AN ABSTRACT OF THE THESIS OF

<u>Kathryn Genevieve Younger</u> for the degree of <u>Master of Science</u> in <u>Animal Science</u> presented on <u>March 17, 2016</u>.

Title: <u>The Effects of Prostaglandin F$_{2\alpha}$ on Reproductive Performance in the Postpartum Dairy Cow and the Mechanism Behind its Benefits</u>

Abstract approved:

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Alfred R. Menino, Jr

During the beginning of the postpartum period, 80 to 100% of lactating dairy cows have bacterial contamination within their uteri. Presence of uterine bacteria may contribute to increased number of days open and services per conception within these cows. Expulsion of uterine contamination is crucial for uterine involution and reproductive health. Susceptibility to bacterial infection within the uterus can be attributed to a decrease in neutrophil function during the first two weeks of the postpartum period. Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) has been found to induce uterine contractility, increase neutrophil propagation and affect the uterine environment. The objective of this study was to determine the effects of a single injection of prostaglandin F$_{2\alpha}$ (Lutalyse, Pfizer Animal Health, New York, NY) on uterine health and reproductive performance in dairy cows. Holstein cows from a commercial dairy were randomly assigned to receive an intramuscular injection of either Lutalyse or saline on Day 7 or 14 postpartum (Day 0= day of parturition). Services per conception and days open were

evaluated to determine the effects on reproductive performance due to treatment. Cytobrush and uterine swabs were performed on a subset of Day 14 postpartum cows at 0 and 24 h after injection to evaluate changes in uterine neutrophil and bacterial populations. The pro-inflammatory cytokines known to induce mobilization of neutrophils, interleukin-1β, interleukin-6, and tumor necrosis factor-α were also measured at 0, 3, 6 and 12 h after injection of Lutalyse or saline on Day 14 postpartum to determine if $PGF_{2\alpha}$ induced an increase in cytokines similar to initial pathogen detection. No significant differences were seen in days open, services per conception, interleukin-1β or tumor necrosis factor-α concentrations. Interleukin-6 concentration was greater in saline cows at 12 h compared to Lutalyse treated cows at 3, 6 and 12 h after injection. Additionally, an increase in uterine neutrophils (P=0.05) and a decrease in bacteria (P=0.05) in response to Lutalyse were observed. Although $PGF_{2\alpha}$ has been shown to have beneficial effects on the postpartum uterus, a single injection protocol may simply not be adequate to induce significant improvement in reproductive performance.

The Effects of Prostaglandin F$_{2\alpha}$ on Reproductive Performance in the Postpartum Dairy

Cow and the Mechanism Behind its Benefits

by
Kathryn Genevieve Younger

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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 thesis to any reader upon request.

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Kathryn Genevieve Younger, Author

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Introduction

Reproductive health is critical to the dairy industry. Dairy production is dependent on lactation occurring in each dairy cow within a herd. However this can only take place after a successful pregnancy and subsequent calving. Therefore, a crucial component of the reproductive lifespan of a dairy cow is the postpartum interval. In this regard, there are many factors that can delay the time and number of services it takes for a cow to conceive. Postponement of rebreeding has financial ramifications, and uterine infection is one of the most expensive impediments of conception for dairy cattle. Though there is pervasive bacterial contamination in the uterus after calving, in 10-17% of cows this contamination becomes a uterine infection that can be detected through physical examination (Sheldon at el., 2006). Despite uterine infections being a relatively common occurrence in the dairy herds, the scope of the problem is larger due to lack of outward signs of infection.

Problems after calving can result in antibiotic use to combat illness. However with most antibiotics available today, this incurs financial loss due to the inability to sell the milk during the milk withdrawal period. If postpartum uterine disease persists despite treatment, or uterine damage occurs that prevents a cow from being rebreed, then the cow is culled, resulting in further financial loss. Furthermore, days open beyond the typical voluntary waiting period can cost a producer $0.42 to $4.95 per day per cow (French and Nobel, 2003).

However severe uterine infection, or metritis, has much farther reaching consequences than simply increasing time before rebreeding. Cows diagnosed with metritis produce 15.1 pounds of milk less per day for their first 30 days of lactation than healthy cows (Overton and Fetrow, 2008). Therefore, on average, $60 is lost per cow with metritis. Due to ailment and decreased milk production, cows with metritis are also at an increased risk of culling during the first 60 days postpartum. This can lead to a loss of $75 per case of metritis (Overton and Fetrow, 2008). Metritis also decreases reproductive efficiency. Only 59% of cows with metritis are able to conceive and maintain the pregnancy for the entire lactation. Treatment cost for this severe of an infection varies depending on the drug utilization and milk withdrawal periods, but can range from $50 to $94 per case (Overton and Fetrow, 2008). With these individual costs of metritis added, the loss approaches $300 per diagnosed case. For a dairy with 2000 milking cows and a 30% incidence rate of metritis, the cost could surpass $180,000 per year (Overton and Fetrow, 2008). Therefore it is essential to reduce factors that negatively impact reproductive health, resulting in an extended postpartum interval and number of days open.

In an attempt to reduce the rate of uterine infection, postpartum injections of prostaglandin $F_{2\alpha}$ have been shown to enhance myometrial contractility which aids in clearing lochia after calving (Nanda et al., 2003). Such treatment decreases the time for uterine involution and induces earlier resumption of postpartum cyclicity, improving reproductive performance (Nanda et al., 2003). It is noteworthy that Lulay et al. (2012)

reported enhanced uterine neutrophil populations within the uterus, resulting in a reduction of uterine bacterial contamination 24 h after $PGF_{2\alpha}$ injection. However, the mechanism by which $PGF_{2\alpha}$ induces neutrophil propagation resulting in an improved uterine environment is still unclear.

Therefore the objective of this research was to investigate the link between $PGF_{2\alpha}$, uterine neutrophils and uterine health in the postpartum dairy cow. Specifically, short-term uterine conditions and long-term animal reproductive performance were measured after a single injection of $PGF_{2\alpha}$.

Literature Review

Estrous Cycle

The bovine estrous cycle is a period of reproductive cyclicity that occurs after puberty. The estrous cycle can be divided into two major phases, namely the follicular and luteal phases. The follicular phase spans from regression of the corpus luteum to ovulation. The primary hormone produced during this phase is estradiol because the dominant ovarian structure during this time is the follicle. Granulosa cells within the follicle produce estrogen. The luteal phase spans from ovulation to when the corpus luteum begins to regress. The dominant ovarian structure is the corpus luteum which produces progesterone (Senger, 2012).

The estrous cycle can be further subdivided into four stages. Proestrus and estrus occur during the follicular phase. In contrast, metestrus and diestrus occur during the luteal phase. Proestrus is characterized by the change in endocrine profile from progesterone to estradiol. The change in hormone production is due to an elevation in plasma follicle stimulating hormone (FSH) and luteinizing hormone (LH). During this time, antral follicles develop and become ready for ovulation. Estrus occurs when peak estradiol levels are reached. Physical signs of estrus in the dairy cow are sexual receptivity, including standing to be mounted. Ovulation occurs during metestrus in cattle. Metestrus is the period of time between the end of sexual receptivity and development of the corpus luteum. During this time estradiol and progesterone concentrations are relatively low, as neither hormone-producing structure is fully

operative. Luteinization of the cells in the ovulated follicle enables the formation of the corpus luteum. Diestrus is the phase when the corpus luteum is completely functional and produces high levels of progesterone. This is the longest stage of the estrous cycle. The end of diestrus is marked by luteolysis by uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Senger, 2012).

Prostaglandin $F_{2\alpha}$ Properties

$PGF_{2\alpha}$ is a lipid compound with hormone-like activity produced by the uterus. This substance is luteolytic in ruminants. Secretion of $PGF_{2\alpha}$ from the endometrium is stimulated by oxytocin production from the corpus luteum, which in turn stimulates further oxytocin production, thereby forming a positive feedback loop. This feedback mechanism continues until luteolysis is complete. This results in decreased progesterone concentration from Day 16 of the estrous cycle until the next estrus (Senger, 2012). Additionally, $PGF_{2\alpha}$ has been found to induce myometrial contractility, proving to be useful in facilitating expulsion of uterine bacteria (Nanda et al., 2003, Lulay, 2011). $PGF_{2\alpha}$ is also pro-inflammatory, and could possibly stimulate production of other pro-inflammatory cytokines. It has also been found to stimulate neutrophil function such as phagocytosis through enhancing uterine production of leukotriene B (Lewis, 2004).

Placentation

The dairy cow has a cotyledonary placenta. Cattle have between 70 and 120 cotyledons, which are of trophoblastic origin and contain blood vessels as well as

connective tissue (Senger, 2012). Cotyledons attach to the endometrium through maternal caruncles. Around Day 25 of gestation, cotyledons on the fetal chorion initiate attachment to the maternal caruncles which enables placentomes to form. Cotyledonary villi project into the crypts of the caruncles forming a specialized form of attachment. Both portions of the placentome grow during gestation, reaching up to 6 cm in diameter by parturition. This growth enables enhanced exchange of nutrients, oxygen and metabolic wastes as the fetus develops (Senger, 2012).

The cow's placenta can also be distinguished by the layers that separate fetal from maternal blood. Cows possess a synepithelochorial placenta (Senger, 2012). This is a less penetrating type of placenta in which there is epithelium in both maternal and fetal components (Senger, 2012). The bovine synepithelochorial placenta is unique because the maternal epithelium layer erodes and regrows intermittently. This causes cycles of exposure of the endometrial capillaries to the chorionic epithelium. Another distinctive characteristic of the bovine placenta is the development of binucleate giant cells. These are large cells containing two nuclei (Senger, 2012). These cells originate from trophoblast cells and appear in the cow at Day 18 of gestation (Senger, 2012). During gestation these cells migrate from the chorion to the maternal epithelium. Binucleate giant cells produce progesterone and estradiol which are important for gestation and pregnancy specific protein B (PSPB), which is used to detect pregnancy in cattle (Senger, 2012).

Postpartum Period

The postpartum period of the dairy cow occurs after parturition up to resumed reproductive function. There are four major events during this period which include: myometrial contractions and expulsion of lochia, endometrial repair, resumed ovarian function and elimination of bacterial contamination in the reproductive tract (Senger, 2012). In the dairy industry, a short postpartum interval is important in order to ensure maximum milk yields. Myometrial contractions are important to aid in the expulsion of fluids and debris from the uterus as well as to decrease time for uterine involution. Suckling by a calf signals the myometrium to contract through oxytocin, however in dairy cows, the calf is taken away from the dam within one day. Without frequent suckling and only being milked two to three times a day, the beneficial postpartum myometrial contractions are dramatically decreased. This can result in an increase in time for uterine involution (Senger, 2012).

The discharge that is expelled from the vulva after parturition is called lochia. This is a fluid that contains remnants of fetal membranes as well as endometrial tissue. Lochia can be seen being expelled from the dairy cow between Days 2 and 9 postpartum. This is due to the very important event of sloughing of caruncles which leaves openings of exposed vasculature, liberating blood. Separation of maternal caruncles from fetal cotyledons occurs 8-12 h after delivery (Senger, 2012). Vasoconstriction occurs around the stalk of the caruncle which leads to necrosis of the

caruncle and its subsequent sloughing. When the necrotic tissue becomes detached, the caruncle is able to heal and will regrow endometrial epithelium (Senger, 2012). This is important for future conception. Lochia production decreases between Days 14 to 18 postpartum in most dairy cows (Senger, 2012).

