AN ABSTRACT OF THE THESIS OF

Adrienne McCracken Lulay for the degree of Master of Science in Animal Science presented on December 14, 2011.
Title: Effects of Prostaglandin F$_{2\alpha}$ on Neutrophil Populations, Uterine Health and Reproductive Performance in Dairy Cows

Abstract approved:

________________________________________________________________________________________

Alfred R. Menino, Jr

Incidences of uterine infections in dairy cattle are high between parturition and Day 21 postpartum. Dairy cows with uterine infections are at risk for prolonged periods of days open and multiple services before becoming pregnant. Neutrophils are the first wave of immune system defense against uterine contamination. Neutrophil function seems to be mediated by reproductive hormones and good uterine health is related to properly functioning neutrophils. To elucidate the interaction between reproductive hormones, neutrophils and uterine health in dairy cows the objectives of this research were to evaluate: 1) changes in circulating white blood cell populations during the estrous cycle, 2) the effects of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on circulating white blood cell populations and 3) the effects of a two-injection PGF$_{2\alpha}$ regimen on uterine neutrophil and bacterial populations and numbers of days open and services per conception. In the first experiment, the effect of stage of the estrous cycle on plasma neutrophil numbers was evaluated. Neutrophils were observed throughout the entire estrous cycle and numbers were greatest (P<0.05) on Day 14 (mid-cycle), when the corpus luteum was the dominant ovarian structure and plasma progesterone was at
In the second experiment, plasma neutrophil numbers were examined in cows after injections of saline or the PGF$_{2\alpha}$ pharmaceutical product, Lutalyse. Compared to saline, numbers of neutrophils were greater ($P<0.05$) 4 and 8 hr after Lutalyse injection. In the third experiment, neutrophil numbers were examined after injections of saline, Lutalyse or the PGF$_{2\alpha}$ analog, Estrumate. Compared to saline, numbers of neutrophils did not differ ($P>0.10$) from cows injected with Lutalyse or Estrumate. In the fourth experiment, uterine bacterial populations and numbers of neutrophils were quantified in cows treated with Lutalyse or saline on Days 0 and 14 or 14 and 28 postpartum. Compared to saline, Lutalyse treatment decreased ($P<0.05$) total bacteria present in the uterus and increased ($P<0.05$) the number of uterine neutrophils. In experiment five, numbers of days open and services per conception were evaluated in cows treated with Lutalyse or saline on Days 0 and 14 or 14 and 28 postpartum. Compared to saline, Lutalyse decreased days open ($154.7 \pm 14.1$ vs. $120.1 \pm 7.9$ days, respectively; $P<0.05$) and services per conception ($3.0 \pm 0.4$ vs. $2.3 \pm 0.2$ services, respectively; $P=0.09$). These results suggest PGF$_{2\alpha}$ treatment can increase neutrophil and depress bacterial cell populations in favor of the dairy cow’s uterine health and may explain why fertility is improved when PGF$_{2\alpha}$ is administered early in the postpartum period.
EFFECTS OF PROSTAGLANDIN F₂₀ ON NEUTROPHIL POPULATIONS, UTERINE HEALTH AND REPRODUCTIVE PERFORMANCE IN DAIRY COWS

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my theses to any reader upon request.

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Adrienne McCracken Lulay, Author
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Well, I guess we will see….
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Introduction

The dairy industry is dependent on a cow’s reproductive fitness. If a dairy cow doesn’t rebreed in a sufficient amount of time, her milk yield declines to a point where she no longer pays for herself. There are many factors affecting how quickly a dairy cow becomes pregnant and the cost involved in achieving that pregnancy. One of the foremost factors is uterine health. Uterine infections are prevalent in dairy cows but are often undiagnosed or untreated. Depending on the type of diagnosis, prevalence of uterine infections range from 10-50% (Markusfeld, 1987; Lewis, 1997; Sheldon and Dobson, 2004).

Economic losses associated with uterine infections in dairy cattle are combinations of cost of diagnosis and treatment of the infection, costs associated with reduction in milk yield, and costs associated with reduced fertility. When considering reproductive parameters alone, reproductive inefficiency beyond 100 days postpartum results in an estimated loss of $2.50 to $3.00 per cow per day, while reproductive inefficiency overall has been estimated to cost dairy producers as much as $5.40 per cow per day (Plaizier et al., 1997). Thus, each incidence of uterine infection could cost between $75 ($2.50 x 30 days) and $162 ($5.40 x 30 days). With approximately 93,000 dairy cows in Oregon, and assuming a 10% incidence of uterine infections, the cost of uterine infections to Oregon dairy producers would be between $697,000 (93,000 cows x 10% x $75/cow) and $1.5 million (93,000 cows x 10% x $162/cow). In a worst case scenario, assuming the high-end 50% infection rate previously cited (Markusfeld, 1987; Lewis, 1997; Sheldon and Dobson, 2004), uterine infections could...
cost Oregon dairy producers from $3.48 million (93,000 cows x 50% x $75/cow) to as much as $7.53 million (93,000 cows x 50% x $162/cow). While the worst case scenario is unlikely, a fairly conservative estimate puts the cost of uterine infections at about $1 million a year to Oregon dairy producers.

It has been known for many years that uterine infections and clearance of such infections are influenced by the reproductive hormonal profile of the cow (Lewis, 1997). It is also known that prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) has the ability to positively affect uterine health (Lewis, 1997; Sheldon and Dobson, 2004). What is unknown is the mechanism of how PGF$_{2\alpha}$ is affecting the immune system and the uterine environment. Prostaglandin F$_{2\alpha}$ treatment protocols have been developed, however their efficacy has been heavily debated and examined with no common consensus.

The objective of this research was to examine the interaction between reproductive hormones, neutrophils and uterine health in dairy cows by evaluating the effects the estrous cycle has on circulating white blood cell populations as well as the effect of PGF$_{2\alpha}$ on those populations. Also, a two-injection PGF$_{2\alpha}$ regimen was tested to determine the effects on uterine neutrophil and bacterial populations and numbers of days open and services per conception.
Review of the literature

Dairy cow reproductive management and consequences of uterine disease

Reproductive success is essential for the dairy industry to survive. For cows to lactate throughout their lifetime they must be rebred so that calving will initiate a new/fresh lactation cycle. There are a multitude of factors affecting reproductive performance in the modern high producing dairy cow. Energy balance, milk yield, disease, estrous synchronization and general reproductive management vary between farms and all affect reproductive performance in different ways (Caraviello et al., 2006).

Dairy cows are highly susceptible to uterine infections because of conditions associated with housing and production demands. Diseases like sub-clinical endometritis, clinical endometritis, metritis and pyometra are associated with sub-fertility and infertility in dairy cows. These conditions manifest as longer intervals from calving to first conception for some animals or ultimately lead to involuntary culling of animals failing to conceive (Sheldon et al., 2008). Postpartum metritis is associated with high economic losses due to the prolonged days open, multiple services and longer inter-calving intervals, which can result in high involuntary culling (LeBlanc et al., 2002; Azawi, 2008; Yavari et al., 2009). When producers were surveyed about reproductive parameters important to them, most indicated that artificial insemination service rate, conception rate, twinning and retained placenta or metritis were of immediate concern. Detection of estrus and early embryonic loss were
of intermediate concern, while ovarian cysts and, especially, reproductive record keeping were not considered problematic (Caraviello et al., 2006).

Cows with uterine infections like endometritis have lower first service conception rates, compared to healthy cows (29.0 vs. 37.9%). Median days open were longer (151 vs. 119 days) and more animals were culled for failure to conceive (6.7 vs. 3.0%) than unaffected animals (Sheldon et al., 2008). Caraviello et al. (2006) reported that failure to conceive was the biggest reason cows were culled from a dairy herd. The effect of reproductive failures is quite measurable. Diseases like metritis had an 8% decrease on time until first conception while dystocia, mastitis and retained placenta had smaller (6.0, 2.8 and 2.5%, respectively) yet measurable effects on conception (Bousquet et al., 2004).

**Uterine infections**

Bacterial contamination of the uterine lumen is ubiquitous from parturition until 2 weeks postpartum (Sheldon et al., 2008; Herath et al., 2009). There are vastly different reports as to the prevalence of the different types of uterine infections. This discrepancy may be due to differences in description of the uterine infections, the period the cow is monitored for infection, parity of the cow and herd management methods (Azawi, 2008). One of most important factors may be how closely each cow is monitored for signs of disease, especially when many of the signs are not seen outwardly. The majority of uterine infections go undiagnosed and untreated. Uterine
infections are classified by degree of severity: endometritis, metritis, toxic puerperal metritis and pyometra.

