulation in vitro (Cocchiara et al., 1992), while both estrogen and progesterone upregulate mast cell migration from the periphery to the uterus (Jensen et al., 2010).

Animal and human studies both suggest that ovarian hormones regulate fluctuations in systemic and uterine Treg cell populations, with an estrogen-regulated increase at the time of ovulation (Arruvito et al., 2007). In mice, direct actions of estrogen on Treg proliferation and suppressive function may contribute to these cycle-related fluctuations (Mao et al., 2010). Also, administration of estrogen after ovariectomy causes an increase in Treg cell numbers and *Foxp3* mRNA expression in the para-aortic lymph nodes draining the uterus of mice (Polanczyk et al., 2004; Kallikourdis and Betz, 2007; Robertson et al., 2009). In women, a similar increase in Treg cells in peripheral blood is observed during the late follicular phase when Treg cell number positively correlate with serum estrogen, followed by a decline during the luteal phase.

Non-Steroidal Hormones

In cows, endometrial concentration of prostaglandins F2α ($PGF_{2\alpha}$) is known to increase temporarily between days 16 and 19 of pregnancy (Seals et al., 1998). Studies in cattle, rabbits, and rats have shown impaired development of embryos following culture in medium supplemented with $PGF_{2\alpha}$ (Maurer and Beier, 1976; Scenna et al., 2004). Interestingly, embryos exposed during later developmental stages have appeared to be less affected by $PGF_{2\alpha}$ (Maurer and Beier, 1976; Scenna et al., 2004). Although gross morphology of blastocysts were classified as normal, their hatching rate, the key criteria determining the quality of embryos, was negatively affected by $PGF_{2\alpha}$ (Maurer and Beier, 1976; Scenna et al., 2004). The mechanism by which $PGF_{2\alpha}$ influences embryonic development remains unclear. It is likely that $PGF_{2\alpha}$ is related to cell-to-cell adhesion, and induction of apoptosis due to altered Na^+ transport within embryos (Chaudhari et al., 2014). In addition, $PGF_{2\alpha}$ affects the expansion of blastocysts and maintenance of the blastocyst cavity, thereby interfering with the hatching process. Biosynthesis of prostaglandins is initially controlled by two types of cyclooxygenases (COX) enzymes, prostaglandin-endoperoxide synthase (PTGS) 1 and PTGS2 which are present in endometrial cells. COX enzymes convert endometrial arachidonic acid to the unstable PGG_2, which is then converted to other PGs by specific endoperoxide isomerase enzymes (Okuda et al., 2002). It has been reported that expression of *PTGS2* is upregulated in the uterine horn ipsilateral to the corpus luteum of ruminants. This suggests that PG is required near the embryo, and likely plays localized role in ovarian function, CL maintenance and embryo development.

The Role of Seminal Plasma in Modulating the Uterine Environment

It is believed that any changes in the uterine cytokine environment, due to infection, stress, or nutrition, may affect embryo implantation success and placenta development. In many species, the uterine environment is thought to be regulated by ovarian hormones or the embryo

itself. However, during natural mating or artificial insemination (AI), the female reproductive tract is exposed to the ejaculate consisting of spermatozoa, paternal cells and seminal plasma (SP). New lines of investigation suggest that SP could play a role in establishing a communication pathway between the sire and dam to modulate the maternal environment and facilitate uterine changes required for pregnancy outline above.

Seminal Plasma as a Vehicle for Sperm Transport

Seminal plasma is complex medium rich with enzymes, mucus, vitamins, proteins, amino acids, ions, minerals, flavins, hormones, electrolytes and sugars (Poiani, 2006). Historically, the primary role of SP who believed limited to the support of sperm viability and to serve as a protective and nourishing source for sperm maturation during their journey through the female reproductive tract (Mann T, 1964; Owen, 2005). Seminal plasma components have been suggested to influence sperm transport in several species (Overstreet and Cooper, 1978; Clavert et al., 1985; Willmen, T. et al., 1991). Troedsson and others have demonstrated that equine SP has a role in transport and survival of viable spermatozoa in the female tract (Troedsson et al., 2005). In pigs, SP facilitates delivery of sperm to the upper reproductive tract through direct effects on uterine contractility mediated by estrogen, which acts through inducing $PGF_{2\alpha}$ release immediately after insemination (Claus, 1990). Secondary effects of SP have demonstrated the capacity to protect sperm from infection and other injurious and toxic agents. Finally, SP allows spermatozoa to overcome the immunological and chemical environment of the female reproductive tract. Seminal plasma helps to neutralize the acidic environment of the vagina to maintain sperm cell function (Vitku et al., 2017).

The Molecular Components of Seminal Plasma

Mammalian SP is the cell free part of the ejaculate which is composed of secretions of cell/tissues along the male reproductive tract, including the epididymis, seminal vesicle glands,

prostate, bulbourethral glands, and sertoli cells. The size, storage capacity, and secretory output of different tissues of the male reproductive tract determine the volume and composition of SP and concentration of sperm in the ejaculate. Profiling of cattle SP reveals a complex medium including peptidase, heparin-binding proteins, spermadhesins, ions (Na^+, K^+, Zn^+ , Ca^{2+}, Mg^{2+}, Cl_2), energy substrates (fructose, sorbitol, glycerylphosphocholine), citric acid, amino acids, lipids, hormones, cytokines, bovine SP proteins, acidic seminal plasma protein (aSPP), glycosidase, exopeptidases, and phospholipases (Juyena and Stelletta, 2012). Among the wide range of components of SP, the peptide and protein elements have specific roles in the regulating fertilization. Proteomic characterization of bovine SP indicates that the proportion of proteins with a molecular weight below 25 kDa represents 80.1% of total proteins. The predominant proteins in bovine SP are binder of sperm protein 1 (BSP1), binder of sperm protein 3 (BSP3), and seminal plasma protein BSP-30 kDa (BSP5) (Druart et al., 2013). A high concentration of pyruvic acid has been observed in bovine seminal vesicle gland plasma, which is a source of energy for the spermatozoa after ejaculation (Marden, 1961). Values for some constituents of SP of the bull are given in Table 1-1 (Juyena and Stelletta, 2012).

Many studies have indicated that cytokines are present in human SP and have been investigated for their association with male and female fertility (Maegawa et al., 2002; Politch et al., 2007; von Wolff et al., 2007; Fraczek and Kurpisz, 2015a). Seshadri and colleagues have reported that cytokines including IL6, IL8, IL10, IL11, IL12, TNFα and IFNγ are present in human SP. Concentrations of SP IL11 are significantly increased in fertile couples. IL11 may have a function in the fertilizing capacity of the spermatozoa (Seshadri et al., 2011). Interferon-γ is detected at low levels in SP but can be significantly elevated in the event of infection (Leutscher et al., 2005). IFNγ has been shown to negatively affect sperm motion (Hill et al.,

1987). One-study reports elevated SP IFNγ in infertile men with poor sperm quality, including low sperm count, low motility and poor morphology (Paradisi et al., 1996). Concentrations of IL-6 in SP are elevated in infertile men compared with fertile controls (Naz and Kaplan, 1994; Sukcharoen et al., 1995; Donnelly et al., 1998). Furthermore, some studies have shown that IL-6 provides a positive signal to enhance the fertilizing capacity of human spermatozoa by increasing capacitation leading to the acrosome reaction (Naz and Kaplan, 1994). Concentrations of IL-8 in human SP are significantly elevated in infertile patients with leukospermia (Shimoya et al., 1993). Human SP also contains a number of adipokines, including leptin and adiponectin (Thomas et al., 2013). Adipokine concentrations in SP are correlated with functional characteristics of spermatozoa, with SP leptin negatively associated with decreased sperm motility (Guo et al., 2014; Elfassy et al., 2017).

Recently, Sharkey and colleagues have evaluated various SP pro-inflammatory cytokines IFNγ and IL8 within individual men over time and found that IFNγ exhibits substantial variation that is independent of duration of abstinence, while sperm motility is inversely correlated with SP IL8 concentrations (Sharkey et al., 2017).

Various hormones are also among the constituents of SP. Progesterone is the most effective hormone at activation of spermatozoa by initiating capacitation, increasing motility, and activating proteolytic enzymes responsible for penetration of the sperm across the ovum membrane (Luconi et al., 2004; Modi et al., 2007). Studies in pigs suggest that SP estrogen has a positive effect on sperm transport and fertilization (Claus, 1990). Both cortisol and dehydroepiandrosterone (DHEA) and its 7-oxygenated metabolites are present in human SP (Hampl et al., 2003). In addition, the presence of catecholamines, gonadotropins, human chorionic gonadotropin (hCG), oxytocin, vasopressin, antimüllerian hormone (AMH), inhibin,

melatonin, calcitonin, relaxin, adrenomedullin, parathyroid hormone, prolactin, $PGF_{2\alpha}$ and PGE_2 have all been reported in human SP, some of which have been correlated with sperm function or male factor infertility.

TGFβ1, TGFβ-2 and TGFβ-3 has been evaluated in human SP (Nocera and Chu, 1995; Loras et al., 1999; Sharkey et al., 2012a). On average the content of SP TGFβ consists of 55% TGFβ-1, 1% TGFβ-2, and 44% TGFβ-3 (Sharkey et al., 2012a). Loras and colleagues have demonstrated that TGFβ content of human SP showed no differences between normal and infertile men (Loras et al., 1999). Both TGFβ-1 and TGFβ-2 are abundant in boar SP, predominantly in the active and not latent form. (O'Leary et al., 2011). It has not yet been demonstrated if bovine SP contains TGFβ.

A Role for Seminal Plasma in Optimizing Pregnancy Outcome

With the use of AI and embryo transfer, it is clear that SP is not mandatory for successful reproduction; however, several studies in different species indicate that pregnancy, and potentially growth and development of the fetus, is compromised if females are not exposed to SP at insemination. Experiments in mice, rats, and hamsters have shown that ablation of SP by surgical removal of the seminal vesicle glands, prostate and coagulating glands reduces pregnancy rates (Pang et al., 1979; F. Queen et al., 1981; Peitz and Olds-Clarke, 1986). Furthermore, in pregnancies sired by seminal vesicles deficient male mice, total and viable implantation sites are reduced (Bromfield et al., 2014). These data are consistent with a major role of SP in rodents as a transport medium for sperm.

In pigs, it has been demonstrated that increased SP exposure around the time of conception by the use of vasectomized boars or intra-uterine SP infusion increases pregnancy rates, litter size and improves farrowing rate (Murray et al., 1983; Mah et al., 1985). Similarly, in

the mare, insemination using sperm suspended in SP increases pregnancy rate compared with mares inseminated with sperm suspended in standard semen extender (Alghamdi et al., 2004).

The effect of surgical seminal vesicle gland removal on bull fertility was tested in 1967. Following breeding of seminal vesicle gland deficient bulls to heifers, a 69% conception rate was observed (Table 1-2). No negative effect on sperm motility or viability were observed following seminal vesicle gland removal; however, an increase in ejaculate sperm concentration, and an approximate halving of the ejaculate volume was observed. This suggests that, contrary to the rodent, the seminal vesicle glands of the bull contributes only 50% of the ejaculate volume (Faulkner et al., 1968). Nevertheless, infusion of additional SP at the time of AI in dairy and beef cattle increases pregnancy rate by 4.6% and 6.7%, respectively; though, this was not statistically significant due to the number of animals in the study (Odhiambo et al., 2009). This suggests that in cattle, exposure to SP has the potential to improve fertility.

In the human, a randomized placebo-controlled clinical trial revealed that vaginal capsules containing SP significantly improved implantation rates (Coulam and Stern, 1995). In a separate study, deposition of semen into the upper vaginal tract of women with tubal occlusion or with no Fallopian tubes during IVF treatment resulted in increased implantation rate (Bellinge et al., 1986). Furthermore, women exposed to semen by intercourse shortly before or after frozen embryo transfer showed improved implantation success (Tremellen, 2000). Epidemiological studies in humans associate a lack of exposure to semen, due to limited sexual experience, use of barrier contraception, or in IVF with increased risk of implantation failure, spontaneous abortion, and pre-eclampsia (Bellinge et al., 1986; Klonoff-Cohen et al., 1989; Robillard et al., 1994). These observations, and those in animals, suggest a potential role for SP in increasing pregnancy success.

Seminal Plasma Modulates the Maternal Reproductive Tract Cellular and Molecular Environment

There are several experiments that lead us surmise that introduction of SP into the female reproductive tract and not sperm or physical stimulation (mating) alter the female reproductive tract environment. Yanagimachi and Chang observed a marked infiltration of leukocytes into the endometrium following coitus, which was greater than that observed at estrus. A series of experiments using mechanical stimulation, washed sperm infusion and uterine ligation allowed the team to identify SP as the principle component of coitus in stimulating this leukocyte infiltrate (Yanagimachi and Change, 1963). Since these studies, other experiments using vasectomized sires or uterine infusion have demonstrated that SP itself initiates a transient inflammatory response in the endometrium of swine, goats, sheep, rodents and the mare, consisting of neutrophil, macrophage, dendritic cell, and lymphocyte accumulation into uterine tissues within hours of insemination (Yanagimachi and Change, 1963; Lovell and Getty, 1968; Phillips and Mahler, 1977; Taylor, 1982; Pandya and Cohen, 1985; De et al., 1991; McMaster et al., 1992; Bischof et al., 1995; Robertson et al., 1996; Engelhardt et al., 1997; Rozeboom et al., 1998; Tremellen et al., 1998; Alghamdi et al., 2004; Johansson et al., 2004; O'Leary et al., 2004). In gilts, inflammatory cells recruited to the endometrium in response to SP influence the capacity of sperm to reach the oocyte, and remove superfluous sperm, microorganisms, and seminal debris (Rozeboom et al., 1999). Similar studies have observed transient leukocyte infiltration into the human cervix following unprotected intercourse, but not condom protected intercourse (Sharkey et al., 2012). This acute inflammatory response to SP dissipates by the time the embryo implants; however, exposure to SP could affect events in the female tract with consequences in later pregnancy.

Cellular changes to the endometrium are initiated when SP interacts with cervical or uterine epithelial cells. This interaction modulates the expression of endometrial or cervical CSF2, IL6 and a other chemokines (Robertson et al., 1996; Sharkey et al., 2012a). This SP induced cytokine cascade elicits recruitment and activation of macrophages and dendritic cells into the endometrial stroma (Watson et al., 1983). Experiments using isolated endometrial or cervical epithelial cells suggest that SP increases expression of epithelial pro-inflammatory cytokines which result in the recruitment of leukocytes to the site of SP exposure (Sharkey et al., 2012a; Tremellen et al., 2012; Elweza et al., 2018) .

Seminal Plasma Modulates the Immune Response to Pregnancy

Seminal plasma stimulates the generation of Treg cells in maternal tissues that protect the semi-allogeneic conceptus from maternal immune attack (Schjenken and Robertson, 2015). Robertson found that exposure to SP increased the absolute number and proportion of $CD25^+$ Treg cells in the uterus and draining lymph nodes of mice, crucial for maternal immune tolerance toward the conceptus in later pregnancy (Robertson et al., 2009). However, it has been suggested that SP and sperm are both necessary for the expansion of $FOXP3^+$ Treg cells in the endometrium during the pre-implantation period (Guerin et al., 2011). The potential for semen to promote functional immune tolerance to male antigens was first proposed by observations that mated mice are not able to reject allogeneic skin grafts of paternal origin (Lengerova and Vojtiskova, 1963). Subsequently, it was demonstrated that survival of allogeneic grafts is dependent on exposure to MHC antigens, but only when sperm is delivered in the context of SP (Beer and Billingham, 1974). Similarly, infusion with washed sperm, but not natural insemination lead to the generation of cytotoxic T-lymphocytes (Hancock and Faruki, 1986). This suggest that the initiating factor(s) for maternal immune tolerance is partly conferred to SP exposure (Robertson et al., 1996; Sharkey et al., 2012a). The initiation of maternal immune

tolerance at insemination would make physiological sense to confer the required immune modulation at the time the embryo implants, 5-19 days following conception.

The relative concentrations of bioactive elements in SP (see below) have been implicated in modifying the female response to insemination, and thus could have beneficial effects on subsequent immune activation and receptivity to pregnancy (Sharkey et al., 2012; Robertson et al., 2013; Robertson and Sharkey, 2016).

Physiological Significance of Seminal Plasma for Embryo Development

While SP alters the cellular and molecular constituents present at conception, these changes may directly affect the development of the embryo. In mice, embryo development to the blastocyst stage is impaired when conception is achieved using seminal vesicle deficient males. Indeed, blastocysts sired by seminal vesicle deficient male mice have recurrent irregularities in blastocoel cavity formation and contain fewer blastomeres compared with control blastocysts (Bromfield et al., 2014). Surprisingly, embryos sired by seminal vesicle deficient males have higher developmental rates when removed from the female tract and cultured in vitro, suggesting that an absence of SP promotes a hostile maternal environment for the developing embryo. To explain why embryo development was perturbed in the absence of seminal plasma, the authors demonstrate a positive effect of SP on oviduct expression of embryotrophic cytokines (*CSF2*, *IL6*, *EGF* and *LIF*), while SP reduced oviductal expression of the embryotoxic cytokines *TRAIL* (Salilew-Wondim et al., 2012; Bromfield et al., 2014). The golden hamster also has increased embryonic loss and slower cleavage rates after mating with seminal vesicle deficient males (O et al., 1988; Chow et al., 2003). In the pig, uterine exposure to SP at the time of AI increased the number, quality and size of embryos recovered 15 days after insemination. The increase in embryo quality may be due to the positive impact that SP administration has on the number of corpus luteum macrophages, corpus luteum steroidogenic function and progesterone secretion

(O'Leary et al., 2004). Furthermore, SP stimulates LIF secretion by human endometrial epithelial cells in vitro, which may then influence embryo development (Gutsche et al., 2003). In mice, embryos transferred to recipients prepared by mating to vasectomized males (resulting in SP exposure) have higher pregnancy rates than pseudopregnant recipient prepared by mechanical means (no SP exposure) (Rafferty, 1970; Watson et al., 1977). To some extent this deficiency of embryo development can be restored by mating of recipient females before embryo transfer in the rat (Carp et al., 1984). Similarly, in cattle, dysregulated fetal and/or placental growth that can occur after embryo transfer can be partially ameliorated by prior exposure to semen (Murray et al., 1983; Stone et al., 1987; Walker et al., 1992).

Together these observations suggest that exposure of the maternal reproductive tract to SP has the capacity to affect embryo development, potentially by modulating the developmental environment of the preimplantation embryo.

Seminal Plasma Molecules Responsible for Observed Uterine Effects

Concentrations of PGE_2 and TGFβ are extraordinarily high in SP of rodents, boars and humans (Robertson et al., 2002; O'Leary et al., 2011). Both PGE_2 and TGFβ have immune-modulating functions. Seminal plasma contains approximately five-fold higher concentrations of TGFβ compared to serum, at comparable concentrations found in colostrum (Pakkanen, 1998). TGFβ has a potent capacity to inhibit the induction of Th1 immunity in several tissue such as the anterior chamber of the eye and other mucosal surfaces (Wilbanks et al., 1992; Letterio and Roberts, 1998; Weiner, 2001). In addition, TGFβ causes phenotype skewing in APC and lymphocytes, and affects the induction and resolution of inflammatory responses. These functions of TGFβ are congruent with the effects of SP observed in maternal tissues following insemination (TM and Nocera, 1993; Robertson et al., 1996; Kelly and Critchley, 1997; Tremellen et al., 1998; Sharkey et al., 2012a). Protein chromatography of semen, neutralizing

antibodies and recombinant proteins have verified SP TGFβ as the principal trigger for the induction of uterine inflammation, expression of pro-inflammatory cytokines and embryotrophic factors following mating in mice (Tremellen et al., 1998). Direct infusion of TGFβ into the uterus of mice increases CSF2 secretion and recruitment of leukocytes into the endometrium, similar to that observed after mating of SP infusion. Exposure of endometrial epithelial cells to SP in the presence of TGFβ neutralizing antibodies inhibits SP induced expression of *CSF2* and other proinflammatory cytokines.

Similarly, in the human, TGFβ alters expression of proinflammatory molecules in primary human cervical epithelial cultures in a similar fashion to SP. Using recombinant protein alone, all three TGFβ isoforms have a similar capacity to increase *CSF2* and *IL6* gene expression in a similar fashion to SP. In parallel, blocking TGFβ in human SP by using TGFβ neutralizing antibodies or inhibitors of TGFβ signaling, prevent SP induced increases of *CSF2* and *IL6* in human ectocervical epithelial cells. Interestingly, the addition of recombinant IL6, IL1α, IL1β, IL12, or TNFα could not mimic SP induced changes in human ectocervical epithelial cells. These studies define SP TGFβ as the key mediator in stimulating of proinflammatory cytokine synthesis in human cervical cells (Sharkey et al., 2012a).

The hormone relaxin is produced by the prostate and seminal vesicle glands and is present in SP (Kohsaka et al., 1992; Gunnersen et al., 1996; Samuel et al., 2003). In the mouse, exogenous administration of relaxin into the uterus increases uterine *Cxcl1* expression. In addition, using stud *Rln* null males results in reduced *Ccl2*, *Csf3*, *Cxcl10*, *Cxcl1*, *Ptgs2* and *Ptprc* (formerly CD45) expression compared to females exposed to wild type males. These data imply that male SP relaxin induces molecular inflammation and may contribute to induction of post-mating uterine inflammation (Glynn et al., 2017).

Ablation of soluble CD38 in semen results in increased fetal loss in mice, which could be rescued by a direct infusion of recombinant soluble CD38 into the uterus. Kim and colleagues have confirmed that exposure of maternal tissues to soluble CD38 promotes maternal immune tolerance and Treg cell proliferation in the mouse (Remes Lenicov et al., 2012; Kim et al., 2015).

Rodriguez and colleagues found that porcine sperm adhesions I and II (PSP-I/PSP-II) heterodimer in boar SP has a predominant role in activating the recruitment of uterine leukocytes and T cells after mating (Rodriguez-Martinez et al., 2010). Furthermore, PSP-I/PSP-II heterodimer stimulates a time-dependent influx of leukocytes into the uterine lumen of estrous sows, at doses 5 times lower than those often present in the SP of boars. These effects are consistent with the results of Assreuy, in which infusion of porcine PSP-I/PSP-II into the peritoneal cavity of rats caused a dose-dependent, time responsive migration of leukocytes (Assreuy et al., 2002). The overall these data suggest the PSP-I/PSP-II sperm adhesion acts as a post-mating inflammation mediator in pigs.

Once identified, the immune modulating components in SP may be utilized in reproductive technologies to help improve fertility and reproductive outcome in other species.

