



## Association between immune function and development of uterine disease in dairy cows

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### Abstract

The transition to lactation (3 weeks before to 3 weeks after calving) is characterized by a decrease in dry-matter intake (DMI), leading to a sharp decrease in glucose and calcium, and an increase in body fat mobilization in the form of non-esterified fatty acids (NEFA). This results in products such as beta-hydroxybutyrate (BHBA) accumulating from incomplete oxidation of NEFA (Vazquez-Añon *et al.*, 1994). Neutrophils (PMN) are the main leukocyte type involved in clearing bacteria after uterine infection; however, during the period of negative energy balance, dairy cows experience a reduction in PMN function, including reduced phagocytosis and killing capacity. This reduction is more pronounced in cows that develop uterine disease. Glycogen is the main source of energy for PMN phagocytosis and killing; calcium is an important second messenger for PMN activation; NEFA is associated with impaired PMN oxidative burst activity; and BHBA reduces PMN phagocytosis, extracellular trap formation, and killing of bacteria. If the immune system is not able to eliminate bacterial infection, disease is established. Pathogenic bacteria associated with metritis and endometritis are *E. coli*, *A. pyogenes*, *F. necrophorum*, and *P. melaninogenicus*. *E. coli* increases the susceptibility of the endometrium to subsequent infection with *A. pyogenes*, and *A. pyogenes* acts synergistically with *F. necrophorum* and *P. melaninogenicus* to enhance the severity of uterine disease. Among their effects, *E. coli* releases bacterial-wall LPS; *A. pyogenes* produces the cholesterol-dependent cytotoxin pyolysin and a growth factor for *F. necrophorum*; *F. necrophorum* produces a leukotoxin; and *P. melaninogenicus* produces a substance that inhibits phagocytosis. A specific *E. coli*, called EnPEC/IUEC, causes uterine disease, and the virulence factor fimH was mostly associated with disease. For *A. pyogenes*, fimA was the only virulence factor associated with uterine disease. The combined effect of bacterial infection and activation of inflammation is damage to the endometrium and embryo, delayed ovulation, shortened or extended luteal phase after ovulation, increased time to first insemination, decreased conception rates, increased time to conception, and increased pregnancy loss.

**Keywords:** dairy cows, endometritis, immune function, metritis, uterine diseases.

### Introduction

The transition to lactation (3 weeks before to 3 weeks after calving) is a challenging period for a high producing dairy cow. This period is characterized by a decrease in dry-matter intake (DMI), leading to a sharp decrease in glucose and calcium, and an increase in body fat mobilization in the form of non-esterified fatty acids (NEFA). This results in products such as beta-hydroxybutyrate (BHBA) accumulating from incomplete oxidation of NEFA (Vazquez-Añon *et al.*, 1994).

Neutrophils (PMN) are the main leukocyte type involved in clearing bacteria after uterine infection (Hussain, 1989; Gilbert *et al.*, 2007); however, during the period of negative energy balance, dairy cows experience a reduction in PMN function, including reduced phagocytosis and killing capacity (Kehrli and Goff, 1989; Gilbert *et al.*, 1993; Cai *et al.*, 1994). The factors that might account for such reduction include decreased PMN glycogen stores (Galvão *et al.*, 2010a), decreased blood calcium concentration (Goff and Horst, 1997; Kimura *et al.*, 2006), and increased NEFA and BHBA (Hammon *et al.*, 2006; Galvão *et al.*, 2010a). In particular, cows that develop uterine disease have a more pronounced decrease in DMI (Huzzey *et al.*, 2007), an increase in NEFA and BHBA, and a decrease in blood PMN pathogen phagocytosis (Kim *et al.*, 2005) and killing (Hammon *et al.*, 2006). In the study by Hammon *et al.* (2006), NEFA was negatively associated with PMN oxidative burst activity. Recently, BHBA was also observed to be negatively associated with PMN phagocytosis, extracellular trap formation, and killing of bacteria (Grinberg *et al.*, 2008).

If the immune system is not able to eliminate bacterial infection, disease is established. Early postpartum (<21 days in milk, or DIM) cows are affected with metritis (severe and acute). Some cows clear the infection but others remain chronically infected (>21 DIM), and the condition is called endometritis. Regardless of the condition, the overall effect of uterine infection is damage to the endometrium and activation of inflammation with release of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1), and interleukin-6 (IL-6), and chemokines, including interleukin-8 (IL-8) (Chapwanya *et al.*, 2009; Galvão *et al.*, 2011). Damage to the endometrium is caused by the bacteria and by neutrophils releasing proteolytic granules and reactive oxygen species.