**Endometritis**

Endometritis is a superficial inflammation of the endometrium, no deeper than the first layer of the uterus (Youngquist and Threlfall, 2006). Endometritis is most often associated with the presence of *Archanobacter pyogenes*, *Fusobacterium necrophorum*, *Prevotella* spp, and *Escherichia coli*. Histologically, there is disruption of the surface epithelium, infiltration of inflammatory cells, and vascular congestion (Sheldon, 2004; Sheldon et al., 2008). Chronic endometritis can lead to scarring, obstruction of the fallopian tubes and adhesions between the ovary and bursa (Sheldon, 2004). Endometritis can be further subdivided into clinical and sub-clinical endometritis. Clinical endometritis is defined by the presence of a purulent (>50% pus) discharge in the vagina, 21 days or more postpartum, and often associated with delayed uterine involution. Mucopurulent (approximately 50% pus, 50% mucus) discharge detectable in the vagina after 26 days is also considered endometritis. Classifying animals as having endometritis less than 21 days postpartum includes many animals spontaneously resolving the bacterial contamination, and so does not accurately reflect the presence of disease. Similarly, using delayed involution alone to diagnose endometritis is unreliable (Sheldon, 2004; Sheldon et al., 2008). Sub-clinical endometritis is defined by >18% neutrophils in uterine cytology samples collected 21-
33 days after calving or >10% neutrophils at 34-47 days postpartum (Sheldon et al., 2008).

As reviewed by Azawi (2008), 90% of postpartum cows will experience some sort of endometritis, mild to severe, in the second through fourth week postpartum. Herath et al. (2009) reported incidences of 40 and 20% for sub-clinical endometritis and endometritis, respectively. Clinical metritis in the first 2 weeks after calving has been reported at 25-40% in dairy cows, but then when the disease persists as clinical endometritis, 20% of the animals are still afflicted (Sheldon et al., 2008). Hammon et al. (2006) found that 21.7% of cows were diagnosed with puerperal metritis (diagnosed by fetid red-brown watery uterine discharge between 0 and 14 days regardless of fever), 51.8% with sub-clinical endometritis and 15.7% with endometritis. Pyometra is relatively rare, comprising <5% of clinical cases of uterine disease (Sheldon et al., 2008).

A factor affecting reports of prevalence of uterine infections is how in depth diagnostic measures are. Various researchers have reported endometritis prevalence at only 4-10% yet when cytological methods were used for diagnosis, 53% of cows were reported with endometritis (Azawi, 2008). Leblanc et al. (2002) reported 16.9% of cows developed clinical endometritis, but 44% of the cows required vaginoscopy to identify the disease. This observation explains why so many infections can go undiagnosed and untreated.
**Metritis**

Metritis is a severe inflammatory reaction involving all layers of the uterus (endometrium, submucosa, muscularis, and serosa). Clinically, metritis is characterized by delayed involution of the uterus, a fetid watery purulent discharge from the vulva, and often pyrexia (≥39.5 °C). Metritis usually occurs in the first week postpartum, and is often associated with retained placenta. Metritis is rare after the second week postpartum (Sheldon, 2004). Animals not systemically ill, but having an abnormally enlarged uterus and a purulent uterine discharge detectable in the vagina, within 21 days after calving, may also be classified as having clinical metritis (Sheldon et al., 2008).

**Puerperal metritis**

Puerperal metritis is defined as animals having an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, with signs of systemic illness and fever >39.5° C, within 21 days after parturition. This condition is severe and can be life threatening (Azawi, 2008; Sheldon et al., 2008). Puerperal metritis is complicated by septicemia, toxemia, reduced milk yield, ataxia or unwillingness to rise, as well as other clinical signs including fever, depression, partial or complete anorexia and laminitis (Azawi, 2008).

**Pyometra**

Pyometra is defined as the accumulation of purulent material within the uterine lumen in the presence of a persistent corpus luteum and a closed cervix (Sheldon et al.,
Pyometra is most likely to develop after the cow’s first postpartum ovulation but before bacterial contaminants are eliminated (Youngquist and Threlfall, 2006).

**Bacteria associated with uterine infection**

Bacterial contamination of the uterus is ubiquitous in the first 2 weeks after parturition because the barriers to microbial invasion are compromised during parturition and remain that way for several days afterwards. Uterine bacterial load can fluctuate during the first 7 weeks postpartum due to spontaneous contamination, clearance and recontamination by bacteria. In fact uterine bacterial load often increases between Days 7-14 postpartum (Sheldon, 2004). Cows that do not develop uterine infections in the first 6 weeks postpartum have spontaneously eliminated microbial contamination (Azawi, 2008).

When pathogenic bacteria do establish themselves in the uterus they cause inflammation, histological lesions of the endometrium, delay in uterine involution and reduction in embryo survival (Azawi, 2008). Numerically, the most prevalent of the pathogens isolated that are associated with uterine infections are *Escherichia coli* (37%) and *Arcanobacterium pyogenes* (49%). *Arcanobacterium pyogenes* was formerly known as *Actinomyces pyogenes*, which was originally named *Corynebacterium pyogenes* (Dhaliwal et al., 2001). Furthermore, *E. coli* infections appear to precede and predispose the uterus for infection by *A. pyogenes* and other bacteria. Other common bacteria isolated from the postpartum uterus are *Streptococci spp.*, *Bacillus licheniformis*, *Prevotella spp* and *Fusobacterium necrophorum*.
(Sheldon, 2004; Herath et al., 2009). Ruder (1981) suggested severe uterine infections depend on pathogenic synergism between \textit{A. pyogenes} and anaerobic organisms, such as \textit{Fusobacterium necrophorum}. \textit{Arcanobacterium pyogenes} produces a growth factor for \textit{Fusobacterium necrophorum} which in turn produces leukotoxin, which can inactivate or destroy white blood cells (WBC).

\textit{Arcanobacterium pyogenes} is considered the most relevant bacterium involved in the pathogenesis of metritis due to its persistence in the contaminated uterus, its broad resistance to antimicrobial agents and its synergistic action with Gram-negative bacteria (Williams et al., 2005; Azawi, 2008). When \textit{A. pygoenes} was isolated from uterine fluids approximately 21 days postpartum, cows developed severe endometritis and usually were infertile at first service (Azawi, 2008). \textit{Arcanobacterium pyogenes} shows a high resistance to many drugs including amoxicillin, chloramphenicol, oxytetracyline and penicillin (Santos et al., 2010).

**The immune system**

There are two major branches of the mammalian immune system, the acquired and the innate immune systems, which collectively are known as WBC. The acquired immune system consists of cells like lymphocytes involved with antigen processing for long term identification of cellular invaders (Tizard, 2008). The innate immune system is primarily responsible for the elimination of bacterial contamination of the animal’s body, including the uterus after parturition. Within the innate immune system there are two main subsets of cells, granulocytes and non-granulocytes. Neutrophils
make up a large percentage of a group of cells called polymorphonuclear granulocytes (PMN), which also include the granulocytes, eosinophils and basophils. Neutrophils are considered the primary phagocytes responding to bacterial challenge, while eosinophils and basophils are involved in parasite interactions and allergic reactions (Tizard, 2008). Blood monocytes and tissue macrophages are non-granular cells that are major phagocytes in the immune defense against pathogenic microorganisms (Dhaliwal et al., 2001). Macrophages are simply monocytes that have migrated into the tissue and matured (Tizard, 2008). Macrophages respond to infection later than neutrophils and are also involved more in triggering the acquired immune system (Tizard, 2008). Neutrophils are the first responders of the immune system which makes them the significant population of WBC to consider when discussing resolution of uterine infections.

Neutrophils are normally short-lived WBC developing from cells in bone marrow. Upon maturation, bone marrow neutrophils are released into the circulation where they marginate on inflamed blood vessel endothelial cells and migrate through them into the area of infection via chemotaxis. Neutrophils are attracted by the invading pathogen under the effect of different substances such as bacterial products, substances released by destroyed cells and components of the complement system (Tizard, 2008). Once migration has occurred, neutrophils do not re-enter the circulation, but rather perform their bactericidal functions, then die by apoptosis in the tissue. The cytokine and hormonal milieu of the blood and extracellular tissue fluid influences neutrophil development and immunity-related activities, but the molecular
basis of these phenotypic changes and the physiological benefits or drawbacks are poorly understood (Burton et al., 2005).

Intravascular neutrophils need to pinpoint the site of infection and adhere to the endothelium of capillaries or venules adjacent to the inflammatory locus. Neutrophils migrate through the vessel wall and interstitial tissues to the infectious site, phagocytose invading microorganisms, and use a process called oxidative burst to kill and digest the microorganism. During this process, neutrophils also need to produce factors to ensure survival in the hostile inflammatory milieu, recruit additional phagocytes, inactivate their own toxic products and eventually induce their own apoptosis to prevent damage to the normal host tissue (van Eeden et al., 1999).

**Changes in uterine physiology by reproductive status**

The animal’s endocrine environment is likely to modulate uterine immunity. It has been known for more than 50 years that risk of uterine infection is greater during the luteal phase of the estrous cycle and induction of luteolysis and estrus is one of the most effective treatments for uterine infection (Sheldon et al., 2008). There are significant differences in hormone profile and immune system function between the postpartum period and the transition into the estrous cycle.

**The Postpartum uterus**

A chain of events must occur in the postpartum uterus in order for the succeeding pregnancy to be successful: involution of the uterus, regeneration of the endometrium, elimination of any bacterial contamination, and a return to ovarian
cyclicity (Sheldon, 2004). In the days immediately after calving the cow sheds any remaining placenta, fetal fluid and blood from her uterus. Expulsion of the majority of lochia from the breakdown (necrosis) of the uterine caruncles and fluids should occur by 12 days postpartum. Lochia may be yellow to brown but should have no unpleasant odor in contrast to discharge from a severe uterine infection (Sheldon, 2004). By Day 18 no discharge should be observed (Sheldon, 2004). By week 4 postpartum the uterus and cervix of the dairy cow should return to almost normal pre-pregnancy size. Uterine involution is not considered complete until 6 weeks after parturition which also defines the postpartum period.