The Utility of Seminal Plasma in Reproductive Technologies

Reproductive technologies including AI and IVF are used commercially to manage the reproduction of domestic species such as poultry, horses, sheep, cows, and pigs (Bromfeld, 2016). The use of these technologies allows for maximal use of the most valuable sires resulting in significant increases to the economic potential of offspring. Approximately 80% of dairy producers in North America use AI for breeding, compared to only 4% of beef producers (Colazo and Mapletoft, 2014). During the processing of semen for AI, to increase the number of applicable inseminations, SP is either removed or significantly diluted. In addition, the volume of semen used during AI is significantly reduced compared with that of natural insemination. These

factors might potentially reduce the efficacy of any active moieties present in semen, and consequently reduce any positive SP effects on reproductive outcomes.

A small number of experiments have been attempted to increase pregnancy outcomes using SP and the premise described above to alter the uterine environment of early pregnancy. Supplementation of TGFβ-1 at AI in the boar failed to improve total or live implantation rates at 80 days of gestation (Rhodes et al., 2006). Similar results have been observed in cattle when supplementation of TGFβ1 at AI had no effect on pregnancy rate, supplementation of SP increased conception by nearly 5% (albeit not significantly) (Odhiambo et al., 2009).

Timed AI is less expensive than natural conception using bulls in the dairy industry and reproductive performance using either AI or natural conception are similar (Lima et al., 2010). Maher and others have suggested that an absence of endometrial cytokines from most embryo culture media might, in part, explain why IVF success is limited, suggesting an importance in modulating these factors within the female tract (Schieve et al., 2002; Maher et al., 2003; Ceelen et al., 2007, 2008). Moore and Haslet in a 100 year review of reproductive technologies in dairy science proposed that one area on which to focus research and discussion is the role of SP on the uterine environment and embryo development (Moore and Hasler, 2017). While the successful use of AI and IVF in assisted reproduction technology indicate that the presence of SP is not a necessary component for pregnancy, the complete biological function of SP at conception and its influence on progeny have not been satisfactorily explored. We suggest that the uterine environment is not optimal when pregnancy is achieved in the absence of SP.

Thesis

Together, the observations discussed above suggest an important role for SP in influencing the periconceptional cytokine environment in mice, pigs, and human. Modulation of the endometrial environment may positively influence pregnancy success and potential fitness of

offspring. To date it is unknown what impact, if any, SP has on modulating the molecular and cellular environment of the endometrium in the bovine. Here, we hypothesis that exposure of endometrial tissue to SP could modulate the expression of factors important in early pregnancy success. Experiments described in chapter 2 aim to define the influence of various semen components on the expression of endometrial genes involved in embryo development, inflammation and ovarian function. Experiments presented here include 1) the use of intact endometrial explants exposed to SP to characterize changes in gene expression, 2) experiments to determine the response of semi-purified endometrial epithelial and stromal cells to SP, and 3) to quantify the content of TGFβ in bovine SP. These studies are an important addition to our understanding of SPs role in modulating the reproductive environment of early pregnancy. As the periconceptional environment influences reproductive success and the phenotype of offspring, these experiments will provide new information in managing fertility and promoting reproductive health outcome in cattle.

Table 1-1. Compositions of bovine seminal plasma.

Component	Concertation (bull)
Fructose	150-900
Glucose	300
Citric acid	340-1150
Total protein, (g/dL)	3.8
Total lipids	29
Cholesterol	312.16
Phospholipids	149.1
Testosterone, (pg/mL)	210-1310
Estrogen, (pg/mL)	20-166
Prostaglandins, (ng/mL)	5-10
Ions Na, K, Cl, Mg, Zn, Ca P,	7-290
Enzymes, ALP, AST, LDH	15-1909

Values are (mg/dL unless otherwise stated) Adapted from (Juyena and Stelletta, 2012). ALP, alkaline phosphatase; AST, aspartate amino transferase; LDH, lactate dehydrogenase.

Table 1-2. Effect of seminal vesicle removal on bull fertility.

Bull ID		Volume (mL)	Concentration score	Heifers bred	Conception rate (%)
2172	Before	2.7	3.2	7	71
	After	1.7*	4.1*		
2240	Before	3.2	2.9	8	75
	After	1.3*	3.9*		
3018	Before	6.2	4.1	16	75
	After	3.5	4.5*		
3024	Before	4.9	4.0	-	-
	After	3.0*	4.9*		
3053	Before	6.3	3.9	5	40
	After	2.5*	3.8		
Average	Before	5.1	3.8	36	69
	After	2.7*	4.8*		

* Significant from 'before' value. (Faulkner et al., 1968)

CHAPTER 2
THE EFFECT OF SEMINAL PLASMA ON MODULATING GENE EXPRESSION IN THE ENDOMETRIUM

Introduction

Seminal plasma (SP) is conventionally thought to have a single purpose, which is as a survival medium for spermatozoa during carriage to the oocyte. However, SPs role is now recognized to extend beyond this process to also optimize reproductive outcomes by targeting the female reproductive tract. The periconceptional and embryo implantation phase of early pregnancy is a vulnerable period of the reproductive process (Norwitz et al., 2001; Cockburn and Rossant, 2010). In livestock species, disruptions to the maternal environment and capacity to support conceptus development are implicated in constraining fertility (Kridli et al., 2016). The cellular and molecular environment of the uterus during the peri-implantation period of early pregnancy is critical for implantation success and optimal fetal and placental development. Despite the fact that SP is not required for successful pregnancy, several studies in multiple species indicate that pregnancy is destabilized if females are not exposed to SP. In mice, surgical removal of the seminal vesicle glands of stud males causes a reduction in subsequent pregnancy rates, due to decreased embryo implantation (Bromfield et al., 2014). In dairy and beef cattle, infusion of SP at the time of AI increases pregnancy rates by 4.6% and 6.7%, respectively (Odhiambo et al., 2009). However, surgical removal of the seminal vesicle gland in the bull maintains a typical 69% conception rate (Faulkner et al., 1968). Seminal plasma acts as a key regulator of the female tract environment. Recent studies in rodents, swine, equine and humans show that SP induces numerous changes within the endometrium or cervix (Lovell and Getty, 1968; Phillips and Mahler, 1977; Pandya and Cohen, 1985; De et al., 1991; McMaster et al., 1992; Robertson et al., 1996; Rozeboom et al., 1998; Alghamdi et al., 2004; Sharkey et al., 2012a). These changes are proposed to occur by activating a cascade of cytokine and leukocyte-

mediated events that appear to contribute to endometrial receptivity for embryo implantation. Epithelial cytokines induced by SP exposure, exert embryotrophic actions on the developing pre-implantation embryo, modulate maternal immunity toward the conceptus and potentially improve pregnancy success. In the human and rodent, SP derived transforming growth factor beta (TGFβ) has been demonstrated to be one of the active molecules within the ejaculate to facilitate these maternal tract changes. Herein, we hypothesize that exposure of endometrial tissue to SP could modulate the expression of factors important in early pregnancy success. Experiments described here aim to define the influence of various semen components on the expression of endometrial genes involved in embryo development, inflammation and ovarian function. Experiments presented include 1) the use of intact endometrial explants exposed to SP to characterize changes in gene expression, 2) experiments to determine the response of semi-purified endometrial epithelial and stromal cells to SP, and 3) to quantify the content of TGFβ in bovine SP. These studies are an important addition to our understanding of SP's role in modulating the female reproductive tract environment of early pregnancy. As the periconceptional environment influences reproductive success, and potentially even the phenotype of offspring, these experiments will provide new information in managing fertility and promoting reproductive health outcomes in cattle.

Materials and Methods

Reagents and Chemicals

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

Semen and Seminal Plasma Collection

Whole semen from commercial bulls was collected during routine breeding soundness evaluations (BSE). Sterility of samples was maintained as best achievable. Whole semen was

collected by electroejaculation from healthy bulls, evaluated and only processed further if the sample was free of blood, urine, and any other visual anomalies. Parameters pertaining to the BSE were recorded for each individual sample; including volume, sperm motility, scrotal circumference, collection date, and bull ID (Table 2-1). On average, semen volume ranged from 7.5 - 15 mL, gross motility ranged from 10-90 %, and scrotal circumference ranged from 31-49 cm. Following collection, whole semen was 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 1,000 x *g* for 10 min at room temperature to facilitate collection of cell free SP and the ejaculate cell pellet. Cell free SP was transferred to new labelled tubes in aliquots of 500 – 1000 μL and stored at -20°C until use. The remaining cell pellet was stored at -20°C.

Preparations of Pooled Semen, Seminal Plasma and Cell Pellet

For in vitro experiments, pools of semen, SP and the semen derived cell pellet were made to assess the capacity of tissues to respond to the various components of semen. A whole semen pool was prepared in a 15 mL tube under aseptic conditions by pooling 100 μL of the designated 11 bulls. Pooled whole semen was stored at -20ºC until use in 500 μL aliquots. Frozen cell pellets from 5 bulls were thawed prior to preparation of the cell pellet pool. The cell pellet from individual bulls was resuspended in sterile phosphate buffered saline (PBS) to the same volume of the original ejaculate (achieving the original ejaculates sperm concentration). The volume of cell suspension from each bull corresponding to 10^8 total cells was pooled in a 15 mL tube, mixed and stored at -20ºC in 500 μL aliquots. Ejaculates that contained a total volume of 5 mL or higher were used to generate the SP pool. The SP pool was prepared from the ejaculates of 21 bulls. Seminal plasma was pooled in a 50 mL tube under aseptic condition by combining 1 or 2

mL of SP from each of the 21 bulls. Pooled SP was mixed and stored at -20ºC in 500 µL aliquots.

Quantification of TGFβ in Seminal Plasma by ELISA

Seminal plasma in other species is known to be a rich source of TGFβ; as such, we assessed the content of TGFβ in bovine SP. Seminal plasma TGFβ content was assessed in 33 bulls by commercial ELISA according to the manufacturer's instructions. Currently, there are no commercially available bovine specific TGFβ ELISA kits. Therefore, we used a human TGFβ-1 and TGFβ-2 ELISA (Human TFGβ-1 DuoSet, and Human TFGβ-2 DuoSet; R&D Systems Minneapolis MN). Human and bovine TGFβ-1 and TGFβ-2 share 95 and 98% homology respectively based on NCBI protein-blast analysis. However, the homology between human and bovine TGFβ-3 is low and was not assessed here with human kits. The concentrations of bioactive TGFβ-1 and TGFβ-2 was determined in untreated SP, while quantification of total TGFβ-1 and TGFβ-2 required acid activation of SP. For acid activation 100 µL of SP was first diluted in 300 µL of PBS before the addition of 50 µL of 1M HCl. Samples were incubated for 20 min at room temperature before neutralizing the pH with the addition of 50 µL of 1M NaOH. Quantification of total TGFβ-1 and total TGFβ-2 was evaluated at 1:300 & 1:600, and 1:5 & 1:10 final dilutions respectively. Quantification of bioactive TGFβ-1 and TGFβ-2 were evaluated at 1:5 & 1:10, and neat respectively. Both TGFβ-1 and TGFβ-2 ELISAs were validated using spike-in/ recovery performance efficiency based on actual and expected recovery of recombinant TGFβ in each ELISA kit. The rate of recovery for TGFβ-1was 82-97% and TGFβ-2 was 75-108%.

Preparation of Female Reproductive Tracts for Endometrial Dissection

Uteri from post pubertal, non-pregnant cattle with no gross evidence of genital disease or microbial infection were collected at the local slaughterhouse. Whole reproductive tracts were

transported to the laboratory within approximately 3 h for further processing at room temperature (RT). Reproductive tracts from a total of 36 animals were used for all experiments. The stage of the reproductive cycle (stage of estrous cycle) was determined by examination of ovarian morphology and vasculature of the corpus luteum (CL). Briefly, the external and internal appearance of the CL, CL diameter and CL surface vasculature is dependent on the stage of estrous cycle (Ireland et al., 1980). Details are described in appendix section 2. Photographs were taken, and all pertinent information was recorded on an evaluation sheet.

Preparation of Whole Endometrial Explants and in Vitro Culture

An endometrial biopsy explant model was utilized to evaluate expression of embryokines and inflammatory factors in response to semen components. We choose to use biopsy explants rather than traditional chopped explants since intact explants maintain endometrial tissue architecture. Methods for culture are previous described with modification (Borges et al., 2012). Briefly, individual uteri were processed and freed of surrounding fat and connective tissue. The external surface of the uterus was washed with ethanol 70%. An incision along the major curvature of the uterine horn was made to expose the endometrium ipsilateral to the corpus luteum. The exposed endometrium was washed twice with Dulbecco's phosphate-buffered saline (DBPS) containing 50 IU/mL of penicillin, 50 µg/mL of streptomycin and 2.5 µg/mL amphotericin B to wash away mucus and potential microorganisms. Endometrial explants were obtained from intra-caruncle tissue using an 8 mm diameter biopsy punch and cut away from the myometrium. The endometrial biopsies were immediately transferred and washed twice with Hank’s balanced salt solution (HBSS). Biopsies were placed in 6-well culture plates in 3 mL of complete culture medium (RPMI 1640 medium, 10% FBS, 2 mM of L-Glutamine, 50 IU/mL of penicillin, 50 µg/mL of streptomycin, and 2.5µg/mL amphotericin B) containing the various treatments (below).

Isolation and Culture of Bovine Endometrial Epithelial and Stromal Cells

To investigate the responsiveness of individual endometrial cell types to SP, endometrial stroma and epithelium were isolated for culture. Endometrial tissue was dissected and processed as described previously (Turner et al., 2014). Uteri were prepared above prior to the endometrium being dissected into thin strips and placed directly into 50 ml pots containing strip wash buffer (HBBS containing 50 IU/mL of penicillin, 50 µg/mL of streptomycin and 250 µg/mL amphotericin B). Tissue pieces were transferred into a centrifuge tube containing HBSS and placed into water bath at 37°C. After 10 min, sterile digestive solution was added to the endometrium pieces (HBSS containing 100 mg BSA, 125 CDU/mg collagenase II, 250 BAEE trypsin and 4% DNase I). Tissue was digested for 1 h in a shaking water bath at 37°C. The cell suspension was then filtered through a 40 µm mesh into a second 50 mL centrifuge tube containing warm stop solution (HBSS containing 10%FBS). The suspension was centrifuged at 700 × *g* for 7 min at RT. The supernatant was discard and the endometrial cells were resuspended in 5 mL of prewarmed complete culture medium (as above). Cells were cultured in 75 cm^2 flasks (Falcon, Becton Dickinson, Franklin Lakes NJ) containing 30 mL of equilibrated complete culture medium. Cells were incubated at 39°C in a humidified atmosphere of air with 5% CO_2. After 18 h of culture unattached epithelial cells in suspension were transferred to a new flask leaving semi-purified stromal cells attached.

After a total of 66 h in culture, cells were detached from flasks by HyQTase treatment. Cells were washed in warm DPBS and resuspended at a final concentration of 1.5×10^5 cells/mL in complete culture medium. Cells were plated in 24-well culture plates in a final volume of 500 µL volume and equilibrated for 24 h before treatment.

Cell Culture Challenge of Endometrial Explants or Endometrial Cells with Semen Components

Treatments were added to complete culture medium according to experimental design and equilibrated to 37°C in the water bath before exposure of explants or cells. Intact endometrial explants or endometrial cells were exposed to either complete culture medium as a negative control or medium containing the various treatments consisting of SP at 1%, 2%, 5%, 10%, or 20%; 5% whole semen (SM); 5% cell pellet (CP); or 100 ng/mL of ultrapure LPS as a positive control (Invivogen, San Diego CA.). Experiments were replicated at least six times, with each replicate representative of endometrial material from individual cows. Explants or endometrial cells were incubated at 39°C in a humidified 5% CO_2 environment during treatment. Dosing experiments using either endometrial explants or cells were performed for a total of 24 h. At the completion of 24 h culture, cell free supernatants were collected and stored at -20°C. Each explant was weighed prior to bisecting and storage in either 1 mL of Trizol or snap frozen in liquid nitrogen. At the completion of culture of individual cell types, cell free supernatants were collected and cells stored in QIAGEN RLT lysis buffer to facilitate extraction of total RNA (QIAGEN, Hilden Germany). Time course experiments of both explants and endometrial cells utilized complete culture medium as a negative control or medium containing 5% SP or 100 ng/ml of ultrapure LPS for 0, 15, 30, 60, 90 and 120 minutes. Both supernatant and total RNA were collected at each time point.

Extraction and Purification of RNA from Endometrial Tissues or Cells

RNA was extracted from endometrial explants using Trizol. Briefly, endometrial explants were homogenized using a plastic pellet pestle. Homogenates were centrifuged at 1200 x *g* for 5 min at 4°C and supernatant was transferred to clean centrifuge tube. A total of 200 µl of chloroform was added to each homogenate and incubated at RT for 5 min. Samples were

centrifuged at 1200 x *g* for 15 min at 4°C. The RNA rich aqueous supernatant was transferred to a new tube and 500 µL of 100% isopropanol was added to the sample and incubated at RT for 10 min. Samples were centrifuged at 1200 x *g* for 15 min at 4°C and supernatant was removed leaving the RNA pellet. The pellet was washed with 1 mL of 75% ethanol and centrifuged at 1200 x *g* for 10 min at 4°C. The RNA pellet was resuspend with 50 µL of RNAase-free water.

Total RNA was extracted from endometrial cells by using the RNeasy Mini Kit according to the manufactures instructions (Qiagen).

Extracted RNA was quantified using a NanoDrop ND1000 spectrophotometer, and the purity of each sample was determined by the ratio A260/A280. A total of 1 µg of RNA was subjected to reverse transcription using the Verso cDNA synthesis kit according to the manufacturers instruction, including a genomic DNA wipe out procedure. The recommended thermal cycling conditions for reverse transcription were inactivation at 95°C for 2 min followed by 1 cycle of cDNA synthesis at 42°C for 30 min.

Quantitative Real-Time RT-PCR (qPCR)

Primers were designed using the NCBI database and initial specificity verified by BLAST to ensure no cross-reactivity with other loci. Primer length less than (200 bp) and GC contents of each primer (50–60%) were selected to avoid primer dimer formation. Amplification efficiency was evaluated for each primer by performing serial dilutions of cDNA. All primers had to meet MIQE guidelines for further use (Pearson correlation coefficient $r > 0.98$ and efficiency between 90%-110%). 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. Primer details are listed in Table 2-2. PCR products were electrophoresed on a 1 % agarose gel containing Diamond Dye and visualized under UV illumination to confirm predicted PCR product size.

Quantitative real-time RT-PCR (qPCR) was performed in 20 μL reactions using iTaq Universal SYBR green chemistry (Bio-Rad, Hercules CA) and 100 nM of each forward and reverse primer. A Bio-Rad CFX Connect light cycler was employed to perform quantitative PCR (Bio-Rad) using a two-step protocol and the recommended thermal cycling conditions outlined below: 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. A 3 step protocol was used for *IL17A1* and *OXTR* primers with an annealing temperature of 63°C. Data were normalized independently to *ACTB* mRNA expression using the ΔCt method.

Evaluating Purity of Endometrial Cell Culture Populations

Epithelial and stromal cell purity was initially evaluated by cellular morphology. Purity of cellular preparations was quantified by flow cytometry using markers for epithelium (cytokeratin), stroma (vimentin) and leukocytes (CD45). Bovine heparinized whole blood was used as a control following red blood cell lysis using ammonium chloride lysis buffer. Leukocytes were centrifuged and resuspend in staining buffer (PBS + 1% FCS) at 10^4 cells/mL. For detection of the cell surface marker CD45, 100 μL of cell suspension was incubated on ice with anti-bovine CD45-RPE monoclonal antibody diluted 1:10 for 30 min in the dark. Cells were washed twice by centrifugation in staining buffer. For detection of the intracellular markers vimentin and cytokeratin, 100 μL of cells were fixed and permeabilized with BD Fixation/ Permeabilization buffer (Becton Dickinson, Franklin Lakes NJ) for 20 min on ice in the dark. Cells were washed twice with permeabilization buffer. Cells were incubated with either anti-human vimentin-Alex488 (1:50) (Cell Signaling Technology, USA) or anti-human pan cytokeratin-APC (1:20) for 30 min on ice, in the dark. Cells were then washed twice as above and resuspended in staining buffer for analysis. A negative control with no addition of antibody was included.

Analysis of cell suspensions was performed on an Accuri C6 flow cytometer (BD Biosciences). A minimum of 10^4 cells were acquired per sample, and dead cells were excluded by selective scatter gating.

Statistical Analysis

Each experiment was repeated six times. SPSS software V24.0 (IBM Analytics, Armonk NY) was used for statistical analysis. Gene expression data was log transformed and analyzed using generalized linear mixed model with pairwise comparisons. Treatment, dose and time were used as fixed factors and replicate was included as a random variable. Interactions were assessed as appropriate in either dose experiments or time course experiments. Pairwise comparisons were made between individual time points or concentrations with vehicle treated controls. Data are presented as mean + standard error of the mean. *P* value of ≤ 0.05 was assumed statistically significant.

Results

The Effect of Semen Components on Ex Vivo Endometrial Explant Gene Expression

To assess the capacity of semen components to modulate expression of endometrial genes important in early pregnancy, intact endometrial explants were exposed to either control medium alone, seminal plasma (SP) at various concentrations, 5% whole semen (SM), 5% semen cell pellet (CP) or 100 ng/ml of LPS for 24 h.

Expression of endometrial *CSF2*, *PTGS*, *PGFS*, *IL6* and *IFNE* were increased following exposure to SP. Exposure to 10% SP increased endometrial expression of *CSF2* maximally by 29.5-fold compared to vehicle treated explants (Figure 2-1A, $P < 0.05$). Exposure to semen components other than SP or LPS did not increase *CSF2* expression. A concentration dependent increase in endometrial *PTGS* and *PGFS* was observed 24 h after exposure to SP compared to vehicle treated control explants (Figure 2-1G and H). Expression of endometrial *PGFS*, but not

PTGS, was also increased following exposure to whole semen compared to vehicle controls. Exposure to LPS or semen cell pellet had no effect on *PTGS* or *PGFS* expression, compared to vehicle treated control explants. Exposure of explants to 5% SP, 20% SP or whole semen reduced endometrial *IL6* expression compared to vehicle treated controls (Figure 2-1B). Interestingly, exposure of explants to 10% SP increase endometrial *IL6* expression 3.8-fold compared to vehicle treated controls. Expression of endometrial *IFNE* increased after exposure to 20% SP or LPS compared to vehicle treated controls (Figure 2-1F).

Exposure of endometrial explants to SP decreased expression of both *IL17A* and *TGFB1* compared to vehicle treated controls (Figure 2-1D and E). Moreover, the reduction of *IL17A* and *TGFB1* was maximal after exposure to 20% SP (9.5- and 37.8-fold respectively, $P < 0.05$). Exposure to other semen components or LPS had no effect on endometrial expression of *IL17A* or *TGFB1* compared to vehicle treated controls. Exposure of explants to 5% whole semen increased *OXTR* endometrial expression compared to vehicle treated controls (4.6-fold), while SP, semen cell pellet or LPS had no effect on *OXTR* expression compared to vehicle treated controls (Figure 2-1I). Exposure to semen components or LPS did not alter endometrial expression of *IL8* compared to vehicle treated controls (Figure 2-1C).

