SEMINAL PLASMA AND FERTILITY OF DAIRY COWS

By

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

UNIVERSITY OF FLORIDA

2019

To my parents, without whose support this achievement would not be possible

ACKNOWLEDGMENTS

First, I would like to thank God for allowing me to be here today and to help me conclude this chapter of my journey. Secondly, I would like to thank my parents for being the warm arms and calm voice when I needed them. For providing me their kindness when needed and their harshness when necessary. This achievement wouldn't be possible without their full support. I also thank my brother Thiago Ortiz for being my best friend during all these years; even when far apart, he always looked forward and cared for me. Great thanks go to all my friends and family, from my home town, college years, and the ones I made during my graduate school period. Naming them all would take a few pages here, but I can not leave without mentioning my beloved cousin Luis Henrique Dias and my brother from another mother Yuri Moreda who both were always there when I needed them the most in life.

I would like to extend my sincere gratitude to Dr. Peter J. Hansen, my major adviser, for believing in me and giving me the opportunity to join his lab when even I doubted myself and thought my dream was done and chances were over. I am lucky and very thankful for having the opportunity to have him as my advisor and sharing some of his knowledge with me. What I valued the most, was his sharing his life and dedicating his time to make each of us a better professional and a better person as well. He is a successful model to be followed, not just in science, but also on how to treat others that are part of the work environment. I can recall as if it was yesterday, in January of 2016 when I first received a call from him to stop by his office to talk with him, when he then offered me to join his team. Until today I am thankful for his patience and dedication towards not only me but all his students. I also appreciate Dr. John Bromfield as a co-adviser. He always had open door policy to share his advice. I also thank Dr. Klibs N. Galvao for serving as a member of my supervisory committee.

I am also grateful to my lab siblings: Eliab Estrada, Adriana Zolini, and Dr. Paula Tribulo who were the pillars to my success as a graduate student. Always collaborating, teaching, and supporting each other no matter what. Couldn't forget to thank Liz, who had great patience with me when she first started teaching me how to perform the proceedures for embryo production. She was always there whenever we needed her, whether at the farm during an experiment, the lab when something didn't look right, or checking on us when traveling to a conference.

I would also like to thank the owners and staff of Alliance Dairies, Trenton, FL, for allowing use of their cows and facilities to conduct my experiment. Thanks also go to the reproduction staff for support and assistance, in particular to Nilo Francisco and Bong Velasquez and the other members of the reproduction team, who performed intrauterine infusions and were patient with the team during the four months the experiment was being conducted. I also thank Adriana Zolini, Paula Tribulo, Eliab Estrada, Luciano Carvalheira, Jason Rizo, Bahroz Ahmed, Thiago Amaral, Guilherme Marquezini, and Fernanda Cavallari de Castro for technical assistance during the farm experiment and data collection when it was done. Thanks also go to Bo Harstine and Select Sire Inc. for providing the treatments and suggestions when asked.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
cDNA	Complementary DNA
Cont	Contrast
Conv	Conventional
CSF1	Colony-stimulating factor 1
CSF2	Colony-stimulating factor 2
d	Day
DPBS	Dulbecco`s phosphate buffered saline
h	Hour
ICM	Inner cell mass
IL1B	Interleukin 1 beta
IL6	Interleukin 6
Int	Interaction
Mult	Multiparous
NGF	Neurve growth factor
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocytes
Prim	Primiparous
RNA	Ribonucleic acid
rtPCR	Real time polymerase chain reaction
Sal	Saline
SAS	Statistical analysis system
SP	Seminal plasma
TAI	Timed artificial insemination

TGFB1	Transforming growth factor beta 1
TNF	Tumor necrosis factor alpha

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

SEMINAL PLASMA AND FERTILITY OF DAIRY COWS

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December 2019

Chair: Peter J. Hansen
Cochair: John J. Bromfield
Major: Animal Sciences

An inflammatory response is induced in the reproductive tract by the deposition of semen during natural mating. It has been proposed that this response could facilitate establishment and maintenance of pregnancy by modifying the microenvironment of the reproductive tract. Here we hypothesized that intrauterine infusion of 0.5 mL seminal plasma at the time of artificial insemination (AI) in first-service lactating Holstein cows will improve pregnancy success after insemination. Cows were inseminated (511 primiparous cows inseminated with X-sorted semen, 554 multiparous cows inseminated with X-sorted semen, and 627 multiparous cows inseminated with conventional semen) using the Double Ovsynch protocol. Cows were randomly assigned to receive intrauterine infusion of either 0.5 mL seminal plasma or vehicle immediately after AI. There was no effect of seminal plasma on the percent of inseminated cows diagnosed pregnant at Day 32 or 60 after AI, pregnancy loss, or percent of inseminated cows calving. Birth weight of heifer calves born using sexed sorted semen, but not conventional semen, were higher when seminal plasma was added. Results do not support a beneficial effect of seminal plasma on pregnancy success after AI, but are suggestive that seminal plasma could program development to affect phenotype at birth.

CHAPTER 1
LITERATURE REVIEW

Inflammatory Response to Semen

Deposition of semen in the female reproductive tract during natural mating not only provides the male gametes for achieving fertilization, but it also induces an inflammatory response. A transient uterine inflammation post-breeding is a normal physiological reaction observed in mouse (Robertson et al., 1992), pig (Kaeoket el at., 2003; Rodriguez-Martinez et al., 2010), horse (Katila et al., 1995; Christoffersen and Troedsson, 2017), human (Sharkey et al., 2007), cow (Howe and Black, 1963; Alghamdi et al., 2009), and sheep (Mattner, 1969).