Factors that may delay uterine involution are dystocia, hypocalcemia, retained placenta, and any sort of uterine infection (Sheldon, 2004). Factors most frequently associated with uterine infection are those that disrupt normal parturition including stillbirth, twins, dystocia or a Caesarean section operation. Retained placenta presumably provides large amounts of necrotic tissue for bacterial to grow. There is also an association between uterine infection and metabolic diseases such as milk fever, ketosis and left displaced abomasums (Sheldon, 2004).

The massive hormonal shift following parturition affects the likelihood of elimination of bacterial contamination (Sheldon, 2004). Progesterone and estrogen decline to extremely low levels. Prostaglandin \( F_{2\alpha} \) metabolite (PGFM) is a marker of tissue remodeling that can be measured to track \( PGF_{2\alpha} \) production and examine uterine involution. Increases in \( PGF_{2\alpha} \) vary depending on whether parturition was complicated or normal. In cows with dystocia or retained placenta, a massive release
of PGF$_{2\alpha}$ occurred compared to normal cows. But, despite the higher concentrations of PGFM, the duration of PGFM was shorter than control cows with normal involution (Nakao et al., 1997). It seems if the duration of PGF$_{2\alpha}$ release postpartum is too short, involution is delayed. If the period is longer, involution is accelerated. However, in cows with metritis or acute endometritis, PGFM in peripheral blood remains elevated longer than in healthy cows and uterine involution is delayed (Dhaliwal et al., 2001). Uterine bacterial contamination also increases plasma concentrations of PGFM and acute phase proteins during the postpartum period (Sheldon, 2004). Persistent and increased production of PGF$_{2\alpha}$ by the infected postpartum uterus initiates debate as to how exogenous prostaglandins eliminate infection (Dhaliwal et al., 2001).

The principal hormones secreted by the endometrium are PGF$_{2\alpha}$ and PGE$_2$, and the secretion of these hormones is modulated by E.coli or lipopolysaccharide (LPS), the E.coli product (Sheldon et al., 2008). Animals with E.coli infection after parturition have more LPS, acute phase proteins and PGE in peripheral circulation. The association between infection and PGE was supported in vitro because LPS treatment of endometrial explants and cells preferentially increased PGE over PGF$_{2\alpha}$ production. Endometrial epithelial cell medium accumulated more PGE than PGF$_{2\alpha}$, whereas the reverse was observed with oxytocin. This switch in prostaglandin accumulation was associated with an increased level of PLA2G6 protein in epithelial cells, rather than changes in the levels of Prostaglandin E synthase (PGES) or Prostaglandin F synthase (PGFS). The switch in steady-state prostaglandin concentrations from the luteolytic F series to the luteotropic E series provides a
mechanism to explain the extended luteal phases associated with uterine disease and infertility in cattle (Sheldon et al., 2008; Herath et al., 2009).

Guidry et al. (1976) found that numbers of peripheral blood neutrophils increased slowly from around 6 weeks prior to parturition to reach a peak on the day of calving. Conversely, maternal and fetal cortisol may suppress neutrophil function at the time of calving (Dhaniwal et al., 2001). During parturition, the reproductive tract requires large numbers of neutrophils to participate in the massive tissue remodeling that ensures the fetus is born alive, even if this occurs at the expense of reduced antibacterial defenses in peripheral tissues (Burton et al., 2005). Tizard (2008) reported a decrease in circulating PMN during uterine infection, and it has been suggested the decrease is because cells move to the site of infection (Azawi, 2008). But despite this influx of neutrophils into the uterus after parturition, their functional capacity is reduced and the reason for this suppression is still unknown. In fact, the function of circulating neutrophils has been reported to begin its decline, prior to parturition and doesn’t return to pre-partum levels until about 4 weeks postpartum (Kehrli et al., 1989). Proposed factors contributing to the decline are the hormonal changes around parturition or suppression by the very bacteria that invade the uterus (Sheldon, 2004). Madsen et al. (2002) proposed neutrophils respond to the physiology of parturition by altering the expression of genes needed for normal cellular functions. Two gene transcripts repressed in neutrophils at parturition have high DNA sequence homology to genes encoding bovine mitochondrial cytochrome b and ribosomal protein S15. These proteins are critical for normal respiratory metabolism and
translation in cells, respectively. Eleven more transcripts repressed in neutrophils at parturition were putatively identified as representing genes of the citric acid cycle and various DNA binding proteins. All of these genes are important for regulating basic life functions of bovine neutrophils and this study confirmed their repression by parturition and the associated changes in steroid hormones (Madsen et al., 2002). An increase in PMN numbers occurs within the uterine lumen after parturition, which may compensate for the reduced activity (Dhaliwal et al., 2001).

Approximately 48 hr after a normal unassisted calving, WBC accumulate in the uterine lumen as well as contaminating micro-organisms. In bovine puerperal metritis there is an initial decrease in phagocytic activity of uterine neutrophils. Two weeks later, when clinical recovery has occurred, phagocytic activity increased and this change coincided with lower numbers of bacteria in the uterine lumen (Dhaliwal et al., 2001).

A factor in parturient cow’s blood promotes extended neutrophil life span. This factor is likely to be cortisol because when added to steroid extracted serum in an in vitro neutrophil experiment, neutrophil survival-inducing capacity was restored back to that of intact parturient serum (Burton et al., 2005). Cows that experience dystocia, retained fetal membranes or metritis demonstrate an even greater reduction of phagocytosis by uterine WBC (Azawi, 2008). Bacteria, such as E.coli or its products, appear to inhibit neutrophils’ ability to phagocytose and generate reactive oxygen species used to neutralize bacteria. Conversely, A. pyogenes stimulated phagocytosis by neutrophils (Sheldon, 2004).
Resumption of estrous cyclicity

Resumption of estrous cyclicity is an important event in the dairy cow. Early return of ovarian cyclic activity is generally accepted to be beneficial for subsequent fertility. However the presence of a corpus luteum resulting from an early postpartum ovulation in the presence of uterine infection can lead to pyometra (Sheldon et al., 2008).

The first follicular wave emerges within 6-8 days after parturition with the dominant follicle reaching dominance about day 10-12 postpartum (Sheldon, 2004). The first follicle has three possible fates: ovulation and formation of the first postpartum corpus luteum (return of ovarian cyclic activity), atresia with the emergence of waves without ovulation (anestrus), or formation of an ovarian follicular cyst (Sheldon et al., 2008). Early follicles often have reduced ovulatory competence until the cow returns to a positive energy balance after parturition. The incidence of postpartum anovulatory anestrus is about 20% and is greater in cows selected for milk production (Sheldon, 2004). This means many high producing dairy cows are not under the influence of any reproductive steroid hormones, positive or negative, until they start cycling again.

Uterine infection perturbs ovarian follicle growth and function. High uterine bacterial load on day 7 postpartum resulted in a smaller first dominant follicle and lower peripheral plasma estradiol (Sheldon, 2004). Bacterial by-products can also inhibit/reduce the pre-ovulatory LH surge (Sheldon et al., 2008).
The estrous cycling uterus

In the cycling cow, the uterus is under the influence of progesterone during the luteal phase for about two thirds of the estrous cycle. The uterus is only under the influence of a significant amount of estradiol (with no progesterone) for 1 day immediately before standing estrus (Azawi, 2008). During the estrogen phase of the ovarian cycle there is increased blood flow to the uterus, increased mucus production, and intensified PMN activity. In the luteal phase there is reduced endometrial epithelial permeability, delayed WBC stimulation and an absence of detoxifying agents in the uterine secretions (Dhaliwal et al., 2001).

During estrus, when progesterone concentrations are decreased and estradiol concentrations are increased, uterine production of PGF$_{2\alpha}$ is increased. Uterine PGF$_{2\alpha}$ decreases to basal levels within a few days after estrus, when progesterone concentrations begin to increase (Azawi, 2008). Uterine infections cause infertility not only by disrupting endometrial health but also by affecting ovarian cycles. Bacterial toxins act at the hypothalamus or pituitary to suppress gonadotrophin release and in the ovary to perturb follicle growth and function (Herath et al., 2009). A common observation is animals with uterine disease have prolonged luteal phases, leading to delayed conception. The length of the luteal phase in ruminants is dependent on oxytocin binding endometrial epithelial cell oxytocin receptors to initiate PGF$_{2\alpha}$ synthesis and luteolysis (Herath et al., 2009). Prostaglandins are produced when arachidonic acid is liberated by phospholipase A2 (PLA2) enzymes. Arachidonic acid is converted into either prostaglandin F$_{2\alpha}$ or prostaglandin E$_2$. Prostaglandin F$_{2\alpha}$ is
luteolytic in ruminants but PGE is luteotropic. Increased concentrations of PGE in the uterine lumen have been reported in cattle with endometrial infections (Herath et al., 2009).

Prostaglandin F\textsubscript{2\alpha} is also a pro-inflammatory molecule that stimulates the production of other pro-inflammatory cytokines. It may also enhance uterine production of leukotriene B(4) which in turn stimulates neutrophil functions like phagocytosis (Lewis, 2004).

