New frontiers in molecular endometritis research  
Robert O. Gilbert*  
College of Veterinary Medicine, Cornell University, Ithaca, NY

Abstract  
Postpartum uterine disease is common in dairy cows. The earliest pathogen seems to be E. coli, but it soon disappears, giving way to Trueperella pyogenes and the gram negative anaerobes, especially Fusobacterium necrophorum, Prevotella melaninogenica and Bacteroides spp. Specific strains of the organisms, expressing specific virulence factors appear to be responsible for mediating uterine disease. Susceptibility to postpartum uterine disease is increased by negative energy balance and deficiency of other nutrients. These deficiencies blunt the postpartum inflammatory response resulting in more sluggish uterine recruitment of leukocytes in the early postpartum period and a more prolonged course of infection and inflammation, with profoundly detrimental consequences for reproduction. Uterine inflammation impairs reproduction locally by influencing sperm function and longevity as well as zygote and embryo development, but also more systemically by reducing pituitary secretion of gonadotrophins and altering follicular function to reduce steroidogenesis and oocyte competence. Some of the pathways accounting for these effects are reviewed here.

Keywords: Cow, embryo, infertility, inflammation, uterus

Introduction  
Postpartum uterine disease is common in dairy cows, with as many as 50% of animals per lactation being affected by at least one of metritis, purulent vaginal discharge, endometritis or cervicitis.1 Since these conditions contribute to infertility and increase the risk of culling they are extremely costly to producers. Furthermore, our current ability to prevent or treat uterine disease is limited. These diseases constitute a welfare issue since they cause discomfort to cows.2 Collectively, the prevalence of postpartum uterine disease, its effect on fertility and welfare and its contribution to antibiotic use in food production make the disease complex important to producers and society as a whole and it is an active field of investigation.

This presentation will focus on a few themes:
1. Which pathogens are primarily responsible for endometritis?
2. Which metabolic and immune factors mediate susceptibility to and pathogenesis of endometritis?
3. How does endometritis mediate infertility?

Which pathogens cause endometritis?  
Most cows have postpartum bacterial infection or contamination of the uterus. The mixed bacterial population characteristic of the early postpartum period was originally judged to be insignificant but persistence of Trueperella pyogenes (formerly Actinobacillus pyogenes, formerly Corynebacterium pyogenes) after 21 days postpartum was shown to be detrimental to subsequent reproduction.3 The synergistic relationship between T. pyogenes and the gram negative anaerobes Fusobacterium necrophorum, Prevotella melaninogenica and Bacteroides spp. in the context of pathological uterine infection was also recognized early. Interestingly, the infection rate increases after the first postpartum day, reaching a maximum at seven to 11 days after parturition.4,5

Classical, culture-based, bacteriological methodology has confirmed the importance of the traditional pathogens in bovine uterine disease, but has also revealed an important role for Escherichia coli. Although E. coli are seldom isolated from diseased uteri, early invasion of the uterus by E. coli

* Current address: Ross University School of Veterinary Medicine, Basseterre, St. Kitts, West Indies
seems to be an important step in paving the way for the traditional pathogens. On the basis of measuring lipopolysaccharide (LPS) concentration in lochia Dohmen et al. concluded that the early presence of E. coli predisposed cows to infection with T. pyogenes and gram negative anaerobes, particularly P. melaninogenica. We found E. coli to be the most common isolate from the bovine uterus in the early postpartum period and that its presence predisposed to later infection with T. pyogenes and F. necrophorum. The mechanism whereby E. coli paves the way for later infection is not known but this organism has been reported to impair neutrophil function. Specific strains of E. coli are associated with bovine uterine disease. Uteropathogenic E. coli belonged to various phylogenetic groups, serotypes and multilocus sequence types (MLST), but showed some characteristics in common: they were more likely to adhere to cultured uterine epithelial cells, and were more likely to invade these cells (becoming intracellular), and they caused more severe disease when infused into the uteri of mice.

Cows are more likely to be infected with uterine E. coli in the early postpartum period if they gave birth to twins or stillborn calves, had retained fetal membranes, or were in poor body condition, all factors known to increase the risk of metritis. Strains of E. coli that were pathogenic in the uterus were more likely to contain the virulence factors fimH, astA, ibeA, hlyA and kpsMII. Several of these virulence factors were found to increase the risk of purulent vaginal exudate later in the postpartum period and to be associated with impaired reproduction.

Metagenomic methods allow identification and quantification of bacteria independent of culture. This means that relative abundance of bacteria, some of which may be difficult to culture in vitro, can be determined accurately. Given the importance of anaerobic organisms in pathogenesis of uterine disease, this is an important attribute. Initial studies showed significantly different bacterial microbiomes in cows with healthy or unhealthy postpartum course. All factors known to increase the risk of metritis. Strains of E. coli that were pathogenic in the uterus were more likely to contain the virulence factors fimH, astA, ibeA, hlyA and kpsMII. Several of these virulence factors were found to increase the risk of purulent vaginal exudate later in the postpartum period and to be associated with impaired reproduction.