The typical inflammatory response is characterized by invasion of polymorphonuclear leukocytes (PMN) that migrate from the blood to the injured site as a consequence of chemo-attractant forces (Kaplanski et al., 2003). In the female, during and post mating, neutrophils are recruited to the reproductive tract to act in sperm selection, and removal of foreign organisms and molecules introduced during mating (Schjenken et al., 2014).

The presence of spermatozoa in the uterus induces rapid chemotaxis of PMN. These cells can be detected in the uterine lumen 30 min after artificial insemination in pig and horses (Katila et al., 1995; Kaeoket el at., 2003), reaching a maximum count 3-6 h (Rodriguez-Martinez et al., 2010) and 4-8 h (Katila et al., 1995) in pig and horses, respectively. Inflammation is normally resolved by 48 h (Katila et al., 1995). Presence of PMN has also been reported in cattle (Howe and Black, 1963) with a maximum count taking place less than 2 h after artificial insemination (Alghamdi et al., 2009).

The mouse has been the most studied model regarding effects of seminal plasma on the female. There is a short uterine inflammatory response for 1 to 3 days after mating with cytokines levels in uterine fluid being of higher concentration than concentrations in non-

pregnant females (Robertson et al., 1992; Sanford et al., 1992). This elevation in the cytokines and chemokines modulates the female immune response and drives recruitment of pro-inflammatory leukocytes into the uterus (mainly composed of neutrophils, macrophage and dendritic cells) (Robertson et al., 1998; Robertson et al., 1996). Robertson's group first described this inflammatory response in the early 1990's. She described an increase in expression of colony-stimulating factor 1 (CSF1) and interleukin 6 (IL6) in the uterus one day after mating when compared with non-pregnant female mice (Robertson et al., 1992). The increase was followed by a rapid leukocyte recruitment to the uterus (Robertson et al., 1996).

The function of the transient post-mating inflammation has been proposed as a mechanism to eliminate contaminating bacteria and excess spermatozoa introduced into the uterine lumen in mares (Troedsson, 2006). More recently, it has been proposed that this inflammatory or immune response to semen could facilitate the establishment and maintenance of pregnancy and alterations in fetal development by modifying the microenvironment of the uterus (Bromfield, 2016).

Nature of the Signal

Semen is composed of spermatozoa and seminal plasma. Both sperm cells and seminal plasma contain a variety of bioactive signaling factors that are delivered during the time of mating to females. When intromission occurs, there is an active response in female reproductive tract to these bioactive factor to propagate cellular changes in the female reproductive tract. It is not clear if sperm itself is the cause of such inflammatory response, or seminal plasma alone. Evidence shows that sperm is critical to trigger an acute leukocyte response in horses, cows, pigs, and sheep (Clark et al., 1976; Katila, 1995; Rozeboom et al., 1999; Mattner, 1969; Scott et al., 2009). The fact that sperm is coated with seminal plasma derived-factors when transitting

through the female reproductive tract, however, means that the original signal could possibly be from seminal fluid (Robertson et al., 2016).

Seminal plasma contains a range of signaling molecules, including cytokines such as transforming growth factor beta (TGFB), CD38, prostaglandins, sex steroid hormones, neuron growth factor (NGF), and proteins (Sharkey et al., 2016; Kim et al., 2015; Remes Lenicov et al., 2012; Ratto et al., 2012). In mice, these factors act on the luminal epithelial cells on the surface of the endometrium causing differential expression of hundreds of mRNA and microRNAs (Robertson, 2007) and leading in many cases to local release of cytokines and chemokines (Maegawa et al., 2002; Mann, 1964; Robertson et al., 2005; Robertson et al., 2014).

Research using artificial insemination in woman shows that when using seminal plasma infusion, there is a leukocyte reaction in the uterine cervix (Thompson et al., 1992). Also, there is a similar response during coitus. It is reported that after unprotected coitus an inflammatory response is observed and cytokines are expressed in larger quantities when compared with samples of protected coitus (Sharkey et al., 2012) discarding the possibility of physical stimulation as a cause of the immune response. The total volume of seminal plasma that actually enters the uterus through the cervix, is not available in the literature.

Does Seminal Plasma Enhance Female Fertility?

Seminal plasma is not required for successful reproduction as indicated by viable pregnancies achieved using epididymal or washed ejaculated sperm in artificial insemination or in vitro fertilization followed by embryo transfer (Pang et al., 1979). Nevertheless, the role of seminal plasma on fertility has been postulated after observing a decrease in pregnancy rate when female mice were mated with male without seminal vesicles (Pang et al., 1979). Moreover, embryo transfer protocols in rodents imply the use of vasectomized males for estrus synchronization of the recipient females, which then experience greater fetal losses than

recipients exposed to seminal plasma (Robertson et al., 2005). Evidence of the role of seminal plasma on fertility are also available in other species. In pigs, for example, conception and farrowing rates were greater in females that received a fertile AI diluted with seminal plasma compared with extender (Rozeboom et al., 2000). In cattle, intracervical deposition of seminal plasma improved pregnancy rates marginally in cows with compromised fertility; i.e. conception rates below 50% (Odhiambo et al., 2009). In horses, suspension of spermatozoa in seminal plasma increased pregnancy rates (Alghamdi et al., 2004).