Prostaglandin F\textsubscript{2\alpha} is the naturally occurring luteolytic agent in non-pregnant ruminants. Oxytocin stimulates uterine secretion of PGF\textsubscript{2\alpha}, which in turn, stimulates oxytocin from the corpus luteum. Luteal oxytocin in turn positively stimulates endometrial secretion of PGF\textsubscript{2\alpha}. This positive feedback mechanism functions until luteolysis is complete. As a result, peripheral blood concentrations of progesterone progressively fall from day 16 of the estrous cycle until estrus. Conversely estradiol concentrations peak when the cow displays estrus (Dhaliwal et al., 2001).

Estradiol and progesterone have both opposing and complementary effects on the female genital tract. Estradiol stimulates vascularization of the endometrium, increases production of cervical mucus and oviductal secretions, enhances uterine contractility, initiates sexual receptivity and affects the immune system. Progesterone aids in endometrial gland differentiation and enhances uterine gland secretions, reduces cervical mucus production, prevents uterine contractility and counters estradiol-induced immune protective responses in the reproductive tract (Azawi, 2008).
When progesterone concentrations are basal, cattle are resistant to uterine infections but when progesterone concentrations increase, susceptibility increases. In fact, uterine infections in cattle usually develop after the formation of the first postpartum corpus luteum, despite bacterial contamination since calving. This has been proven under experimental conditions where cows received intrauterine infusions of *A. pyogenes* and *E.coli*. If cows were estrual and progesterone concentrations were basal, either no infection occurred or there was enhancement of the elimination of bacteria. However, if progesterone was elevated, bacterial growth was facilitated, and uterine infection was able to develop (Lewis, 2003; Sheldon, 2004).

When considering the effects of estrogen and progesterone on the immune system, they appear to be antagonistic, yet overall favor animal health. Uterine immune function is up-regulated at estrus when there are many opportunities for pathogens to be introduced and down-regulated during the luteal phase when the uterus is capable of supporting the conceptus, and needs to tolerate a fetal allograft to have a successful pregnancy (Azawi, 2008).

High estradiol concentrations at estrus and parturition cause changes in numbers and proportions of circulating WBC with a relative neutrophilia. Estradiol also causes an increase in quantity and alters composition of vaginal mucus. Vaginal mucus plays an important role in defense of the uterus against bacteria by providing a protective physical barrier and by flushing and diluting bacterial contaminants (Azawi, 2008).
Fluctuations in peripheral blood steroid hormone concentrations during the estrous cycle may influence uterine WBC activity. For example, elevated blood progesterone concentrations inhibit both uterine and peripheral blood neutrophil phagocytic activities (Dhaliwal et al., 2001). During estrus there is clear evidence uterine immune response is enhanced or heightened (Sheldon, 2004). However, it is unclear whether the effect the hormones are having is direct or indirect. In studies using exogenous estradiol or progesterone to measure changes in peripheral or uterine hormones the evidence is inconsistent (Sheldon, 2004). When neutrophils were opsonised with uterine secretions obtained from cows during the follicular phase rather than the luteal phase, there was a significant increase in phagocytic activity (Sheldon, 2004).

A handful of researchers have reported an increase in the number of neutrophils during diestrus compared to estrus (Subandrio and Noakes, 1997). This is in disagreement with the long-accepted view that the uterus of the cow is more susceptible to infection during the luteal phase than at estrus. The reason for this discrepancy may be due to the suppression of other uterine defense mechanisms present at diestrus by progesterone and therefore the neutrophil response is a compensatory one. Alternatively, it may be that other neutrophil functions, e.g., phagocytosis and killing of bacteria are depressed by progesterone, as has been suggested by Roth et al. (1983) using neutrophils obtained from peripheral blood. Da Costa et al. (2003) found no significant difference in migration, phagocytosis or
oxidative burst at either phase of the estrous cycle in the mare although there was a tendency for blood neutrophils to increase in number under progesterone influence.

**Diagnosis**

Although most cows clear microorganisms contaminating the uterus by 6 weeks postpartum, the ones that fail to do so develop endometritis. Diagnosis of uterine infection usually occurs during a routine examination of cows after calving or when a cow is bred (Dhaliwal et al., 2001). If the cow is not identified as having an infection/disease until breeding then many weeks can be lost. It may take several more weeks to clear the infection before she can be bred again and her days open are further extended. Normal postpartum discharge ranges in color from dark brown to red or white and usually should not be considered abnormal unless the fluid is malodorous or other aberrant clinical signs are observed (Azawi, 2008).

Endometritis in dairy cows is one of the most controversial topics discussed among practitioners due to the lack of a diagnostic gold standard (Youngquist and Threlfall, 2006). Definitive diagnosis of endometritis can be made by histologic examination of endometrial biopsies. However, biopsies and swabs are rarely used because they require extended clinical diagnosis which prevents prompt treatment. Transrectal palpation for delayed involution is too subjective for diagnosis because uterine involution is variable. A diagnosis at cow-side is a goal for the producer and herd manager. Examining the contents of the vagina by manually palpating the reproductive tract is cheap, rapid and can be performed without introducing any
additional bacterial contamination. It also won’t provoke an acute phase protein response or affect uterine horn diameter (Sheldon et al., 2008). Some believe that rectal palpation of uterine size is too subjective to use as a reliable diagnosis because it fails to take into account normal events and variability in uterine involution. Lewis (1997) reported that vaginoscopy, for the detection of pus and cervical contents, is the most useful tool for the diagnosis of endometritis. Sheldon (2004) also advocates examination of the vaginal contents to diagnose endometritis. If vaginoscopy is too inconvenient, the contents of the vagina can be manually withdrawn for examination. Sheldon (2004) categorized the character, color and odor of the vaginal mucus to produce endometritis clinical scores. The scoring system for character and color includes a mucus character score: 0, clear or translucent mucus; 1, clear mucus containing flecks of white pus; 2, <50 ml exudates containing ≤50% white or cream pus; and 3, ≥50mL exudates containing ≥ 50% white, cream, or bloody pus. Vaginal mucus odor ranges from 0 for no odor to 3 if a fetid odor is present. Character and odor scores are summed to give an endometritis clinical score ranging from 0 to 6. A fetid odor of the vaginal mucus is also associated with a greater load of recognized uterine pathogens, but not other bacteria. Presence of vaginal lacerations should also be noted for diagnosis of disease (Sheldon, 2004). The endometrial clinical scoring evaluating character, color and odor of the discharge, has been highly correlated with the growth density of pathogenic bacteria in the uterus (Azawi, 2008).

Treatment of all animals with endometritis more than 3 weeks postpartum is probably justified. However, mucus containing only flecks of pus (score 1) has
similar numbers of bacteria as clear, normal mucus (score 0) 3 or 4 weeks postpartum. Thus treatment of animals with an endometritis score of 1 could be questioned (Sheldon, 2004). Williams and Sheldon (unpublished observations) recommended treating these cows because they have longer calving to conception intervals than normal cows.

Azawi (2008) reported that cytological evaluation of a cervical smear at freshening is suitable for diagnosis of sub-clinical endometritis, planning for treatment and prognosis of fertility after a voluntary waiting period. Endometrial biopsies have been shown to have predictive value for reproductive performance, but the technique itself is associated with deleterious effects on fertility (Azawi, 2008). Ultrasonography is a useful diagnostic technique for identification of subclinical endometritis. Kasimanickam et al. (2004) concluded intrauterine fluid volume identified using ultrasonography was significantly correlated with uterine swab bacterial growth and infection significantly impaired uterine involution.

Subclinical endometritis, characterized by an increased proportion of neutrophils in uterine cytology, is prevalent in high producing dairy cows and is associated with both decreased pregnancy per artificial insemination and extended interval to pregnancy (Kasimanickam et al., 2004). When cows were classified as having subclinical endometritis based on the following neutrophil numbers: $\geq 8.5$, 6.5 and 4.0% neutrophils at 21, 35 and 49 days in milk (DIM), respectively; 66.0, 38.2 and 37.2% of 406 cows in the study were diagnosed, respectively (Galvão et al., 2009).
Endometrial cytological examination is useful because the presence of a uterine infection initiates recruitment of neutrophils from the circulation into the uterine lumen where they can be sampled (Azawi, 2008). Neutrophil presence is an excellent indication of an active inflammatory process because neutrophils are absent in the normal uterus (Azawi, 2008).

Endometrial and inflammatory cells can be collected by guarded cotton swab, uterine biopsy, uterine lavage or cytobrush. The cytobrush technique is the most effective because it recovers an in situ sample which may represent the inflammatory nature of the endometrium, compared to the uterine lavage technique, which yields a diluted sample of luminal contents. The cytobrush also results in less distortion of cells compared to lavage. The technique is easy, more consistent and produces rapid results (Kasimanickam et al., 2005). The prepared smear must contain epithelial cells to confirm the correct site of collection. If no epithelial cells are seen, there is no assurance that the sample was taken from the uterus. A ratio of neutrophils to epithelial cells should be calculated. If the ratio is more than one neutrophil to ten epithelial cells, inflammation may be judged significant (Azawi, 2008).

