UTERINE DISEASE; UNDERSTANDING THE UNDERLYING CAUSES AND ENVISIONING SOLUTIONS

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UTERINE DISEASE; UNDERSTANDING THE UNDERLYING CAUSES AND ENVISIONING SOLUTIONS

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This thesis was carried out to improve our understanding regarding uterine disease in dairy cattle. Several independent research projects were conducted to address the following aims: i) Evaluate the association between the pattern of pro-inflammatory and anti-inflammatory cytokine gene expression by blood monocytes and uterine tissue, and incidence of uterine disease, ii) Evaluate the association between cellular and systemic energy status, and incidence of uterine disease, iii) Evaluate the association between a single nucleotide polymorphism in the neutrophil IL-8 receptor and incidence of uterine disease, iv) Evaluate the efficacy of prostaglandin F2α (PGF2α) in treating uterine disease, v) Evaluate the effect of early ovulation on the prevalence of uterine disease and fertility.

Chapter 2 describes the association between uterine disease and cytokine expression in blood monocytes from lactating Holstein cows. Cows that developed metritis had decreased gene expression and secretion of pro-inflammatory cytokines compared to cows that had subclinical endometritis (SCE) or healthy cows. The lower level of expression of pro-inflammatory cytokines around calving may impair activation of inflammation and predispose cows to the development of metritis.

Chapter 3 describes the association between SCE and cytokine expression by uterine tissue from Holstein cows. Gene expression of the main pro-inflammatory cytokines was decreased in the first week after calving but was increased at the time of

diagnosis of SCE in cows that had SCE.

Chapter 4 describes the association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. Cows that developed uterine disease experienced a greater degree of negative energy balance and had decreased ability to maintain PMN glycogen levels which could be the predisposing factor for disease because of decreased availability of oxidative fuels.

Chapter 5 describes the association between a single nucleotide polymorphism in the neutrophil IL-8 receptor (SNP +735) and incidence of uterine disease. Cows with the SNP +735 were not more likely to develop uterine disease.

Chapter 6 describes Effect of PGF2α on SCE and Fertility in Dairy Cows. Administration of PGF2α did not affect prevalence of SCE but increased hazard of pregnancy in cows without SCE and cows with low body condition score.

Chapter 7 describes the effect of Early Postpartum Ovulation on SCE and Fertility in Dairy Cows. Early postpartum ovulation was associated with decreased prevalence of SCE and improved fertility.

BIOGRAPHICAL SKETCH

Klibs Galvão was born in Iporá, a small town in the state of Goiás, which is located in the central west region of Brazil. He is the son of Osvaldina Alves Galvão and Edelvan de Sousa Galvão. Klibs' parents were farmers and taught him early on the value of hard work. He has one sister, Liliane Alves Galvão, who he grew up with, climbing trees and playing with dirt. As the older brother, Klibs always thought that was his responsibility to watch out for her. He was also the oldest grandson so it was also his responsibility to teach his young cousins how to fish, milk cows, and ride horses or other farm animals that they could put their hands on.

Growing up on a farm undoubtedly influenced his decision to become a veterinarian and later to pursue a career in science. Klibs was always curious and wanted to know and see how chickens laid eggs, how chicks developed and came out of the egg, and how cows gave birth. His interest in discovery was slowly being embedded in his professional life and became an integral part of his career. He attended the Federal university of Goiás and got his degree as a Doctor of Veterinary Medicine in 2002. The first two years of veterinary school were marked by parties and lots of "beverage", which helped to develop strong friendships that would last for his lifetime. However, things changed drastically in the third year when in a Friday evening he met his soul mate. Jackellyne was this gorgeous, vivacious, and fun brunette that was probably a bit out of her mind when she decided to go out with him that evning. All of a sudden, parties were no fun and Klibs wanted to spend every little second of his life with her.

Although his desire was to stay close to Jackellyne, the prospect of a career in Brazil was bleak; therefore, Klibs decided to go to the USA for an externship in a dairy farm in Maine. While the prospects in Brazil were bad, the prospects for his externship in Maine were even worse. Klibs was saved by Dr. Jose Santos, who

offered him an externship at UC-Davis. During the externship, Klibs was exposed to the large dairy operations in California as well as applied research. This experience further increased his interest in food animal production systems and science. This led him to go back to the USA to work with Dr. Santos, which he did for a year, before being accepted for a residency in Dairy Production Medicine at UC-Davis.

The 18 month period between his externship and acceptance for his residence was a great challenge for his relationship with Jackellyne. They spent most of the time away from each other, with her in Brazil studying to become a physical therapist and him in the USA. However, this test strengthened their love and increased their bond. Now they truly understood the importance and value of the presence of one another. The beginning of his residency was also the beginning of a new life; Klibs and Jackellyne were now married. The challenges of marriage were no less than the challenges of being apart but once again they came out stronger and were blessed with a daughter, Camila, in May of 2006. In August of 2006 Klibs finished his residency and they moved to Ithaca where Klibs was starting his PhD at Cornell University. Life in Ithaca was more serene; Klibs worked mostly regular hours but still managed to complete the requirements for his PhD by August of 2009. Now Camila was talking, maybe even talking too much, and Jackellyne was expecting their second daughter, Isabela. They were very happy and anxious to start a new chapter in their lives.

To my wife Jackellyne Galvão, for her love, dedication, and unconditional support.

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I am very grateful to my whole family. I am the son of many mothers and many fathers who have each contributed to the formation of my character and education. If it wasn't for their individual contributions this work would probably not exist. I am forever in debt with my mother who fought with all her strength and might to give me and my sister the best opportunities in life. She taught us the value of honesty and hard work. I thank my grandmother who helped to raise me and imprinted in me an almost unbearable sense of organization. I thank my aunt Elione who taught me good manners, helped me with homework in elementary school, which was a terrifying experience, and supported me in my early days in California. I am thankful to my uncle and aunt Valsuir and Deusarina, who were always close to me but became even more important after my dad past away, and I moved with them during high school. My uncle was definitely an inspiration, a model, and at times a father to me.

I extend my gratitude to my entire graduate committee: Dr. Julia Flaminio who helped me to take the first steps into the field of immunology, Dr. Hollis Herb, who has greatly helped to sharpen my epidemiological and analytical skills as well as my critical thinking, and Dr. Ron Butler who was a model for research excellence.

I thank my former mentor Dr. Jose Santos for his devotion and for his grueling way of teaching, as well as for all the opportunities that were opened to me because of his relentlessness. I am deeply grateful to my PhD mentor Dr. Rob Gilbert for believing in me and for giving me all the conditions to perform my work. The independence acquired while working with Rob will be invaluable for my career after my PhD.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BCS = body condition score

BHBA = beta-hydroxybutyrate

CXCR1 = interleukin-8 receptor-a

DIM = days in milk

DMI = dry-matter intake

DNA = deoxyribonucleic acid

ELISA = Enzyme Linked Immunosorbent Assay

HMS = hexose monophosphate shunt

HR = hazard ratio

IL = interleukin

LPS = lipopolysaccharide

MØ = macrophage

MyD88 = myeloid-differentiation factor 88

NEFA = nonsterified fatty acids

n-fold = fold change in gene expression

NRC = national research council

OR = odds ratio

P4 = progesterone

PBS = phosphate buffered saline

$PGF_{2\alpha}$ = prostaglandin F2α

PMN = polymorphonuclear

SCE = subclinical endometritis

RNA = ribonucleic acid

RNS = reactive nitrogen species

ROC = receiver-operating characteristic curve

ROS = reactive oxygen species

RT-PCR = real-time reverse transcriptase polymerase chain reaction

SNP = single-nucleotide polymorphism

TBS = tris-buffered saline

TLR = toll-like receptor

TMR = total-mixed ration

LIST OF SYMBOLS

α = alpha

β = beta

ε = error term

ρ = Spearman's rank correlation coefficient

CHAPTER ONE

INTRODUCTION

The relevance of uterine disease

Uterine diseases can be classified as puerperal metritis, clinical metritis, clinical endometritis and subclinical endometritis (SCE) (Sheldon et al., 2006). Puerperal metritis is characterized by the presence of an abnormally enlarged uterus and a fetid watery red-brownish uterine discharge, associated with signs of systemic illness and fever (> 39.5 ^{o}C), within 21 days in milk (DIM). Animals without systemic signs but with an enlarged uterus and a purulent uterine discharge within 21 DIM may be classified as having clinical metritis. Clinical endometritis is characterized by the presence of purulent uterine discharge after 21 DIM. Subclinical endometritis is defined by the presence of >10% neutrophils (PMN) in uterine cytology samples colleced between 34 and 47 DIM (Sheldon et al., 2006). Uterine diseases are highly prevalent in high-producing dairy cows. Metritis affects about 20.0% of lactating dairy cows, with the incidence ranging from 8 to > 40% in some farms (Curtis et al., 1985; Galvão et al., 2009; Goshen and Shpigel, 2006; Hammon et al., 2006; Huzzey et al., 2007; Markusfeld, 1984). Clinical endometritis also affects about 20.0% of lactating dairy cows, with the prevalence ranging from 5.0 to >30% in some herds (Galvão et al., 2009; LeBlanc et al., 2002; McDougall et al., 2007). Subclinical endometritis is the most prevalent of all uterine diseases; it affects ~ 40.0 to 50.0% of lactating dairy cows, with the prevalence ranging from 30.0 to >70.0% in some herds (Galvão et al., 2009; Gilbert et al., 2005; Hammon et al., 2006; Kasimanickam et al., 2004; Kasimanickam et al., 2006).

Uterine diseases cause decreased pregnancy per AI, extended interval to pregnancy (Fourichon et al., 2000; Galvão et al., 2009; Gilbert et al., 2005; McDougall

et al., 2007) and decreased milk yield (Goshen and Shpigel, 2006; Fourichon et al., 1999; Rajala and Gröhn, 1998), which leads to increased involuntary culling and economic loss (Bartlett et al., 1986; Sheldon, 2004). The cost per metritis case is US $106.00 (Bartlett et al., 1986). In the USA there are ~ 9 x 10^6 dairy cows. With an incidence of ~ 30%, the direct cost to the dairy producers would be ~ US$ 285 million per year. The decreased fertility is because of negative effects in the uterus and in the ovary. Uterine diseases cause lesions in the endometrium (Bonnett et al. 1991), disrupt endometrial function (Sheldon and Dobson, 2004), and impair embryo development (Soto et al., 2003; Hill and Gilbert 2008). Uterine diseases decrease luteinising hormone, first dominant follicle size and growth, follicular ability to secrete estradiol (which impairs ovulatory capacity) (Peter et al. 1989; Sheldon et al. 2002; Williams et al., 2008). After postpartum ovulation resumes, cows that developed uterine disease present prolonged luteal phases (Opsomer et al. 2000; Mateus et a., 2002), which can decrease time to insemination and conception. Besides economic losses, there is concern for animal welfare. Metritis clearly affects animal well being; affected cows lose appetite, become dehydrated, and show clear signs of pain such as an elevated tail head, arched back and vocalization during palpation of the uterus (Chenault et al., 2004; Huzzey et al., 2007; Sheldon et al., 2006).

The etiology of uterine disease

Uterine disease has a multifactorial etiology. Around calving and early in lactation, dairy cows undergo a period of negative energy balance in which the energy consumed does not meet the energy demands for growth, maintenance and milk production (Butler et al., 1981, Doepel et al., 2002). This period is characterized by a decrease in dry-matter intake (DMI), leading to a sharp decrease in glucose, an increase in body-fat mobilization in the form of nonsterified fatty acids (NEFA), and

accumulation of products of incomplete oxidation of NEFA such as beta-hydroxybutyrate (BHBA) (Vazquez-Añon et al., 1994). The metabolic and the hormonal changes that occur around calving are believed to compromise immune function and predispose cows to disease (Goff and Horst, 1997). The uterine bacterial load depends on the balance between bacterial contamination and the animal's immune-defense mechanisms; however, during the period of negative energy balance, dairy cows experience a reduction in PMN chemotaxis, phagocytosis and killing capacity (Cai et al., 1994; Kehrli and Goff, 1989; Gilbert et al., 1993). In particular, cows that develop uterine disease have a more pronounced decrease in DMI (Huzzey et al., 2007), an increase in NEFA and BHBA, and a decrease in blood PMN pathogen phagocytosis (Kim et al., 2005) and killing (Hammon et al., 2006). The high concentrations of cortisol and estradiol are also believed to contribute to the overall state of immunosuppression around calving (Goff and Horst, 1997).

The dairy cow is unique in the sense that virtually all cows are infected with bacteria right after calving (Shendon and Dobson, 2004). Bacterial culture of the postpartum uterus yields a wide range of isolates (Elliot et al., 1968; Galvão et al., 2009; Griffin et al., 1974; Sheldon et al., 2002); however, the main bacterial strains involved in uterine disease are *Arcanobacterium pyogenes* (*A. pyogenes*), *Escherichia coli* (*E. coli*), *Fusobacterium necrophorum* (*F. necrophorum*), and *Prevotella melaninogenicus* (*P. melaninogenicus*) (Bondurant et al., 1999; Bonnett et al., 1991; Gilbert et al., 2007; Huszenicza et al., 1999). In fact, *E. coli* might increase the susceptibility of the endometrium to subsequent infection with *A. pyogenes* (Gilbert et al., 2007; Williams et al., 2007) and that *A. pyogenes* might act synergistically with *F. necrophorum* and *P. melaninogenicus* to enhance the severity of uterine disease (Bonnett et al., 1991; Griffin et al., 1974; Ruder et al., 1981). Among their effects, *E. coli* releases bacterial-wall LPS (Williams et al., 2008); *A. pyogenes* produces an

exotoxin (Miller et al., 2007) and a growth factor for *F. necrophorum* (Sheldon and Dobson, 2004); *F. necrophorum* produces a leukotoxin; and *P. melaninogenicus* produces a substance that inhibits phagocytosis (Sheldon and Dobson, 2004).

Energy balance complications around calving

Although plasma NEFA (Hammon et al., 2006) and BHBA (Suriyasathaporn, et al., 2000) are associated with PMN function, glucose is the immediate source of energy for PMN functions such as chemotaxis, phagocytosis and microbial killing (Babior, 1984). Glucose serves as energy source for housekeeping functions through the ATP-forming glycolysis and also for NADPH formation through the hexose monophosphate shunt (HMS). Glucose is first metabolized by hexokinase to form glucose-6-phosphate; then, it can enter glycolysis or the HMS. The NADPH formed by the HMS is used by NADPH oxidase to generate superoxide anions (Babior, 1984). Most of the superoxide is used to form other ROS including H2O2, hydroxyl radicals, and HOCl, which function as microbicidal oxidants. The production of other microbicidals such as RNS also relies on NADPH from the HMS for the function of NO synthase which forms NO (which in turn reacts with ROS to form the microbicidal peroxynitrite) (Hancock, 1997). Neutrophils rely on different glucose sources for different functions; they depend mainly on extracellular glucose (but can use glycogen under hypoglycemic conditions) for the energy required for chemotaxis while they depend mainly on intracellular glycogen, and glycogenolysis, for the glucose necessary for phagocytosis and killing (Kuehl and Egan, 1980; Weisdorf et al., 1982a; Weisdorf et al., 1982b). Whereas chemotactic stimuli (such as FMLP, C5a, and aracdonic acid) accelerate glucose uptake, phagocytic stimuli (such as opsonized zymosan particles and cytochalasin B) failed to increase glucose uptake but increased glycogen breakdown (Weisdorf et al., 1982a; Weisdorf et al., 1982b). Therefore, the

low glucose concentrations observed in the first 10 days of lactation (Vazquez-Añon et al., 1994) might directly impair PMN chemotaxis and could lead to decreased PMN glycogen stores leading to decreased phagocytic and killing capability (and possibly chemotaxis), which would predispose cows to disease.

Glycogen-storage disease, a human condition characterized by the inability of leukocytes to process intracellular glucose (Kim et al., 2006), leads to defects in neutrophil respiratory burst, chemotaxis and calcium influx in response to bacterial stimulation that are very similar to the PMN defects observed in high-producing dairy cows early in lactation (Cai et a., 1994; Hammon et al., 2006; Kehrli et al., 1989a). Moreover, in a mouse model of glycogen-storage disease, peritoneal inflammation, macrophage production of chemokines and peritoneal PMN accumulation were decreased which suggests that all leukocyte types might be affected (Kim et al., 2006).

Immunological complications around calving

Neutrophils (PMN) are the main leukocyte type involved in bacterial clearance after uterine infection (Hussain, 1989; Gilbert et al., 2007). Phagocytes (macrophages and PMN) recognize pathogen-associated molecular patterns (PAMPs) present in microbial invaders through pattern-recognition receptors (PRRs). The most important group of PRRs is the toll-like receptors (TLRs) family. There are 10 well characterized and widely expressed TLRs in mammals (Akira et al., 2006); TLR1, TLR2, and TLR6 recognize lipids found in bacteria and fungi; TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids from viruses and bacteria; TLR4 recognizes lipopolysaccharide (LPS) and polysaccharides found in gram-negative bacteria (LPS), fungi (mannan), and parasites (glycoinositolphospholipids from *Trypanosoma*); TLR5 recognizes flagellin in flagellated bacteria; and TLR10 still has no recognized ligand

(Akira et al., 2006). After macrophages or PMN bind to microbes through recognition of PAMPs by TLRs, the plasma membrane flows around the microbial surface until the microbe is completely enclosed in an intracellular compartment called "phagosome" (Stossel, 1993). It is noteworthy that most phagocytic binding cannot occur without opsonization of the antigen. Phagocytic cells express receptors for the complement protein C3b (CR1) for the Fc portion of antibodies that will bind to opsonized antigens. With the antigen coated in these molecules, binding of the antigen to the phagocyte is greatly enhanced. Granules containing digestive and antimicrobial compounds such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are then emptied into the phagosomes (Hampton et a., 1998). The phagosome containing the ingested particle fuses with the lysosome and creates the phagolysosome for safe digestion.

Resident macrophages (MØs) and the uterine endometrium participate in the initial immune response against bacterial invasion. After contact with bacteria through TLRs, macrophages are stimulated to produce and release pro-inflammatory cytokines and chemokines [including tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8)] (Tzianabos, 2000). Binding of TLRs to PAMPs result in triggering of downstream signaling cascades and production of proinflammatory cytokines and chemokines. TLRs activate the same signaling pathway used for IL-1 receptor signaling, including recruitment of adaptor proteins such as myeloid-differentiation factor 88 (MyD88) and activation of downstream signaling cascade (Akira and Takeda, 2004). Later in the inflammatory process, macrophages release the anti-inflammatory cytokine interleukin-10 (IL-10) (Tzianabos, 2000). Importantly, TNFα and IL-1 stimulate the expression of adhesion molecules on endothelial cells and IL-8 for neutrophil and monocyte diapedesis and chemoattraction. Moreover, TNFα activates neutrophils and macrophages (promoting

increased phagocytosis and bacterial killing).

Likewise, the uterine endometrium can recognize PAMPs and are stimulated to produce pro-inflammatory cytokines and chemokines to attract and activate neutrophils and monocytes (Davies et al., 2008; Herath et al., 2006; Tzianabos, 2000). The uterine endometrium also provide the initial response against microbes by producing antimicrobial peptides and Mucin-1 (Selsted and Ouellette, 2005; Cornican et al., 2008; Davies et al., 2008). Resident MØs also contribute to the inflammatory process by release of cytokines and chemokines after phagocytosis of bacterial invaders (Tzianabos, 2000).

Neutrophils (PMN) are the main leukocyte type involved in bacterial clearance after uterine infection (Hussain, 1989; Gilbert et al., 2007). Recently, single-nucleotide polymorphisms (SNPs) in the neutrophil IL-8 receptor-a (CXCR1) were identified (Youngerman et al., 2004a). Cows with the genotype CC for the nonsynonymous SNP at position +777 (now known to be at position +735) relative to the published sequence (GenBank Accession No. U19947) had increased incidence of subclinical mastitis (Youngerman et al., 2004b). Cows might have the SNP in both (CC), one (GC), or neither (GG) of the alleles. The substitution of the nucleotide G with C results in a glutamine-to-histidine substitution which compromises receptor affinity for IL-8 and function. Neutrophils from cows with the genotype CC have decreased receptor affinity for IL-8, decreased calcium signaling, impaired migration, and reduced killing ability (Rambeaud and Pighetti, 2005; Rambeaud and Pighetti, 2006; Rambeaud and Pighetti, 2007). Interestingly, neutrophil function (such as killing ability and survival) was intermediate for cows with the genotype GC compared with CC and GG (Rambeaud and Pighetti, 2006) and migration was actually impaired compared with genotype GG (Rambeaud and Pighetti, 2005). Therefore, cows with a SNP +777 could have increased susceptibility to uterine disease because of reduced

neutrophil chemotaxis and function.

Neutrophils are involved not only in bacterial clearance after uterine infection (Hussain, 1989; Gilbert et al., 2007) but also in the release of the placenta after calving. Retained placenta (a major risk factor for metritis) is characterized by a reduction in chemotaxis of PMNs to the site of placental attachment and a reduction in PMN killing ability (Kimura et al., 2002). The reduction in chemotaxis was suggested to be a result of lower concentrations of interleukin-8 (IL-8) at the fetal and maternal placental interface (Kimura et al., 2002). Regarding uterine disease, cows with the greatest influx of neutrophils into the uterus have reduced incidences of bacterial infection and SCE (Gilbert et al., 2007). Therefore, the defect in PMN migration and killing ability that predisposes cows to retained placenta might also predispose cows to uterine disease, explaining the positive association between retention of placenta and metritis.

Treatment of uterine disease

The most common method of treatment of uterine disease is either intrauterine (Galvão et al., 2009; Goshen and Shpigel, 2006; Kasimanickam et al., 2005; LeBlanc et al., 2002; Thurmond et al., 1993) or systemic (Chenault et al., 2004) antibiotic administration. Currently in the USA, there is no approved antibiotic for intrauterine administration in dairy cows. Therefore, the method of choice is ceftiofur hydrochloride (Excenel RTU EZ, Pfizer Animal Health, New York, NY), a broad-spectrum third-generation cephalosporin in an oil suspension, which is approved for systemic administration for treatment of metritis in postpartum dairy cows at a dose of 2.2 mg/Kg of body weight. Although systemic administration of ceftiofur hydrochloride improves clinical signs of metritis (Chenault et al., 2004), the effects on fertility have not been evaluated. On the other hand, intrauterine treatment with 5 g

chlortetracycline twice weekly for 2 weeks prevented the negative effects of metritis on fertility and on milk yields in multiparous cows (Goshen and Shpigel, 2006).

A formulation containing 500 mg of cephapirin benzathine in 19 g emulsifier (Metricure®, Intervet, Boxmeer, The Netherlands) is approved for treatment of clinical endometritis by intrauterine administration in Canada, Europe, New Zealand, Australia, and other countries around the world. Intrauterine infusion of Metricure improved reproductive performance of cows with clinical endometritis (LeBlanc et al., 2002), and cows with a history of retained fetal membranes, stillbirths, or a vulval discharge after 13 DIM (McDougall, 2001).

Although there is no approved treatment for SCE, Metricure also improved reproductive performance of cows with SCE (Kasimanickam et al., 2005). Interestingly, in that study, prostaglandin F2α (PGF2α) had a similar beneficial effect (Kasimanickam et al., 2005). The benefit from PGF2α administration is believed to arise from induction of estrus in cows having a PGF2α-responsive corpus luteum; the estrus leads to physical expulsion of bacterial contaminants and inflammatory products as well as a possible improvement in the uterine defenses under low progesterone (Kasimanickam et al., 2005). It is generally agreed that a high-progesterone environment suppresses cervical mucus production, myometrial contractility, uterine-gland secretion and the phagocytic activity of uterine neutrophils (Frank et al., 1983; Hussain, 1989; Bondurant, 1999), and is therefore permissive of uterine infection. PGF2α is not only luteolytic but also appears to have pro-inflammatory actions that might enhance neutrophil function (Lewis, 2004). Because there is increased concern about bacterial acquisition of antibiotic resistance, PGF2α might provide an efficacious and safer method of treatment of SCE.

General aims

This thesis was carried out to improve our understanding regarding uterine disease in dairy cattle. We approached the problem (uterine disease) on three fronts: 1 – I evaluated the cow's ability to mount an inflammatory response. We hypothesized that cows that develop uterine disease would have decreased ability to mount an inflammatory response early in lactation (which could lead to poor PMN influx to the uterus and predispose to disease). To investigate that, I evaluated the association between the pattern of pro-inflammatory and anti-inflammatory cytokine gene expression by blood monocytes (chapter 2) and uterine tissue (chapter 3), and incidence of uterine disease. 2 – I evaluated factors that could affect the ability of PMNs to respond to an inflammatory process. I hypothesized that PMNs from cows that develop uterine disease would have impaired ability to respond to an inflammatory process (which could lead to poor PMN chemotaxis and function and predispose to disease). To investigate that, I evaluated the association between cellular (PMN) and systemic energy status with incidence of uterine disease (chapter 4), and evaluate the association between SNP in the neutrophil IL-8 receptor and incidence of uterine disease (chapter 5). 3 – I evaluated methods of treatment or prevention of uterine disease. I hypothesized that PGF2α would be an effective treatment for SCE because of physical cleansing and improved uterine immune defense during estrus. Furthermore, because cows that ovulate early in lactation have better energy balance, I hypothesized that those cows would also have improved uterine health. To investigate that, I evaluated the efficacy of PGF2α treatment in treating uterine disease (chapter 6), and also evaluated the effect of early ovulation on uterine health and fertility (chapter 7).

CHAPTER TWO

Association between uterine disease and cytokine expression in monocytes from lactating Holstein cows

Interpretive Summary: Monocyte gene expression and uterine disease. Galvão.

Cytokine gene expression and secretion by monocytes was evaluated in 42 cows from calving until 42 d after calving. Cows that developed metritis and subclinical endometritis (SCE) were compared to healthy cows. *E. coli*-stimulated monocytes from metritis cows had lower gene expression of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) when compared to SCE or healthy cows from calving until 14 d after calving; however, gene expression did not differ between SCE and healthy cows. Concentration of TNFα was decreased in the culture medium of *E. coli*-stimulated monocytes metritis cows compared to SCE or healthy cows at calving and at 7 and 21 d after calving. The lower level of expression of pro-inflammatory cytokines around calving might result in lower activation of inflammation and predispose cows to the development of metritis.

ABSTRACT

The objectives of this study were to compare monocyte gene expression and protein secretion of the main cytokines from calving until 42 d after calving in cows that do and do not developed uterine disease. Metritis was characterized by a fetid vaginal discharge and rectal temperature ≥ 39.5 ^{o}C in the first 14 d after calving. Subclinical endometritis (SCE) was determined at 42 d after calving when low-volume uterine lavage cytologic results indicated the presence of ≥ 10% neutrophils. Gene expression of cytokines was measured by real-time quantitative RT-PCR in *E. coli*-stimulated monocytes (target) in contrast to non-stimulated monocytes (calibrator), normalized to GAPDH (endogenous control). ELISA was performed with the culture medium of stimulated cells to quantify TNFα, IL-1β, and IL-8 production, and with plasma to measure IL-8. The final analysis included 42 cows. 8 with metritis, 14 with SCE, and 20 healthy controls. *E. coli*-stimulated monocytes from metritis cows had lower ($P < 0.05$) gene expression of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) when compared to SCE or healthy cows from calving until 14 d after calving; however, gene expression did not differ between monocytes from SCE and healthy cows ($P > 0.15$). Concentration of TNFα was decreased in the culture medium of *E. coli*-stimulated monocytes from metritis cows compared to SCE or healthy cows at calving, 7 and 21 d after calving. The lower expression of pro-inflammatory cytokines around calving might decreae activation of inflammation and predispose cows to the development of metritis.

Key words: metritis, subclinical endometritis, cytokines, gene expression, dairy cows

INTRODUCTION

The periparturient or transition period (3 wk before to 3 wk after calving) is critical in determining the health status of a dairy cow. The incidence of infectious diseases such as metritis, endometritis, and mastitis is the highest during this time (Goff and Horst, 1997). These diseases lead to major economic losses not only by negatively affecting milk productivity and production costs, but also by having long-term detrimental effects on fertility (Loeffler, et al., 1999; Jorritsma, et al., 2000; Cook, et al., 2001). Cows with metritis or endometritis have decreased pregnancy per artificial insemination (AI) and increased time to pregnancy (Gilbert et al., 2005; Goshen and Shpigel, 2006; Galvao et al., 2009).

Neutrophils are the main leukocyte type involved in bacterial clearance during uterine infection (Hussain, 1989; Gilbert et al., 2007). Retained placenta (a major risk factor for metritis) is characterized by a reduction in chemotaxis of neutrophils to the site of placental attachment as evidenced by lower levels of interleukin-8 (IL-8) (Kimura et al., 2002). Data in our laboratory indicate that influx of neutrophils into the uterus is critical to preventing uterine disease. Cows with the greatest influx of neutrophils into the uterus have reduced risk of bacterial infection and incidence of subclinical endometritis (SCE; Gilbert et al., 2007).

Resident macrophages participate in the initial immune response against bacterial invasion. After contact with bacteria, macrophages are stimulated to produce and release pro-inflammatory cytokines and chemokines, including tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8) and later to release the anti-inflammatory cytokine interleukin-10 (IL-10) (Tzianabos, 2000). Importantly, TNFα and IL-1 stimulate the expression of adhesion molecules on endothelial cells and IL-8 for neutrophil and macrophage diapedesis and

chemoattraction. Moreover, TNFα activates neutrophils and macrophages, promoting increased phagocytosis and bacterial killing.

We hypothesized that a decreased production of pro-inflammatory cytokines around calving would result in decreased neutrophil influx and bactericidal function in the uterus, and predispose to disease. The objective of this study was to evaluate monocyte gene expression or protein production of the main pro-inflammatory (TNFα, IL-1 beta, IL-6) and anti-inflammatory (IL-10) cytokines, and the main neutrophil chemokine (IL-8) between calving and 42 d after calving in dairy cows; in addition, compare these expression and secretion values between healthy cows and cows that developed uterine disease (either metritis or subclinical endometritis). Monocytes are the circulating pool of cells that will become macrophages. Macrophages were not derived from monocytes to avoid the confounding effect of the long period of culture necessary for differentiation of monocytes into macrophages.

MATERIALS AND METHODS

Animals, Housing, and Feeding

Forty-four cows (21 primiparous and 23 multiparous) from a commercial Holstein dairy farm located in the Cayuga County, New York, USA were enrolled in the study from April to September of 2007. The herd had 3,000 milking cows with a rolling herd milk production average of ~ 12,000 kg of milk per year. Cows were housed in freestall facilities and milked three times daily. The mean ± S.D. milk yield for primiparous and multiparous cows during the study period was 30.3 ± 4.1 and 36.9 ± 3.8 Kg/d, respectively. All cows were fed the same TMR tgat was formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% fat corrected milk.

Evaluation of Uterine Diseases

Cows were evaluated daily for signs of metritis in the first 14 DIM. Metritis was characterized by the presence of watery, fetid vaginal discharge and rectal temperature > 39.5 ^{o}C. Cows with clinical metritis received 2 boluses of aspirin (15.6 g of acetylsalicyclic acid per bolus; AgriLabs, St. Joseph, MO) until the fever was lowered or for a maximum of 3 days; and ceftiofur hydrochloride (Excenel® RTU EZ, ceftiofur hydrochloride sterile suspension, Pfizer Animal Health, NY) for 5 consecutive days at a dose of 2.2 mg/kg body weight. At 42 DIM, a low-volume uterine lavage was performed in all cows for diagnosis of SCE based on the proportion of PMNs out of a total of 200 cells, including all leukocyte types and epithelial cells but excluding erythrocytes, as previously described (Gilbert et al., 2005). Based on a larger experiment (Cheong et al., 2008), the proportion of PMNs used for classifying cows as having SCE was ≥ 10%. None of the cows classified as having SCE had overt reproductive tract exudate, but vaginoscopic examination was not performed.

Blood Collection, and Monocyte Isolation and Stimulation

From calving until 42 DIM blood (120 mL) was collected weekly from the jugular vein into two 60 mL syringes containing 3 mL of EDTA as anticoagulant. Blood was transported to the laboratory within 2 h and centrifuged at 1200 g for 25 min at room temperature. The buffy coat was harvested (around 5 mL), diluted 1:2 with phosphate buffered saline (PBS), overlaid on 10 mL of Ficoll-Paque Plus® (density = 1.077; GE Healthcare, Chalfont St. Giles, United Kingdom), and centrifuged at 1200 x g for 25 min at room temperature. The mononuclear-cell layer was collected, washed with PBS, and resuspended in a hypotonic lysis solution of 0.2% saline for 30 s to eliminate erythrocytes and thrombocytes; isotonicity was reconstituted with 1.6% saline solution. The leukocytes were washed with PBS and

resuspended to a concentration of 10 x 10^6 mononuclear cells/mL in culture medium [88% DMEM F12 (1:1) (Gibco-Invitrogen, Life Technologies Corporation, Carlsbad, California, USA), 10% bovine growth serum (Hyclone, Thermo Fisher Scientific Inc., Waltham, MA, USA), and 2% antibiotic/antimycotic (Gibco-Invitrogen)]. The proportion of monocytes and lymphocytes was measured from a slide prepared using a cytospin centrifuge (Cyto-Tek®, Sakura Finetek, Torrance, CA), air dried and stained using a Romanowski type of staining (CAMCO® STAIN PAK, Cambridge Diagnostic Products, Inc. Fort Lauderdale, FL). During cell counting, trypan blue (Gibco-Invitrogen) exclusion was used to describe cell viability. Typically, 10 to 12% of the cells were monocytes (the rest lymphocytes), and > 95% of the cells were viable. One milliliter of the cell suspension was added to each of 6 wells of a 24-well flat-bottom plate (Nunc, Thermo Fisher Scientific Inc., Waltham, MA, USA), and incubated for 1 h at 37°C, 5% CO_2. Subsequently, cells were washed with pre-warmed medium to remove non-adherent cells, and the adherent monocytes were incubated overnight at 37°C, 5% CO_2. The cells were washed again twice with pre-warmed medium without antibiotics to remove non-adherent cells, and 1 mL of the medium without antibiotics was added to each well. *E. coli* were added to 3 of the 6 wells for each cow at a ratio of 10 bacteria per adherent monocyte (assuming around 10^6 monocytes per well and adjusting for monocyte density). The other 3 wells for each cow were left non-stimulated. Based on a kinetic study to determine peak gene expression, one pair of wells with *E. coli*-stimulated (EC) and non-stimulated (NS) cells was harvested for mRNA isolation at 4 h to test pro-inflammatory cytokines, and at 8 h for the anti-inflammatory cytokine IL-10. The medium from the third pair of wells was harvested at 12 h after stimulation to measure cytokine concentrations by ELISA.

RNA Isolation and Real-Time RT-PCR

Total RNA was extracted using a commercial kit (RNeasy Mini kit, Qiagen, Hilden, Germany). For the first step (cell lysis), 600µL of the lysis buffer plus 2-mercaptoethanol (10µL 2ME/ 1mL RLT) was added directly into the wells after removing the medium. The rest of the protocol was followed according to the manufacturer instructions; the isolated RNA was eluted in 35µL of RNA free water, and treated for genomic DNA contamination using a commercial kit (DNase I kit, Gibco-Invitrogen). The samples were diluted to a final concentration of 5 ng RNA/µL water and stored at -80°C.

For the real-time reverse transcriptase PCR (RT-PCR), sets of primers and probes for all the cytokines and endogenous control (glyceraldehyde-3-phosphate (GAPDH)) were designed using published sequences and the Primer Express software (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) (**Table 2.1**). For the one-step method, the reaction mixture containing the universal mastermix (Applied Biosystems Cat# 4309169, TaqMan One-Step RT-PCR Master Mix, MultiScribe Reverse Transcriptase and AmpliTaq GOLD DNA Polymerase), forward and reverse primers, Taqman probe, and RNAse inhibitor was added to 10ng of total RNA. The fold difference (n-fold) in gene expression was calculated using the relative quantitation method (2^{-ddCt}) having GAPDH as the endogenous control and the NS cells as the calibrator for each EC sample.