Taken together, we observed an effect of SP on *CSF2*, *IL6*, *IL17A1*, *TGFB1*, *IFNE*, *PTGS*, and *PGFS* endometrial explant gene expression following 24 h culture.

The Acute Effects of Semen Components on Ex Vivo Endometrial Gene Expression

To investigate the acute responsiveness of intact endometrial explants to SP, a time course experiment over a 2 h period was performed. Intact endometrial explants were exposed to 5% SP, 100 ng/mL of LPS or control medium alone for 0, 15, 30, 60, 90, and 120 min (Figure 2-2; LPS data is shown in Figure A-1).

There was an observed treatment effect of SP exposure on the expression of endometrial *CSF2, IL6, IL8, TGFB1, IFNE,* and *PTGS.* An effect of culture time was observed for expression of endometrial *PGFS,* and a treatment by culture time interaction for *IFNE*.

Seminal plasma significantly increased endometrial expression of *CSF2, TGFB1, IFNE* and *PTGS* after 120 min of culture compared to vehicle treated controls at the same time point (Figure 2-2A, E, F and G). Exposure to SP reduced endometrial *IL6* expression from 30 min through 120 min, compared to vehicle treated controls (Figure 2-2B). Endometrial expression of *PGFS* was also reduced following exposure to SP at 90 min, compared to vehicle treated controls (Figure 2-2H). Endometrial expression of *IL17A1* was increased only at 30 min following SP exposure compared to vehicle treated controls (Figure 2-2D). There was no observed effect of SP exposure on endometrial expression of *OXTR* (Figure 2-2C and I).

Exposure of explants to LPS was used as a positive control (Figure A-1). There was an observed time effect of LPS exposure on the expression of endometrial *IL6, IFNE* and *PGFS,* consistent with previous studies (Borges et al., 2012). A treatment by culture time interaction was observed for expression of endometrial *PGFS* and *IFNE*. Treatment with LPS increased endometrial expression of *CSF2, TGFB1, IFNE* and *PGFS* compared with vehicle treated control with maximal response at 120 min. There was no treatment or time effect on endometrial expression of *IL8* or *PTGS.*

Taken together we observed an effect of SP on *CSF2, IL6, IL8, IL17A1, TGFB1, IFNE,* and *PTGS* endometrial gene expression during an acute 2 h period.

Purity of Isolated and Cultured Epithelial and Stromal Endometrial Cells

To determine the ability of semen components to modulate gene expression of specific endometrial cell types, we isolated and cultured semi-purified epithelium and stromal cells from the endometrium.

Epithelial and stromal cell purity were confirmed by observation of cellular morphology and flow cytometry. Epithelial cultures were confirmed as small round cells with epithelial-like morphology (Figure 2-3A). Stromal cells had a typical fibroblast-like morphology (Figure 2-3B). To quantify cell purity, flow cytometry using cell specific markers for epithelial (cytokeratin) and stromal (vimentin) cells was performed, in addition to identification of contaminating leukocytes (CD45). Endometrial cell cultures contained minimal leukocytes in either stromal (4 ± 1% $CD45^+$ total cells) or epithelial cell cultures (5 ± 3% $CD45^+$ total cells) (Figure 2-3E and F). Stromal cell cultures were 95 ± 3% vimentin positive (Figure 2-3D), while purity for epithelial cell cultures was lower at 56 ± 16% cytokeratin positive (Figure 2-3C). Using vimentin and cytokeratin as markers for cell purity, these cultures are consistent with those reported by others (Turner et al., 2014).

The Ability of Semen Components to Alter Gene Expression of Isolated Endometrial Epithelial and Stromal Cells

To explore whether semen components can modulate gene expression of endometrial cells, semi-purified epithelial or stromal cells were exposed to 1%, 2%, 5% SP, 5% whole semen (SM), 5% semen cell pellet (CP) or 100 ng/ml of LPS for 24 h (Figure 4 through 6).

Exposure to semen components increased expression of *CSF2*, *IL6*, *IL8*, *IL17A1*, *TGFB1*, *IFNE*, *PTGS*, *PGFS* and *OXTR* in both epithelial and stromal cells compared to vehicle treated controls. Specifically, expression of epithelial *CSF2*, *IL8*, *TGFB1*, *PTGS* and *PGFS* were all increased after exposure to SP compared to vehicle treated cells. Expression of stromal *CSF2*, *IL6*, *IL8*, *IL17A1*, *TGFB1*, *PTGS* and *PGFS* were all increased after exposure to SP compared to vehicle treated cells. Interestingly, exposure of epithelial cells to semen cell pellet promoted the largest increase of *CSF2*, *IL6*, *IL8*, *IL17A1*, *IFNE*, *PTGS*, *PGFS* and *OXTR* expression compared to controls. Exposure of stromal cells to SP or whole semen induced the largest increase in

expression of *CSF2*, *IL6*, *IL8*, *IL17A1*, *IFNE*, *PTGS*, and *PGFS* compared to controls. Exposure to semen cell pellet induced maximal expression of *TGFB1* and *OXTR* in stromal cells.

When comparing epithelial and stromal cell response to semen components, it appears that the primary source of *CSF2*, *IL6*, *IL8*, *TGFB1*, *IFNE*, and *PGFS* expression is the epithelium, while stromal cells are responsible for maximal *IL17A1*, *PTGS* and *OXTR* expression.

Expression of *CSF2* was increased in both cell types following exposure to SP compared to vehicle treated controls (Figure 2-4A and D). Maximal expression of *CSF2* in stromal cells was achieved following exposure to whole semen, which only mildly increased *CSF2* expression in epithelial cells compared to vehicle controls. Expression of *IL6* was increased in stromal cells only following exposure to SP compared to vehicle treated controls (Figure 2-4B and E). Maximal expression of *IL6* in epithelial cells was achieved following exposure to semen cell pellet, which had no effect on stromal cells, while stromal cells increased *IL6* expression in response to 1% or 2% SP. Expression of *IL8* was increased in both cell types following exposure to SP compared to vehicle treated controls (Figure 2-4C and F). Maximal expression of *IL8* in stromal cells was achieved following exposure to 2% SP. While no effect of whole semen or semen cell pellet was observed in stromal cells, epithelial cells increased expression of *IL8* in response to both whole semen and semen cell pellet compared to vehicle controls.

Expression of *IL17A1* was increased in stromal cells following exposure to SP or whole semen, compared to vehicle treated controls (Figure 2-5A and D). *IL17A1* expression in epithelial cells increased in response to whole semen or semen cell pellet compared to vehicle controls. Expression patterns of *TGFB1* in epithelial or stromal cells were similar according to treatment, with both cell types increasing expression to all semen components compared to

vehicle (Figure 2-5B and E). Stromal cells increased *IFNE* expression in response to whole semen compared to vehicle controls, while epithelial cells increased *IFNE* expression in response to whole semen or semen cell pellet compared to vehicle treated controls. Neither cell type increased *IFNE* expression in response to SP treatment (Figure 2-5C and F).

Expression patterns of *PTGS* in epithelial or stromal cells were similar according to treatment, with both cell types increasing expression to all semen components compared to vehicle (Figure 2-6A and D). Expression of *PGFS* in epithelial cells was increased following exposure to all semen components compared to vehicle (Figure 2-6B), while stromal cells increased *PGFS* expression to SP or whole semen compared to vehicle treated cells (Figure 2-6E). Epithelial and stromal cells had a similar pattern of *OXTR* expression (Figure 2-6C and F), both increasing expression in response to semen cell pellet and no other treatments compared to vehicle treated controls.

The Acute Effects of Semen Components on Purified Endometrial Epithelial and Stromal Cells

A time course experiment was performed to investigate the acute responsiveness of endometrial epithelial or stromal cells to SP over a 2 h period. Endometrial epithelial or stromal cells were exposed to 5% SP, 100 ng/mL of lipopolysaccharide or control medium alone for 0, 15, 30, 60, 90, and 120 min (Figure 7 through 9; LPS data is shown in Figure A-2 and 3).

There was an observed treatment effect of SP exposure in both endometrial epithelial and stromal cells for expression of *CSF2*, *IL6*, *IL17A1, TGFB1*, *IFNE, PTGS* and *PGFS*. There was no observed effect of culture time or treatment by culture time interaction for any measured transcript.

Expression of *CSF2* was increased in both epithelial and stromal cell types following exposure to SP from 15 min through 120 min, compared to vehicle treated controls (Figure 2-7A

and D, $P < 0.05$). Expression of *IL6* was increased in stromal cells from 15 min through 120 min compared to vehicle treated controls (Figure 2-7E, $P < 0.05$). While a treatment effect was observed for *IL6* expression in epithelial cells, no specific time points was significantly elevated above control treated cells (Figure 2-7B). Endometrial stromal cells increased *IL8* expression following exposure to SP at 30 min (40-fold increase; Figure 2-7F, $P < 0.05$). No effect of SP was observed on endometrial epithelial cell expression of *IL8* (Figure 2-7C).

Endometrial epithelial cell expression of *IL17A1* was increased at 15 min and 120 min following SP exposure compared to vehicle treated controls (Figure 2-8A, $P < 0.05$). Similarly, stromal cell *IL17A1* expression was increased at 30 min, 60 min and 120 min following SP exposure compared to vehicle treated controls (Figure 2-8D, $P < 0.05$).

Expression of *TGFB1* was increased in both epithelial and stromal cells at each time point compared to vehicle treated controls (Figure 2-8B & E, $P < 0.05$). Epithelial cell expression of *IFNE* was increased following expose to SP for 120 min, compared to vehicle treated controls (21-fold increase; Figure 2-8C). Stromal cell expression of *IFNE* was increased following 60 min of SP exposure (67-fold increase; Figure 2-8F, $P < 0.05$).

Expression of *PTGS* was similar in both epithelial and stromal cells, with increased expression at each time point from 15 min to 120 min of SP exposure (Figure 2-9A & D, $P < 0.05$). Epithelial cell expression of *PGFS* was increased following 15, 30, 90 and 120 min of SP exposure (Figure 2-9B). Stromal cell expression of *PGFS* was not significantly increased at any single time point compared to vehicle treated controls (Figure 2-9E). Stromal cell expression of *OXTR* was increased following 60 min of SP exposure compared to vehicle treated controls (Figure 2-9F, $P < 0.05$), while epithelial cells did not alter *OXTR* expression in response to SP over the 120 min exposure period (Figure 2-9C).

Following exposure to LPS, we observed a treatment effect on gene expression of *IL8* in both epithelial and stromal cells. Exposure to LPS increased expression of *IL6* in stromal cells. Exposure to LPS significantly increased *IL6* expression above controls treated cells consistent with previous reports (Turner et al., 2014). There was no observed effect of culture time or treatment by culture time interaction for *CSF2*, *IL17A* and *PTGS* in either epithelial or stromal cells. Stromal cell expression of *IFNE* and *TGFB1*was increased following exposure to LPS for 120 min. However, there was no observed effect of culture time or treatment by culture time interaction for *TGFB1* or *IFNE* epithelial cell expression. Epithelial cell expression of *OXTR* was increased following 60 min of LPS exposure. Stromal cell expression of *PGFS* and *OXTR* was not significantly altered. A time effect was observed for *PGFS* expression in epithelial cells (Figure A-2 and 3).

Taken together, we observed differential acute effects of SP exposure on the expression of multiple genes in endometrial epithelial or stromal cells**.**

Quantification of Total and Bioactive TGFβ-1 and TGFβ-2 in Bovine Seminal Plasma

Transforming growth factor-beta (TGFβ) has been shown to be one of the active molecules in SP to elicit pro-inflammatory responses of the female reproductive tract of rodents and humans (Tremellen et al., 1998; Sharkey et al., 2012a). Here, we quantified total and bioactive TGFβ-1 and TGFβ-2 in SP of 33 individual bulls using a commercial ELISA (Figure 2-10).

Bull SP contains an average of 7.11 ± 1.55 ng/mL of total TGFβ-1 (range 0.16 – 33.31 ng/mL), while the average concentration of bioactive TGFβ-1 was 5.3 ± 6.1 pg/mL (range 0.0 – 28.6 pg/mL). An average of 0.37% ± 0.12% of TGFβ-1 in SP is present in the bioactive form.

The average concentration of total TGFβ-2 in SP was 6.07 ± 1.16 ng/mL (range 0 – 27.36 ng/mL), while the concentration of bioactive TGFβ-2 was 330 ± 500 pg/mL (range 0.0 – 4398.2

pg/mL). An average of 4.15% ± 1.62% of TGFβ-2 is present in SP in the bioactive form. Taken together, SP contains active moieties that may interact with endometrial cells lining the female reproductive tract to influence the uterine environment.

Table 2-1. Parameters pertaining to bulls undergoing BSE and semen collection.

Bull ID	Volume (ml)	Pool Preparation	Motility (%)	Scrtl. Circ. (cm)	Breed	Collection Date
14-064	7.5	SP	90	40	Angus	3/18/2015
12-062	10	SP	60	43	Angus	3/18/2015
13-083	14	SP	70	43	Sim-Angus	3/18/2015
10-024	7.5	SP	90	42.5	Angus	3/18/2015
13-097	7.5	SP	50	45	Braford	3/18/2015
10-045	9.5	SP	80	47	Angus	3/18/2015
12-802	12	SP	70	39	Angus	3/18/2015
12-035	11.5	SP	70	46	Angus	3/18/2015
10-050	9	SP	90	44	Angus	3/18/2015
12-093	8.5	SP	60	49	Braford	3/18/2015
10-020	12.5	SP	60	42	Angus	3/18/2015
11-048	11.5	SP	90	42	Angus	3/18/2015
14-151	10.5	SP	50	36	Braford	3/18/2015
13-102	10.5	SP	40	40	Braford	3/18/2015
14-273	8.5	SP	50	38	Angus	3/18/2015
B-250	8	SP/ SM	40	40	Brangus	4/09/2015
B-55	10	CP/ SM	10	35	Angus	4/09/2015
B-278	10	CP/ SM	50	36	Brangus	4/09/2015
B-49	10	SM	30	40.5	Angus	4/09/2015
B-236	8	CP	50	39	Brangus	4/09/2015
B-238	10	SP/ SM	40	38	Brangus	4/09/2015
B-256	10	SP/ SM	50	33.5	Brangus	4/09/2015
B-245	10	SP/ SM/ CP	40	37	Brangus	4/09/2015
B-62	12	SP/ SM	50	31	Angus	4/09/2015
B-29	10	SM	10	39.5	Angus	4/09/2015
B-23	10	SP/ SM	40	37.5	Angus	4/09/2015
B-47	15	CP/ SM	50	36.5	Angus	4/09/2015

SP, seminal plasma; CP, cell pellet; SM, semen; Scrtl. Circ, Scrotum circulation; Sim-Angus, are genetic crossbreeds of Simmental & Angus cattle.

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

Gene	Primer	Sequence (5'–3')	GenBank accession no.	Product size (bp)
ACTB	Forward	CAGAAGCACTCGTACGTGGG	NM_173979.3	200
	Reverse	TTGGCCTTAGGGTTCAGGG		
IL6	Forward	ATGACTTCTGCTTTCCCTACCC	XM_015468553.1	180
	Reverse	GCTGCTTTCACACTCATCATTC		
IL8	Forward	GCAGGTATTTGTGAAGAGAGCTG	NM_173925.2	149
	Reverse	CACAGAACATGAGGCACTGAA		
CSF2	Forward	TTCCTGTGGAACCCAGTTTATC	NM_174027.2	114
	Reverse	TTTGGCCTGCTTCACTTCT		
PTGS	Forward	CGTGAAAGGCTGTCCCTTTA	XM_010813540.2	97
	Reverse	ATCTAGTCCAGAGTGGGAAGAG		
IL17A1	Forward	GCCCACCTACTGAGGACAAG	NM_001008412.2	134
	Reverse	ATTGCGGTGGAGAGTCCAAG		
PGFS	Forward	TGCAACCAGGTGGAATGTCA	NM_181027.2	122
	Reverse	ACCCATTCTTTTAGTCGTTGGGA		
OXTR	Forward	AAGATCCGCACGGTCAAGAT	NM_174134.2	127
	Reverse	TGAAAGGTGAGGCTTCCTTG		
TGFB1	Forward	AAGGGGTACCACGCCAATTT	NM_001166068.1	79
	Reverse	CCAGGACCTTGCTGTACTGTG		
IFNE	Forward	GCACTCATGAGACTGCAAGC	XM_005209901.2	223
	Reverse	TGGGTCAAGTTTCCATGCCC		

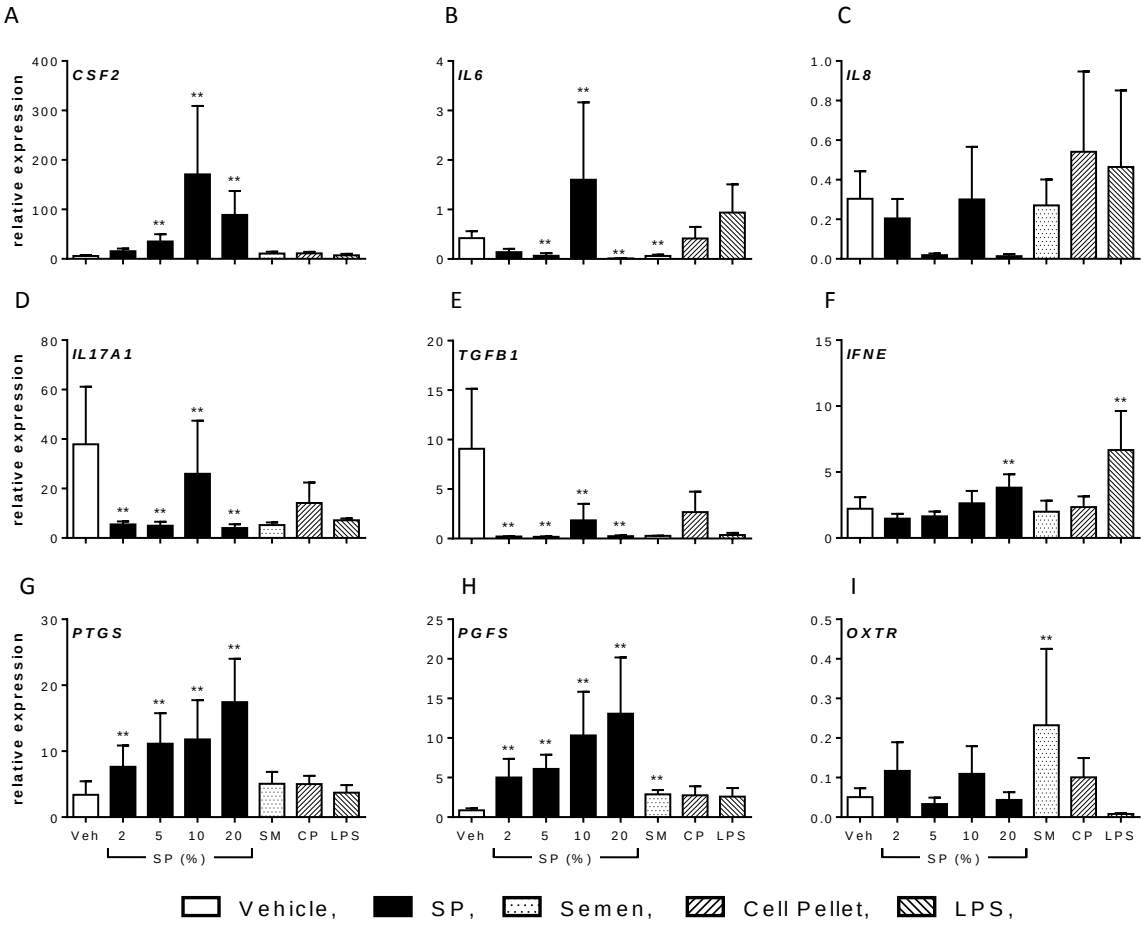


Figure 2-1. Effect of semen components on endometrial explant gene expression. Intact endometrial explant expression of CSF2, IL6, IL8, IL17A, TGFB1, IFNE, PTGS, PGFS and OXTR in response to seminal plasma (SP; 2%, 5%, 10%, 20%), 5% semen cell pellet (CP), 5% whole semen (SM), 100 ng/mL of lipopolysaccharide (LPS) or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. **, P value of ≤ 0.05.

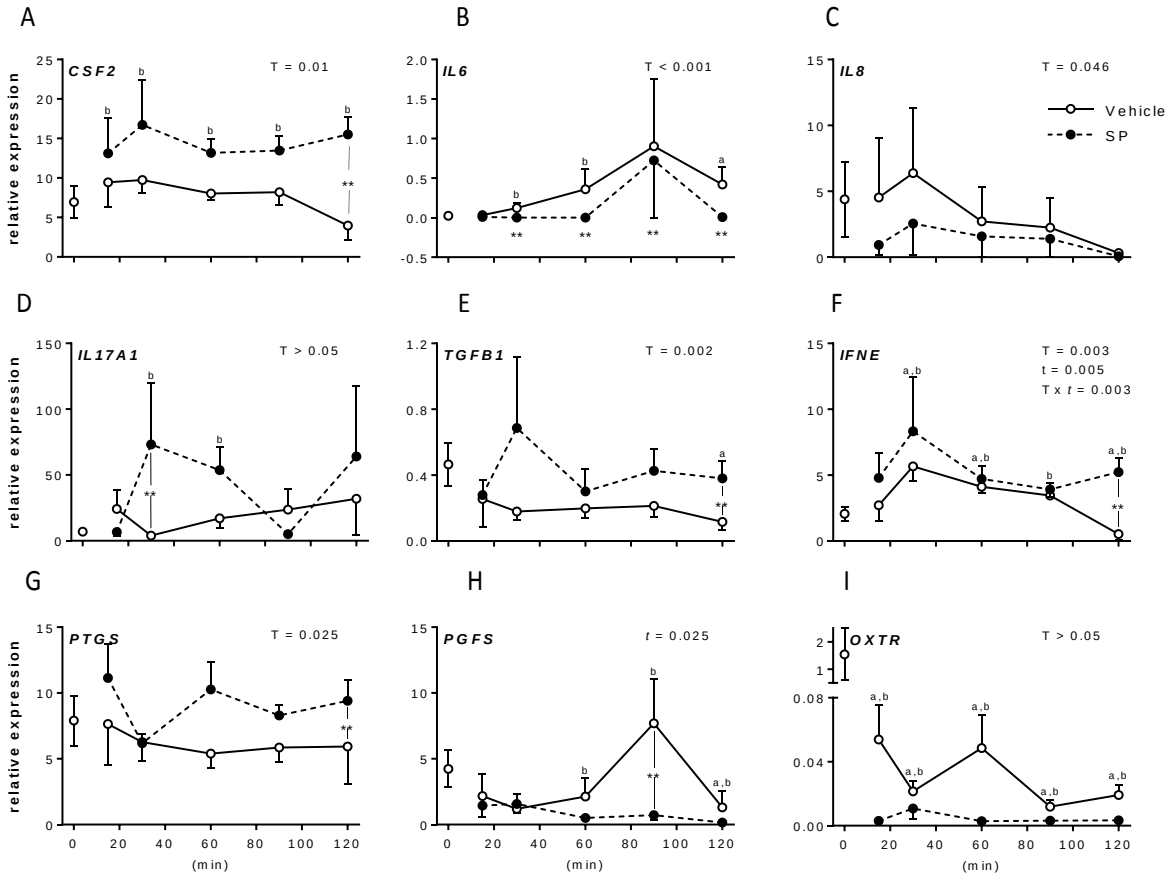


Figure 2-2. Acute effects of semen components on endometrial explant gene expression. Intact endometrial explant expression of *CSF2*, *IL6*, *IL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS*, *PGFS* and *OXTR* in response to 5% seminal plasma (SP) or control medium alone (Veh) period from 15-120 min. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control within each time point. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; ^{a}, difference in vehicle treated controls compared to 0 min; ^{b}, difference in SP treated compared to 0 min; T, treatment; t, time; T x t, Treatment by time interaction.