Seminal plasma may act as a positive or negative regulator of the expression of cytokines that affects embryo development. There is evidence that seminal plasma has a beneficial status to fertility in multiple species including bovine (Odhiambo et al., 2009), equine (Alghamdi et al., 2004), swine (O'Leary et al., 2004), human (Coulam and Stern, 1995). The outcomes of these different cytokines present in seminal plasma and the inflammatory response generated after mating are different in between species, but it is a mediator to fertilization across them.

The role of seminal plasma in fertility may be a consequence of its effects on spermatozoa. Upon ejaculation, spermatozoa are exposed to a set of surface re-modeling components derived from the accessory glands. Thus, secretions from the male reproductive tract participate in biological processes spermatozoa have to undergo during their transit through the female genital tract (Poliakov et al., 2009). Positive effects of exposure of spermatozoa to seminal plasma include prolonged survival time (Du et al., 2016), improved membrane integrity (Du et al., 2016), and fertility in vivo (Barranco et al., 2016). The exposure of sperm cells to seminal plasma, however, may also have negative effects. For example, it has been shown that the presence of some metal, such as arsenic and cadmium, in seminal plasma has an inverse correlation with progressive and total sperm motility (Wang et al., 2017).

Besides the role of seminal plasma on spermatozoa, the role of seminal plasma on fertility may be mediated by its interaction with the female genital tract. Consequences of such interactions include induction of endometrial secretion that either prepare the uterine environment for implantation, and/or regulate embryonic development. To illustrate, a study in mice showed that females mated with males lacking seminal vesicles had reduced conception rates, embryos with poor development to the blastocyst stage and low implantation rates, and offspring which experienced sex-dependent changes in postnatal phenotypes (Bromfield et al. 2014). In addition, intrauterine infusion of seminal plasma increased the number and viability of embryos recovered at Days 5 and 9 after artificial insemination in gilts (O'Leary et al., 2004).

There is some indication that seminal plasma may play a modulatory role in the cow to create a uterine environment conducive to fertility. Odhiambo et al. (2009) observed that pregnancy per AI was numerically higher for lactating dairy cows receiving 0.5 ml seminal plasma at the time of insemination than for cows receiving no treatment. In the same study, pregnancy per AI for beef cows was also numerically higher for cows receiving seminal plasma infusion than for cows receiving infusion of bovine serum albumin or no treatment.

Possible Mechanisms for the Increase in Fertility Caused by Seminal Plasma

The mechanism by which intrauterine infusion of seminal plasma can improve fertility is not known but could be the result of changes that seminal plasma triggers within the endometrium. A number of studies in different species show that seminal plasma induces numerous changes within the endometrium or cervix. In the pig, seminal plasma modulates the cytokine milieu in uterine tissue (Waberski et al., 2015). In the rabbit, intrauterine presence of seminal plasma induces leukocyte migration in the vagina (Phillips et al., 1977). In the human, seminal plasma causes cervical leukocyte invasion (Pandya and Cohen, 1985). In mice, there is also a well characterized inflammatory response to seminal plasma (De et al., 1991; McMaster et

al., 1992, Robertson et al., 1996), and in cattle seminal plasma modulates expression of genes encoding endometrial inflammatory mediators (Ibrahim et al., 2018).

In mice, seminal plasma increased the tolerance to paternal alloantigen's leading authors to propose that the improvement of fertility driven by seminal plasma may be a consequence of enhanced maternal tolerance to the embryo (Robertson et al., 2009). A study in horses showed that suspension of spermatozoa in seminal plasma results in acceptable pregnancy rates compared to the low pregnancy rates obtained after suspension of spermatozoa in extender (Alghamdi et al., 2004). The mechanism by which seminal plasma increases fertility in this study was proposed to be due to reduction of sperm binding to leukocytes.

One of the factors identified in seminal plasma as the inducer of the inflammatory response to semen in the female reproductive tract is transforming growth factor beta 1 (TGFB1). This molecule initiates endometrial leukocyte infiltration by up-regulating expression of colony stimulating factor 2 (CSF2), among others (Robertson et al, 1997). It has been shown that CSF2 can cause several effects on embryonic development, including greater competence to develop to the blastocyst stage (de Moraes et al., 1997; Loureiro et al., 2009; Dobbs et al., 2013), reduced apoptosis (Loureiro et al., 2011), increased survival of inner cell mass (ICM) in a pluripotent state in culture (Dobbs et al., 2013), and alteration in gene expression of both cell types present in the blastocyst, ICM and trophectoderm (Ozawa et al., 2016). Moreover, embryos exposed to CSF2 have greater competence to establish and maintain pregnancy (Loureiro et al., 2009; Denicol et al., 2014). All this information positions CSF2 as the best studied embryokine and raises the possibility that seminal plasma alters the expression of genes in the endometrium to improve the uterine environment that better supports embryonic development.

CHAPTER 2
EFFECTS OF INTRAUTERINE INFUSION OF SEMINAL PLASMA AT ARTIFICIAL INSEMINATION ON FERTILITY OF LACTATING HOLSTEIN COWS

Introduction

An inflammatory response is induced in the reproductive tract by the deposition of semen during natural mating (see reviews by Robertson, 2005; Schuberth et al., 2008; Katila, 2012; Bromfield, 2016). In cattle, the inflammatory response to mating is less well described than in some other species but includes accumulation of neutrophils (Howe and Black, 1963; Mattner, 1968) and, based on responses to seminal plasma, changes in gene expression in the endometrium (Ibrahim et al., 2019).

