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Genes and environmental factors that influence disease resistance to microbes in the female reproductive tract of dairy cattle

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Abstract

Microbes commonly infect the female reproductive tract of cattle, causing infertility, abortion, and postpartum uterine diseases. When organisms reach the uterus the resistance to disease depends on the balance between the classic triad of the virulence of the microbes, the host defence systems, and the environment. The present review considers each aspect of this triad, using postpartum uterine disease as an exemplar for understanding disease resistance. The bacteria that cause postpartum uterine disease are adapted to the endometrium, and their microbial toxins cause tissue damage and inflammation. However, non-specific defence systems counter ascending infections of the female reproductive tract and inflammatory responses in the endometrium are driven by innate immunity. Disease resistance to bacterial infection involves many genes involved with maintaining or restoring tissue homeostasis in the endometrium, including antimicrobial peptides, complement, cytokines, chemokines, and Toll-like receptors. The most important environmental factors facilitating the development of postpartum uterine disease are related to trauma of the reproductive tract and to the metabolic stress of lactation in dairy cows. Long-term solutions for uterine disease will include genetic selection for disease resistance, and optimising the care of the animal before, during, and after parturition.

Introduction

Microbes commonly infect the female reproductive tract of cattle, and these infections cause infertility, abortion, and postpartum uterine diseases. The species of microbes that infect the reproductive tract span the full range of organisms from bacteria to viruses, and mycoplasma to fungi. Some of the microbes reach the reproductive tract by the haematogenous route, whilst many others ascend the reproductive tract after coitus or after parturition. Several microbes are sexually transmitted, although their clinical importance has waned since the introduction of artificial insemination. Others, particularly viruses, are transmitted from animal to animal, whilst the environment and the skin of cattle are the most common source of bacteria. Within the reproductive tract, the vagina has a persistent microbiome, whilst the uterus is probably mainly contaminated with bacteria when the cervix is patent, and the ovary is usually considered to be sterile. When organisms reach the uterus the resistance to disease depends on the balance between the classic triad of the virulence of the microbes, the host defence systems, and the environment. The present review considers each aspect of this triad, using postpartum uterine disease as an exemplar for understanding disease resistance. Postpartum uterine disease was selected because it is common in dairy cows with high milk yields (Dobson *et al.* 2007; Sheldon *et al.* 2009); endometrial inflammation and infertility persist for several weeks after the clinical signs of uterine disease resolve (Borsberry and Dobson 1989; Bonnett *et al.* 1991a); and, uterine disease has wider impacts on reproduction than just damaging the endometrium, including perturbations of the ovary, oocyte, hypothalamus and pituitary (Karsch *et al.* 2002; Sheldon *et al.* 2002; Herath *et al.* 2007; Bromfield and Sheldon 2011).

Postpartum uterine disease

To maximise milk production, many farmers aim to calve cows every 12 months, and so the uterus must recover rapidly after parturition in preparation for the next 9-month gestation. However, parturition carries considerable risks to health and fertility, which is a major challenge for the dairy industry (Sheldon and Dobson 2003). The recovery processes for the uterus after parturition requires tissue repair, regeneration of the endometrial epithelium, and elimination of bacteria that always contaminate the uterus around the time of parturition. The elimination of invading bacteria is most problematic in dairy cattle, where the incidence of uterine disease has increased along with rising milk yields over the last 50 years. The common postpartum manifestations of postpartum uterine disease in cattle include metritis, endometritis, and pyometra. The characteristics of these diseases have been defined previously (LeBlanc *et al.* 2002; Sheldon *et al.* 2006; Sheldon *et al.* 2008; Sheldon *et al.* 2009). However, all these diseases are associated with the accumulation of pus in the reproductive tract, inflammation and pain. The incidence of uterine disease in many dairy herds in Europe and the USA is remarkably high compared with beef cows or other domesticated species. Up to 40% of animals develop disease within 10 days of parturition, and uterine disease persists beyond 3 weeks postpartum in 20% of cows as endometritis, or occasionally pyometra, with subclinical endometritis affecting an additional 15% of animals (Sheldon *et al.* 2009).