As mentioned, the type of placentation cattle possess is cotyledonary in which caruncles attach to codelydons to form placentomes. Though this is one of the least invasive types of placentation, uterine involution still occurs over a 40-day period. Uterine involution consists of the reduction in uterine size, clearing bacterial contamination, sloughing of caruncles and regeneration of the endometrium (Sheldon et al., 2008). Resumption of cyclicity also occurs during this interval, with the first ovulation occurring around Days 21 to 30 postpartum (Kaminmura et al., 1993). However, this interval can be affected by nutrition, body condition, parity and can be extended due to uterine disease (Sheldon et al., 2002). For this reason dairy producers practice a voluntary waiting period of typically 60 d before rebreeding.

Resumption of Estrous Cyclicity

After parturition, an important event of the reproductive life of the dairy cow is the resumption of estrous cyclicity. However the timing of this occurrence is crucial for healthy reproductive performance. Generally, early resumption of cyclicity has been deemed positive and beneficial for reproductive health and successive fertility. Though this may generally be advantageous, returning to cyclicity too early in the postpartum period can be detrimental. Pyometra can occur if a corpus luteum is formed due to

ovulation early in the postpartum period when a uterine infection is present (Sheldon et al., 2008). The negative effects of the corpus luteum on the early postpartum uterus can be attributed to progesterone. Progesterone induces relaxation of the myometrium which can be deemed immunosuppressive, and can inhibit clearing of bacterial contamination of the uterus (Bonafos et al., 1995). Additionally, elevated concentrations of plasma progesterone inhibit phagocytic activity of both uterine and peripheral blood neutrophils (Dhaliwal et al., 2001).

At around Days 6-8 postpartum, the first follicular wave occurs, with one follicle reaching dominant follicle status by Days 10-12 postpartum (Sheldon, 2004). What becomes of this follicle determines if the cow resumes cyclity. There are three possible outcomes for this dominant follicle. First, the follicle can ovulate, resulting in the formation of a corpus luteum and therefore resumption of estrous cyclicity. Second, the follicle can become atretic, regressing with other follicles rather than ovulating. Lastly, this follicle can become cystic (Sheldon et al., 2008). Generally, a positive energy balance must be reached in a cow in order to increase the otherwise reduced ovulatory competence of postpartum early follicles. This can be more difficult for cows selected for high milk production, increasing their incidence rates of postpartum anestrus to over 20% (Sheldon, 2004).

Resumption of ovarian cyclicity can also be hindered by uterine infection, which can occur do to the ubiquitous bacterial contamination during the postpartum period. This occurs because uterine disease can prevent follicular growth and subsequent

ovulation, leading to an increased postpartum anovulatory period. The bacterial by-products such as lipopolysaccharide can reduce and even inhibit the LH surge required for ovulation (Sheldon et al., 2008). Furthermore, a high amount of uterine bacteria on Day 7 postpartum induced smaller first dominant follicles and subsequently lower plasma estradiol concentrations (Sheldon, 2004).

Periparturient Immunosuppression

Periparturient immunosuppression occurs during the peripartum period in the dairy cow (Zoldan et al., 2014). This condition is associated with calving and milk production, where both physiological and metabolic changes negatively affect functional properties of immune cells (Ingvartsen and Moyes, 2013). Calving is a stressful process which results in the production of immunosuppressive glucocorticoids by the adrenal cortex (Coutinho and Chapman, 2011). The suppressive effects of glucocorticoids on the immune system can be attributed to alterations in transcription of leukocyte genes in response to binding of glucocorticoid receptor. Glucocorticoids inhibit many of the initial events that occur during inflammatory response and up-regulate the production of anti-inflammatory proteins. The vasodilation and vascular permeability necessary for immune response are inhibited by glucocorticoids. This reduces neutrophil migration to sites of infection during the postpartum period (Coutinho and Chapman, 2011).

During the early postpartum period not only are higher proportions of apoptotic neutrophils reported, but neutrophil functions such as reactive oxygen species

production, random migration and phagocytosis are also impaired (Zoldan et al., 2014). Neutrophils are part of the innate immune system and the first defense against invading microorganisms. Innate immune cells are the primary cells responsible for elimination of infection-causing bacteria within the postpartum uterus (Singh et al., 2008). Due to this periparturient immunosuppression, a rise in severity of disease, such as mastitis, is observed during this early postpartum stage.

Negative effects on these innate immune cells, which are the first line of defense against invading pathogens can be seen as devastating when one considers the lower number of neutrophils the dairy cow possesses. In regard to cell counts, neutrophils only account for 20-25% of white blood cells (Cunningham and Klein, 2007). This may not seem to be too few, however when compared with other mammals, such as the human, the comparison becomes concerning. In healthy humans 60% of their total white blood cells are neutrophils (Murphy et al., 2008). This allows for more immediate action when any antigen is sensed. During the postpartum period cows with endometritis have been shown to have lower neutrophil counts and furthermore, these neutrophils have a decreased ability to migrate after parturition (Kim et al., 2005). Not only do cows have fewer neutrophils immediately ready during this peripartum period, but the function of these innate cells is diminished.

During the postpartum period, toll-like receptor-4 (TLR-4) expression on neutrophils is down-regulated which may be a critical dysfunctional factor during Days 7-14 postpartum (Cui et al., 2011). TLR-4 is well known to recognize lipopolysaccharide

(LPS), a pathogen associated molecular pattern (PAMP) seen in Gram-negative bacteria. When TLR-4 is activated, a signaling cascade is initiated resulting in the production of pro-inflammatory cytokines and chemokines. This results in the mobilization and activation of other innate immune cells (Murphy et al., 2008). The amount of TLR-4 molecules involved in recognition of LPS is critical in initiating signals leading to activation of the neutrophils and the innate immune response (Triantafilou and Triantafilou, 2005). If TLR-4 production is down-regulated in these already few neutrophils, the ability to identify pathogenic bacteria during the postpartum period would be further decreased.

Neutrophil Origin

Neutrophil progenitors originate from bone marrow, as are all immune cells derived from bone marrow stem cells. Pluripotent hematopoietic stem cells become myeloid progenitors for cells destined for the blood stream. For neutrophils in particular, these cells then become granulocyte progenitors. These granulocytes are so named due to the granules which store substances used to kill pathogens that can be seen within the cell when stained and viewed microscopically. Finally these progenitors differentiate into various types of polymorphonuclear leukocytes, specifically of interest, the neutrophil (Murphy et al., 2008).

Neutrophil Role in the Immunological Response

Neutrophils are ephemeral cells containing granules with immediate anti-microbial effectors. These cells can be found in the blood, but migrate to tissue when an

infection within the body is detected. Granules possessed by the neutrophils have a potent antibacterial agent activated after phagocytosis occurs. The neutrophil is the only myeloid cell that has pre-formed granules for immediate response. This cell has a number of antimicrobial mechanisms including enzymes, such as lysozymes which digest cell walls of some Gram-positive bacteria and acid hydrolases which break down ingested microbes. Neutrophils also contain nitric oxide, superoxide, hydrogen peroxide, hydroxyl radicals and hypohalite. Furthermore, proton pumps in phagosomes lead to acidification and a pH between 3.5-4. When a neutrophil detects a pathogen via various innate immune receptors, a pseudopod is formed, extending the membrane of the cell around the pathogen. The bacteria is then engulfed and brought into the cell within a phagosome. Upon this activation, lysosomes and granules containing enzymes fuse in order to produce superoxide and activate the killing mechanisms against the invading microbes. The phagosome containing the invading bacteria can then fuse to these granules resulting in the death of the microbes (Murphy et al., 2012).

When a pathogen is recognized, innate immune cells are recruited to the site through chemoattractants and adhesion molecules on the endothelia. Chemokines released in response to pathogen invasion cause rapid unfolding of integrins to expose the active binding sites. As neutrophils roll along the vascular endothelia surface, these activated integrins bind intercellular adhesion molecules (ICAMs) causing a tight connection. The neutrophil can then squeeze through the tight junctions of the endothelium using platelet endothelial cell adhesion molecules (PECAMs) found on the

cell surface. This action is referred to as diapedesis. The neutrophil can then migrate through the tissue along the chemotactic gradient to the site of infection, in this case, the endometrium (Murphy et al., 2008).

Macrophages are another leukocyte activated in response to a pathogen, though neutrophils are the cells that initially respond. Macrophages are important for releasing a range of cytokines and initiating tissue inflammation to stop the spread of the unwanted pathogen. These cytokines have a diverse effect on the body, but specifically interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) are of importance. When acting upon the bone marrow, these cytokines induce neutrophil mobilization. Cytokines can also signal for the granulocyte progenitors to differentiate into neutrophils (Murphy et al., 2008).

Uterine Disease

During the first two weeks of the postpartum period, bacterial contamination of the uterus is a natural consequence of parturition (Sheldon et al., 2008). However, the rate at which these contaminations develop into uterine infections has been a source of disagreement in the scientific community. An important factor in this discrepancy is the lack of a uniform definition of uterine infections and the period of time the cow should be monitored (Azawi, 2008). In fact, a majority of uterine diseases remain undiagnosed and untreated because many cows do not show obvious outward signs of infection. Uterine infections can be classified by degree of severity and therefore some are more difficult to identify. Uterine infections are classified as puerperal metritis, clinical

endometritis, subclinical endometritis and pyometra (Sheldon, 2004; Sheldon et al., 2008).

Puerperal metritis

Metritis is a severe inflammatory response that occurs within all the layers of the uterus, including the endometrium, submucosa, myometrium and perimetrium. Puerperal metritis usually occurs within Day 10 postpartum and is defined as an acute systemic illness caused by an infection of the uterus (Sheldon et al., 2006). This type of infection is characterized by a fetid reddish brown liquid uterine discharge and pyrexia (Drillich et al., 2001). Severe cases of puerperal metritis can lead to reduced milk production, elevated heart rate, dehydration and anorexia (Sheldon et al., 2006). An important characteristic of puerperal metritis, as defined by Sheldon et al. (2006), is the addition of a fever greater than 39.5°C within 21 d after calving. Factors increasing the risk of developing puerperal metritis are: dystocia, retained placenta, still birth or calving twins (Drillich et al., 2001). Cows without systemic signs of illness but having delayed uterine involution and a purulent uterine discharge within the vagina within 21 d postpartum are diagnosed as having clinical metritis (Sheldon et al., 2006).

