Future concepts in combating uterine disease depend on understanding the mechanisms of infection and immunity

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ABSTRACT

Dairy cows help feed the world by converting proteins from plants to higher value proteins in milk, which have a more appropriate essential amino acid profile for human consumption and are more readily digestible. Lactation depends on pregnancy but following uterine disease after parturition many dairy cows are infertile, which compromises global food security. Uterine disease is caused by *Escherichia coli, Trueperella pyogenes*, anaerobic bacteria and viruses. Specific strains of microbes are tropic for the uterus, including endometrial pathogenic *E. coli* (EnPEC) and Bovine Herpesvirus-4. In the endometrium, epithelial and stromal cells are the first line of defence against microbes and they have key roles in innate immunity. Endometrial cells possess Toll-like receptors to detect pathogen-associated molecular patterns, leading to the secretion of chemokines and cytokines, which attract macrophages and neutrophils. Uterine disease also compromises the function of ovarian follicles and the corpus luteum. Granulosa cells from ovarian follicles express Toll-like receptors, and pathogen-associated molecular patterns perturb their endocrine function, stimulate the secretion of inflammatory mediators and damage oocytes. The inflammatory response to microbes can be limited by treating endometrial cells with inhibitors that target intracellular signalling pathways. Understanding the mechanisms of infection and immunity in the female genital tract is driving smarter discovery of therapeutics to combat uterine disease.

KEYWORDS: Cow, endometritis, metritis, uterus, infection, innate immunity, TLR, bacteria

INTRODUCTION

Feeding the ever expanding population of the world is the grand challenge facing plant and animal scientists (Campbell 2010). Dairy cows help to feed the world population by converting low quality protein from plants to higher value proteins in milk, which have a more appropriate essential amino acid profile for human consumption and are more readily digestible. The protein digestibility-corrected amino acid score, adopted by the FAO/WHO as the preferred method for the measurement of protein value in human nutrition, calculates that milk has at least twice the value compared with plant proteins in cereal-based diets (Schaafsma 2000). Furthermore, milk is a tradable commodity that is easily processed into many foods and products. Lactation, of course, depends on fertility and pregnancy – no calf, no milk. However, global dairy production is facing a major obstacle because the fertility of dairy cows is at an all time low, due in a large part to post partum uterine disease (Sheldon and others 2009).

Bacteria contaminate the uterine lumen of about 90% of dairy cattle after parturition and half of these animals subsequently develop clinical disease. Uterine disease ranges from metritis within the first two weeks post partum to endometritis that can persist for several weeks. These clinical diseases affect more than one million cows each year in the UK alone. An emerging challenge is the recognition that there are also many animals that have subclinical endometritis. However, the extent of the problem is not obvious to most clinicians or farmers because currently the diagnosis of subclinical uterine disease depends on laboratory assessment of the proportion of neutrophils in endometrial cytobrush samples.

Uterine disease causes infertility, not just sub-fertility, and is therefore a significant burden on the dairy industry. The cost of replacing infertile animals, reduced milk yields and treatments for uterine disease is €1.4 billion each year in the EU (Sheldon and others 2009). Additionally, there is a wider environmental impact incumbent on keeping more replacement animals which use more land, increase water degradation and increase greenhouse gas emissions. Restoring cattle fertility to levels attained in 1995 would reduce methane emissions by 10% (Garnsworthy 2004).

If uterine diseases are so common and important one could ask why they have remained relatively neglected compared with similar endemic diseases such as mastitis or calf pneumonia? Probable answers to this question include: the "hidden" nature of the disease in an organ inside the animal; an acceptance of the disease as a part of the routine of keeping dairy cows; more effective therapeutics have been hard for industry to find; few veterinarians or pharmaceutical companies have championed combating the disease; and basic or applied research was rather limited in the last century. This situation, however, has

recently changed resulting in dairy cow fertility and productivity moving up the agenda of the dairy industry, pharmaceutical companies, research institutes and governments. Trailblazers for the fight against uterine disease have also emerged in several regions including North America, New Zealand, Japan and Europe. Finally, research groups are starting to explore the fundamental biology of uterine disease as well as the applied aspects of countering the problem.