The inflammatory response to mating helps clear microorganisms and spermatozoa (Hansen et al., 1987; Alghamdi et al., 2009) from the reproductive tract. In addition, it has been proposed that inflammatory or immune responses to semen could facilitate the establishment and maintenance of pregnancy by modifying the microenvironment of the reproductive tract (Robertson, 2005; Bromfield, 2016). In the mouse, females mated with males lacking seminal vesicles had reduced conception rates, embryos with poor development to the blastocyst stage and low implantation rates, and offspring which experienced sex-dependent changes in postnatal phenotypes (Bromfield et al. 2014).

For other species, the importance of seminal plasma for modulating female fertility is unclear. Removal of individual accessory sex glands had no effect on fertilization in the golden hamster but embryonic death at day 9 of pregnancy was increased when females were mated with males in which either the ampullary gland or ventral prostate were ablated (Chow and O, 1989). Removal of seminal vesicles had no effect on fertility of bulls (Faulkner et al., 1968) or boars (Davies et al., 1975). However, intrauterine infusion of seminal plasma increased the number and viability of embryos recovered at Days 5 and 9 after artificial insemination in gilts

(O'Leary et al., 2004). In studies in the pig (Rozeboom et al., 2000) and mare (Alghamdi et al., 2004), addition of seminal plasma to extended semen improved fertility in gilts in which inflammation was induced by intrauterine infusion of lipopolysaccharide injection or killed sperm (Rozeboom et al., 2000) and mares in which inflammation was induced by intrauterine infusion of killed sperm (Algamdi et al., 2004). The observation that there was no beneficial effect of seminal plasma in the absence of inflammation in gilts (Rozeboom et al., 2000) is suggestive that beneficial effects of seminal plasma may depend on the degree of inflammation in the uterus.

The effectiveness of artificial insemination using extended semen in cattle is indicative that deposition of more than miniscule amounts of seminal plasma are not required for establishment of pregnancy. There is some indication, however, that seminal plasma may play a modulatory role in the cow to create a uterine environment conducive to fertility. Odhiambo et al. (2009) observed that pregnancy per AI was numerically higher for lactating dairy cows receiving 0.5 mL seminal plasma at the time of insemination (37.8%) than for cows receiving no treatment (33.2%). In the same report, pregnancy per AI for beef cows was also numerically higher for cows receiving seminal plasma infusion than for cows receiving infusion of bovine serum albumin (58.1% vs 55.1% in one series of studies and 67.0 vs 59.0% in another study) or no treatment (61.4% vs 52.4%).

The present experiment was performed to further evaluate the efficacy of seminal plasma for increasing fertility of cows. The hypothesis was that intrauterine infusion of 0.5 mL seminal plasma at the time of AI in first-service lactating Holstein cows will improve the percent of cows pregnant to first-service AI, reduce subsequent pregnancy losses and increase the percent of

inseminated cows that calf. Effects of intrauterine infusion of seminal plasma on birth weight of heifer calves was also evaluated.

Materials and Methods

The experiment was performed on a commercial dairy farm in north-central Florida (Alliance Dairy, Trenton, FL, 29°36′54″N 82°49′4″W) from November 2017 to February 2018. Cows were housed in free-stall barns equipped with fans and soakers, milked three times a day and fed a total mixed ration. The experiment followed the standard reproductive management protocols used in this farm. All procedures, including injections, pregnancy diagnosis, and timed artificial insemination (TAI) were performed while cows were restrained in self-locking head gates at the feed line. A total of 1692 first-service lactating Holstein cows [511 primiparous cows inseminated with X-sorted semen, 554 multiparous cows inseminated with X-sorted semen, and 627 multiparous cows inseminated with conventional semen] were subjected to TAI using the Double Ovsynch procedure (Souza et al., 2008) for first service at 75-82 d.

Each cow was inseminated with a straw of semen of the farm's choice. Bulls used for sexed semen were different than bulls used for conventional semen. The total number of sires used were 18 for sexed semen and 21 for conventional semen. Cows were assigned randomly to receive immediately after insemination an intrauterine infusion of either a straw of pooled seminal plasma (n=860) or saline [0.9% (w/v) NaCl] (n=832). In addition, for 5 weeks of the study (January-February), a third group of cows was included where cows were inseminated without infusion of seminal plasma or saline (n=249). Treatments were packaged in 0.5 cc straws by Select Sires Inc. (Plain City, Ohio) following all the Certified Semen Services minimum requirements for disease control of semen produced for artificial insemination. Semen was obtained from 44 different bulls collected on a single day. Semen was pooled, antibiotics (Shin et al., 1988) added and then semen was centrifuged at 1500 g for 15 min. The seminal plasma

supernatant was collected, passed through a 3 µm pleated filter to eliminate remaining sperm and then packaged in straws and frozen in liquid nitrogen. The sterile saline control solution was packaged in 0.5 cc semen straws and frozen in liquid nitrogen. The seminal plasma and saline were packaged in straws of different colors; treatments were blinded.