Uterine disease is associated with endometrial inflammation, infiltration of immune cells such as neutrophils and macrophages, and tissue damage (Archbald *et al.* 1972; Bonnett *et al.* 1991a; Herath *et al.* 2009b). Samples collected from the endometrium of diseased animals, compared with unaffected animals, have more abundant gene transcripts for: cytokines *IL1A, IL1B, IL6* and *TNF;* chemokines *CXCL8* and *CXCL5*; and receptors involved in innate immunity, such as *TLR4* and *IL1R* (Gabler *et al.* 2009; Herath *et al.* 2009b; Fischer *et al.* 2010; Ghasemi *et al.* 2012; Kasimanickam *et al.* 2014)**.** Furthermore, there are temporal changes in the expression of transcripts for *IL1B, IL6, CXCL8, TNF, CXCL5*, *HP* and *PTGS2* during the postpartum period, with the highest gene expression on day 17 postpartum (Gabler *et al.* 2010). Similarly, *ex vivo* organ cultures of endometrium produce IL-1α, IL-1β, IL-6, IL-8 and prostaglandin E_2 when challenged with bacteria that cause uterine disease (Borges *et al.* 2012).

Postpartum uterine disease causes infertility, with conception rates about 20% lower for cows with endometritis, the median calving to conception interval is 30 days longer and there are at least 3% more animals culled for failure to conceive (Borsberry and Dobson 1989; LeBlanc *et al.* 2002). As uterine disease causes infertility, animals are routinely treated with antibiotics or hormones. Although these treatments improve the clinical signs, they do not improve fertility. The use of antibiotics and hormones in food-producing animals also raises concerns in the European Union and World Health Organization about drug residues in milk, and the spread of antimicrobial resistance. The cost of treatment, reduced milk yields, and replacing infertile animals is about €1.4 billion each year for the dairy industry in the European Union (Sheldon *et al.* 2009). It is assumed that uterine disease also contributes to the overall problem of dairy cow infertility in the EU and USA, with conception rates <40% to the first insemination after parturition (Chagas *et al.* 2007; Kerestes *et al.* 2009; Sheldon *et al.* 2009). However, the reason for this lack of resistance to uterine disease in dairy cows is not known. Potentially each of the triad of microbial virulence, host defence systems, and the environment could contribute to this propensity for uterine disease to develop after parturition.

Microbes causing postpartum uterine disease

Disease of the endometrium is caused most commonly by *Escherichia coli* and *Trueperella pyogenes*, with *E. coli* isolated from the uterus in the first few weeks postpartum, followed subsequently by *T. pyogenes* and other anaerobic bacteria (Sheldon *et al.* 2002; Williams *et al.* 2007; Sheldon *et al.* 2009; Sheldon *et al.* 2010; Westermann *et al.* 2010). The endometrial pathogenic *E. coli* (EnPEC) causing uterine disease are novel strains that are adapted to the endometrium, and they differ from enteric, mastitis or urinary *E. coli* strains (Sheldon *et al.* 2010). In particular, although EnPEC possess adhesion factors such as the gene FimH for a fimbrial adhesin like most strains of *E. coli*, many other genes for virulence factors typical of enteric strains are absent (Sheldon *et al.* 2010; Goldstone *et al.* 2014b).