THE NEED FOR NOVEL THERAPEUTICS FOR UTERINE DISEASE

Dairy cows are usually treated with parenteral antimicrobials for metritis and intra-uterine antimicrobials for endometritis. In addition, hormones such as prostaglandin F are used to induce luteolysis and oestrus to treat endometritis. The success rates for treatments are higher for less severe disease, and grading systems for metritis and endometritis have been devised to assist veterinary surgeons with prognosis as well as add value to their clinical evaluation of disease. The most widely used grading system from Sheldon and others (2009) is available Open Access to everyone *via* the veterinarians' page at www.crib.swansea.ac.uk. Overall clinical cure rates for endometritis over a 2-week period are about 65% but the hidden issue here is that many of these animals remain sub-fertile even though they appear healthy. Furthermore, whilst subclinical endometritis likely compromises fertility it is not clear how the condition should be treated and existing strategies are not effective. However, compromise of uterine function is only part of the problem as uterine disease also perturbs ovarian function. Cows with uterine disease have smaller and less functional ovarian follicles, and are less likely to conceive (Sheldon and others 2002).

There is a clear need for the design of smarter treatments and prevention strategies for infertility caused by uterine disease, an approach which at its root requires better understanding of the uterine disease. The present article will discuss the basic features of the mechanisms of uterine disease, but further detail is available in multiple reviews (Sheldon and others 2009, Gilbert 2011, LeBlanc and others 2011).

MICROBES CAUSE POSTPARTUM UTERINE DISEASE

The bacteria that are consistently associated with uterine disease are *Escherichia coli, Trueperella pyogenes* (formerly *Arcanobacterium pyogenes* and before that *Corynebacterium pyogenes*) and a range of anaerobic bacteria such as *Fusobacterium pyogenes, Bacteroides* and *Prevotella* species. Uterine disease is associated with specific strains of *E. coli*, called endometrial pathogenic *E. coli* (EnPEC) that were identified by molecular typing techniques, and are distinct from strains of *E. coli* that cause diarrhoea or mastitis (Sheldon and others 2010). These EnPEC strains are more adherent and invasive to bovine endometrial cells than other strains of *E. coli*. As well as the role of EnPEC in disease, it is well established that the presence of *T. pyogenes* is correlated with the extent of endometrial pathology (Bonnett and others 1991). Both *E. coli* and *T. pyogenes* stimulate inflammatory responses in the endometrium (Herath and others 2009b, Borges and others 2012). The genotyping and phenotyping of bacteria that contribute to uterine disease remains an area of active investigation. In particular, a multitude of anaerobic bacteria have been identified in the endometrium using molecular biology rather than standard microbiology techniques. There is also an emerging role for viruses that contribute to uterine disease with bovine herpesvirus-4 the main candidate because the virus is tropic for bovine endometrial cells (Donofrio and others 2007).

IMMUNITY AND INFLAMMATION IN THE UTERUS

The two main arms of immunity are innate and adaptive immunity. Innate immunity provides non-specific, immediate alarm-type responses to microbes, initiates inflammation and directs the adaptive immune response. Adaptive immunity provides a slower but more sophisticated and antigen specific response against matter recognised as "non-self". However, the recognition of non-self in the endometrium is a problem after conception because the embryo is semi-allogeneic being derived from the father as well as the mother. So, the adaptive immune response is highly regulated in the uterus to tolerate the semi-allogeneic embryo. On the other hand, innate immunity rapidly responds to pathogens without being triggered by the embryo, fetus or placenta.

Innate immunity has multiple systems for the non-specific defence of the host against microbes, including antimicrobial peptides, the epithelial barrier, and the complement cascade. Impetus for the expansion of research in innate immunity came from seminal discoveries made in the laboratories of Jules Hoffmann and Bruce Beutler of specific cellular "pattern recognition receptors" that detect pathogen-associated molecular patterns (PAMPs) commonly associated with

microbes (Ronald & Beutler 2010, Ferrandon and others 2007), which led to the award of the 2011 Nobel Prize in Physiology or Medicine. The first discovery was that the "Toll" protein involved in embryonic development of the fruitfly *Drosophila melanogaster*, was also required for an effective immune response to the fungus *Aspergillus* (Ronald & Beutler 2010). The next major advance was the discovery that Toll-like receptor 4 (TLR4) in mammals was necessary for the inflammatory response to the lipopolysaccharide (LPS) cell wall component of bacteria, which acts as a PAMP (Ferrandon and others 2007). Interestingly, whilst TLR4 binding to LPS should lead to an appropriate immune response to defend against bacteria, in some situations over exuberant TLR4-dependent inflammation exacerbates disease, which is why LPS is also commonly called "endotoxin".