Table 2.1. Primer and probe sequences used for real time RT-PCR.

Cytokine	Primer sequence (forward and reverse; 5' – 3')	Accession #
IL-1β	AGCATCCTTTCATTCATCTTTGAAG	M37211
	GGGTGCGTCACACAGAAACTC	
IL-6	GGGCTCCCATGATTGTGGTA	BC123577
	GTGTGCCCAGTGGACAGGTT	
IL-10	AGAACCACGGGCCTGACAT	U00799
	AGCTCACTGAAGACTCTCTTCACCTT	
TNFα	TCTTCTCAAGCCTCAAGTAACAAGT	NM_173966
	CCATGAGGGCATTGGCATAC	
IL-8	TGCTTTTTTGTTTTCGGTTTTTG	AF232704
	AACAGGCACTCGGGAATCCT	
GAPDH	GCATCGTGGAGGGACTTATGA	BC102589
	GGGCCATCCACAGTCTTCTG	
Cytokine	Probe sequence (5' – 3')	Accession #
IL-1β	CCTGTCATCTTCGAAACGTCCTCCGA	M37211
IL-6	TTCCTGGGCATTCCCTCCTCTGGT	BC123577
IL-10	TTCTGCCCTGCGAAAACAAGAGCAA	U00799
TNFα	AGCCCACGTTGTAGCCGACATCAACTC	NM_173966
IL-8	TAATCTTGCAACCCTCACCTGCTGGC	AF232704
GAPDH	CACTGTCCACGCCATCACTGCCA	BC102589

ELISAs for TNFα, IL-1β, and IL-8

ELISA for TNFα, IL-1β, and IL-8 concentrations was performed on the culture medium collected 12h after stimulation with *E. coli*. Also, IL-8 concentration in plasma from blood samples collected from calving until 21 DIM was determined using a human IL-8 ELISA kit (R&D Systems, Inc., Minneapolis, MN) validated for use in bovine (Shuster et al., 1996; Galligan and Coomber, 2000).

For TNFα and IL-1β ELISAs, flat-bottom 96-well plates (Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated overnight at 4 ^{o}C with 100 µL of 0.05 M sodium carbonate, pH 9.6, containing either rabbit monoclonal anti-bovine TNFα (1µg/mL) or rabbit polyclonal anti-bovine IL-1β (5µg/mL) antibodies (Pierce-Endogen, Thermo Fisher Scientific Inc., Waltham, MA, USA). The plates were allowed to warm to room temperature for 1 h, washed 3 times with 0.05% Tween 20 diluted in 50 mM Tris-buffered saline (TBS), pH 8.0, and subsequently blocked with 10% (diluted with TBS) Seablock blocking buffer (Pierce-Endogen) for 1 h at room temperature. The plates were washed 5 times with 0.05% Tween 20 diluted in 50 mM TBS, and 100 µL of culture medium or plasma were added to each well in duplicate. Recombinant bovine TNFα and IL-1β (Pierce-Endogen) were diluted to form a standard curve within each plate. The plates were incubated for 1.5 h and then washed 5 times as described above. Mouse monoclonal anti-bovine TNFα (AbD Serotec Inc. Kidlington, UK) or rabbit biotinylated polyclonal anti-bovine IL-1β (Pierce-Endogen) were diluted to a concentration of 5 µg/mL using 10% blocking buffer, and 100 µL were added to each well. The plates were then incubated for 1 h at room temperature, washed 5 times as described, 100 µL of Streptavidin (AbD Serotec) diluted 1:1000 in 10% blocking buffer were added to each well, and plates were incubated for 1 h at room temperature, in the dark. Following incubation, the plates were washed 5 times, and 100 µL of 3,3', 5,5' tetramethylbenzidine substrate solution (Sigma-Aldrich, St.

Louis, MO, USA) was added to each well; the reaction was allowed to proceed for 20 mins and stopped by adding 100 µL of 2 M H_2SO4. The absorbance was read at 450 nm on a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). A background correction reading at 570 nm was subtracted from the 450 nm absorbance reading.

Concentrations of TNFα, IL-1β, and IL-8 (ng/mL) in the medium were later normalized to the average concentration of total RNA (µg) at 4 and 8 hours after stimulation with *E. coli*. Results are expressed as ng/ml/µg of total RNA. The normalization was performed to adjust for individual variation in the number of adherent monocytes among cows. Simple linear regression showed that number of isolated monocytes was directly correlated ($R^2 = 0.99$) with the total RNA isolated when number of monocytes went from 100,000 to 500,000 (total RNA yield from 0.55 to 10.97 µg); however, the RNA yield plateaued after 500,000 monocytes. Our total RNA yield ranged from 0.5 to 10 µg. Within cow, there was little variation: the total RNA isolated from wells at 4 h after stimulation was highly associated with the total RNA isolated from wells at 8 h after stimulation ($P < 0.0001$; $R^2 = 0.73$); therefore, the average total RNA concentration between 4 and 8 h would be a good predictor of the total RNA at 12 h after stimulation.

Statistical Analyses

This was an observational cohort study. Two out of the 21 primiparous cows enrolled in the study were culled before the end of the observation period and were excluded from the analysis. Therefore, 42 cows (19 primiparous and 23 multiparous) were used for the final analysis for the association between uterine disease and cytokine gene expression and production. For statistical analysis, cows were classified into three categories: 1) cows that developed metritis in the first 14 DIM; 2) cows that

did not develop metritis but were diagnosed with subclinical endometritis at 42 DIM; 3) healthy control cows. Fold difference (n-fold) in cytokine gene expression (PCR), cytokine concentration in the culture medium (ELISA), from calving until 42 DIM, and cytokine concentration in plasma from calving until 21 DIM were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS (Version 9.1; SAS Inst. Inc., Cary, NC). A first-order autoregressive covariance structure was used. Because n-fold was not normally distributed, the statistical analysis was performed on the delta delta Ct (ddCt) values (normally distributed) and then converted to n-fold (2^{-ddCt}) for data presentation. Cytokine data from ELISAs were skewed; therefore, they were transformed to their natural logarithm for data analysis, and back transformed for data presentation. Normality of standardized residuals was evaluated by inspection of normal probability plots. Models included the effects of uterine disease, parity (primiparous vs. multiparous), time (day of blood collection; 0, 7, 14, 21, 28, 35, and 42), and 2- and 3-way interactions between uterine disease, parity and time. Metritis and parity were forced into the models but interactions were removed if $P > 0.15$. The cow was included in the analysis as a random effect. When either an effect of uterine disease or an interaction between metritis and time was observed, post-hoc multiple comparisons were performed using the Bonferroni adjustments in SAS.

Differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered a tendency. Interactions were significant when $P \leq 0.15$.

RESULTS

Of the 42 cows used in the study, 8 (19%) were diagnosed with metritis and classified as metritis for the statistical analysis; 18 (43%) were diagnosed with SCE, but only 14 were classified as SCE for the statistical analysis because 4 of them also

had metritis and therefore were classified as metritis; and 20 (48%) were not diagnosed with metritis or SCE and were classified as healthy controls. Mean and median day of diagnosis of metritis were 5 and 5 DIM, with a range from 3 to 8 DIM.

There was an interaction between uterine disease and time on TNFα gene expression ($P < 0.001$), and monocytes from cows that developed metritis had lower TNFα gene expression than monocytes from cows that had SCE ($P = 0.001$) or healthy cows ($P = 0.005$) at 7 and 14 d after calving; however, TNFα gene expression did not differ ($P = 0.32$) between monocytes from cows that had SCE and healthy cows (**Figure 2.1a**). TNFα gene expression decreased from calving until 14 d after calving, and then returned to the levels of calving by 28 d (time effect, $P < 0.001$; **Figure 2.1a**).

There was an interaction between uterine disease and time ($P = 0.10$), and monocytes from cows that developed metritis had lower IL-1β gene expression than monocytes from cows that had SCE ($P = 0.003$) or healthy cows ($P = 0.005$); however, IL-1β gene expression did not differ ($P = 0.53$) between monocytes from cows that had SCE and healthy cows (**Figure 2.1b)**. There was also an interaction between parity and time ($P = 0.03$) with monocytes from multiparous cows presenting lower IL-1β gene expression 7 d after calving than monocytes from primiparous cows (2.6 ± 1.0 vs. 10.0 ± 3.2; $P = 0.006$). IL-1β gene expression decreased from calving until 14 d after calving, returning to the levels of calving by 28 d (time effect, $P < 0.001$; **Figure 2.1b**).

There was an interaction between uterine disease and time ($P = 0.07$), and monocytes from cows that developed metritis had lower IL-6 gene expression than monocytes from cows that had SCE ($P = 0.007$) or healthy cows ($P = 0.01$); however, IL-6 gene expression did not differ ($P = 0.62$) between monocytes from cows that had SCE and healthy cows (**Figure 2.1c)**. There was also an interaction between parity and

time ($P = 0.07$) with monocytes from multiparous cows presenting lower IL-6 gene expression 7 d after calving than monocytes from primiparous cows (2.6 ± 0.9 vs. 7.1 ± 2.2; $P = 0.03$). IL-6 gene expression decreased from calving until 14 d, and then returned to the levels of calving by 28 d (time effect, $P < 0.001$; **Figure 2.1c**).

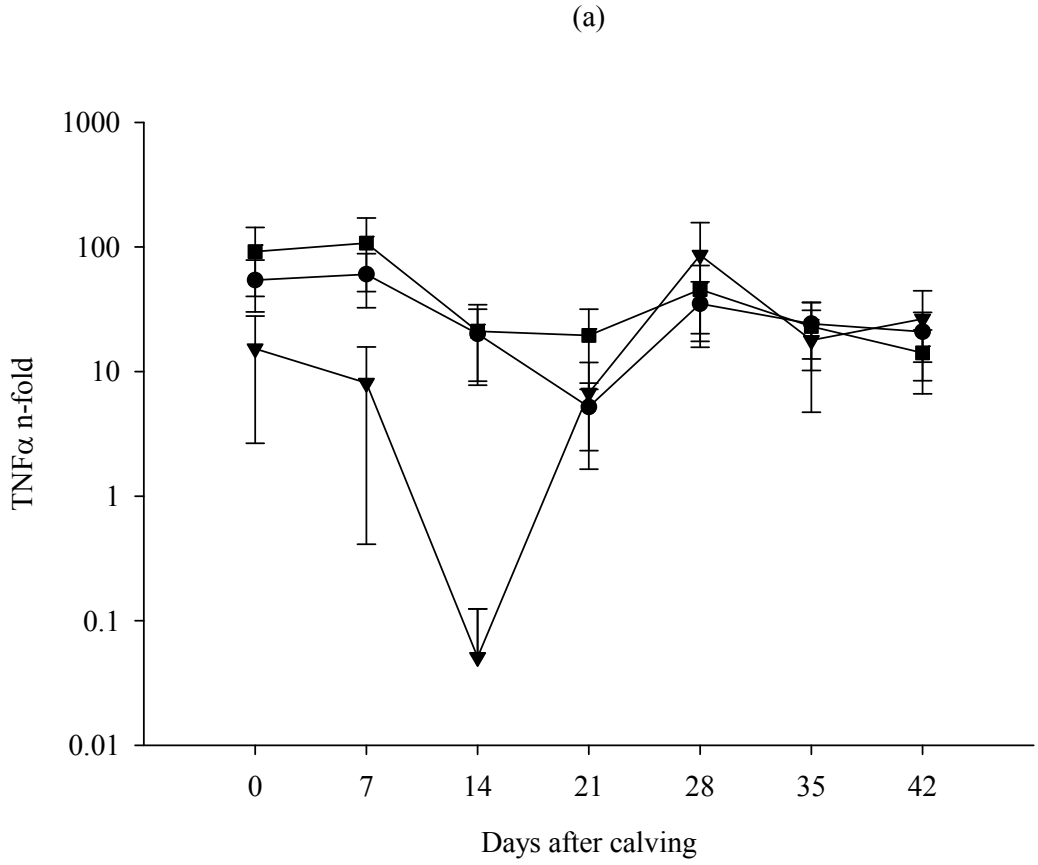

Figure 2.1a. Fold difference in **TNFα** mRNA expression for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

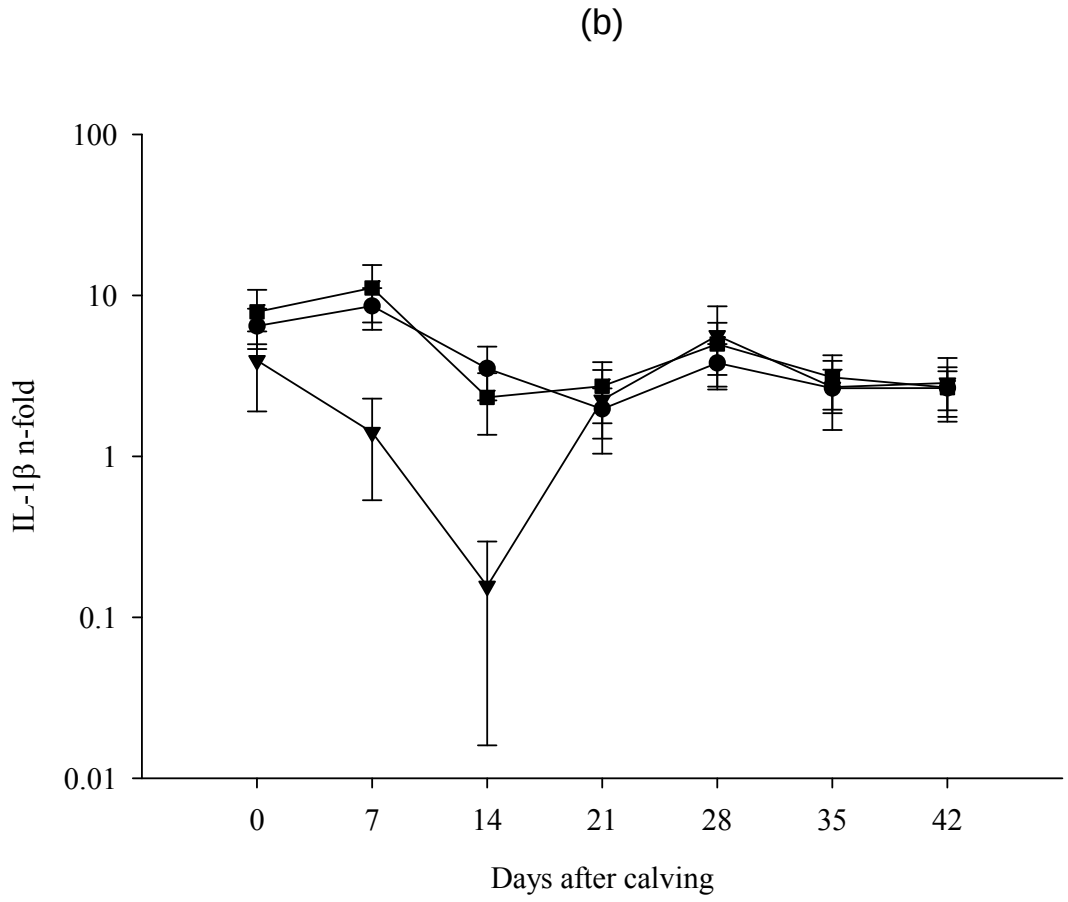

Figure 2.1b. Fold difference in IL-1β mRNA expression for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

(c)

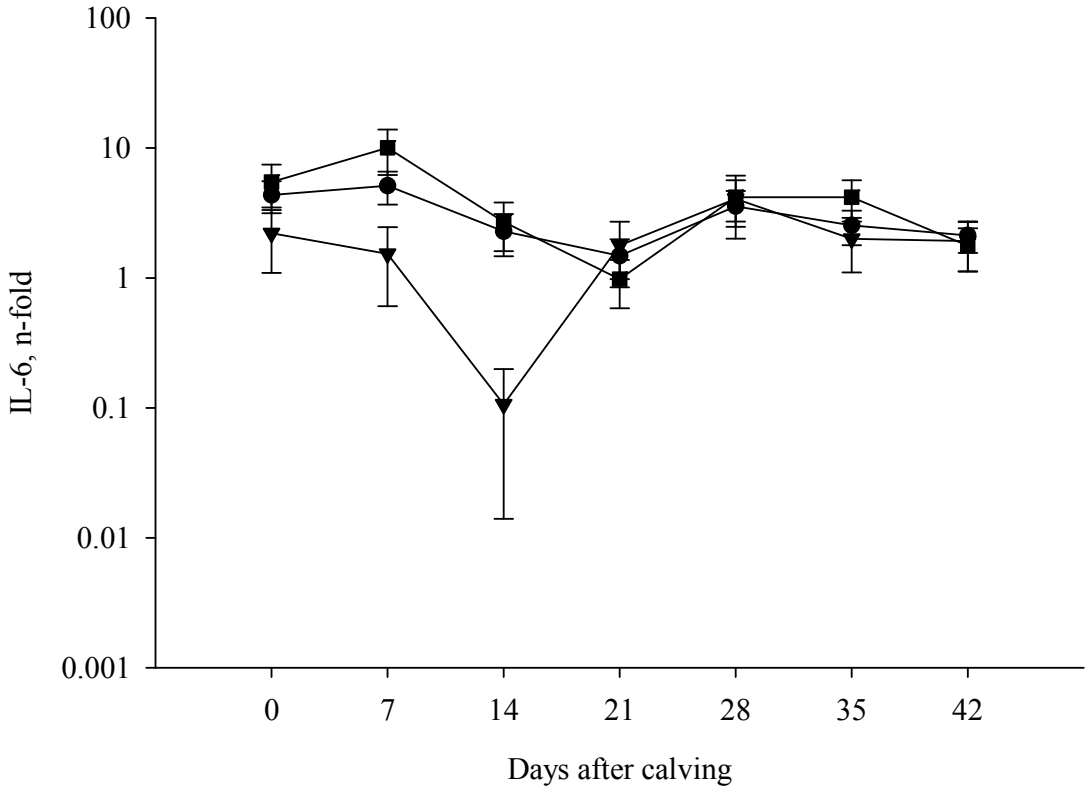

Figure 2.1c. Fold difference in **IL-6** mRNA expression for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was no effect of uterine disease ($P = 0.16$) or interaction between uterine disease and time ($P = 0.74$) on IL-8 gene expression (**Figure 2.2)**. There was an interaction between parity and time ($P = 0.10$) with monocytes from multiparous cows presenting lower IL-8 gene expression 7 d after calving than monocytes from primiparous cows (2.6 ± 0.7 vs. 6.8 ± 1.6; $P = 0.009$). IL-8 gene expression decreased from calving until 14 d, returning to the levels of calving by 28 d (time effect, $P = 0.004$; **Figure 2.2**).

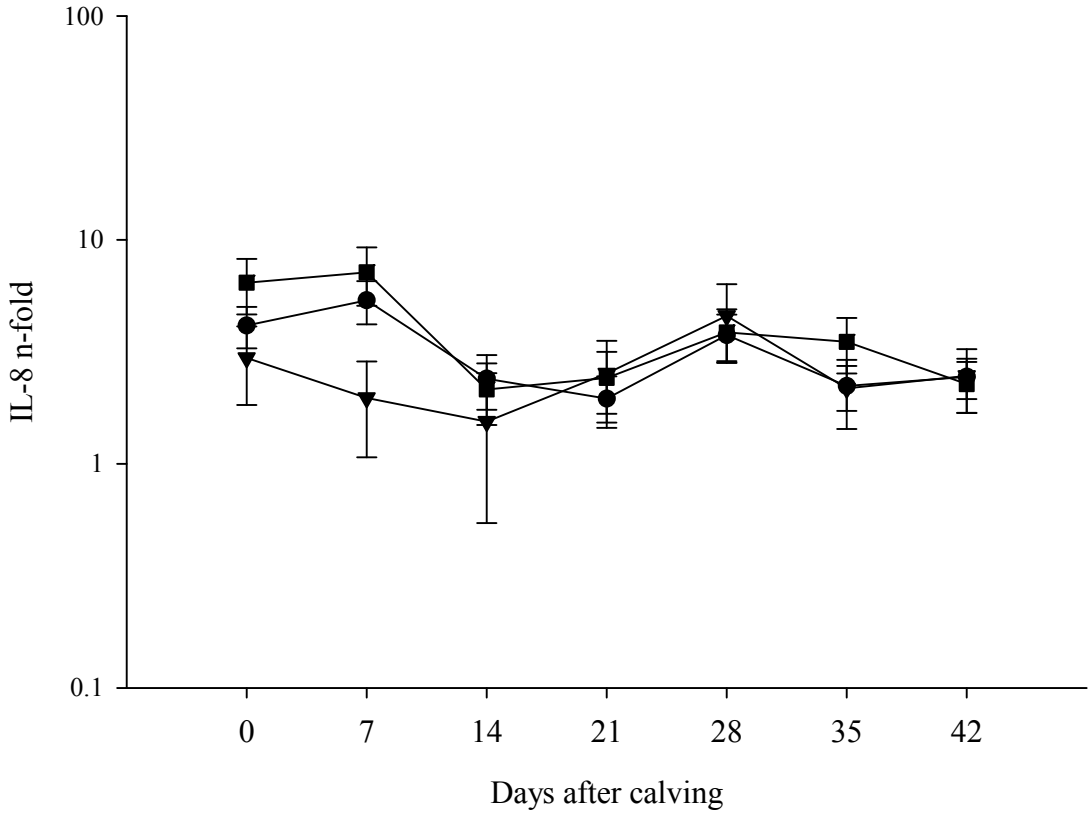

Figure 2.2. Fold difference in **IL-8** mRNA expression for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was no effect ($P = 0.68$) of uterine disease or interaction between uterine disease and time ($P = 0.60$) on IL-10 gene expression (**Figure 2.3)**. IL-10 gene expression was increased at 14 and 28 d after calving (time effect; $P = 0.03$; **Figure 2.3**).

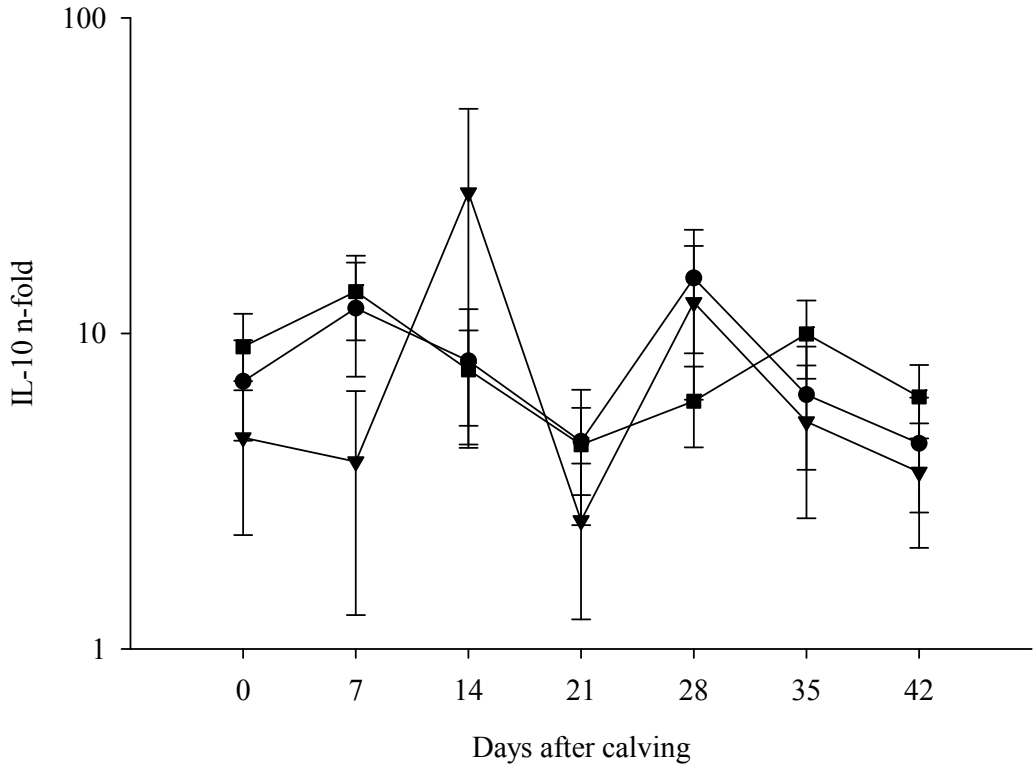

Figure 2.3. Fold difference in **IL-10** mRNA expression for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was an interaction between uterine disease and time ($P = 0.11$) on the TNFα concentration in the monocyte culture medium from calving until 42 d after calving. TNFα concentration in the medium used to culture monocytes from cows that developed metritis tended to be decreased at calving compared to cows that had SCE ($P = 0.06$) or healthy cows ($P = 0.09$); in addition, TNFα was decreased ($P < 0.05$) at 7 and 21 d after calving (**Figure 2.4a**). There was an effect of parity (P = 0.07) and TNFα concentration tended to be increased in the medium used to culture monocytes from primiparous cows compared to multiparous cows (10.2 ± 2.4 vs. 5.5 ± 1.4

ng/mL/µg of total RNA). TNFα concentration was the lowest at calving and at 21 DIM (time effect, $P = 0.02$; **Figure 2.4a**). There was no effect of uterine disease ($P = 0.94$) or interaction between uterine disease and time (P = 0.20) on the IL-1β concentration in the monocyte culture medium from calving until 42 d after calving (**Figure 2.4b**). IL-1β concentration increased from calving until 42 d after calving (time effect, $P = 0.10$; **Figure 2.4b**).

(a)

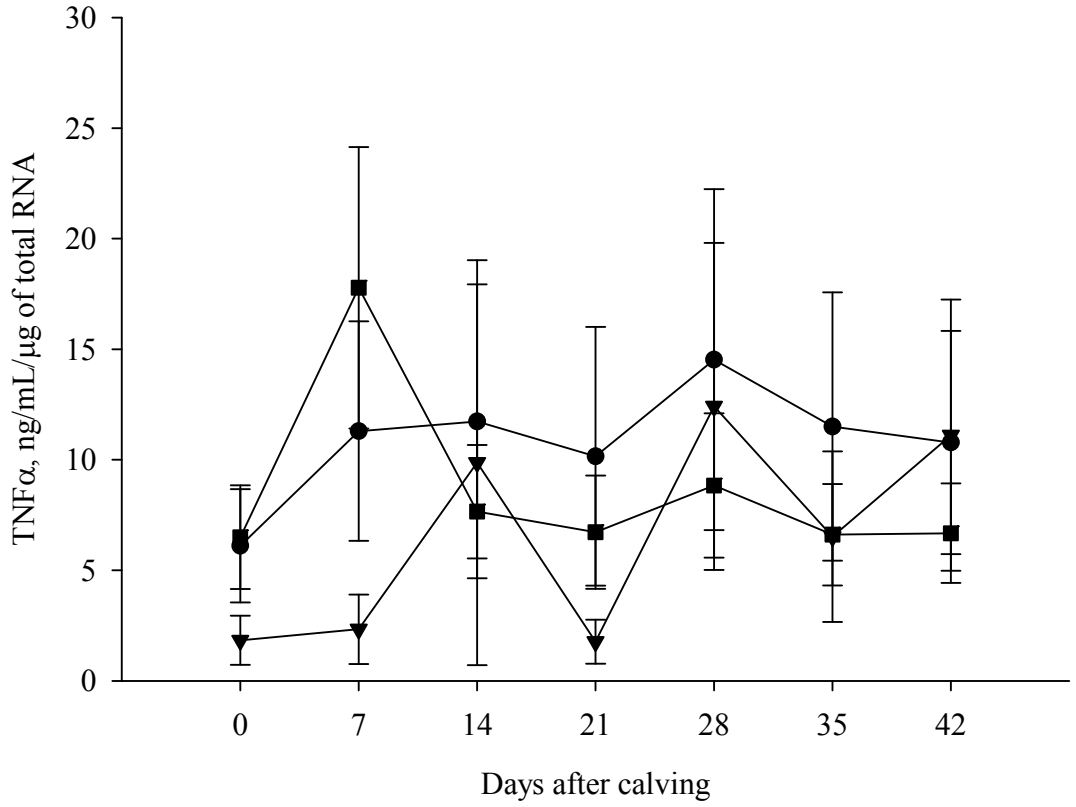

Figure 2.4a. Concentration of **TNFα** in the monocyte culture medium collected 12 hours after stimulation with *E. coli* for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

(b)

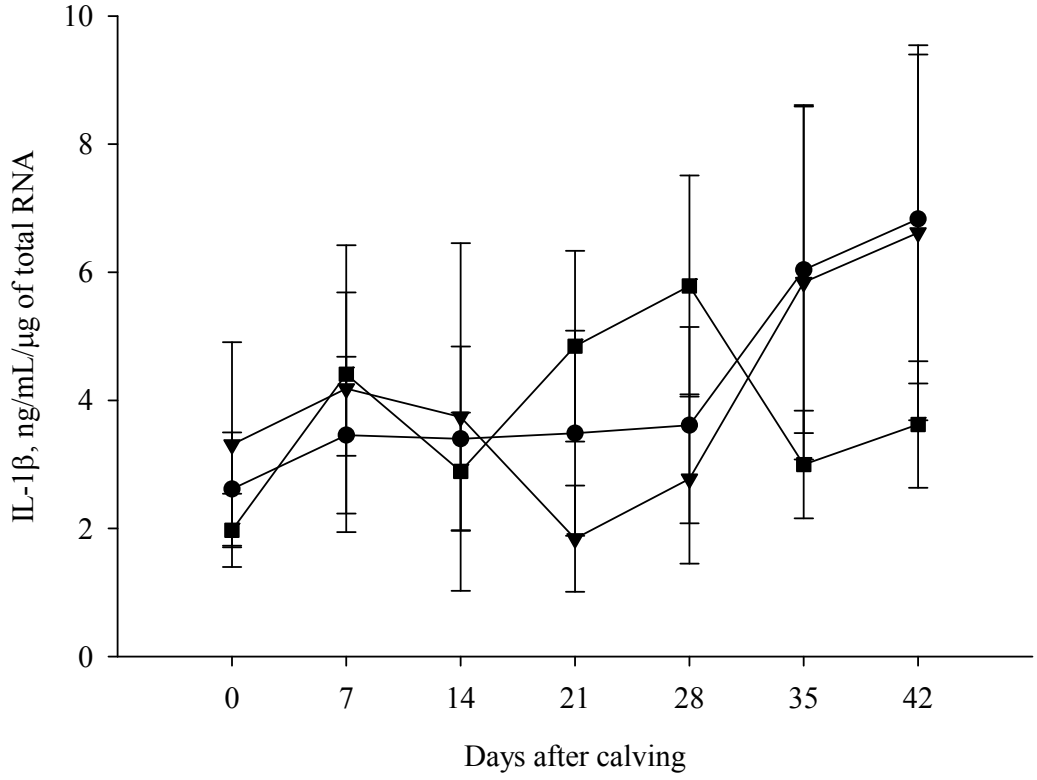

Figure 2.4b. Concentration of **IL-1β** in the monocyte culture medium collected 12 hours after stimulation with *E. coli* for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was no effect of uterine disease ($P = 0.71$), time ($P = 0.58$) or interaction between uterine disease and time ($P = 0.88$) on the IL-8 concentration in the monocyte culture medium from calving until 42 d after calving (**Figure 2.5)**. There was an effect of parity ($P = 0.02$), and IL-8 concentration was increased in the medium used to culture monocytes from primiparous cows compared to multiparous cows (0.40 ± 0.04 vs. 0.29 ± 0.03 ng/mL/µg of total RNA).

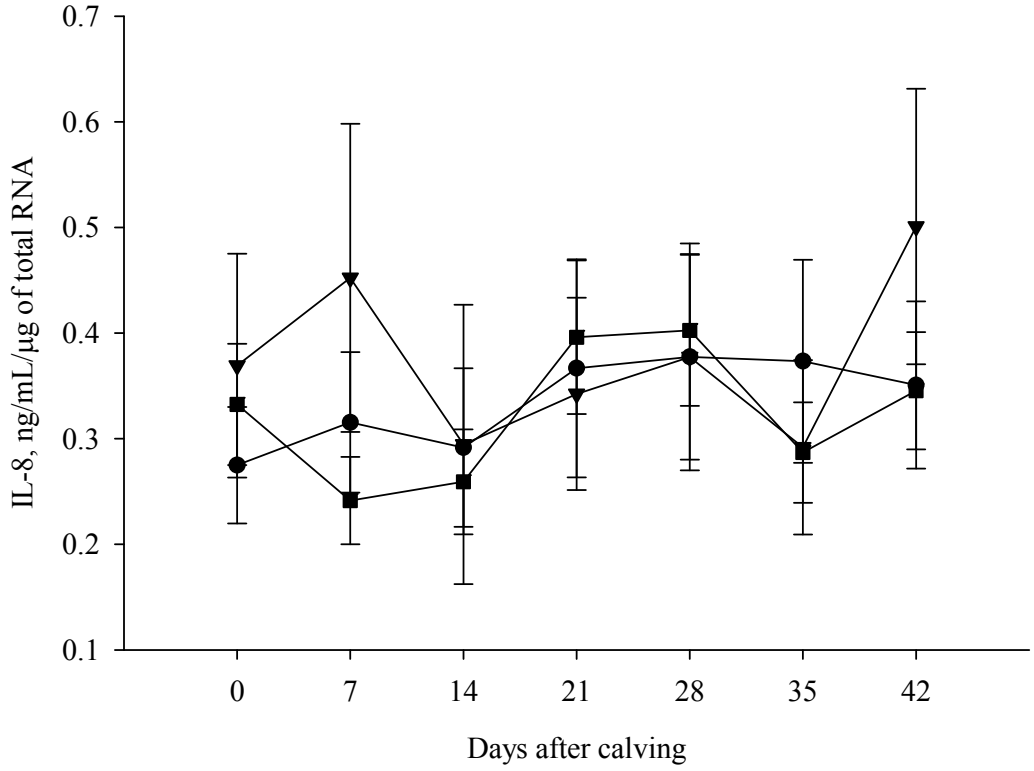

Figure 2.5. Concentration of **IL-8** in the monocyte culture medium collected 12 hours after stimulation with *E. coli* for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was no effect of uterine disease ($P = 0.94$), time ($P = 0.20$) or interaction between uterine disease and time ($P = 0.97$) on the IL-8 concentration in plasma from calving until 21 d after calving (**Figure 2.6**).