Whilst *E. coli* is isolated from the uterus in the first two weeks postpartum, it is *T. pyogenes* that causes most pathology (Sheldon *et al.* 2002; Sheldon *et al.* 2009; Westermann *et al.* 2010). Infection of the endometrium with *T. pyogenes* lasts several weeks postpartum and the presence of *T. pyogenes* is associated with pus in the uterus, the severity of clinical signs, and extent of infertility (Rowson *et al.* 1953a; Sheldon *et al.* 2002; Sheldon *et al.* 2009; Westermann *et al.* 2010). Furthermore, the presence of *T. pyogenes* is particularly correlated with histological and cytological evidence of endometrial pathology (Bonnett *et al.* 1991a; Westermann *et al.* 2010). A heat-labile exotoxin and a member of the cholesterol-dependent cytolysin family of pore-forming toxins, called pyolysin is the major virulence factor of *T. pyogenes* (Billington *et al.* 1997; Jost and Billington 2005). Cholesterol-dependent cytolysins are secreted in a water-soluble form but convert into amphipathic multimers in cholesterol-rich domains of the plasma membrane of mammalian cells to create 30-50 nm diameter transmembrane pores, which then disrupt ion balances and cause osmotic cytolysis (Gurcel *et al.* 2006; Gonzalez *et al.* 2011). The pyolysin gene is universal to all isolates of *T. pyogenes* (Silva *et al.* 2008); and all isolates express pyolysin protein (Amos *et al.* 2014). Furthermore, the pyolysin gene sequence is identical and the gene promoter is highly similar amongst clinical isolates of *T. pyogenes* (Amos *et al.* 2014; Goldstone *et al.* 2014a). Bacteria-free filtrates of the *T. pyogenes* cultures cause hemolysis and endometrial cytolysis, and pyolysin is the main cytolytic agent, because addition of anti-pyolysin antibody prevents cytolysis. Endometrial stromal cells are far more sensitive to cytolysis than epithelial cells or immune cells, when challenged with recombinant pyolysin or with native pyolysin in bacteria-free filtrates of *T. pyogenes* cultures. Stromal cells contain more cholesterol than epithelial cells, and reducing stromal cell cholesterol using cyclodextrins protects against pyolysin. The marked sensitivity of stromal cells to pyolysin-mediated cytolysis also provides an explanation for how *T. pyogenes* acts as an opportunistic pathogen to cause pathology of the endometrium only once the protective epithelium is lost after parturition.

An increased incidence of disease in dairy cows might be associated with changes in the microbes or their virulence. However, the microbes cultured from the uterus of diseased cows is similar now to those found throughout the last 50 years (Rowson *et al.* 1953b; Elliot *et al.* 1968; Griffin *et al.* 1974; Noakes *et al.* 1991; Williams *et al.* 2005). On the other hand, a range of less abundant or uncultivable bacteria are emerging as potentially important for uterine disease (Santos *et al.* 2011; Machado *et al.* 2012). Perhaps the interaction between multiple species of bacteria may be important for the onset of disease. This concept is not novel, and associations between anaerobic bacteria and aerobic bacteria in the uterus may underpin an increased risk of disease (Ruder *et al.* 1981). Similarly, there are synergistic interactions between bacteria in the endometrium and infection with bovine herpesvirus-4 (Donofrio *et al.* 2007; Donofrio *et al.* 2008). Conversely, lactobacilli and an acid pH in the vagina are protective against pathogens in many species (Ravel *et al.* 2011). Although, knowledge about the role of commensal bacteria to counter pathogens in the bovine reproductive tract is at an early stage, there is a deeper understanding of the host defense systems against microbes.

Host defence systems and immunity

Physical barriers

Host defence systems encompass several mechanisms for protection against microbes and tissue damage in the female reproductive tract (Figure 1). The obvious anatomical barriers include the vulva, vagina and cervix, and they counter microbial infections ascending the reproductive tract to reach the uterus; beyond the uterus the uterine tube (oviduct) impedes bacteria reaching the ovary. Other physical barriers in the reproductive tract include the stratified squamous epithelium of the vagina, the columnar epithelium of the endometrium, the surface epithelium of the ovary, the basement membrane of ovarian follicles, and the zona pellucida of the oocyte.

Non-specific defences

Organisms respond to challenges such as tissue damage and infection, with a coordinated sequence of local and systemic changes, which are often manifest as inflammation, influx of immune cells, changes in metabolism, activation of the complement system and innate immunity, and induction of acute phase proteins. The bovine reproductive tract is no exception and there are multiple defence systems against microbes (Fig. 1). The aim of all these responses is to return the cells, tissue and the animal to a state of homeostasis (Medzhitov 2008). Several studies have examined the expression of genes in endometrial biopsies or in cytobrush samples of the surface of the endometrium, and compared gene expression between animals with uterine disease or inflammation and normal animals (Table 1).