Machado, et al. obtained uterine samples at 35 days postpartum and identified bacteria by individual amplification of 16S rRNA followed by pyrosequencing. Bacteroides spp., Ureaplasma spp., Fusobacterium spp., Peptostreptococcus spp., Sneathia spp., Prevotella spp. and Trueperella spp. prevalence was significantly (P < 0.05) higher in samples derived from cows that had concurrent endometritis. Bacteroides spp., Ureaplasma spp., Fusobacterium spp., and Trueperella spp. prevalence was significantly (P < 0.05) higher in samples derived from cows that were not pregnant by 200 DIM. Subsequent studies have yielded similar, but not identical results, suggesting some farm-specific or regional variation in pathogen importance. For example, Jeon, et al. reported that Bacteroides spp. was much more important than F. necrophorum in the mediation of metritis. Interestingly, Knudsen, et al., while reporting presence of generally similar bacterial phyla, showed a difference in the microbiota of the endometrium and the uterine lumen, a finding that should receive more attention.

Virulence factors for major bacteria involved in pathogenesis of postpartum uterine disease have been investigated. By using major virulence factors as markers for specific species of bacteria, increasing diversity of pathogen load from calving to ten DIM was again confirmed. The E. coli virulence factor fimH, when present in the early postpartum period increased the odds of developing metritis by almost 5-fold while the lktA virulence factor (F. necrophorum) increased odds by 2.6. Virulence factors associated with risk of development of purulent vaginal discharge diagnosed at 35 DIM included E. coli fimH at 1-3 DIM and T. pyogenes fimA at 8-10 or 34-36 DIM. Cows in which the E. coli virulence factor fimH was detected at 1-3 DIM were significantly less likely to become pregnant, regardless of other findings in the postpartum period.

The close association of specific virulence factors with development of postpartum uterine disease prompted us to attempt a vaccine trial. We tested several permutations of bacteria and virulence factors (recombinant virulence factors alone, inactivated bacteria alone, or a combination of bacteria and virulence factors) and two routes of administration (subcutaneous or intravaginal). All three vaccines, when administered subcutaneously but not intravaginally, reduced incidence of metritis and postpartum fever and improved subsequent reproduction. Follow up testing is under way. Additional research should be devoted to sequential changes in uterine microbiome in the healthy or complicated postpartum
period, with a view to understanding mechanisms of synergism and antagonism between bacteria and potentially also viruses.

**Metabolic and immune mechanisms mediating endometritis**

Epidemiologic and experimental studies consistently demonstrate that negative energy balance in the periparturient period is a major risk factor for endometritis. The mechanisms linking energy deficit and endometritis are emerging. Cows that develop metritis or endometritis have diminished intracellular reserves of glycogen in circulating neutrophils in the period preceding development of disease. This may directly reflect lack of available energy substrates and, since neutrophils depend on intracellular energy sources for migration, phagocytosis and killing, it directly impairs immune function. We have recently shown that cows with the healthiest postpartum course recruit neutrophils to the uterus in greater numbers in the immediate postpartum period. These cows have fewer intrauterine bacteria and resolve postpartum infection and inflammation more promptly than cows with a more sluggish initial recruitment of leukocytes to the uterus, in which infection and inflammation linger, eventually being manifest as cytological endometritis. This robust recruitment of neutrophils to the postpartum uterus is reflected in increased endometrial expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) or interleukin (IL) 1β in the immediate postpartum period. The effect may be generalized as circulating monocytes of cows destined to develop metritis similarly express less mRNA for proinflammatory cytokines when challenged in vitro with *E. coli*. Consistent with these results from our group, others have also shown that endometrial gene expression is pro-inflammatory in the early postpartum period but changes to a predominantly tissue-remodeling environment by about 21 days postpartum in cows with a healthy postpartum course compared to diseased cows in which the proinflammatory environment persists. Other workers have also linked this persistent state of inflammation with severe negative energy balance and have confirmed that cows with more severe negative energy balance recruit uterine neutrophils less effectively in the first week postpartum. Several investigators have investigated the pathways linking negative energy balance, metabolic status and immune function, with particular reference to the uterus. Uterine inflammatory responses are diminished by lack of glucose availability. Restricting glucose resulted in dose responsive reduction in IL-1β, IL-6 and IL-8 in response to LPS challenge. This effect appears to be mediated by AMP-activated protein kinase (which senses the AMP:ATP ratio as a measure of energy availability). mTOR, a more general sensor of nutrient deficit did not affect response to LPS in this experiment. Importantly, this paper also showed that uterine response to LPS is energetically expensive. Interestingly, many differences have emerged between first lactation cows and multiparous animals, and these differences should be accounted for in future studies.

Fundamental to the function of the innate immune system is recognition of potential pathogens by so-called pattern recognition receptors, the Toll-like receptors (TLR), NOD-like receptors (NLR), retinoic acid-inducible gene I (RIG-1)-like receptors and C-type lectin receptors (CLR). Expression of most of the pattern recognition receptors has been found in uterine tissue. Interestingly, purified populations of uterine epithelial or stromal cells also express many of these molecules, meaning that these cells themselves, and not just professional immune cells within the uterus, can respond directly to pathogens. Stimulation of the pattern recognition receptors in the bovine endometrium results in marked increases in MAPK p38 and ERK 1/2 and nuclear translocation of NFκB, and increased expression of IL-1β, IL-6 and IL-8. Accumulation of IL-6 in peripheral circulation stimulated production of acute phase proteins in the liver. Cows express α1-acid glycoprotein, haptoglobin and ceruloplasmin after parturition, and their increase is further stimulated in cattle experiencing severe uterine infection.