At the time of treatment, straws of seminal plasma or saline were thawed at 36.5ºC, loaded in AI guns and kept in warmer pockets until time of infusion. Treatments were infused in the uterine body. Pregnancy diagnosis was performed by transrectal ultrasonography at day 32 (+-4) and day 60 (+-7) after insemination. A cow was determined pregnant when an embryonic vesicle with a viable embryo (presence of heartbeat) was detected. Birth was recorded and body weights of heifer calves determined using an electronic WeightSouth VS-2501 scale (WeightSouth, Inc. Asheville, NC, USA).

The bioactivity of seminal plasma used for intra-uterine infusion was confirmed using in vitro culture. Briefly, 10 straws of seminal plasma were thawed and pooled under aseptic conditions, 10 straws of saline were also thawed and pooled. Bovine Endometrial Epithelial (BEND) cells (ATCC, Manassas VA) were cultured following the retailer's instruction in a 1:1 mixture of Dulbecco's Minimum Essential Media (DMEM) and Ham's F12 Nutrient Mixture medium supplemented with 1.5 g/L sodium bicarbonate, 0.034 g/L D-valine, 10% fetal bovine serum, and 10% horse serum (all from Fisher Scientific, Waltham MA). Cells were plated at 105 cells/mL in 24-well culture plates (TPP, Trasadingen Switzerland) at a final volume of 500 µL for 24 h at 38.5°C in a humidified 5% CO2 environment. Treatments containing either 1% v/v saline, 1% v/v seminal plasma or culture medium alone were applied to BEND cells for 24 h. Following treatment cells were washed with warm Dulbecco`s phosphate buffered saline (DPBS)

and stored in RLT Lysis Buffer (Qiagen, Hilden, Germany) at -80°C until processing to collect total cellular RNA.

RNA extraction was performed using the RNeasy Mini Kit according to manufacturer's instructions (Qiagen). RNA concentration was quantified with a NanoDrop ND1000 (Fisher Scientific), and a total of 1 μg of RNA was used for reversed transcription using the Verso cDNA kit (Fisher Scientific) according to manufacturer's instructions. Reverse transcription was performed for 1 cycle at 42°C for 30 min, followed by enzyme inactivation at 95°C for 2 min. The resultant cDNA was diluted 1:3 in molecular grade water and stored at -20°C.

Quantitative real time RT-PCR was performed in 20 μL reactions using iTaq Universal SYBR green chemistry (Bio-Rad, Hercules CA) with 100 nM of each forward and reverse primer. Primer details are described in Figure 2-2. A Bio-Rad CFX Connect light cycler was employed to perform quantitative PCR using a two-step protocol. Thermal cycling conditions were: 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. 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. Relative expression for genes of interest were calculated using the 2-ΔCt method relative to the housekeeping gene (GAPDH).

Statistical Analysis

Gene expression data were analyzed on log-transformed values by analysis of variance using the GLM procedure of the Statistical Analysis System (SAS) v 9.4 (SAS Institute, Cary, NC, USA). The model included treatment, which were partitioned into individual degree-of-freedom comparisons using orthogonal contrasts to compare no treatment and saline to seminal plasma and no treatment to saline.

Data on pregnancy per AI and pregnancy loss were analyzed with the GLIMMIX procedure of the Statistical Analysis System (SAS) v 9.4 (SAS Institute, Cary, NC, USA) with the dependent variable, pregnancy outcome (pregnant, non-pregnant), modeled as having a binomial distribution. Two analyses were performed. The first included all cows assigned to saline or seminal plasma treatments. The model included effects of group (primiparous-sexed semen; multiparous-sexed semen; multiparous-conventional semen), seminal plasma treatment, parity and the interaction. Contrasts were used to separate variation due to group and the treatment x group interactions into effects due to parity, (primiparous-sexed semen vs multiparous-sexed semen), semen type (multiparous-sexed semen vs multiparous-conventional semen) as well as treatment interactions with parity and semen type. The second analysis included cows from the weeks in which three treatment groups (no treatment, saline, seminal plasma) were included. The model was the same as above except orthogonal contrasts were also used to partition effects of treatment into the following comparisons: no treatment vs (saline + seminal plasma) and saline vs seminal plasma.

The GLIMMIX procedure was also used to analyze effects of treatment on gestation length and calf birth weight. Variables were considered continuous. Models were the same as described above except that sire was also included in the model as a random variable.

Results

The Effect of Seminal Plasma on BEND Cell Gene Expression

BEND cells were exposed for 24 h to either 1% (v/v) saline, 1% (v/v) seminal plasma or complete culture medium alone as a negative control (Figure 2-1). Exposure to seminal plasma increased expression of interleukin 1 B (IL1B) and IL6 by 8.9 and 22.8-fold compared to the negative control or saline, respectively (Figure 2-1 C-D; $P < 0.0001$). Expression of CSF2 or

tumor necrosis factor (TNF) were unaffected by seminal plasma treatment (Figure 2-1 A-B; P > 0.10). Exposure of BEND cells to saline had no effect on gene expression.

Pregnancy per AI

Pregnancy outcomes for each of the three groups of cows used (primiparous-sexed semen, multiparous-sexed semen and multiparous-conventional semen) are shown in Table 2-1. Group affected pregnancy per AI at d 32 (P=0.0017) and 60 (P=0.004) after insemination but not calving per AI (P=0.427). Orthogonal contrasts were used to partition effects of group into an effect of parity (primiparous-sexed semen vs multiparous-sexed semen) and semen type (multiparous-sexed semen vs multiparous conventional semen). There was a tendency (P=0.098) for pregnancy per AI at d 32 to be lower for primiparous cows than multiparous cows but effects of parity were not significant for pregnancy outcomes at other times. Semen type tended (P=0.056) to affect pregnancy per AI at d 60 but not at other times, with pregnancy per AI tending to be lower for cows inseminated with sexed semen. There was no main effect of treatment with seminal plasma at any time measured. There was, however, an interaction between seminal plasma treatment and semen type for pregnancy per AI at d 32 (P=0.041) and calving (P=0.098) because seminal plasma reduced pregnancies if cows were inseminated with conventional semen but not if cows were inseminated with sexed semen.