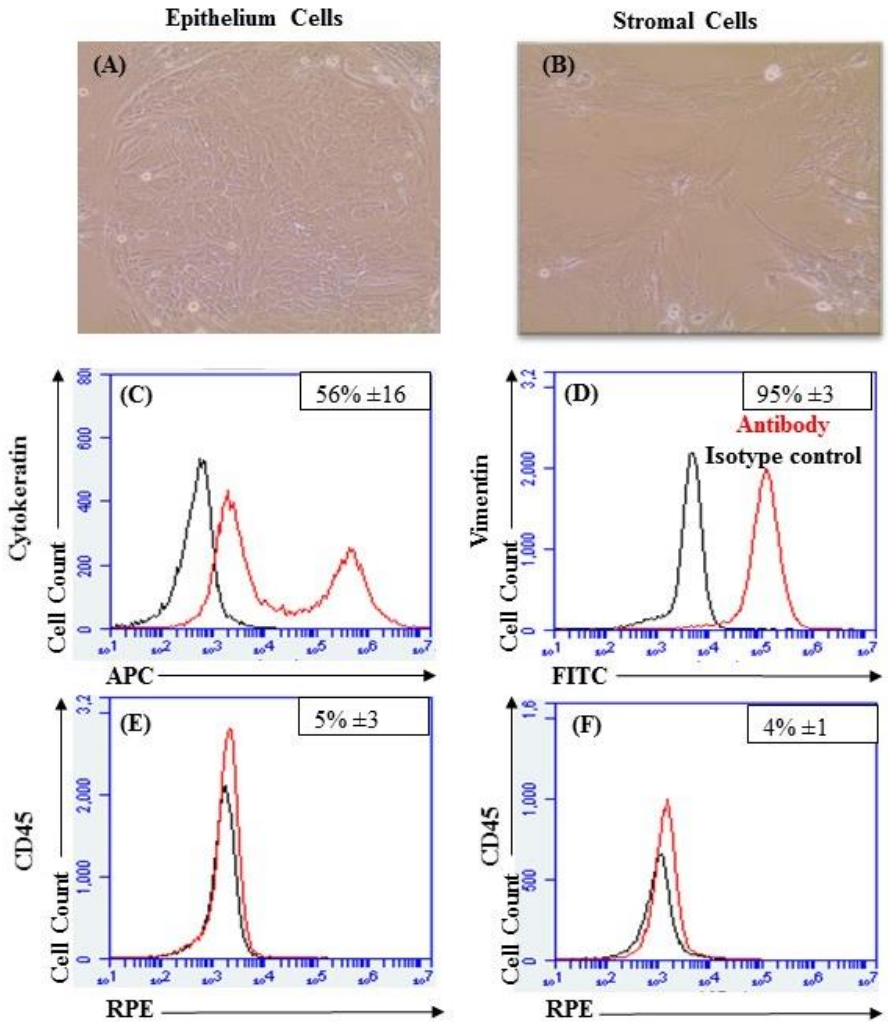


Figure 2-3. Cell purity of isolated endometrial epithelial and stromal cells. Photomicrographs of bovine endometrial epithelial (A) and stromal (B) cells. Epithelial cells appear in a regular coble stone pattern, while stromal cells have a typical fibroblast-like morphology. Total magnification is 200x. Bovine endometrial cell purity was quantified by flow cytometry using markers for epithelium (C, cytokeratin), stroma (D, vimentin) and $CD45^{+}$ immune cells (E-F). Representative histograms of 3 isolations using 10,000 cells are shown. Black lines represent negative cells (no antibody) and red lines are positive labeled cells. Values in boxes represent the mean ± SEM positive cells from 3 independent isolations.

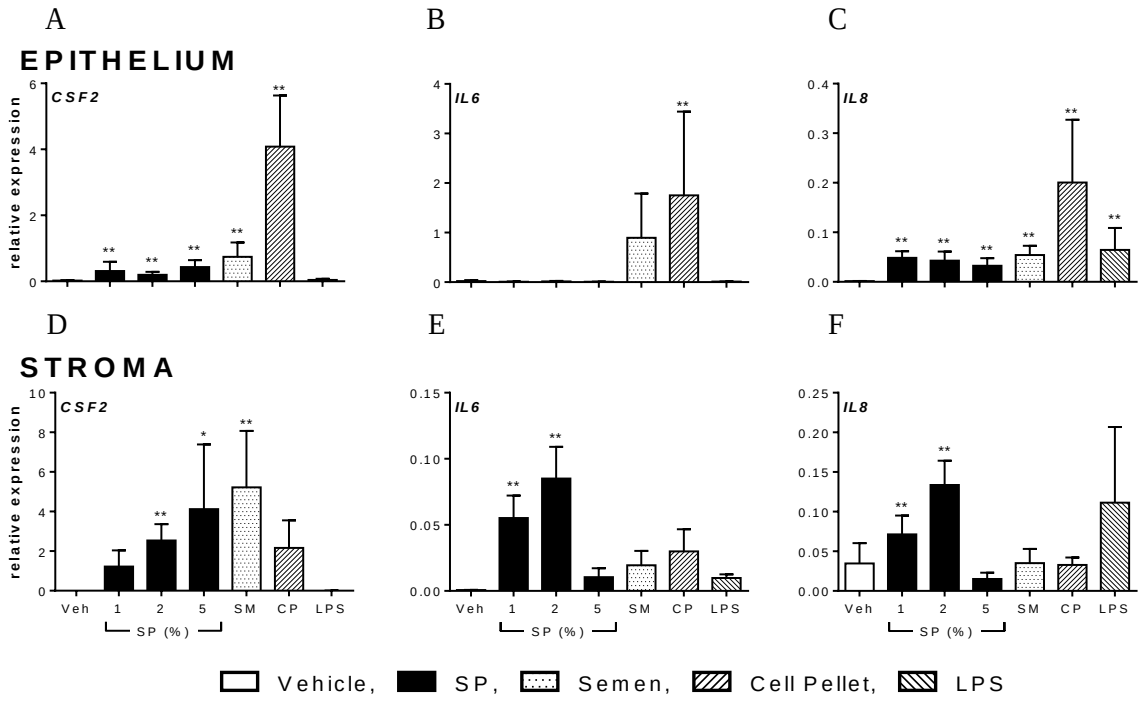


Figure 2-4. Ability of semen components to alter embryokine gene expression of isolated endometrial epithelial or stromal cells. Purified endometrial epithelial (A-C) or stroma cell (D-F) expression of *CSF2*, *IL6* and *IL8* in response to seminal plasma (SP; 1%, 2%, 5%), 5% semen cell pellet (CP), 5% whole semen (SM), 100 ng/mLof lipopolysaccharide (LPS) or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *, $P \leq 0.08$; **, $P \leq 0.05$.

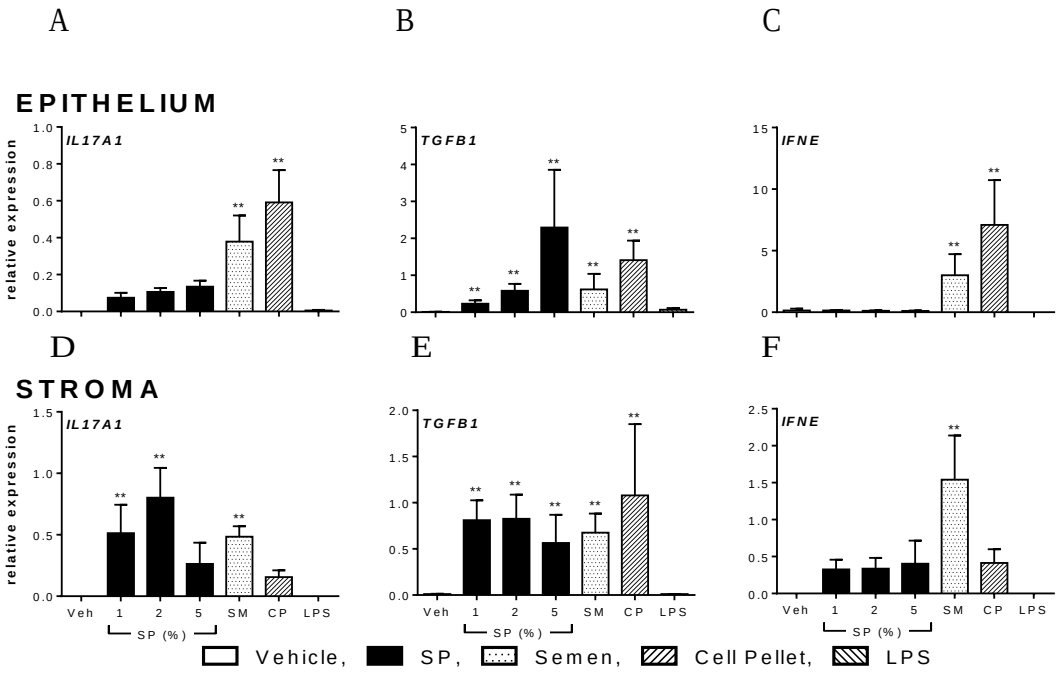


Figure 2-5. Ability of semen components to alter inflammatory gene expression of isolated endometrial epithelial or stromal cells. Purified endometrial epithelial (A-C) or stroma cell (D-F) expression of *IL17A1*, *TGFB1* and *IFNE* in response to seminal plasma (SP; 1%, 2%, 5%), 5% semen cell pellet (CP), 5% whole semen (SM), 100 ng/mL of lipopolysaccharide (LPS) or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. **, $P \leq 0.05$.

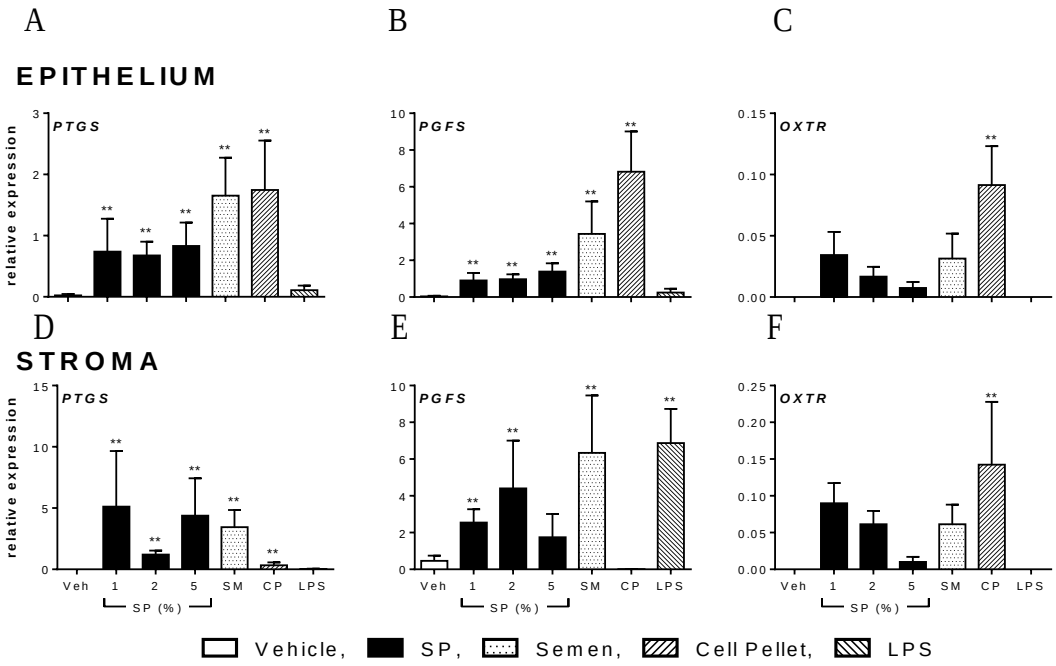


Figure 2-6. Ability of semen components to alter gene expression of isolated endometrial epithelial or stromal cells. Purified endometrial epithelial (A-C) or stroma cell (D-F) expression of *PTGS*, *PGFS* and *OXTR* in response to seminal plasma (SP; 1%, 2%, 5%), 5% semen cell pellet (CP), 5% whole semen (SM), 100 ng/mL of lipopolysaccharide (LPS) or vehicle medium alone (Veh) after 24 h. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. **, $P \leq 0.05$.

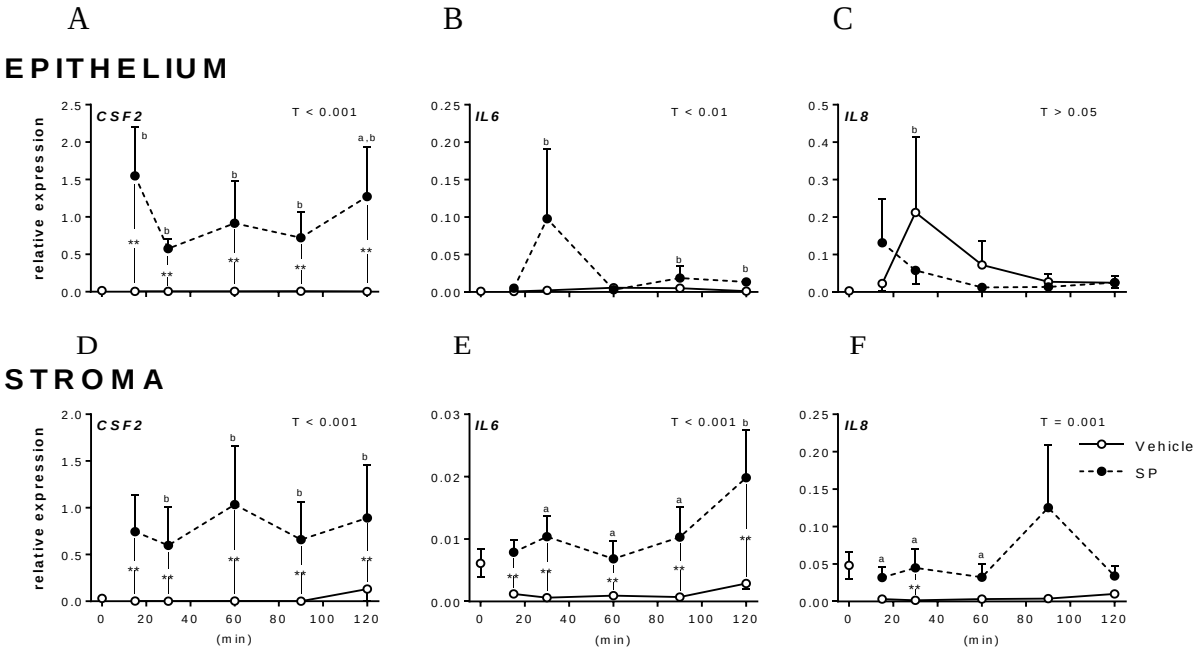


Figure 2-7. Acute effects of semen components on purified endometrial epithelial or stromal cell embryokine gene expression. Purified endometrial epithelial (A-C) and stromal (D-F) cell expression of *CSF2*, *IL6* and *IL8* in response to 5% seminal plasma (SP) or vehicle medium alone (Veh) period from 15-120 min. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; ^{a}, difference in vehicle treated controls compared to 0 min; ^{b}, difference in SP treated compared to 0 min; T, treatment; t, time; T x t, Treatment by time interaction.

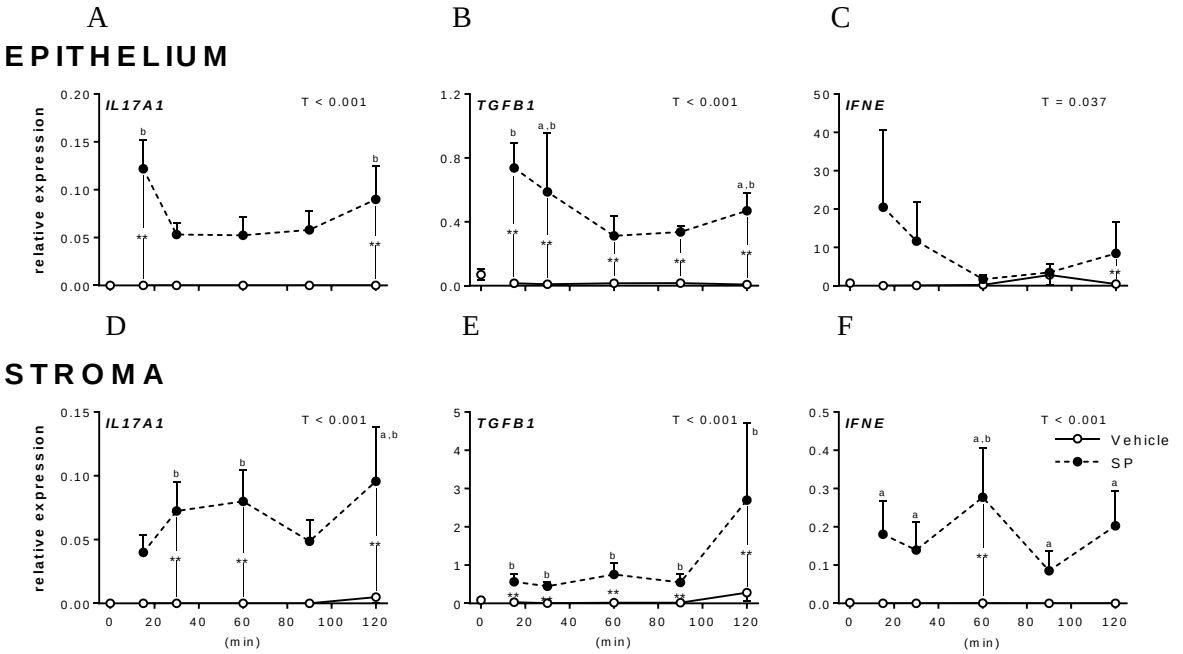


Figure 2-8. Acute effects of semen components on purified endometrial epithelial or stromal cell inflammatory gene expression. Purified endometrial epithelial (A-C) and stromal (D-F) cell expression of *IL17A1*, *TGFB1* and *IFNE* in response to 5% seminal plasma (SP) or vehicle medium alone (Veh) period from 15-120 min. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; ^{a}, difference in vehicle treated controls compared to 0 min; ^{b}, difference in SP treated compared to 0 min; T, treatment; t, time; T x t, Treatment by time interaction.

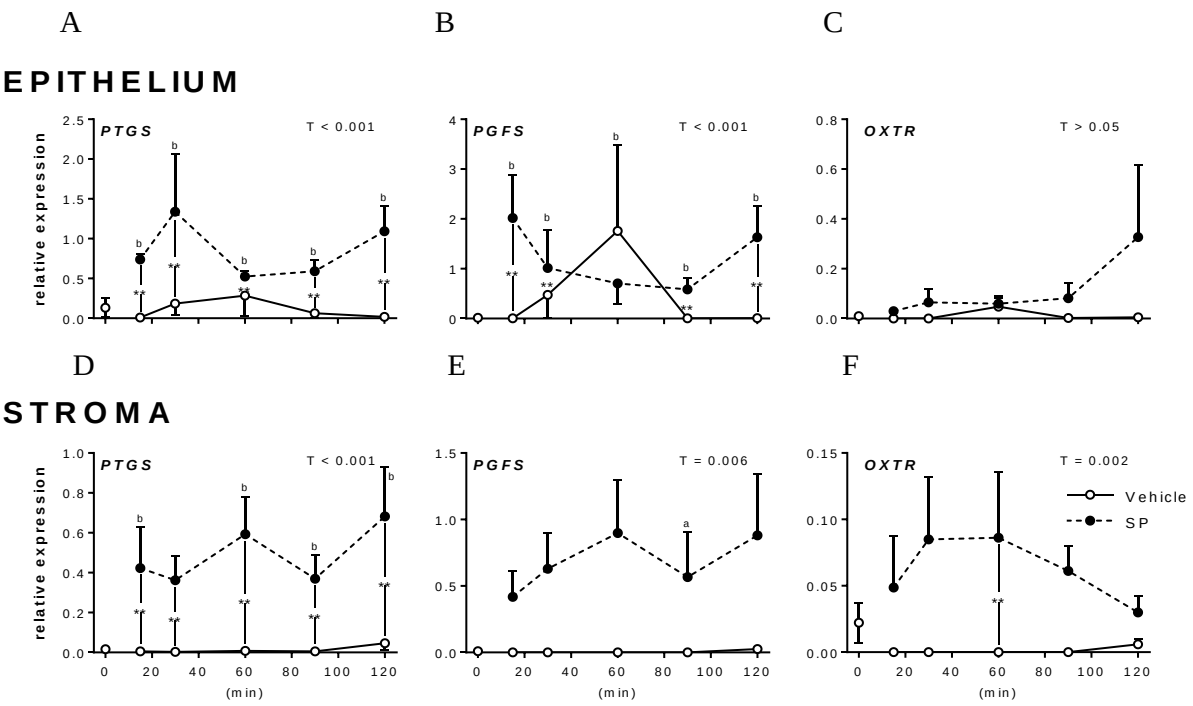


Figure 2-9. Acute effects of semen components on purified endometrial epithelial or stromal cell gene expression. Purified endometrial epithelial (A-C) and stromal (D-F) cell expression of *PTGS*, *PGFS* and *OXTR* in response to 5% seminal plasma (SP) or vehicle medium alone (Veh) period from 15-120 min. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; ^{a}, difference in vehicle treated controls compared to 0 min; ^{b}, difference in SP treated compared to 0 min; T, treatment; t, time; T x t, Treatment by time interaction.