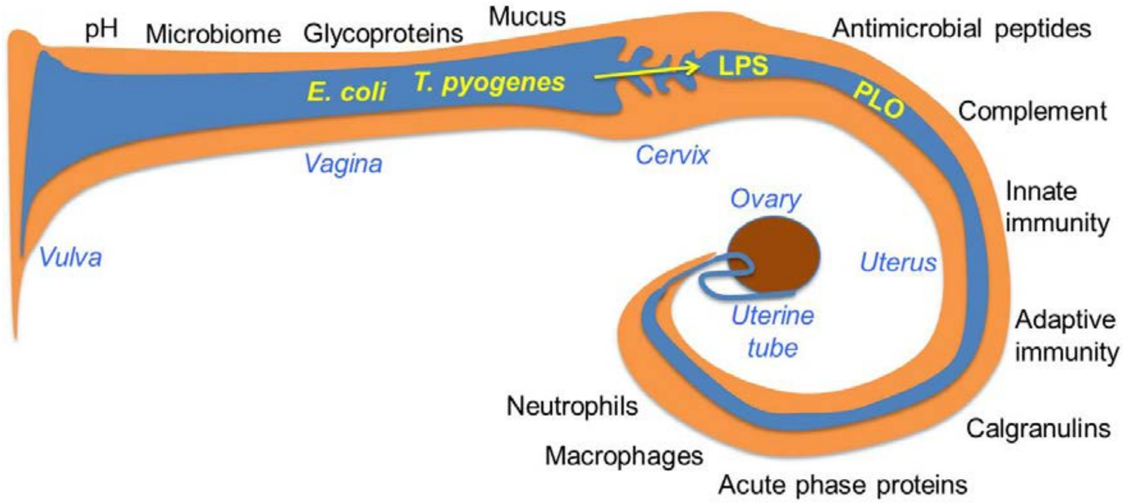

Figure 1 Defence systems in the bovine female reproductive tract
Microbes such as E. coli and T. pyogenes infect the postpartum uterus, where their toxins LPS and PLO cause inflammation and tissue damage. However, multiple defence systems protect the female reproductive tract against these microbes, including the physical barriers of the vulva and cervix, the microbiome, vaginal mucus, and a range of non-specific immune defences. In the endometrium components of the innate and adaptive immune systems counter the microbes and their toxins, including influx of neutrophils and macrophages to clear the invading bacteria.

Antimicrobial peptides and mucosal glycoproteins cover the mucosa of the vagina, cervix and endometrium, where they neutralise bacteria and prevent them reaching the plasma membrane of the epithelia. The principal cysteine-rich, cationic, antimicrobial peptides expressed in the bovine endometrium include β-defensins, lingual antimicrobial peptide (LAP) and tracheal antimicrobial peptide (TAP), and several gene transcripts for antimicrobial peptides are more abundant in the face of microbial challenge (Davies *et al.* 2008; Chapwanya *et al.* 2009).

Another group of molecules that may help protect the endometrium include the mucins and enzymes in mucus. Evidence for their role includes genetic deletion of *Muc1* in mice, which is associated with chronic inflammation of the lower female reproductive tract by opportunistic bacterial infections (DeSouza *et al.* 1999). The expression of MUC1 is induced when endometrial epithelial cells are treated with endotoxin from *E. coli* (Davies *et al.* 2008); and, in cytobrush samples of the endometrial epithelium of cows with uterine disease (Kasimanickam *et al.* 2014). Lysozyme gene expression is also increased in the endometrium of cows with uterine inflammation, and lysozyme digests the peptidoglycans found in the cell walls of bacteria (Hoelker *et al.* 2012).

Acute phase proteins
Acute phase proteins are synthesised in the liver, often in response to increased peripheral plasma concentrations of cytokines such as IL-6, and they have important functions in restoring homeostasis after infection or tissue damage (Baumann and Gauldie 1994). These functions include haemostasis typified by the action of fibrinogen, anti-microbial effects, and the attraction and activation of phagocytes. The severity of bacterial contamination is associated with the concentrations of the acute phase proteins in peripheral plasma, including α_1-acid glycoprotein, haptoglobin and ceruloplasmin, particularly in the presence of uterine infections with *E. coli* and *T. pyogenes* (Smith *et al.* 1998; Sheldon *et al.* 2001). However, the acute phase response is not only initiated by infection but also by trauma (Baumann and Gauldie 1994). Indeed, as uterine involution progresses there is a decrease in the concentrations of α_1-acid glycoprotein, haptoglobin and ceruloplasmin (Sheldon *et al.* 2001). The expression of genes encoding acute phase proteins has also been noted in the uterus and ovary, which may be of interest because they may provide further localized protection (Chapwanya *et al.* 2009; Fischer *et al.* 2010; Lecchi *et al.* 2012). However, it is possible that the more usual and more abundant hepatic production of acute phase proteins may be more relevant *in vivo*.