Pregnancy Loss

Data on pregnancy loss are also in Table 2-1. Group affected, or tended to affect, pregnancy loss from d 32 to 60 (P=0.073), 60 to calving (P=0.007) and d 32 to calving (P=0.096). The effect of group on pregnancy loss from day 32 to 60 was largely due to effect of semen type, with sexed semen being associated with higher pregnancy loss (P=0.064). Effect of group on pregnancy loss at later times was due to higher pregnancy loss for multiparous cows inseminated with sexed semen than for primiparous cows inseminated with sexed semen,

whether measured from d 60 to calving (P=0.024) or from d 32 to calving (P=0.048). Pregnancy loss was not affected by seminal plasma treatment. There was a tendency (P=0.095) for an interaction with semen type for pregnancy loss from d 32 to 60, but not at other times. The interaction involved seminal plasma increasing pregnancy loss in multiparous cows treated with sexed semen but not in primiparous cows inseminated with sexed semen.

Effect of Intrauterine Infusion on Fertility

To determine whether infusion itself affected fertility, a third group of cows that did not receive intrauterine infusion was included in the experiment for 5 weeks of the study. There was no difference between pregnancy outcomes for this group as compared to the two groups receiving infusions. Overall, the fraction and percent of cows pregnant at day 32 was 165/369 (44.7%) for vehicle, 165/368 (44.8%) for seminal plasma, and 116/249 (46.6%) for no infusion. For pregnancy at day 60, values were 162/369 (43.9%) for vehicle, 156/368 (42.4%) for seminal plasma, and 115/249 (46.2%) for no infusion. For calving, values were 135/369 (36.6%) for vehicle, 133/368 (36.1%) for seminal plasma, and 92/249 (36.9%) for no infusion.

Gestation Length and Birth Weights of Heifer Calves

Results are shown in Table 2-2. Gestation length was not affected by group, treatment or the interaction. Birth weight was affected by group (P=0.025). Use of orthogonal contrasts indicated that the group effect was due to birth weights being heavier for calves born from cows inseminated with sexed semen as compared to cows inseminated with conventional semen (P=0.007). Birth weight also tended to greater for calves born from cows treated with seminal plasma as compared to cows treated with saline (P=0.097). Although the interaction was not significant, examination of means was indicative that the effect of sexed semen occurred only for calves from cows inseminated with sexed semen. Moreover, the effect of sexed semen was significant (P=0.019) if analysis was limited to the subset of calves born using sexed semen.

CHAPTER 3
CONCLUSION

Results reported here do not support the hypothesis that seminal plasma improves fertility in first-service lactating Holstein cows. In general, there were no effects of seminal plasma on fertility with the exception for a tendency for seminal plasma to reduce pregnancy per AI at d 32 and calving per AI for multiparous cows inseminated with conventional semen. Among cows inseminated with sexed semen, however, there was a difference in birth weight between heifer calves born from cows treated with seminal plasma vs heifer calves born from cows treated with saline. This result suggests that changes in the endometrial environment caused by seminal plasma may change the developmental program of the resulting embryo in a way that caused increased birth weight. The lack of effect of seminal plasma on birth weight of calves derived from conventional semen may reflect the presence of residual amounts of seminal plasma in straws of conventional semen.

The lack of a positive effect of seminal plasma on fertility may reflect that seminal plasma does not have a biological role in regulating female reproductive function in cattle. Perhaps differences in the timing of development relative to insemination in the cow vs mouse makes inflammation important in one species but not another. For example, the blastocyst forms at day 3.5 in the mouse and implantation into the uterus takes place at day 4.0 (Kojima et al., 2014). These events occur later after insemination in the cow so that, perhaps, inflammatory responses in the uterus are less important for subsequent development. The embryo does not enter the uterus until day 4 or 5 after ovulation (Hackett et al., 1993), the blastocyst typically forms at day 7 (Betteridge and Fléchon, 1988) and attachment of the embryo to the endometrial epithelium is first initiated around day 20 of gestation (King et al., 1981).

It may also be that inflammation associated with semen deposition during natural mating is important for establishment of pregnancy in cattle but the treatment regimen used in the present experiment did not mimic the inflammation typically induced by natural mating. In the mare, intrauterine infusion of spermatozoa and seminal plasma provoked a greater infiltration of polymorphonuclear leukocytes in the uterus than did seminal plasma alone (Kotilainen et al., 1994). In the gilt, seminal plasma reduced the leukocytic response to intrauterine deposition of spermatozoa (Rozeboom et al., 1999).

The overall fertility in the herd used in the current experiment was high; the pregnancy per AI at d 30 was 45.4%. Perhaps, disorders in uterine function corrected by seminal plasma infusion were of low incidence in the herd so that seminal plasma was of limited value. If so, seminal plasma might be more beneficial for improving fertility in herds of cows in which fertility is low. In the study of Odhiambo et al. (2009), where there was a non-significant tendency for seminal plasma to increase fertility of lactating cows, pregnancy per AI for control cows was 33.2%.