Another metabolic indicator of increased susceptibility to postpartum uterine disease is cholesterol. Diminished circulating cholesterol has long been associated with uterine disease and poorer fertility and cholesterol, in turn, is reduced in cows with more severe negative energy balance. It has recently been shown that manipulation of the mevalonate pathway, specifically inhibition of squalene synthase, dampens inflammatory response to LPS in cultured bovine endometrium, providing a novel
potential therapeutic or preventive approach. Dietary manipulation of periparturient lipid metabolism may also hold potential for management of postpartum disease.42

How does endometritis mediate infertility?

It may seem intuitive that endometritis should be associated with infertility since loss of function is a hallmark of inflammation in general. However, much work has been devoted to understanding mechanisms of endometritis-related infertility with some interesting results. Detrimental effects may be mediated by direct effect of pathogens themselves, or indirectly by the effects of inflammatory mediators such as cytokines, eicosanoids, nitric oxide and oxidative stress on sperm, ovarian, uterine and embryonic and endocrine function.43

Uterine infection and inflammation have several effects at distant sites. Uterine infection is associated with slower postpartum follicular growth, diminished follicular estradiol synthesis, increased risk of anovulation.8,44-46 Delayed postpartum ovulation is associated with impaired reproductive performance,47,48 and this effect is independent of and additive to, the effect of endometritis.49 The effects of uterine infection on ovarian function are mediated in part by an effect at the hypophysis. Uterine infection of administration of exogenous LPS reduce luteinizing hormone (LH) pulsatility.50-52 However, LPS from the uterus has a direct effect on follicular function.53 Follicular cells express pattern recognition molecules, and their stimulation can perturb follicular steroid synthesis. Lipopolysaccharide has been found in follicular fluid and its concentration was proportional to the degree of endometrial inflammation.53

It also seems likely that endometritis may directly affect oocyte quality, an effect that persists after resolution of the active inflammation. Evidence for this lies in the observation that fertilization failure, rather than (or in addition to) embryonic death, is frequently observed in cows that previously had endometritis.54,55 A similar observation has been made in embryo donors with mastitis; fertilization rate improves once mastitis is resolved (R.J. Mapleton, personal communication, 2011). We have also observed an increased number of unfertilized oocytes and fewer transferrable embryos from donor cows with cytologic evidence of endometritis (R.O. Gilbert and M. Frajblat, unpublished observations). Exposure of bovine oocytes to LPS during in vitro maturation reduced the proportion developing to blastocysts but exposure to LPS after fertilization had no effect.56 In consideration of the effect of endometritis on follicular function it is important to recall the nature of the utero-ovarian circulation and the ease with which inflammatory products could be transferred from uterine vein to ovarian artery, using the mechanism described for ipsilateral transfer of prostaglandin.57-60 The basement membrane of the follicle is porous to diffusion of molecules of up to 850 kD.61 In addition to LPS, inflammatory mediators TNFα and IL-1 inhibit follicular steroidogenesis.62,63

Local inflammation in the uterus could affect sperm survival and function. Sperm motility and zona binding are impaired in an inflammatory environment63,64 and sperm phagocytosis is increased65 (in women and horses). Bovine sperm exposed to oxidative stress suffer impaired motility, oocytes exposed to such sperm are less likely to undergo cleavage, and those that do cleave are less likely to result in blastocysts.66

In vitro produced bovine embryos co-cultured with neutrophils, even for as long as six days, suffer only very minor developmental impediment67 while those exposed even briefly to cell-free uterine secretion of cows with experimentally induced aseptic endometritis have fewer trophoeotdorm cells, possibly resulting in defective pregnancy recognition.68 Several inflammatory mediators impair embryo development. Exposure to nitric oxide completely inhibits development to blastocyst stage in vitro69 and development may be improved in the presence of a nitric oxide scavenger.69 TNFα increases blastocyst apoptosis.70 Loss of established pregnancy (after 28 days) is common in dairy cows71 and is increased in cows previously diagnosed with uterine disease.72

Conclusion

Postpartum uterine disease in cattle is common and appears to be mediated by specific pathogens expressing specific virulence factors. Susceptibility to such infection is greatest in cows suffering deficits
in energy and other nutrients. The effect of these deficiencies seems to be to blunt the immediate postpartum recruitment of leukocytes to the uterus, with a consequently prolonged course of interaction with pathogens and longer residual inflammation. Endometritis exerts detrimental effects on inflammation at the pituitary and ovarian levels as well as in the uterus itself with consequences on fertility that persist after the resolution of the initial inflammation and may be seen as fertilization failure, early or late embryonic death. Progress in understanding of mechanisms involved is likely to enhance our ability to prevent endometritis and mitigate its effects.

References