Complement
The Complement system is expressed in the female reproductive tract, and this series of related proteins opsonise infected cells to attract immunoglobulin and drive the formation of the membrane attack complex leading to cytolysis (Morgan 1995). However, normal cells in the reproductive tract are protected against formation of the complement complex by complement regulatory proteins including CD46, CD55 and CD59 (Jensen *et al.* 1995). Components of the complement system, such as the genes *C1QA*, *C1QB*, *C1QC*, *C3* and C8 are differentially expressed in the endometrium of postpartum cows with more severe negative energy balance (Wathes *et al.* 2009).

Table 1 Differentially expressed genes in the endometrium or endometrial cytology samples between normal animals and postpartum cows with uterine disease

Ontology	**Differentially expressed Genes**	**Supporting references**
Cytokines	*IL1A, IL1B, IL6, TNF, IL12A, IL1R1, IL1R2*	Chapwanya *et al.* 2009; Gabler *et al.* 2009; Herath *et al.* 2009b; Fischer *et al.* 2010; Gabler *et al.* 2010; Galvao *et al.* 2011; Ghasemi *et al.* 2012; Kasimanickam *et al.* 2014
Chemokines	*CXCL5, CXCXL8*	Fischer *et al.* 2010; Gabler *et al.* 2010; Galvao *et al.* 2011; Ghasemi *et al.* 2012; Kasimanickam *et al.* 2014
Prostaglandins	*PTGS1, PTGS2, PTGDS*	Gabler *et al.* 2009; Gabler *et al.* 2010
Innate Immunity	*TLR4, NFKB1*	Chapwanya *et al.* 2009; Herath *et al.* 2009b; Kasimanickam *et al.* 2014
Mucins	*MUC1*	Kasimanickam *et al.* 2014
Antimicrobial peptides	*TAP, DEFB5, DEFB1*	Chapwanya *et al.* 2009
Acute phase proteins	*HP, SAA3*	Chapwanya *et al.* 2009
Metabolism	*IGF1*	Kasimanickam *et al.* 2014

Innate immunity

Innate immunity provides immediate defence responses against bacteria and tissue damage. In particular, cellular receptors such as Toll-like Receptors (TLRs) and NOD-like Receptors (NLRs) bind pathogen-associated molecular patterns (PAMPs) (Takeuchi and Akira 2010; Moresco *et al.* 2011). For example, TLR2 on cells binds to bacterial lipopeptides, whilst TLR4 binds to the lipopolysaccharide (LPS) of Gram-negative bacteria, such as *E. coli*. Binding of PAMPs to TLRs activates intracellular signalling pathways involving mitogen-activated protein kinases (MAPK; p38, JNK, ERK1/2), and nuclear factor kappa-B (NF-κB), resulting in the production of inflammatory mediators. This innate immune response typically induces the secretion of IL-1β, IL-6, IL-8 and prostaglandin E_2, which attract and activate immune cells such as neutrophils and macrophages to clear the bacteria (Takeuchi and Akira 2010; Moresco *et al.* 2011). The release of mature IL-1β is dependent on activation of the multiprotein inflammasome complex, often containing NLRP3 (nucleotide-binding domain and leucine rich repeat pyrin 3 domain), leading to caspase-1 activation to cleave pro-IL-1β to mature protein (Schroder and Tschopp 2010). Interestingly, the inflammasome may also be activated by cholesterol-dependent cytolysins or the ion fluxes they induce (Gurcel *et al.* 2006; McNeela *et al.* 2010); this may be important for *T. pyogenes* infections because the bacteria secretes pyolysin (Amos *et al.* 2014).