Endometritis

Endometritis is the inflammation of the endometrium that penetrates no deeper than the first layer of the uterus (Youngquist et al., 2006). This can be seen histologically which shows the surface epithelium becomes disturbed, followed by an infiltration of inflammatory cells (Sheldon, 2004; Sheldon et al., 2008). Scarring and blockage of the

fallopian tubes as well as the bursa adhering to the ovary can occur due to chronic

endometritis (Sheldon, 2004). Clinical endometritis occurs at or beyond 21 d

postpartum and is defined by the presence of purulent or a 50/50 mix of pus and mucus

uterine discharge within the vagina, without systemic signs of illness (LeBlanc et al.,

2002). Clinical endometritis can be diagnosed by: visualizing the purulent vaginal mucus,

measuring a cervical diameter greater than 7.5 cm at or beyond 21 d postpartum, or

identifying mucopurulent discharge in the vagina 26 d after calving (Sheldon et al.,

2006). Defining clinical endometritis before 21 d postpartum may misdiagnosing cows as

having an infection, when they simply need more time to spontaneously resolve the

uterine bacterial contamination (Sheldon et al., 2006). Subclinical endometritis is

endometrial inflammation that can also typically be diagnosed through cytology if

purulent discharge is absent in the vagina (Gilbert et al., 1998). At 20-33 d after calving,

this infection can be defined by greater than 18% neutrophils in uterine cytology

samples or greater than 10% neutrophils at 34-47 d (Sheldon et al., 2006).

Pyometra

Pyometra occurs in the presence of a corpus luteum on the ovary and is defined

by the buildup of a purulent or mucopurulent substance within the uterus and

subsequent distention of the uterus (Sheldon et al., 2006). An increased amount of

pathogenic bacteria reside within the uterus when the corpus luteum forms and results

in pyometra (Noakes et al., 1990). Though the cervix is typically occluded during this

time, it may be sufficiently open to allow some pus into the vagina (Sheldon et al.,

2006). This uterine disease can be diagnosed using ultrasonography to find a corpus luteum as well as fluid accumulation with mixed echodensity within the uterine lumen and distension of the uterus (Sheldon et al., 2006).

Effects of Postpartum Uterine Disease

Eighty to 100% of cows will have bacterial contamination within the uterus after parturition. Under homeostatic conditions, most cows may be able to combat this contamination, however due to a period of immunosuppression, around 20% of these contaminations result in infection (Sheldon et al., 2009). Cows with abnormal vaginal discharge have been found to be more likely to have an increased anovulatory period or extended postpartum luteal phases. Additionally, cows diagnosed with endometritis have 20% lower conception rates and the average interval from calving to conception is 30 days longer. This results in a 3% increase in culling rates due to failure to breed back. The decrease in reproductive performance is even seen after cows are successfully treated for endometritis. Subclinical endometritis can persist after outward signs resolve which result in an increase in number of days open and more services per conception (Sheldon et al., 2009).

The monetary effect of endometritis and other uterine disease take multiple aspects of the production life of the dairy cow into account. Reduced fertility can increase the amount of hormones used for synchronization as well as multiple doses of semen due to increased services per conception. Milk production will be reduced due to the disease as well. Furthermore, treatments not only have a cost individually, but can

further reduce the amount of saleable milk due to milk-withdrawal periods. Culling rates can also be increased when the cow fails to conceive. This results in the need to replace that cow with a heifer which is an additional cost. With these aspects taken into account, and considering an incidence rate of 20% for metritis, the annual cost would be $650 million for the 8,495,000 dairy cows in the United States (Sheldon et al., 2009).

Pathogenic Bacteria Associated with Uterine Disease

After parturition the uterine lumen is almost always contaminated with bacteria. However, whether the cow succumbs to this bacteria with a clinical disease, depends on an increase in risk factors. Such risk factors resulting in an increase in chance of clinical disease are: retained placenta, dystocia, twins and stillbirth calves (Kim et al., 2003; Grohn et al., 2000). However these issues are not ones that can be easily intervened in order to reduce the risk of disease. If the factors do align to produce an environment in which the immune system cannot combat the already present pathogens, there are some pathogens that most commonly cause postpartum disease. *Escherichia coli (E. coli)* and *Arcanobacterium pyogenes (A. pyogenes)* are the most abundant bacteria found within the uterine lumen of a cow ailed with uterine disease (Sheldon, et al. 2009). Uterine infections with *E. coli* allow for successive infection with other pathogens (Williams et al., 2007; Donofrio et al., 2008). Infection with *E. coli* also produces negative effects on the ovary, the hypothalamic-pituitary axis and systemic health (Williams et al., 2007). Cows with *E.coli* infections after calving have been found to have more LPS, acute phase proteins and prostaglandin E_2 (PGE) within the peripheral circulation

(Sheldon et al., 2009). LPS is a PAMP that is produced by *E. coli*. An association between

uterine infections and PGE has been supported through in vitro studies showing

treatment of endometrial cells with LPS resulted in an increase in PGE production. The

medium in which the endometrial cells were cultured accumulated more PGE than

$PGF_{2\alpha}$. Treatment of endometrial cells with oxytocin showed the reverse effect,

increasing $PGF_{2\alpha}$ production. Interestingly, the switch in production of prostaglandin

type was attributed to an increase in phospholipase A2 B6 (PLA2B6) protein in the

epithelial cells and not changes in levels of prostaglandin E synthase or prostaglandin F

synthase. PGE is luteotropic rather than luteolytic as $PGF_{2\alpha}$ and explains the extended

luteal periods associated with uterine infection in cattle (Sheldon et al., 2009).

Additionally, it is believed PGE and LPS may stimulate viral replication in macrophages

persistently infected with bovine herpesvirus 4. Bovine herpesvirus 4 can subsequently

infect the endometrial stromal and epithelial cells. This results in further tissue damage

and infertility (Sheldon et al., 2009).

A. pyogenes is another opportunistic pathogen that can infect the uterus after

E.coli infection. *A. pyogenes* causes concerning effects on the postpartum uterus as well.

A. pyogenes causes the most severe endometrial lesion, which are due to the virulence

gene Plo produced by the bacteria. This gene encodes a cholesterol-dependent

cytotoxin called pyolysin, which is attracted to cholesterol-rich domains in the

endometrial cell membranes. Pyolysins aggregate at the cell membranes forming a pore,

killing endometrial epithelia and stromal cells in vitro (Miller, 2009). When *A. pyogenes*

was found in uterine fluids 21 d after calving, cows developed severe endometritis and were usually found to be infertile at first breeding (Azawi, 2008). *A. pyogenes* has shown a high resistance to many treatments including amoxicillin, chloramphenicol, penicillin and oxytetracyline (Santos et al., 2010). Factors mentioned above, such as retained placenta, can provide a suitable environment for these pathogens. The necrotic lochia created by the retained placenta allows for an ideal medium for bacterial growth, and any damage done to the endometrium can also allow for adhesion and subsequent invasion of these pathogens (Sheldon et al., 2009).

Treatment of Uterine Disease

Postpartum uterine disease can be difficult to treat. The success of the treatment is dependent on a number of factors. Lochia that can provide an ideal breeding environment for bacteria, must be eliminated from the uterus (Azawi, 2008). Expulsion of uterine fluid can be achieved by repeated palpations of the uterus, which releases prostaglandins and results in myometrial contractility, or injections of hormones to induce the same contractions (Azawi, 2008). Drugs used must be able to effectively target and eradicate the pathogen (Azawi, 2008). Additionally, the entire endometrium must be targeted by the drug in order to ensure the treatment is adequate (Azawi, 2008). Concentration and amount of administrations of the drug can also affect the success of the treatment. Treatment efficiency is also dependent on the severity of inflammation in the uterus, the time in which the treatment is given during the postpartum period and if a corpus luteum is present (LeBlanc, 2008).

The use of antibiotics as a treatment for uterine disease is controversial due to the concern of multidrug resistance of pathogenic bacteria as well as the debatable efficacy. Intrauterine infusions of antibiotics have been credited in some studies as successfully treating endometritis, however the beneficial effects could have been due to a stimulated immune system because of an irritation to the endometrium (Azawi, 2008). Infusions have been shown to have the possibility of causing endometrial fibrosis (Dhaliwal et al., 2001). Additionally, intrauterine application of Lugol's iodine was found to destroy phagocytic activity of the white blood cells within the uterus for several days (Azawi, 2008). Though a consensus has not been met regarding the effectiveness of treating postpartum uterine infections with antibiotics, the concern of the development of antibiotic resistance in bacteria is universally shared among the scientific community.

Oxytetracycline, ceftiofur, cephapirin , enrofloxacin and cefquiome are drugs that meet the minimum inhibitory concentration as antibiotics for *E. coli, A. pyogenes, Fusobacterium necrophorum and Prevotella melaninogenicus.* Unfortunately, evidence of bacterial resistance to oxytetracycline has been found which diminishes its efficacy as a treatment for endometritis (Sheldon, 2004). Furthermore Kasimanickam et al. (2016) determined that *Trueperella pyogenes* multidrug resistance gene and *E.coli* biofilm virulence factor were present in severe forms of uterine disease in cattle. Specifically, 35% of the *T. pyogenes* isolates were positive for a gene cassette linked to antibiotic resistance and 33% of the *E.coli* isolates were positive for genes for the virulence factor associated with biofilm production. Biofilm exists naturally within the body which is

produced by free-floating bacteria that latch onto a surface area and secrete

extracellular polymeric substances. Typically the biofilm is produced by symbiotic

bacteria protecting the body from potential harm. However, this film can isolate the

bacteria from antibiotics, and if it is disturbed or unbalanced, pathogenic bacteria can

take up residence in biofilm (Kasimanickam et al., 2013). Evidence of antibiotic

resistance raises concern about antibiotic use and shows these bacterial infections may

require a more innovative treatment or, perhaps, prevention.

Prostaglandin $F_{2\alpha}$ Administration and Postpartum Disease Prevention

Though the mechanism in which $PGF_{2\alpha}$ exerts some of its beneficial effects is still

unknown, exogenous $PGF_{2\alpha}$ injections have become an accepted treatment for bovine

endometritis (Dhaliwal et al., 2001; Lewis, 2003). $PGF_{2\alpha}$ has been shown to enhance

neutrophil chemotaxis and phagocytic capacity, which is crucial during the

immunosuppressed early postpartum period, presumably by increasing uterine

leukotriene B_4 (LTB$_4$) production (Lewis 2003). LTB$_4$ stimulates chemotaxis, antibody-

independent cell-mediated cytotoxicity and random migration (Hoedemaker et al.,

1992). These data show $PGF_{2\alpha}$ not only induces propagation of neutrophils but also

enhances their function, thereby increasing the capability to fight off pathogens.

Seals et al. (2002) compared endogenous hormone levels in cows developing

endometritis to healthy cows. The hormone of interest in this study was $PGF_{2\alpha}$ which

was measured through the $PGF_{2\alpha}$ metabolite 12, 14-dihydro-15-keto- $PGF_{2\alpha}$ (PGFM) and

collected by jugular venipuncture. Endometritis was clinically diagnosed at 18 d

postpartum and PGFM was measured from 0 to 35 d postpartum. PGFM concentrations were significantly lower in cows diagnosed with endometritis compared to healthy cows during the time period in which blood was collected, except on Days 15 to 21. This indicated the reduced PGFM concentrations, and therefore endogenous $PGF_{2\alpha}$ production, during the first 14 d postpartum could be a predictor of susceptibility to uterine infection in dairy cows. One may then reason that the first two weeks of the postpartum period is an important interval for prevention of uterine disease. Furthermore, reduced levels of $PGF_{2\alpha}$ culminating to uterine infection could suggest that a treatment or possibly multiple treatments of exogenous $PGF_{2\alpha}$ may be an effective preventative measure to reduce postpartum maladies.