It is possible that the amounts of seminal plasma introduced in the uterus do not mimic exposure of the uterus to seminal plasma after natural mating, where semen deposition occurs in the vagina and an unknown amount of seminal molecules is transferred to the uterus, either bound to spermatozoa (Plante et al., 2016) or transported by contractions of the reproductive tract (Hawk, 1987). Conceivably, introduction of too much seminal plasma into the uterus results in changes in endometrial function different than what is induced by exposure to molecules in seminal plasma after natural mating. Regardless, our in vitro data support a functional response of endometrial cells to the seminal plasma used for intra-uterine infusion. The data observed here confirm that the seminal plasma used was bioactive because of the increase in IL1B and IL6

expression in BEND cells. Changes in gene expression in BEND cells caused by seminal plasma were less than for cultures of endometrial explants cells (Ibrahim et al., 2018). In particular, there was no effect of seminal plasma on CSF2. This inconsistency could reflect that BEND cells, although derived from endometrium, are immortalized, and that regulatory interactions between various cell types present in endometrial explants is not possible for cultured BEND cells.

There are several reports that alterations of maternal physiology around the time of conception can modify development of the embryo to affect postnatal phenotype (Hansen et al., 2016; Fleming et al., 2018). Indeed, seminal plasma may function to program postnatal development as reported for exposure of female mice to secretions of male seminal vesicles (Bromfield et al., 2014). Consistent with this idea was the finding that heifer calves born from females treated with seminal plasma were larger at birth than heifers from females treated with saline, provided dams were inseminated with sexed semen. One needs to be cautious in interpreting interactions with semen type because different sires were used for sexed semen than conventional semen. It is possible, however, that a developmental programming effect of seminal plasma would be greater for calves derived from cows inseminated with sexed semen because of the loss of seminal plasma during the sex sorting process. Perhaps, the lack of effect of seminal plasma on birth weight in calves from conventional semen was because some seminal plasma remains in extended semen. Further longitudinal studies or the calves born in this study will reveal additional information regarding consequences of exposure of the dam to seminal plasma on postnatal development of the resultant offspring.

It is notable that there were differences in birth weights between calves derived from sexed semen vs conventional semen. It is possible that this effect simply reflects sire. However, there is one report that use of reverse-sorted semen for in vitro production of embryos has several

consequences for the offspring including reduced milk yield when female offspring themselves calve (Siqueira et al., 2017). Further work to evaluate whether use of sexed semen causes a change in the developmental program of the embryo is warranted.

Table 2-1. Pregnancy outcomes as affected by intrauterine infusion of saline or seminal plasma at the time of insemination. a

Group	Treatment	Pregnancy per AI, % (n/n)			Pregnancy loss, % (n/n)		
		Day 32	Day 60	Calving	d 32 to 60	D 60 to calving	D 32 to calving
Prim – sexed semen	Saline	42.5 (108/254)	39.4 (100/254)	35.0 (89/254)	7.4 (8/108)	11.0 (11/100)	17.6 (19/108)
	Seminal plasma	38.5 (99/257)	35.8 (92/257)	31.9 (82/257)	7.1 (7/99)	10.9 (10/92)	17.2 (17/99)
Multi – sexed semen	Saline	43.0 (114/265)	41.3 (109/264)	33.0 (87/264)	3.5 (4/113)	20.2 (22/109)	23.0 (26/113)
	Seminal plasma	48.1 (139/289)	42.6 (123/289)	35.0 (101/289)	11.5 (16/139)	17.9 (22/123)	27.3 (38/139)
Multi – conv. semen	Saline	52.4 (164/313)	51.1 (160/313)	40.6 (127/313)	2.4 (4/164)	20.6 (33/160)	22.6 (37/164)
	Seminal plasma	45.5 (143/314)	43.8 (137/313)	33.2 (104/313)	3.5 (5/142)	24.1 (33/137)	26.8 (38/142)
P-values	Treatment	0.425	0.185	0.233	0.126	0.953	0.429
	Group	0.017	0.004	0.427	0.073	0.007	0.096
	Cont: parity	0.098	0.148	0.866	0.754	0.024	0.048
	Cont: semen type	0.242	0.059	0.304	0.064	0.356	0.890
	Treatment x group	0.107	0.336	0.254	0.241	0.719	0.826
	Cont: treatment x parity	0.136	0.416	0.377	0.095	0.812	0.581
	Cont: treatment x semen type	0.041	0.141	0.098	0.320	0.424	0.992

[a] Cont = contrast

Table 2-2. Characteristics of heifer calves as affected by intrauterine infusion of saline or seminal plasma at insemination. a

Group	Treatment	Gestation length, heifers, days	Birth weight, heifers, kg
Prim – sexed semen	Saline	272.5 + 0.6	32.2 + 0.6
	Seminal plasma	272.6 + 0.6	33.9 + 0.6
Multi – sexed semen	Saline	273.3 + 0.6	33.3 + 0.6
	Seminal plasma	273.6 + 0.5	33.9 + 0.6
Multi – conv. semen	Saline	272.7 + 0.6	31.8 + 0.6
	Seminal plasma	273.9 + 0.7	31.6 + 0.7
P-values	Treatment	0.240	0.097
	Group	0.335	0.025
	Cont: Parity	0.155	0.258
	Cont: Semen type	0.800	0.007
	Treatment x group	0.694	0.246
	Cont: treatment x parity	0.919	0.283
	Cont: treatment x semen type	0.470	0.488