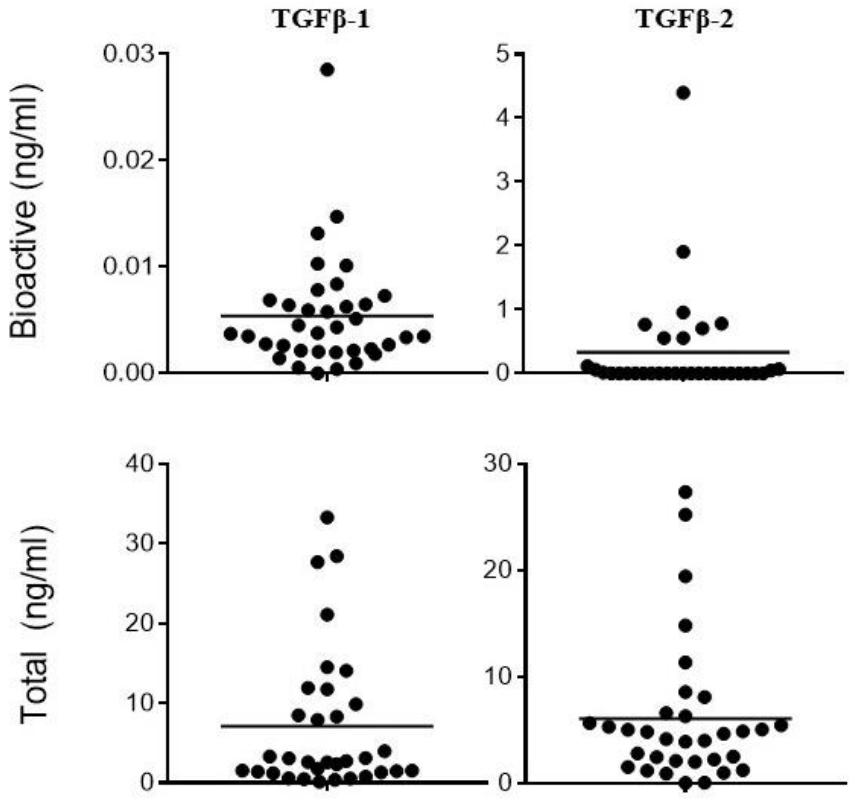


Figure 2-10. Quantification of total and bioactive TGFβ isoforms in bovine seminal plasma. The concentration of TGFβ in seminal plasma of 33 individual bulls was evaluated by isoform-specific ELISA. Each data point represents an individual bull and the line represents the mean for the population. The limits of detection were 1.7 pg/mL (TGFβ-1) and 7 pg/mL (TGFβ-2). Concentrations of total TGFβ-1 and TGFβ-2 were determined after transient acid activation of SP to activate latent cytokine. Bioactive TGFβ-1 and TGFβ-2 were determined by analysis without prior acid activation.

CHAPTER 3
DISCUSSION AND CONCLUSIONS

Discussion

Pregnancy is routinely achieved in livestock species without SP priming of the female by using AI or embryo transfer. However, the addition of SP during AI in cattle increases pregnancy rates by nearly 5%, although not significantly (Odhiambo et al., 2009). A 5% increase in pregnancy rates for dairy cattle would have vast economic and production influences for producers. In a number of species, SP is not just a transport medium for sperm, but contains active molecules that interact with epithelial cells lining the female reproductive tract to orchestrate the optimal environment for embryo development and implantation (Tremellen et al., 1998; Sharkey et al., 2012a). The physiological influence of SP on the bovine female reproductive tract and its influence on embryo development have not been satisfactorily examined. Thus, the present study tested the hypothesis that exposure of endometrial tissue to SP could modulate the expression of factors important in early pregnancy success. Our main objective of this thesis work was to study how SP (and semen components) affect maternal expression of embryokines, inflammatory mediators and factors regulating ovarian function. Using this information, we then aimed to characterize which endometrial cells (epithelial or stromal) were responsive to semen components and finally evaluate the presence of TGFβ in SP from a select population of bulls.

The natural site of semen deposition in cattle is the fornix vagina and sperm travel through the cervix into the uterus leaving the majority of SP behind. It is unclear how much, if any, SP is delivered into the uterus in cattle, whereas horses and swine receive a large dose of SP directly into the uterine lumen. These anatomical differences of conception could influence the dynamics by which the female reproductive tract communicates with SP or semen components.

However, artificial insemination bypasses the cervix and results in extended semen being delivered directly into the uterus. Current semen extension protocols result in commercial semen doses containing variable concentrations of SP (Bergeron et al., 2004). In cattle, little is known about the relationship between the endometrium and semen components.

Seminal plasma is a potent inducer of many of endometrium genes related to early pregnancy success. Here, we demonstrate an effect of SP endometrial explant *CSF2*, *IL6*, *IL8*, *IL17A1*, *TGFB1*, *IFNE*, *PTGS*, and *PGFS* gene expression.

Endometrial epithelial cells are first to encounter the luminal contents of the endometrium, and are the origin of cytokines with an innate immunity role, while endometrial stromal cells make up the majority of the cellular volume that consist of the endometrium (Giudice, 2003; Aplin et al., 2008). Here we show that endometrial epithelial cells are the major source of semen stimulated *CSF2*, *IL6*, *IL8*, *TGFB1*, *IFNE*, and *PGFS* expression, while endometrial stromal cells are the primary source for *IL17A1*, *PTGS* and *OXTR* expression. Collectively, endometrial epithelial and stromal cells respond differently to semen components.

Other have demonstrated that endometrial cytokines exert embryotrophic actions on the developing embryo to modulate maternal immunity toward the conceptus and potentially improve pregnancy success (Loureiro et al., 2011; Siqueira and Hansen, 2016). In gilts, SP induces embroykine production in the uterus, including CSF2 which increases viability of the preimplantation embryo by inhibiting apoptosis.

Our findings are consistent with what has been reported previously in human and rodent studies, where SP exposure increases expression of *CSF2*, *IL6* and *IL8* in human ectocervical epithelial cells or mouse endometrium (Sharkey et al., 2012). In general, *IL8* is produced mainly by activated monocytes and macrophages (Monaco et al., 2004). In humans, *IL8* has been

detected in the endometrium, specifically luminal and glandular epithelial cells (Arici et al., 1998). IL8 is known to act as a chemotactic factor, whereas IL6 has multiple roles including activation of neutrophils and stimulation of acute-phase immune responses (Beutler, 2009; Takeuchi and Akira, 2010; Moresco et al., 2011). It has reported that induction of CXC chemokines, including IL8, promote an influx of neutrophils from the peripheral circulation to the primary site of inflammation (Hoch et al., 1996; Laan et al., 1999; Song et al., 2016). We found epithelial cells increased expression of *IL8* in response to SP, semen cell pellet (sperm) and whole semen. This is consistent with previous finding which found a sperm dose dependent response to increase uterine epithelial *IL8* expression (Elweza et al., 2018). Neutrophil influx is dependent on the production (secretion) of other proinflammatory cytokines such as IL-1β, TNF-α, and G-CSF (Ye et al., 2001a). IL-17R deficient mice have a delay in neutrophil recruitment into the alveolar space associated with a significant decrease in G-CSF and macrophage-inflammatory protein-2 (MIP-2) (Ye et al., 2001b). We show that *IL6* and *IL8* expression was increased in endometrial tissues following exposure to LPS, consistent with previous reports describing increased expression in cows with uterine infection, or in endometrial explants treated with LPS (Fischer et al., 2010; Borges et al., 2012; Silva et al., 2012; Turner et al., 2014). Interestingly, estradiol or progesterone does not influence endometrial inflammatory responses to LPS (Saut et al., 2014; Turner et al., 2014). Exposure of human epithelial or stromal cells to SP increases expression of genes and secreted proteins related with cellular migration, proliferation, viability and inhibition of apoptosis (Chen et al., 2014).

We observed SP induced *IFNE* expression in explants and increased expression in purified cells following whole semen exposure. Interferons, and in particular IFNε, are critical cytokines involved in host defense against pathogens, due to their capacity to stimulate effector

cells of the innate and adaptive immune response. Epithelial cells of both human and mouse express *IFNE* in the uterus, cervix, vagina, and ovary (Fung et al., 2013). Importantly, *IFNE* expression in mice and human is hormonally regulated and controlled by the stage of the estrous cycle. Seminal plasma increases endometrial expression of *IFNE* and may play a role in innate immunity of the female tract following insemination required for clearance of potential male derived pathogens.

We observed a reduction in *IL17A* and *TGFB1* explant expression following SP exposure, and surprisingly increases in purified endometrial cells. In mice TGFβ signaling is required to limit IL-17α production by T-cells (Konkel et al., 2017). Similarly, the presence of TGFβ, IL-6 and/or IL-21 influence IL-17α secretion (Dong, 2008). We observed increased expression of *TGFB1* in purified cells after exposure to SP, semen cell pellet and whole semen. This is agreeance with Yousef et al who demonstate a significant increase in bovine oviduct epithelial cells *TGFB1* expression after culture with sperm at 12 and 24 h (Yousef et al., 2016). However, Elweza and colleagues suggest that semen cell pellet did not affect *TGFB1* expression in bovine uterine epithelial cells, suggesting cell type may influence TGFβ responsiveness (Elweza et al., 2018). In the mouse SP can stimulate γδ T cells to secret IL-17α. In non-reproductive tissues IL-17α regulates inflammation, secretion of pro-inflammatory cytokines (IL-1β and TNF-α), expression of matrix metalloproteinases (MMP), and chemokines (CXCL-1, -2, -5 IL-2, IL-5 and CCL-20) (Jovanovic et al., 1998; Laan et al., 1999; Martel-Pelletier et al., 1999; Park et al., 2005). As discussed above chemokines then promote the recruitment of neutrophils to promote uterine inflammation (Zhi-Hui Song et al., 2016). Many subsets of immune cells can secret IL-17α, such as $CD8^+$ T cells (TC17), $CD4^+$ T cells (Th17), γδ T cells (γδ T17), iNKT cells, and NK cells (Korn et al., 2009; Cua and Tato, 2010), while Th17 cells

and γδ T cells, eosinophils, macrophages, DCs and B cells are potential sources of IL-17α during LPS-induced endotoxin shock (Shimura et al., 2014). During pregnancy, the balance between Treg and Th17 cells facilitates maternal tolerance to the fetus, a dominance of Treg cells is required for fetal survival, while IL-17α Th17 cells are elevated in women with recurrent pregnancy loss (Aluvihare et al., 2004; Wang et al., 2010; Lee et al., 2011; Figueiredo and Schumacher, 2016). Here, SP increased endometrial expression of *PTGS* and *PGFS* expression in explants and purified cells. Prostaglandin synthesis is critical for inflammation to occur; however, prostaglandins have secondary roles in regulating luteal function, progesterone secretion, and maintenance of gestation. In swine, SP has been shown to increase expression of endometrial *PTGS* and is associated with increased plasma progesterone (O'Leary et al., 2004). The effects of SP on prostaglandin secretion needs to be evaluated to determine if they play a role in localized inflammation or potentially also ovarian function.

Our data show that endometrial *OXTR* expression is upregulated by whole semen exposure in explants and purified cells. In mammals, signaling by the neuropeptide oxytocin via receptors in the uterus results in the initiation of parturition and contributes to luteolysis in ruminant species (Kamel, 2010; Lee et al., 2010). During the estrous cycle of cattle, oxytocin produced by large luteal cells of the CL activates OXTR signaling in the endometrium resulting in secretion of PGF2α and subsequent luteal regression (Fields and Fields, 1986; Niswender et al., 2007). Conversely in the pregnant cow, the conceptus produces IFNτ that reduces endometrial OXTR expression, preventing PGF2α secretion and maintaining the corpus luteum (Spencer et al., 2007). In cattle and pigs OXTR signaling also stimulates expression of *PTGES* and subsequent secretion of luteotrophic PGE_2 by endometrial cells during early pregnancy to promote progesterone secretion (Madore et al., 2003; Waclawik, Blitek, & Ziecik, 2010). Our

data may implicate SP as playing a role in supporting the CL and progesterone secretion in early pregnancy before maternal recognition of pregnancy.

Seminal plasma has immune modulatory properties and has been shown to both suppress and stimulate immune function (Alghamdi et al., 2004; Anderson & Tarter, 1982; O'Leary et al., 2004; Schuberth et al., 2008). Within SP, TGFβ has been identified as a primary molecule to regulate maternal response in various species. Murine SP contains predominately TGFβ-1 and TGFβ-2 isoforms and elicits endometrial inflammation similar to that induced by SP exposure (Robertson, 2005). Human SP TGFβ-1, TGFβ-2 and TGFβ-3 are plentiful and stimulate inflammatory changes in ectocervical epithelial cells, mimicking the post coital environment (Sharkey et al., 2012a). We found bull SP contains TGFβ-1 and TGFβ-2 in both form active and latent. Following insemination in mice, 70% of TGFβ in uterine fluid is present in the active form, suggesting that activation of TGFβ occurs at the time of ejaculation or after deposition in the female tract (Tremellen et al., 1998). In human, the acidic environment of the vagina (pH 5.0) could be enough to remove TGF-binding protein and activate latent protein. Latent SP TGFβ may also involve SP enzymes, including plasmin, subtilisin-like endoproteases and urokinase type plasminogen activator (Chu et al., 1998; Robertson et al., 2002).

Our data demonstrate that bull SP contains active moieties that may modulate the bovine uterine environment. These SP induced changes may aid in embryo development, ovarian function, tissue remodeling and maternal immune tolerance to the conceptus.

Conclusions

In the dairy cow, the majority of pregnancy loss occurs during the first week of gestation for unknown reasons. Here, our data reveal the importance of semen components (including SP) to modulate expression of endometrial factors important in early pregnancy, related to embryo development, ovarian function, and maternal immune modulation. Preliminary examination of

bull semen demonstrates the presence of TGFβ, the active molecule responsible for SP induced endometrial alterations in other species. These new data are important in the context of domestic species production where routine breeding occurs by AI using extended semen containing diluted SP and fewer sperm cells number.

It is unclear whether the developmental environment of the oviduct and uterus of the cow is similar following natural live cover or artificial insemination. We propose that SP contributes to alteration in the developmental environment of the bovine endometrium important in early pregnancy. Further studies are required to identify the active components of SP responsible for endometrial alterations during early pregnancy. Ultimately, supplementation of these SP molecules to semen will be utilized to develop new AI protocols to optimize reproductive outcomes in commercial dairy herds.

APPENDIX A
ACUTE EFFECTS OF LPS ON EX VIVO ENDOMETRIAL, ENDOMETRIAL STROMAL CELL OR ENDOMETRIAL EPITHELIAL CELL GENE EXPRESSION

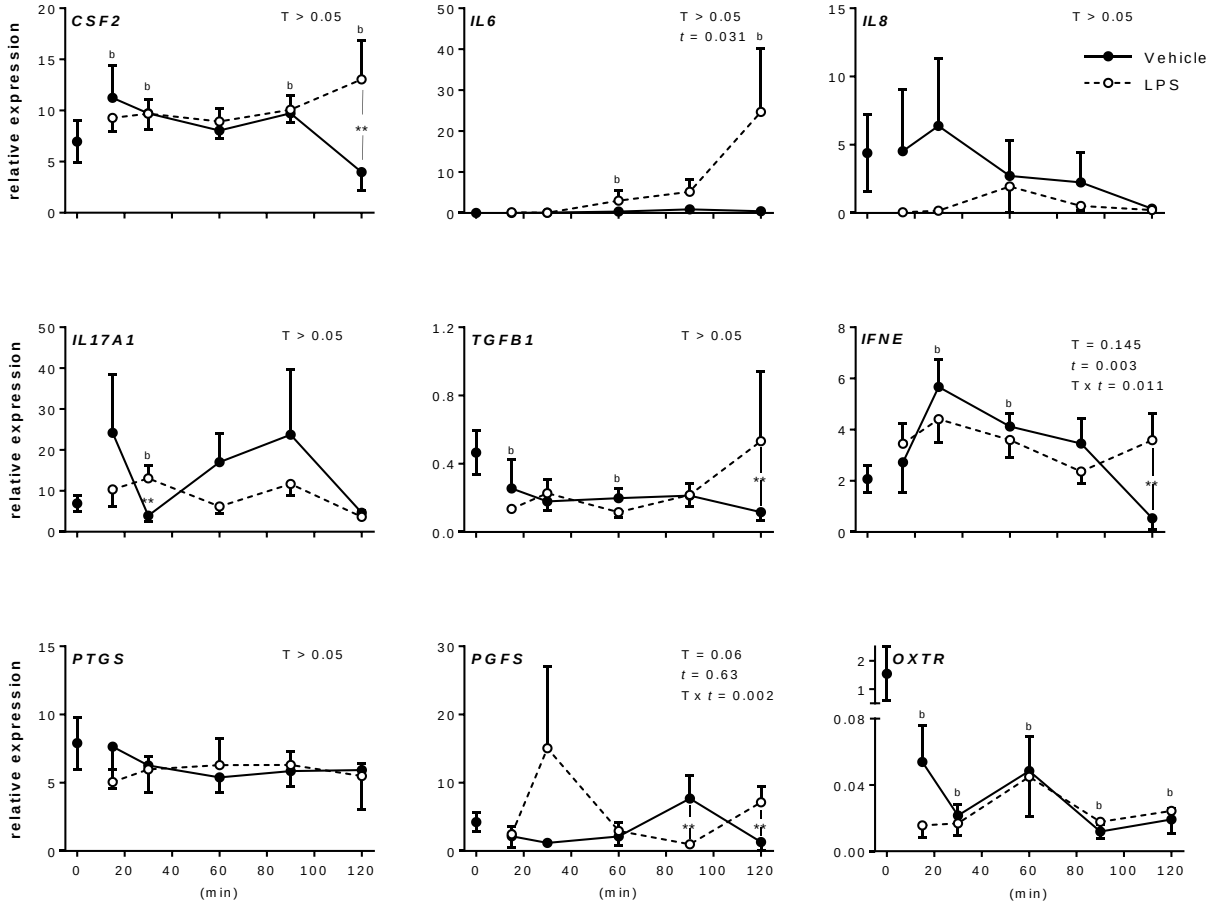


Figure A-1. Acute effects of LPS on ex vivo endometrial gene expression. Intact endometrial explant expression of *CSF2*, *IL6*, *IL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS*, *PGFS* and *OXTR* in response to 100ng/mL of lipopolysaccharide (LPS) or control medium alone. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; [a], difference in vehicle treated controls compared to 0 min; [b], difference in LPS treated compared to 0 min. T, treatment; t, time; T x t, Treatment by time interaction.

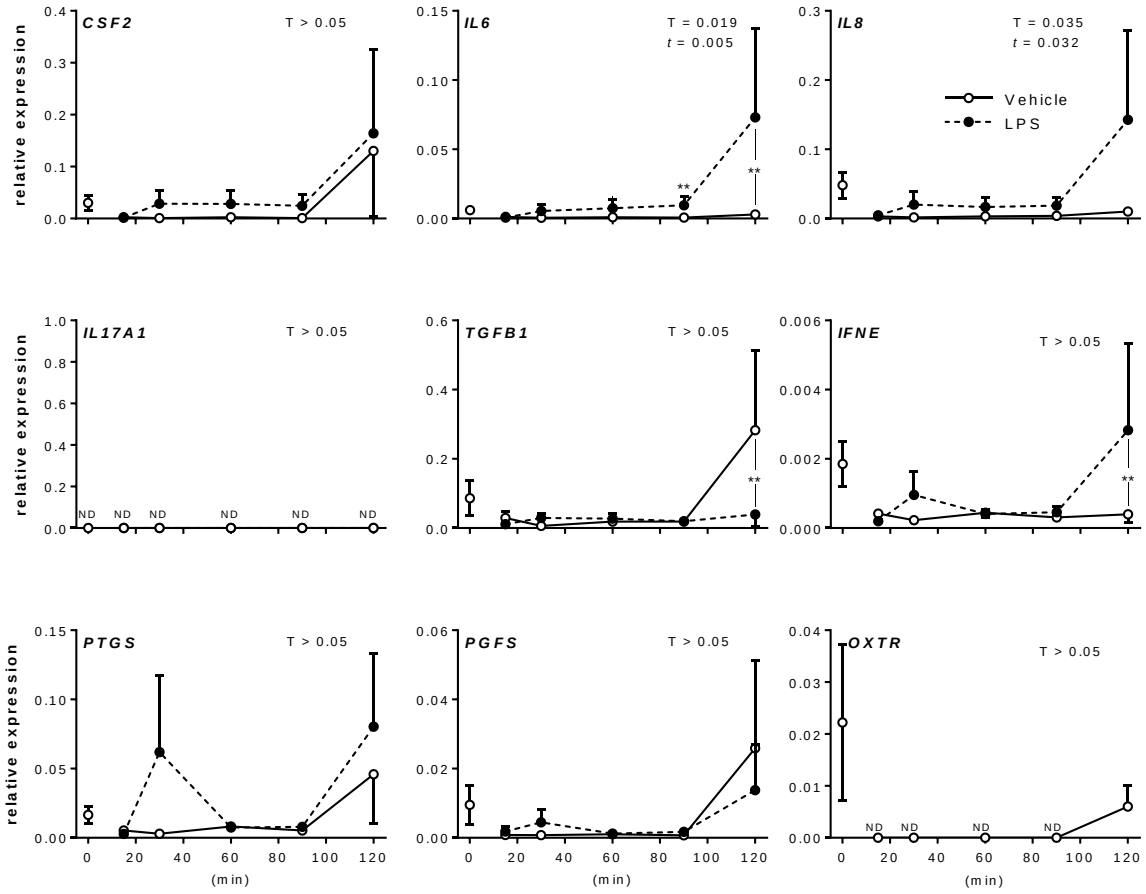


Figure A-2. Acute effects of LPS on purified endometrial stromal cell embryokine and inflammatory gene expression. Endometrial stromal cell expression of *CSF2*, *IL6*, *IL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS*, *PGFS* and *OXTR* in response to 100ng/mL of lipopolysaccharide (LPS) or control medium alone. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; [a], difference in vehicle treated controls compared to 0 min; [b], difference in LPS treated compared to 0 min. T, treatment; t, time; T x t, Treatment by time interaction.