Herath at el. (2013) discovered additional findings supporting the importance of prostaglandins during the postpartum period. Endometrial samples were collected from the uteri of dairy cows, and both anaerobic and aerobic bacteria were cultured. Growth densities were graded based on the number of colony-forming units with 0 representing no colony formation and 4 representing greater than 500 colony-forming units. When cows had an *E.coli* infection and corresponding higher levels of LPS, a higher level of prostaglandin E_2 could be measured systemically. Due to the luteotropic nature of PGE, the study suggested the switch from luteolytic $PGF_{2\alpha}$ could elucidate the mechanism behind the extended luteal phase occurring with postpartum uterine disease and infertility in dairy cattle. Furthermore, this study showed the switch to PGE induced an endocrine environment more likely to sustain a uterine infection. Administration of

exogenous $PGF_{2\alpha}$ would help resolve the uterine infection and consequent negative effects, such as the prolonged luteal phase (Herath et al., 2013).

A study performed on mice showed promising results of using $PGF_{2\alpha}$ to increase neutrophil populations (Ulich et al., 1986). The neutrophil population induced did not contain banded neutrophils, which suggested that while $PGF_{2\alpha}$ mobilized neutrophils from the bone marrow, they were not so heavily elicited as to release an immature subset. This further validated the use of $PGF_{2\alpha}$ as a method for prevention, as the responding neutrophils were of a mature population and were ready to respond to detected pathogens immediately.

As well as decreased neutrophil function, impaired lymphocyte function has also been found during the peripartum period (Galvão et al., 2012). This may be an indicator of a depressed function of monocytes and macrophages during this period. Monocytes and macrophages also play important roles in the elimination of bacteria. These cells are immunomodulators stimulated by contact with bacteria and produce pro-inflammatory cytokines and chemokines (Butterfield et al., 2006). Pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 stimulate neutrophil diapedesis and chemoattraction to the area of the detected pathogen and increase the phagocytic ability of the neutrophils (Butterfield et al., 2006). A reduction in cytokines during the peripartum period points towards reduced monocyte function as well as neutrophil function. Galvão et al. (2012), investigated responsiveness of cytokines in healthy postpartum cows and postpartum cows with uterine infections. Monocytes collected from cows that developed metritis

had elevated gene expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 when unstimulated. TNF-α expression was found to be lower at calving, and Days 7 and 14 postpartum by monocytes isolated from cows with metritis compared to monocytes from healthy cows when the cells were stimulated with *E. coli*. This suggests during the first two weeks of the postpartum period, innate immune cells such as monocytes have reduced function and ability to signal for neutrophil recruitment.

Multiple studies have connected $PGF_{2\alpha}$ treatments to reduced postpartum issues and increased reproductive performance. Of interest, Lulay et al. (2012) conducted research testing the effects of a double injection protocol of $PGF_{2\alpha}$ (Lutalyse, Pfizer Animal Health, New York, NY) on reproductive performance in postpartum dairy cows. Cows were injected at either Day 0 and 14 or Day 14 and 28 postpartum. Lutalyse increased uterine neutrophils and decreased uterine bacteria. The increase in these beneficial immune cells to fight off bacteria that can infect the uterus translated into a decreased number of days open as well as a decrease in services per conception. Though improvements were seen in both intervals of injections within this study, greater improvements were seen in the Day 0 and 14 protocol. This seems to align with numerous studies pointing towards the early postpartum period, particularly the first two weeks, being the ideal time to take preventative measures.

Further research has been conducted to determine the ability and limitations of $PGF_{2\alpha}$ as a postpartum treatment. Sharawy et al. (2015) investigated the effectiveness of a single treatment of synthetic $PGF_{2\alpha}$ on postpartum cows. The first group was treated

with a synthetic $PGF_{2\alpha}$ (Estrumate) immediately after calving, the second group was

treated with GnRH (Receptal) on Days 13-15 postpartum and the third group was

administered synthetic $PGF_{2\alpha}$ (Cloprostenol) immediately after calving and GnRH

(Buserelin) on Days 13-15 postpartum. The fourth group served as the control and did

not receive any treatment. The study found the time required for uterine involution was

shorter in groups receiving synthetic $PGF_{2\alpha}$ as well as $PGF_{2\alpha}$ plus GnRH than the groups

receiving only GnRH or no treatment. Despite these results, it was found that

administration of $PGF_{2\alpha}$ and GnRH did not significantly improve reproductive

performance.

These findings may seem to suggest that a single injection of $PGF_{2\alpha}$ is not

adequate for a preventative postpartum treatment. However Sharawy et al. (2015) used

synthetic $PGF_{2\alpha}$ which has been shown to have reduced effects when compared to

endogenous $PGF_{2\alpha}$. Lulay (2011) found that Lutalyse induced a systemic increase in

white blood cells in cows, while Estrumate, a synthetic analogue of the hormone,

resulted in no difference in neutrophil counts when compared with saline treated cows.

This shows the importance of using endogenous $PGF_{2\alpha}$ rather than an analogue in order

to achieve the full beneficial effects of the hormone. This may be particularly important

when administering a single injection of $PGF_{2\alpha}$ as a preventative postpartum treatment.

The mechanism behind increased neutrophil propagation in response to $PGF_{2\alpha}$ is

still unknown. Additionally, little research has been done to attempt to elucidate why

this response occurs. Therefore, a reasonable area to start would be to identify how

neutrophil aggregation and proliferation occurs under normal physiologic conditions. As stated before, neutrophils are the first line of defense from invading pathogens. Studies exploring the physiologic response to something like an induced bacterial infection could give beneficial insight. Lee (1998) induced mastitis in Holstein dairy cows by E.coli infusion through the teat and measured levels of known pro-inflammatory cytokines from 0 to 24 h. The pro-inflammatory cytokines of interest were IL-1β, TNF-α and IL-6. These cytokines are known to induce mobilization of neutrophils from the bone marrow in response to a detected pathogen. A substantial increase in gene expression was seen for all cytokines evaluated by 6 h and remained elevated through the 24 h period. These findings hold true to the general understanding of the initial innate immune response to an invading pathogen. However, it is possible this mechanism could be induced by PGF$_{2\alpha}$, resulting in the increase in uterine neutrophil populations by 24 h post-injection, seen by Lulay et al. (2012). The objectives of this study were to determine the effectiveness of a single injection of PGF$_{2\alpha}$ (Lutalyse) at either Days 7 or 14 postpartum in improving reproductive performance as well as to illuminate the mechanism by which neutrophil propagation occurs within the uterus in response to treatment.

Materials and Methods

Animals

Cattle used for this experiment were from the Konyn Dairy. This dairy has approximately 3,000 Holstein cows with 1,500 in milking. Cows were supplied with water using a free-choice system. The ration fed varied as prices of feed fluctuated but the basic composition of the feed included corn silage, grass silage, alfalfa, corn, cotton seed and soy bean meal. At the beginning of this project, the Konyn Dairy was experiencing a higher than average postpartum infection rate, with roughly 30% of cows having visual signs of infection. This was believed to have been caused by poor up-keep of the bedding by the employees. This was alleviated by switching management and maintaining cow beds, keeping them packed high enough to keep the cows off the alley floors as well as keeping them cleaner to prevent infection.

The breeding program at the Konyn Dairy involved a 55 d voluntary waiting period. They used genomic breeding, matching cows genetically to the best possible sire. The synchronization program used was Ovsynch which involved injecting thirty cows at a time with GnRH on the Monday after the 55 d voluntary waiting period.

Pregnancy determination was performed by a veterinarian at approximately 40 days of gestation. Cows were examined for pregnancy and ovarian structures using rectal palpation and ultrasound. If there was no pregnancy and no ovarian structures present, the cow was given a Controlled Internal Drug Release (CIDR) device. Only two

cows had CIDRs inserted per month. No live cover was used at this dairy and all cows were bred through artificial insemination.

Experiment 1: Effects of Lutalyse treatment on reproductive performance in postpartum dairy cows

A total of 145 cows at Day 14 postpartum were given a 5 mL intramuscular (IM) injection of 25 mg Lutalyse or saline. The first cow given an injection was chosen at random, and the following cows injected were given alternating treatments. This was done to insure randomization in the experiment and an even number of cows receiving Lutalyse or saline.

A total of 98 cows at Day 7 postpartum were given a 5 mL IM injection of 25 mg Lutalyse or saline. Randomization was performed as described above.

Number of days open and services per conception were the reproductive performance of interest and were determined by examination of herd records.

Experiment 2: Effects of Lutalyse treatment on uterine neutrophil and bacterial populations in postpartum dairy cows

This experiment was performed on a subset of cows (n=15) to evaluate the changes in uterine environment after an injection of Lutalyse at Day 14 postpartum. Before administering injections of either saline or Lutalyse, uterine swabbing was used to collect bacterial samples and endometrial cytological samples were recovered for neutrophils using a Cytobrush. After samples were recovered, cows were given their respective injections. Twenty-four hours after the injection, sampling was conducted to determine any changes resulting from the injection.

Bacterial Assessment

Uterine bacteria samples were performed through rectal palpations and swabbing the inside of the uterine body. First the uterus was palpated rectally, and once the cervix was grasped securely, the vulva was wiped clean using paper towels and spread open as the first guarded tube of the swab was inserted into the vagina. This tube was inserted into the cervical os, followed by pushing the second guarded tube through the cervix. Once the second guarded tube was in the uterine body approximately 2 cm, the swab was pushed out of the second guard and spun gently on the uterine body. The swab was retracted back into the second guard and the guarded tubes were removed from the vagina. The swab with the uterine sample was broken off and placed in a tube containing 10 mL of Dulbecco's phosphate buffered saline (DPBS). A 1/10 dilution was made from the sample collected in the field using DPBS as the diluting fluid. One hundred microliters of each dilution (0 and 1/10) were spread onto each side of a Blood/ MacConkey Agar bacteriological culture biplate (Hardy Diagnostic, Santa Marta, CA). Colony counts from the blood agar portion of the plate gave the total bacteria count and the MacConkey's agar side, which is a selective medium, provided the number of *E.coli*. Plates were covered and left to sit facing up for 1 h at RT. Biplates were placed face down in an incubator at 37°C for 24 h. Numbers of colonies growing on either side of the biplates were counted.

Endometrial Sampling

Endometrial samples for cytologic determination of neutrophil numbers were collected using a double-guarded Cytobrush. The procedure for recovering an

endometrial sample using the double-guarded Cytobrush was similar to the bacterial swabbing. From the point of palpating the uterus and inserting the Cytobrush into the uterus, techniques were similar between both collections. Once the second guarded tube of the Cytobrush was inserted into the uterus, the Cytobrush was gentle rolled against the endometrium, retracted back into the guard and removed from the reproductive tract. The Cytobrush was then rolled onto a clean glass microscope slide and the sample was fixed to the slide using cytofixative spray. Slides were then stained using the Diff-Quick staining kit.

Assessment of the endometrial sample was done by counting a minimum of one-hundred cells at 400X magnification in an area on the slide that was most representative of the whole slide. This was done to determine the numbers of neutrophils, lymphocytes, monocytes and epithelial cells.