Innate immunity is conserved across species from insects to mammals. The genes of the receptors used by the innate immune system are principally expressed by hematopoietic cells such as macrophages and neutrophils (Takeuchi and Akira 2010; Moresco *et al.* 2011). However, bovine endometrial epithelial and stromal cells express most *TLR* genes (Davies *et al.* 2008). Furthermore, using siRNA to test functionality, these epithelial and stromal cells respond to bacteria, LPS and lipopeptides, via TLR1, TLR2, TLR4 and TLR6 by secreting IL-1β, IL-6, IL-8 and prostaglandin E_2 (Herath *et al.* 2006; Cronin *et al.* 2012; Turner *et al.* 2014). Furthermore, the gene expression of *IL1B, IL6, CXCL8* (encoding IL-8), and *TLR4* is increased *in vivo* in the endometrium of cows with uterine disease (Herath *et al.* 2009b; Kasimanickam *et al.* 2014). Innate immunity also has roles in the inflammatory response to tissue damage, when receptors bind damage-associated molecular patterns (DAMPs) (Chen and Nunez 2010). This may be relevant to the uterus, and the role of DAMPs needs urgent investigation because parturition and *T. pyogenes* both cause damage to the endometrium (Bonnett *et al.* 1991a).

Changes in the expression of genes associated with innate immunity have been studied in tissues or cells collected from the endometrium of cows with uterine disease and compared with normal animals (Table 1). Probably the most consistent change in gene expression is increased expression of *CXCL8*, which is a chemokine that attracts immune cells, particularly neutrophils. The increased expression of *CXCL8* is consistent with the accumulation of neutrophils and formation of pus in diseased animals. The expression of several other cytokines, chemokines and genes associated with innate immunity are also usually increased in diseased endometrium, including *IL1A*, *IL1B*, *IL6*, *CXCL8*, *CXCL5*, *NFKB1*, and *TLR4* (Table 1). For some gene transcripts, such as *TNF*, the picture is more confusing with some reports of increased gene expression (Fischer *et al.* 2010; Gabler *et al.* 2010; Ghasemi *et al.* 2012; Kasimanickam *et al.* 2014); whereas others find reduced gene expression (Chapwanya *et al.* 2009; Galvao *et al.* 2011). Furthermore, at the protein level, PAMPs do not stimulate detectable concentrations of TNFα from endometrial epithelial or stromal cells *in vitro* (Turner *et al.* 2014). Prostaglandins are essential hormones for normal physiological function in the endometrium but they also have roles in innate immunity, and genes for rate limiting enzymes in prostaglandin synthesis are up-regulated in diseased endometrium (Table 1). These findings are supported by *in vitro* evidence for the importance of prostaglandin E_2 synthesis in bovine endometrial cells (Herath *et al.* 2009a).

Adaptive immunity

The role of adaptive immunity in the endometrium is more implied than explicit for postpartum uterine disease, although areas rich in T cells and B cells are evident in the postpartum endometrium, often as lymphocytic foci within the stroma (Wagner and Hansel 1969; Bonnett *et al.* 1991b).

Clearly adaptive immunity must play a role and preliminary data on a vaccine for metritis suggests that immunoglobulins must provide some protection against disease (Machado *et al.* 2014).

Environmental risk factors for uterine disease

The environment is an important determinant of susceptibility to uterine disease, and many risk factors have been associated with uterine disease (Table 2). Indeed, these environmental factors may be more important than genetic factors. For example, although some polymorphisms in genes for bovine *TLR2*, *TLR4*, *TLR6*, and *TLR9* may elicit small effects on uterine health in dairy cows, environmental factors such as dystocia, parity, and ketosis are more predictive for the incidence of uterine disease than the genetic markers evaluated so far (Pinedo *et al.* 2013).