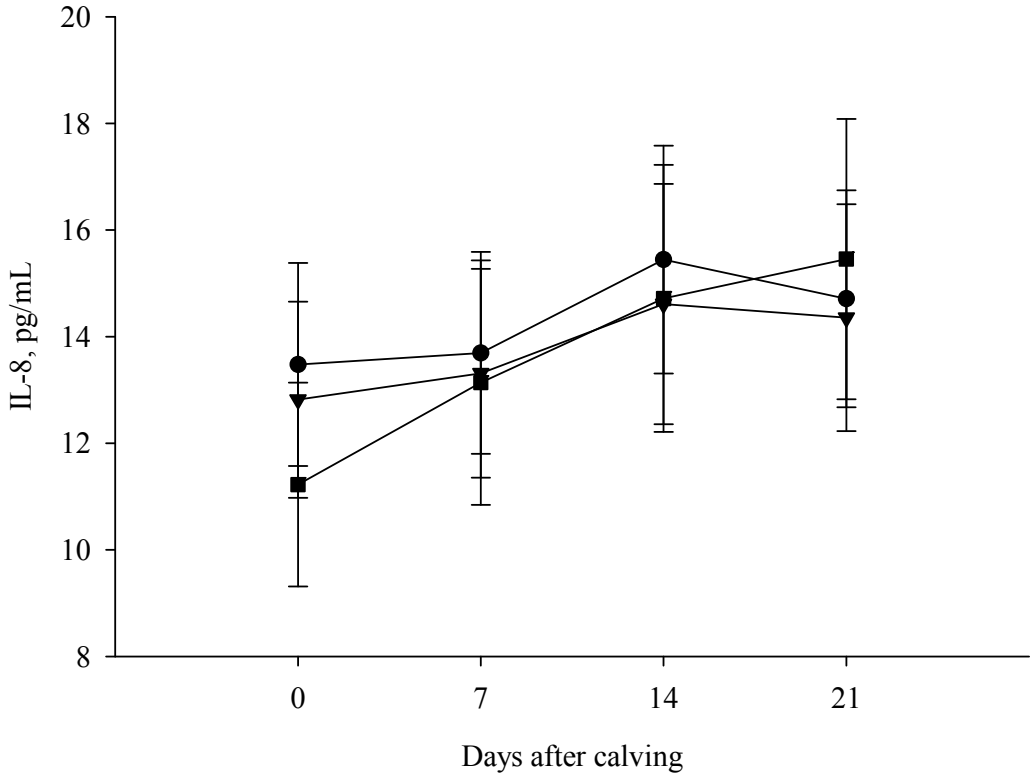

Figure 2.6. Concentration of **IL-8** in plasma of cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

DISCUSSION

Neutrophil function (chemotaxis, phagocytosis and killing ability) is decreased around the time of calving in high-producing dairy cows (Gilbert et al., 1993; Goff and Horst, 1997; Kehrli et al., 1989a; Kimura et al., 1999) particularly in cows that develop uterine disease (Cai et al., 1994; Hammon et al., 2006; Kim et al., 2005). Decreased lymphocyte function was also observed around calving (Kehrli et al., 1989b; Kehrli and Goff, 1989; Mallard et al., 1998). Nonetheless, Limited information is available about the role of monocytes and macrophages in modulating the immune response around calving. Karcher et al., (2008) evaluated modulation of cytokine gene expression and secretion during the periparturient period in dairy cows naturally infected with Mycobacterium; however, no information is available about uterine disease.

Overall, we observed a sharp decrease in pro-inflammatory cytokine gene expression from calving until 14 d after calving. This drop was mainly because cows that developed metritis had a decrease in all pro-inflammatory cytokines compared to healthy cows and SCE cows. Metritis is an acute and severe process caused by bacterial contamination around calving, and characterized by fetid uterine discharge and systemic complications such as loss of appetite and fever (Bondurant, 1999, Sheldon and Dobson, 2004). Subclinical endometritis can be considered a chronic condition in which cows fail to completely clear bacterial contaminants (Gilbert et al.,2007). Neutrophils from cows that developed metritis and cows that had SCE have had a loss in function around calving (Hammon et al., 2006); therefore, loss of neutrophil function might be the main cause for the development of SCE, and decrease in function of monocytes might also be involved in the development of metritis.

Although gene expression for all pro-inflammatory cytokines was decreased for cows that developed metritis, we only detected a difference in secretion of TNFα

using ELISA. TNFα is the main cytokine involved in stimulation of expression of cell adhesion molecules that are specific to neutrophils (E-selectin), the induction of production of the neutrophil chemokine IL-8 by endothelium, and the activation of neutrophils and monocytes (Roach et al., 2002). Therefore, a decrease in secretion of TNFα could impair neutrophil migration to sites of bacterial infection, as well as affect their phagocytic and killing ability. The decrease in gene expression and secretion of pro-inflammatory cytokines in cows that developed metritis could be due to an intrinsic defect in their leukocyte function (Mallard et al., 1998; Nino-Soto et al., 2008) or an extrinsic mechanisms affecting their activity, such as a greater degree of negative energy balance that these cows experience (Hammon et al., 2006). Because differences in gene expression and secretion among cows that developed metritis and cows that did not occurred mainly when negative energy balance would have expected to be most severe (Beam and Butler, 1998), it is likely that the state of negative energy balance is affecting the ability of these cows to regulate gene expression.

Downregulation of inflammation could result from a decreased in the production of pro-inflammatory cytokines or from the upregulation of anti-inflammatory regulatory cytokines (such as IL-10, as it has been shown in cows infected with clinical Johne's disease; Khalifeh and Stabel, 2004). We did not observe any difference in gene expression according to disease status; therefore, downregulation of pro-inflamatory cytokines may be the main factor affecting the ability to mount a proper inflammatory process in cows that develop metritis.

Applying our *in vitro* observations applied to the *in vivo* condition, the development of metritis in cows could be explained in part by a decrease in gene expression and secretion of TNFα at calving, which consequently fails to induce the expression of selectins, and the secretion of IL-8 by the vascular endothelium in the uterus for appropriate neutrophil extravasation. Cows that develop retained placenta (a

major risk factor for metritis) have decreased chemotaxis to the sites of placental attachment in the uterus, along with decreased plasma IL-8 concentrations (Kimura et al., 2002). Nonetheless, we did not detect any difference in plasma IL-8 concentration among cows that developed metritis and cows that did not. Chemokines act locally and in minute concentrations at the site of inflammation; therefore, although we did not detect a systemic difference in IL-8 concentration, a local difference could still occur.

In summary, monocyte gene expression of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6), and secretion of the main pro-inflammatory cytokine (TNFα) were decreased from calving until 2 to 3 wk after calving in cows that developed metritis. Cows that had SCE did not differ from healthy cows. Decreased expression of pro-inflammatory cytokines could lead to poor chemotaxis and activation of neutrophil, which would impair diapedesis to the site of infection, bacterial clearance and predispose cows to the development of metritis.

CHAPTER THREE

Association between subclinical endometritis and cytokine expression in uterine tissue from Holstein cows

Interpretive summary: Cytokine expression by uterine tissue. Galvão.

Uterine tissue cytokine gene expression was evaluated in 28 Holstein cows from calving until 7 wk after calving. ELISA was performed on serum to measure IL-8. Pro-inflammatory (TNFα and IL-1β) cytokine gene expression in uterine tissue was decreased in cows that had SCE compared to healthy cows at 1 week after calving. IL-1β, IL-6, and IL-8 gene expression in uterine tissue were increased in cows that had SCE compared to healthy cows at 5 or 7 wk after calving. Multiparous cows had increased IL-1β, IL-6 and IL-8 gene expression and increased IL-8 in serum compared to primiparous cows. The lower expression of pro-inflammatory cytokines early after calving indicates poor activation of inflammation and might impair clearance of bacteria and lead to the development of subclinical endometritis.

ABSTRACT

The objective of this study was to evaluate uterine tissue gene expression of the main pro-inflammatory (TNFα, IL-1 beta, Il-6) and anti-inflammatory (IL-10) cytokines and the main neutrophil chemokine (IL-8) from calving until 7 wk after calving in dairy cows and later to compare healthy cows with cows that developed subclinical endometritis (SCE). Uterine biopsies were collected at calving and at 3, 5, and 7 wk after calving. SCE was evaluated 5 wk after calving by uterine lavage and cytology; cows having ≥ 10% neutrophils were considered to have SCE. Real-time RT-PCR threshold values (Ct) were used to calculate the fold difference in gene expression, using the 2^{-ddCt} method, normalized to GAPDH and calibrated to the average dCt at calving. ELISA was performed on serum to measure IL-8. All 44 cows (20 had SCE) enrolled in the study were used for ELISA, but only 28 cows (11 had SCE) had uterine biopsies and were used PCR analysis. TNFα gene expression in uterine tissue was decreased in cows that had SCE compared to healthy cows at 1 week after calving. IL-1β gene expression in uterine tissue tended to be decreased (P = 0.08) in cows that had SCE compared to healthy cows at 1 week after calving, but then tended to be increased (P = 0.10) at wk 5 and 7 after calving. Cows that had SCE had an overall increase (P = 0.008) in IL-6 gene expression in uterine tissue compared to healthy cows. IL-8 gene expression was increased (P = 0.03) in cows that had SCE compared to healthy cows at week 7 after calving. Uterine disease was not associated with IL-10 gene expression. The lower level of expression of pro-inflammatory cytokines early after calving indicates poor activation of inflammation and might impair clearance of bacteria and lead to the development of subclinical endometritis.

Key words: subclinical endometritis, cytokines, gene expression, dairy cows

INTRODUCTION

Uterine diseases such as metritis and endometritis are highly prevalent in high-producing dairy cows (Gilbert et al., 2005; Goshen and Shpigel, 2006; Leblanc et al., 2002) and lead to economic loss due to decreased milk yield and fertility (Bartlett et al., 1986; Gilbert et al., 2005; Goshen and Shpigel, 2006). Metritis is an acute inflammatory process caused by bacterial infection of the uterus in the first 14 d after calving. Subclinical endometritis (SCE) can be considered a chronic condition in which cows fail to completely clear bacterial contaminants (Sheldon and Dobson, 2004; Gilbert et al., 2007) and is defined by an increased number of neutrophils in the uterus (Gilbert et al., 2005).

Neutrophils are the main leukocyte type involved in bacterial clearance after uterine infection (Hussain, 1989; Gilbert et al., 2007). Most cows are infected around calving (Dohmen et al., 2000; Sheldon and Dobson, 2004); therefore, prompt neutrophil response after infection is critical for bacterial clearance and prevention of disease. Cows with the greatest influx of neutrophils to the uterus on the day of calving have the lowest rates of positive bacterial culture and prevalence of SCE subsequently (Gilbert et al., 2007).

Tissue macrophages are the main regulators of the initial immune response against pathogens. After contact with bacterial invaders, macrophages are stimulated to produce and release pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6) which stimulate the expression of cell adhesion molecules and induce production of IL-8 (a potent neutrophil chemoattractant) by the vascular endothelium. Pro-inflammatory cytokines activate monocytes and neutrophils to increase their phagocytic and killing capacity. After infection is cleared, macrophages release anti-inflammatory cytokines such as interleukin-10 (IL-10) to decrease inflammation (Tzianabos, 2000). The uterine

endometrium also recognizes bacterial lipopolysaccharide through its toll-like receptor 4 and is stimulated to produce cytokines such as TNFα (Herath et al., 2006).

In summary, cows that remain healthy have an increased influx of neutrophils into the uterus at calving and low neutrophil counts later in lactation whereas SCE cows have low influx of neutrophils at calving and increased neutrophil influx into the uterus later in lactation. Therefore, we hypothesized that uterine tissue from SCE cows would have decreased gene expression of pro-inflammatory cytokines around calving, and increased gene expression of pro-inflammatory cytokines at the time of diagnosis of SCE. Also, because pro-inflammatory cytokines induce the production of IL-8, we hypothesized that an increase in the level of gene expression in pro-inflammatory cytokines would be associated with an increase in serum IL-8. Therefore, our objectives were to compare uterine tissue gene expression of the main pro-inflammatory (TNFα, IL-1 beta, IL-6) and anti-inflammatory (IL-10) cytokines and the levels of IL-8 in serum (from calving until 7 wk after calving) between healthy cows and SCE cows.

MATERIALS AND METHODS

Animals, Housing, and Feeding

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals in this experiment. Fourty-four cows (15 primiparous and 29 multiparous) from the Cornell herd were enrolled in the study from December of 2004 to October of 2005. The herd had 75 milking cows and the rolling herd average was ~ 11,500 kg of milk per year. Cows were housed in freestall facilities and milked three times daily. All cows were fed the same TMR that was formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% fat corrected milk.

Evaluation of Uterine Diseases

Cows were evaluated for presence of SCE at 5 wk after calving and then rechecked at 7 wk after calving. A low-volume uterine lavage was performed as previously described (Gilbert et al., 2005) and the percent neutrophils out of a total of 200 cells (including all leukocyte types and epithelial cells but excluding erythrocytes) was used as diagnosis for SCE. Cows having ≥ 10% or ≥ 8% neutrophils at 5 or 7 wk after calving, respectively, were considered to have SCE. The percent neutrophil used as cutoff for diagnosis of SCE was based on receiver-operating characteristic analysis from data from a larger experiment that included 780 cows (Cheong et al., 2008).

Blood and Biopsy Collection

Blood was collected from all cows at calving and at 3, 5, and 7 wk after calving by puncture of coccygeal vessels into Vacutainer tubes without anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The samples were immediately placed on ice and transported to the laboratory within 4 hours, where serum was separated by centrifugation at 2000 g for 15 min, frozen at -80º C, and later analyzed for IL-8 concentration by ELISA.

Twenty-eight out of the 44 cows used for this study were randomly selected to have a uterine biopsy collected at calving then at 3, 5, and 7 wk after calving. For biopsy collection, an epidural was performed using 4 mL of 2% lidocaine; a biopsy tool was protected with a sanitary chemise, which was broken immediately before the biopsy tool was passed through the cervical oz. After passing the cervix, the biopsy tool was pressed against the uterine wall; a biopsy sample was collected, immediately placed in 1 mL of RNA later (Ambion-Applied Biosystems Inc., Foster City, CA, USA), and frozen at -80 ^{o}C until RNA isolation. At each time point 2 biopsies were

performed. Each tissue sample weighed between 100 and 250 mg.

RNA Isolation and real-time RT-PCR

Total RNA was extracted using the Trizol method (Chomczynski and Sacchi, 2006). Uterine-tissue samples were homogenized using a bead mill homogenizer (Mini-Beadbeater-8, BioSpec Products, Inc., Bartlesville, OK, USA). A 2-mL screw-cap microtube was filled to two-thirds full with 2.3-mm stainless steel beads then about 100 mg of uterine tissue was added, the microtube was filled almost to the top with Trizol® (Gibco-Invitrogen, Life Technologies Corporation, Carlsbad, California, USA), and homogenized for 3 min. After homogenization, the protocol was followed as reported previously (Chomczynski and Sacchi, 2006). The total RNA was eluted in 50 µL, and the RNA yield ranged from 100 to 1000 µg/mL; the 260 to 280 nm ratio from the spectrophotometric reading ranged from 1.8 to 2.2. Part of the total RNA (1 µg) was treated for genomic DNA contamination using a commercial kit (DNase I kit, Gibco-Invitrogen), and stored at -80°C for real-time reverse transcriptase PCR (RT-PCR). For the RT-PCR, sets of primers and probes for all the cytokines and endogenous control (glyceraldehyde-3-phosphate (GAPDH)) were designed using the primer express software (Applied Biosystems, Life Technologies Corporation, Carlsbad, California, USA) (**Table 3.1**). The one-step method was used and the reaction mixture (Applied Biosystems Cat# 4309169, TaqMan® One-Step RT-PCR Master Mix) contained 2 µL (37 ng/ µL) of the DNAse treated RNA, universal mastermix (with MultiScribe Reverse Transcriptase and AmpliTaq GOLD DNA Polymerase), forward and reverse primers, Taqman® probe, and RNAse inhibitor. The fold difference (n-fold) in gene expression was calculated using the relative quantitation method (2^{-ddCt}), with GAPDH as the endogenous control and the average dCt for samples collected at calving as the calibrator for each sample.

Table 3.1. Primer and probe sequences used for real time RT-PCR.

Cytokine	Primer sequence (forward and reverse; 5' – 3')	Accession #
IL-1β	AGCATCCTTTCATTCATCTTTGAAG	M37211
	GGGTGCGTCACACAGAAACTC	
IL-6	GGGCTCCCATGATTGTGGTA	BC123577
	GTGTGCCCAGTGGACAGGTT	
IL-10	AGAACCACGGGCCTGACAT	U00799
	AGCTCACTGAAGACTCTCTTCACCTT	
TNFα	TCTTCTCAAGCCTCAAGTAACAAGT	NM_173966
	CCATGAGGGCATTGGCATAC	
IL-8	TGCTTTTTTGTTTTCGGTTTTTG	AF232704
	AACAGGCACTCGGGAATCCT	
GAPDH	GCATCGTGGAGGGACTTATGA	BC102589
	GGGCCATCCACAGTCTTCTG	
Cytokine	Probe sequence (5' – 3')	Accession #
IL-1β	CCTGTCATCTTCGAAACGTCCTCCGA	M37211
IL-6	TTCCTGGGCATTCCCTCCTCTGGT	BC123577
IL-10	TTCTGCCCTGCGAAAACAAGAGCAA	U00799
TNFα	AGCCCACGTTGTAGCCGACATCAACTC	NM_173966
IL-8	TAATCTTGCAACCCTCACCTGCTGGC	AF232704
GAPDH	CACTGTCCACGCCATCACTGCCA	BC102589

ELISA for IL-8

Concentration of IL-8 in serum samples collected from calving until 7 wk after calving was determined using a commercially available human IL-8 ELISA kit (R&D Systems, Inc., Minneapolis, MN) validated for use in bovine (Shuster et al., 1996; Galligan and Coomber, 2000).

Statistical Analyses

This was an observational cohort study. For the statistical analysis, the diagnosis (yes, no) performed at 5 wk was used to classify cows as having SCE. Nonetheless, there was high correlation between diagnosis at 5 and 7 wk (Pearson correlation = 0.6; $P < 0.0001$). Fold difference (n-fold) in cytokine gene expression (PCR) and the cytokine concentration in serum (ELISA) from calving until 7 wk after calving were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS (Version 9.1; SAS Inst. Inc., Cary, NC). A first-order autoregressive covariance structure was used. Because n-fold was not normally distributed, the statistical analysis was performed on the delta delta Ct (ddCt) values and then converted to n-fold (2^{-ddCt}) for data presentation. Data on IL-8 concentration in serum were skewed and were transformed to their natural logarithm for data analysis and then back-transformed for data presentation. Normality of standardized residuals was evaluated by inspection of normal probability plots. Models included the effects of uterine disease (healthy or SCE), parity (primiparous or multiparous), time (week of sample collection; 0 (calving), 1, 3, 5, and 7), and 2-way interactions between uterine disease and parity or time. Uterine disease, interaction between uterine disease and time, and parity were forced into the models, but other interactions were removed if $P > 0.15$. The cow was included in the analysis as a random effect. When either an effect of uterine disease or an interaction between uterine disease and time was observed,

post-hoc multiple comparisons were performed using the Bonferroni adjustments in SAS.

Differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered a tendency toward statistical difference. Interactions were considered to be significant when $P \leq 0.15$.

RESULTS

Of the 44 cows used in the study, 20 (46%) were diagnosed with subclinical endometritis; 5 primiparous (33%; 5/15) and 15 multiparous (52%; 15/29).

There was an interaction between uterine disease and time ($P = 0.15$) on TNFα gene expression, which revealed that TNFα gene expression in uterine tissue was decreased ($P < 0.05$) in cows that had SCE compared to healthy cows at 1 week after calving. There was no effect of time ($P = 0.81$) on TNFα gene expression in uterine tissue (**Figure 3.1a**).

There was an interaction between uterine disease and time ($P = 0.02$) on IL-1β gene expression, which revealed that IL-1β gene expression tended to be increased ($P = 0.08$) in cows that had SCE compared to healthy cows at 1 week after calving, but tended to be decreased ($P = 0.10$) at wk 5 and 7 after calving (**Figure 3.1b**). There was no effect of time ($P = 0.27$) on IL-1β gene expression in uterine tissue.

Cows that had SCE had on overall increased IL-6 gene expression in uterine tissue compared to healthy cows ($P = 0.008$). Multiple comparisons showed that differences were significant ($P \leq 0.05$) at calving and at 7 wk after calving (**Figure 3.1c**). IL-6 gene expression increased from calving until week 7 after calving (time effect, $P = 0.05$; **Figure 3.1c**).

There was an interaction between uterine disease and time ($P = 0.06$) on IL-8 gene expression, which showed that IL-8 gene expression was increased ($P = 0.03$) in

cows that had SCE compared to healthy cows at week 7 after calving (**Figure 3.1d**). IL-8 gene expression decreased from calving until week 7 after calving (time effect, P = 0.009; **Figure 3.1d**).

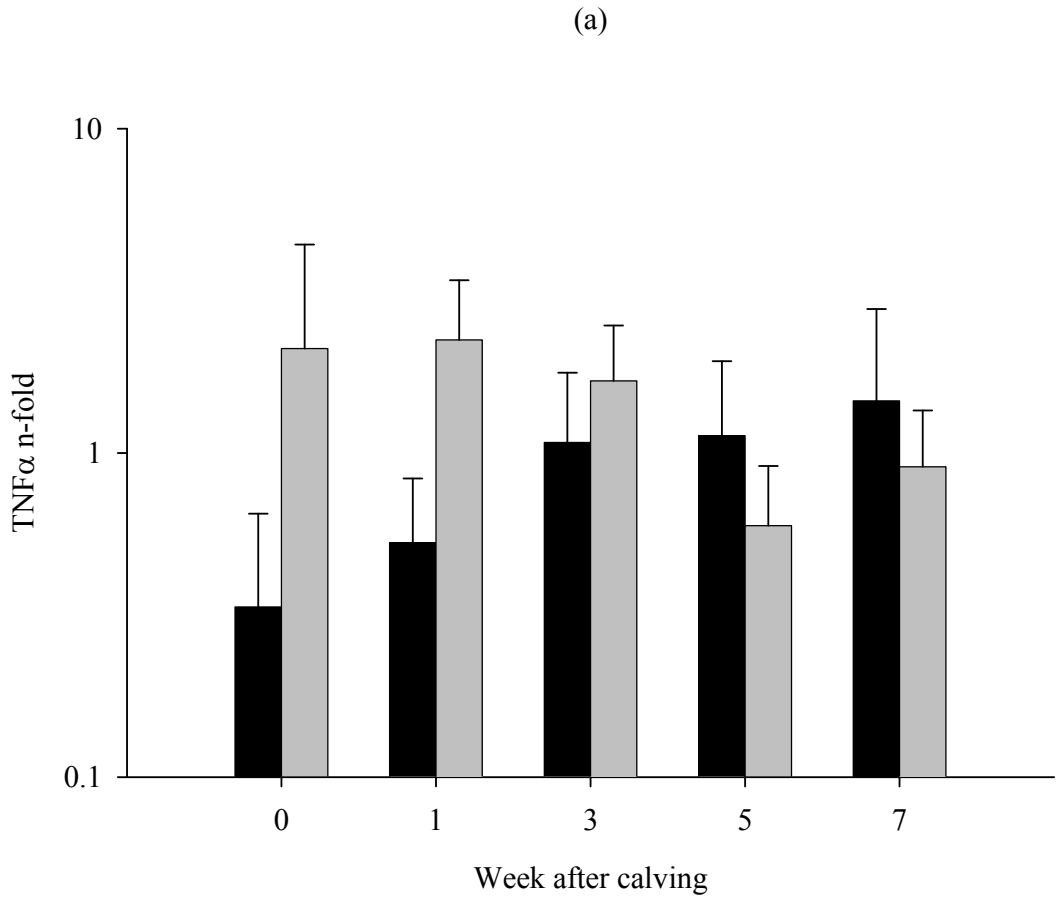

Figure 3.1a. Fold difference (n-fold) in **TNFα** mRNA gene expression in cows that had subclinical endometritis at week 5 (black bars) and healthy cows (gray bars) from calving (0) until week 7 after calving.

(b)

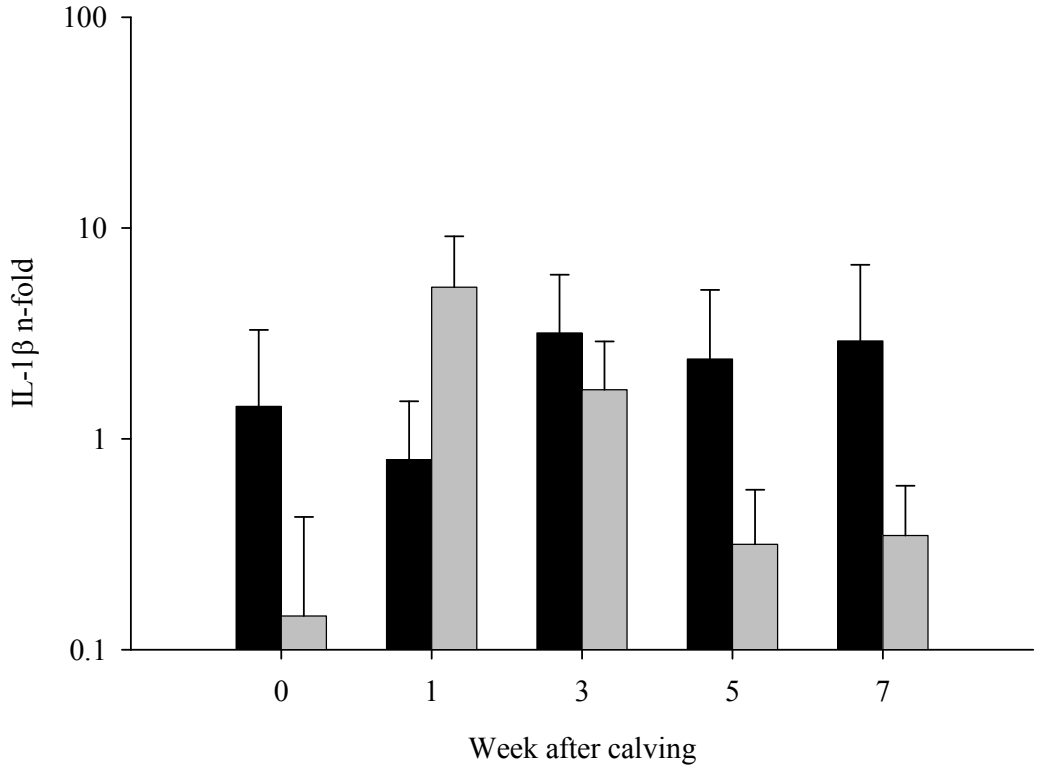

Figure 3.1b. Fold difference (n-fold) in **IL-1β** mRNA gene expression in cows that had subclinical endometritis at week 5 (black bars) and healthy cows (gray bars) from calving (0) until week 7 after calving.

(c)

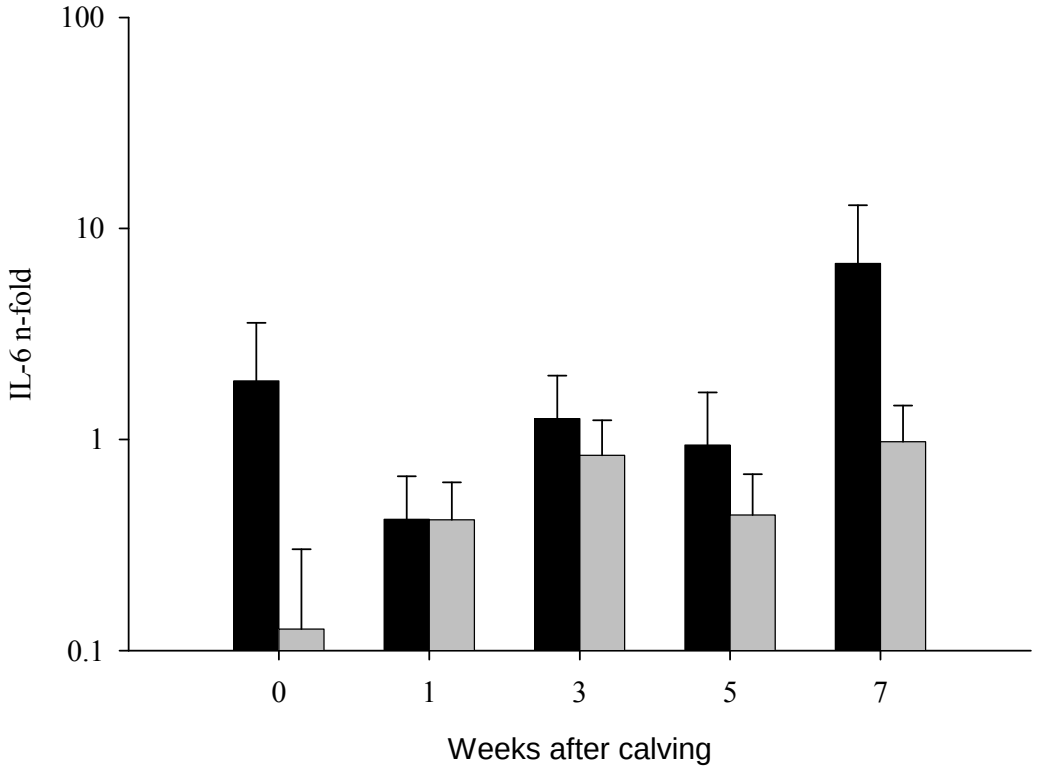

Figure 3.1c. Fold difference (n-fold) in **IL-6** mRNA gene expression in cows that had subclinical endometritis at week 5 (black bars) and healthy cows (gray bars) from calving (0) until week 7 after calving.

(d)

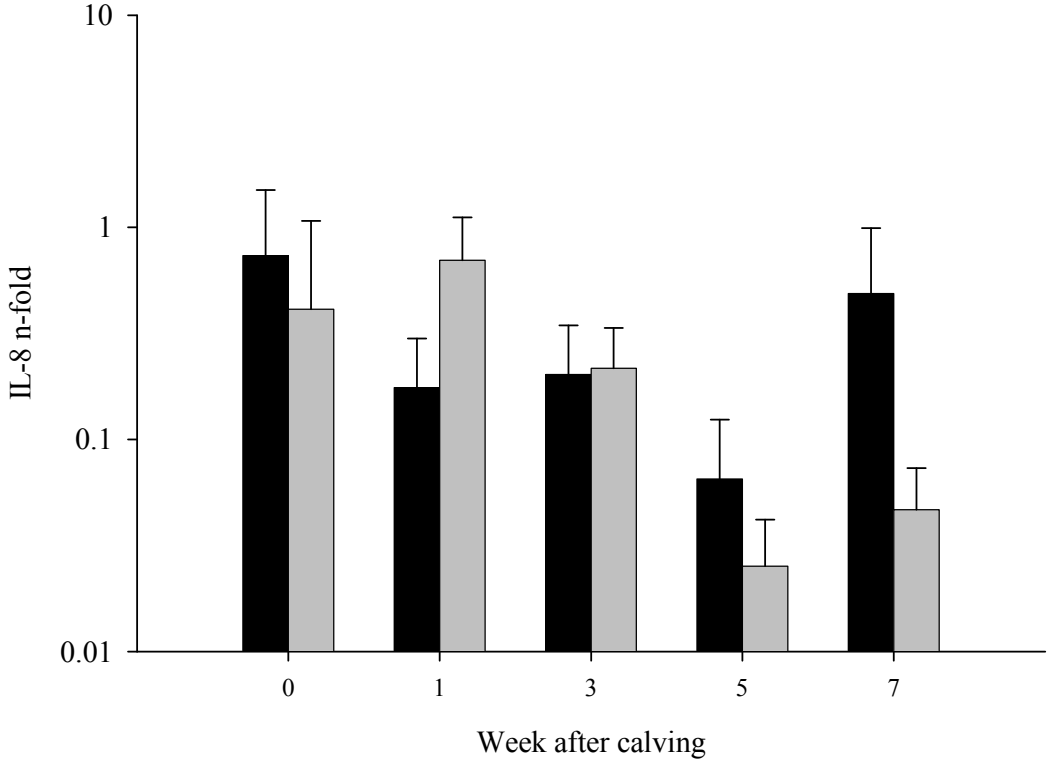

Figure 3.1d. Fold difference (n-fold) in **IL-8** mRNA gene expression in cows that had subclinical endometritis at week 5 (black bars) and healthy cows (gray bars) from calving (0) until week 7 after calving.

Parity did not affect (P = 0. 11) TNFα gene expression (**Figure 3.2a**) but affected gene expression of all other cytokines. Multiparous cows had greater IL-1β (P < 0.001; **Figure 3.2b**), IL-6 (P < 0.001; **Figure 3.2c**), and IL-8 (P = 0.003; **Figure 3.2d**) gene expression than primiparous cows.

(a)

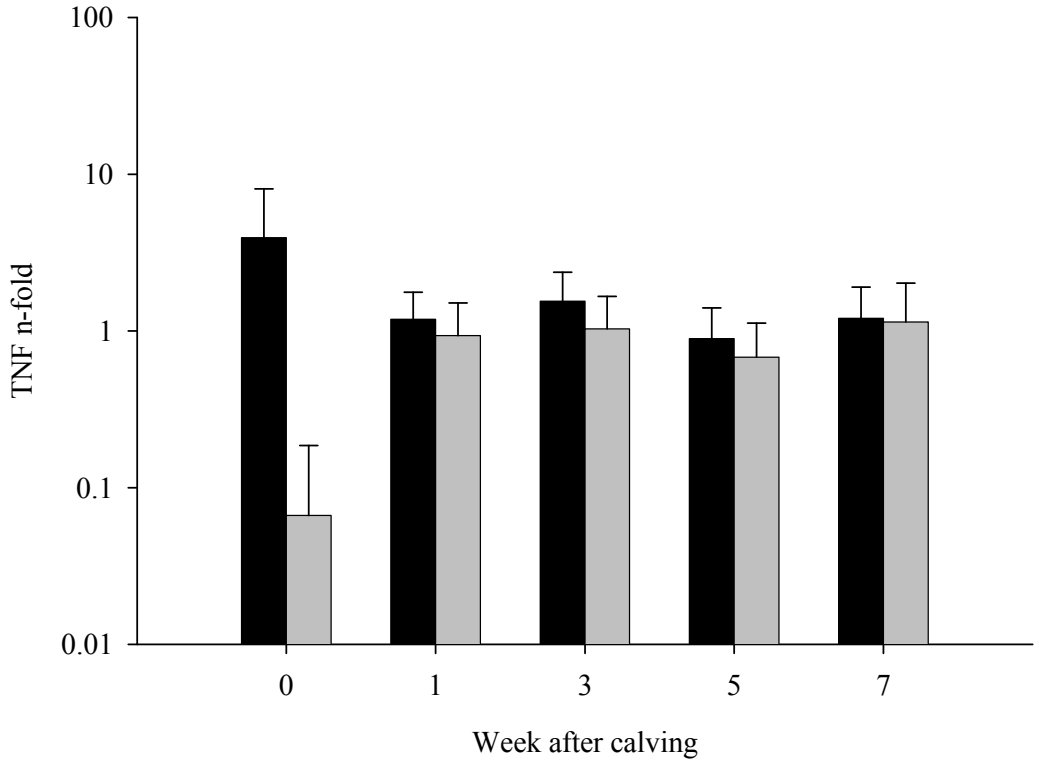

Figure 3.2a. Fold difference (n-fold) in **TNFα** mRNA gene expression in multiparous cows (black bars) and primiparous cows (gray bars) from calving (0) until week 7 after calving.

(b)

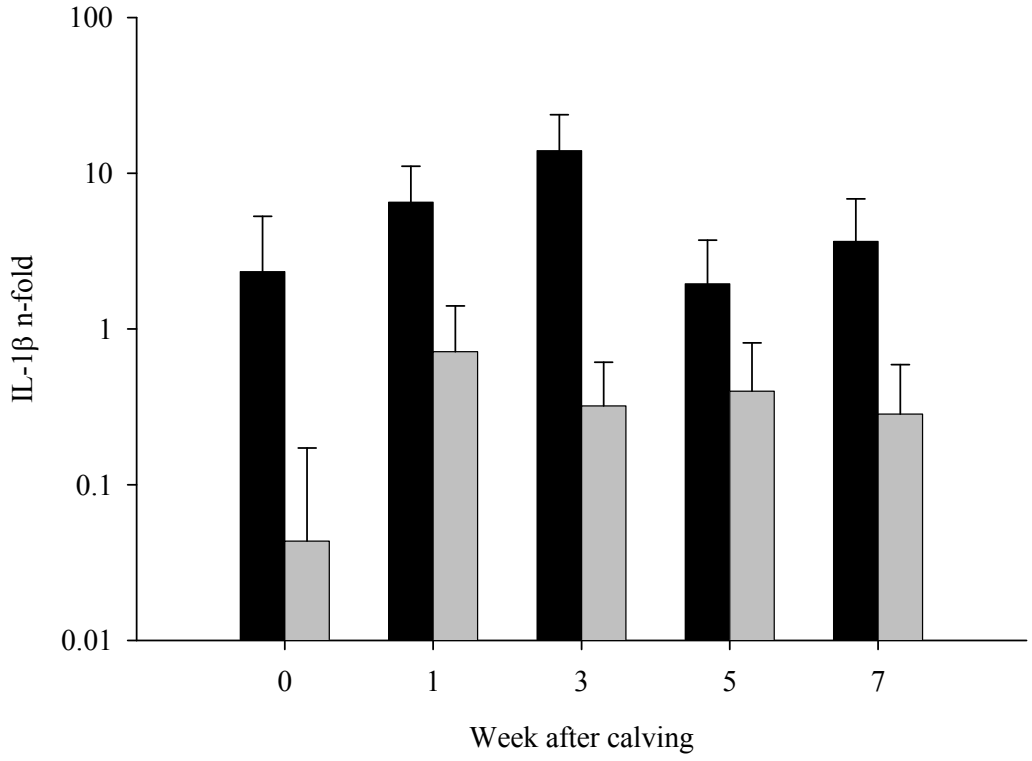

Figure 3.2b. Fold difference (n-fold) in **IL-1β** mRNA gene expression in multiparous cows (black bars) and primiparous cows (gray bars) from calving (0) until week 7 after calving.