Experiment 3: Effects of Lutalyse on systemic pro-inflammatory cytokines

Blood was collected via caudal venipuncture from Day 14 postpartum cows at 0, 3, 6 and 12 h after injection of Lutalyse or saline. After each sample collection, blood was allowed to clot and centrifuged at 3000x g for 2-5 min to separate the serum. Serum was collected and placed in 0.5 mL tubes for a total of 4 replicates for each time point for every cow. Samples were frozen immediately until assays were ready to be performed. Serum concentration of the pro-inflammatory cytokines IL-6, IL-1β and TNFα were determined using ELISA for the bovine proteins (Genorise Scientific, Inc., Berwyn, PA).

Statistical analyses:

In Experiments 1 and 2, differences due to treatment in days open and services per conception and differences in numbers of bacteria, epithelial cells, neutrophils and lymphocytes between the 0 and 24 h sampling times were analyzed by one-way analysis of variance (ANOVA). In Experiment 3, differences in the changes in pro-inflammatory cytokine concentrations in response to Lutalyse and saline, relative to the 0 h concentrations, were analyzed by repeated measures ANOVA. Sources of variation were treatment, time and the treatment x time 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: Effects of Lutalyse treatment on reproductive performance in postpartum dairy cows

A total of 97 Day 14 postpartum cows (Lutalyse=46; saline=51) conceived by the conclusion of this study. No differences (P=0.96) were observed for days open between cows treated with Lutalyse or saline (Figure 1). Mean days open for Lutalyse and saline cows were nearly equivalent (117 ± 8 vs 118 ± 8 days, respectively). No differences (P=0.7) were observed in services per conception between cows treated with Lutalyse or saline (2.5 ± 0.3 vs 2.4 ± 0.2 services, respectively; Figure 2).

A total of 82 Day 7 postpartum cows were pregnant by the conclusion of this study (Lutalyse=43; saline=39). No differences (P=0.59) were observed in number of days open between cows treated with Lutalyse or saline (103 ± 7 vs 108 ± 7 days, respectively; Figure 3). Likewise, Lutalyse treatment did not produce a difference (P=0.98) in services per conception compared to saline (2.3 ± 0.3 vs 2.4 ± 0.2 services, respectively; Figure 4).
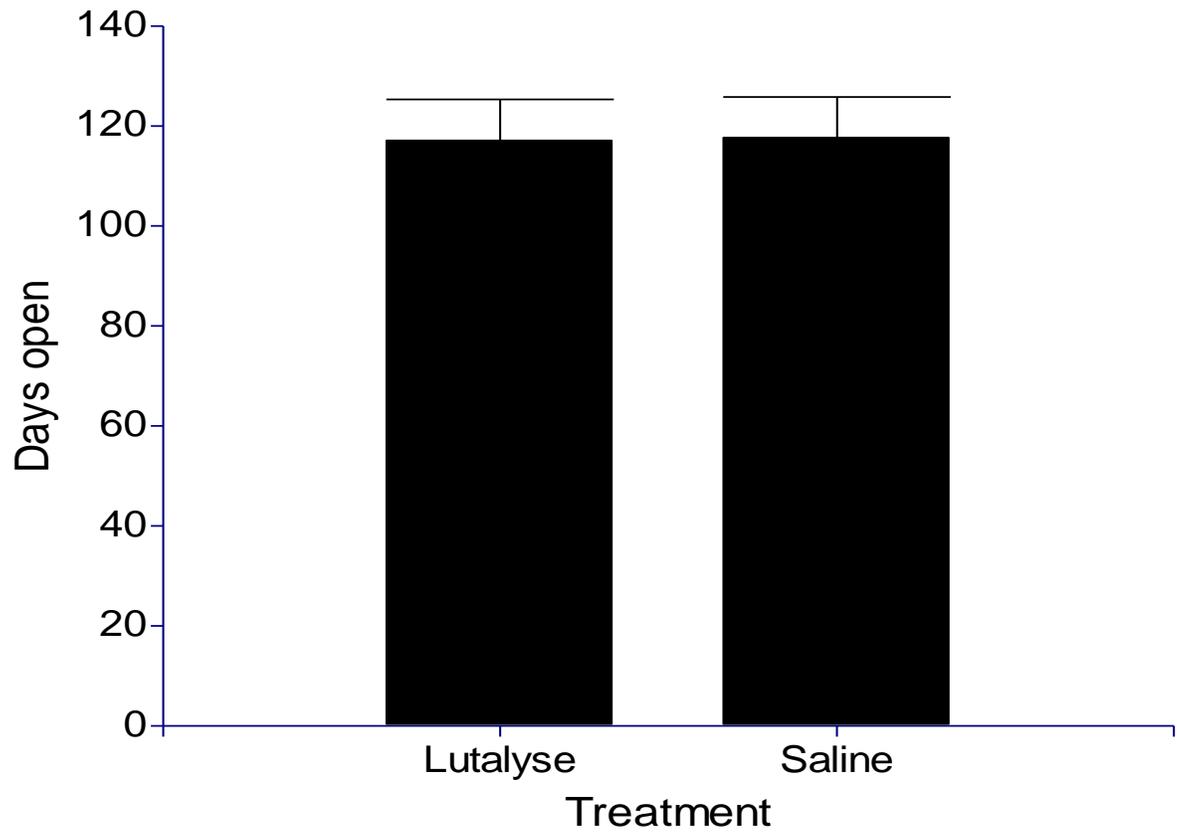
Figure 1: Number of days open (mean ± SE) for cows injected with Lutalyse (n=46) or saline (n=51) on Day 14 postpartum.
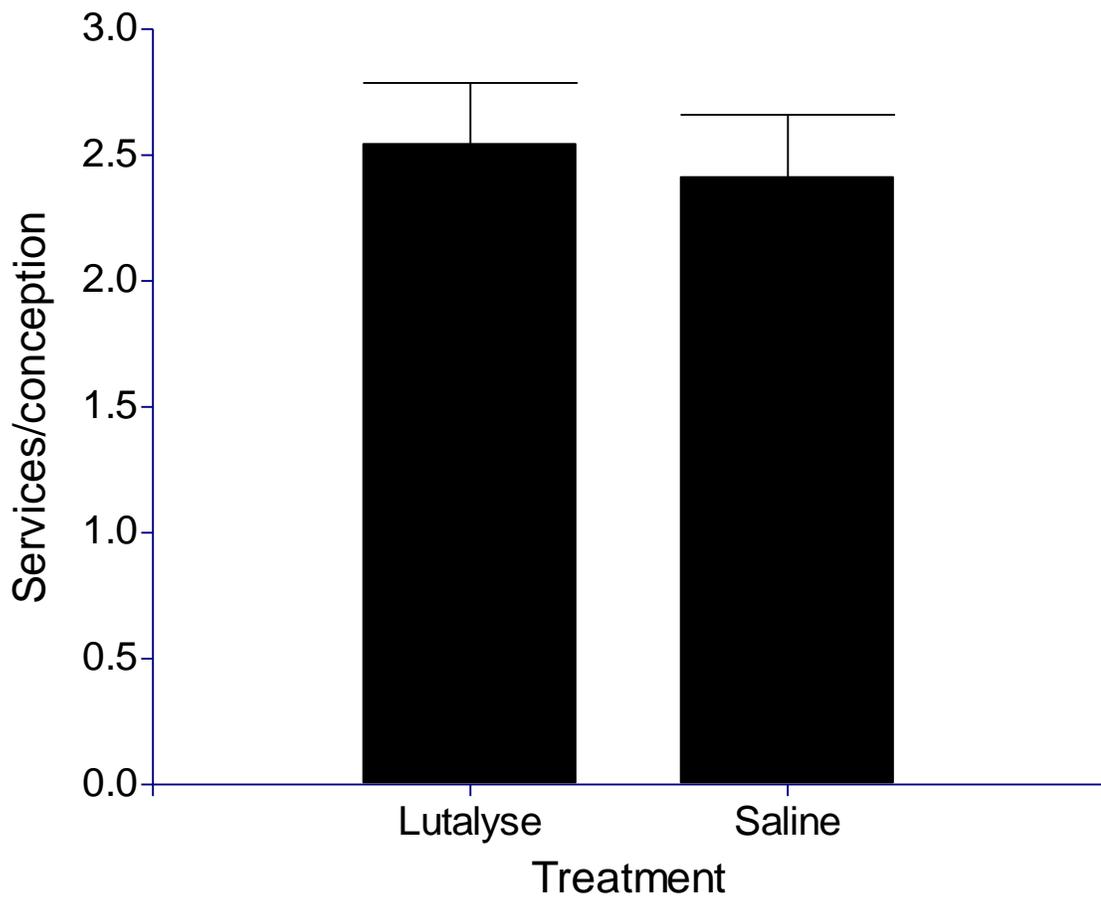
Figure 2. Number of services per conception (mean ± SE) for cows injected with Lutalyse (n=46) or saline (n=51) on Day 14 postpartum.
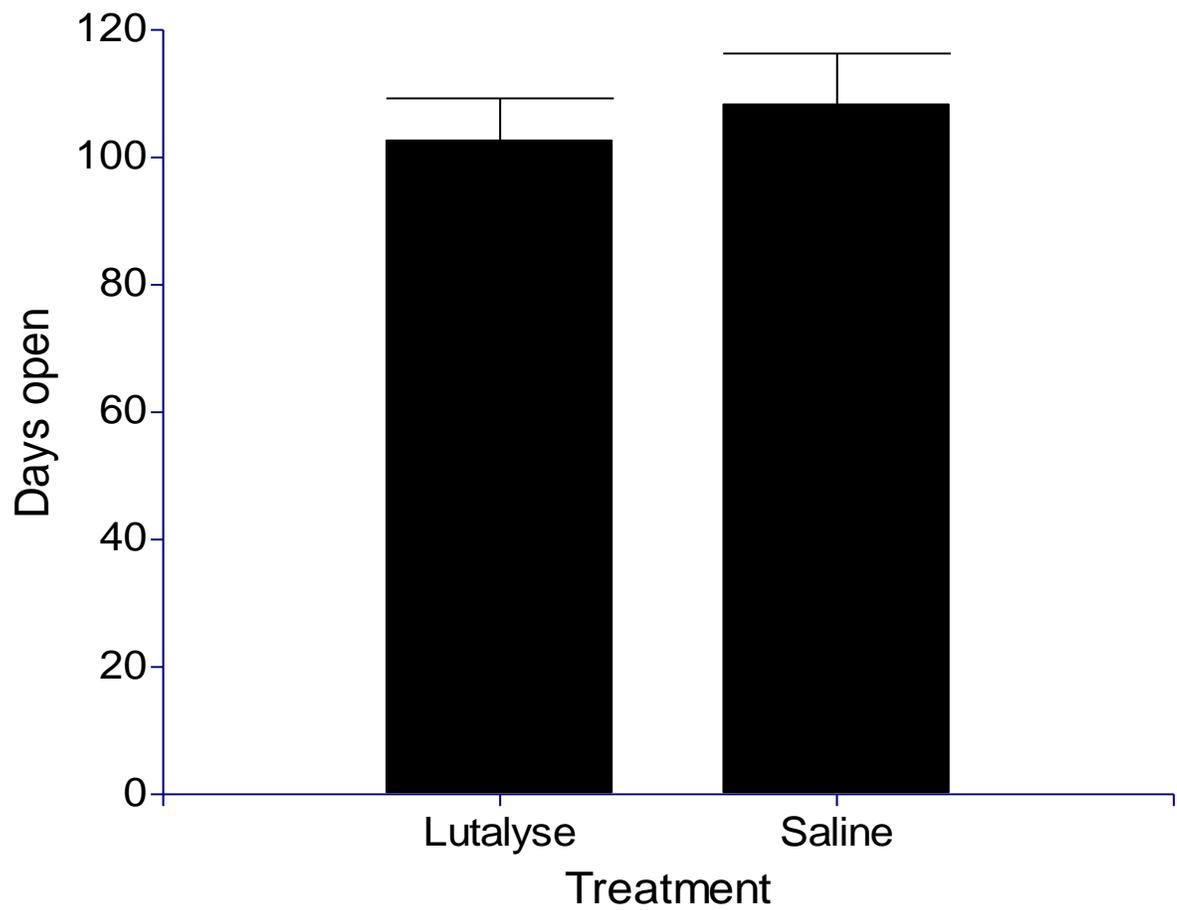
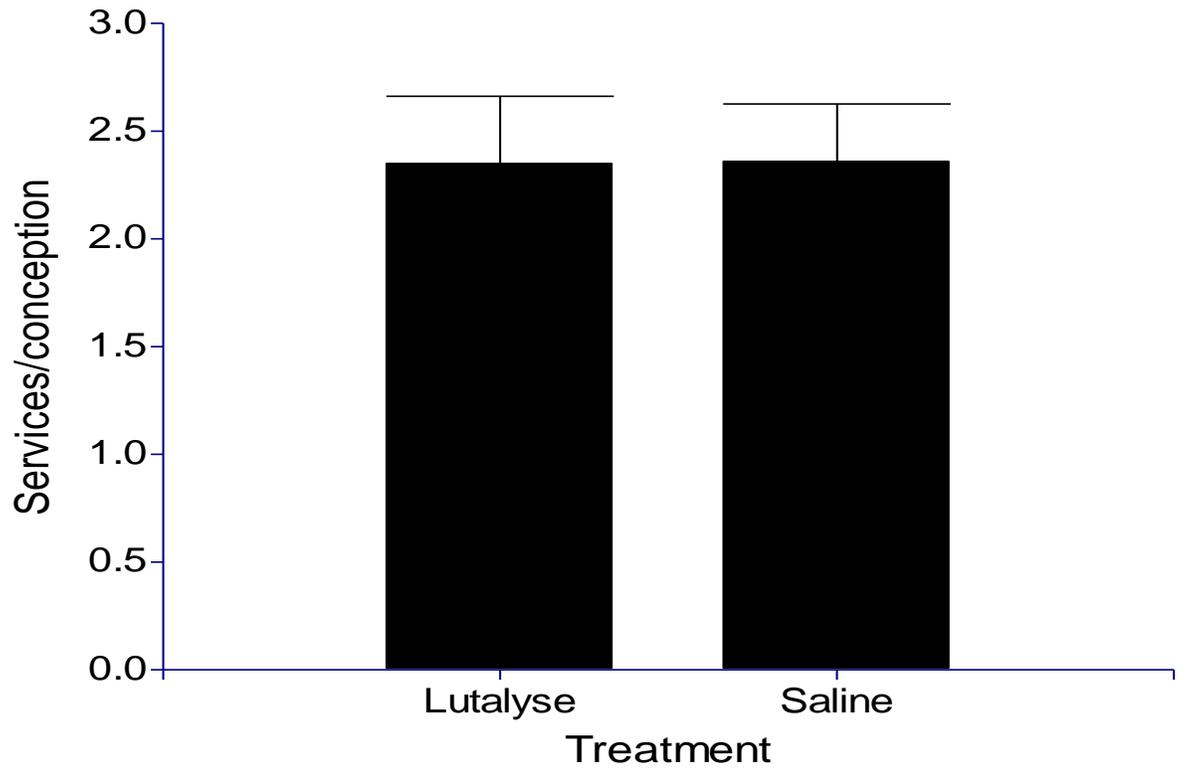**Figure 3. Number of days open (mean ± SE) for cows injected with Lutalyse (n= 43) or saline (n= 39) on Day 7 postpartum.**