Table 2 The environment and uterine disease

Environment	Risk factor	Example references
Trauma and tissue damage	Retained fetal membranes	Paisley *et al.* 1986; Bruun *et al.* 2002; Kim and Kang 2003; Han and Kim 2005; Dubuc *et al.* 2010; Potter *et al.* 2010
	Male calf	Potter *et al.* 2010
	Stillbirth	Markusfeld 1984; Potter *et al.* 2010
	Twins	Markusfeld 1984; Dubuc *et al.* 2010; Potter *et al.* 2010
	Dystocia	Dubuc *et al.* 2010; Potter *et al.* 2010; Pinedo *et al.* 2013
	Induction of parturition	Markusfeld 1984
	Parity	Markusfeld 1984; Kim and Kang 2003
Metabolism	Milk fever	Bruun *et al.* 2002; Whiteford and Sheldon 2005
	Reduced feed intake ante partum	Huzzey *et al.* 2007
	Ketosis	Markusfeld 1984; Bruun *et al.* 2002; Dubuc *et al.* 2010; Pinedo *et al.* 2013
	Left displaced abomasum	Markusfeld 1984
	Metabolic disorder	Kim and Kang 2003
Hygiene	Calving season	Markusfeld 1984; Bruun *et al.* 2002
	Angle of vulva	Potter *et al.* 2010

Trauma

Several of the environmental risk factors are associated with tissue trauma and disruption to the normal processes of parturition. Obvious causes of trauma include dystocia, a large male calf, stillbirths, twins, first parity and induction of parturition (Table 2). However, retained foetal membranes are the most important risk factor for uterine disease and have by far the greatest impact on the likelihood of disease (Paisley *et al.* 1986; Kim and Kang 2003; Potter *et al.* 2010). The necrotic material associated with retained fetal membranes provides a favourable environment for bacterial growth in the uterine lumen, and retained membranes negate the physical barrier provided by the cervix, and delay uterine involution. Unfortunately, the causes of retained fetal membranes are multifactorial, including a genetic component (Joosten *et al.* 1991; Laven and Peters 1996). Tissue repair is obviously important to counter the negative effects of trauma and as part of the healing process in the endometrium after parturition.

Metabolism

Uterine disease is associated with changes in metabolism after parturition or diseases that disrupt metabolism, such as left displaced abomasum (Table 2). Dairy cows are under metabolic stress because they cannot consume enough food to meet the substantial extra demand for nutrients that are required for lactation. At the whole animal level, the metabolisable energy required every day to produce 40 litres of milk is about 200 MJ; three times the 65 MJ needed for normal resting metabolism. Consequently, postpartum dairy cows lose weight as tissues are broken down to satisfy the dietary energy and protein deficits (Chagas *et al.* 2007). The animals also develop insulin resistance; and have reduced blood concentrations of insulin-like growth factor (IGF-1) and glucose, and the mobilization of fat reserves increases the concentration of ketones such as acetoacetate and β-hydroxybutyrate (Chagas *et al.* 2007; Wathes *et al.* 2011). It is thought that the metabolic stress facing postpartum dairy cows compromises their peripheral blood immune cell function; although the biochemical and molecular mechanisms are not always clear (Hammon *et al.* 2006; Mendonca *et al.* 2013). A group of genes that are up-regulated in the endometrium of animals with more severe negative energy balance are the antimicrobial calgranulins of the S100 family, and their increased gene expression is supported by changes in protein abundance in the endometrium (Wathes *et al.* 2009; Swangchan-Uthai *et al.* 2013). The S100A8 and S100A9 proteins attract neutrophils to sites of inflammation and stimulate neutrophil activity. Similarly, genes associated with adaptive immunity such as *HLA-DQB1*, and genes involved in extracellular matrix homeostasis, such as the matrix metallopeptidases *MMP1*, *MMP3*, *MMP9* and *MMP13*, are differentially regulated in cows with severe negative energy balance compared with more normal animals (Wathes *et al.* 2009).

Hygiene

It is intuitive that the hygiene of the calving environment and the postpartum housing should be important for uterine disease. This might be reflected in the association between calving season and uterine disease, and an angle of the vulva that allows faecal contamination of the vagina (Table 2). However, direct evidence for the importance of hygiene in the postpartum environment is limited and some studies find that the level of hygiene is relatively unimportant (Noakes *et al.* 1991; Potter *et al.* 2010).