The pattern recognition receptors of the innate immune system appear to have some importance for the detection of microbial infection and PAMPs in the uterus of cattle. Using endometrial biopsies from postpartum dairy cows we confirmed that the Toll-like receptors (TLRs) were expressed in the bovine endometrium and that genes encoding inflammatory mediators were activated during bacterial infection post partum (Herath and others 2009b, Herath and others 2006). Surprisingly TLRs were not only expressed by immune cells such as neutrophils but were also present in purified populations of epithelial and stromal cells isolated from the endometrium of dairy cattle (Herath and others 2009b, Davies and others 2008). Furthermore TLR4 is functionally active in endometrial epithelial and stromal cells from many species and is able to detect LPS from *E. coli* (Sheldon & Roberts 2010, Sheldon and Bromfield 2011, Cronin and others 2012). To investigate the importance of TLR4 expression in endometrial cells, the production of the TLR4 protein was inhibited at the cellular level using short interfering RNA (siRNA) technology (Mello & Conte 2004). Indeed, the inhibition of TLR4 in endometrial cells ameliorated the effects of LPS, confirming the essential role of TLRs in the recognition and response to microbes in the bovine endometrium (Cronin and others 2012).

Exploring the interactions between immunity and endocrine function further extended the clinical relevance of the discovery that endometrial epithelial cells have roles in innate immunity. The TLR4-mediated response to LPS in the endometrium also switches prostaglandin production by the epithelial cells from the luteolytic F series to the luteotrophic E series (Herath and others 2009a). The increased prostaglandin E_2 produced in response to LPS from *E. coli* may help explain the established clinical observation that uterine disease can delay luteolysis. Conversely, if animals ovulate but still have microbes in the uterus, then progesterone perturbs the TLR4 mediated response to LPS. Perhaps removing the negative impact of progesterone on immunity, when animals are treated with prostaglandin $F_{2\alpha}$, helps to resolve clinical endometritis. Beyond the existing treatments, researchers are now targeting the components of the TLR cell signalling pathway using small molecules to regulate the inflammatory response (Cronin and others 2012).

IMMUNITY AND INFLAMMATION IN THE OVARY

A novel aspect of our work was the discovery that bacterial infection of the uterus perturbs postpartum ovarian function. Specifically, the presence of bacterial infection in the uterine lumen reduced the rate of ovarian follicle growth and perturbed follicle function as determined by reduced oestradiol secretion (Sheldon and others 2002). Furthermore, the peripheral plasma concentrations of progesterone are lower in cows with uterine disease than those in normal fertile animals (Williams and others 2007). The mechanisms linking uterine disease to ovarian function were not obvious because bacteria are rarely found in the ovary and ovarian follicles are devoid of immune cells such as macrophages. However, LPS was found in the follicular fluid of diseased cows, perhaps reaching the ovary by the same vascular mechanisms used by prostaglandin. Furthermore, the granulosa cells that line ovarian follicles expressed TLRs, and PAMPs perturb their endocrine function as well as causing inflammation (Herath and others 2007, Bromfield & Sheldon 2011). The essential role of granulosa cells in innate immunity was confirmed by targeting TLR4 using siRNA, and this reduced TLR4 expression limited the expected cytokine response to LPS (Bromfield & Sheldon 2011).

A further question was whether perturbations of the ovarian follicle were the only mechanism linking uterine disease to lower ovarian fertility, or whether there may also be a direct impact on the egg? The cumulus granulosa cells in the ovarian follicle nurture the oocyte, and the cumulus-oocyte complex mounts a cytokine response to LPS (Bromfield & Sheldon 2011). Furthermore, we are the first to show in any species that the meiotic spindle, containing the chromosomes that should form the embryo, was disrupted in oocytes treated with LPS (Bromfield & Sheldon 2011). This

damage of oocytes by PAMPs may help explain how infertility persists beyond the duration of clinical uterine disease in dairy cows.

CONCLUSIONS

Uterine disease is a common and important cause of infertility in cattle, which compromises global food security and negatively impacts the environment. The current treatments and veterinary management of uterine disease leaves considerable room for improvement, yet little progress has been made through applied research. An alternative approach to developing new therapeutics is to understand the fundamental biology of the host-pathogen interactions that lead to uterine disease in postpartum cattle. The knowledge from the interface between microbiology and reproductive immunology is already yielding new target therapeutic pathways, which the pharmaceutical industry is exploring toward smarter ways of treating or preventing uterine disease.