(c)

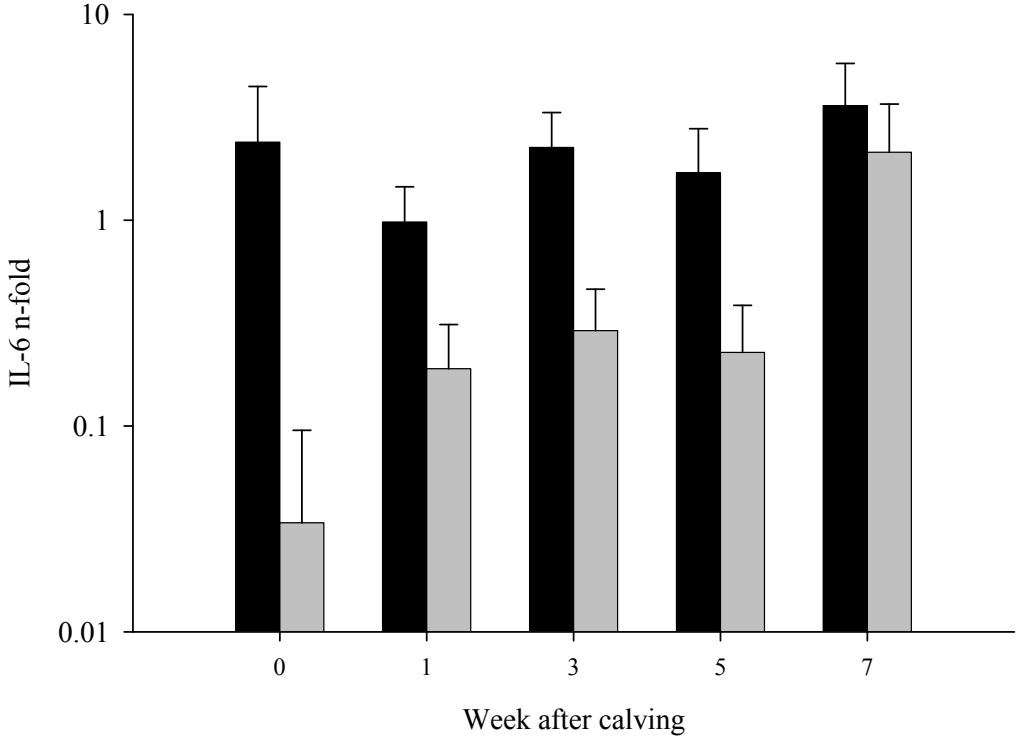

Figure 3.2c. Fold difference (n-fold) in **IL-6** mRNA gene expression in multiparous cows (black bars) and primiparous cows (gray bars) from calving (0) until week 7 after calving.

(d)

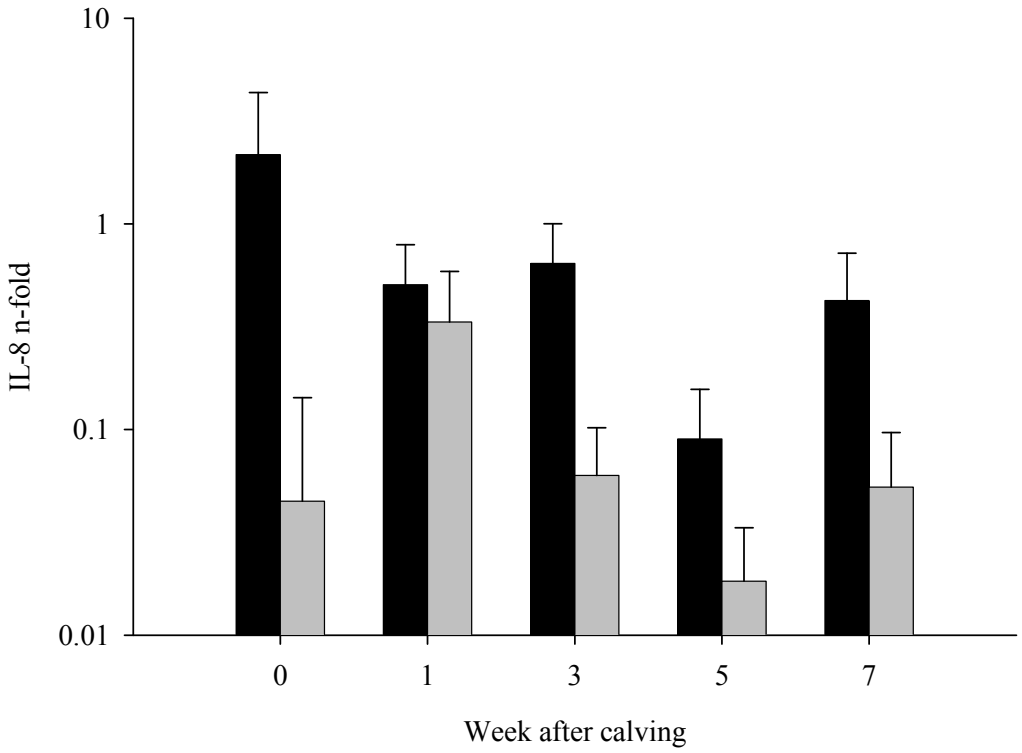

Figure 3.2d. Fold difference (n-fold) in **IL-8** mRNA gene expression in multiparous cows (black bars) and primiparous cows (gray bars) from calving (0) until week 7 after calving.

There was no effect ($P = 0.88$) of uterine disease, time ($P = 0.44$) or interaction between uterine disease and time ($P = 0.44$) on IL-10 gene expression in uterine tissue (**Figure 3.3**). Also, there was no effect of parity on IL-10 gene expression.

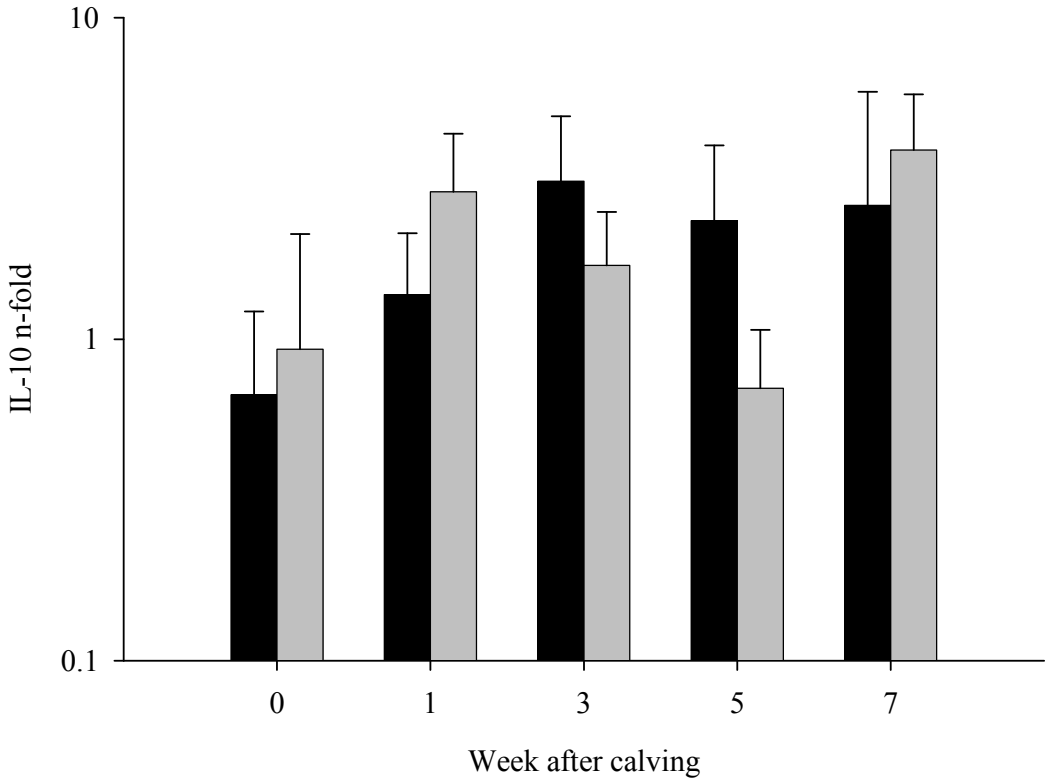

Figure 3.3. Fold difference (n-fold) in **IL-10** mRNA expression in cows that had subclinical endometritis at week 5 (black bars) and healthy cows (gray bars) from calving (0) until week 7 after calving.

There was no effect of uterine disease ($P = 0.30$) or interaction between uterine disease and time ($P = 0.55$) on IL-8 concentration. IL-8 concentration in serum increased from calving to the first week after calving and then decreased steadily from the first week after calving until week 7 (time effect, $P < 0.001$; **Figure 3.4**). Multiparous cows had greater ($P = 0.01$) concentration of IL-8 in serum than primiparous cows (**Figure 3.5**).

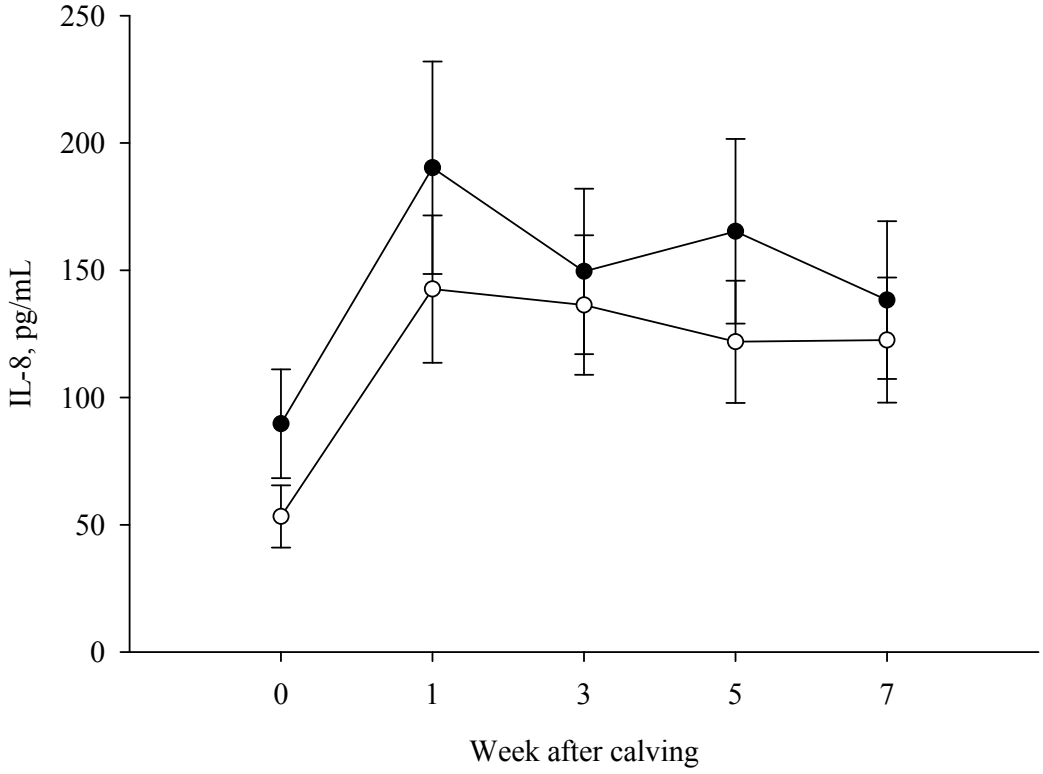

Figure 3.4. Concentration of **IL-8** in serum in cows that had subclinical endometritis at week 5 (black circles) and healthy cows (open circles) from calving (0) until week 7 after calving.

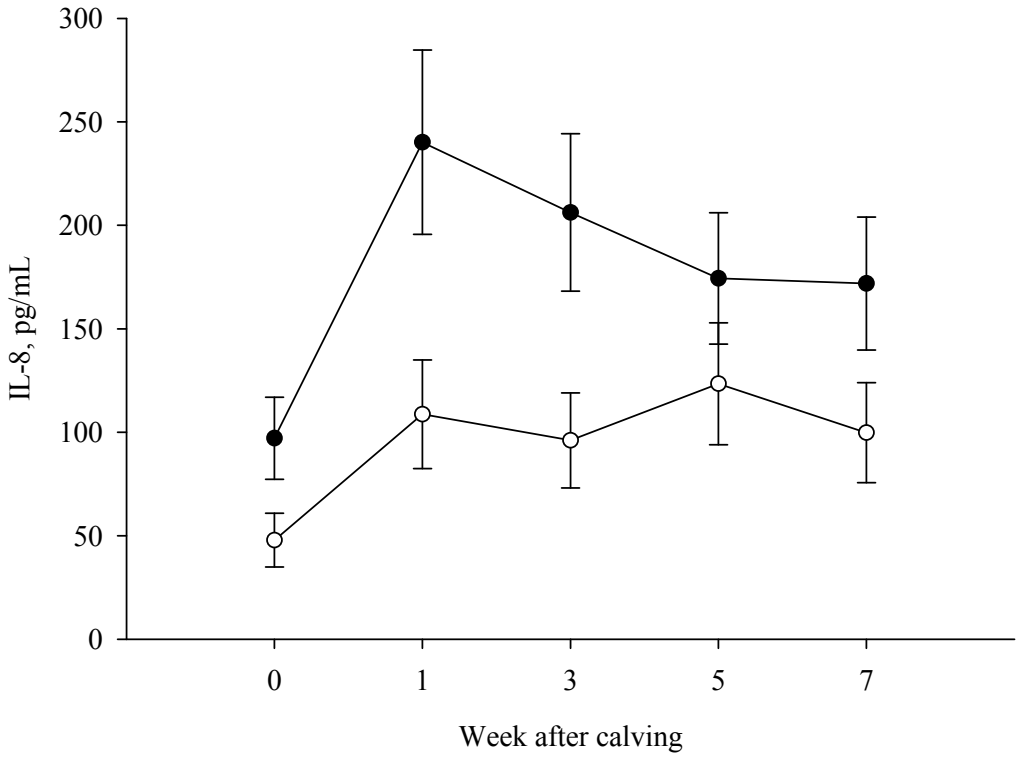

Figure 3.5. Concentration of **IL-8** in serum in multiparous cows (black circles) and primiparous cows (open circles) from calving (0) until week 7 after calving.

DISCUSSION

Little information is in the literature regarding the role of the uterine endometrium and resident MØs to induce an inflammatory process. Resident MØs and the uterine endometrium recognize bacterial invaders and respond by producing cytokines and chemokines to attract and activate neutrophils and monocytes. Herein, we focused on the ability of the endometrium to mount an inflammatory process and observed a decrease in TNFα and IL-1β in the first week after calving. The decrease in expression of TNFα and IL-1β in uterine tissue around calving could compromise the leukocyte response to bacterial invaders by reducing chemotaxis and activation of

neutrophils and monocytes. This would lead to impairment in bacterial clearance and establishment of disease.

The decrease in gene expression of pro-inflammatory cytokines in cows that developed SCE could be due to an intrinsic defect in leukocyte function, (Mallard et al., 1998; Nino-Soto et al., 2008) or extrinsic mechanisms affecting leukocyte activity, such as a greater degree of negative energy balance that these cows experience (Hammon et al., 2006). Because differences in gene expression between cows that developed SCE and healthy cows occurred mainly when negative energy balance was expected to be most severe (Beam and Butler, 1998), it is likely that the state of negative energy balance is affecting the ability of these cows to regulate gene expression. The impairment in leukocyte function around calving is thought to arise from deficit in energy, vitamins or minerals, and an increase in estradiol and glucocorticoids (Goff and Horst, 1997). Therefore, it appears that not only individual leukocytes can be affected during transition into lactation but also other cells with immune capabilities such as endometrial cells.

The pattern of IL-6 gene expression at calving and in the first week after calving was different from TNFα and IL-1β. Whereas TNFα and IL-1β were not affected at calving and were decreased in week 1 in cows that were later diagnosed with SCE. The pattern for IL-6 was reversed. Although IL-6 is an important pro-inflammatory cytokine (aiding in several aspects of inflammation such as induction of fever, increase in vascular permeability and induction of acute phase proteins by the liver), IL-6 is not involved in induction of expression of cell adhesion molecules involved in chemotaxis or induction of chemokine synthesis by vascular endothelium. Therefore, the practical effect of increased IL-6 gene expression at calving in cows that later developed SCE is unclear at this point.

Because we observed a decrease in TNFα and IL-1β gene expression in the

first week after calving in cows that later developed SCE, it was expected that IL-8 gene expression by uterine endometrium and IL-8 secretion by the endometrium vascular endothelium would also be decreased. Nonetheless, we did not detect differences in IL-8 gene expression or IL-8 concentration in serum between healthy cows and SCE cows. It is possible that a lack of power precluded the observation of a statistical difference in gene expression and secretion. Because chemokines act locally and in minute concentrations, a localized difference could occur without a detectable systemic difference.

Because SCE is believed to be caused by failure to clear bacterial contamination (Gilbert et al., 2007; Sheldon and Dobson, 2004), cows that developed SCE were expected to have increased gene expression of pro-inflammatory cytokines and chemokines later in lactation. Although there was no difference in TNFα gene expression, gene expression for IL-1β, IL-6, and IL-8 were increased at 5 and or 7 wk after calving in cows that were diagnosed with SCE at week 5 after calving. Increases in IL-1β alone could have induced up-regulation of both IL-6 and IL-8. Despite up-regulation of IL-8 around the time of diagnosis of SCE, serum concentration of IL-8 was not increased.

There was an increase in gene expression of IL-1β, IL-6, and IL-8 in multiparous cows that was associated with increased concentration of IL-8 in serum. Multiparous cows have a decreased incidence of metritis (Goshen and Shpigel, 2006) but we find either no difference or an increased incidence of SCE in multiparous cows (Cheong et al., 2008; Galvão et al., 2008; Galvão et al., 2009, unpublished). Milk production has a detrimental effect in leukocyte function (Kimura et al., 1999; Nonnecke et al., 2003); therefore, leukocytes from multiparous cows would be expected to be more severely affected because of greater milk yields. In fact, phagocytic activity of neutrophils from older cows is more markedly reduced after

calving compared to younger cows (Kehrli et al., 1989a; Gilbert et al., 1993), and we have observed that multiparous cows have increased bacterial contamination of the uterus at 51 d after calving (Galvão et al., 2009). Therefore, it seems that increased levels of pro-inflammatory cytokine production in the uterine endometrium might help to prevent metritis but because multiparous cows have greater demands for milk yield, they might be less able to clear infection completely and therefore might be more likely to have SCE. Another important factor that might be involved in the susceptibility to metritis is the circulating levels of immunoglobulins. Immunoglobulins work as opsonins, which greatly enhance phagocytic capacity. Primiparous cows have lower immunoglobulin content in colostrums which indicate lower circulating immunoglobulin levels (Muller and Ellinger, 1981); therefore, phagocytosis might not be optimal in early lactation in primiparous cows.

Although a downregulation in pro-inflammatory cytokines would lead to a decreased inflammatory process, an upregulation in anti-inflamatory cytokines, such as IL-10, as it has been shown in cows infected with clinical Johne's disease (Khalifeh and Stabel, 2004), would have the same effect. Nonetheless, we did not observe any difference in gene expression of IL-10 according to disease status; therefore, down-regulation of pro-inflamatory cytokines may be the main factor affecting the ability to mount a proper inflammatory process in cows that develop SCE.

In summary, TNFα and IL-1β gene expression in uterine tissue of cows that developed SCE were decreased in the first week after calving while the gene expression of IL-1β, IL-6 and IL-8 were increased at wk 5 and or 7 after calving. Although TNFα and IL-1β gene expression were decreased in the first week after calving and increased at wk 5 and 7 in cows that developed SCE, serum concentrations of IL-8 were not affected. Multiparous cows have increased uterine gene expression of pro-inflammatory cytokines and chemokines which may prevent

metritis but does not prevent SCE. Cows that develop SCE have compromised ability to up-regulate the gene expression of the main pro-inflammatory cytokines (TNFα and IL-1β) early in lactation. Decreased expression of pro-inflammatory cytokines could lead to poor chemotaxis and activation of neutrophils and monocytes, which would impair bacterial clearance and predispose cows to the development of SCE.

CHAPTER FOUR

Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows

Interpretive summary: Uterine disease and indicators of energy status. Galvão. Neutrophil function depends on glucose for the energy required for chemotaxis, and glycogen stores for phagocytosis and microbial killing. Early in lactation, there is a decrease in glucose availability, which could decrease PMN glycogen stores, and contribute to the loss of PMN function around calving and the development of uterine disease. We evaluated PMN glycogen concentration and blood glucose in cows that developed metritis or subclinical endometritis, and cows that remained healthy. We observed that cows that developed uterine disease were less able to maintain their neutrophil glycogen stores compared to healthy cows, which could be a predisposing factor for the disease.

ABSTRACT

The objective of this study was to evaluate the association between uterine disease and indicators of neutrophil (PMN) and systemic energy status in dairy cows. Peripheral blood (120 mL) was collected weekly from 84 Holstein cows for PMN isolation and plasma collection from calving until 42 d in milk (DIM). The final analysis included 80 cows. Of those, 20 cows were classified as having metritis (fetid uterine discharge and fever), 15 others as having SCE (≥ 10% PMN on uterine cytology) and 45 as healthy controls. Plasma haptoglobin concentration was increased only in cows that developed metritis. Neutrophil glycogen content was less for metritis cows than for healthy cows on the day of calving and at 7 and 42 DIM. SCE cows had lower PMN glycogen content than healthy cows at 7, 28, and 42 DIM. Blood glucose was affected by disease status within parity. Primiparous metritis cows had greater blood glucose concentrations than healthy primiparous cows. Multiparous metritis cows tended to have lower blood glucose concentration than multiparous SCE cows. Cows that developed metritis and SCE tended to have or had greater NEFA and BHBA than healthy cows, mainly around calving. At calving, cows that developed metritis had greater plasma estradiol concentration than healthy cows and greater plasma cortisol than cows that had SCE. Plasma insulin was not affected. Plasma glucagon was increased for SCE cows. Cows that developed uterine disease experienced a greater degree of negative energy balance and had decreased ability to maintain PMN glycogen levels; these could predispose to disease because of decreased availability of oxidative fuels.

Key words: Metritis, glycogen, energy balance, dairy cows

INTRODUCTION

Early in lactation, dairy cows undergo a period of negative energy balance in which the energy consumed does not meet the energy demands for growth, maintenance and milk production (Butler et al., 1981, Doepel et al., 2002). This period is characterized by a decrease in dry-matter intake (DMI), leading to a sharp decrease in glucose and an increase in body fat mobilization in the form of nonsterified fatty acids (NEFA), and resulting in accumulation of products of incomplete oxidation of NEFA such as beta-hydroxybutyrate (BHBA) (Vazquez-Añon et al., 1994).

Neutrophils (PMN) are the main leukocyte type involved in bacterial clearance after uterine infection (Hussain, 1989; Gilbert et al., 2007). However, during the period of negative energy balance, dairy cows experience a reduction in PMN function and killing capacity (Cai et al., 1994; Kehrli and Goff, 1989; Gilbert et al., 1993). In fact, multiparous cows that develop uterine disease have a more pronounced decrease in DMI, an increase in NEFA and BHBA, and a decrease in blood PMN killing ability (Hammon et al., 2006) and phagocytosis (Kim et al., 2005).

Although plasma NEFA (Hammnon et al., 2006) and BHBA (Suriyasathaporn, et al., 2000) are associated with PMN function, PMNs depend mainly on glucose for the energy required for chemotaxis, and on glycogen stores for phagocytosis and microbial killing (Kuehl and Egan, 1980; Weisdorf et al., 1982a; Weisdorf et al., 1982b). Therefore, it is possible that the low glucose levels observed in the first 10 days of lactation (Vazquez-Añon et al., 1994) directly impairs PMN chemotaxis and leads to decreased PMN glycogen stores (leading to decreased phagocytic and killing capability, which would predispose cows to disease).

Glycogen-storage disease, a human condition characterized by the inability of leukocytes to process intracellular glucose (Kim et al., 2006), leads to defects in neutrophil respiratory burst, chemotaxis and calcium influx in response to bacterial

stimulation. Moreover, in an experimental model of peritoneal inflammation, macrophage production of chemokines and peritoneal PMN accumulation were decreased in mice with glycogen-storage disease (Kim et al., 2006).

Therefore, we hypothesized that lactating dairy cows that end up developing uterine disease would have lower glucose and/or decreased PMN glycogen stores in early lactation, compared to healthy cows. The objective was to compare plasma glucose, intracellular PMN glycogen concentration, and indicators of energy balance (NEFA and BHBA) in cows that develop uterine disease and healthy cows in the first 42 DIM. Because glucose can be increased on the day of calving in response to cortisol, and glucose homeostasis is mainly controlled by insulin and glucagon, those hormones were also evaluated. Because estradiol is believed to affect PMN function negatively, this hormone was also evaluated. Because milk production can deplete blood glucose in early lactation, milk yield was also evaluated.

MATERIALS AND METHODS

Animals, Housing, and Feeding

Eighty-four cows (38 primiparous and 46 multiparous) from a commercial Holstein dairy farm located in Cayuga County, New York, USA were enrolled in the study from April to November of 2007. A physical exam was performed at calving and only cows appearing healthy were enrolled. Cows that had uterine prolapses, cesarean section, or were severely lame were not enrolled. Cows were not excluded if they developed retained placenta, ketosis, mastitis or mild lameness. Two out of the 38 primiparous and 2 out of the 46 multiparous cows enrolled in the study were culled before the end of the observation period and excluded from the analysis. One of the primiparous was culled because of low milk yield after a surgical correction of left-displaced abomasum and the other because of herpes virus teat lesions. The

primiparous cow that had left-displacement of the abomasum also had metritis. The multiparous cows were culled because of severe lameness. None of the other cows that were culled had metritis. Because they were culled before 42 DIM, they were not sampled for SCE. Therefore, 80 cows (36 primiparous and 44 multiparous) were used for the final analysis of the association between uterine disease and the outcomes of interest. The herd had 3,000 milking cows and the rolling herd averages was about 12,000 kg of milk. Primiparous and multiparous cows were housed separately in freestall facilities. Cows were moved to far-off pens 60 days before expected calving date, and moved to close-up pens 21 days before expected calving date. Cows were moved to calving pens that would hold up to 5 cows 3 days before expected calving or when imminent signs of calving (such as exposure of uterine membranes) appeared. After calving, cows were milked and then transferred to fresh-cow pens where they stayed for about 30 days, and subsequently moved to a mid-lactation pen for the remainder of the experiment. Lactating cows were milked three times daily. All cows were fed the same total mixed ration formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% fat corrected milk.

Sample Collection

All cows had 120 mL of blood collected weekly from the jugular vein, using two 60 mL syringes with 3 mL of 0.5M EDTA each, and a 12-gauge needle, from the day of calving until 42 DIM. For blood collection, cows had their head locked into headlock stanchions and tied to one side using a halter; the hair over the jugular vein was clipped and the skin disinfected using iodine solution. The side of blood collection was alternated after each sampling to prevent phlebitis. Blood was collected immediately after the morning milking. At the time of blood collection, body

condition was scored using a 5-point (1 = thin to 5 = fat) system (Ferguson et al., 1994). Evaluator of body condition score was not blinded to uterine health status.

Glycogen, Glucose, Hormones, and other metabolites

After blood collection, syringes were gently inverted 10 times to allow thorough mixing with EDTA, blood glucose was measured using a portable glucometer (Accu-Check® Active, Roche Diagnostics, Indianapolis, IN), and the syringes were placed on ice and transported to the laboratory within 4 hours. To validate the use of the glucometer for dairy cows, we compared the results from the glucometer with that of a biochemistry analyzer (YSI-Life Sciences, BioAnalyzer model 7100, Yellow Springs, OH) using EDTA plasma samples from 52 lactating cows from the Cornell University dairy herd. The two methods were highly associated ($P < 0.001$) and the coefficient of determination (R^2) was 66%. Nonetheless, the values given by the BioAnalyzer were on average 9.6 points lower than the glucometer (62.3 vs. 71.9 mg/dL). The following is a Bland-Altman plot of the plasma glucose measured with the BioAnalyzer by the blood glucose measured with the glucometer; **Figure 4.1**). This plot is used to assess agreement between the two methods of measurement. As you can see, most of the squares (data points) lie within the 95% confidence limits.

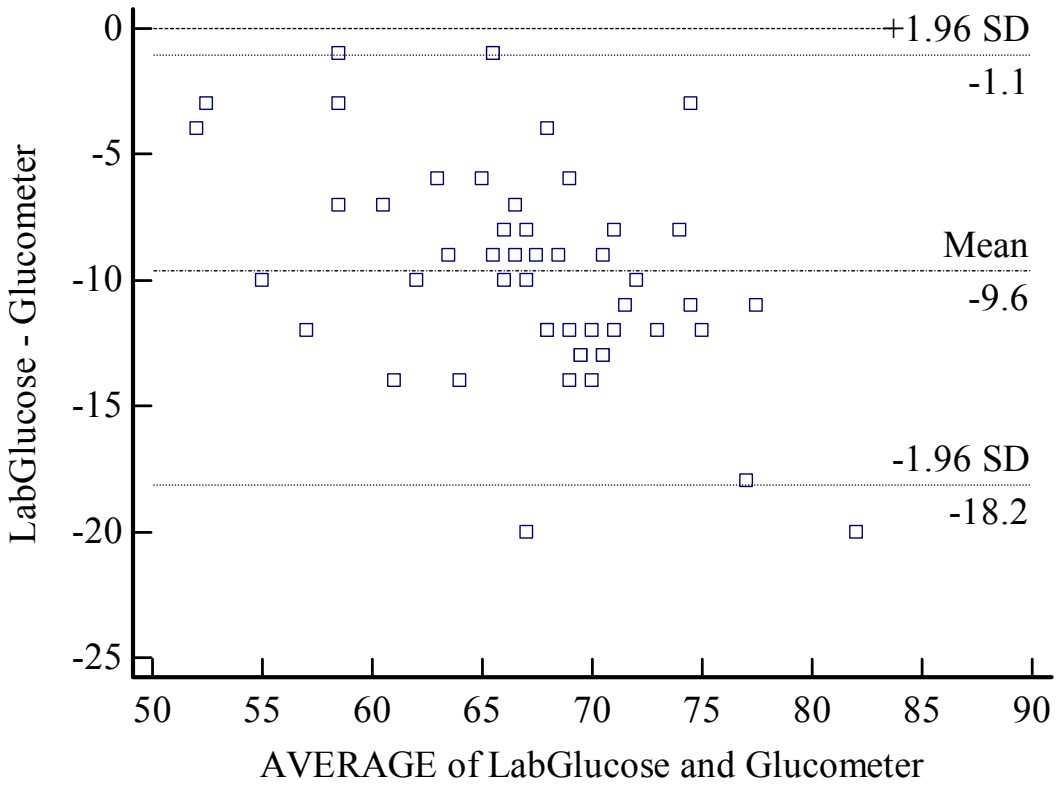

Figure 4.1. Bland-Altman plot of the plasma glucose measured with a biochemistry analyzer by the blood glucose measured with a blood glucometer.

In the laboratory, 40 mL of blood were transferred into three 50 mL polypropylene centrifuge tubes (Corning® Life Sciences, Lowell, MA) and centrifuged at 1200 g for 30 min to separate the plasma; 10 mL of plasma were harvested and frozen at -20 ^{o}C for hormone and metabolite assays. Blood PMNs were isolated as described previously (Roth and Kaeberle, 1981). After isolation, 10^7 PMNs were frozen in duplicates for later glycogen determination using a micromodification of the method of Keppler and Decker (1974) as previously described (Weisdorf et al., 1982a). Briefly, glycogen was hydrolyzed to glucose using amyloglucosidase; available glucose was determined by reacting 50 µL of supernatant with a 1 mL mixture of 1 mM ATP, 0.9 mM NADP, 5 µg G6DP, 0.3 M triethanolamine, 4 mM

MgSO4, and recording the appearance of NADPH after the addition of 5 μL of hexokinase (2 mg/ml) as ΔOD at 340 nm on a spectrophotometer. This ΔOD was compared to a standard curve of oyster glycogen assayed in similar fashion, and results were expressed as μg glycogen/10^6 PMN.

Plasma concentrations of NEFA were analyzed by enzymatic analysis (NEFA-C®; Wako Pure Chemical Industries, Osaka, Japan) using modifications described by McCutcheon and Bauman (1986) and Sechen et al. (1990). Plasma concentrations of BHBA were quantified (BHBA dehydrogenase) using a commercial kit (kit 310-UV®; Sigma Chemical). All spectrophotometric measurements were conducted using a microplate reader (BioTek Instruments, Model EL 340, Winooski, Vermont). In addition, plasma was analyzed for concentrations of estradiol and cortisol by radioimmunoassay (RIA) using the Coat-A-Count® estradiol and cortisol assay kits (Siemens Medical Solutions Diagnostics, Los Angeles, CA), respectively. Plasma concentrations of insulin we determined by RIA (Ehrhardt et al., 2001) using bovine insulin (Elanco Animal Health, Greenfield, IN) and glucagon (Linco RIA kit, Linco Research, St. Charles, MO) kits. Intra- and interassay coefficients of variation were 1.8 and 2.6%, 5.1 and 6.7%, 6.1 and 9.6%, 3.0 and 9.8%, 5.6 and 7.2%, 6.5 and 8.1%, for NEFA, BHBA, estradiol, cortisol, insulin, and glucagon, respectively.

Evaluation of Uterine and Metabolic Diseases

Cows were evaluated daily by the herd personnel for signs of metritis in the first 14 DIM. Metritis was characterized by the presence of watery, fetid vaginal discharge and rectal temperature > 39.5 ^{o}C. Cows with clinical metritis received 2 boluses of aspirin (15.6 g of acetylsalicyclic acid per bolus; AgriLabs, St. Joseph, MO) until the fever improved or for a maximum of 3 days and ceftiofur hydrochloride for 5 consecutive days at a dose of 2.2 mg/kg body weight (Excenel® RTU EZ, ceftiofur

hydrochloride sterile suspension, Pfizer Animal Health, NY). Febrile or lethargic cows were checked for acetonuria (Acetoacetic Acid) as an indicator of ketosis using ketone strips (Ketostix®; Bayer Health Care, Tarrytown, New York). Cows with moderate or high ketones were treated with 500 mL 50% dextrose intravenously (AgriLabs, St. Joseph, MO) until urine ketones were lowered. Blood sampling was performed before treatment administration. At 42 DIM, cows had a low-volume uterine lavage for diagnosis of subclinical endometritis based on the proportion of PMN out of a total of 200 cells, including all leukocyte types and epithelial cells but excluding erythrocytes, as previously described (Gilbert et al., 2005). Based on a larger experiment (Cheong et al., 2008), the proportion of PMN that classified cows as having subclinical endometritis was ≥ 10%.