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### Neutrophil function

Neutrophils rely on different glucose sources for different functions. They depend mainly on extracellular glucose (but can use glycogen under hypoglycemic conditions) for the energy required for chemotaxis, while they depend mainly on intracellular glycogen and glycogenolysis for the glucose necessary for phagocytosis and killing (Kuehl and Egan, 1980; Weisdorf *et al.*, 1982a, b). Whereas chemotactic stimuli (such as N-formyl-methionine-leucine-phenylalanine, C5a, and arachidonic acid) accelerate glucose uptake, phagocytic stimuli (such as opsonized zymosan particles) failed to increase glucose uptake but increased glycogen breakdown (Weisdorf *et al.*, 1982a, b). Therefore, the low glucose concentrations observed in the first 10 days of lactation (Vazquez-Añón *et al.*, 1994) might directly impair PMN chemotaxis and could lead to decreased PMN glycogen stores. In turn, the impaired PMN chemotaxis and decreased PMN glycogen stores lead to decreased phagocytic and killing capability (and possibly chemotaxis), which would predispose cows to disease. In a recent study, PMN glycogen stores were found to be reduced during early postpartum and such reduction was more pronounced in cows that developed uterine disease (Galvão *et al.*, 2010a). Calcium is an important second messenger for PMN activation. In a recent study, cows that developed uterine disease had a greater reduction in calcium concentration than healthy cows (Martinez-Patino *et al.*, 2012). Furthermore, the odds of developing metritis decreased by 62% for every 1 mg/dl increase in serum Ca in the first 3 days after calving (Martinez-Patino *et al.*, 2012).

### Effect of cortisol and estradiol

The high concentrations of cortisol and estradiol are also believed to contribute to the overall state of immunosuppression around calving (Wyle and Kent, 1977; Goff and Horst, 1997). Cortisol is a known immunosuppressive hormone (Roth and Kaerberle, 1982). We recently observed that primiparous cows had increased cortisol concentration compared to multiparous (Galvão *et al.*, 2010a). Greater cortisol concentrations in primiparous cows is probably the consequence of stressful events such as frequent pen moves, more interactions with the herd personnel, calving itself, and milking for the first time, and could be contributing to the overall higher metritis incidence observed in primiparous cows (Goshen and Shpigel, 2006). Furthermore, it was observed that cows that developed metritis had greater cortisol concentrations at calving than cows that had subclinical endometritis (SCE); however, concentrations were not different from healthy cows. Plasma estradiol on the day of calving is also thought to contribute to the overall immunosuppression observed in dairy cows around

calving (Goff and Horst, 1997; Wyle and Kent, 1977). Recently, we observed that cows that developed metritis had greater estradiol concentrations than healthy cows on the day of calving. This might be contributing to the state of immunosuppression and predisposing cows to disease.

### Effect of pathogenic bacteria

Pathogenic bacteria associated with metritis and endometritis are *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum*, and *Prevotella melaninogenicus* (Bonnett *et al.*, 1991; BonDurant *et al.*, 1999; Huszenicza *et al.*, 1999; Földi *et al.*, 2006; Gilbert *et al.*, 2007). These four main bacteria are believed to work synergistically to cause uterine disease (Griffin *et al.*, 1974; Ruder *et al.*, 1981; Bonnett *et al.*, 1991). In fact, *E. coli* might increase the susceptibility of the endometrium to subsequent infection with *A. pyogenes* (Olson *et al.*, 1984; Gilbert *et al.*, 2007; Williams *et al.*, 2007), while *A. pyogenes* acts synergistically with *F. necrophorum* and *P. melaninogenicus* to enhance the severity of uterine disease (Griffin *et al.*, 1974; Ruder *et al.*, 1981; Bonnett *et al.*, 1991). Among their effects, *E. coli* releases bacterial-wall lipopolysaccharides (LPS; Williams *et al.*, 2008); *A. pyogenes* produces the cholesterol-dependent cytotoxin pyolysin (Miller *et al.*, 2007) and a growth factor for *F. necrophorum* (Sheldon and Dobson, 2004); *F. necrophorum* produces a leukotoxin; and *P. melaninogenicus* produces a substance that inhibits phagocytosis (Sheldon and Dobson, 2004).

*Escherichia coli* and *A. pyogenes* have been more extensively studied than the other bacteria. Recently, it was observed that a specific *E. coli* causes uterine disease, which is different from known diarrhoeic or extra-intestinal pathogenic *E. coli* (Sheldon *et al.*, 2010). This specific *E. coli* was named endometrial pathogenic *E. coli* or EnPEC. EnPEC was found to be more adherent and invasive to endometrial cells and also to stimulate greater production of prostaglandin E2 and interleukin 8 (Sheldon *et al.*, 2010). Interleukin 8 is the main neutrophil chemokine. In another study, six virulence factors were found to be associated with metritis and endometritis: fimbriae components (fim) fimH, hemolysin A (hlyA), cytolethal distending toxin (cdt), group II capsule (kpsMII), invasion of brain endothelium (ibeA), and arginine succinyltransferase (astA). However, fimH was the most prevalent and significant factor (Bicalho *et al.*, 2010). The authors concluded that *E. coli* carrying fimH and at least one of the other factors were pathogenic to dairy cows. The authors called this virulent strain of intrauterine *E. coli* (IUEC).