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REFERENCES

Bonnett, B.N., Martin, S.W., Gannon, V.P., Miller, R.B., Etherington, W.G. (1991) Endometrial biopsy in Holstein-Friesian dairy cows. III. Bacteriological analysis and correlations with histological findings. Canadian Journal of Veterinary Research **55:** 168-173

Borges, A.M., Healey, G.D., Sheldon, I.M. (2012) Explants of intact endometrium to model bovine innate immunity and inflammation ex vivo. American Journal of Reproductive Immunology **67:** 526-539

Bromfield, J.J., Sheldon, I.M. (2011) Lipopolysaccharide Initiates Inflammation in Bovine Granulosa Cells via the TLR4 Pathway and Perturbs Oocyte Meiotic Progression *in Vitro*. Endocrinology **152:** 5029-5040

Campbell, P. (2010) Food: The growing problem. Nature **466:** 546-547

Cronin, J.G., Turner, M.L., Goetze, L., Bryant, C.E., Sheldon, I.M. (2012) Toll-Like Receptor 4 and MYD88-Dependent Signaling Mechanisms of the Innate Immune System Are Essential for the Response to Lipopolysaccharide by Epithelial and Stromal Cells of the Bovine Endometrium. Biology of Reproduction **86:** 51, 51-59

Davies, D., Meade, K.G., Herath, S., Eckersall, P.D., Gonzalez, D., White, J.O., Conlan, R.S., O'Farrelly, C., Sheldon, I.M. (2008) Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. Reproductive Biology and Endocrinology **6:** 53

Donofrio, G., Herath, S., Sartori, C., Cavirani, S., Flammini, C.F., Sheldon, I.M. (2007) Bovine herpesvirus 4 (BoHV-4) is tropic for bovine endometrial cells and modulates endocrine function. Reproduction **134:** 183-197

Ferrandon, D., Imler, J.L., Hetru, C., Hoffmann, J.A. (2007) The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology **7:** 862-874

Garnsworthy, P.C. (2004) The environmental impact of fertility in dairy cows: a modelling approach to predict methane and ammonia emissions. Animal Feed Science and Technology **112:** 211-223

Gilbert, R.O. (2011) The effects of endometritis on the establishment of pregnancy in cattle. Reproduction, Fertility, and Development **24:** 252-257

Herath, S., Fischer, D.P., Werling, D., Williams, E.J., Lilly, S.T., Dobson, H., Bryant, C.E., Sheldon, I.M. (2006) Expression and function of Toll-like receptor 4 in the endometrial cells of the uterus. Endocrinology **147:** 562-570

Herath, S., Lilly, S.T., Fischer, D.P., Williams, E.J., Dobson, H., Bryant, C.E., Sheldon, I.M. (2009a) Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F_{2a} to prostaglandin E_2 in bovine endometrium. Endocrinology **150:** 1912-1920

Herath, S., Lilly, S.T., Santos, N.R., Gilbert, R.O., Goetze, L., Bryant, C.E., White, J.O., Cronin, J., Sheldon, I.M. (2009b) Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. Reproductive Biology and Endocrinology **7:** 55

Herath, S., Williams, E.J., Lilly, S.T., Gilbert, R.O., Dobson, H., Bryant, C.E., Sheldon, I.M. (2007) Ovarian follicular cells have innate immune capabilities that modulate their endocrine function. Reproduction **134:** 683-693

LeBlanc, S.J., Osawa, T., Dubuc, J. (2011) Reproductive tract defense and disease in postpartum dairy cows. Theriogenology **76:** 1610-1618

Mello, C.C., Conte, D., Jr. (2004) Revealing the world of RNA interference. Nature **431:** 338-342

Ronald, P.C., Beutler, B. (2010) Plant and animal sensors of conserved microbial signatures. Science **330:** 1061-1064

Schaafsma, G. (2000) The protein digestibility-corrected amino acid score. Journal of Nutrition **130:** 1865S-1867S

Sheldon, I.M., Bromfield, J.J. (2011) Innate immunity in the human endometrium and ovary. American Journal of Reproductive Immunology **66**(1): 63-71

Sheldon, I.M., Cronin, J., Goetze, L., Donofrio, G., Schuberth, H.J. (2009) Defining Postpartum Uterine Disease and the Mechanisms of Infection and Immunity in the Female Reproductive Tract in Cattle. Biology of Reproduction **81:** 1025-1032

Sheldon, I.M., Noakes, D.E., Rycroft, A.N., Pfeiffer, D.U., Dobson, H. (2002) Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. Reproduction **123:** 837-845

Sheldon, I.M., Roberts, M.H. (2010) Toll-like receptor 4 mediates the response of epithelial and stromal cells to lipopolysaccharide in the endometrium. PLoS ONE 5: e12906

Sheldon, I.M., Rycroft, A.N., Dogan, B., Craven, M., Bromfield, J.J., Chandler, A., Roberts, M., H., Price, S.B., Gilbert, R.O., Simpson, K.W. (2010) Specific strains of *Escherichia coli* are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. PLoS ONE **5:** e9192

Williams, E.J., Fischer, D.P., Noakes, D.E., England, G.C., Rycroft, A., Dobson, H., Sheldon, I.M. (2007) The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology **68:** 549-559