Statistical Analyses

This was an observational cohort study. 80 cows (36 primiparous and 44 multiparous) were used for the final analysis of the association between uterine disease and the outcomes of interest. The cows were classified into three categories: 1) cows that developed metritis in the first 14 DIM; 2) cows that did not develop metritis and were diagnosed with subclinical endometritis at 42 DIM; 3) healthy control cows. All outcomes (PMN glycogen concentration, hormones, and metabolite) were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS (Version 9.1; SAS Inst. Inc., Cary, NC). Each outcome was analyzed in separate models over the whole experimental period. Models included the effects of uterine disease, parity (primiparous vs. multiparous), mean BCS (< 2.75 vs. ≥ 2.75), BCS change from calving to 42 DIM (< 0.5 vs. > 0.5 points), time (day of blood collection; 0, 7, 14, 21, 28, 35, and 42), and interactions between uterine disease and other covariates. Uterine disease and parity were forced into the models but other covariates

were manually removed if $P > 0.15$ bases of Wald's statistic criterion. Because data were collected longitudinally, data points were correlated within each cow; therefore, cow was included in the analysis as a random effect. Because all the outcomes evaluated were biological samples collected at even intervals, a first-order autoregressive covariance structures used. When either an effect of metritis or subclinical endometritis, or an interaction between metritis or subclinical endometritis and time was observed, post-hoc multiple comparisons were performed using the Bonferroni adjustments in SAS.

Evaluation of normality of the residuals was performed in Minitab (Version 15; Minitab Inc., State College, PA) by inspection of standardized residuals plotted against predicted values for the residuals using the regression option in Minitab. Residuals fit to a normal distribution were also evaluated. Standardized residuals for glycogen, glucose, and glucagon were found to be normally distributed; however, raw data for haptoglobin, NEFA, BHBA, cortisol, and insulin, were transformed to their base-10 logarithm to achieve normality. After statistical analysis, transformed data were back-transformed to report least-squares means. Differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered a tendency. Interactions were considered to be significant when $P \leq 0.15$.

RESULTS

Of the 80 cows used in the final analysis, 20 (25%) were diagnosed with metritis and classified as metritis for the statistical analysis; 23 were diagnosed with SCE but only 15 were classified as SCE for the statistical analysis (because 8 of them had metritis and therefore were classified as metritis) and 45 were not diagnosed with metritis or SCE and were classified as healthy controls. Although 8 out of 20 cows

diagnosed with metritis were also diagnosed with SCE, cows that had metritis were not more likely to be diagnosed with SCE than cows that did not have metritis (40% (8/20) vs. 25% (15/60); $P = 0.20$). Incidence of metritis was similar between multiparous and primiparous (23% (10/44) vs. 28% (10/36); P = 0.60); but prevalence of SCE tended to be greater in multiparous compared to primiparous (36% (16/44) vs. 19% (7/36); $P = 0.09$). Mean and median day of diagnosis of metritis was 7 DIM (range 3 to 12 DIM). Cows that had metritis were more likely to be diagnosed with clinical ketosis based on ketone strips compared to healthy cows (35 (7/20) vs. 2% (1/45); $P < 0.01$; Fisher's exact test) and tended to be more likely than cows that had SCE (35 (7/20) vs. 7% (1/15); $P = 0.10$). Mean and median day of diagnosis of ketosis was 6 DIM (range 2 to 10 DIM).

Cows that developed metritis had greater plasma concentration of haptoglobin then cows that had SCE ($P = 0.05$) or healthy cows ($P = 0.02$). Haptoglobin concentration was similar between cows that had SCE and healthy cows ($P = 0.87$). There was also an interaction between uterine disease and time ($P < 0.001$), in which cows that developed metritis had greater plasma haptoglobin concentration than cows that had SCE (P = 0.005) or healthy cows ($P < 0.001$) at 7 DIM. Plasma haptoglobin concentrations peaked at 7 DIM then dropped sharply until 21 DIM (time effect, P < 0.001; **Figure 4.2**). No other variable affected plasma haptoglobin concentrations.

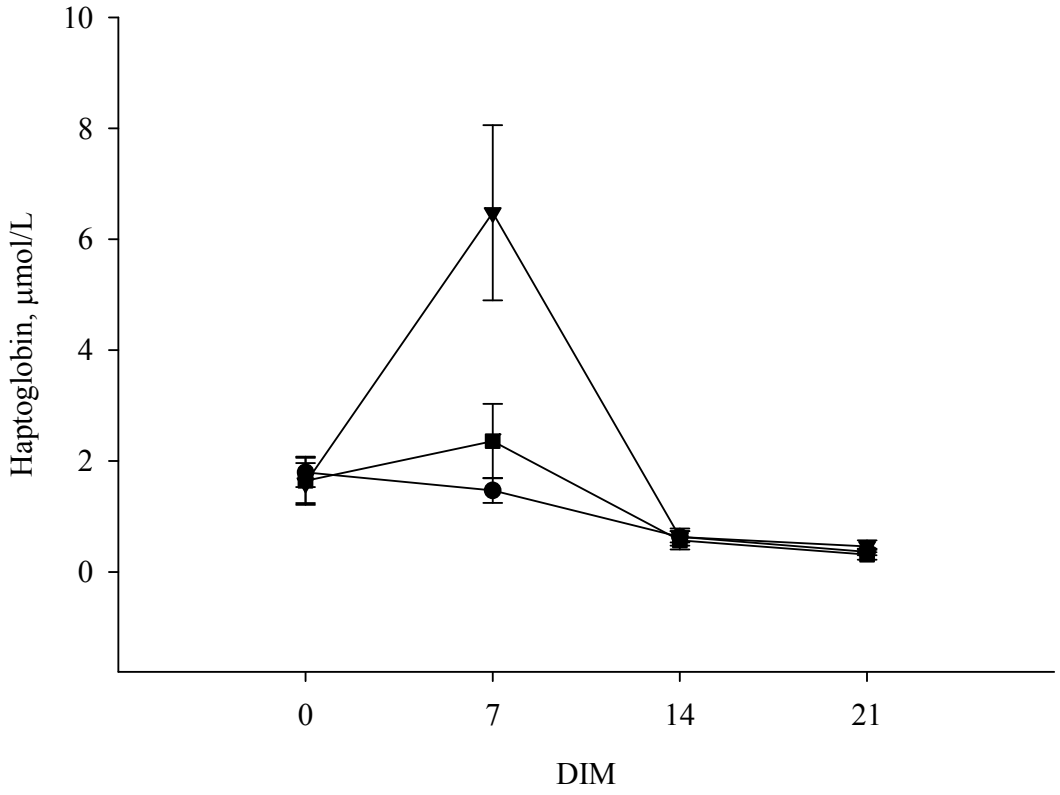

Figure 4.2. Least-squares means ± S.E.M. for plasma **haptoglobin** concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

Cows that had SCE had an overall greater ($P = 0.04$) plasma NEFA concentration than healthy cows while cows that developed metritis tended to have greater NEFA concentrations on the day of calving ($P = 0.08$) and at 35 DIM ($P = 0.06$) than healthy cows (**Figure 4.3a**). Primiparous cows had greater ($P = 0.02$) NEFA concentration compared to multiparous cows (mean ± S.E.M.; 302 ± 21 vs. 241 ± 15 µeq/L). Plasma NEFA concentrations were greatest at calving and at 7 DIM, and then dropped steadily from 7 DIM until 42 DIM (time effect, $P < 0.001$; **Figure 4.3a**). No other variable affected plasma NEFA concentrations.

(a)

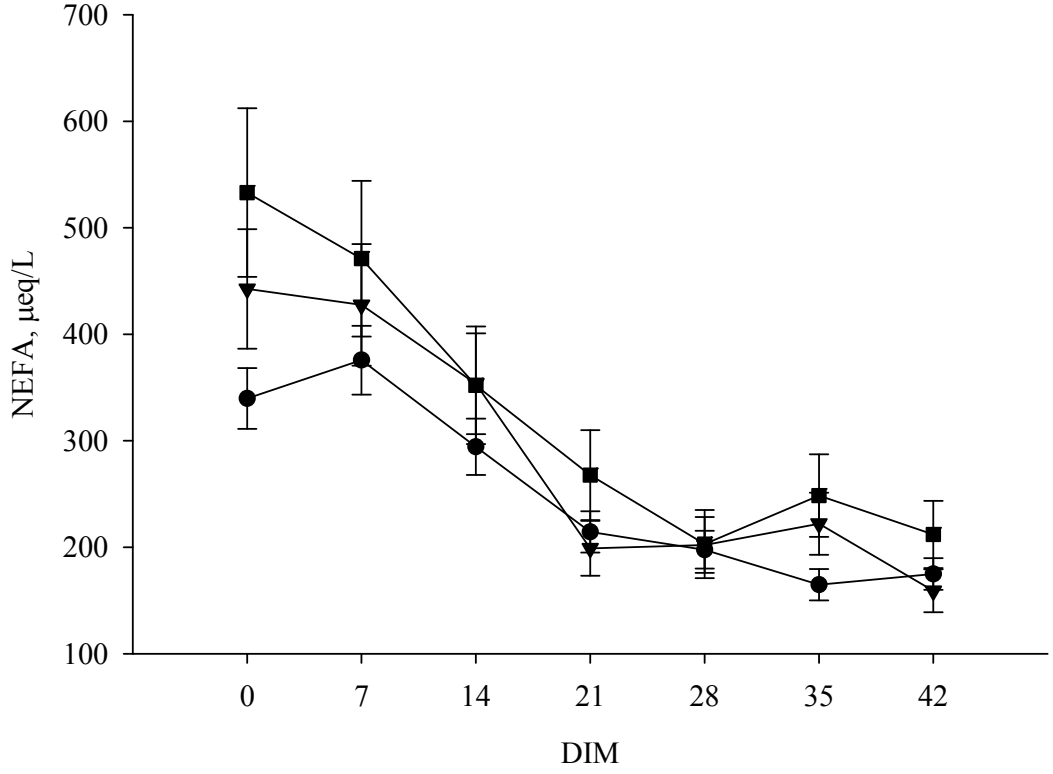

Figure 4.3a. Least-squares means ± S.E.M. for plasma **NEFA** concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was an interaction between uterine disease and time ($P = 0.12$), in which cows that developed metritis had greater ($P = 0.03$) BHBA concentrations on the day of calving compared to healthy cows and SCE cows tended to have greater BHBA concentrations at 7 ($P = 0.10$) and 14 DIM ($P = 0.07$) compared to healthy cows (**Figure 4.3b**). Plasma BHBA concentrations peaked at 7 DIM, then dropped from 7 DIM to 14 DIM, and remained constant until 42 DIM (time effect; $P = 0.05$; **Figure**

4.3b). No other variable affected plasma BHBA concentrations.

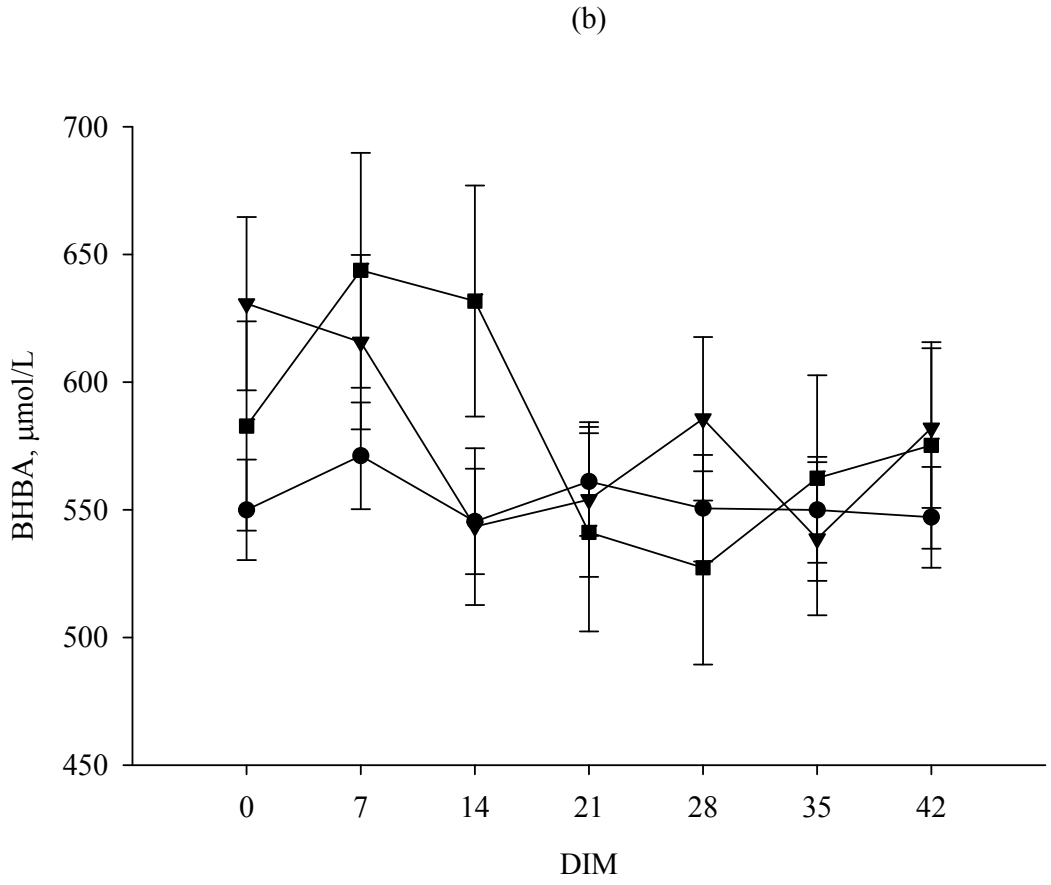

Figure 4.3b. Least-squares means ± S.E.M. for plasma **BHBA** concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was an interaction ($P = 0.001$) between uterine disease and time on blood PMN glycogen concentration (**Figure 4.4**). Metritis cows had lower ($P = 0.001$) blood PMN glycogen concentration than cows that had SCE on the day of calving and lower than healthy cows on the day of calving ($P = 0.03$), at 7 ($P = 0.10$) and 42 DIM ($P = 0.05$). SCE cows had lower blood PMN glycogen than healthy cows at 7 (P =

0.06), 28 (P = 0.01), and 42 DIM (*P* = 0.02). Blood PMN glycogen concentrations decreased from calving until 14 DIM, then recovered to the level of calving by 42 DIM (time effect; *P* = 0.001; **Figure 4.4**). No other variable affected PMN glycogen concentrations.

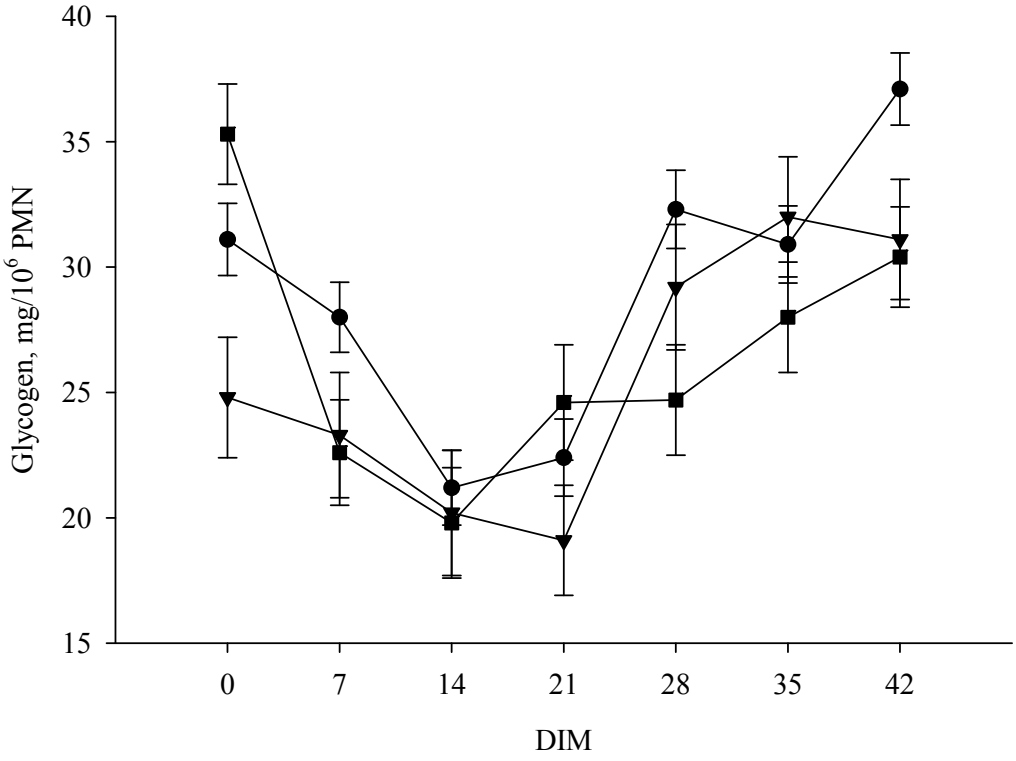

Figure 4.4. Least-squares means ± S.E.M. for blood PMN **glycogen** concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There were interactions between uterine disease and parity (*P* = 0.03), and a three-way interaction among uterine disease, parity and time (*P* = 0.07) on blood glucose concentrations (**Figures 4.5a, 4.5b**). Primiparous metritis cows had greater (*P* ≤ 0.05) glucose concentrations than primiparous SCE cows or healthy primiparous

cows on the day of calving and at 14 DIM, and greater than SCE cows at 42 DIM (**Figure 4.5a**); while multiparous metritis cows had lower glucose concentrations than multiparous SCE cows or healthy multiparous cows at 28 and 42 DIM (**Figure 4.5b**). Overall, primiparous cows had greater ($P < 0.001$) blood glucose concentration than multiparous cows (3.9 ± 0.1 vs. 3.5 ± 0.04 mmol/L). Blood glucose concentrations dropped sharply from the day of calving to its lowest value at 7 DIM, and recovered from 7 to 42 DIM; however, blood glucose at 42 DIM was still lower than on the day of calving (time effect, $P < 0.001$; **Figure 4.5a, 4.5b**). No other variable affected blood glucose concentrations. Spearman's rank correlation showed a positive correlation between PMN glycogen concentration and plasma glucose concentration (correlation coefficient $\rho = 0.2$; $P < 0.001$); however, the correlation was low (< 0.3).

(a)

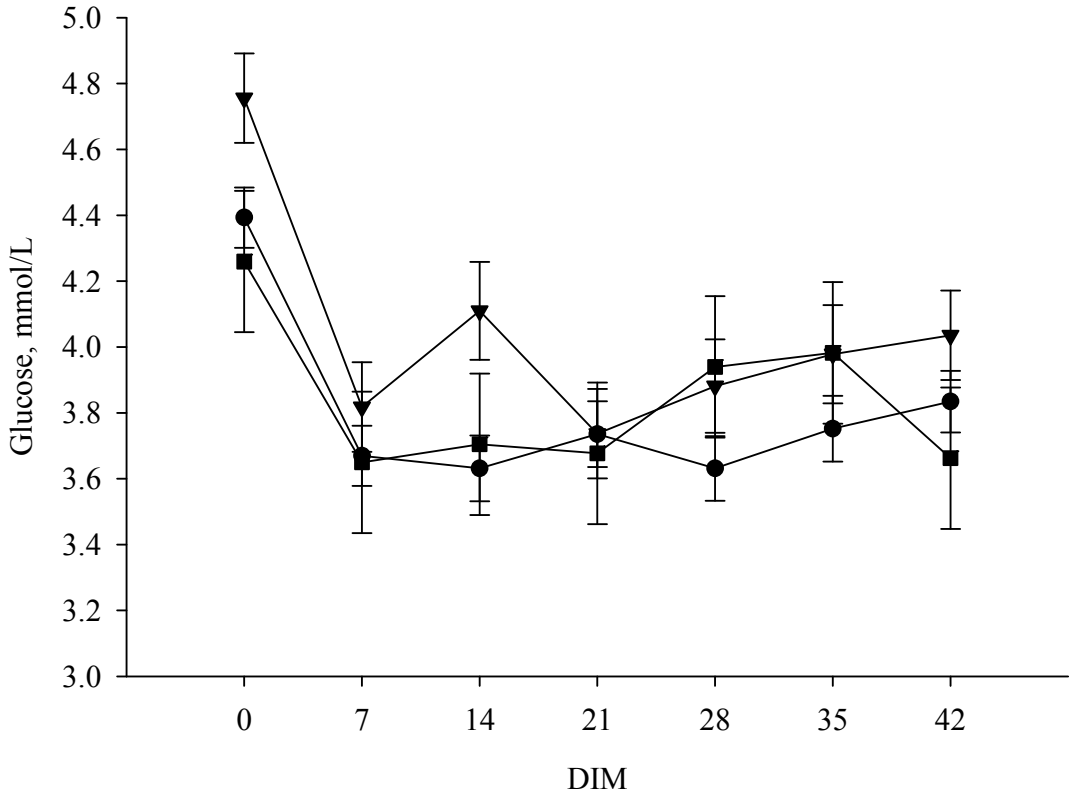

Figure 4.5a. Least-squares means ± S.E.M. for blood **glucose** concentrations for primiparous cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

(b)

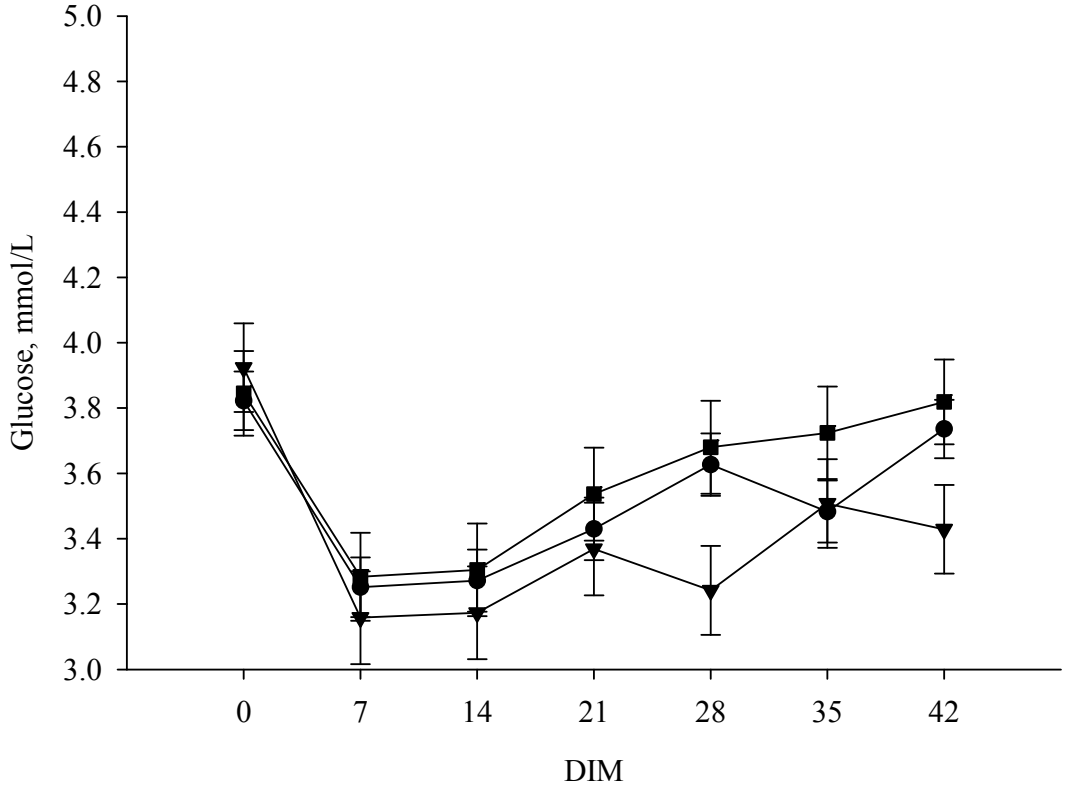

Figure 4.5b. Least-squares means ± S.E.M. for blood **glucose** concentrations for multiparous cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was an interaction between uterine disease and time ($P = 0.14$) on plasma cortisol concentrations (**Figure 4.6**); metritis cows had greater ($P = 0.04$) plasma cortisol concentration than SCE cows on the day of calving and had lesser ($P = 0.03$) cortisol concentration than healthy cows at 14 DIM. There was also, a tendency ($P = 0.06$) for primiparous cows to have greater plasma cortisol concentration than multiparous cows (25.4 ± 2.4 vs. 20.3 ± 1.5 nmol/L). Plasma cortisol concentration decreased from calving until 21 d after calving (time effect, $P = 0.10$; **Figure 4.6**) Spearman's rank correlation showed positive correlation between plasma cortisol and blood glucose concentration ($\rho = 0.2$; $P < 0.001$); however, the correlation was low (< 0.3).

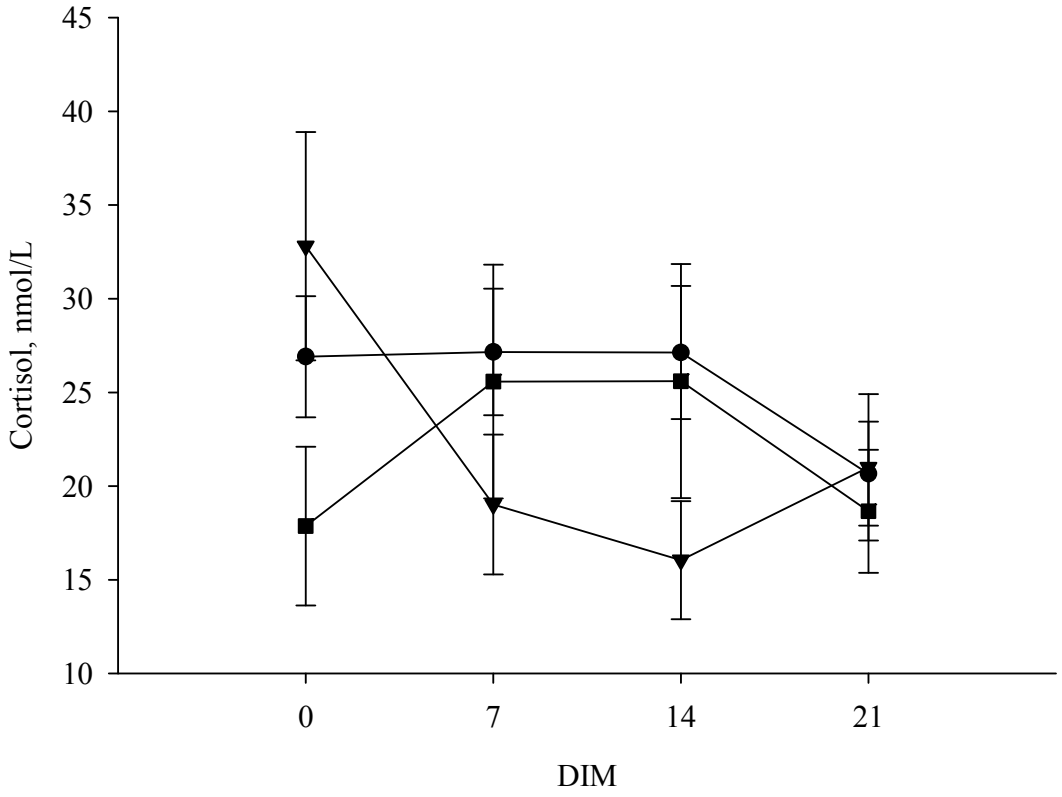

Figure 4.6. Least-squares means ± S.E.M. for plasma **cortisol** concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical

endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was a three-way interaction among uterine disease, parity and time ($P < 0.001$) on milk yield concentrations (**Figure 4.7a, 4.7b)**. Weekly average for the daily milk yield was lesser ($P < 0.10$) for primiparous metritis cows compared to primiparous SCE cows from the second (8 to 14 DIM) until the fourth week (22 to 28 DIM) of lactation and lesser than healthy primiparous cows for the third and fourth week (15 to 21 and 22 to 28 DIM) of lactation. Weekly average for the daily milk yield was grater ($P \leq 0.05$) for multiparous metritis cows than multiparous SCE cows and healthy multiparous cows for the fifth and sixth week (29 to 35 and 36 to 42 DIM) of lactation. Healthy multiparous cows also had greater milk yield than multiparous SCE cows for the fifth week (29 to 35 DIM) of lactation (**Figure 4.7b**). Overall, primiparous cows had lower ($P < 0.001$) milk yield than multiparous cows (29 ± 1 vs. 39 ± 1 Kg/d). Milk yield increased sharply from the first 7 DIM until 28 DIM and then plateau until 42 DIM (time effect; $P < 0.001$; **Figure 4.7a, 4.7b**). Spearman's rank correlation showed that there was a negative correlation between milk yield and blood glucose concentration ($\rho = - 0.27$; $P < 0.001$); however, the correlation was low.

(a)

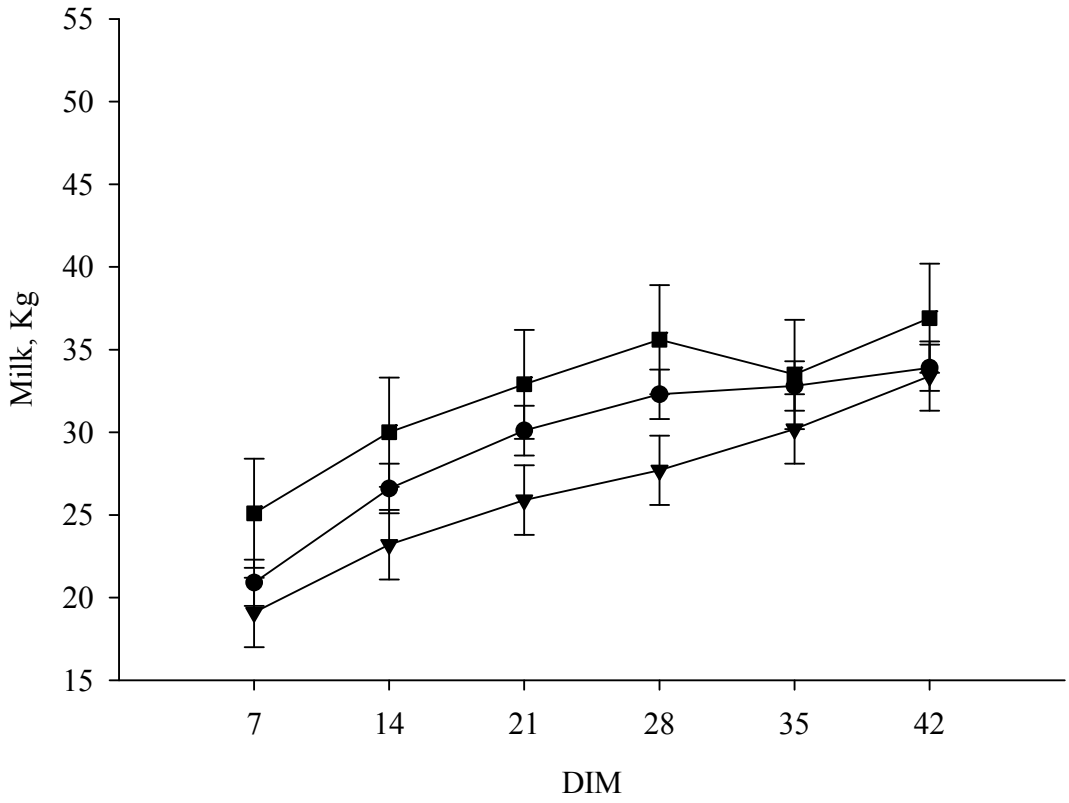

Figure 4.7a. Least-squares means ± S.E.M. for weekly **milk yield** for primiparous cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles). Milk yields are the daily average for the week, starting at calving.

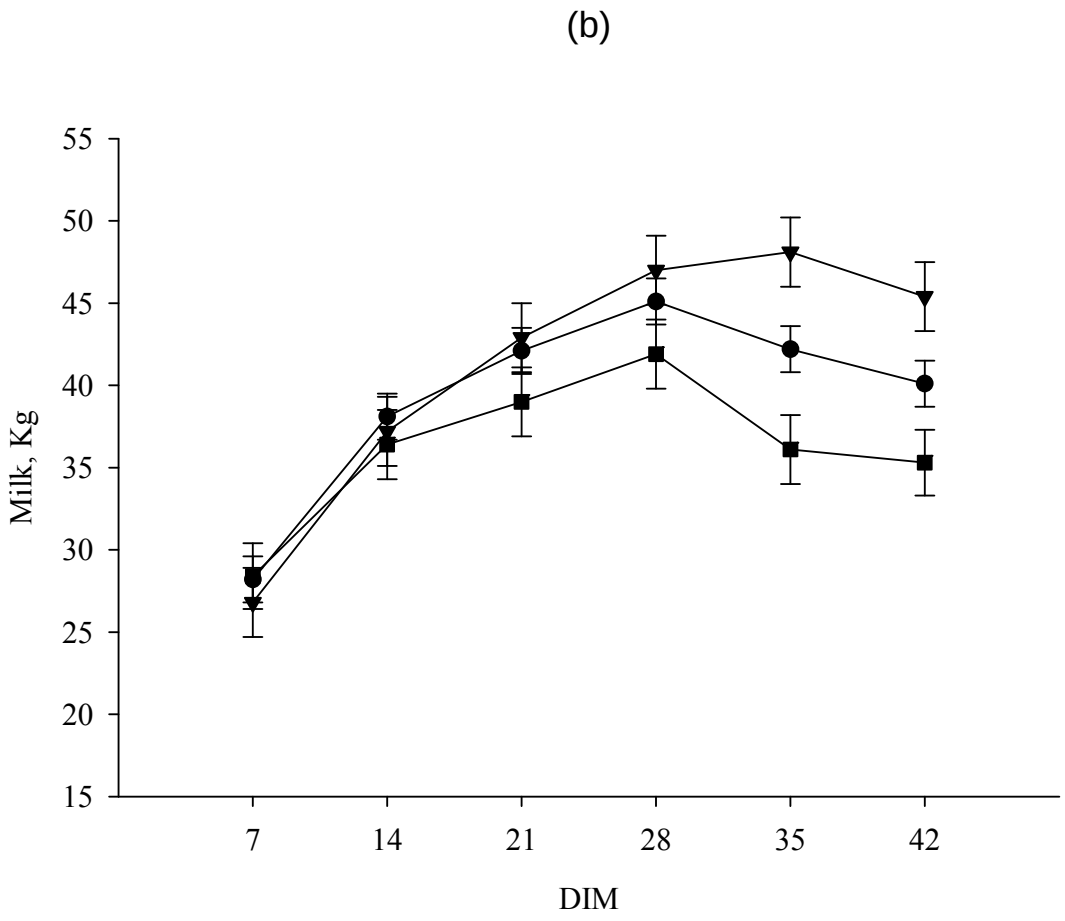

Figure 4.7b. Least-squares means ± S.E.M. for weekly **milk yield** for multiparous cows (b) that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles). Milk yields are the daily average for the week, starting at calving.

There was a three-way interaction among uterine disease, parity and time (P = 0.02) on plasma insulin concentration (**Figures 4.8a, 4.8b).** Plasma insulin was lower for primiparous SCE cows with SCE compared with primiparous metritis cows (27 ± 8 vs. 66 ± 13; P = 0.01) at 42 DIM. Plasma insulin concentrations were high on the day of calving and dropped to its lowest levels at 7 DIM and increased from 7 DIM to the levels at calving by 42 DIM (time effect, $P < 0.001$; **Figures 4.8a, 4.8b**). Plasma

insulin concentrations were high on the day of calving and dropped to its lowest levels at 7 DIM and increased from 7 DIM to the levels at calving by 42 DIM (time effect, *P* < 0.001; **Figures 4.8a, 4.8b**). No other variable affected plasma insulin concentrations. Also, there was no effect of uterine disease (P = 0.67) or interaction between uterine disease and time (P = 0.27) on glucose to insulin ratio. Spearman's rank correlation showed a positive correlation between blood glucose and plasma insulin concentration (ρ = 0.29; P < 0.001); however, the correlation was low.