The % PMN in the uterus is negatively associated with DIM. This is in agreement with the findings of Bonnett et al. (1990) and Gilbert et al. (1993) who showed a decrease in neutrophil number as the postpartum period approached the completion of histological involution at 40 days post calving (Kasimanickam et al., 2005).
Treatment

Success in the treatment of uterine infections depends on: evacuation of uterine fluids, susceptibility of the infectious agents to the drugs used, concentration and number of times the drug is used and exposure of the entire endometrium to the drug (Azawi, 2008). Evacuation can be done by repeated palpations of the uterus or hormones to expel the fluid or hasten the onset of estrus. Estrus is usually the best way of stimulating uterine contractions and expelling fluids (Azawi, 2008).

Use of antibiotics and antiseptics is controversial because of the question of their effectiveness. Intrauterine infusions may sometimes be credited with successful antimicrobial treatments of endometritis when the beneficial effect was actually an irritation of the endometrium, which in turn stimulates the natural immune response (Azawi, 2008). However their effects may be detrimental. Lugol’s iodine and polyvinyl-pyrrolidone-iodine both cause necrosis of the endometrial epithelium with subsequent release of PGF$_2$$\alpha$. Infusions can also cause endometrial fibrosis (Dhaliwal et al., 2001). Most antiseptics, and many antibiotics, have been shown to depress phagocytosis for several days after intrauterine administration. In one study, Lugol’s iodine destroyed phagocytic activity of uterine WBC for several days after intrauterine application (Azawi, 2008). Their effects are most pronounced because high concentrations of the antibiotics must be achieved in the uterine lumen for it to be effective (Azawi, 2008).

Oxytetracycline, the broad spectrum antibiotic, which is indicated for treatment and control of many infections caused by gram-positive and negative bacteria, is often
infused directly into the uterus. This intrauterine route is useful because the drug is administered directly to the caruncles and endometrium at therapeutic concentrations. However, tetracyclines are known to be partially inactivated by purulent material and cell debris at a pH found in afflicted uteri. While intrauterine administration may be effective against endometritis, systemic treatment is necessary if the cow has toxic puerperal metritis, where microorganisms have invaded the deeper layers of the uterus (Azawi, 2008).

Oxytetracycline, enrofloxacin, cefquioime, ceftiofur, cepaparin and a mixture of cepaparin and mecillinam are drugs meeting the minimum inhibitory concentration as antibiotics for *E coli*, *A pyogenes*, *F necrophorum* and *P melaninogenicus*. However, there is evidence for bacterial resistance to oxytetracycline which reduces its use as a treatment for endometritis (Sheldon, 2004). Antimicrobial resistance in pathogenic bacteria has become a severe problem and may be partially attributed to the use of antimicrobials as growth promoters or prophylactic agents in animal agriculture (Santos et al., 2010).

**Treatment with hormones**

In the past estradiol has been recommended as a treatment for uterine infections to stimulate myometrial contractions, phagocytosis by immune cells and mucus production by the reproductive tract (Sheldon, 2004). However others have
concluded estradiol has no beneficial effects on metritis. In fact, some authors have suggested estradiol can have a negative effect by propelling the uterine inflammatory exudate into the oviduct. Therapeutic use of estradiol has been banned in some countries due to high tissue residues (Azawi, 2008).

Administration of PGF$_{2\alpha}$ is the treatment of choice for cases of endometritis in which a corpus luteum is present and progesterone is high. Prostaglandin F$_{2\alpha}$ is also recommended for non-cycling cows, but its mode of action in this group of cows is not fully understood (Dhaliwal et al., 2001). There are three proposed actions for PGF$_{2\alpha}$ on the reproductive tract which may make it an effective treatment for uterine infections. First, in cows with a functional CL, administration of PGF$_{2\alpha}$ decreases plasma progesterone and increases plasma estrogen concentrations. This removes the suppressive effect of progesterone on the immune system and allows for maximal resistance of the uterus to bacterial infection (Dhaliwal et al., 2001; Azawi, 2008). However, PGF$_{2\alpha}$ administration can clear uterine bacterial infections even when circulating progesterone was maintained at luteal phase or greater concentrations (Del Vecchio et al., 1994; Lewis, 2003). Second, PGF$_{2\alpha}$ can stimulate myometrial contractions which may in turn expel debris and microorganisms from the uterus. Although in an equine study, PGF$_{2\alpha}$-stimulated contractions reduced the volume of uterine fluid present during infection without eliminating the bacteria (Nikolakopoulos and Watson, 1999). Third, PGF$_{2\alpha}$ may have a stimulatory effect on the phagocytic activity of uterine PMN (Dhaliwal et al., 2001).
Typically, administration of exogenous PGF$_2$α is followed by an increase in endogenous uterine PGF$_2$α production (Azawi, 2008). Prostaglandin F$_2$α, which is considered a pro-inflammatory molecule, may stimulate the production of pro-inflammatory cytokines that enhance phagocytosis and lymphocyte function. Prostaglandin F$_2$α enhanced neutrophil chemotaxis and stimulated phagocytosis by bovine neutrophils in vitro (Hoedemaker et al., 1992; Azawi, 2008).

There have been numerous studies examining different PGF$_2$α treatment protocols with varying results on efficacy. The biggest differences seem to be when and how many PGF$_2$α injections cows receive. Bonnet et al. (1990) assessed histologic characteristics and bacterial presence by examining endometrial biopsies and clinical findings at Day 40 in cows receiving PGF$_2$α treatment on Day 26 postpartum. Cows receiving treatment had less vaginal discharge, smaller postpartum uterine horn diameter, less inflammation and fibrosis in the endometrium, and were less likely to have *A. pyogenes* present. What was most interesting was the effects were independent of plasma progesterone concentrations at the time of treatment (Bonnett et al., 1990).

Del Vecchio et al. (1994) examined whether uterine infections were associated with elevated plasma concentration of a PGF$_2$α metabolite (PGFM). Cows were selected based on presence of uterine infection between Days 21 and 28 postpartum. *Archanobacter pyogenes* was most prominent in infected cows while *Streptococcus* and *Pasturella* were also prevalent. *Escherichia coli* was actually found in infected and control cows not displaying signs of infection. Infected cows had significantly more neutrophils, plasma cells and lymphocytes in the endometrium than control
cows. Also, 83 and 50% of control and infected cows, respectively, had luteal function during the 2-week collection period (21-28 days postpartum to 14 days later). The PGFM was greater in infected cows.

Several studies have described routine administration of PGF$_{2\alpha}$ to healthy cows after calving. Prostaglandin F$_{2\alpha}$ initiated early onset of ovarian activity, independent of peripheral blood progesterone concentrations, at the time of treatment and improved reproductive efficiency (Dhaliwal et al., 2001). McClary et al. (1989) administered one luteolytic dose of PGF$_{2\alpha}$ to cows Days 14-16 post-calving. Days to first service and first service pregnancy rates were not different, but mean days open were 20 days less for treated cows and services per conception were improved in treated vs. untreated cows (1.64 vs. 2.33 services, respectively). Archbald (1990) selected cows for a single PGF$_{2\alpha}$ treatment on Day 0 based on calving ease. This approach provided no beneficial effects on fertility during the succeeding postpartum period. There may be a viable strategy for treating cows with more than one PGF$_{2\alpha}$ injection during the postpartum period. Salasel and Mokhtari (2011) treated cows with two injections of PGF$_{2\alpha}$ 8 h apart on Day 20 postpartum and observed an increase in first service conception rate and decreases in services per conception and days open. Treatment with PGF$_{2\alpha}$ also increased the percentage of cows pregnant by 150 days in milk and decreased the prevalence of repeat breeders. This study was especially significant because cows were selected for the study if they experienced dystocia, retained placenta, twinning, abortion or postpartum uterine infection (Salasel and Mokhtari, 2011).
A definitive study examining the effects of the hormonal milieu on WBC populations and administration of a PGF$_{2\alpha}$ regimen during a fixed period postpartum on uterine WBC and bacterial populations and reproductive performance has not been conducted. Therefore, the objectives of the research conducted in this thesis were to elucidate the interaction between reproductive hormones, neutrophils and uterine health in dairy cows. We evaluated the changes in circulating WBC populations during the estrous cycle, the effects of PGF$_{2\alpha}$ on circulating WBC populations and the effects of a two-injection PGF$_{2\alpha}$ regimen on uterine neutrophil and bacterial populations and numbers of days open and services per conception.
Materials and Methods

Animals

A total of 55 Holstein and Jersey cows from the Oregon State University (OSU) Dairy Center were used for Experiments 1-4. Cows were housed in free stall facilities and milked 2 times daily. Water was available ad libitum. Cows were fed the same total mixed ration (TMR), consisting of alfalfa hay, haylage, grass silage, cottonseed, corn silage and grain mix, formulated to meet NRC requirements for lactating cows. Estrus detection was conducted using the Pedometer Plus and Afimilk program (Afikim, Massillion, OH). Voluntary waiting period was set at 60 days in milk (DIM). Cows were examined for pregnancy by palpation per rectum and ultrasound by OSU veterinarians at least >35 days following artificial insemination. Cows were examined again for pregnancy prior to dry-off at 220 days carrying calf. Physical activity suggesting return to estrus or abortion was also monitored by pedometer.