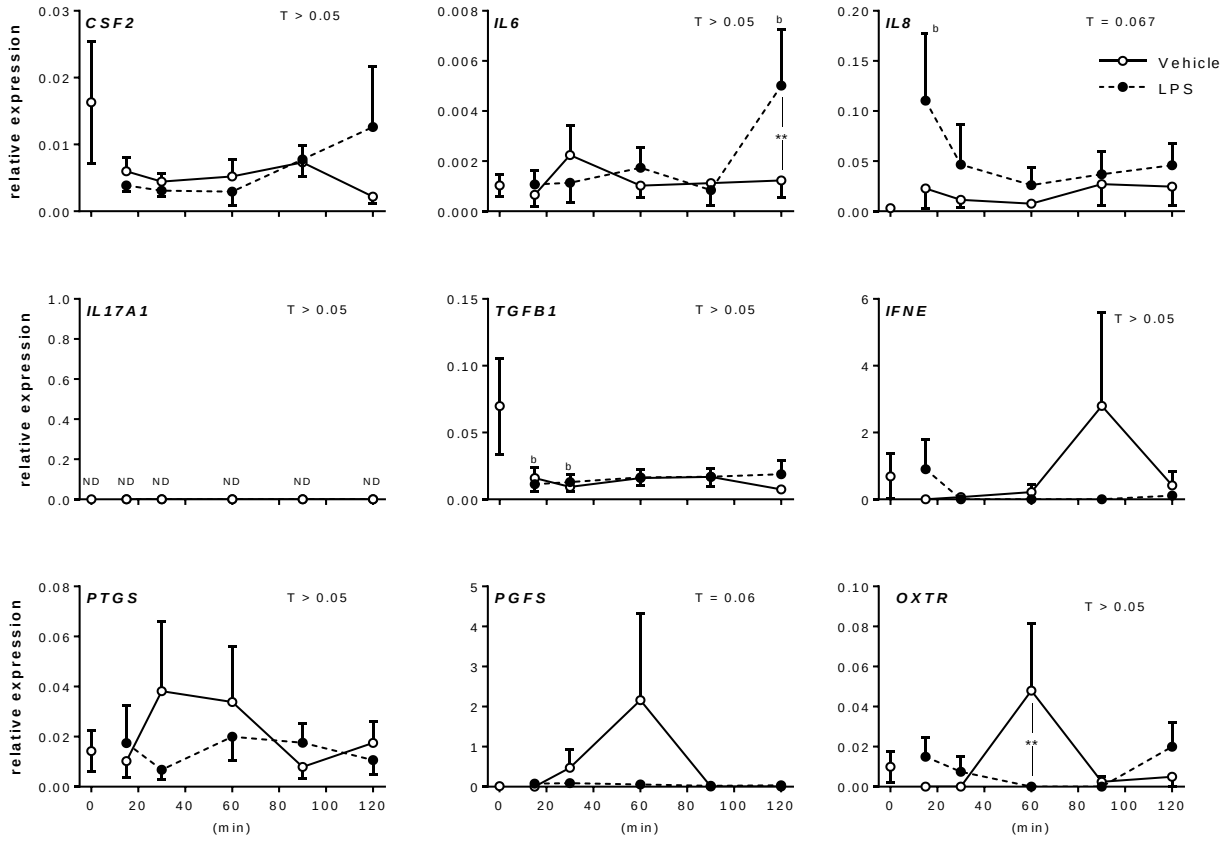


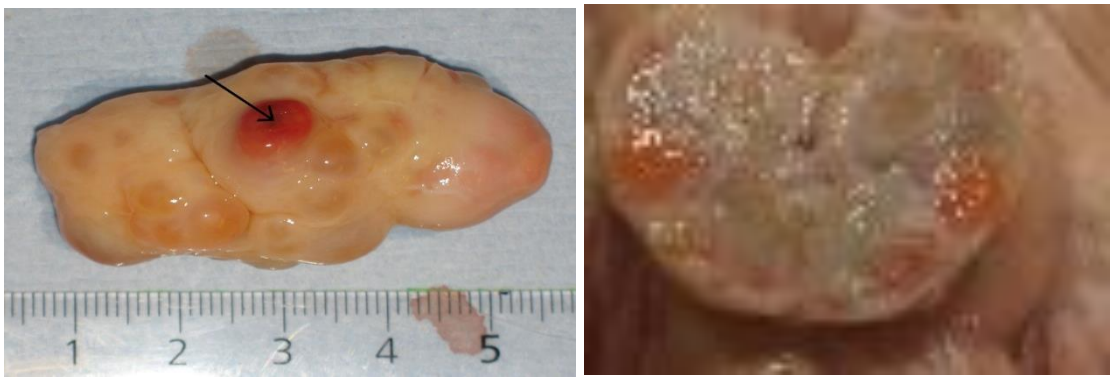
Figure A-3. Acute effects of LPS on purified endometrial epithelial cell embryokine and inflammatory gene expression. Semi-purified endometrial epithelial cell expression of *CSF2*, *IL6*, *IL8*, *IL17A*, *TGFB1*, *IFNE*, *PTGS*, *PGFS* and *OXTR* in response to 100ng/mL of lipopolysaccharide (LPS) or control medium alone. Data are presented as the mean relative expression + S.E.M. from six independent experiments. Data were log transformed and analyzed using generalized linear mixed model with pairwise comparisons made with vehicle treated control. *P* value of ≤ 0.05 was assumed statistically significant. **, difference between treatment groups within a time point; [a], difference in vehicle treated controls compared to 0 min; [b], difference in LPS treated compared to 0 min. T, treatment; t, time; T x t, Treatment by time interaction.

APPENDIX B
STAGING THE BOVINE CORPUS LUTEUM.

Reference: J. Ireland, R.L. Murphee and P.B. Coulson (1980). Accuracy of Predicting Stages of Bovine Estrous Cycle by Gross Appearance of the Corpus Luteum. *Journal of Dairy Science* **63:** 155-160.

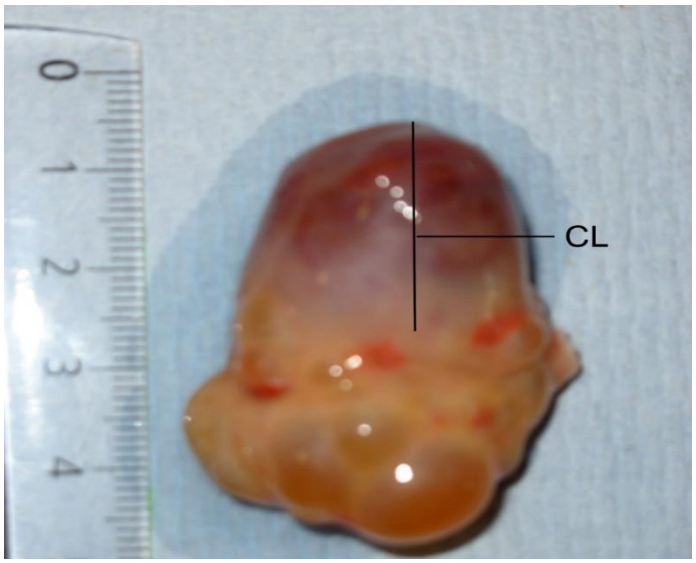
Characteristics	Stage of Estrous Cycle			
	I	II	III	IV
Appearance of CL (external)	Red, recently ovulated, point of rupture not covered over.	Point of rupture covered over. Apex red or brown	Tan – orange	Light yellow – white
Appearance of CL (internal)	Red, occasionally filled with blood cells.	Red or brown at apex only – remainder is orange.	Orange	Orange to yellow
CL Diameter	0.5 - 1.5 cm	1.6 – 2 cm	1.6 – 2 cm	<1 cm
Follicles	Absent	Present	May be present or absent	Present

Stage I CL: -

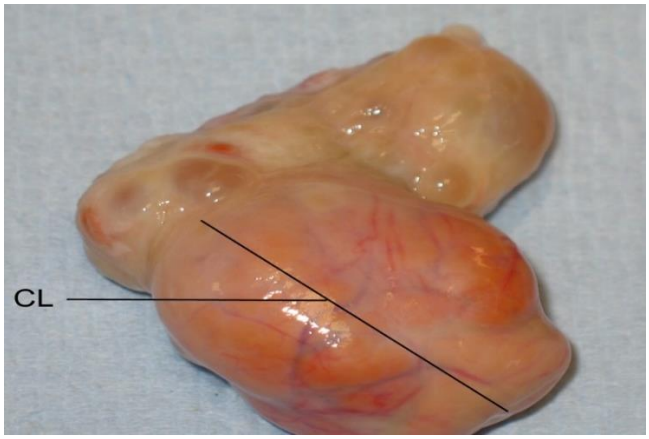


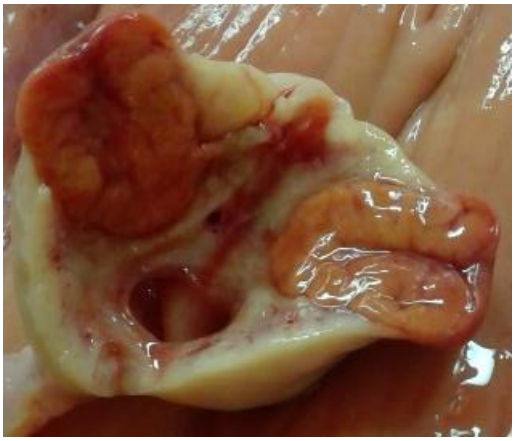
Arrow points to the site of follicle rupture.

Stage II CL: -

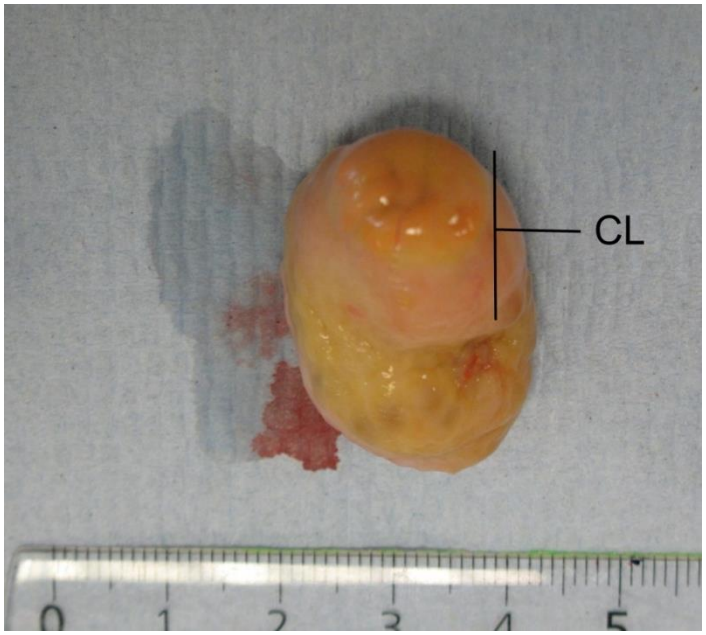


Stage III CL: -





Stage IV CL: -



APPENDIX C
DISSECTION OF BOVINE ENDOMETRIUM

Figure C-1. The Bovine Reproductive Tract

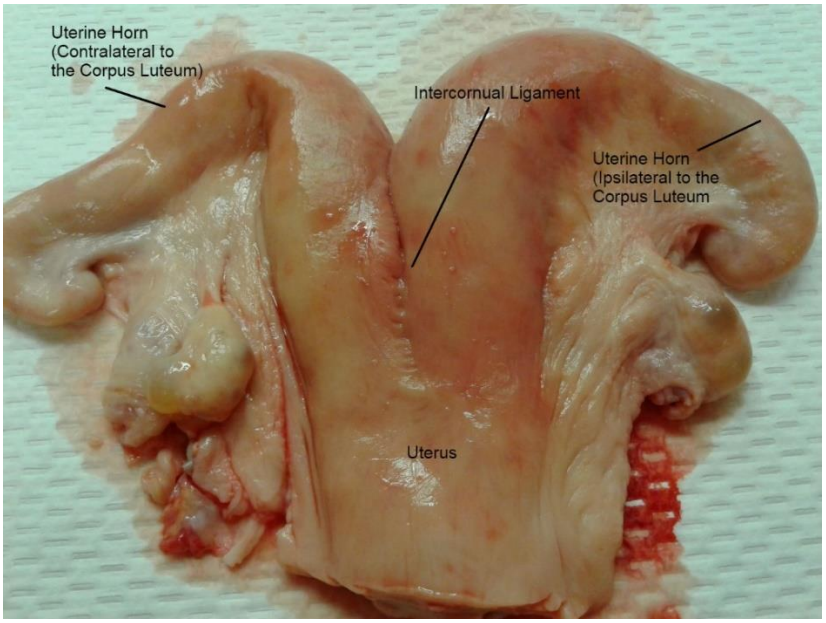


Figure C-2. Transverse cut across the uterine horn

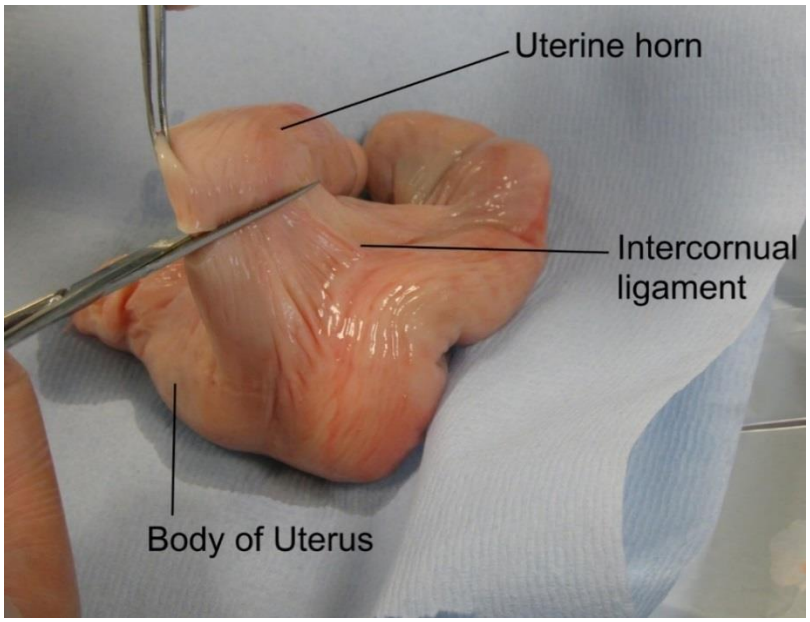


Figure C-3. Longitudinal cut along the uterine horn

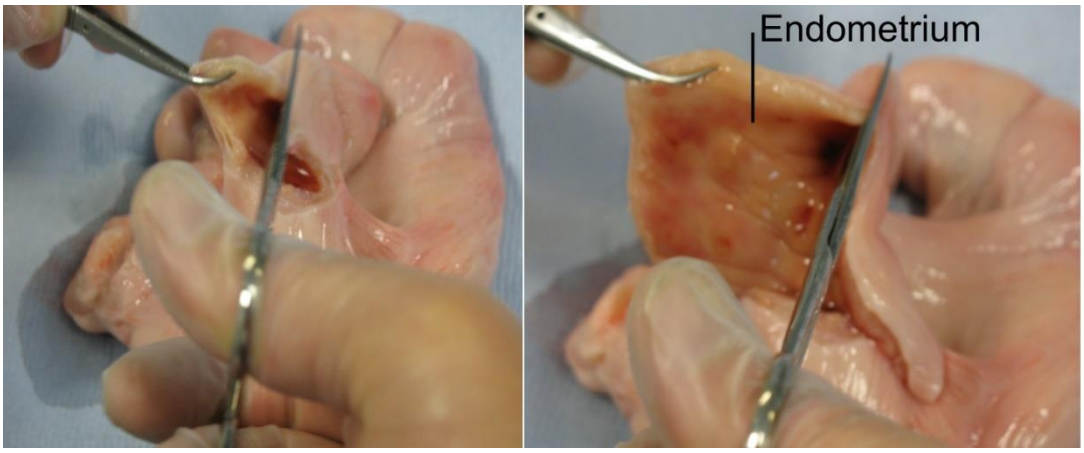
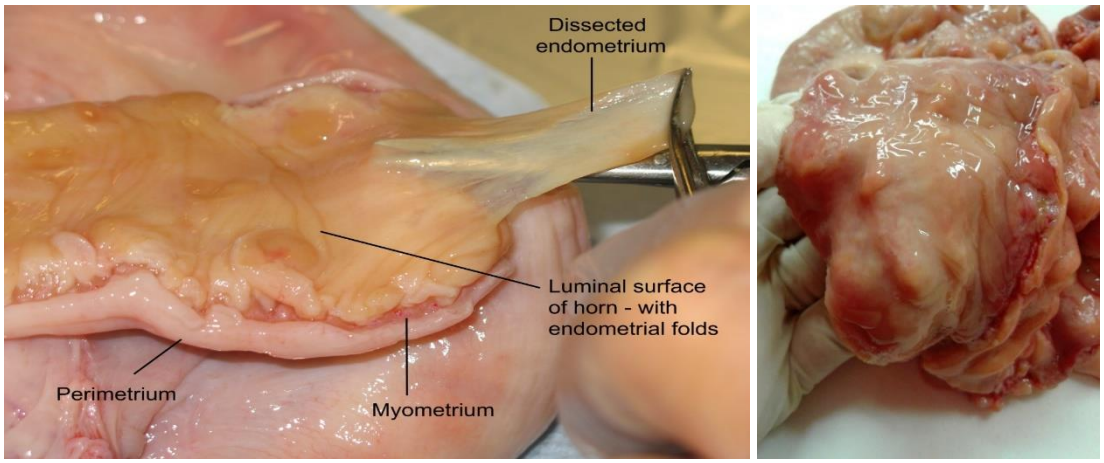


Figure C-4. Dissection of endometrium, showing the various layers of the uterus.



APPENDIX D
HUMAN GRANULOSA–LUTEAL CELLS INITIATE AN INNATE IMMUNE RESPONSE TO PATHOGEN-ASSOCIATED MOLECULES

REPRODUCTION
RESEARCH

Human granulosa–luteal cells initiate an innate immune response to pathogen-associated molecules

Laila A Ibrahim[1,3], Joseph M Kramer[1,2], R Stan Williams[1,2] and John J Bromfield[1,3]

[1]*D. H. Barron Reproductive and Perinatal Biology Research Program, Gainesville, Florida, USA,* [2]*Department of Obstetrics and Gynecology, College of Medicine, University of Florida, Gainesville, Florida, USA, and* [3]*Department of Animal Sciences, University of Florida, Gainesville, Florida, USA*

Correspondence should be addressed to J J Bromfield; Email: jbromfield@ufl.edu

Abstract

The microenvironment of the ovarian follicle is key to the developmental success of the oocyte. Minor changes within the follicular microenvironment can significantly disrupt oocyte development, compromising the formation of competent embryos and reducing fertility. Previously described as a sterile environment, the ovarian follicle of women has been shown to contain colonizing bacterial strains, whereas in domestic species, pathogen-associated molecules are concentrated in the follicular fluid of animals with uterine infection. The aim of this study is to determine whether human granulosa–luteal cells mount an innate immune response to pathogen-associated molecules, potentially disrupting the microenvironment of the ovarian follicle. Human granulosa–luteal cells were collected from patients undergoing assisted reproduction. Cells were cultured in the presence of pathogen-associated molecules (LPS, FSL-1 and Pam3CSK4) for 24 h. Supernatants and total RNA were collected for assessment by PCR and ELISA. Granulosa–luteal cells were shown to express the molecular machinery required to respond to a range of pathogen-associated molecules. Expression of *TLR4* varied up to 15-fold between individual patients. Granulosa–luteal cells increased the expression of the inflammatory mediators *IL1B*, *IL6* and *CXCL8* in the presence of the TLR4 agonist *E. coli* LPS. Similarly, the TLR2/6 ligand, FSL-1, increased the expression of IL6 and CXCL8. Although no detectable changes in *CYP19A1* or *STAR* expression were observed in granulosa–luteal cells following challenge, a significant reduction in progesterone secretion was measured after treatment with FSL-1. These findings demonstrate the ability of human granulosa–luteal cells to respond to pathogen-associated molecules and generate an innate immune response.

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Introduction

Sexually transmitted infections in women of reproductive age are very common in the USA, with most infections occurring in young women aged between 15 and 24 years. The Gram-negative bacteria *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are the leading causes of sexually transmitted bacterial infections with a combined total of 3.6 million new cases/year in the USA (277 million worldwide; Centers for Disease Control and Prevention). Pelvic inflammatory disease (PID) is caused primarily by untreated chlamydia or gonorrhea of the upper reproductive tract, with approximately 10–15% of women with chlamydia developing PID (reviewed in (Mitchell & Prabhu 2013)). Direct medical expenditures for PID and its sequelae were estimated at $1.88 billion in 1998 (Rein *et al.* 2000). Although PID is a leading cause of gynecological hospitalization of reproductive aged women in the USA, 10% of PID patients become infertile primarily due to pelvic adhesions and tubal obstruction (Velebil *et al.* 1995). Although PID is a cause of pelvic pain and infertility, little is known about the effects on the ovary and female gametes. However, it has been shown that patients with PID have reduced follicle numbers and increased cystic follicles and corpora lutea after the resolution of the initial infection (Weiner & Wallach 1974, Bychkov 1990). Retrospective analysis of patients undergoing fertility treatment suggest that increased endometrial LPS reduces IVF success (Kamiyama *et al.* 2004). More recently, it has been demonstrated that bacteria can be isolated from the follicular fluid of women undergoing infertility treatment, with the extent of bacterial colonization of the follicle being correlated with the success of fertility treatment (Neuer *et al.* 1997, Pelzer *et al.* 2013).

The initial immune response to pathogens is driven by the innate immune system. Innate immune responses to bacteria depend on conserved pathogen-associated molecules being recognized by receptors of the innate immune system, such as Toll-like receptors (TLRs). Toll-like receptors are typically found in hematopoietic immune cells in addition to non-immune cells of the gut, which readily encounter bacteria. Previous work has demonstrated the ability of primary human granulosa–luteal cells (GLCs) to bind LPS (Sanchotello *et al.* 1992), whereas the granulosa cell line COV434

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Table 1 Primer sequences for PCR.

Gene	Sequence (5′–3′) Sense	Antisense	Accession number
ACTB	ACAGAGCCTCGCCTTTGCCG	TTGCACATGCCGGAGCCGTT	NM_001101.3
CD14	CAGAACCCTAGATGCCCTGC	CGCGCTCCATGGTCGATA	NM_000591.3
CYP19A1	GACGTCGCGACTCTAAATTGC	TGGGAGATGAGGGGTCCAAT	NM_000103.3
IL1B	AACCTCTTCGAGGCACAAGG	GTCCTGGAAGGAGCACTTCAT	NM_000576.2
IL6	CAGTTCCTGCAGAAAAAGGCAA	GCTGCGCAGAATGAGATGAG	NM_000600.3
CXCL8	CAGAGACAGCAGAGCACACA	GGCAAAACTGCACCTTCACA	NM_000584.3
LY96 (MD2)	ACACCTACTGTGGGAGAGAT	CGTCATCAGATCCTCGGCAA	NM_001195797.1
MYD88	TCCTGCTGCTGCTTCAAGAT	GACTGCTCGAGCTGCTTACC	NM_001172567.1
PTPRC (CD45)	TGAAAGAGTGAGAGTGGACGA	TTCTGGTGTCTGCCTGCTTC	NM_001267798.1
STAR	TGTCATCAGGGCCGGAGCAC	CAGGACCTGGTTGATGATGCT	NM_000349.2
TLR4	CTGCCACATGTCAGGCCTTAT	AATGCCCACCTGGAAGACTCT	NM_138554.4

possess TLRs and initiate an innate immune response to pathogen-associated molecules (Price *et al.* 2012). Whether human GLCs can initiate an innate immune response to pathogen-associated molecules and potentially alter the microenvironment of the follicle remains unknown.