(a)

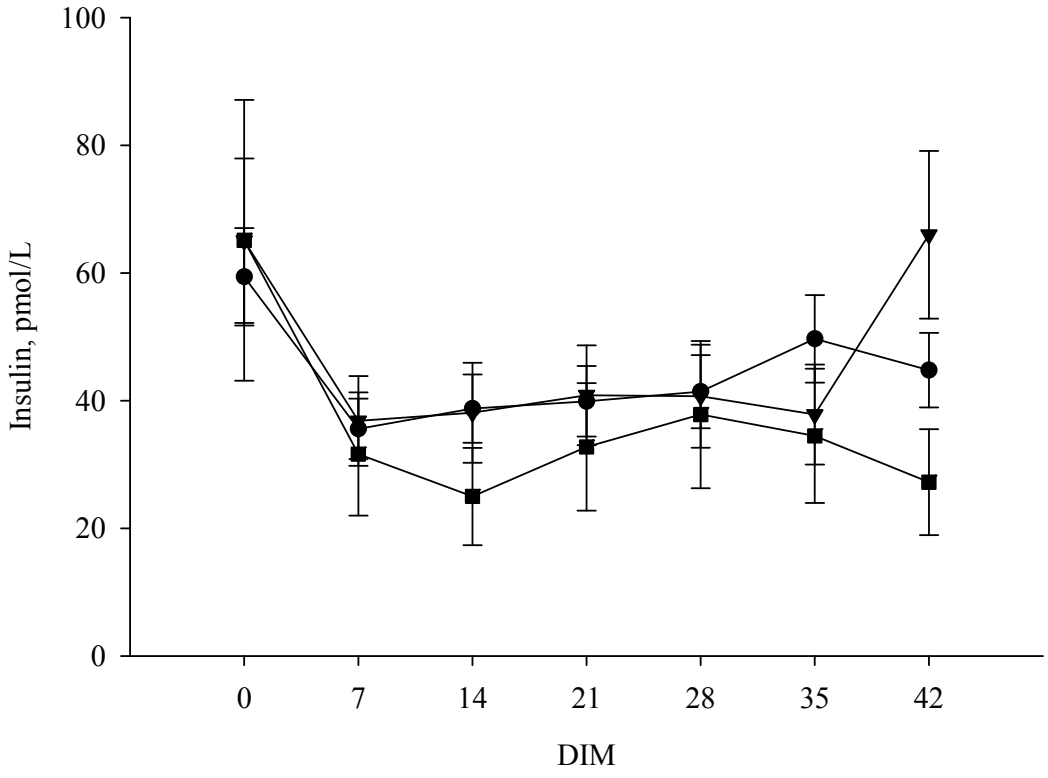

Figure 4.8a. Least-squares means ± S.E.M. for plasma **insulin** concentration for primiparous cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM

(circles).

(b)

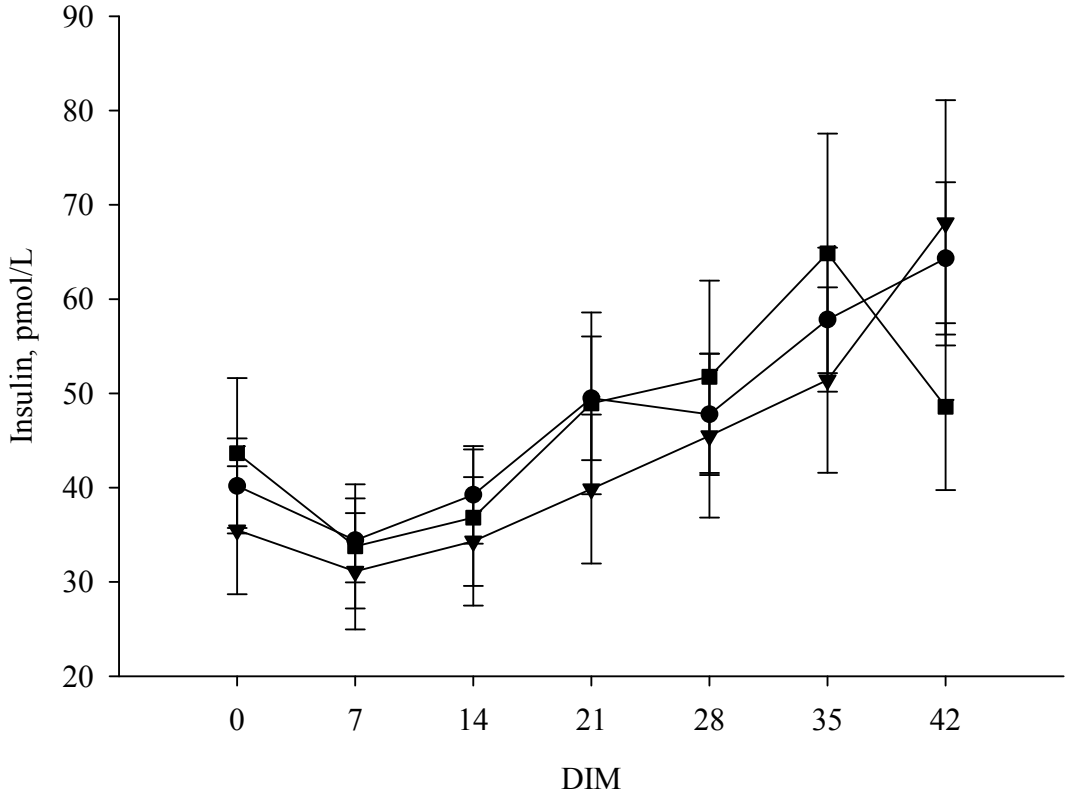

Figure 4.8b. Least-squares means ± S.E.M. for plasma **insulin** concentration for multiparous cows (b) that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

There was an interaction between uterine disease and time ($P = 0.003$) on plasma glucagon concentration (**Figure 4.9**). Cows that had SCE had greater ($P < 0.05$) plasma glucagon concentration than healthy cows and metritis cows at 7, 28 and 35 DIM. Multiparous cows had greater ($P < 0.001$) glucagon concentration than

primiparous cows (109 ± 3 vs. 92 ± 4 ng/L). Plasma glucagon concentrations were lowest on the day of calving and increased from calving until 28 DIM and then decreased slightly until 42 DIM (time effect, $P < 0.001$; **Figure 4.9**). No other variable affected plasma glucagon concentrations. Spearman's rank correlation showed a negative correlation between blood glucose and plasma glucagon concentration ($\rho = -0.29$; $P < 0.001$); however, the correlation was low.

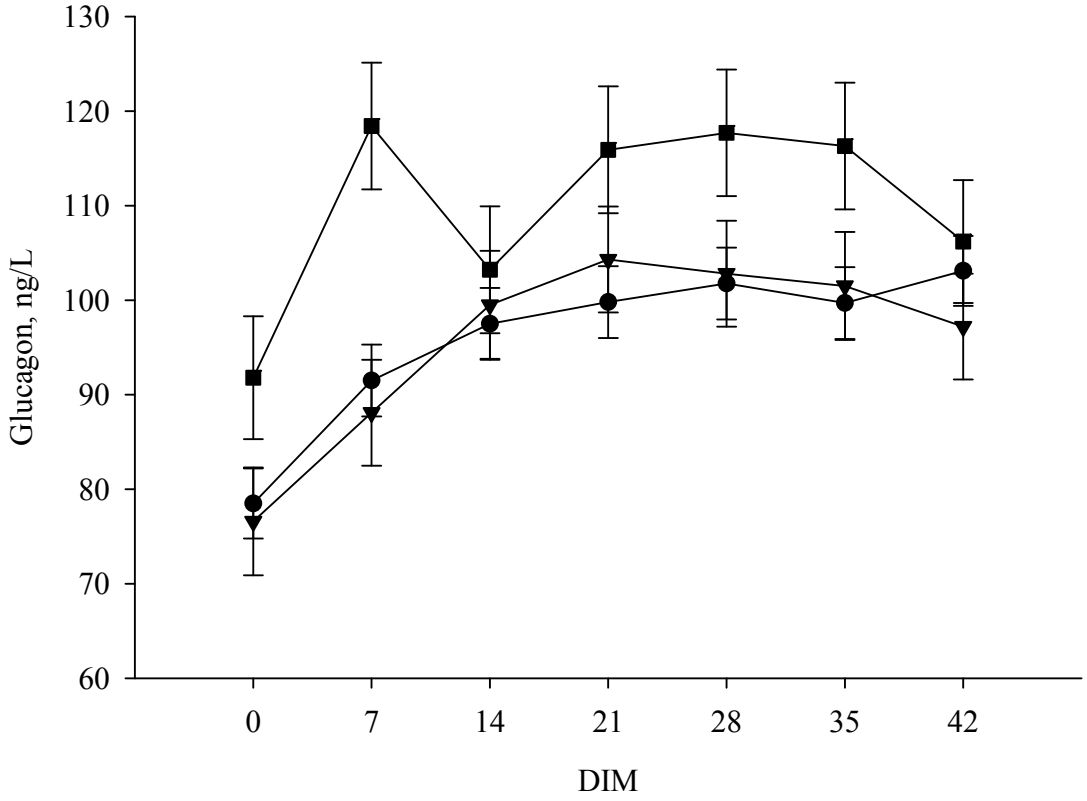

Figure 4.9. Least-squares means ± S.E.M. for plasma **glucagon** concentrations for cows that developed metritis up to 14 days in milk (DIM) (triangles), had subclinical endometritis at 42 DIM (squares) or remained healthy up to 42 DIM (circles).

Cows that developed metritis had greater ($P = 0.02$) plasma estradiol concentration on the day of calving than healthy cows (median = 29.7 vs. 1.3 pmol/L);

however, concentration in both groups were similar ($P > 0.38$) to cows that had SCE (median = 24.0 pmol/L).

DISCUSSION

This study tried to elucidate the link between negative energy balance and immunosuppression, which seems to be at the root of the problem of increased risk of infectious diseases around parturition (Goff and Horst, 1997). Therefore, we investigated cellular and metabolic parameters that could account for the decrease in PMN function in high-producing dairy cows that develop uterine disease. While metritis is an acute process caused by bacterial contamination around calving and characterized by fetid uterine discharge and fever (Bondurant, 1999, Sheldon and Dobson, 2004), SCE can be considered a chronic condition in which cows fail to clear bacterial contaminants completely (Gilbert et al.,2007). To evaluate the response to inflammation and to support our clinical diagnosis of metritis, we evaluate the acute phase protein haptoglobin. Cows that developed metritis had elevated haptoglobin levels around the time of metritis diagnosis (Skinner et al., 1991). Although SCE is usually diagnosed after 35 DIM (Gilbert et al., 2005), the slight increase in haptoglobin concentration at 7 DIM in cows that were later diagnosed with SCE indicate that a mild inflammatory process may be taking place early in lactation; however, haptoglobin concentrations were still similar to control cows, which agrees with Skinner et al. (1991).

Mmultiparous cows that developed metritis or SCE had decreased DMI and greater NEFA and BHBA plasma concentrations (Hammon et al., 2006). Our findings confirmed that cows that develop uterine disease experience a greater degree of negative energy balance, although differences in NEFA and BHBA were mainly in the first 2 wk of lactation. Our data contrasts with the results in the study by Hammon et

al. (2006) where the differences persisted until the forth week of lactation. Hammon et al. (2006) also observed that DMI was affected in cows that developed uterine disease until the fifth week of lactation; it is possible that, in our study, DMI for cows that developed uterine disease was not so severely affected. Also, dextrose treatment of cows diagnosed with ketosis could have narrowed the differences between cows that developed metritis and healthy cows.

High-producing dairy cows (Goff and Horst, 1997; Mallard et al., 1998; Gilbert et al., 1993; Kehrli et al., 1989), and especially cows that develop uterine disease (Cai et al., 1994; Hammon et al., 2006; Kim et al., 2005) experience a reduction in neutrophil function (such as chemotaxis, phagocytosis and killing ability) around calving. Factors that could account for the loss in PMN function include increases in blood estradiol and cortisol concentrations around calving, and deficits in nutrients and minerals such as vitamins A and E, calcium and selenium (Goff and Horst, 1997; Kimura et al., 2006; Rutigliano et al., 2008). For this work, we focused on the immediate source of energy for PMN function such as glucose, especially its storage form glycogen (Kim et al., 2006; Weisdorf et al., 1982a). We also evaluated hormones such as estradiol, cortisol, insulin and glucagon that could affect PMN function and/or are involved in glucose homeostasis. We observed that PMN glycogen concentration was greatly reduced from the first to the third week after calving. Importantly, cows that developed uterine disease had decreased PMN glycogen levels around calving and later in lactation. Most cows are infected around calving (Sheldon and Dobson, 2004); therefore, prompt PMN response after infection is critical for bacterial clearance and prevention of disease. In fact, data from our laboratory indicate that cows with the greatest influx of PMN to the uterus on the day of calving have the lowest prevalences of positive bacterial culture and SCE (Gilbert et al., 2007). Blood glucose concentration was positively associated with PMN glycogen concentration;

therefore, it seems plausible that glucose deficiency could lead to a decrease in PMN glycogen. Nonetheless, it seems that other factors associated with the state of negative energy balance or even the inherited ability to maintain PMN glycogen stores in cows that developed uterine disease could account for our observations because of the low positive correlation between blood glucose and PMN glycogen concentrations.

A challenge to our hypothesis was that blood glucose concentration was greater in cows that developed metritis, mainly because of greater levels around calving in primiparous cows. Nevertheless, low blood glucose concentration preceded low PMN glycogen concentration in all groups during the study; likewise, lower PMN glycogen on the day of calving could have been caused by lower glucose before calving (Vasquez-Añon et al., 1994). Both observations deserve further investigation. Greater glucose levels around calving could have been caused by an increase in cortisol levels, which would induce gluconeogenesis in the liver. Furthermore, exposure to high levels of cortisol could exacerbate the state of immunosuppression. Indeed, we did observe greater cortisol concentrations at calving in cows that developed metritis compared to cows that had SCE; however, concentrations were not different from healthy cows. Cortisol concentrations were decreased at 14 DIM for cows that developed metritis compared to healthy cows, which might be a consequence of the disease because metritis occurred before 14 DIM. Greater cortisol concentrations in primiparous cows were expected as these cows are experiencing stressful events (such as frequent pen moves, more interactions with the herd personnel, the calving itself, and milking) for the first time. Greater cortisol levels in primiparous cows could be contributing to the overall greater metritis incidence observed in primiparous cows (Goshen and Shpigel, 2006). Although glucose is an important energy source for leukocytes (Ohtsuka et al., 2006; Weisdorf et al., 1982a; Weisdorf et al., 1982b), acute hyperglycemia (as is observed around calving) also

impairs leukocyte function and increases the risk for infection (Blondet and Beilman, 2007). Therefore, while low glucose shortly after calving might impair the ability of PMN to maintain intracellular glycogen and affect PMN function, high glucose at calving might also be detrimental.

While cortisol might be the main determinant of blood glucose levels at calving, glucose utilization for milk synthesis may be the main factor affecting blood glucose concentrations shortly after calving (Bell, 1995; Guo et al, 2008; Vasquez-Añon et al., 1994). Indeed, milk yield was negatively associated with blood glucose concentrations. Milk yield according to disease status followed a pattern that was the opposite of the pattern for blood glucose concentration and may help to explain some of the differences observed in glucose levels. Also, as observed for blood glucose concentration, there were major differences in milk yield according to disease status between primiparous and multiparous cows. Milk yield is usually decreased for primiparous and multiparous cows that develop metritis (Goshen and Shpigel; Huzzey et al., 2007; Rajala and Gröhn, 1998); however, some studies have reported no difference (Fourichon et al., 1999). Milk yields for cows that develop SCE had not been reported previously. Whereas no difference was observed between SCE and healthy primiparous cows, SCE multiparous cows produced less milk than metritis and healthy multiparous cows. It is possible that other factors that affect milk yield, such as subclinical mastitis, ruminal acidosis, or lameness could be associated with SCE; therefore, further experiments need to be conducted to confirm our findings.

During late gestation and early lactation, there is establishment of insulin resistance in peripheral tissues (Bell and Bauman 1997), which is caused by an increase in growth hormone (Bell and Bauman 1997) and NEFA concentrations in the blood (Pires et al., 2007); therefore, greater NEFA concentrations in cows that developed uterine disease and in primiparous cows in general could have induced

insulin resistance and accounted for the observed differences in glucose levels. Nonetheless, glucose to insulin ratio, an indicator of insulin resistance, was not associated with uterine disease. Insulin followed a pattern very close to the glucose pattern. The only difference observed was lower plasma insulin concentration at 42 DIM for cows that had SCE. Accordingly, those cows also had decreased concentration of glucose at that time.

In combination with insulin, glucagon is responsible for glucose homeostasis and can also affect neutrophil function (Al-essa et al., 1993; Deitch and Bridges, 1987; Petersen et al., 1978). Because of its role in promoting gluconeogenesis to maintain normoglycemia, plasma glucagon concentration was negatively associated with blood glucose concentration; therefore, as glucose decreased, glucagon increased. Therefore, greater glucagon levels in multiparous cows were a consequence of lower glucose in those cows. Cows that had SCE had greater glucagon concentration than healthy cows or metritis cows. Glucagon acts by binding to its receptor on PMN (Al-essa et al., 1993) and impairs PMN chemotactic and bactericidal activity (Deitch et al. 1987), which could predispose to the development of SCE.

Lastly, plasma estradiol on the day of calving is also thought to contribute to the overall immunosuppression observed in dairy cows around calving (Goff and Horst, 1997; Wyle and Kent, 1977); indeed we observed higher estradiol concentrations on the day of calving for cows that developed uterine disease. Nonetheless, only plasma estradiol concentrations for cows that developed metritis were different from healthy controls. Plasma estradiol peaks right before calving and drops quickly after calving (Radcliff et al., 2003) but we were still able to detect significant differences in cows that developed metritis; therefore, this seems to be an important aspect involved in immunosuppression in cows that develop uterine disease and deserves further investigation.

In conclusion, we confirmed that cows that develop uterine disease experience a greater degree of negative energy balance as indicated by greater levels of NEFA and BHBA around calving. All cows experienced a decrease in PMN glycogen concentration in the first 3 weeks after calving, which could account for the overall immunosuppression observed at this time. Furthermore, cows that developed uterine disease were not as able to maintain PMN glycogen stores as cows that remained healthy. At this point it is not clear why cows that developed uterine disease were less able to maintain their PMN glycogen stores but it seems that blood glucose is involved. Glucose was mainly affected by cortisol levels around calving and by milk yields later on. Greater cortisol levels in primiparous cows could be contributing to their greater incidence of metritis; therefore, strategies to decrease stress around calving may have the potential to decrease the incidence of metritis. We were not able to find any association between uterine disease and plasma insulin. Glucagon on the other hand was increased in cows that developed SCE, which could impair their function and predispose these cows to SCE. Greater plasma estradiol levels at calving in cows that developed metritis could be another contributing factor to their immunosuppression. Cows that developed metritis or SCE had several metabolic and hormonal changes that could account for their exacerbated immunosuppression including greater degree of negative energy balance, an inability to maintain neutrophil glycogen concentration, alteration in glucose homeostasis around calving, and increased levels of estradiol, cortisol, and glucagon.

CHAPTER FIVE

Association between CXCR1 polymorphism and uterine disease in Holstein cows

Interpretive summary: Polymorphism and uterine disease. Galvão.

Association between uterine disease and the SNP +735 in CXCR1 was evaluated in 350 dairy cows. 36 (10%) were diagnosed with retained placenta, 86 (25%) were diagnosed with metritis, and 125 (36%) were diagnosed with SCE. There was no association of SNP +735 with retained placenta, metritis or SCE. Therefore, SNP +735 is not relevant to uterine health.

ABSTRACT

Our objective was to evaluate the association between uterine disease and SNP +735 in CXCR1 in dairy cows. Metritis was characterized by a fetid vaginal discharge and rectal temperature > 39.5 ^{o}C in the first 14 DIM. Subclinical endometritis (SCE) was evaluated at 42 DIM by uterine lavage and cytology; cows having ≥ 10% PMN were considered to have SCE. The final analysis included 350 Holstein cows; 36 (10%) were diagnosed with retained placenta, 86 (25%) were diagnosed with metritis, and 125 (36%) were diagnosed with SCE. Frequency of genotypes CC, GC, and GG were 22, 50, and 28%, and tended (P = 0.06) to be different between primiparous and multiparous (16, 55, and 29% vs. 27, 46, and 28 %) mainly because of an increase (P = 0.02) in the frequency of genotype CC in multiparous compared to primiparous. There was no association of SNP +735 with retained placenta (P = 0.95), metritis (P = 0.38) or SCE (P = 0.91). Therefore, SNP +735 is not relevant to uterine health.

Key words: Metritis, subclinical endometritis, single-nucleotide polymorphism, dairy cows

INTRODUCTION

Recently, single-nucleotide polymorphisms (SNPs) in the neutrophil (PMN) interleukin-8 (IL-8) receptor were identified (Youngerman et al., 2004a) and cows with the genotype CC had increased incidence of subclinical mastitis (Youngerman et al., 2004b). Initially, the SNP was reported to be in the IL-8 receptor-b (CXCR2) gene (Youngerman et al., 2004b), but was later recognized to be in the IL-8 receptor-a (CXCR1) gene (Pighetti and Rambeaud, 2006). This nonsynonymous SNP was reported to be at position +777 relative to the published sequence (GenBank Accession No. U19947) but is now known to be at position +735. Cows can have the

SNP in both (CC), one (GC), or none (GG) of the alleles. The substitution of nucleotide G with C results in a glutamine to histidine substitution which might compromise receptor affinity for IL-8 and function. In fact, PMNs from cows with the genotype CC had decreased receptor affinity to IL-8, decreased calcium signaling, impaired migration, and reduced killing ability (Rambeaud and Pighetti, 2005; Rambeaud and Pighetti, 2006; Rambeaud and Pighetti, 2007). Neutrophil function such as killing ability and survival was intermediate for cows with the genotype GC compared with CC and GG (Rambeaud and Pighetti, 2006) and migration was impaired compared with genotype GG (Rambeaud and Pighetti, 2005).

Neutrophils are the main leukocyte type involved in placental release (Kimura et al., 2002) and in bacterial clearance after uterine (Hussain, 1989). Cows that retain their fetal membranes present reduced PMN migration to sites of placental attachment (Kimura et al., 2002) cows with the greatest influx of PMN into the uterus have reduced risk of bacterial infection and reduced incidence of SCE (Gilbert et al., 2007). IL-8 is the main chemoattractant for PMN; binding of IL-8 to its receptors (CXCR1 and CXCR2) in the PMN induces PMN activation, stimulates chemotaxis, and increases phagocytosis and killing ability (Mitchell et al., 2003).

We hypothesized that dairy cows with the genotype CC and or GC for the SNP in the position +735 in CXCR1 would have increased incidence of retained placenta, metritis, and SCE. Therefore, the objective was to genotype cows for the CXCR1 SNP in the position +735 (GG, GC, or CC) and compare the prevalence of retained placenta, incidence of metritis, and the prevalence of SCE among cows with different genotypes.

MATERIALS AND METHODS

Animals, Housing, and Feeding

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals in this experiment. Three hundred and fifty cows (147 primiparous and 203 multiparous) from 23 commercial Holstein dairy farms located in the state of New York, USA were used in the study. Cows used for this study were part of a larger study evaluating the prevalence of SCE in dairy cows in the state of New York (Cheong et al., 2008) and a study evaluating the association between subclinical endometritis and glycogen concentration in neutrophils of lactating Holstein cows (Galvão et al., 2008). Herd sizes ranged from 500 to 2400 in the study by Cheong et al. (2008) and 3000 cows in the study by Galvão et al. (2008). Ten to 20 cows were used from each of the 22 herds from the study by Cheong et al. (2008) and 80 cows from the study by Galvão et al. (2008). In the study by Cheong et al. (2008) cows were sampled randomly between 40 and 60 DIM and in the study by Galvão et al. (2008) cows were sampled randomly at calving. The rolling herd averages were ~ 11,500 kg of milk. Primiparous and multiparous cows were housed separately in freestall facilities in all herds. All cows were fed TMR that was formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% fat-corrected milk.

Blood Sample Collection, DNA Isolation, and CXCR1 Genotyping

All cows had a blood sample collected from 40 to 60 d after calving by puncture of coccygeal vessels into Vacutainer® tubes containing EDTA as anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The samples were immediately placed on ice and transported to the laboratory within 4 hours and submitted to the DNA bank at Cornell University for DNA was isolation.

Genotyping for SNP +735 in CXCR1 was performed as previously reported (Youngerman et al., 2004).

Evaluation of Uterine Diseases

Cows were evaluated daily by the herd personnel for signs of metritis in the first 14 DIM. Metritis was characterized by the presence of watery, fetid vaginal discharge and rectal temperature > 39.5 ^{o}C. At 42 d after calving, cows had a low-volume uterine lavage for diagnosis of SCE based on the proportion of PMNs out of a total of 200 cells (including all leukocyte types and epithelial cells but excluding erythrocytes) as previously described (Gilbert et al., 2005). Based on a larger experiment (Cheong et al., 2008), the proportion of PMNs used as cutoff for classifying cows as having SCE was ≥ 10%.

Statistical Analyses

This was an observational cohort study. Association between retained placenta, metritis, and SCE was evaluated by chi-square using the FREQ procedure in SAS (Version 9.1; SAS Inst. Inc., Cary, NC). Association between retained placenta, metritis, SCE, or mastitis and CXCR1 genotypes (CC, GC, or GG) was evaluated by mixed logistic regression using the GLIMMIX procedure of SAS. The model included the fixed effects of CXCR1 genotypes and parity, and herd as a random effect. Differences with $P \leq 0.05$ (2-sided) were considered significant based on Wald's statistic criterion and $0.05 < P \leq 0.10$ were considered a tendency toward statistical difference.

RESULTS

Of the 350 cows, 36 (10%) were diagnosed with retained placenta, 86 (25.0%) were diagnosed with metritis, 125 (36%) were diagnosed with SCE. Cows that had retained placenta were more likely to have metritis (56 vs. 21%; $P < 0.001$) and SCE (64 vs. 33%; $P < 0.001$); however, cows that had metritis were not more likely to have SCE (37 vs. 35%; $P = 0.74$). Multiparous cows tended to have greater prevalence of retained placenta (13 vs. 7%; $P = 0.06$), had lesser incidence of metritis (19 vs. 32%; $P = 0.01$); and had greater prevalence of SCE (40 vs 29%; $P = 0.03$). Mean and median day of diagnosis of metritis was 6 d after calving with a range from 2 to 14 d after calving. Frequency of genotypes CC, GC, and GG were 22, 50, and 28%, and tended ($P = 0.06$) to be different between primiparous and multiparous (16, 55, and 29% vs. 27, 46, and 28 %) mainly because of an increase ($P = 0.02$) in the frequency of genotype CC in multiparous compared to primiparous.

Table 5.1 shows the prevalences of retained placenta, metritis, and SCE according to SNP +735, adjusted for the effect of parity. There was no association of SNP +735 with retained placenta ($P = 0.95$), metritis ($P = 0.38$) or SCE ($P = 0.91$).

Table 5.1. Association between single nucleotide polymorphism (SNP) +735 and metritis, analyzed by logistic regression (adjusting for parity; AOR = adjusted odds ratio).

Variable	Genotype	Cows	Affected, %	*P*	AOR	95% CI
Retained placenta	CC	78	11.5	0.78	1.2	0.4 – 3.1
	GC	174	10.3	0.77	1.1	0.5 – 2.7
	GG	98	9.2	-	-	-
Metritis	CC	78	20.5	0.84	1.1	0.5 – 2.3
	GC	174	28.2	0.21		0.8 – 2.7
	GG	98	21.4	-	-	-
SCE	CC	78	38.5	0.66	1.2	0.6 – 2.2
	GC	174	36.2	0.79	1.1	0.6 – 1.9
	GG	98	32.7	-	-	-

[1] For the logistic regression, cows that had the SNP +735 in at least one allele (CC or GC = Yes) were combined and compared against cows without a SNP +735 (GG = No).

DISCUSSION

High-producing dairy cows (Goff and Horst, 1997; Mallard et al., 1998; Gilbert et al., 1993; Kehrli et al., 1989). Especially cows that develop uterine disease (Cai et al., 1994; Hammon et al., 2006; Kim et al., 2005) experience a reduction in PMN function around calving. This reduction is associated with a more severe negative-energy balance around calving (Hammon et al., 2006). We investigated the association between SNP +735 in CXCR1 and the prevalence of retained placenta,

incidence of metritis and prevalence of SCE to identify other possible culprits for the high prevalence of uterine diseases in dairy cows (Galvão et al., 2009a; Gilbert et al., 2005; Goshen and Shpigel, 2006).

Although PMNs with the SNP +735 have impaired migration and reduced killing ability (Rambeaud and Pighetti, 2005; Rambeaud and Pighetti, 2006) and cows with genotype CC have increased incidence of subclinical mastitis (Youngerman et al., 2004), we did not observe any association between SNP +735 genotype and prevalence of uterine diseaese. Because PMN migration and function is equally important for bacterial clearance in the mammary gland as well as for bacterial clearance in the uterus and release of the placenta, we hypothesized that uterine health could also be compromised in cows with a SNP in position +735. Nonetheless, our results indicate that the uterus is not as sensitive to defects in CXCR1 as the mammary gland is. It is possible that factors other than the defect in CXCR1 might be more important for uterine health. In the case of retained placenta, the reduction in chemotaxis was suggested to be a result of lower concentrations of interleukin-8 (IL-8) at the fetal maternal placental interface (Kimura et al., 2002). Therefore, it is possible that the concentrations of IL-8 are more important than the SNP in CXCR1. Because retained placenta is a major risk factor for metritis and SCE, it is possible that IL-8 concentration in the endometrium would also be more relevant for the development of uterine disease than the SNP in CXCR1.

Although we did not observe lower IL-8 mRNA expression by blood monocytes or by the endometrium of cows that developed uterine disease, the levels of pro-inflammatory cytokines (TNFα and IL-1β) were decreased around calving (which could lead to lower IL-8 production locally). After disease takes place, IL-8 mRNA expression does increase in the uterus; however, the levels before disease might be more important (Chapwanya et al., 2009). Also, DMI and the degree of negative-

energy balance (Hammon et al., 2006; Huzzey et al., 2007) greatly affect the cow's susceptibility to disease and might be more important for PMN function and uterine health then the SNP in CXCR1.

We have observed in other studies that cows that develop uterine disease have decrease monocyte and endometrial gene expression of pro-inflammatory cytokines (such as TNFα and IL1-β). These cytokines stimulate the expression of cell-adhesion molecules in the vascular endothelium and activate neutrophils and monocytes (promoting increased chemotaxis, phagocytosis and bacterial killing). IL-8 is not the only chemokine for neutrophils. Complement split products (C3a, C4a and C5a, which are formed after tissue damage) also act as chemotactic agents by binding to their own receptors in leukocytes (Gerard and Gerard, 1994; Hopken et al., 1996). The anaphylatoxin C5a has the most diverse properties, including stimulation of leukocyte chemotaxis and activation, enhancement of neutrophil-endothelial cell adhesion, induction of granule secretion in phagocytes, as well as induction of pro-inflammatory cytokines from leukocytes (Hopken et al., 1997). Furthermore, in the uterus, it is possible that binding of IL-8 to CXCR2 could compensate for the defect in CXCR1. Therefore, several mechanisms that might be specific to the uterus could help to explain the lack of association between SNP +735 genotype and uterine health. Frequency of genotypes at SNP +735 in Holstein cows differed slightly from an earlier report (Youngerman et al., 2004) but followed the same pattern. There was a shift in prevalence of SNP +735 genotypes from primiparous to multiparous. There was an increase in the prevalence of genotype CC in multiparous compared to primiparous, which indicates that cows with genotype CC might have survival advantage. Nonetheless, a longitudinal study would be warranted to confirm our observation in this crossectional study.

Although multiparous cows have increased incidence of retained placenta (a

major risk factor for metritis), they have decreased incidence of metritis (Curtis et al., 1985; Goshen and Shpigel 2006). We found either no difference or an increased incidence of SCE in multiparous cows (Cheong et al., 2008; Galvão et al., 2008; Galvão et al., 2009, unpublished). Increased prevalence of SCE in multiparous cows could be the result of impaired ability to eliminate bacterial infection from the uterus. Multiparous cows have much greater demands for milk production; therefore, this could impair the ability of their immune system to function properly. In 2 studies conducted concurrent by the present study, we observed that multiparous cows produced almost 10 kg more milk and had reduced ability to maintain normoglycemia in the first 2 to 3 weeks of lactation, and that their monocytes had decreased ability to produce pro-inflammatory cytokines after stimulation with *E. coli* (which could reduce inflammation and allow bacterial colonization). In fact, we observed that multiparous cows have increased bacterial contamination of the uterus at 51 d after calving (Galvão et al., 2009).

In conclusion, uterine health was not affected by different SNP +735 genotypes. Therefore, the uterus is not as sensitive to defects in CXCR1 as the mammary gland is. Factors such as energy balance, concentration of IL-8 in the uterus, chemotaxis induced by complement split products, and binding of IL-8 to CXCR2 might be more important than the defect in CXCR1 or might compensate for the defect in CXCR1.

CHAPTER SIX

Effect of PGF2α on Subclinical Endometritis and Fertility in Dairy Cows.

Interpretive summary: $PGF_{2\alpha}$ and subclinical endometritis. Galvão.

Administration of PGF2α improves reproductive performance of dairy cows because of increased performance in cows with subclinical endometritis (SCE). Nevertheless, effect of PGF2α treatment on prevalence of SCE was not evaluated. In this trial, treatment with PGF2α resulted in increased first-service pregnancy per AI in all cows and increased hazard of pregnancy in cows with low body-condition score; however, the positive effect of PGF2α on hazard of pregnancy was only in cows without SCE. Furthermore, PGF2α administration did not decrease prevalence of SCE. Administration of PGF2α does not affect prevalence of SCE but can increase hazard of pregnancy in cows without SCE and with low body condition score.

ABSTRACT

Our objectives were to determine the effects of $PGF_{2\alpha}$ treatment on the prevalence of subclinical endometritis (SCE) and breeding performance of dairy cows. We used 406 Holstein cows (167 primiparous and 239 multiparous) from 5 herds. Uterine lavage for diagnosis of SCE, $PGF_{2\alpha}$ treatment, body condition scores (BCS), and collection of blood samples for cyclicity determination were performed at 21, 35, and 49 DIM. Polymorphonuclear cells (PMNs) were quantified and cutoffs for diagnosing SCE were selected by ROC analysis. Cows were classified as having SCE 21 DIM with ≥ 8.5% PMNs, at 35 DIM with ≥ 6.5% PMNs and at 49 DIM with ≥ 4.0% PMNs Median days to pregnancy were delayed by 30 (151 vs. 121 days) and 40 (169 vs. 129) days for cows classified as having SCE at 35 and 49 DIM, respectively, but not by SCE at 21 DIM. Treatment with $PGF_{2\alpha}$ did not affect the prevalence of SCE either at 35 (38 vs. 38%) or at 49 DIM (34 vs. 40%). Treatment with $PGF_{2\alpha}$ did not affect time to first insemination. Nonetheless, $PGF_{2\alpha}$ treatment increased pregnancy to first AI in all the cows (36 vs. 24%) and hazard of conception in cows with BCS ≤ 2.5. when only cows without SCE were evaluated (HR = 1.8; 95% CI = 1.2 – 2.7) but when only SCE-positive cows were evaluated (HR = 0.9; 95% CI = 0.6 – 1.3). Treatment with $PGF_{2\alpha}$ does not affect SCE prevalence or time to first insemination, but can increase first service pregnancy per AI and decrease time to pregnancy in cows with low BCS.