A total of 64 cows at a cooperating local dairy farm were used for Experiment 5 and details of the herd management are presented in that section. All work conducted with animals in this research was approved by the OSU Institutional Animal Care and Use Committee.
Experiment 1: Changes in circulating white blood cell populations during the estrous cycle

The objective of this experiment was to evaluate changes in total WBC, neutrophils and lymphocytes by stage of the estrous cycle in dairy cows. Seven cows were estrous-synchronized with two 25-mg (5 mg/ml) injections (im) of PGF$_{2\alpha}$ (Lutalyse; Pfizer Animal Health, New York, NY) 10 days apart. Cows were observed for estrus twice daily for 20 minutes. Two 10-ml blood samples were collected by coccygeal venipuncture into EDTA-containing Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) on Days 0 (where Day 0 = onset of estrus), 7, 14 and 21 of the estrous cycle. One 10-ml blood sample was transported on ice to the OSU Veterinary Diagnostic Laboratory for a complete blood count (CBC) evaluation. The second sample was transported on ice to the laboratory and centrifuged at 3000Xg and plasma was recovered and frozen at -20° C for progesterone analysis.

Experiment 2: Changes in circulating white blood cell populations following injection of Lutalyse or saline

The objective of this experiment was to evaluate changes in total WBC, neutrophils and lymphocytes following injection of Lutalyse in dairy cows. Eight luteal cows were randomly assigned to one of two treatments, Lutalyse or Saline (four cows per treatment). Lutalyse or Saline cows were injected (im) with 5 ml of Lutalyse or physiologic saline, respectively. Two 10-ml blood samples were collected by
coccygeal venipuncture into Vacutainer tubes containing EDTA at 0, 1, 4 and 8 hr post-injection for CBC and progesterone measurements as described in Experiment 1.

**Experiment 3: Changes in circulating white blood cell populations following injection of Lutalyse, saline or a PGF₂α analog**

The objective of this experiment was to evaluate changes in total WBC, neutrophils and lymphocytes following injection of Lutalyse or the PGF₂α analog, cloprostenol sodium (Estrumate; Merck and Co., Inc., Summit, NJ) in dairy cows. Nine luteal cows were randomly assigned to one of three treatments: Lutalyse, Saline or Estrumate (three cows per treatment). Lutalyse, Saline or Estrumate cows were injected (im) with 5 ml of Lutalyse, 5 ml of physiologic saline or 2 ml of Estrumate (250 µg/ml), respectively. Two 10-ml blood samples were collected by coccygeal venipuncture into Vacutainer tubes containing EDTA at 0, 1, 2, 4, 8 and 16 hr post-injection for CBC and progesterone measurements as described in Experiment 1.

**Experiment 4: Effects of Lutalyse treatment on uterine neutrophil and bacterial populations in postpartum dairy cows**

The objective of this experiment was to evaluate the effects of a two-injection Lutalyse protocol administered at two intervals postpartum on uterine neutrophil populations and bacterial loads in dairy cows. Twenty-eight postpartum cows were assigned randomly to one of four treatments. Treatments consisted of two injections (im) of: 1) saline (5 ml) on Days 0 or 1 and 14 postpartum (where Day 0 = day of
calving), 2) saline (5 ml) on Days 14 and 28 postpartum, 3) Lutalyse (5 ml) on Days 0 or 1 and 14 postpartum or 4) Lutalyse (5 ml) on Days 14 and 28 postpartum. For all treatments, on the day of the second injection (Day 14 or 28), the uterus was sampled for neutrophils and bacteria (0 hr). Sampling consisted of an endometrial cytological sample for neutrophils obtained using a Cytobrush (Reproduction Resources, Walworth, WI) and an uterine swab to obtain a bacterial sample. Twenty-four hours (24 hr) after the 0 hr sampling, a second uterine sampling was conducted for neutrophils and bacteria. Blood (10 ml) was collected by coccygeal venipuncture into Vacutainer tubes containing EDTA at the 0 and 24-hr sampling times for progesterone measurements as described in Experiment 1 to verify the presence or absence of a corpus luteum.

**Endometrial sampling**

Endometrial samples for cytologic examination of neutrophils were collected using a double guarded Cytobrush as described by Kasimanickam et al. (2005). Slides were prepared for cytological examination by rolling the Cytobrush onto a clean glass microscope slide and the tissue sample was fixed using a cytofixative spray (Cytoprep; Fisher Scientific, Pittsburgh, PA). Slides were brought to the laboratory within 2 hr of collection and stained using the Diff-Quick staining kit/protocol (Hardy Diagnostic, Santa Marta, CA).

Cytological assessment was performed by counting a minimum of 100 cells at 400X and 1000X magnification to determine the numbers of neutrophils, endometrial
epithelial cells, lymphocytes, macrophages and other nucleated cells. Initially the whole slide was assessed and a representative area was selected to determine the cell counts. Slides for cytologic examination were assessed twice by one individual and once by a second individual who was blinded regarding the sampling (Kasimanickam et al., 2005).

**Bacterial assessment**

For sampling uterine bacteria, the vulva was cleaned with paper towels and the swab was passed through the vagina up to the external cervical os. The first guarded tube was inserted into the os, and the second guarded tube was pushed through the first tube, advanced through the cervix and into the uterine horn approximately 2 cm. The swab was advanced out of the second guarded tube and manipulated to ensure contact with the uterine fluid. The swab was retracted into the two guarded tubes and removed from the reproductive tract. The swab was then removed from the guarded tubes and placed into 10 ml of Dulbecco’s phosphate buffered saline (DPBS) and transported to the laboratory within 1 hr of collection for culture. Serial dilutions using DPBS as the diluting fluid (0 and 1/10) were made from the original uterine sample and 100 µl of each dilution was plated onto each side of a Blood Agar/MacConkey Agar bacteriological culture biplate (Hardy Diagnostic, Santa Marta, CA). Biplates were incubated for 1 hr at room temperature, placed into a 37°C incubator upside down for 24 hr and evaluated for number of colonies on both types of agar. Colony
counts from the Blood Agar side provided the total number of bacteria while counts from the MacConkey’s Agar side, a selective medium, provided the number of *E.coli*.

**Experiment 5: Effects of Lutalyse treatment on reproductive performance in postpartum dairy cows**

The objective of Experiment 5 was to evaluate the effects of the two-injection Lutalyse protocol administered at two intervals postpartum on reproductive performance in a commercial dairy herd. A total of 64 Holsteins at a cooperating local dairy were used for this experiment. Cows were assigned randomly to one of 4 treatment groups regardless of parity or calving ease. Treatments consisted of two injections (im) of: 1) saline (5 ml) on Days 0 or 1 and 14 postpartum (where Day 0 = day of calving), 2) saline (5 ml) on Days 14 and 28 postpartum, 3) Lutalyse (5 ml) on Days 0 or 1 and 14 postpartum or 4) Lutalyse (5 ml) on Days 14 and 28 postpartum.

Cows were managed at the cooperating dairy under the following standard operating procedures. Cows were housed in free stalls and received *ad libitum* water and a TMR formulated to meet the NRC recommended diet for high producing cows. Starting at Day 50 postpartum, cows were administered two 25-mg injections (im) of Lutalyse two weeks apart as an estrous pre-synchronization program. After the second injection, estrus was monitored twice daily and determined based on the removal of tail chalk applied using paint sticks (All-Weather Paintstik; LA-CO, Chicago IL). When estrus was observed, cows were artificially inseminated 12 hr later. Cows not observed in estrus within 2 weeks were started on an Ovsynch timed artificial
insemination program consisting of: 2 ml of GnRH (gonadorelin diacetate
tetrahydrate; 100 µg; Cystorelin; Merial LLC, Duluth, GA) on Day 0 (im), 5 ml of
Lutalyse on Day 7 (im), 2 ml of GnRH on Day 10 (im) and timed artificial
insemination 24 hr after the second GnRH injection. All cows remaining on the farm
until 100 days postpartum were artificially inseminated the first time between 65 and
91 days postpartum.

Pregnancy status was monitored by rectal palpation by a veterinarian at 6, 17
and 29 weeks post-insemination. Cows were injected with 2 ml of GnRH (im) 14 days
before the scheduled pregnancy diagnosis so the Ovsynch program described earlier
could be started immediately if a cow was diagnosed as not pregnant. If a cow was
observed in estrus after a confirmed pregnancy diagnosis, she was re-examined and if
not pregnant recorded as aborted, and re-bred on the next scheduled Ovsynch
program. At 34 weeks postpartum, cows were moved to a pen containing two bulls for
breeding by natural service.

**Progesterone assay**

Plasma progesterone concentration in cows from Experiments 1-4 were
quantified using ELISA (Calbiotech, Spring Valley, CA). Cross-reactivity of the
antiserum was 100% to progesterone with the next highest cross-reactivities of: 0.74%
to corticosterone, 0.11% to cortisone, 0.1% to testosterone and less than 0.1% for all
other steroids. Concentrations less than 1.5 ng/ml were considered non-luteal. Intra-
and inter-assay coefficients of variation were 7.7 and 12.1, respectively. The limit of sensitivity was 0.22 ng/ml.