We hypothesized that human GLCs isolated from infertility patients would initiate an innate immune response to pathogen-associated molecules similar to those found on sexually transmitted pathogens. Innate immune-mediated changes to the microenvironment of the ovarian follicle may help to further explain infertility associated with PID or sexually transmitted infections.

Materials and methods

Tissue collection and granulosa–luteal cell isolation

Human follicular aspirates were collected from women attempting conception through IVF, as well as from oocyte donors, at the University of Florida Reproductive Medicine Clinic. The collection of follicular aspirates for this study was reviewed and approved by the Institutional Review Board at the University of Florida (#IRB201400399). All patients provided non-written consent to provide tissues as the University of Florida Institutional Review Board considered the follicular aspirates to be non-human waste collected during normal procedures and subsequently discarded and no patient information was obtained. Consenting patients underwent ovarian stimulation using standard long Lupron suppression or GNRH antagonist protocols. Oocyte retrieval was scheduled for 36 h after administration of an ovulation trigger. After oocytes were collected from the aspirate, the remaining follicular cells were collected by centrifugation at 800 **g** for 10 min. Cell pellets were then resuspended in warm PBS and washed by centrifugation at 800 **g** for 10 min. Cell pellets were resuspended in complete culture medium containing DMEM: Hams F12 (1:1), 10% fetal bovine serum, ITS, and 1% penicillin/streptomycin (all Thermo Fisher Scientific). A 1:1 (vol:vol) Percoll:PBS solution was prepared and the cell suspension layered on top of the Percoll solution and centrifuged at 800 **g** for 10 min. Cells were aspirated from a single layer in Percoll and washed in 10 mL warm PBS by

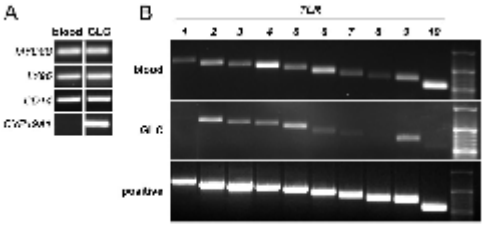

Figure 1 Granulosa–luteal cells express the TLR4 co-receptors and Toll-like receptors required to initiate an innate immune response. GLCs were collected from patients undergoing IVF. (A) After cellular purification, expression of the TLR4 co-receptors *MYD88*, *LY96*, and *CD14* were assessed by RT-PCR in addition to the granulosa cell-specific marker *CYP19A1* (aromatase). (B) Patient GLCs were assessed for the expression of all ten TLRs using RT-PCR after cellular purification. Commercial primer sets were utilized for the detection of individual TLR members. dsDNA was used as positive controls to demonstrate target amplification. RNA collected from whole blood was used as a positive control for all PCR. Each amplification was performed on a minimum of four patient samples.

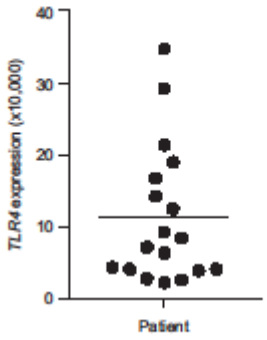

Figure 2 Expression of granulosa–luteal cell *TLR4* varies between individual fertility patients. Following cellular purification, expression of *TLR4* was assessed in individual patients using real-time RT-PCR. A total of 18 patients were assessed for the expression of *TLR4* after confirming cultures were free of immune cell marker. Data were normalized to the housekeeper *ACTB*.

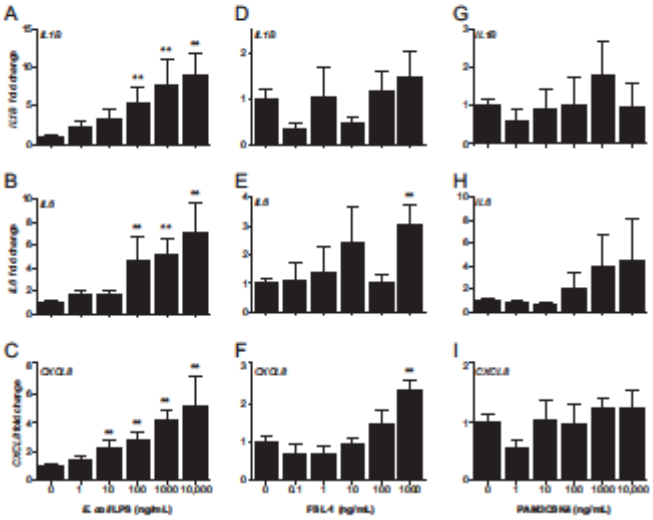

Figure 3 Granulosa–luteal cells increase expression of *IL1B*, *IL6*, and *CXCL8* in response to pathogen-associated molecules. Following a 24-h treatment of GLCs with tenfold increasing concentrations of *E. coli* LPS (A, B and C), FSL-1 (D, E and F), or Pam3CSK4 (G, H and I), expression of *IL1B* (A, D and G), *IL6* (B, E and H), and *CXCL8* (C, F and I) was assessed using real-time RT-PCR. Data are presented as mean ± S.E.M. from four to eight independent experiments. $^{**}P \leq 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett's pairwise *post hoc* tests.

centrifugation at 800 *g*. Cellular aggregates of GLCs were disassociated in 1 mL of 300 µg/mL of hyaluronidase for 10 min at 37°C following a brief vortex. Cells were washed twice in complete medium by centrifugation at 800 *g* for 10 min. Cells were resuspended and plated at 10^5 cells/mL in 500 µL aliquots in 24-well plates (TPP; Switzerland) and incubated at 37°C with 5% CO_2 in a humidified environment.

Granulosa–luteal cell culture and challenge

Granulosa–luteal cells were allowed a 48-h period of equilibration before treatment. Each treatment was performed in a single well and repeated a minimum of four times on different days from different patients. Following 48 h of culture, medium was replaced with fresh medium containing either ultrapure *Escherichia coli* O111:B4 LPS (TLR4 agonist; 1 mg/mL is equivalent to 10^6 EU/mL), FSL-1 (TLR2/6 agonist; LPS ≤ 0.001 EU/µg), Pam3CSK4 (*N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine) (synthetic ligand for TLR1/2; LPS ≤ 0.001 EU/µg), or ultrapure *Rhodobacter sphaeroides* LPS (LPS-RS; TLR4 antagonist) (all from Invivogen, San Diego, CA, USA). Treatments were performed at tenfold increasing doses between 100 pg/mL and 10 µg/mL. After culture for 24 h, cell-free supernatants and total RNA were collected and stored at −20°C. A time–course experiment involved the treatment of cells with 100 ng/mL *E. coli* LPS between 15 and 360 min.

THP-1 cell line culture

The human monocyte cell line THP-1 (ATCC, Manassas, VA, USA) was used as a positive control throughout. THP-1 cells were authenticated by ATCC using short tandem repeat (STR) profiling and confirmed a 100% identity with THP-1 cells according to ATCCs STR database. Cells were cultured in RMPI-1640 containing 10% FCS and 0.05 mM 2-mercaptoethanol. When required, cells were plated at a density of 10^5 cells/mL in 500 µL aliquots in 24-well plates and incubated at 37°C with 5% CO_2 in a humidified environment. The addition of 50 ng/mL phorbol myristate acetate (PMA) facilitated the differentiation of monocytes to adherent macrophage-like cells that were used in experimental treatments (Park *et al.* 2007). Cells were allowed 48 h for differentiation and equilibration before treatment as per the methods described above.

RNA isolation and PCR

Cells were washed in warm PBS and lysed in RLT buffer to collect total RNA. Extraction of RNA was performed using the RNA Easy Mini kit according to the manufacturer's instruction (Qiagen). Total RNA was subjected to reverse transcription using the Verso cDNA synthesis kit according to the manufacturer's instruction (Thermo Fisher Scientific). Primers were designed using the NCBI database and initial specificity verified by BLAST to ensure no cross-reactivity with other loci (Table 1). Real-time PCR was performed in 25 µL reactions using Maxima SYBR Green chemistry containing 0.5 µM of each forward and reverse primer (Thermo Fisher Scientific). A CFX Connect light cycler was employed to perform quantitative PCR (Bio-Rad). Data were normalized independently to *ACTB* mRNA expression and plotted as expression relative to the mean of the untreated control group using the ΔΔCt method. To examine cell-specific markers (*PTPRC* and *CYP19A1*) and the presence of receptors required for innate immune recognition of pathogen-associated molecules (*MYD88*, *LY96* and *CD14*), PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized under

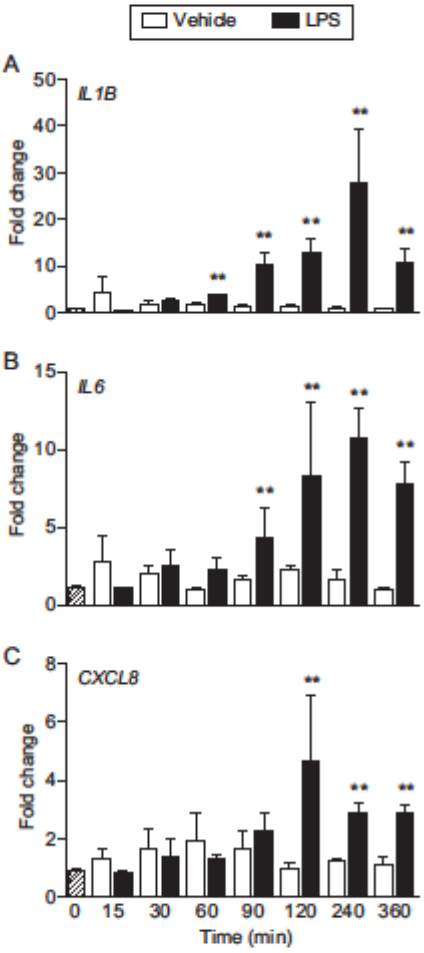

Figure 4 Acute response of granulosa–luteal cells to lipopolysaccharide. Acute responses of GLCs were measured following treatment with 100 ng/mL *E. coli* LPS. Cells were treated for 15–360 min before expression of *IL1B* (A), *IL6* (B), and *CXCL8* (C) was measured using real-time RT-PCR. Data are presented as mean ± S.E.M. from three to six independent experiments. $^{**}P \leq 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett's pairwise *post hoc* tests.

UV illumination. Detection of the TLR family was performed using commercial primer sets from Invivogen according to the manufacturers' instructions and internal positive controls (Invivogen). Whole-blood RNA was purchased and used as an internal control for immune cell markers (Thermo Fisher Scientific).

ELISA

Progesterone and 17β-estradiol were measured in cell-free supernatants using commercially available ELISA kits according to the manufacturer's instructions (DRG Instruments, GmbH, Germany). The limits of detection for 17β-estradiol and progesterone were 10.7 and 0.3 pg/mL respectively; the intra-assay coefficients of variance were 1.1 and 6.4%, and the inter-assay coefficients of variance were 1.2 and 6.63% respectively.

Immunocytochemistry and fluorescence microscopy

Cells were plated onto sterile cover glass within 6-well culture plates for 24 h. On the day of labeling, cells were washed twice in warm PBS containing 0.5% BSA and subsequently blocked in 20% normal goat serum for 15 min in the incubator. Cells were again washed in warm PBS+BSA before application of a 1:10 dilution of anti-human CD45-FITC antibody (Miltenyi Biotec, San Diego, CA, USA) in combination with 1 μg/mL Hoechst (Thermo Fisher Scientific) for 30 min in the incubator. Cells were washed twice in PBS + BSA before immediate live cell image acquisition.

Cells were observed immediately using a Zeiss Axioplan 2 epifluorescence microscope and Axiovision software. Images were acquired using a Zeiss MRm digital camera. A minimum of 200 cells in five separate fields for each sample were assessed for Hoechst staining (total cells) and CD45-positive immunoreactivity (positive cells).

Statistical analysis

SPSS version 20.0 software was used for statistical analysis. Gene expression measured by real-time RT-PCR and concentrations of steroid hormones were analyzed using ANOVA, and comparisons were made with control using the Dunnett's pairwise *post hoc* test. If data were not normally distributed, it was log transformed for analysis. Data are arithmetic mean ± S.E.M., and a P value of ≤0.05 was assumed to be statistically significant.

Results

Granulosa–luteal cells are free of immune cells

Contamination of GLCs with immune cells was assessed to ensure that any response measured was due to target cells and not that of contaminating immune cells. Immunocytochemistry was performed to detect immunoreactivity to the immune cell marker PTPRC (CD45). The human monocyte cell line, THP-1, was 91.7% positive for PTPRC, whereas only 0.45% of GLCs displayed PTPRC immunoreactivity, suggesting a 99.5% immune cell marker-free preparation (Supplementary Fig. 1A and B, see section on supplementary data given at the end of this article). Following 40 cycles of RT-PCR, expression of *PTPRC* (*CD45*) was detected in blood while minimally visible in in GLC cultures

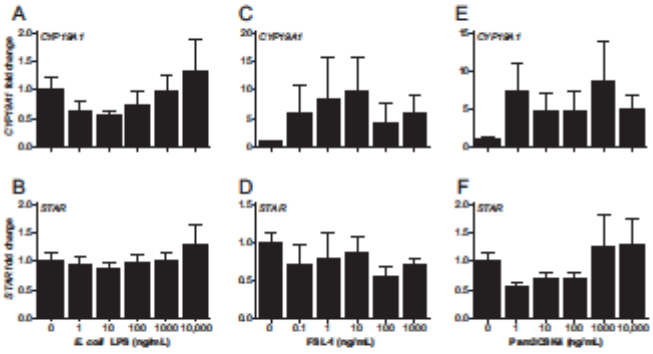

Figure 5 Pathogen-associated molecules do not change expression of *CYP19A1* or *STAR* in granulosa–luteal cells. Following a 24-h treatment of GLCs with tenfold increasing concentrations of *E. coli* LPS (A and B), FSL-1 (C and D) or Pam3CSK4 (E and F) expression of *CYP19A1* (A, C and E) and *STAR* (B, D and F) was assessed using real-time RT-PCR. Data are presented as mean ±S.E.M. from four to eight independent experiments. Compared with untreated controls; analysis by ANOVA followed by Dunnett's pairwise *post hoc* tests.

(Supplementary Fig. 1C). All samples were routinely screened for the presence of *PTPRC* at the initiation of culture to ensure an immune cell marker-free culture; any cultures shown to express *PTPRC* were excluded from analysis.

Granulosa–luteal cells express the receptors required to initiate an innate immune response

Initiation of innate immune signaling is dependent on the presence of TLR co-receptors MYD88, CD14, and LY96 (formerly MD2). Expression of *MYD88*, *LY96*, and *CD14* were all detected in GLC and blood (Fig. 1A). In addition, the expression of the granulosa cell-specific marker *CYP191A* (aromatase) was shown to be present in GLCs and absent in blood (Fig. 1A). A PCR screen of all known human TLRs was performed to determine the capacity of GLCs to detect various pathogen-associated molecules. Expression of all ten TLRs was detected in blood, while GLCs had a distinct absence of *TLR1*and *TLR8* expression (Fig. 1B).

Quantitative PCR was used to assess the variable expression of the prototypic receptor, *TLR4*, on GLCs between 18 individual patients. Analysis showed that GLC expression of *TLR4* varied 15-fold between the highest and lowest expressing patients (Fig. 2).

Granulosa–luteal cells increase expression of inflammatory mediators in response to pathogen-associated molecules

Granulosa–luteal cells were challenged for a period of 24 h with one of the three pathogen-associated molecules: *E. coli* LPS (endotoxin, TLR4 agonist), FSL-1 (synthetic lipoprotein, TLR2/6 agonist), or Pam3CSK4 (synthetic triacylated lipopeptide, TLR1/2 agonist). Expression of the proinflammatory cytokines *IL1B* and *IL6* and the chemokine *CXCL8* was evaluated. Challenge of GLCs with LPS showed a dose-dependent increase in *IL1B*, *IL6*, and *CXCL8* expression after 24 h (Fig. 3A, B and C; $P \leq 0.05$). Challenge with FSL-1 showed a significant increase in the expression of *IL6* and *CXCL8*, but only at higher concentrations (Fig. 3D, E and F; $P \leq 0.05$). Challenge of GLCs with Pam3CSK4 showed some numerical increase in the expression of *IL6* (Fig. 3G, H and I; not statistically significant). The human monocyte cell line, THP-1, was also challenged with pathogen-associated molecules under the same conditions as GLCs (Supplementary Fig. 2). THP-1 cells displayed very strong dose response to all three pathogen-associated molecules with increases to *IL1B*, *IL6* and *CXCL8* expression ($P \leq 0.05$).

Granulosa–luteal cells display an acute response to pathogen-associated molecules

Using LPS as the prototypical TLR4 agonist, a time–course experiment was performed to assess the acute responsiveness of GLCs. Following exposure to 100 ng/mL of LPS, GLCs displayed an increased expression of *IL1B* by 60 min, *IL6* by 90 min, and *CXCL8* by 120 min, which was maintained for 360 min (Fig. 4). Following treatment for 120 min, expression of *IL1B*, *IL6*, and *CXCL8* was higher than that observed in GLCs following 24 h (Fig. 3). The human monocyte cell line, THP-1, had a similar acute response to LPS compared with GLCs (Supplementary Fig. 3). THP-1 cells displayed increased expression of *IL1B* after 90 min and elevated *CXCL8* after only 60 min. Acute expression of *IL6* in THP-1 cells was muted. THP-1 cells had higher expression of both *IL1B* and *CXCL8* at 240 min compared with 24 h.

Effect of pathogen-associated molecules on granulosa–luteal cell expression of the steroidogenic enzymes CYP19A1 and STAR

Granulosa cells of bovine have been shown to alter the expression of steroidogenic enzymes after challenge with pathogen-associated molecules (Williams *et al.* 2008, Price *et al.* 2013), whereas rats

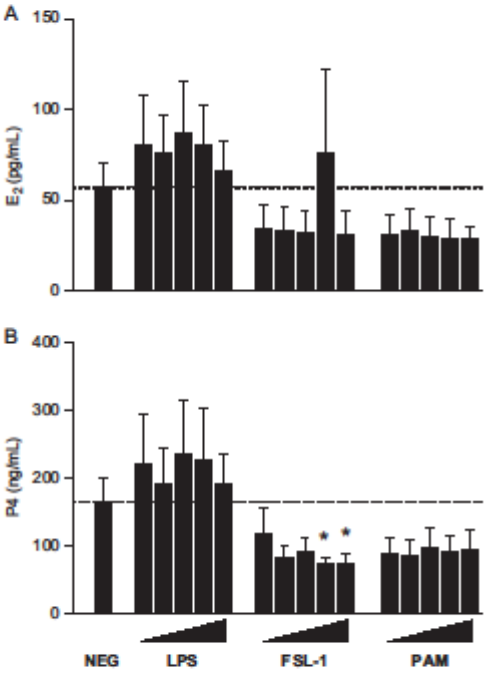

Figure 6 Pathogen-associated molecules alter the accumulation of steroid hormone in granulosa–luteal cells. Following a 24-h treatment of GLCs with tenfold increasing concentrations of *E. coli* LPS, FSL-1, or Pam3CSK4 (PAM), accumulation of estradiol (A) or progesterone (B) was assessed in cell-free supernatants using ELISA. Data are presented as mean ±S.E.M. from six independent experiments. *$P \leq 0.08$ compared with untreated controls; analysis by nonparametric Mann–Whitney *U* test.

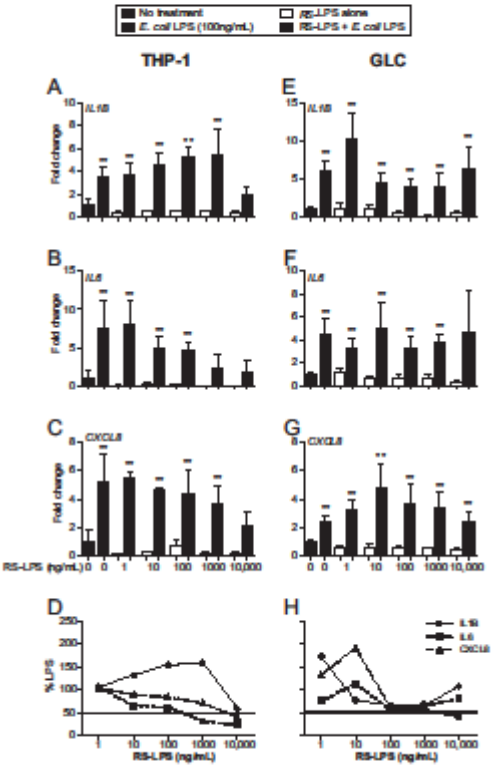

Figure 7 The TLR4 antagonist *Rhodobacter sphaeroides* LPS does not block the expression of *E. coli* LPS-induced inflammatory mediators in granulosa–luteal cells. GLCs were cultured in the presence of *E. coli* LPS alone ■, the TLR4 antagonist *Rhodobacter sphaeroides* LPS alone (RS-LPS; □), RS-LPS in combination with 100 ng/mL of *E. coli* LPS ■, or control culture medium ■. Following a 24-h treatment, the expression of the inflammatory mediators *IL1B* (A and E), *IL6* (B and F), and *CXCL8* (C and G) was assessed using real-time RT-PCR in THP-1 cells (A, B, C and D) or GLCs (E, F, G and H). Percent change of expression in cells exposed to RS-LPS in combination with *E. coli* LPS compared with LPS alone are presented for THP-1 (D) and GLCs (H). Data are presented as mean ±S.E.M. from three to eight independent experiments. **$P \leq 0.05$ compared with untreated controls; analysis by ANOVA followed by Dunnett's pairwise *post hoc* tests.

reduce estradiol secretion following exposure to LPS (Taylor & Terranova 1996). Interestingly, the human granulosa cell line (COV343) showed no change after challenge with pathogen-associated molecules (Price *et al.* 2012). We measured the expression of *CYP19A1* and *STAR* in GLCs following challenge with pathogen-associated molecules (Fig. 5). We did not detect any changes in the expression of either *CYP19A1* or *STAR* following any treatment.

Effect of pathogen-associated molecules on granulosa–luteal cell secretion of 17β-estradiol and progesterone

Although no effect on *CYP19A1* or *STAR* expression was observed following exposure of GLCs to pathogen-associated molecules, we measured accumulation of 17β-estradiol (Fig. 6A) and progesterone (Fig. 6B) in supernatants to confirm any effect on GLC function. Exposure of GLCs to higher concentrations of FSL-1 reduced progesterone secretion when compared with untreated controls (2.2-fold reduction; $P \leq 0.08$). In addition, exposure of GLCs to either FSL-1 or Pam3CSK4 resulted in a numerical reduction in the secretion of both 17β-estradiol and progesterone. Challenge of GLCs with LPS had no effect on either 17β-estradiol or progesterone accumulation.