[a] Cont = contrast

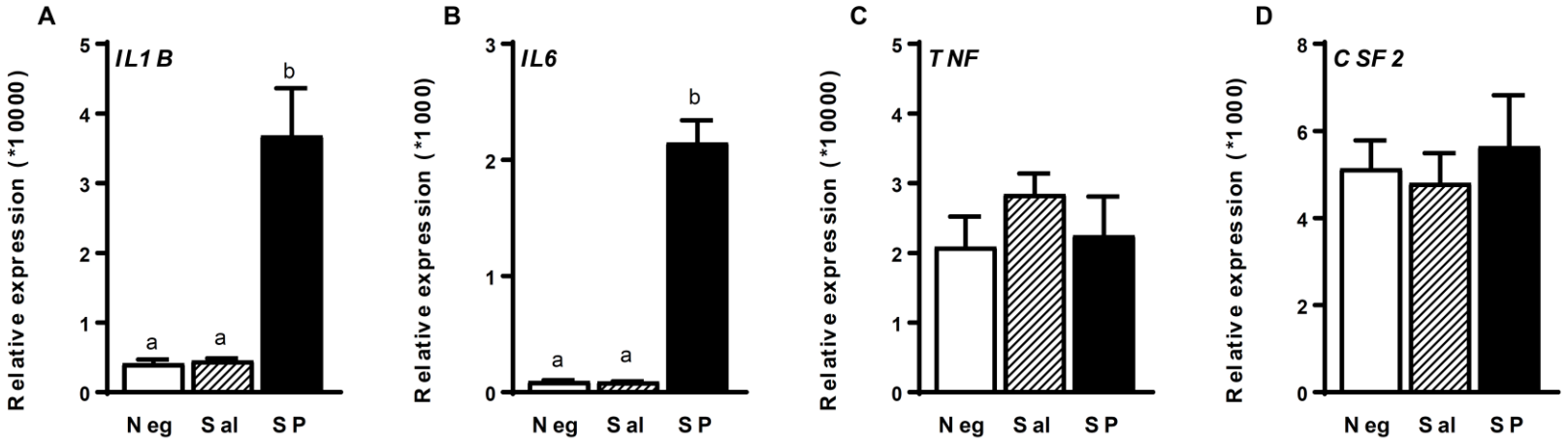

Figure 2-1. Effect of treatment of BEND cells with seminal plasma on expression of selected genes. Expression of IL1B (A), IL6 (B), TNF (C), and CSF2 (D) in BEND cells treated for 24 h with 1% (v/v) seminal plasma (SP), 1% v/v saline (Sal) or complete culture medium as a negative control (Neg) control. Data are presented as the mean relative expression + SEM from seven independent experiments. Data were log transformed and analyzed using the general linear model with pairwise comparison, compared to vehicle treated control only. Superscripts above each bar differ ($P<0.0001$).

Gene	Primer	Sequence (5'–3')	GenBank accession number	Product size (bp)
GAPDH	5'	AGGTCGGAGTGAACGGATTC	NM_001034034.2	114
	3'	ATGGCGACGATGTCCACTTT		
CSF2	5'	TTCCTGTGGAACCCAGTTTATC	NM_174027.2	114
	3'	TTTGGCCTGCTTCACTTCT		
IL1B	5'	CTTCATTGCCCAGGTTTCTG	NM_174093.1	142
	3'	CAGGTGTTGGATGCAGCTCT		
IL6	5'	ATGACTTCTGCTTTCCCTACCC	XM_015468553.1	180
	3'	GCTGCTTTCACACTCATCATTC		
TNF	5'	CACATACCCTGCCACAAGGC	NM_173966.3	261
	3'	CTGGGGACTGCTCTTCCCTCT		

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

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BIOGRAPHICAL SKETCH

William Vinícius Bueno Góis Ortiz was born in 1991, in Ji-Paraná, Rondônia, Brazil. He is the oldest son of Edson and Renata Ortiz. He is also the oldest of the fourth generation to continue the legacy of his family tradition and involvement in the Brazilian cattle industry. He attended CEDUSP high school where he graduated in 2007. Following graduation, his family moved to the United States of America where William started his undergraduate studies at Southern Illinois University – Carbondale. During his undergraduate education, he pursued an interniship in California where he was introduced to the American dairy industry. He graduated in Animal Sciences in 2014 with a focus in beef production, but with an interest in dairy as well. As a result, after graduating, he accepted a job offer to work at Green Hill Dairy in Quitman, Georgia where he learned more of the management aspects of a grazing dairy. After observing the need and potential to bring this technology to Rondônia, William pursued a master's degree at the University of Florida in the Department of Animal Sciences under the supervision of Dr. Peter J. Hansen and John J. Bromfield. His main area of research was the use of seminal plasma during the time of insemination to improve dairy reproduction and alter the phenotype of the offspring. During this time, he was always continuously involved with the embryo production procedures in Dr. Hansen's laboratory and participated in many studies involving embryology and reproductive physiology of cattle. After completion of the requirements of the master's degree, William plans to return to Rondônia to start a career in his family's ranch by switching the focus of the ranch to a cow-calf beef operation and also improving reproductive management and technologies as an advisor and facilitator to local cattleman.