**Statistical analyses**

In Experiment 1, differences in numbers of total WBC, neutrophils and lymphocytes, expressed relative to 0 hr concentrations, were analyzed by one-way analysis of variance (ANOVA) where day of the estrous cycle was the source of variation. In Experiments 2 and 3, plasma progesterone and differences in numbers of total WBC, neutrophils and lymphocytes, expressed relative to 0 hr concentrations, were analyzed by repeated measures ANOVA. For Experiments 2 and 3, sources of variation in the ANOVA were treatment (Lutalyse or Saline and Lutalyse, Saline or Estrumate, respectively), time and the treatment x time interaction. In Experiment 4, differences in numbers of bacteria, neutrophils, endometrial epithelial cells and lymphocytes and plasma progesterone concentrations between the 0 and 24 hr sampling times were analyzed by ANOVA for a 2 x 2 factorial design. Sources of variation in the ANOVA included treatment (Lutalyse or Saline), interval (Days 0 and 14 or Days 14 and 28) and the treatment x interval interaction. In Experiment 5, differences between days open and services to conception were analyzed by ANOVA for a 2 x 2 factorial design where sources of variation included treatment (Lutalyse or Saline), interval (Days 0 and 14 or Days 14 and 28) and the treatment x interval interaction. If significant effects were observed in the ANOVA, differences between means were evaluated by Fisher’s least significant differences procedures. All
analyses were performed using the NCSS statistical software program (Number Cruncher Statistical System; 2000, Jerry Hintze, Kaysville, UT).
Results

Experiment 1: Changes in circulating white blood cell populations during the estrous cycle

Two cows were omitted from this experiment because progesterone analysis revealed aberrant estrous cycles hence data were recovered from five cows. Numbers of total WBC and lymphocytes did not differ (P>0.10) by the day of the estrous cycle (Figure 1). Numbers of neutrophils were similar (P>0.10) on Days 0, 7 and 21 of the estrous cycle, however, a significant increase was observed on Day 14 (mid-cycle) when the corpus luteum was dominant.

Experiment 2: Changes in circulating white blood cell populations following injection of Lutalyse or saline

Progesterone analysis confirmed all cows were in the luteal phase of their estrous cycles and no differences (P>0.10) in plasma progesterone concentrations were observed between the two treatments at the time of injection. Compared to Saline cows, numbers of total WBC in cows injected with Lutalyse were similar (P>0.10) at 1 hr but greater (P<0.05) at 4 and 8 hr post-injection (Figure 2). Neutrophils followed a similar pattern as total WBC where numbers were similar (P>0.10) at 1 hr and greater (P<0.05) at 4 and 8 hr in Lutalyse compared to Saline cows (Figure 3). Lutalyse injection exerted no effect (P>0.10) on lymphocyte populations and numbers were similar (P>0.10) to Saline cows within the 8-hr sampling period (Figure 4).
Numbers of total WBC, neutrophils and lymphocytes did not differ (P>0.10) over the sampling period in Saline cows.
Figure 1. Changes in total white blood cell (WBC), neutrophil and lymphocyte populations over the estrous cycle of the cow.

a,b Means without common superscripts on a specific day of the estrous cycle differ (P<0.05).
Figure 2. Changes in circulating total white blood cells (WBC) after injection of Lutalyse or saline.

\[ \text{a,b} \text{ Means without similar superscripts at a specific hr post-injection differ (P<0.05).} \]
Figure 3. Changes in circulating neutrophils after injection of Lutalyse or saline.

a,b Means without similar superscripts at a specific hr post-injection differ (P<0.05).
Figure 4. Changes in circulating lymphocytes after injection of Lutalyse or saline.
Experiment 3: Changes in circulating white blood cell populations following injection of Lutalyse, saline or a PGF\(_{2\alpha}\) analog

Progesterone analysis confirmed cows were in the luteal phase of their estrous cycles however differences (P<0.05) in plasma progesterone concentrations were observed among the three treatments at the time of injection (Lutalyse < Estrumate < Saline; 2.1, 4.8 and 8.5 ng/ml, respectively; pooled SE = 0.5 ng/ml).

Total WBC in cows injected with Lutalyse increased (P<0.05) compared to Saline cows at 2 and 4 hr then returned to near 0-hr values at 8 and 16 hr post-injection (Figure 5). Interestingly, despite the changes in total WBC observed with Lutalyse, no differences (P>0.10) in total WBC were observed in cows injected with Estrumate, the PGF\(_{2\alpha}\) analog, compared to Saline cows. No differences (P>0.10) in total WBC were observed in Saline cows over the 16-hr sampling period.

Numbers of neutrophils observed over the 16-hr sampling period did not differ (P>0.10) between cows injected with Lutalyse, Saline or Estrumate (Figure 6). Although more neutrophils were observed in Lutalyse-treated cows, no significant differences were detected. Neutrophil counts in this experiment were fraught with significant variation between animals, as evidenced by the large standard error bars, which no doubt contributed to the lack of statistical significance in the analysis.

Numbers of lymphocytes were similar (P>0.10) between Lutalyse, Saline and Estrumate cows during the first 8 hr of sampling (Figure 7). However, both prostaglandins increased lymphocyte numbers at 16 hr post-injection with Lutalyse inducing a significantly greater response compared to Saline.
Figure 5- Changes in circulating total white blood cells (WBC) after injection of Lutalyse, saline or Estrumate.

a,b Means without similar superscripts at a specific hr post-injection differ (P<0.05).
Figure 6. Changes in circulating neutrophils after injection of Lutalyse, saline or Estrumate.
Figure 7. Changes in circulating lymphocytes after injection of Lutalyse, saline or Estrumate.

\[^{a,b}\] Means without similar superscripts at a specific hr post-injection differ (P<0.05).
Experiment 4: Effects of Lutalyse treatment on uterine neutrophil and bacterial populations in postpartum dairy cows

Because of difficulty encountered in obtaining uterine samples using the bacterial swab or the Cytobrush, data were recovered from a total of 24 cows [Treatment (n): Lutalyse 0-14 (6), Lutalyse 14-28 (7), Saline 0-14 (6) and Saline 14-28 (5)]. Lutalyse treatment increased (P< 0.05) the number of uterine neutrophils between the 0 and 24 hr samplings compared to cows injected with saline (23.4 ± 5.5 vs. -2.5 ± 9.7 cells, respectively). Interval and the treatment x interval interaction were not significant sources of variation in number of neutrophils (Figure 8). Lutalyse treatment decreased (P= 0.06) the number of endometrial epithelial cells between the 0 and 24 hr samplings compared to cows injected with saline (-26.4 ± 6.2 vs. -2.2 ± 11.3 cells, respectively). Interval and the treatment x interval interaction were not significant sources of variation in number of endometrial epithelial cells (Figure 9). Greater numbers of uterine lymphocytes were associated with cows injected with saline and the 14-28 day interval however treatment, interval and the treatment x interval interaction were not significant sources of variation (Figure 10).

Lutalyse treatment decreased (P< 0.05) total bacteria present in the uterus compared to cows injected with saline (-1300 ± 843 vs. 1127 ± 526 cells, respectively). Interval and the treatment x interval interaction were not significant sources of variation for total uterine bacteria (Figure 11). Bacterial colony growth on MacConkey’s agar, presumably *E. coli*, was infrequent in the cows sampled hence no analyses were conducted.
Cows injected with Lutalyse experienced reduced plasma progesterone concentrations compared to cows injected with saline (-2.0 ± 1.1 vs. 0.2 ± 0.4 ng/ml, respectively) however no significant differences were observed. Plasma progesterone concentration decreased (P<0.05) in cows injected during the 14-28 compared to the 0-14 interval (-2.1 ± 1.1 vs. 0.4 ± 0.1 ng/ml, respectively) and the treatment x interval interaction was a significant source of variation (Figure 13).
Figure 8. Changes in uterine neutrophils in cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.
Figure 9. Changes in endometrial epithelial cells in cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.
Figure 10. Changes in uterine lymphocytes in cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.
Figure 11. Changes in total uterine bacteria in cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.
Figure 12. Changes in plasma progesterone in cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.

a,b Means without similar superscripts differ (P<0.05).
Experiment 5: Effects of Lutalyse treatment on reproductive performance in postpartum dairy cows

Of the 64 cows initially committed to this experiment, seven were not pregnant at the conclusion of the study hence data were recovered from 57 cows. Lutalyse treatment reduced (P<0.05) the number of days open compared to saline (Figure 13). Interval (127.3 ± 11.5 vs. 146.6 ± 11.8 days for 0-14 vs. 14-28 days postpartum, respectively) and the treatment x interval interaction were not significant factors in reducing days open (Table 1). Likewise, Lutalyse treatment reduced (P<0.10) the number of services per conception compared to saline (Figure 14). Interval (2.3 ± 0.3 vs. 3.0 ± 0.3 services for 0-14 vs. 14-28 days postpartum, respectively) and the treatment x interval interaction were not significant sources of variation in services per conception (Table 1).
Figure 13. Number of days open for cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.

\[\text{Days open} \]

\[\text{Lutalyse} \quad \text{Saline} \]

\[\text{Treatment} \]

\[a, b\] Means without similar superscripts differ (P<0.05).
Table 1. Number of days open and services per conception for dairy cows injected with Lutalyse or Saline Days 0-14 or 14-28 postpartum

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days open (n)</th>
<th>Services per conception (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-14</td>
<td>14-28</td>
</tr>
<tr>
<td>Lutalyse</td>
<td>102.6± 8.3 (12)</td>
<td>132.4 ± 11.3 (17)</td>
</tr>
<tr>
<td>Saline</td>
<td>145.8 ±18.0 (16)</td>
<td>166.6 ± 23 (12)</td>
</tr>
</tbody>
</table>
Figure 14. Number of services per conception for cows injected with Lutalyse or saline Days 0-14 or 14-28 postpartum.