*Arcanobacterium pyogenes* has been highlighted in several studies as the main causative agent of endometrial damage and infertility (Bonnett *et al.*, 1991; Ruder *et al.*, 1991; Dohmen *et al.*, 2000;



BonDurant *et al.*, 1999). A recent study also tried to find specific virulence factors associated with uterine disease (Silva *et al.*, 2008). They evaluated a series of virulence factors including pyolysin (plo), neuraminidases (nan) nanP, nanH, collagen-binding protein A (cbpA), fimA, fimC, fimE, and fimG, but were unable to find any association with incidence of metritis. They concluded that synergism between *A. pyogenes* and other bacteria and differential gene expression of virulence factors might be more important for establishment of infection. In another study, only fimA was found to be overrepresented in cows with metritis, while the other virulence factors were similarly found in both healthy and metritic cows (Santos *et al.*, 2010).

Infection with these pathogenic bacteria will induce the release of pro-inflammatory cytokines such as TNF $\alpha$ , which have been found to stimulate the release of prostaglandin-F2 $\alpha$  (PGF2 $\alpha$ ) from the endometrium and luteal cells and to induce luteolysis (Skarzynski *et al.*, 2005; Kaneko and Kawakami, 2008, 2009). On the other hand, IL-1 and IL-6 have been found to decrease the expression of oxytocin receptors in endometrial cells, which could impair the mechanism of luteolysis (Leung *et al.*, 2001). Therefore, inflammation could have a bimodal effect on the length of the estrous cycle.

Although the initial response to infection might be to lyse the corpus luteum, another response observed in cows having uterine disease is a prolonged luteal phase. Prolonged luteal lifespan was observed when the first ovulation postpartum occurred in the presence of a heavily contaminated uterus (Olson *et al.*, 1984), or when *A. pyogenes* was infused into the uterine lumen (Farin *et al.*, 1989). *Escherichia coli* releases the endotoxin LPS, which impairs the release of both gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH; Peter *et al.*, 1989), decreases aromatase activity (Herath *et al.*, 2009), and increases the prostaglandin-E2 (PGE) to PGF2 $\alpha$  ratio (Herath *et al.*, 2009). The consequences of decreased aromatase activity are decreased follicular growth and estradiol production (Williams *et al.*, 2007, 2008). Decreased GnRH/LH release leads to decreased ovulation rate (Peter *et al.*, 1989), and an increase in the ratio of PGE to PGF2 $\alpha$  leads to extended luteal phase when ovulation does occur (Farin *et al.*, 1989). *A. pyogenes* releases the cholesterol-dependent cytotoxin pyolysin, which damages the endometrium and leads to the production of pro-inflammatory cytokines, decreased endometrial oxytocin receptors, and impairment of embryo development (Hansen *et al.*, 2004; Hill and Gilbert, 2008). The combined effect of bacterial infection and activation of inflammation is damage to the endometrium and embryo, delayed ovulation, shortened or extended luteal phase after ovulation, increased time to first insemination, decreased conception rates, increased time to conception, and increased pregnancy loss (Opsomer *et al.*, 2000, Galvão *et al.*, 2009, 2010b).

## Conclusion

In summary, PMN are the main leukocyte type involved in clearing bacteria after uterine infection; however, during the period of negative energy balance, dairy cows experience a reduction in PMN function, including reduced phagocytosis and killing capacity. This reduction is more pronounced in cows that develop uterine disease. Glycogen is the main source of energy for PMN phagocytosis and killing; calcium is an important second messenger for PMN activation; NEFA is associated with impaired PMN oxidative burst activity; and BHBA reduces PMN phagocytosis, extracellular trap formation, and killing of bacteria. If the immune system is not able to eliminate bacterial infection, disease is established. Pathogenic bacteria associated with metritis and endometritis are *E. coli*, *A. pyogenes*, *F. necrophorum*, and *P. melaninogenicus*. *E. coli* increases the susceptibility of the endometrium to subsequent infection with *A. pyogenes*, and *A. pyogenes* acts synergistically with *F. necrophorum* and *P. melaninogenicus* to enhance the severity of uterine disease. Among their effects, *E. coli* releases bacterial-wall LPS; *A. pyogenes* produces the cholesterol-dependent cytotoxin pyolysin and a growth factor for *F. necrophorum*; *F. necrophorum* produces a leukotoxin; and *P. melaninogenicus* produces a substance that inhibits phagocytosis. A specific *E. coli*, called EnPEC, causes uterine disease, and the virulence factor fimH was mostly associated with disease. For *A. pyogenes*, fimA was the only virulence factor associated with uterine disease. The combined effect of bacterial infection and activation of inflammation is damage to the endometrium and embryo, delayed ovulation, shortened or extended luteal phase after ovulation, increased time to first insemination, decreased conception rates, increased time to conception, and increased pregnancy loss.

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