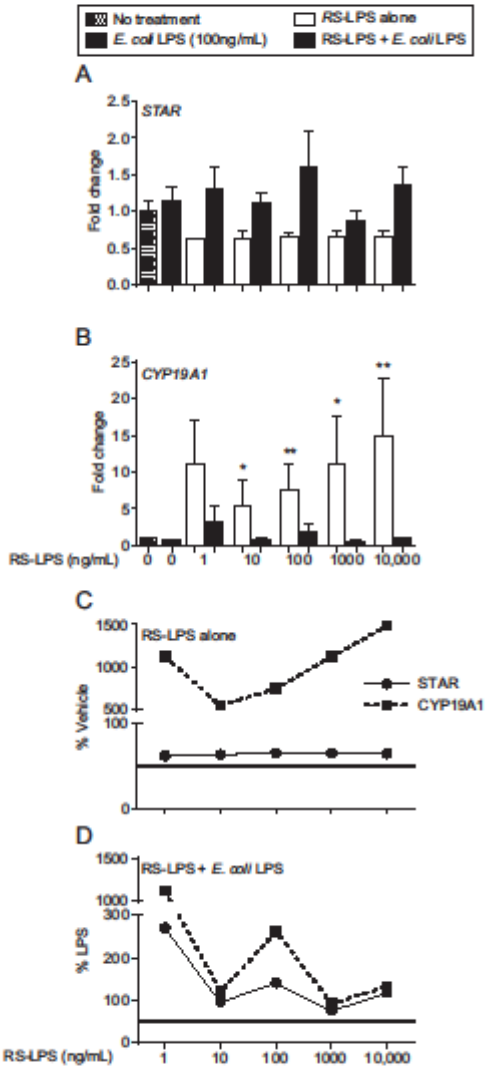

Figure 8 The TLR4 antagonist *Rhodobacter sphaeroides* LPS increases *CYP19A1* expression. GLCs were cultured in the presence of *E. coli* LPS alone ■, the TLR4 antagonist *Rhodobacter sphaeroides* LPS alone (RS-LPS; RS-LPS in combination with 100 ng/mL of *E. coli* LPS, or control culture medium). Following a 24-h treatment expression of steroidogenic factors, *STAR* (A) and *CYP19A1* (B) expressions were assessed using real-time RT-PCR. Percent change of RS-LPS alone-treated cells compared with untreated controls (C) and RS-LPS treated in combination with *E. coli* LPS compared with LPS alone (D) are presented. Data are presented as mean ± S.E.M. from three to eight independent experiments. $^{**}P \leq 0.05$; $^{*}P \leq 0.08$ compared with untreated controls; analysis by nonparametric Mann–Whitney *U* test.

The TLR4 antagonist Rhodobacter sphaeroides LPS did not block expression of E. coli LPS-induced inflammatory mediators

Lipopolysaccharide derived from the bacterium *Rhodobacter sphaeroides* (RS-LPS) blocks pathogenic LPS/TLR4 activation by competitive binding of the TLR4 co-receptor LY96 (MD2) at 100-fold excess concentrations (Rallabhandi *et al.* 2012). Here, we attempted to block pathogenic LPS/TLR4 activation by adding RS-LPS in the presence of *E. coli*-derived LPS and assessing the inflammatory markers above. To confirm the validity of using RS-LPS to block TLR4 signaling, the monocyte cell THP-1 was challenged with *E. coli* LPS in combination with RS-LPS (Fig. 7A, B, C and D). Expression of LPS-induced *IL1B*, *IL6*, and *CXCL8* was reduced to vehicle-treated control when exposed to a 100-fold excess of RS-LPS in THP-1 cells. Expression of LPS-induced *IL1B*, *IL6*, and *CXCL8* was reduced to 58.2, 23.1, and 41.7% respectively of LPS alone treatment when THP-1 cells were exposed to 100-fold excess of RS-LPS (Fig. 7D). Challenge of GLCs with RS-LPS alone had no effect on the expression of the inflammatory mediators *IL1B*, *IL6*, or *CXCL8* (Fig. 7E, F, G and H). The addition of RS-LPS did not block the increased expression of *E. coli* LPS-induced inflammatory mediators, even at 100-fold excess concentrations in GLCs. Expression of LPS-induced *IL1B*, *IL6*, and *CXCL8* was 106.9, 79.9 and 40.0% respectively of LPS alone-treated GLCs when exposed to 100-fold excess of RS-LPS (Fig. 7H).

The TLR4 antagonist Rhodobacter sphaeroides *LPS increases CYP19A1 expression in granulosa–luteal cells*

The expression of GLC *STAR* was not affected by either *E. coli* LPS or RS-LPS (Fig. 8A). As previously observed, *CYP19A1* expression was not affected in GLCs treated with *E. coli* LPS alone. However, challenge of GLCs with RS-LPS alone increased the expression of *CYP19A1* up to 14.8-fold over untreated controls (Fig. 8B). When GLCs were treated in combination with *E. coli* LPS and RS-LPS, expression of *CYP19A1* was comparable to untreated controls. Expression of RS-LPS-induced *CYP19A1* was between 549 and 1485% of untreated controls (Fig. 8C). Expression of *CYP19A1* or *STAR* in GLCs treated with *E. coli* LPS alone was comparable to GLC-treated cells in combination with RS-LPS (Fig. 8D).

Discussion

Infertility associated with the sequelae of bacterial infections of the upper reproductive tract is highly prevalent among women, estimated to represent approximately 20–30% of all infertility. Previous work has demonstrated that tubal pathologies associated with chlamydia or gonorrhea are the primary cause of infertility in these patients. However, more recent work has demonstrated that bacteria colonize the

ovarian follicle and that patients suffering PID show ovarian pathologies (Bychkov 1990, Pelzer *et al.* 2011, Pelzer *et al.* 2013). The data presented here suggest that GLCs can initiate an innate immune response to pathogen-associated molecules, potentially altering the microenvironment of the follicle. Although it is important to demonstrate the action of GLCs in initiating the immune response from a mechanistic stand point, it is also physiologically relevant as the ovarian follicle is normally replete of hematopoietic immune cells before ovulation when immune cells infiltrate the follicle to aid in the ovulatory process and control tissue damage (Brannstrom *et al.* 1994). Previous reports indicate that the cellular makeup of preovulatory human follicular fluid contains approximately 10% macrophages (Baranao *et al.* 1995), whereas in earlier follicle stages, immune cells are restricted to the ovarian theca (Brannstrom *et al.* 1993) and corpus luteum after ovulation (Petrovska *et al.* 1992). The potential of granulosa cells to alter secretion of inflammatory mediators in response to pathogen-associated molecules could alter the follicular environment or affect neighboring follicles. It is intriguing to consider the redundancies that exist between inflammatory pathways active during normal ovarian physiology and innate immune responses to pathogens. It may be that inappropriate activation of these pathways play a role in reducing ovarian function or oocyte quality following infection. A role of the chemokine IL-8 in promoting macrophages migration to preovulatory follicles has been suggested and is considered critical to facilitate the process of ovulation (Runesson *et al.* 1996). It could be presumed that inappropriate granulosa cell IL-8 secretion could recruit immune cells to the follicle before preparation for ovulation (Goto *et al.* 1997). Inflammatory mediators such as IL-1β and IL-6 have been demonstrated to be important to oocyte developmental competence and ovulation, as such inappropriate concentration or temporal expression may have negative consequences on fertility (Machelon *et al.* 1994, Passos *et al.* 2016). The TLR4 pathway has also been demonstrated to be important in physiological ovulation in mice, again suggesting that inappropriate activation of the pathway could have negative consequences on ovarian function (Shimada *et al.* 2008).

Previous work in both bovine (Price *et al.* 2013) and rodent (Taylor & Terranova 1996) models indicates that pathogen-associated molecules have the ability to induce changes in granulosa cell steroid hormone production. Although we did not observe any changes in the expression of the steroidogenic enzymes *CYP19A1* or *STAR* in GLCs following challenge with pathogen-associated molecules, we observed a small reduction in progesterone secretion from GLCs treated with the TLR2/6 ligand FSL-1. This discrepancy from previously reported changes to steroid production is likely related to gonadotropin-induced steroid production, which was not examined in these current studies. However, LPS does not alter FSH-induced steroid production in the human granulosa cell line COV343 (Price *et al.* 2012). Future experiments will determine the effect of pathogen-associated molecules on gonadotropin-dependent steroid hormone production in GLCs. Interestingly here, the TLR4 antagonist (RS-LPS) increased the expression of *CYP19A1* in GLCs. Currently, we have no clear explanation for this phenomenon; however, it has been shown that LPS from distinct pathogens can induce different cellular responses in other cell types (Pulendran *et al.* 2001). The mode of action for RS-LPS in this model system requires further investigation.

Although sexually transmitted infection-induced infertility is of great concern to the human population, animal models demonstrate the significant effect of upper reproductive tract infections on the ovary. Uterine infections are ubiquitous in dairy cows with approximately 40% of animals suffering significant bacterial uterine infections after parturition, many of which become infertile after the resolution of infection (reviewed in (Sheldon *et al.* 2009)). Cows that suffer these infections display disrupted ovarian function (Williams *et al.* 2008), whereas *in vitro* studies reveal that oocyte maturation is perturbed in the presence of LPS and granulosa cells initiate a significant inflammatory response to pathogen-associated molecules (Herath *et al.* 2007, Bromfield & Sheldon 2011). Similarly, in rodent models, exposure to LPS alters steroidogenesis of granulosa cells (Taylor & Terranova 1996) and significantly reduces the follicular reserve in a TLR4-dependent manner (Bromfield & Sheldon 2013). Currently, there are a limited number of studies that have investigated the effect of upper reproductive tract infections on the ovary in humans.

Studies investigating the effect of pathogen-associated molecules on fertility treatments have demonstrated a clear association between the presence of LPS in culture components, follicular fluid, or menstrual effluent to poor reproductive outcomes (Snyman & Van der Merwe 1986, Fishel *et al.* 1988, Nagata & Shirakawa 1996, Kamiyama *et al.* 2004). Although these associations have been made in clinical care, it is interesting to see in this study that patients displayed significant variability in the expression of *TLR4* in GLCs. Although this study does not have details regarding infection status of patients, which may result in variable expression of TLR4, it is routine for all patients undergoing fertility treatment to be free of any clinical upper reproductive tract infection. However, patient variability may be a result of subclinical infection or various single-nucleotide polymorphisms present in the human populations. Human polymorphisms of *TLR4* have been associated with the severity of inflammatory conditions such as asthma and arthritis (Zhang *et al.* 2011, Perica *et al.* 2015). Similarly, TLR4 polymorphisms have also been associated with a decreased incidence of infections such as periodontitis (Sellers *et al.* 2015).

Although infection of the ovary itself is rare, it is proposed that systemic LPS exposure, or infection of tissues distant from the reproductive tract, can have impacts on the ovary. The presence of LPS-binding protein (LBP) in circulation is indicative of systemic LPS exposure by infection or possibly a "leaky gut wall", which allows the translocation of LPS from the gut to circulation. In women undergoing IVF treatment, a positive correlation between the presence of LBP in circulation and follicular fluid IL-6 accumulation suggests that systemic exposure to LPS affects the follicular microenvironment. The same study revealed a negative correlation between systemic LBP and serum progesterone, similar to what was observed in this study (Tremellen *et al.* 2015). In dairy cows, bacterial infections of the mammary gland result in altered steroidogenesis, ovarian function, and oocyte quality following natural or induced disease (Lavon *et al.* 2008, 2011, Roth *et al.* 2013, Asaf *et al.* 2014).

To conclude, human GLCs from fertility patients express the molecular machinery necessary to initiate an innate immune response to pathogen-associated molecules, increasing expression of inflammatory mediators. These studies build on those previously performed in human's investigating systemic endotoxemia and animal models of clinical disease. These data suggest that granulosa cells have the potential to respond to pathogens and consequently alter the microenvironment of the follicle, perturb oocyte quality, and potentially affect subsequently fertility.

Supplementary data

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Declaration of interest

The authors have no conflict of interest to report that could be perceived as prejudicing the impartiality of the research reported.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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APPENDIX E
SEMINAL VESICLE GLAND OVERVIEW

Seminal Vesicle Gland—Overview

John J Bromfield, Laila A Ibrahim, and Jason A Rizo, University of Florida, Gainesville, FL, United States

Email addresses: jbromfield@ufl.edu (J.J. Bromfield); lailaaibrahim@ufl.edu (L.A. Ibrahim); jason.rizo@ufl.edu (J.A. Rizo)

Development and Structure

The development of the seminal vesicle glands is testosterone dependent and follows a similar pattern in most mammals. The formation of the seminal vesicle glands occurs in parallel with the development of the mesonephros, a primitive excretory organ of the early embryo. The embryonic mesonephros consists of numerous tubules that form the urogenital ridge. Each tubule connects a vascular glomerulus at one end, and opens into the Wolffian excretory duct at the other. The cranial tubules and glomeruli of the Wolffian duct undergoes atrophy and the corporal section develops into the epididymis (Sadler, 2012). By the thirteenth week of development in the human an invagination forms in the caudal portion of the Wolffian duct close to the urogenital sinus, dividing it into the deferent duct and the ejaculatory duct. This invagination constitutes the earliest form of the seminal vesicle gland which is at first a straight tube that branches into numerous convoluted ducts later in development.

In rodents, the formation of the seminal vesicle glands begins at the end of gestation, around day 19. At this point in development the seminal vesicle gland epithelium increases in height and forms a lumen (Fig. 1). The density of granular endoplasmic reticulum in the epithelium, important for secretion, increases (Aumuller and Riva, 1992). These changes constitute early fetal preparations for the main adult secretory activity of the epithelium. In human embryos, seminal vesicles differentiation in completed by the seventh month of gestation.

Fully developed seminal vesicle glands are paired structures consisting of convoluted and curved tubular glands with numerous lateral projections located posterior to the prostate and urinary bladder, superior to the distal ureter, and posterior to the rectum (Fig. 1). Laterally, the ampulla of the vas deferens and the veins of the prostatic venous plexus lie along its medial margins. The principal cells of the seminal vesicle glands are slender columnar epithelium, containing numerous mitochondria and a well-developed rough-endoplasmic reticulum important for protein secretion. Basal cells of the seminal vesicle gland are small stellate cells with a compact cytoplasm and few organelles (Aumuller and Riva, 1992).

The growth, development, and differentiation of the seminal vesicle glands from birth to the onset of puberty is slow. Before birth the seminal vesicle gland epithelium is a hollow tube surrounded by a layer of mesenchyme. Following birth the epithelium undergoes dramatic growth and branching (Settle et al., 2001) (Fig. 1). Buds from the epithelium grow laterally into the mesenchyme as systemic androgen concentration increases, the elongating buds bifurcate, and the epithelium folds upon itself. After puberty, maximal androgen levels provoke growth of the seminal vesicles that triggers functional differentiation of the secretory epithelium. Finally, the glands develop to form sac-like structures which, in humans, have a capacity to store a volume of about 3.4–4.5 mL (Aumuller and Riva, 1992).

In humans, the duct of the seminal vesicle and the vas deference share a common ejaculatory duct that opens into the urethra (Hafez, 2000); however, the ejaculatory duct is not present in any of the domestic mammals, where each duct opens separately into the urethra (Setchell, 1991). Dogs and cats have no seminal vesicle glands, while boars are considered to have the largest seminal vesicle glands among domestic animals. In humans, there are some individual variations in size and storage capacity of the seminal vesicle glands; two to three ejaculations may deplete the seminal vesicle glands to such an extent that a refractory period of at least 2 days is required before the glands fully restore sufficient fluid for a normal ejaculate. Conversely, in the bull the seminal vesicle glands are capable of storing up to 50 mL of secretions, sufficient to provide fluid for up to a dozen ejaculates.

Secretion of Seminal Vesicles

The seminal vesicle glands are an androgen dependent organ that secretes a significant fraction of the fluid that eventually becomes semen (seminal fluid). In most species the major contribution to semen volume is provided by the seminal vesicle glands. For example, human seminal vesicle glands contribute between 70% and 85% of ejaculate volume, bull seminal vesicle glands produce approximately 50% of ejaculate volume, and in the mouse seminal vesicle glands produce approximately 90% of the ejaculate volume (Kierszenbaum and Tres, 2011). Seminal vesicle fluid is a thick, viscous, alkaline fluid, resulting in semen having a mild alkaline pH. The alkalinity of semen helps neutralize the acidic environment of the vagina to maintain sperm viability in the female reproductive tract.

Seminal vesicle gland secretions contain various proteins, enzymes, mucus, vitamins, amino acids, ions, minerals, flavins, and hormones. Seminal vesicle gland secretions are characterized by a high content of fructose. Fructose is the primary source of energy for

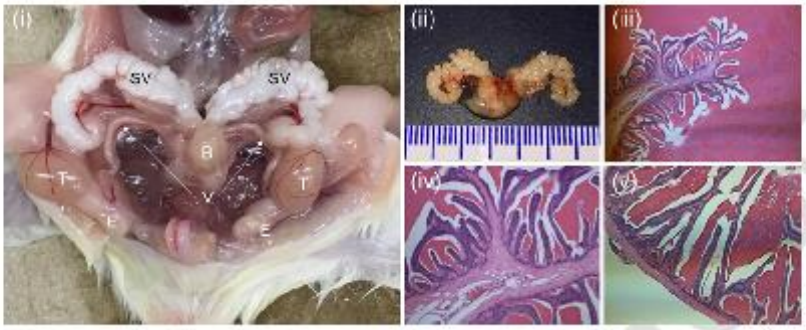

Fig. 1 *Mouse seminal vesicle glands*. Gross morphology of the male reproductive tract (i) showing the placement of the large seminal vesicle glands (SV; ii) above the bladder (B) and meeting at the juncture of the vas deferance (V). The relative size of the seminal vesicles is evident in comparison to the testicles (T) and tail of the epididymis (E). Photomicrographs showing the internal (iii, iv) and capsular (v) epithelium of the seminal vesicle gland at 10 × and 40 × magnifications. The columnar epithelium (*purple*) is evident against the dense seminal vesicle gland fluid (*pink*).

spermatozoa within the ejaculate. Other carbohydrates, inositol, glucose, fucose, ribose and sorbitol are also present in small amounts in seminal vesicle gland secretions. The lipid hormone, prostaglandin is found in all accessory sex glands but have their highest concentration in the seminal vesicle glands. Four types of prostaglandin are present in seminal vesicle gland secretions; prostaglandin A, B, E, and F. The secretions of the seminal vesicle glands contain ions including high concentration potassium, lower concentration sodium, while chloride is almost absent. The role of these ions is unclear but may center on sperm motility or contraction of muscle in male and/or female reproductive tracts. Large scale proteomic studies have identified upwards of 2000 seminal vesicle derived proteins in humans (Duncan and Thompson, 2007). Proteins required for the coagulation and liquefaction of semen such as semenogelins I, II, and kallikrein are secreted from the seminal vesicle glands and prostate and are integral to the functionality of semen (Lilja et al., 1987). Seminal vesicle secretions of induced ovulatory species such as camelids (including the llama) contain specific ovulation-inducing factors like β-nerve growth factor (β-NGF), which acts at the level of the brain to induce ovulation (Tribulo et al., 2015).

It would seem that while the seminal vesicle glands secrete a complex fluid, the main role of this fluid is to facilitate sperm survival and transport through both male and female reproductive tracts to enable fertilization. However, the high number of compounds in seminal vesicle secretions may suggest secondary roles in communication between sire and dam at the time of conception.

Role of the Seminal Vesicle Gland in Reproduction

The primary role of the seminal vesicle gland is to produce a supportive fluid to maintain sperm viability and functionality during transport through the male reproductive tract at ejaculation and female reproductive tract to facilitate fertilization of the ovum. Seminal vesicle fluid is rich in energy substrates, buffering agents and antioxidants expressly for the purpose of maintaining the functional capacity of the sperm. As sperm utilize carbohydrate for their energy to traverse the female reproductive tract they produce large amounts of reactive oxygen intermediates which can potentially harm the valuable paternal DNA contained within the sperm nucleus. High concentrations of antioxidant present in seminal vesicle gland fluid help to reduce potential oxidative stress and subsequent DNA damage. A major role of seminal vesicle gland derived prostaglandins is smooth muscle contraction. It is surmised that prostaglandins in semen aid in sperm transport in both male and female reproductive tracts by stimulating contraction of the testicular capsule, seminiferous tubules, epididymis, vas deferens, cervix and uterine body (da Silva et al., 2016). As semen is a coagulatory fluid the process of liquefaction is a necessity to release sperm to become free swimming in the ejaculate. The process of liquefaction requires seminal vesicle gland derived proteases to allow sperm to pass through the cervix after vaginal deposition at coitus.

The role of seminal vesicle gland fluid may not be as simple as solely maintaining sperm viability and facilitating sperm transport. Invertebrates including mosquitoes, crickets and flies use seminal vesicle gland fluid as a means to alter reproductive characteristics in mated females. In mice, horses, pigs and humans, seminal vesicle gland fluid induces significant changes to the maternal cellular environment of the endometrium or cervix. Interestingly, seminal vesicle gland fluid is nearly indispensable in natural conception, however assisted reproductive technologies such as artificial insemination and in vitro fertilization prove that seminal vesicle gland fluid is not a requirement for fertilization or successful pregnancy outcomes. Nevertheless, evidence in mice, cattle and women suggest exposure to seminal vesicle gland fluid may improve fertility and benefit pregnancy outcomes (Odhiambo et al., 2009, Bromfield et al., 2014, Tremellen et al., 2000).

While seminal vesicle gland fluid is no longer a requirement for fertilization due to new technologies it is interesting to consider its potential as a mediator of pregnancy success in a number of species.

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Glossary

Epithelium Cell layer that is commonly found lining glandular structures and mucosal surfaces. Mostly secretory in nature.
Fertilization The process of haploid sperm and egg forming a new diploid cell.
Mesonephros Excretory organ similar to the kidney active during fetal development that forms part of the male reproductive tract.

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BIOGRAPHICAL SKETCH

Laila Awad Ibrahim was born in Benghazi, Libya, in 1982. In September 2000, she began her studies in the Zoology Department at University of Benghazi, and she graduated with a Bachelor of Science in July 2004. She began a Master program in March 2005 and received her Master of Science degree in Zoology from the University of Benghazi in February 2009. She then worked at the University of Benghazi as a teaching assistant from 2005 to 2008. In 2009, she began work as a lecturer in the Zoology Department within the Embryology and Histology Program at the University of Benghazi. In January 2016, she moved to Gainesville, Florida, USA to join the Animal Molecular and Cell Biology Graduate Program in the Department of Animal Sciences at University of Florida as a Master of Science student under the supervision of Dr. John Bromfield. She obtained her Master of Science degree in May 2018.