\(^a,b\) Means without similar superscripts differ (P<0.10).
Discussion

Postpartum dairy cows are highly susceptible to uterine infections because of bacterial contamination and a massive flux of hormones. The annual incidence of uterine infections in dairy cattle ranges from 10-50%. In cows where bacterial infections persist, sub-clinical endometritis can affect uterine health, conception rates and ovarian, hypothalamic and pituitary function. Neutrophils play an important role in clearing uterine infections and it is known PGF$_{2\alpha}$ can enhance neutrophil function. How PGF$_{2\alpha}$ affects the immune system is not completely understood and although there is a great deal of speculation as to the reasons for the effectiveness of exogenous PGF$_{2\alpha}$ as a treatment for uterine infections, the mechanism of action is still unclear.

The objectives of this study were to examine the interaction between reproductive hormones, mainly PGF$_{2\alpha}$, neutrophils and uterine health in dairy cows by evaluating changes in circulating white blood cell populations during the estrous cycle and after PGF$_{2\alpha}$ administration. A two-injection PGF$_{2\alpha}$ regimen was also evaluated to determine the efficiency of such a treatment on uterine neutrophil and bacterial populations and reproductive performance in a commercial dairy herd.

In Experiment 1, neutrophil numbers increased during diestrus (mid-cycle) when progesterone was high. The results of the current study are in agreement with Subandrio and Noakes (1997), who reported an increase in the number of circulating neutrophils during diestrus compared to estrus. Subandrio et al. (2000) also observed that the concentration of peripheral WBC was not influenced by the reproductive status of the cow. However, the authors reported a significant difference in mean
neutrophil concentrations between reproductive states and between individual cows. Little difference in the function of the peripheral and uterine neutrophils was observed, but there was high variation in all aspects of neutrophil function between reproductive states and individual cows (Subandrio et al., 2000). High variation in circulating numbers of WBC among cows was also observed in the present study. These results disagree with the long-accepted view that the uterus of the cow is more susceptible to infection during the luteal phase than at estrus (Lewis, 2003). The reason for this discrepancy may be due to the suppression of other uterine defense mechanisms present at diestrus by progesterone, and therefore the neutrophil response is compensatory (Dhaliwal et al., 2001). Alternatively, other neutrophil functions, e.g., phagocytosis and killing of bacteria, are depressed by progesterone as reported in an in vitro study by Roth et al. (1983) using neutrophils obtained from peripheral blood. Apoptotic gene expression may also be stimulated in neutrophils by progesterone.

Neutrophils express two main isoforms of glucocorticoid receptors (GR), GR\(\alpha\) and GR\(\beta\), and progesterone acts as a GR\(\alpha\) antagonist (Burton et al., 2005).

In Experiment 2, a significant increase in circulating neutrophils was observed 4 hr after injection of PGF\(_{2\alpha}\). Because PGF\(_{2\alpha}\) is a pro-inflammatory molecule and has been shown to enhance neutrophil chemotaxis, it may be inducing release of neutrophils from the bone marrow (Hoedemaker et al., 1992; Azawi, 2008). In Experiment 3, a significant increase in circulating total WBC was observed 2 and 4 hr after injection of Lutalyse, similar to the results of Experiment 2. However, unlike Experiment 2, no significant differences in neutrophil populations were observed.
between cows receiving saline or either of the PGF$_{2\alpha}$ drugs. Close inspection of the data does show greater numbers of neutrophils 2 and 4 hr after injection of Lutalyse compared to saline, but again, no significant differences were detected. Considerable variation was observed in neutrophil populations in the cows sampled in Experiment 3 which contributed to the lack of statistical significance. A factor which may have contributed to this variability was the plasma progesterone status of the cows comprising the three treatments. Unlike the cows in Experiment 2, progesterone concentrations significantly differed between the three treatments at the time of injection. This difference may have affected neutrophil numbers similar to the effect seen in Experiment 1 where more circulating neutrophils were associated with higher plasma progesterone. Why Estrumate, a PGF$_{2\alpha}$ agonist, did not mirror the effects of Lutalyse more closely is difficult to explain. Equivalent luteolytic doses of the two drugs were administered however it is possible the aromatic ring on C-16 of Estrumate interfered with the immune system responses observed with Lutalyse. The increase in lymphocytes 16-hr post-injection in PGF$_{2\alpha}$–treated cows was novel and may suggest a delayed response of this cell-type to the hormone.

Prostaglandin F$_{2\alpha}$ treatment increased uterine neutrophils and decreased total uterine bacteria 24 hr after injection in Experiment 4. The reduction in bacteria 24 hr after Lutalyse injection corresponds well with the increase in uterine neutrophils. Bonnet et al. (1990) observed cows were less likely to have *A. pyogenes* present on the day of sampling following PGF$_{2\alpha}$ treatment 14 days earlier compared to control cows. Chemotaxis toward the reproductive organs by neutrophils is due to bacteria or
trauma, and administering exogenous PGF$_{2\alpha}$ may induce greater neutrophil release from the bone marrow, thereby making more neutrophils available to enter the uterus.

Plasma progesterone concentrations were higher in cows receiving treatments at Days 14-28 compared to Days 0-14 postpartum in Experiment 4 suggesting many of the cows had resumed ovarian cyclicity despite incomplete uterine involution. Many consider the postpartum period in dairy cows to be 6 weeks in duration, while others consider it to be until the cow resumes ovarian cyclicity, despite incomplete uterine involution (Youngquist and Threlfall, 2006). Resumption of cyclicity may have a greater negative impact on uterine health because in cows with uterine infections the presence of a corpus luteum resulting from an early postpartum ovulation can lead to pyometra (Sheldon et al., 2008). Exogenous PGF$_{2\alpha}$ at this time would have a dual effect on uterine health by stimulating the immune system, maybe directly, and reducing the suppressive effect of endogenous progesterone through its luteolytic action on an early postpartum corpus luteum. Furthermore, administration of exogenous PGF$_{2\alpha}$ is followed by an increase in endogenous uterine PGF$_{2\alpha}$ production thereby prolonging the beneficial effects of PGF$_{2\alpha}$ on the immune system (Azawi, 2008).

The beneficial effects of PGF$_{2\alpha}$ were also observed on reproductive performance of cows receiving treatment at the commercial dairy in Experiment 5. Cows receiving the two injection Lutalyse protocol had reduced days open and number of services per conception compared to saline injection. Salasel and Mokhrari (2011) reported similar results where cows treated with two injections of PGF$_{2\alpha}$ 8 h
apart on Day 20 postpartum experienced increases in first service conception rates and
decreases in services per conception and days open.

Results from the current experiments suggest PGF$_{2\alpha}$ exerts direct effects on
neutrophil proliferation. This mechanism may be how PGF$_{2\alpha}$ functions to clear uterine
infections. Considerable speculation exists as to how PGF$_{2\alpha}$ works to clear uterine
infections. One popular notion is that PGF$_{2\alpha}$ exerts a purgative effect on the uterus by
causing uterine contractions. However, in an equine model, PGF$_{2\alpha}$-stimulated
contractions reduced the volume of uterine fluid present during an infection without
eliminating the bacteria (Nikolakopoulos and Watson, 1999). It has also been
hypothesized that PGF$_{2\alpha}$ resolves uterine infections through its luteolytic activity
because it causes regression of the corpus luteum, thereby reducing circulating
progesterone concentrations. However, PGF$_{2\alpha}$ administration resulted in clearance of
bacterial infections even when circulating progesterone concentrations were
maintained at luteal phase or greater concentrations (Del Vecchio et al., 1994; Lewis,
2003). Lewis (2003) established uterine infections in ewes by bacterial infusions and
demonstrated resolution of the infections when PGF$_{2\alpha}$ was administered during luteal
phase plasma concentrations of progesterone. Hence, the ability of PGF$_{2\alpha}$ to enhance
immune function is not solely due to removing the source of progesterone. Lastly,
Dhaliwal et al. (2001) have suggested PGF$_{2\alpha}$ may have a stimulatory effect on
phagocytic activity of uterine PMN.

Significant research effort has been directed towards studying the pathogenic
microorganisms causing uterine infections and the effects these microorganisms have
on impairment of fertility in cattle. However, despite this research, preventative practices and new treatment strategies to alleviate the problem have not been developed, and the incidence of uterine infection has not changed significantly in cattle over the previous 30 years. New knowledge in this area is a necessary first step towards development of new and practical methods for preventing uterine infections and novel therapeutic regimes not requiring antibiotics for treatment of uterine infections in cattle. The reduction in days open was 35 days which provides an estimated savings of approximately $75 per cow. The reduction in services per conception was about one, which can amount to approximately $10-$20 in savings on semen costs and labor for breeding and heat detection. The two Lutalyse injection protocol would definitely pay for itself if a single dose of Lutalyse is under $3/dose. Lutalyse is commonly used in dairy operations to synchronize estrous cycles for artificial insemination hence this regimen can be readily implemented with relatively little cost and effort. Treating cows with Lutalyse early in the postpartum period could provide an inexpensive method not requiring antibiotics for preventing uterine infections in cattle.
Bibliography