Figure 4. Number of services per conception (mean ± SE) for cows injected with

Lutalyse (n=43) or saline (n= 39) on Day 7 postpartum.

Experiment 2: Effects of Lutalyse treatment on uterine neutrophil and bacterial populations in postpartum dairy cows

Data were recovered from a total of 15 cows for bacterial sampling (n=8 7 for Lutalyse and saline, respectively) and 8 cows for endometrial sampling (n=4 and 4 for Lutalyse and saline respectively). Lutalyse decreased (P=0.05) the total number of uterine bacteria on the blood agar portion of the biplate compared to saline (-188 ± 293 vs 729 ± 313 cells, respectively; Figure 5). Although a similar decrease with Lutalyse was observed for bacteria on the MacConkey's agar (-26 ± 17 vs 20 ± 17 cells, respectively), the difference was not significant (P=0.08). Lutalyse treatment increased (P<0.05) the difference in uterine neutrophil population when compared to saline (11 ± 13 vs -35 ± 13, respectively; Figure 6). Lutalyse treatment had no effect (P>0.10) on lymphocytes within the uterine cell population compared to saline (2 ± 6 vs 3 ± 6, respectively; Figure 6). Though not significant, the difference in epithelial cells decreased (P=0.08) after 24 h of Lutalyse treatment compared to saline (Lutalyse vs saline; -26 ± 17 vs 20 ± 17 cells, respectively; Figure 7). No difference (P>0.10) was observed in monocytes within the cell populations after injection (Figure 7).
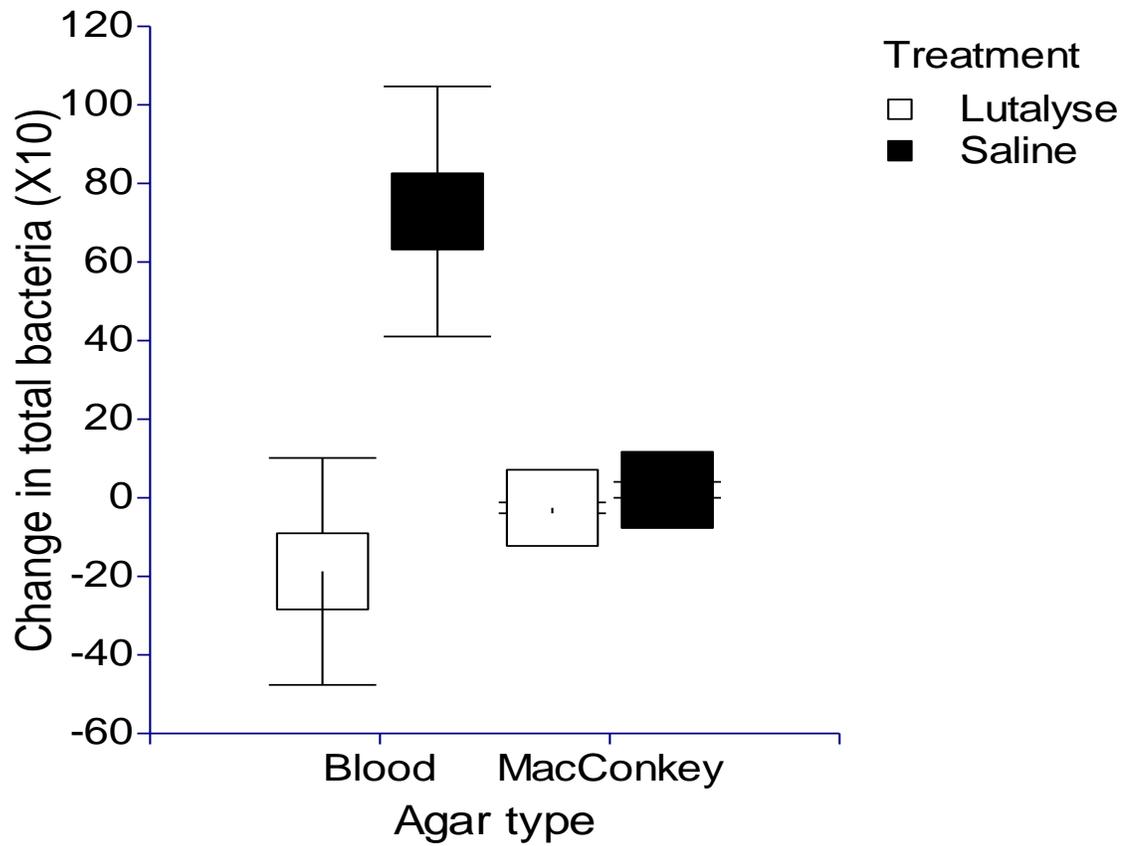
Figure 5. Changes (mean± SE) in uterine bacteria in cows injected with Lutalyse (n=8) or saline (n= 7) on Day 14 postpartum.
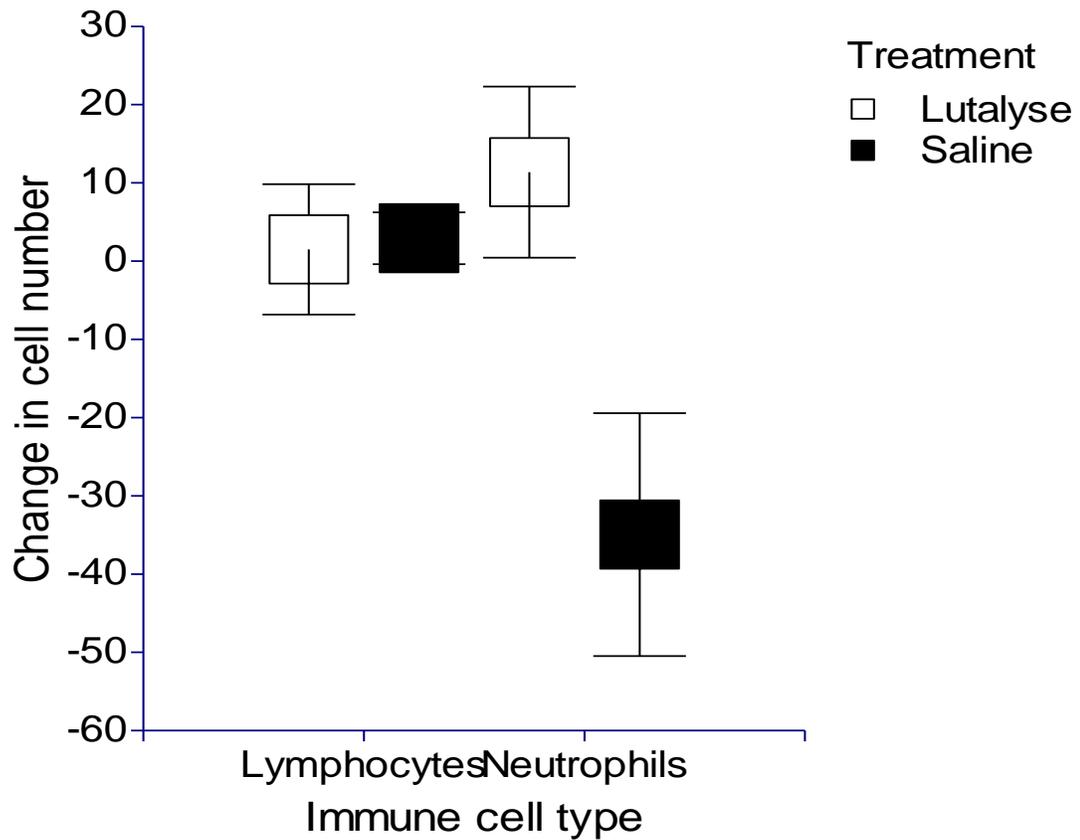
Figure 6. Changes (mean ± SE) in uterine lymphocytes and neutrophils in cows injected