Key words: Subclinical endometritis, prostaglandin treatment, dairy cows

INTRODUCTION

Subclinical endometritis (SCE), characterized by increased proportion of polymorphonuclear cells (PMNs) in uterine cytology (Gilbert et al., 1998;

Kasimanickam et al., 2004; Gilbert et al., 2005), is prevalent in high-producing dairy cows and is associated with both decreased pregnancy per AI and extended interval to pregnancy (Gilbert et al., 1998; Kasimanickam et al., 2004; Gilbert et al., 2005). Administration of a $PGF_{2\alpha}$ analogue to cows with SCE was as efficacious as intrauterine infusion of cephapirin benzathine in improving reproductive performance (Kasimanickam et al., 2005).

The benefit from $PGF_{2\alpha}$ administration is believed to arise from induction of estrus in cows having a $PGF_{2\alpha}$-responsive corpus luteum; the estrus leads to physical expulsion of bacterial contaminants and inflammatory products as well as a possible improvement in the uterine defenses under low progesterone (Kasimanickam et al., 2005). Nonetheless, although a positive effect of $PGF_{2\alpha}$ on reproductive performance was observed, it was not determined whether the prevalence of SCE was reduced or whether the effect was in cows with an active corpus luteum.

We hypothesized that SCE would be detrimental to fertility, but treatment with $PGF_{2\alpha}$ would reduce the prevalence of SCE and improve reproductive performance. Therefore, our objectives were to determine the effects of $PGF_{2\alpha}$ on uterine health, pregnancy to first AI, and intervals from calving to insemination and to pregnancy in lactating dairy cows.

MATERIALS AND METHODS

Animals, Housing, Feeding, and Reproductive Management

We enrolled 445 cows (185 primiparous and 260 multiparous) from 5 commercial Holstein dairy farms located in Cayuga County, New York, USA. The herds ranged from 70 to 1,500 milking cows and the rolling herd averages were ~ 11,500 kg of milk/cow/year. Lactating dairy cows were housed in freestall facilities and milked three times daily. Within herd, all cows were fed the same TMR that was

formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% fat corrected milk.

Reproductive management varied among farms; however, all dairies relied on estrus detection (by visual observation) for most of their breedings and their voluntary waiting periods were set to 49 DIM. All the cows were examined for pregnancy by one of the investigators (C. L. Guard) by palpation per rectum at 38 ± 3 d after AI. Cows not conceiving to the first service were reexamined for pregnancy after each subsequent insemination until 300 DIM. Pregnancy per AI was defined as the number of pregnant cows divided by the number of cows receiving AI in each treatment at 38 d after insemination.

Treatments and Sample Collection

Figure 1 illustrates activities during the study. During the routine biweekly herd visits, cows at 21 ± 3 DIM were assigned to one of 2 treatments. In a blinded design, cows with even-numbered identification ear tags received 5 mL of solution B (25 mg $PGF_{2\alpha}$, Lutalyse®, 5 mg/mL dinoprost tromethamine, Pfizer Animal Health, New York, NY) at 21, 35, and 49 ± 3 DIM (PGF; n = 218), and cows with odd-numbered identification ear tags received 5 mL of solution A (sterile saline solution) at 21, 35, and 49 DIM (Control; n = 227). Solutions A and B were bottled at the Pharmacy of the College of Veterinary Medicine at Cornell University and were indistinguishable. Their identities were revealed to investigators only after completion of the trial.

A low-volume uterine lavage for diagnosis of SCE was performed immediately before treatment administration at 21, 35, and 49 ± 3 DIM. Cows with reproductive-tract disorders such as pyometra and uterine adhesions or abscesses that could compromise sampling for SCE evaluation or future inseminations were not enrolled in

the study. Cows that could not be sampled at 21 DIM were also not enrolled in the study. Of the 445 cows that were initially sampled at 21 DIM, 39 cows were either missed at 35 or at 49 DIM or at both samplings; therefore, only 406 cows (PGF = 203; Control = 203) were sampled at all time points and included in the final analysis. The diagnosis of SCE was based on the proportion of PMN out of a total of 200 cells, including all leukocyte types and epithelial cells (but excluding erythrocytes) as previously described (Gilbert et al., 2005). A single investigator read all the slides. This observer was unaware of treatments and slides were ordered by sampling date within each farm, and not by sampling date for each cow, which avoided any bias of knowing the counts of a previous slide for each cow.

All cows used for this study had a blood sample collected at 21, 35, and 49 ± 3 DIM. Blood was collected by puncture of coccygeal vessels into Vacutainer® tubes without anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The samples were immediately placed on ice and transported to the laboratory within 4 h, where serum was separated by centrifugation at 2000 g for 15 min and frozen at -25º C and later analyzed for progesterone by radioimmunoassay (Beam and Butler, 1997). Inter-assay and intra-assay CV were 7.5 and 6.0%, respectively. Cows having serum progesterone (P4) concentration ≥ 1 ng/mL were assumed to have a functional CL at the time of sampling and therefore, considered to be cyclic (Galvão et al., 2004). Body-condition score (BCS) was evaluated in all cows using a 5-point (1 = thin to 5 = fat) system (Ferguson et al., 1994) at each sampling.

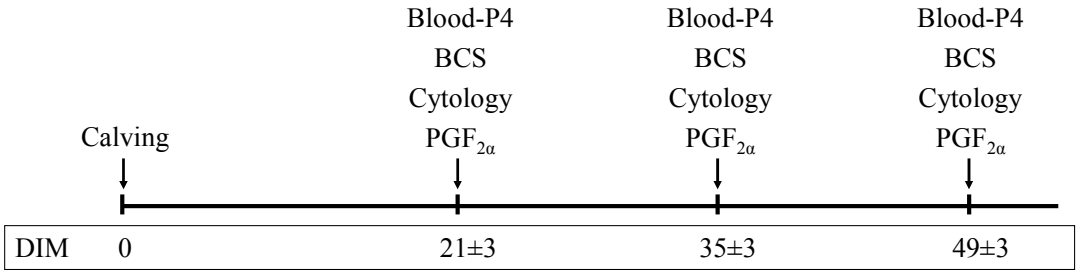

Figure 6.1. Timeline of activities during the study. Blood-P4 = blood sampling for progesterone measurement; BCS = body-condition score; Cytology = low-volume uterine lavage for cytological examination; $PGF_{2\alpha}$ = treatment administration; cows with even-numbered ear tags received 25 mg $PGF_{2\alpha}$ (Lutalyse®, 5 mg/mL dinoprost tromethamine, Pfizer Animal Health, New York, NY) and cows with odd-numbered ear tags received sterile saline solution.

Statistical Analyses

The PMN cutoffs for classification of cows as having SCE were selected using receiver-operating characteristics (ROC) analysis using the MedCalc® software version 9.2 for Windows (MedCalc Software, Mariakerke, Belgium). Only the control cows were used for selection of the cutoff because $PGF_{2\alpha}$ could affect the proportion of PMN at 35 and 49 DIM and bias our estimate. The dichotomous outcome used for selection of the cutoffs was nonpregnancy by 150 DIM (one-half of the observation period). In MedCalc, a cutoff is selected automatically based on the best combined sensitivity and specificity. It is important to notice that one could use a different approach such as choosing a cutoff based on high sensitivity or high specificity depending on the goals of diagnosing cows with SCE. After selection of a cutoff, cows were classified as above or below the PMN cutoff and Kaplan-Meyer survival curves were used to evaluate whether cows above the cutoff had increased time to conception

based on log-rank test. If cows classified as having SCE had increased time to conception (Kaplan-Meier), the PMN cutoff for SCE was considered as validated for indicating negative effects on fertility.

The outcomes SCE at 35 and 49 DIM, and first-service pregnancy per AI were analyzed by mixed logistic regression using the GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC). The models included the fixed effects of $PGF_{2\alpha}$ treatment at 21 DIM for SCE at 35 DIM and $PGF_{2\alpha}$ treatment at both 21 and 35 DIM for SCE at 49 DIM (yes or no), cyclic by the time of $PGF_{2\alpha}$ administration (yes or no), parity (primiparous vs. multiparous), season of calving [winter (Dec, Jan, Feb), spring (Mar, Apr, May), summer (Jun, Jul, Aug), fall (Sep, Oct, Nov)], mean BCS for the three observations (< 2.5 vs. ≥ 2.5), and 2-way interactions between $PGF_{2\alpha}$ treatment and other covariates. Herd was included as a random effect. For "SCE at 49 DIM", the GLIMMIX model also included the effect of SCE presence at 35 DIM and the interaction between $PGF_{2\alpha}$ treatment and SCE at 35 DIM. Effect of SCE presence at 49 DIM on first service pregnancy per AI was also included in the multivariable analysis.

The hazard of insemination up to 150 DIM and hazard of pregnancy up to 300 DIM were analyzed by Cox proportional-hazard model using the PHREG procedure of SAS. The effect of $PGF_{2\alpha}$ on the hazard of pregnancy was evaluated in all cows and then in cows with SCE and in cows without SCE at 49 DIM, separately to investigate differential effect of $PGF_{2\alpha}$ in these 2 populations (Kasimanickam et al., 2005). The hazard ratio (HR) is the conditional daily probability of a given event (insemination or pregnancy) and may be interpreted as the insemination rate or pregnancy rate (speed at which cows are submitted for insemination or become pregnant). The time variable for the hazard of insemination was the days between calving and first insemination and for hazard of pregnancy was the days between calving and pregnancy (detected 38 ± 3

d after AI). Cows that were not inseminated by 150 DIM or were sold or died were censored in the analysis of time to first insemination. Cows that were not inseminated or not pregnant by 300 DIM or were sold or died were censored in the analysis of time to pregnancy. The Cox models included the same variables cited for the GLIMMIX model; however, cyclic by 49 DIM was used and data were stratified by herd, using the STRATA option, to control for clustering as previously reported (Bicalho et al., 2007). For the strata option in the PHREG procedure, each stratum has its likelihood function derived from its hazard function but partial likelihoods are multiplied to generate a single likelihood function to allow estimation of a single coefficient for each variable in the model. Proportionality of hazard rate between groups was assessed by inclusion of interaction of $PGF_{2\alpha}$ treatment with time and by evaluation of Kaplan-Meier curves. The median days to first insemination or to pregnancy were obtained by survival analysis from the Kaplan-Meier model using the LIFETEST procedure of SAS. The survival plot was generated using the survival option of MedCalc.

For all statistical tests, a two-sided hypothesis was considered. Differences with $P \leq 0.05$ were considered significant. When an interaction between 2 dichotomous variables was observed, a new variable containing the 4 possible combinations (dummy variables: {0,0}; {0,1}; {1,0}; {1,1}) was created and the model was re-run including only the new variable. For data presentation, only biologically relevant comparisons are shown. For both the logistic (GLIMMIX) and Cox proportional-hazard models (PHREG), a hierarchical backward elimination was performed; variables were manually removed when $P > 0.05$. Treatment was forced into the final model in both types of models.

RESULTS

The PMN cutoffs selected by the ROC analysis for classifying cows as having SCE were ≥ 8.5, 6.5, and 4.0% at 21, 35, and 49 DIM, respectively. Using these cutoffs, 67 % (n = 271), 38 % (n = 155), and 37% (n = 151) of the 406 cows would be classified as having SCE at the three examination days. Diagnosis of SCE at 35 DIM was not correlated with diagnosis at 49 DIM (Pearson correlation = 0.06; $P = 0.29$. The PMN cutoff at 21 DIM was not associated with time to pregnancy by Kaplan-Meyer survival analysis. Higher cutoffs were tested (≥ 15, 20, and 30% PMN); however, none was associated with time to pregnancy (all $P > 0.50$). On the other hand, the cutoffs used for classifying cows as having SCE at 35 and 49 DIM led to increased ($P < 0.05$) time to pregnancy. Median days to pregnancy were delayed by 30 (151 vs. 121 days), and 40 (169 vs. 129) days for cows classified as having SCE at 35 and 49 DIM, respectively. **Figures 6.2 and 6.3** compares the survival curves for cows having or not having SCE at 35 and 49 DIM, respectively.

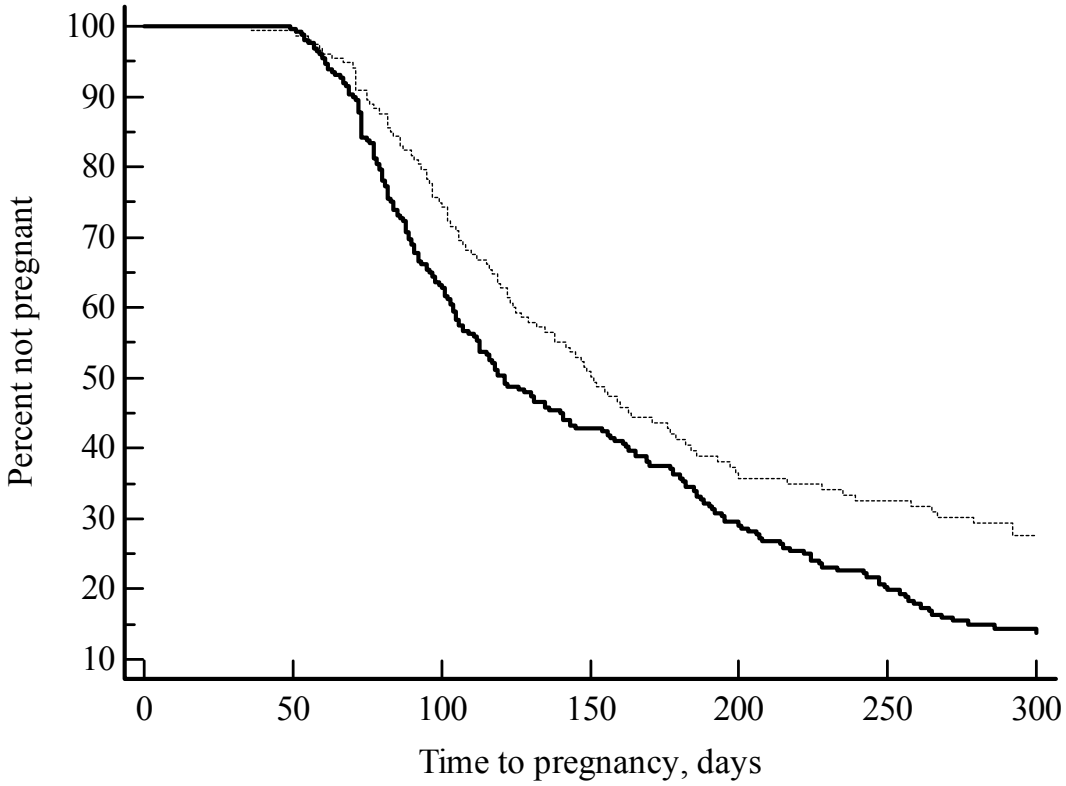

Figure 6.2. Time to pregnancy in dairy cows that were classified as having (n = 155; dashed line; median = 151 d), or not having (n = 251; solid line; median = 121 d; *P* = 0.003) subclinical endometritis at **35 DIM**.

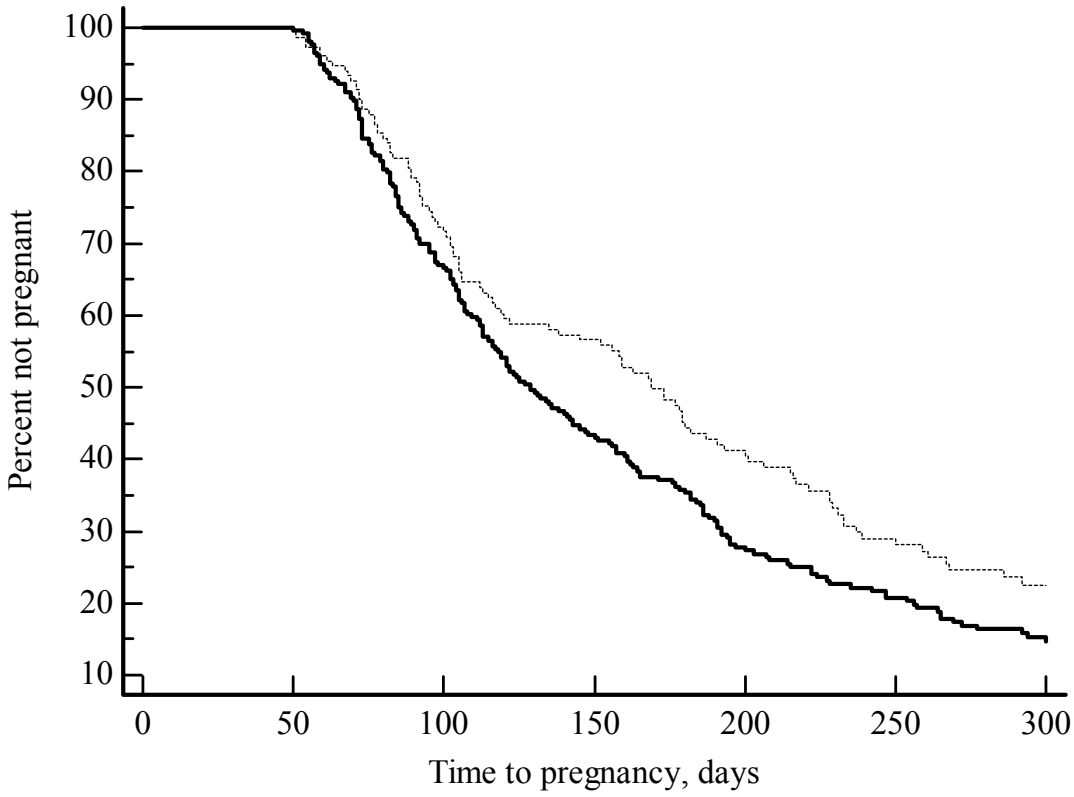

Figure 6.3. Time to pregnancy in dairy cows that were classified as having (n = 151; dashed line; median = 169 d), or not having (n = 255; solid line; median = 129 d; *P* = 0.03) subclinical endometritis at **49 DIM**.

Treatment with $PGF_{2\alpha}$ did not affect the prevalence of SCE either at 35 or at 49 DIM (**Table 6.1)**. The only variable affecting prevalence of SCE at 35 and 49 DIM was that cows with an active CL (n = 106) at 21 DIM had lower ($P < 0.05$) prevalence of SCE at 35 DIM (26.4 vs. 42.3%) and 49 DIM (25.5 vs. 41.3%). There was no interaction (P = 0.57) between presence of an active CL at the time of $PGF_{2\alpha}$ administration and $PGF_{2\alpha}$ treatment on the prevalence of SCE at 35 or 49 DIM. Furthermore, there was no interaction (P = 0.20) between $PGF_{2\alpha}$ treatment and presence of SCE at 35 DIM on the prevalence of SCE at 49 DIM.

Table 6.1. Effect of $PGF_{2\alpha}$ treatment on subclinical endometritis (SCE) at 35 and 49 DIM.

Outcome	Level	Cows	SCE, %	*P*	OR	95% CI
SCE 35 DIM[1]						
Treatment[2]	$PGF_{2\alpha}$	203	37.9	0.76	0.9	0.6 – 1.5
	Control	203	38.4	-	-	-
SCE 49 DIM[1]						
Treatment[2]	$PGF_{2\alpha}$	203	34.0	0.18	0.8	0.5 – 1.1
	Control	203	40.4	-	-	-

[1] Subclinical endometritis was characterized by the presence of ≥ 6.5 and 4.0% PMN in the uterine cytology at 35 and 49 DIM, respectively.

[2] Cows in the $PGF_{2\alpha}$ treatment group received an i.m. injection of 25 mg $PGF_{2\alpha}$ at 21, 35, and 49 ± 3 DIM and Control cows received sterile saline solution. Only $PGF_{2\alpha}$ treatment at 21 DIM was considered in the model for SCE at 35 DIM and only $PGF_{2\alpha}$ treatment at 21 and 35 DIM was considered in the model for SCE at 49 DIM.

Hazard of first insemination was not affected by either SCE at 49 DIM (HR = 0.9; 95% CI = 0.7 – 1.1; *P* = 0.40) or $PGF_{2\alpha}$ treatment at 21, 35, and 49 DIM (HR = 1.1; 95% CI = 0.9 – 1.3; *P* = 0.63). Median days to first insemination was 78 days for cows with or without SCE and 76 and 79 days for cows that did or did not receive $PGF_{2\alpha}$ treatment, respectively. The only variable affecting the hazard of first insemination was cyclicity by 49 DIM; cows that were cyclic (n = 315) had increased hazard of insemination (HR = 1.5; 95% CI = 1.2 – 2.0; *P* = 0.003) leading to a 21-day

shorter median time to first insemination compared to noncyclic cows (74 vs. 95 days) (**Figure 6.4**). There was no interaction ($P > 0.05$) between $PGF_{2\alpha}$ treatment and SCE, cyclicity by 49 DIM or any other variable on the hazard of first insemination.

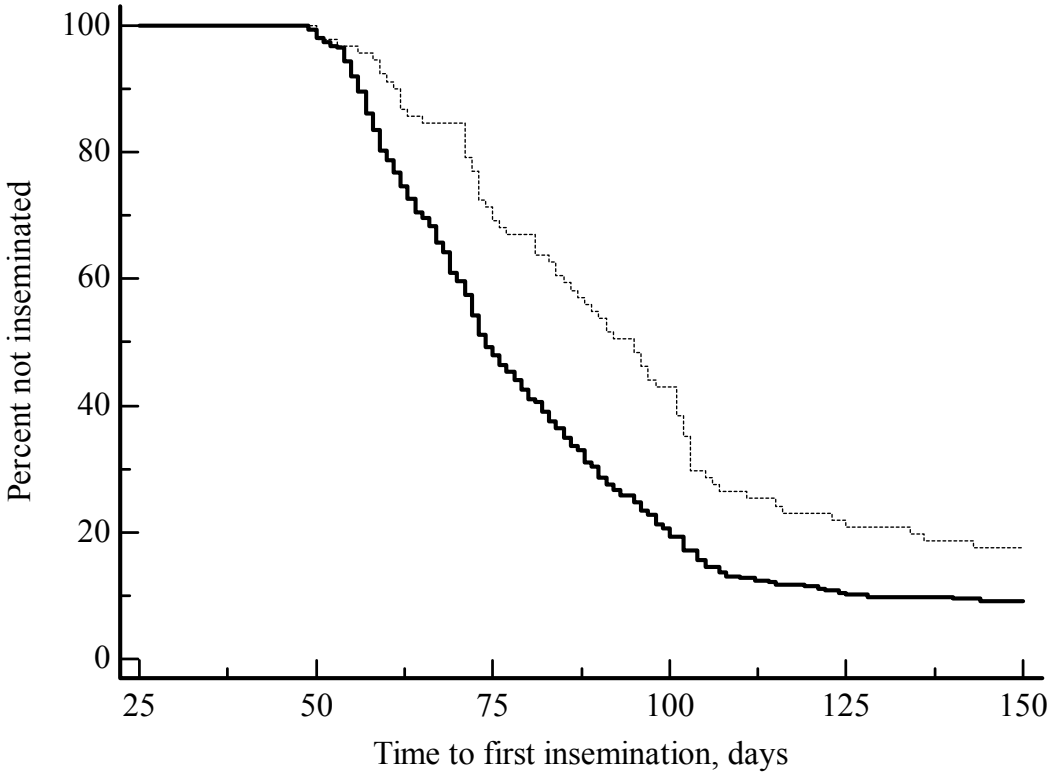

Figure 6.4. Time to first insemination in dairy cows that were cyclic (n = 315; solid line; median = 74 d) or noncyclic (n = 91; dashed line; median = 95 d; $P = 0.003$) by 49 d postpartum.

Treatment with $PGF_{2\alpha}$ increased ($P = 0.01$) first service pregnancy per AI (**Table 6.2)**. No other variable affected first-service pregnancy per AI. There was no effect of SCE or interaction ($P \geq 0.11$) between $PGF_{2\alpha}$ treatment and SCE, cyclicity by 49 DIM or any other variable on first-service pregnancy per AI.

Table 6.2. Effect of $PGF_{2\alpha}$ treatment on first-service pregnancy per AI (PRAI).

Level	Treatment[1]	Cows	PRAI, %	*P*	OR	95% CI
Treatment[1]	$PGF_{2\alpha}$	203	35.5	0.01	1.7	1.1 – 2.7
	Control	203	24.1	-	-	-

[1] Cows in the $PGF_{2\alpha}$ treatment group received an i.m. injection of 25 mg $PGF_{2\alpha}$ at 21, 35, and 49 ± 3 DIM and Control cows received sterile saline solution.

There was an interaction (*P* = 0.02) between $PGF_{2\alpha}$ treatment and BCS (**Table 6.3)**; treatment with $PGF_{2\alpha}$ increased (P = 0.02) the hazard of pregnancy in cows with BCS ≤ 2.5 which led to a decrease of 45 d in median time to pregnancy (**Figure 6.5**); however, $PGF_{2\alpha}$ treatment did not affect (P = 0.34) the hazard of pregnancy in cows with BCS > 2.5. No other variable or interaction affected the hazard of pregnancy up to 300 DIM.

Table 6.3. Effect of $PGF_{2\alpha}$ treatment on hazard of pregnancy up to 300 DIM.

Variable[1]	Level[2]	Cows	*P*	HR	95% CI
$PGF_{2\alpha}$ x BCS[3]	BCS (0), PGF (0)	96	-	-	-
	BCS (0), PGF (1)	121	0.02	1.5	1.1 – 2.0
$PGF_{2\alpha}$ x BCS[4]	BCS (1), PGF (0)	107	-	-	-
	BCS (1), PGF (1)	82	0.34	0.9	0.6 – 1.2

[1] PGF = Cows in the $PGF_{2\alpha}$ treatment group received an i.m. injection of 25 mg $PGF_{2\alpha}$ at 21, 35, and 49 ± 3 DIM and Control cows received sterile saline solution.

[2] BCS (0) = BCS ≤ 2.5; BCS (1) = BCS > 2.5; PGF (0) = Control; PGF (1) = $PGF_{2\alpha}$

treatment.

[3] An interaction between $PGF_{2\alpha}$ treatment and BCS was observed. Comparison between cows with BCS ≤ 2.5 that received $PGF_{2\alpha}$ (BCS (0), PGF (1)) and cows with BCS ≤ 2.5 that did not receive $PGF_{2\alpha}$ (BCS (0), PGF (0); reference group).

[4] Comparison between cows with BCS > 2.5 that received $PGF_{2\alpha}$ (BCS (1), PGF (1)) and cows with BCS > 2.5 that did not receive $PGF_{2\alpha}$ (BCS (1), PGF (0); reference group).

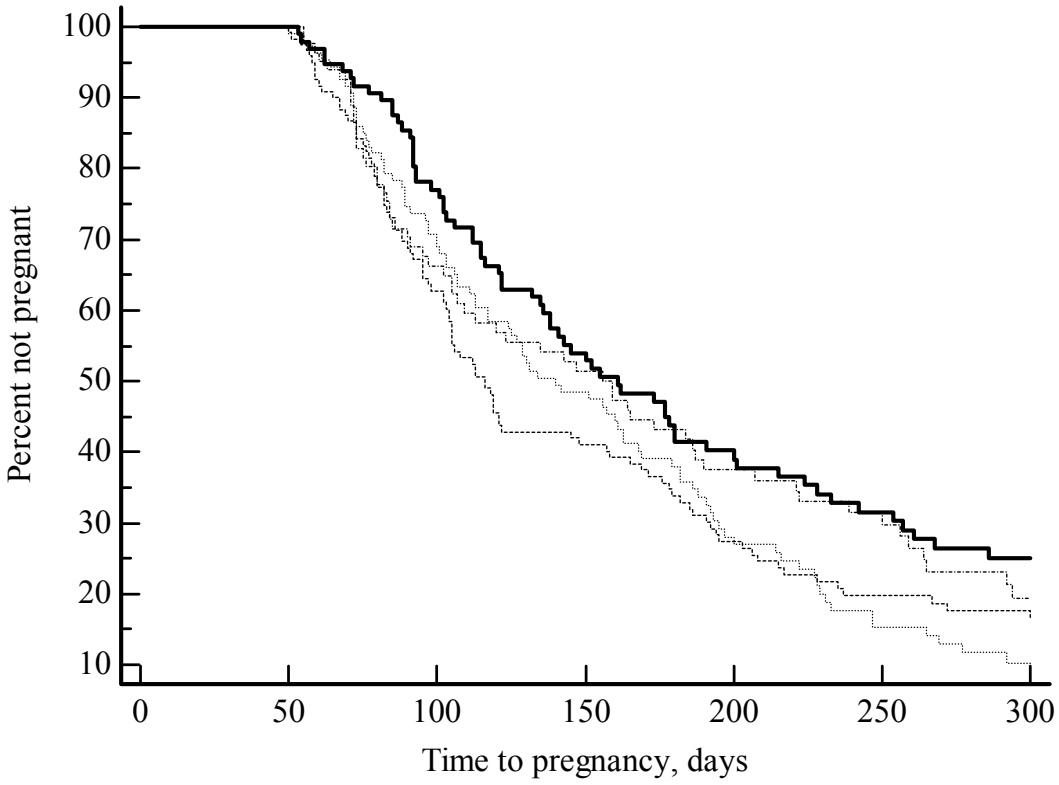

Figure 6.5. Time to pregnancy in dairy cows with BCS ≤ 2.5 that received $PGF_{2\alpha}$ treatment (n = 121; dashed line) or sterile saline (n = 96; solid line) at 21, 35, and 49 DIM and in dairy cows with BCS > 2.5 that received $PGF_{2\alpha}$ treatment (n = 82; dashed-dotted line) or sterile saline (n = 107; dotted line) at 21, 35, and 49 DIM. Median days

to pregnancy was decreased by $PGF_{2\alpha}$ treatment in cows with BCS ≤ 2.5 (116 vs. 161 days), but not in cows with BCS > 2.5 (159 vs. 140 days).

Treatment with $PGF_{2\alpha}$ had no effect ($P = 0.45$) on the hazard of pregnancy up to 300 DIM when only cows with SCE were evaluated (**Table 6.4)**. Median days open was exactly the same (169 days) for $PGF_{2\alpha}$-treated and Control cows. No variable or interaction affected the hazard of conception in cows with SCE. However, when only cows without SCE were evaluated, there was again an interaction between $PGF_{2\alpha}$ treatment and BCS (P = 0.01) on the hazard of pregnancy. Similar to when all cows were evaluated, treatment with $PGF_{2\alpha}$ increased (P = 0.006) the hazard of pregnancy in cows with BCS ≤ 2.5 which led to a decrease of 49 d in median time to pregnancy; however, $PGF_{2\alpha}$ treatment did not affect (P = 0.48) the hazard of pregnancy in cows with BCS > 2.5. No other variable or interaction affected the hazard of pregnancy up to 300 DIM in cows without SCE.

Table 6.4. Effect of $PGF_{2\alpha}$ treatment and other significant covariates on the hazard of pregnancy up to 300 DIM in cows with or without subclinical endometritis (SCE).

Stratum Variable	Level[2]	Cows	*P*	HR	95% CI
Cows **with** SCE[1]					
Treatment	$PGF_{2\alpha}$	69	0.45	0.9	0.6 – 1.3
	Control	82	-	-	-
Cows **without** SCE					
$PGF_{2\alpha}$ x BCS[3]	BCS (0), PGF (0)	53	-	-	-
	BCS (0), PGF (1)	82	0.006	1.8	1.2 – 2.71
$PGF_{2\alpha}$ x BCS[4]	BCS (1), PGF (0)	68	-	-	-
	BCS (1), PGF (1)	52	0.48	0.9	0.6 – 1.3

[1] Subclinical endometritis was characterized by the presence of ≥ 4.0% PMN in the uterine cytology at 49 DIM.

[2] Cows in the $PGF_{2\alpha}$ treatment group received an i.m. injection of $PGF_{2\alpha}$ at 21, 35, and 49 ± 3 DIM and Control cows received sterile saline solution. BCS (0) = BCS ≤ 2.5; BCS (1) = BCS > 2.5; PGF (0) = Control; PGF (1) = $PGF_{2\alpha}$ treatment.

[3] An interaction between $PGF_{2\alpha}$ treatment and BCS was observed. Comparison between cows with BCS ≤ 2.5 that received $PGF_{2\alpha}$ (BCS (0), PGF (1)) and cows with BCS ≤ 2.5 that did not receive $PGF_{2\alpha}$ (BCS (0), PGF (0); reference group).

[4] Comparison between cows with BCS > 2.5 that received $PGF_{2\alpha}$ (BCS (1), PGF (1)) and cows with BCS > 2.5 that did not receive $PGF_{2\alpha}$ (BCS (1), PGF (0); reference group).

DISCUSSION

This study was designed to evaluate the effect of a treatment regimen with $PGF_{2\alpha}$ at 21, 35, and 49 DIM on the prevalence of SCE at 35 and 49 DIM and on subsequent fertility. Herein, PMN cutoff (8.5%) at 21 DIM was not diagnostic for SCE based on negative effects on time to pregnancy. However, because previous reports indicated that 18% PMN at cytological examination between 20 and 33 DIM resulted in increased time to pregnancy (Kasimanickam et al., 2004), we evaluated higher cutoffs (15, 20, and 30% PMN), again with no effect. It is noteworthy that only cows with no abnormal uterine discharge were enrolled in the study by Kasimanickam et al. (2004). We did not use such an exclusion criterion. In defining clinical endometritis from 20 to 33 DIM based on hazard of pregnancy, LeBlanc et al. (2002) concluded that cows with more severe forms of uterine discharge (such as foul and purulent discharge) should be included in the case definition from 20 to 33 DIM; however, less severe forms (such as mucopurulent discharge) should only be included from 27 to 33 DIM. Another difference in our study is that cows were examined at 21 ± 3 days, rather than 20 – 33 as in the study of Kasimanickam et al. (2004). When no cows are excluded from evaluation for SCE, it is likely that presence of PMN in the uterus around 21 DIM is part of the physiological process of uterine involution, not a pathological finding. In the early postpartum period, neutrophils are active in phagocytosing bacteria and debris to prevent excessive bacterial colonization and disease and to restore normal uterine anatomy and histology (Foldi et al., 2006). Nonetheless, the cutoffs at 35 and 49 DIM, although slightly lower then previous reports (Kasimanickam et al., 2004; Gilbert et al., 2005), resulted in reduced fertility; the presence of PMN above a certain threshold at those times may be considered pathological.

Herein, cyclicity at 21 DIM decreased the prevalence of SCE at 35 and 49

DIM; however, cyclicity by 35 DIM (Cycling at 21 or 35 DIM) did affect SCE at 49 DIM, which indicates that early cyclicity (21 DIM) is the main determinant of later uterine health. Early cyclicity (Beam and Butler, 1997) and uterine diseases (Hammon et al., 2006) are associated with the energy status of dairy cows; therefore, cyclicity at 21 DIM might be an indicator of overall good health and uncomplicated transition.

Although $PGF_{2\alpha}$ administration did not affect the prevalence of SCE, it was effective in increasing first service pregnancy per AI in all the cows and the hazard of pregnancy in cows with low BCS when all the cows were evaluated or when only cows without SCE at 49 DIM were evaluated. However, treatment with $PGF_{2\alpha}$ had no effect in cows with subclinical endometritis at 49 DIM. In the present study, $PGF_{2\alpha}$ treatment appears to be exerting a positive effect on fertility but not via attenuating incidence of SCE. Kasimanickam et al. (2005) evaluated the effect of a single treatment with $PGF_{2\alpha}$ on fertility and reported an overall increase in pregnancy to first AI and on the hazard of pregnancy; however, in contrast to our findings, the positive effect of $PGF_{2\alpha}$ treatment on fertility was attributed to an improvement in cows with SCE not in cows without SCE at the time of $PGF_{2\alpha}$ treatment. Although treatment regimens differed considerably (a single dose vs. 3-dose regimen), the discrepancy in findings is not readily explained. In our study, the improvement in hazard of pregnancy in cows with low BCS was not expected. Cows with low BCS are more likely to be noncyclic by 65 DIM and to have decreased pregnancy per AI (Santos et al., 2008). However, a hypothesis to explain the differential effect in this particular group of cows could not be crafted at this point. Prostaglandin $F_{2\alpha}$ is thought to act mainly by inducing estrus in cows with a responsive CL and promoting physical clearance of the uterus and possibly by promoting an improvement in uterine defenses under low progesterone (Kasimanickam et al., 2005). Nevertheless, the effect of $PGF_{2\alpha}$ did not depend on presence of an active CL at the time of treatment.