Impact of uterine disease on the ovary

Whilst the triad of microbes, immunity and the environment, dictate the severity of uterine disease, infertility is also dependent on the impact of that disease on ovarian function. Dairy cows with postpartum uterine infections have a slower growth of the dominant follicle, lower peripheral plasma oestradiol concentrations, and are less likely to ovulate (Sheldon *et al.* 2002). At the herd level, a history of uterine infection or problem calving are associated with a delay in the return to ovarian cyclic activity, and with prolonged luteal phases (Opsomer *et al.* 2000). One possible mechanism linking uterine disease to ovarian dysfunction is that the cytokines associated with the host defence response to bacteria in the uterus may reach the ovary via the localised counter-current mechanism, as used by prostaglandin $F_{2\alpha}$ during luteolysis. For example, cytokines such as IL-6 and TNFα perturb bovine ovarian follicular cell steroidogenesis (Alpizar and Spicer 1994; Spicer 1998). A second mechanism is that cytokines and PAMPs perturb the endocrine function of the hypothalamus and the pituitary, reducing the release of GnRH and LH, which impacts ovarian function (Peter *et al.* 1989; Karsch *et al.* 2002). A third mechanism linking infection of the endometrium with ovarian function is that, PAMPs might also reach the ovary from the uterus, and the concentrations of LPS in follicular fluid aspirated from dominant follicles is correlated with the severity of uterine disease (Herath *et al.* 2007). Notably, healthy ovarian follicles do not contain hematopoietic immune cells, so ovarian follicle responses to cytokines or PAMPs must rely on the granulosa cells and oocyte (Herath *et al.* 2007; Bromfield and Sheldon 2011). Indeed, LPS limits granulosa cell oestradiol production by reducing *CYP19A1* gene expression and aromatase protein levels (Herath *et al.* 2007; Price *et al.* 2013). Interestingly, granulosa cells isolated from growing or dominant ovarian follicles express most of the TLRs (Bromfield and Sheldon 2011; Price *et al.* 2013). Furthermore, PAMPs such as bacterial LPS or bacterial lipopeptides stimulate an inflammatory response by granulosa cells, with the secretion of IL-1β, IL-6, CXCL1, CXCL2, CXCL3 and IL-8 protein (Bromfield and Sheldon 2011; Price *et al.* 2013). Inhibiting *TLR4* or *TLR2* gene expression in bovine granulosa cells using siRNA reduced the secretion of IL-6 in response to their cognate PAMPs (Bromfield and Sheldon 2011; Price *et al.* 2013). So, granulosa cells in antral follicles clearly have roles in innate immunity. Furthermore, LPS reduces the primordial ovarian follicle pool, with an associated increase in primordial follicle activation, and loss of primordial follicle expression of phosphatase and tensin homolog (PTEN) and cytoplasmic translocation of forkhead box O3 (FOXO3) proteins (Bromfield and Sheldon 2013). However, not all stages of follicle development are sensitive to PAMPs, and LPS did not affect the growth and viability of individually cultured secondary follicles or their enclosed oocytes (Bromfield and Sheldon 2013).

At later stages of ovarian follicle development, LPS stimulates IL-6 secretion from cumulus-oocyte complexes and activates cumulus expansion *in vitro* (Bromfield and Sheldon 2011). Inappropriate timing of cumulus expansion may contribute to infertility because expansion is normally closely coordinated with ovulation. Furthermore, LPS or IL-6 might reach the oocyte via the cytoplasmic trans-zonal projections from granulosa cells that synapse on the oolema. Indeed, LPS increases the incidence of meiotic arrest and germinal vesicle breakdown failure in bovine oocytes (Bromfield and Sheldon 2011). Furthermore, treatment of cumulus-oocyte complexes with LPS or PAM perturbed expression of genes such as *GDF9* and *NLRP5*, which are involved in oocyte maturation (Sheldon *et al.* 2014).

Conclusion

Resistance to development of uterine disease depends on the pathogenicity of the microbes infecting the endometrium, the host defence responses to those microbes, and environmental factors that impact the balance between microbes and immunity. Non-specific defence systems counter ascending infections of the female reproductive tract after parturition. However, the microbes that cause uterine disease are adapted to the endometrium and their toxins cause tissue damage and inflammation. Much of the inflammatory response in the postpartum endometrium is driven by innate immunity, and many of the genes differentially expressed when there is infection of the endometrium are linked to the innate immune response. However, the greatest factor facilitating the development of postpartum uterine disease appears to be related to trauma to the reproductive tract and to the metabolic stresses of lactation in dairy cows. Selection for disease resistance and optimising the care of the periparturient animal are likely important for long-term solutions to uterine disease.

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