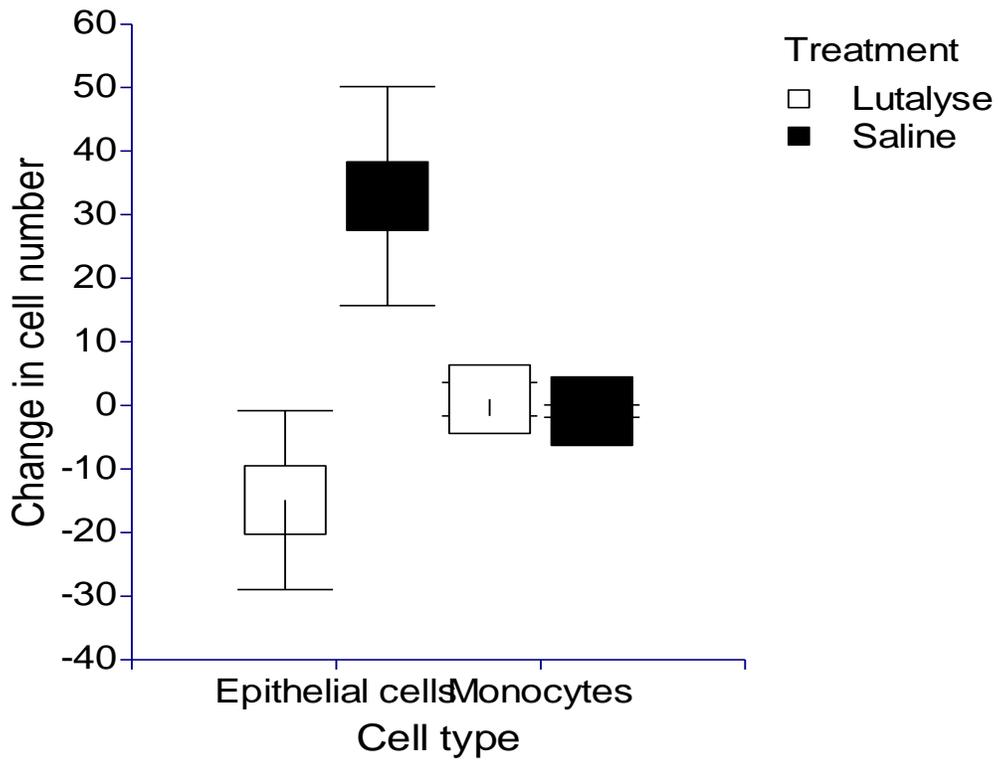with Lutalyse (n=4) or saline (n=4) on Day 14 postpartum.

Figure 7. Changes (mean ± SE) in uterine epithelial cells and monocytes in cows injected with Lutalyse (n=4) or saline (n=4) on Day 14 postpartum.

Experiment 3: Effects of Lutalyse on systemic pro-inflammatory cytokines

A total of 12 Day 14 postpartum cows were used for this study. One cow was found to have considerably higher TNF-α concentrations than her herdmates and inspection of the health records indicated this cow had been treated for metritis 4 d after blood was collected. This cow was omitted from the statistical analysis. Wide variability was observed among cows within treatment groups for serum cytokine concentrations, hence data were expressed as fold changes relative to the 0 h concentration. No significant differences between Lutalyse and saline treatment were observed in serum concentrations of IL-1β (1.0 ± 0.1 vs 1.1 ± 0.1, respectively) and TNF-α (1.1 ± 0.2 vs 1.1 ± 0.2, respectively; Figures 8 and 9). The treatment x time interactions were also not significant. Fold changes in IL-6 (P=0.09) were lower after Lutalyse injection compared to saline (0.7 ± 0.2 vs 1.1 ± 0.2, respectively; Figure 10). The treatment x time interaction was significant where fold change in IL-6 at 12 h following saline injection was greater compared to 3, 6 and 12 h after Lutalyse.
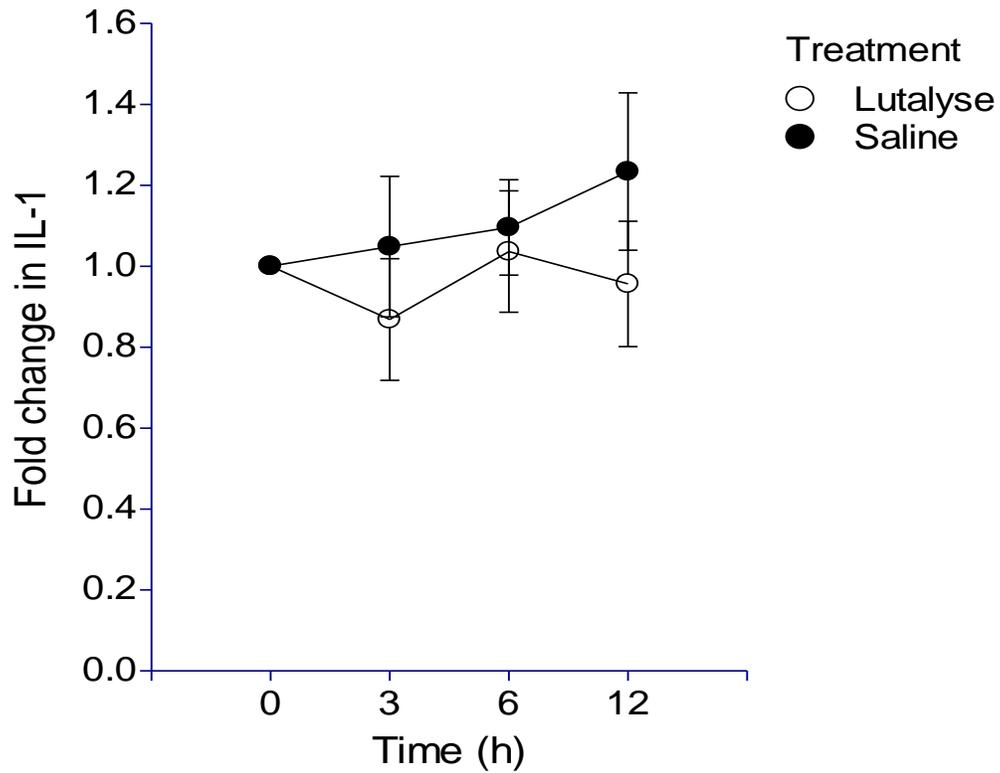
Figure 8. Fold changes (mean ± SE) in serum IL-1β (IL-1) concentrations following injection of Lutalyse (n=5) or saline (n=5) on Day 14 postpartum.

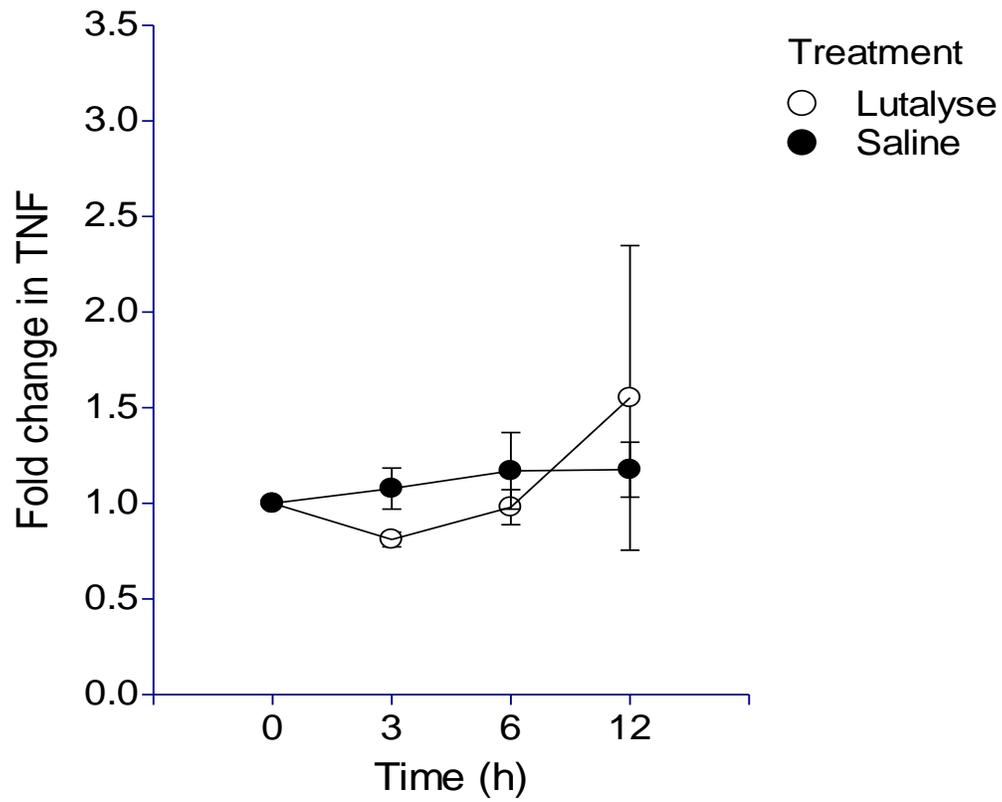**Figure 9. Fold changes (mean ± SE) in serum TNF-α (TNF) concentrations following injection of Lutalyse (n=5) or saline (n=5) on Day 14 postpartum.**
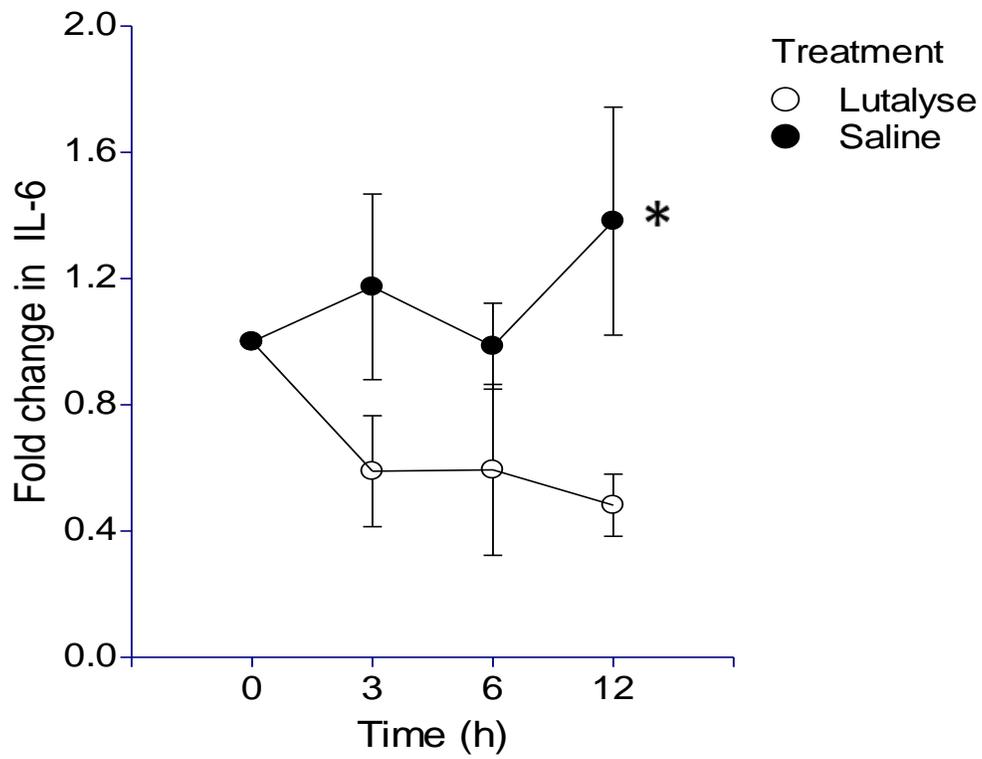
Figure 10. Fold changes (mean ± SE) in serum IL-6 concentrations following injection of Lutalyse (n=5) or saline (n=5) on Day 14 postpartum. * Greater than fold changes in Lutalyse treated cows at 3, 6 and 12 h after injection.