Although a decrease in time to first insemination could be expected as one of the immediate benefits from PGF, no difference was observed in time to first AI. Time to first AI probably was not affected by $PGF_{2\alpha}$ treatment because $PGF_{2\alpha}$ did not appear to particularly benefit cyclic cows; cyclic cows were inseminated at a faster rate then noncyclic cows independently of $PGF_{2\alpha}$ treatment. This response was expected because all dairies relied upon estrus detection for most of their inseminations. Therefore, the benefit from $PGF_{2\alpha}$ on time to pregnancy was probably because of increased pregnancy per AI. Furthermore, SCE (which also affected time to pregnancy) did not affect time to first insemination. This finding suggests that detrimental effects from SCE are also on pregnancy per AI and might be mediated by local effects on the uterus and or embryo. Subclinical endometritis was associated with pathogenic bacteria such as *Arcanobacterium pyogenes* (Gilbert et al., 2007) that can damage the uterus and lead to infertility (BonDurant, 1999). Furthermore, induction of inflammation in the absence of bacteria can lead to reduced embryo quality (Hill and Gilbert, 2008).

In conclusion, proportion of PMN at cytological examination at 21 DIM was not diagnostic of SCE based on negative effect on time to pregnancy; however, 6.5% PMN at 35 DIM and 4.0% PMN at 49 DIM resulted in increased time to pregnancy and were considered diagnostic for SCE at those time points. Treatment with $PGF_{2\alpha}$ at 21, 35, and 49 DIM was not found to decrease prevalence of SCE evaluated at 35 or 49 DIM or to increase hazard of pregnancy in cows diagnosed with SCE at 49 DIM. However, $PGF_{2\alpha}$ treatment increased first service pregnancy per AI in all cows and hazard of pregnancy up to 300 DIM in cows with low BCS when all cows were evaluated or when only cows without SCE at 49 DIM were evaluated. Treatment with $PGF_{2\alpha}$ did not decrease SCE at the time points evaluated, but improved pregnancy per AI and hazard of pregnancy particularly in cows with low BCS.

CHAPTER SEVEN

Effect of early postpartum ovulation on subclinical endometritis and fertility in dairy cows

Interpretive summary: Early Ovulation and Fertility. Galvão.

Resumption of cyclicity before the first AI is one of the main determinants of first-service conception risks; however, cows that ovulate early in lactation might have better reproductive outcomes than cows that ovulate later. Cows were detected to be cyclic at 21, 49, or not cyclic by 49 DIM and subclinical endometritis was diagnosed at 49 DIM. Cows ovulating early in lactation had better first-service pregnancy per AI, shorter time to insemination and to pregnancy, and lower prevalence of subclinical endometritis compared to cows ovulating later in lactation and noncyclic cows.

ABSTRACT

Our objectives were to determine the effects of early ovulation on fertility and uterine health of dairy cows. We used 445 Holstein cows (185 primiparous and 260 multiparous) from 5 herds. Blood samples were collected at 21, 35, and 49 DIM and cows were considered to be cyclic at 21 DIM (Cyc21) if serum progesterone (P4) concentration was ≥ 1 ng/mL, cyclic by 49 DIM (Cyc49) if P4 concentration was ≥ 1 ng/mL at 35 or 49 DIM, or not cyclic (NotCyc) if P4 concentration was < 1 ng/mL in all samplings. Subclinical endometritis was diagnosed by uterine cytology at 49 DIM in a subset of 414 cows. Cows in the group Cyc21 had increased ($P < 0.05$) hazard of insemination, for the first service, compared to cows in Cyc49 (HR = 1.4) and NotCyc (HR = 2.1). Cows in the Cyc49 group also had increased ($P < 0.05$) hazard of insemination compared to cows in the NotCyc group (HR = 1.5). Median days to insemination were, respectively, 71, 76, and 96 for the Cyc21, Cyc49, and NotCyc groups. Cows in Cyc21 had greater ($P < 0.05$) first service pregnancy per AI than Cyc49 (39 vs. 28%) and NotCyc (24%). Pregnancy per AI was similar in Cyc49 and NotCyc cows. Cows in Cyc21 had increased hazard of pregnancy up to 300 DIM compared to Cyc49 (HR = 1.5) and NotCyc (HR = 2.0). Cows in Cyc49 tended ($P = 0.09$) to have increased hazard of pregnancy compared to NotCyc (HR = 1.3). Median days to pregnancy were, respectively, 103, 147, and 173 for cows in Cyc21, Cyc49, and NotCyc groups. Cows in the Cyc21 group had decreased prevalence of subclinical endometritis compared to cows in the NotCyc group (30 vs 44%); however, the prevalence did not differ from the Cyc49 group (39%). Cyc49 cows had similar prevalence of subclinical endometritis compared to NotCyc cows. Early postpartum ovulation was associated with improved uterine health and fertility.

Key words: early ovulation, subclinical endometritis, fertility, dairy cows

INTRODUCTION

Virtually all Holstein dairy cows have the first wave of follicle growth commencing 2 wk postpartum with about 40 to 50% of these cows ovulating the dominant follicle of the first follicular wave within 21 days in milk (DIM). Another 30 to 40% of the cows will ovulate follicles from subsequent follicular waves from 30 to 50 DIM and about 10 to 30% remain anovulatory by 50 DIM (Beam and Butler, 1997; Beam and Butler 1998). On average, first ovulation postpartum occurs from 27 to 30 DIM (Darwash et al., 1997; McCoy et al., 2006).

Resumption of ovarian cyclicity before the first AI is a positive determinant of first-service pregnancy per AI (Chebel et al., 2006; Galvão et al., 2004; Santos et al., 2009). Furthermore, anovulation before the first AI has been associated with increased pregnancy loss (Galvão et al., 2004; Santos et al., 2004). Prevalence of anovulation at 50 to 65 DIM affects > 20% of the lactating dairy cows (Cerri et al., 2004; Moreira et al., 2001; Santos et al. 2009), with some herds having > 40% of noncyclic cows around that time (Chebel et al., 2006; El-Zarkouny et al., 2004; Santos et al., 2009).

Studies evaluating fertility of high-producing Holstein cows (≥ 11,500 kg/cow/year) in the USA have focused on ovarian cyclicity right before first AI and usually evaluate only conception to the first (Galvão et al., 2004; El-Zarkouny et al., 2004; Santos et al., 2009) and sometimes to the second service (Chebel et al., 2006). Although anovulation at 50 to 65 DIM is negatively associated with fertility outcomes, studies in British-Friesian (5,193 to 6,008 kg/cow/year) and Holstein-Friesian cows (7,229 to 7,559 kg/cow/year) in the UK have shown that cows that ovulate early in lactation have better reproductive outcomes than cows that ovulate later (Darwash et al., 1997; McCoy et al., 2006). Increased fertility in cows that ovulate early in lactation is believed to be because of increased numbers of estrous cycles before the first insemination (Thatcher and Wilcox, 1973; Darwash et al., 1997), which would

provide progesterone priming and uterine cleansing during estrus.

Furthermore, cows ovulating the dominant follicle of the first postpartum follicular wave have a lower degree of negative energy balance and especially a shorter interval to its nadir (Beam and Butler, 1997; Beam and Butler, 1998). Indicators of energy balance such as non-esterified fatty acids and beta-hydroxybutyrate are positively associated with uterine diseases such as clinical metritis and subclinical endometritis (SCE) (Hammon et al., 2006), which in turn lead to long-term negative effects on fertility (Gilbert et al., 2005). Early ovulation may be an indicator of good overall health and uneventful transition; therefore, cows that ovulate their first dominant follicle might have better long-term fertility than cows ovulating later in lactation or cows not ovulating before their first service.

We hypothesized that cows that ovulate their dominant follicle of the first follicular wave would have better uterine health and overall fertility than cows ovulating follicles from subsequent follicular waves and better than cows not ovulating by the end of the voluntary waiting period. The objectives were to evaluate time to first insemination, first-service pregnancy per AI, time to pregnancy, and prevalence of subclinical endometritis among cows ovulating within 21 ± 3 DIM, those ovulating from 21 to 49 ± 3 DIM and those not ovulating by 49 ± 3 DIM. With the chosen intervals, we tried to identify cows that ovulated a dominant follicle from the first follicular wave (21 ± 3 DIM), those that ovulated a follicle from the second or subsequent follicular waves (21 to 49 ± 3 DIM) and those that had not ovulated by the end of a typical voluntary waiting period in the USA (49 ± 3 DIM).

MATERIALS AND METHODS

Animals, housing, feeding, and reproductive management

We enrolled 445 (185 primiparous and 260 multiparous) from 5 commercial

Holstein dairy farms located in Cayuga County, New York, USA. The herds ranged in size from 70 to 1,500 milking cows and the rolling herd averages were ~ 11,500 kg of milk/cow/year. Lactating dairy cows were housed in freestall facilities and milked three times daily. Within herd, all cows were fed the same TMR that was formulated to meet or exceed the NRC (2001) nutrient requirements for lactating Holstein cows weighing 680 kg and producing 45 kg of 3.5% fat corrected milk.

Cows used for this study were part of a trial evaluating the effect of $PGF_{2\alpha}$ administration at 21 ± 3, 35 ± 3, and 49 ± 3 DIM on SCE and subsequent fertility (Galvão et al., 2009). Of the 445 cows enrolled in the study, 218 received $PGF_{2\alpha}$ and 227 remained as controls. All dairies relied on estrus detection for most of their breedings and the voluntary waiting period was set at 49 DIM. All the cows were examined for pregnancy by palpation per rectum at 38 ± 3 d after AI. Cows not conceiving to the first service were reexamined for pregnancy after each subsequent insemination until 300 DIM. Pregnancy per AI was defined as the number of pregnant cows divided by the number of cows receiving AI in each treatment at 38 d after insemination.

Sample collection, cyclicity determination and subclinical endometritis diagnosis

All cows used for this study had a blood sample collected before $PGF_{2\alpha}$ administration at 21, 35, and 49 ± 3 DIM. Blood was collected by puncture of coccygeal vessels into Vacutainer® tubes without anticoagulant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The samples were immediately placed on ice and transported to the laboratory within 4 hours, where serum was separated by centrifugation at 2000 g for 15 min and frozen at -25º C and later analyzed for progesterone, by RIA, as previously reported (Beam and Butler 1997). Inter-assay and intra-assay CV were 7.5 and 6.0%, respectively. Cows having a concentration of

serum P4 ≥ 1 ng/mL were assumed to have a functional corpus luteum/corpora lutea (**CL**) at the time of sampling (Galvão et al., 2004) and therefore, considered to be cyclic.

Body condition was scored on all cows using a 5-point (1 = thin to 5 = fat) system (Ferguson et al., 1994) at each sampling and a subset of 414 cows were diagnosed for SCE using a low volume uterine lavage at 49 DIM as previously described (Gilbert et al., 2005). The cutoff used for classification of cows as having SCE was the presence of ≥ 4% neutrophils in the uterine cytology. The cutoff was based on receiver-operating characteristics analysis as previously reported (Kasimanickam et al., 2004) and is presented in a separate report (Galvão et al., 2009).

Statistical analyses

The current manuscript describes an observational cohort study. Cows determined to be cyclic at 21 DIM (Cyc21) were compared with cows that started cycling between 21 and 49 DIM (Cyc49) and with cows that were not cycling by 49 DIM (NotCyc). Outcomes first-service pregnancy per AI and subclinical endometritis were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Inst. Inc., Cary, NC). The model included the main effect of cyclicity group and controlled for the effects of herd, parity (primiparous vs. multiparous), and $PGF_{2\alpha}$ treatment by forcing these variables into the model. Other important variables such as season of calving (summer, fall, winter, and spring), body-condition score (< 2.5 vs. ≥ 2.5), and the interactions between cyclicity group and other covariates were offered to the model and maintained if $P \leq 0.15$. The hazard of insemination and hazard of pregnancy among the groups were analyzed by Cox's proportional-hazard model using the PHREG procedure of SAS. The hazard ratio (HR) is the daily probability of a given event (insemination or pregnancy) and may be interpreted as the insemination

rate or pregnancy rate (speed at which cows are submitted for insemination or become pregnant). The time variable used in the model to evaluate the hazard of insemination was the interval in days between calving and first insemination and to evaluate hazard of pregnancy was the interval in days between calving and pregnancy, which was detected 38 ± 3 d after AI. Cows that were not inseminated by 150 DIM or were sold or died were censored in the analysis of time to first insemination. Cows that were not inseminated or not pregnant by 300 DIM or were sold or died were censored in the analysis of time to pregnancy. The Cox model included the same variables cited for the logistic model plus the interaction of cyclicity group with time to check proportionality of hazard rate between groups. Proportionality was also assessed by inspection of Kaplan-Meier curves. The median days to first insemination or to pregnancy were obtained by survival analysis from the Kaplan-Meier model using the LIFETEST procedure of SAS. The survival plot was generated using the survival option of MedCalc version 9.2 for Windows (MedCalc Software, Mariakerke, Belgium). Differences with $P \leq 0.05$ (2-sided) were considered significant and $0.05 < P \leq 0.10$ were considered as a tendency towards statistical difference.

RESULTS

Based on progesterone concentrations, 26% (114), 54% (242), and 20% (89) of the 445 cows used in the study were in groups Cyc21, Cyc49, and NotCyc, respectively.

Hazard of insemination was affected ($P < 0.05$) by cyclicity group. Cows in the group Cyc21 had increased hazard of insemination compared to cows in Cyc49 (HR = 1.4; 95% CI = 1.1 – 1.8; $P = 0.006$) and NotCyc groups (HR = 2.1; 95% CI = 1.5 – 2.8; $P < 0.001$). Cows in the Cyc49 group also had increased hazard of

insemination compared to cows in the NotCyc group (HR = 1.5; 95% CI = 1.1 – 1.9; *P* = 0.005). Median days to insemination were, respectively, 71, 76, and 96 for cows in Cyc 21, Cyc49, and NotCyc groups (**Figure 7.1**).

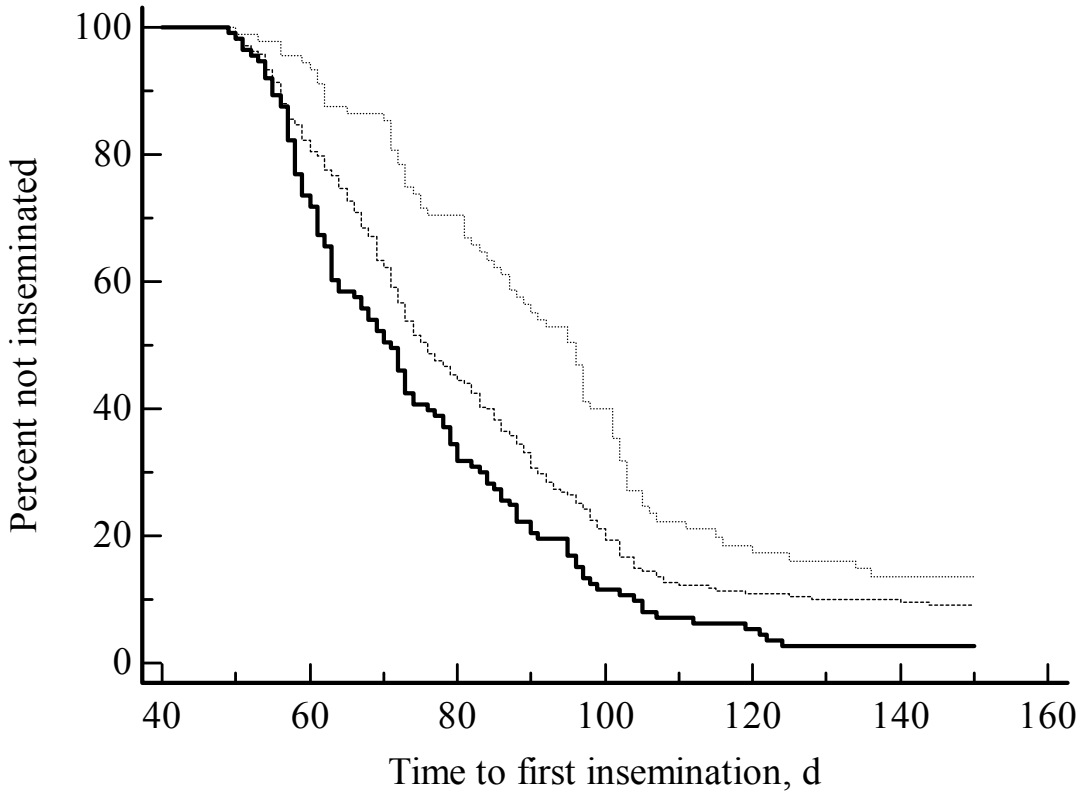

Figure 7.1. Survival curves for time to first insemination in dairy cows that were cycling at 21 DIM (Cyc21; n = 114; solid line), or started cycling from 21 to 49 d (Cyc49; n = 242; dashed line) or were not cycling by 49 DIM (NotCyc; n = 89; dotted line).

First-service pregnancy per AI was affected by cyclicity group ($P < 0.05$) and cows in Cyc21 had greater pregnancy per AI than cows in Cyc49 (39 vs. 28%; Adjusted odds ratio (AOR) = 1.7; 95% CI = 1.0 – 2.8; $P = 0.04$) and NotCyc cows (39

vs. 24%; AOR = 2.1; 95% CI = 1.1 – 4.0; $P = 0.03$). Pregnancy per AI was similar between cows in Cyc49 and NotCyc cows (AOR = 1.3; 95% CI = 0.7 – 2.2; P = 0.45).

Hazard of pregnancy by 300 DIM was also affected ($P < 0.05$) by cyclicity group; cows in Cyc21 had increased hazard of pregnancy compared to cows in the Cyc49 (hazard ratio = 1.5; 95% CI = 1.2 – 2.0; $P = 0.002$) and NotCyc groups (HR = 2.0; 95% CI = 1.4 – 2.8; $P < 0.001$). Cows in the group Cyc49 tended to have increased hazard of pregnancy compared to cows in the NotCyc group (HR = 1.3; 95% CI = 0.96 – 1.8; $P = 0.09$). Median days to pregnancy were, respectively, 103, 147, and 173 for cows in Cyc 21, Cyc49, and NotCyc groups (**Figure 7.2**).

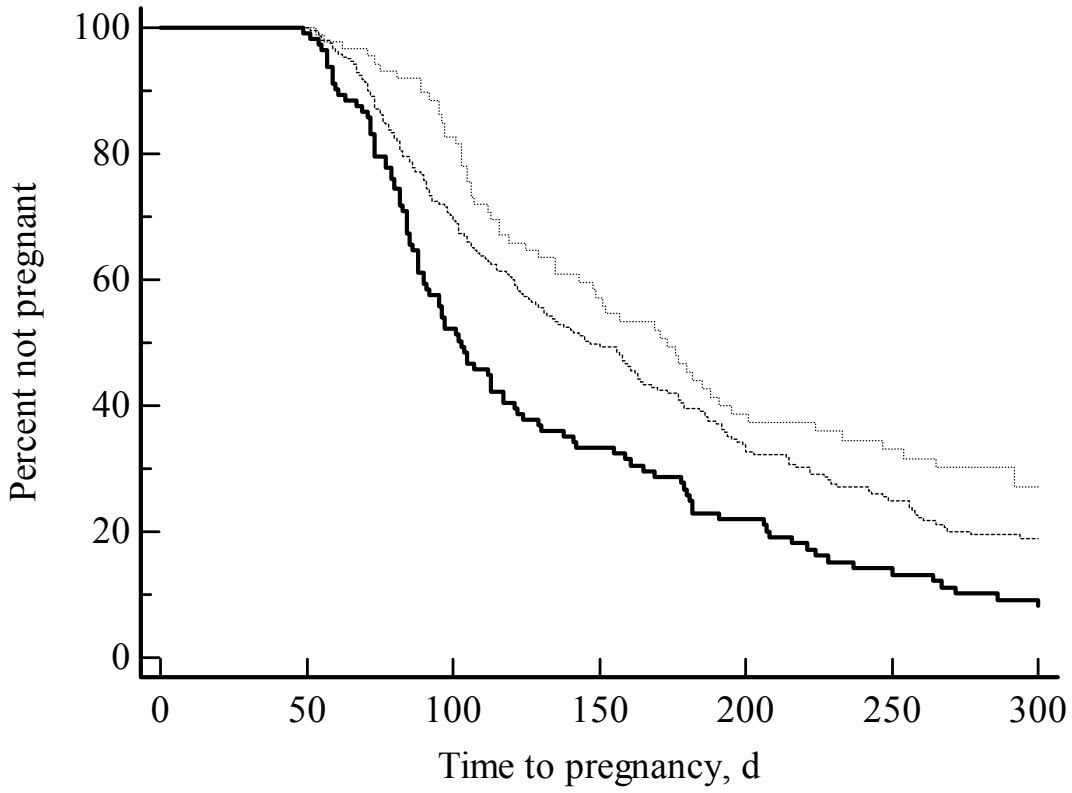

Figure 7.2. Survival curves for time to pregnancy in dairy cows that were cycling at 21 DIM (Cyc21; n = 114; solid line), or started cycling from 21 to 49 DIM (Cyc49; n

= 242; dashed line) or were not cycling by 49 DIM (NotCyc; n = 89; dotted line).

Of the 414 cows evaluated for SCE at 49 DIM, 26% (107), 53% (220), and 21% (87), were from groups Cyc21, Cyc49, and NotCyc, respectively. Overall prevalence of SCE was 38%. Cyclicity group was associated ($P = 0.10$) with prevalence of SCE; cows in the Cyc21 group had decreased odds for SCE compared to cows in the NotCyc group (30 vs. 44%; AOR = 0.5; 95% CI = 0.3 – 1.0; $P = 0.04$); however, the odds did not differ from the Cyc49 group (AOR = 0.68; 95% CI = 0.4 – 1.1; $P = 0.15$). Cows in the Cyc49 group had similar odds of having SCE compared to cows in NotCyc group (AOR = 0.8; 95% CI = 0.5 – 1.3; $P = 0.32$).

DISCUSSION

Anovulation by 49 DIM was within reported ranges (Cerri et al., 2004; Moreira et al., 2001; Santos et al., 2009); however, ovulation by 21 DIM was lower than previous reports (Darwash et al., 1997; Beam and Butler, 1998; McCoy et al., 2006). Beam and Butler (1998) reported that cows ovulated their first dominant follicle at 16.1 ± 3.5 (S.D.) days; therefore, it is possible that sampling cows from 18 to 24 d could misclassify some cows that ovulated up to 3 days before sampling. Nonetheless, the observed differences in fertility outcomes between cows in Cyc21 and Cyc49 indicate that most cows were probably correctly classified.

We believe this is the first time that survival analysis was used to compare hazard of first service insemination and pregnancy and time to first service insemination and time to pregnancy among high-producing Holstein cows ovulating early or late in lactation or remaining anovulatory by the end of the voluntary waiting period. Results for hazard of first service were really surprising to us because although cows in Cyc21 and Cyc49 were cyclic by the end of the voluntary waiting period,

cows in Cyc21 were inseminated for the first time at a faster rate than cows in Cyc49. As expected, both Cyc21 and Cyc49 cows had greater insemination rates than NotCyc cows. Herein, cows in Cyc21 had decreased prevalence of SCE compared with NotCyc cows, while incidence in Cyc49 cows was intermediate. Uterine diseases such as metritis and SCE have been associated with a greater degree of negative energy balance (Hammon et al., 2006), which affects resumption of postpartum cyclicity (Beam and Butler, 1997; Beam and Butler, 1998). Furthermore, bacterial infection of the uterus is associated with a decrease in luteinising hormone, and a decrease in the first dominant follicle size and growth as well as the ability to secrete estradiol; individually or in combination, these effects might impair ovulatory capacity (Peter et al., 1989; Sheldon et al., 2002). After postpartum ovulation resumes, cows that developed uterine disease present prolonged luteal phases (Opsomer et al., 2000), which is believed to be caused by a shift in production of the luteolytic prostaglandin $F_{2\alpha}$ to the luteotrophic prostaglandin E_2 (Herath et al., 2008), and might help to explain the differences in time to first insemination among Cyc21, Cyc49, and NotCyc cows.

Effects of cyclicity group on first-service pregnancy per AI and hazard of pregnancy followed a similar pattern as the hazard of first service. Cows in Cyc21 performed the best, NotCyc cows performed the worst and Cyc49 were intermediate. Nonetheless, although Cyc49 cows had greater hazard of first-service compared to NotCyc cows, they did not have greater first service pregnancy per AI and only tended to have greater hazard of pregnancy up to 300 DIM. This finding indicate that the observed benefit of being cyclic before first AI on first service pregnancy per AI (Galvão et al., 2004; El-Zarkouny et al., 2004; Santos et al., 2009) resulted mainly from cows that ovulate their dominant follicle of the first postpartum follicular wave. This is in agreement with early findings that cows having more estrus cycles before the first AI have improved pregnancy per AI (Thacher and Wilcox, 1973; Darwash et

al., 1997). Furthermore, cows showing estrus before 30 DIM had fewer services per conception than anestrus cows but cows showing estrus from 31 to 60 DIM were similar to anestrus cows (Thacher and Wilcox, 1973).

Decreased prevalence of SCE in Cyc21 cows likely contributed to the increased first-service pregnancy per AI and increased hazard of pregnancy compared to Cyc49 and NotCyc cows. Cows with SCE have both decreased first service pregnancy per AI and decreased hazard of pregnancy (Gilbert et al., 2005; Kasimanickam et al., 2005). Endometritis and associated bacteria such as *E. coli* and *Arcanobacterium pyogenes* (Gilbert et al., 2007) induce inflammation, cause lesions in the endometrium (Bonnett et al., 1991), disrupt endometrial function (Sheldon and Dobson, 2004), alter follicular and luteal function (Sheldon et al., 2002; Williams et al., 2007; Williams et al., 2008), and impair embryo development (Soto et al., 2003; Hill and Gilbert, 2008), which might have immediate and long-term negative effects on fertility.

The increase in insemination rate combined with the increase in pregnancy per AI (probably due to more estrus cycles before first AI and decreased prevalence of SCE) likely led to increased hazard of pregnancy in Cyc21 cows compared to Cyc49 and NotCyc cows. Therefore, it seems that early ovulation is an indicator of good overall health.

We conclude from this experiment that ovulation of the dominant follicle of the first follicular wave is a good marker for successful transition into lactation because cows detected to have ovulated their dominant follicle of the first follicular wave had improved uterine health, and fertility compared to cows that ovulated later in lactation and cows that did not ovulate by the end of the voluntary waiting period.

CHAPTER EIGHT

FINAL CONSIDERATIONS

The main objective of this dissertation was to add information to the understanding of the underlying causes of uterine disease (with a focus on metritis and subclinical endometritis; SCE), as well as to test treatment for these diseases in dairy cows. This dissertation presented data on the association between the pattern of pro-inflammatory and anti-inflammatory cytokine gene expression by monocytes (chapter 2) or uterine tissue (chapter 3) and incidence or prevalence of uterine diseases; the evaluation of the association between cellular and systemic energy status and incidence or prevalence of uterine diseases (chapter 4); the association between a single-nucleotide polymorphism in the neutrophil IL-8 receptor and incidence or prevalence of uterine diseases (chapter 5), the efficacy of prostaglandin F2α (PGF2α) in treating uterine disease (chapter 6), or the effect of early ovulation on the prevalence of uterine disease and fertility (Chapter 7).

In chapter 2, I observed that monocyte gene expression of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) and secretion of the main pro-inflammatory cytokine (TNFα) were decreased from calving until 2 to 3 wk after calving in cows that developed metritis. Cows that had SCE did not differ from healthy cows. Decreased expression of pro-inflammatory cytokines could lead to poor chemotaxis and activation of neutrophils, which would impair diapedesis to the site of infection, bacterial clearance and predispose cows to the development of metritis.

In chapter 3, I observed decreased TNFα and IL-1β gene expression in the first week after calving in uterine tissue of cows that developed SCE, while the gene expression of IL-1β, IL-6 and IL-8 were increased at wk 5 and or 7 after calving. Cows that develop SCE seem to have a compromised ability to up-regulate the gene expression of the main pro-inflammatory cytokines (TNFα and IL-1β) early in

lactation. These results corroborate with the findings using monocytes, and would support the thought that decreased expression of pro-inflammatory cytokines could lead to poor chemotaxis and activation of neutrophils, which would impair bacterial clearance and predispose cows to the development of SCE.

In chapter four, I confirmed that cows that develop uterine disease experience a greater degree of negative energy balance as indicated by greater concentrations of NEFA and BHBA around calving. All cows experienced a decrease in PMN glycogen concentration in the first 3 wk after calving, which could account for the overall immunosuppression observed at this time. Furthermore, cows that developed uterine disease were not as able to maintain PMN glycogen stores as cows that remained healthy. At this point, it is not clear why cows that developed uterine disease were less able to maintain their PMN glycogen stores, but it seems that blood glucose and plasma glucagon are involved. Glucose was mainly affected by cortisol concentrations around calving and by milk yields later on. Greater cortisol concentrations in primiparous cows could be contributing to their greater incidence of metritis. Another important factor that might be involved in the susceptibility to metritis is the circulating levels of immunoglobulins. Immunoglobulins work as opsonins, which greatly enhance phagocytic capacity. Primiparous cows have lower immunoglobulin content in colostrums which indicate lower circulating immunoglobulin levels (Muller and Ellinger, 1981); therefore, phagocytosis might not be optimal in early lactation in primiparous cows. We were not able to find any association between uterine disease and plasma insulin. Glucagon, on the other hand, was increased in cows that developed SCE which could impair neutrophil function and predispose cows to SCE. Greater plasma estradiol concentrations at calving in cows that developed metritis could be another factor contributing to their immunosuppression. Cows that developed metritis or SCE had several metabolic and hormonal changes (including greater degree

of negative energy balance, an inability to maintain neutrophil glycogen concentration, alteration in glucose homeostasis around calving, and increased concentrations of estradiol, cortisol, and glucagon) that could exacerbate immunosuppression. Therefore, strategies to increase dry-matter intake, improve energy balance, and decrease stress-related hormone changes around calving have the potential to decrease the incidence of uterine disease.

The most important finding from chapters 2 and 3 was the fact that both monocyte and endometrial gene expression of pro-inflammatory cytokines was decreased in cows that develop uterine disease. Activation of inflammation is critical for pathogen elimination and prevention or cure of disease. Combined with the findings in chapter 4 where cows that developed uterine disease were in more severe negative energy balance, it is reasonable to speculate that the state of negative energy balance compromises the ability of leukocytes and endometrial cells to mount an inflammatory response. The fact that cows that developed uterine disease had decreased ability to maintain PMN glycogen stores was really interesting to me and deserves further investigation, both to confirm my findings and to discover mechanisms for this inability to maintain PMN glycogen. I observed that glucose concentration around calving is one of the main players but other factors such as NEFA or BHBA might be involved. A future study should include samples pre-partum and also evaluate function (phagocytosis and killing ability) of PMN with low glycogen content. In chapter 4 I postulated that hyperglycemia at calving could compromise PMN function. This fact is observed in humans and should be better evaluated in periparturient cows. Although the association between uterine disease and cortisol concentrations was not very strong, it seems that cortisol is an important player (mainly in primiparous cows); therefore, the effect of cortisol in the development of uterine disease (mainly metritis) deserves further investigation. This is

an important point because management strategies to decrease stress around calving (such as: decrease the number of pen moves, decrease overcrowding, and milking pre-partum could be easily implemented. Greater estradiol concentration at calving in cows that developed metritis was another interesting observation and deserves further attention. It would be valuable to characterize the rise and fall in estradiol concentration better around calving and to evaluate PMN function in cows with high or low estradiol.

In chapter five I observed that single-nucleotide polymorphism (SNP) at position +735 in the IL-8 receptor CXCR1 did not affect the incidence of retained placenta or metritis, or the prevalence of SCE. No effect on incidence of uterine disease contrasts with findings of increased incidence of subclinical mastitis in cows with genotype CC for the SNP +735. Therefore, the uterus is not as sensitive to defects in CXCR1 as is the mammary gland. Factors such as energy balance, concentration of IL-8 in the uterus, chemotaxis induced by complement split products, and binding of IL-8 to CXCR2 might be more important for the development of uterine disease than the defect in CXCR1 or might compensate for the defect in CXCR1.

The finding of no effect of SNP genotype on uterine health (chapter 5) indicates that activation of inflammation might be different in different organs and tissues. Nonetheless, it seems important to reevaluate the effect of SNP genotypes on mastitis and also to characterize the effects of different genotype on milk yield, reproductive performance (time to pregnancy), and survival better. The initial finding that cows with genotype CC had more subclinical mastitis is relevant, but ultimately, dairy farmers are more concerned about milk production, reproduction and survival in the herd.

In chapter six I observed that the proportion of PMN in uterine cytological

examination at 21 DIM was not diagnostic of SCE. There was no negative effect on time to pregnancy; however, ≥ 6.5% PMN at 35 DIM and ≥ 4.0% PMN at 49 DIM resulted in increased time to pregnancy and therefore were considered diagnostic for SCE at those time points. Treatment with $PGF_{2\alpha}$ at 21, 35, and 49 DIM did not decrease prevalence of SCE evaluated at 35 or 49 DIM or increase hazard of pregnancy in cows diagnosed with SCE at 49 DIM. However, $PGF_{2\alpha}$ treatment increased first-service pregnancy per AI in all cows and hazard of pregnancy up to 300 DIM in cows with low BCS when only cows without SCE at 49 DIM were evaluated. Treatment with $PGF_{2\alpha}$ did not decrease SCE at the time points evaluated, but improved pregnancy per AI and hazard of pregnancy (particularly in cows with low BCS).

My attempt to improve reproductive performance of cows with SCE with PGF2α treatment (chapter 6) was not successful. Given that one study showed improvement in reproductive performance of cows with SCE after PGF2α treatment, a final verdict has not been reached and maybe this question needs to be addressed once more. Increase in first-service pregnancy per AI and increased reproductive performance in cows with low BCS that received PGF2α raises questions about the mechanism of action. My hypothesis that PGF2α would decrease prevalence of SCE mainly by acting in cows with a PGF2α-responsive corpus luteum and the decrease in prevalence of SCE would lead to increased reproductive performance was not confirmed. Therefore, further studies to confirm our findings and to investigate the effect of PGF2α in thin cows are warranted.

In chapter seven, I observed that ovulation of the dominant follicle of the first follicular wave is a good marker for successful transition into lactation because cows detected to have ovulated their dominant follicle of the first follicular wave had improved uterine health and fertility compared to cows that ovulated later in lactation

(and compared to cows that did not ovulate by the end of the voluntary waiting period). Hormonal treatment to induce ovulation early postpartum has the potential to decrease the prevalence of uterine disease and to improve fertility.

Improved uterine health in cows that ovulate early in lactation (chapter 7) raises the question whether uterine health can be improved by inducing ovulation early in lactation. The natural path would be that cows that ovulate early in lactation are the ones that are in better energy balance and therefore are the ones that did not have metritis and are not going to have SCE later on. Nonetheless, re-examining the benefit of induction of ovulation early in lactation could give us insights toward the effect of progesterone exposure on subsequent uterine health.

Therefore, regarding our main objectives for improving our understanding about uterine disease in dairy cattle and to test a treatment for SCE, I believe I added to the body of knowledge by showing that monocyte and endometrial gene expression of the main pro-inflammatory cytokines, and PMN glycogen are decreased around calving, which seems to be related to the state of negative energy balance. I did not confirm that PGF2α is a suitable treatment for SCE but raised the question whether induction of ovulation in early lactation can reduce prevalence of SCE, thereby improve fertility.

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