Discussion

The postpartum period is an extremely important interval within the reproductive lifespan of the dairy cow. Bacterial contamination is ubiquitous within the postpartum uterus, specifically within the first two weeks after calving. The dairy cow experiences a peripartum immunosuppression that can increase the likelihood of these animals succumbing to the bacteria and developing a uterine infection. Cattle can experience subclinical endometritis, which is difficult to detect. Though no outwards signs of infection may be seen, persistence of bacteria within the uterus can result in devastating effects. Bacteria such as *E.coli* remaining in the uterus over a period of time can result in reduced ovarian function such as decreased follicular growth and function as well as reduced corpora lutea size and therefore less progesterone production. In addition to endometrial damage, the presence of *E.coli* can allow for subsequent infections of other detrimental bacteria. Neutrophils are innate immune cells that are essential for clearing bacteria within the uterus, regardless of whether the bacteria is the source of a clinical or subclinical infection. An exogenous injection of $PGF_{2\alpha}$ has been shown to increase myometrial contractility, inducing physical expulsion of unwanted bacterial contamination as well as enhancing neutrophil function and propagation. Though the mechanism in which $PGF_{2\alpha}$ induces contractions of the myometrium is known, how neutrophil propagation and function is increased in response to $PGF_{2\alpha}$ still remains unclear.

The objectives of this study were to determine the effectiveness of a single injection of Lutalyse at either Days 7 or 14 postpartum in improving reproductive performance as well as to elucidate the mechanism in which the neutrophil population increases within the uterus in response to the treatment. In Experiment 1, no significant differences were found in days open or services per conception between cows treated with Lutalyse or saline. Though no differences in reproductive performance were observed, this does not suggest that $PGF_{2\alpha}$ is an ineffective preventative method or treatment. A single injection during the early postpartum period simply could not be enough to combat pathogens present in the uterus during the first two weeks in which immunosuppression occurs. It is possible that the number of injections administered during the postpartum period could be adjusted to the incidence rate of uterine infections at the individual dairy to maximize its effectiveness. The commercial dairy in which this research was conducted experienced a higher than average incidence rate of uterine infection at various periods during the study. This could account for the apparent ineffectiveness of the single Lutalyse injection to improve reproductive performance in cows from this dairy. A double injection protocol where Lutalyse was administered on either Days 0 and 14 or 14 and 28 postpartum has been successful in reducing days open and services per conception in a commercial dairy (Lulay et al., 2012). Reproductive improvements have also been shown by administering two injections of $PGF_{2\alpha}$ 8 h apart on Day 8 postpartum in cows diagnosed with acute puerperal metritis (Melendez et al., 2004). An increase in conception rate at first service was observed for this protocol. The findings from these studies suggest that a double

injection protocol could be used both as a treatment and as a preventative measure to combat uterine infections. Further research should be conducted to find the optimal days during the postpartum period for the two injections of $PGF_{2\alpha}$. Additionally, a third injection could be beneficial for cows experiencing more severe infections or cows still experiencing difficulty clearing the infection. However, for dairies not experiencing a high incidence of uterine infections, a single injection could be sufficient to prevent uterine infections in the few cows succumbing to the bacteria present during the first weeks postpartum. Considering Lutalyse costs $3.00 a dose, even the suggestion of a triple injection protocol of the drug would cost dramatically less than the detrimental effects on reproductive performance and the resulting cost of uterine disease. Research should be conducted to determine the effectiveness of $PGF_{2\alpha}$ on improving reproductive performance with the number of injections varied, and initial incidence rates of uterine infections taken into account.

In Experiment 2, uterine bacteria were reduced in cows treated with Lutalyse. An increase in uterine neutrophils was seen in cows treated with Lutalyse while cows treated with saline experienced a drop in intrauterine neutrophil population. This finding emboldens the notion that although $PGF_{2\alpha}$ has been proven to have beneficial effects, a single injection protocol simply could not be enough to increase reproductive performance. One possible reason for the decrease in uterine neutrophils in cows treated with saline may be due to prostaglandins released during manual stimulation of the uterus. Rectal palpation of the uterus has been show to induce the release of prostaglandins, which could aid in the expulsion of neutrophils and uterine bacteria. In

this experiment, collecting uterine bacterial and cytological samples required intense manipulation of the uterus. It is reasonable to assume this action was sufficient to cause the release of prostaglandins by the uterus in both saline and Lutalyse-treated cows. However, uterine neutrophils increased in response to Lutalyse while neutrophils decreased in cows treated with saline. This observation could be explained by the inability of the prostaglandins produced by the uterus due to manipulation in the cows injected with saline to induce neutrophil propagation. Neutrophils present in the uterus before uterine samples and injection of saline occurred could have been flushed out along with the bacteria. Neutrophil populations were replenished as well as increased in cows administered 2 injections of Lutalyse due to the known effects of $PGF_{2\alpha}$ (Lulay et al., 2012). Bacterial populations however, increased in cows injected with saline. It is possible passage of the swab and Cytobrush into the uterus aggravated the uterine environment such that bacterial proliferation was stimulated. Furthermore, as a decrease in neutrophil population was observed in cows treated with saline, resident bacteria adhering to the endometrium would be able to proliferate at a greater rate not inhibited by the presence of the neutrophils.

An adjustment could be made to this study design to determine whether $PGF_{2\alpha}$ is working directly to improve reproductive performance and uterine health. Postpartum dairy cows from the same commercial dairy would be divided into three treatments groups. These groups would be injected with saline, $PGF_{2\alpha}$ or $PGF_{2\alpha}$ plus a $PGF_{2\alpha}$ blocker such as ibuprofen. Ibuprofen is a non-steroidal anti-inflammatory drug that inhibits cyclooxygenase-1, cyclooxygenase-2 and the enzyme prostaglandin H synthase (Rao and

Knaus, 2008). Therefore, treatment with PGF$_{2\alpha}$ and ibuprofen would serve to inhibit pro-inflammatory effects of PGF$_{2\alpha}$ such as increased neutrophil population.

Experiment 3 attempted to elucidate the mechanism in which neutrophil propagation is induced in response to Lutalyse. No significant differences were observed in IL-1β and TNF-α following injection of Lutalyse but IL-6 decreased. However, as stated before, the dairy was experiencing a higher incidence of disease than usual. As mentioned in the results, one cow had extremely high levels of TNF-α and upon referencing her health records, it was found she had been treated for metritis 4 days after blood was collected. Normal serum concentrations for IL-6 in a healthy lactating dairy cow are 5-6 ng/ml (Hagiwara et al., 2001). IL-1β levels ranges between 1-3 ng/mL in Day 14 postpartum cows (Goto et al., 1997). Plasma TNF-α levels are approximately 0.41 ng/mL in cows with a normal body condition score (O'Boyle et al., 2006). Cows in this study had lower concentrations of IL-1β and IL-6 than the reported baseline levels of healthy lactating dairy cows. However, as reported by Galvão et al. (2012), monocytes isolated from cows that developed metritis produced higher pro-inflammatory cytokine mRNA when unstimulated but, gene expression of pro-inflammatory cytokine mRNA by those monocytes decreased from calving to 14 d postpartum when stimulated with *E.coli.* This suggests an inability for the innate immune system to properly respond to infection causing bacteria in cows to develop severe uterine infections. Interestingly, a decrease in IL-6 concentration from 0 h of injection was observed in cows treated with Lutalyse. PGE is known to stimulate IL-6 production, and has been shown to be elevated in cows with *E.coli* infections. An injection of exogenous PGF$_{2\alpha}$ could have induced a

higher systemic PGF$_{2\alpha}$ to PGE ratio, decreasing IL-6 production. It is possible the presence of infection resulted in both lower serum concentration and wide variation of pro-inflammatory cytokines within the cows in this study. For this reason, it may be more feasible to repeat this study, observing changes in pro-inflammatory cytokines as before, but in dry, healthy cows. This would help reduce the variability as seen in the animal model used in this study.

The connection between pro-inflammatory cytokines and PGF$_{2\alpha}$ is clearly demonstrated by luteolysis in the bovine corpus luteum (CL) (Shirasuna et al., 2012). PGF$_{2\alpha}$ is produced by the uterus and acts directly on the CL through a countercurrent exchange in the cow, rather than being released systemically. The luteolytic cascade is remarkably similar to that of a general acute inflammation and immune response. Dramatic changes in vascular diameter and blood flow as well as infiltration of immune cells such as neutrophils, macrophages and T-lymphocytes occur during this time. It has been suggested that PGF$_{2\alpha}$ released from the uterus could be the first stimulus for the acute vasoconstriction occurring in the CL due to its vascular constricting property. A significant increase in neutrophil numbers within the CL has been shown 5 min after PGF$_{2\alpha}$ administration. This could partly be due to the hormone's ability to stimulate expression of P-selectin on luteal endothelial cells, resulting in increased neutrophil adhesion. Additionally, inflammatory cytokines TNF-α and IL-1β as well as chemokines have been shown to be involved in luteal regression. Macrophages and T-lymphocytes, which are found in high numbers in the regressing CL of the cow, have the potential to produce cytokines such as the ones previously mentioned. TNF-α, presumably produced

by one of these myeloid cells, induces interferon gamma and Fas-mediated apoptotic

cell death of the bovine luteal and endothelial cells. This is due to an increase in

caspase-3 activity (Shirasuna et al., 2012). Therefore, injection of exogenous $PGF_{2\alpha}$ may

enable the hormone to exert its effects systemically by initiating production of pro-

inflammatory molecules resulting in mobilization of neutrophils from the bone marrow.

If this notion holds true, the next step would be to determine which cells respond to the

exogenous $PGF_{2\alpha}$ injection and release these pro-inflammatory cytokines, resulting in

neutrophil propagation. Perhaps the neutrophils themselves possess $PGF_{2\alpha}$ receptors.

More research needs to be conducted to determine the mechanism by which $PGF_{2\alpha}$

increases both systemic and intrauterine neutrophil populations.

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