Changes induced by pathogens and metabolic stress on endometrial function in cattle: possible impacts of increased inflammation on fertility

Mudanças induzidas por patógenos e estresse metabólico na função endometrial de bovinos: possíveis impactos do aumento da inflamação na fertilidade

Y. Guo[1], W. Chankeaw[1,2], M. Chanrot[2], J.F. Valarcher[1], P. Chantarapratep[2], R. Bage[1], E. Bongcam-Rudloff[3], G. Andersson[3], G. Charpigny[4], Patrice Humblot[1, £]

[1]Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden.
[2]Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Nakhon si thammarat, Thailand.
[3]Department of Animal Breeding and Genetics, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden.
[4]Biologie du Development et Reproduction, INRA Domaine de Vilvert, Jouy en Josas, France.

Abstract

Harmonized embryo maternal relationships, are necessary at the beginning of pregnancy to insure the development of the young embryo and of the placenta. Specific mechanisms modulate the immune balance towards immune-tolerance for the embryo to be accepted by the endometrium. By the same time this tissue is submitted to intense remodelling, under complex signalling, allowing implantation.

Some pathogens and metabolic stress, especially excessive mobilisation of fat tissue, disturb this delicate balance. This review describes how these stressors can alter endometrial function through pro-inflammatory mechanisms and by inducing changes of specific signals possibly altering the establishment of contacts and functional interactions between the embryo and the endometrium around time of implantation.

Keywords: cow, uterine inflammation, endometritis, LPS, metabolic stress, NEFA, epithelial cells.

Embryo maternal interactions at time of implantation

The bovine endometrium is a highly dynamic and heterogeneous tissue, which comprises caruncular and inter-caruncular regions that present different features. Caruncular tissue is mainly composed of stromal cells whereas the inner part of inter-caruncular tissue contains a large amount of glands composed of glandular epithelial cells. Both parts are recovered by the luminal epithelium and these tissues also comprise vascular and immune cells in various numbers and proportions depending on the stage of the oestrous cycle and pregnancy (Chanrot et al., 2017b). As in other mammals, immune cells such as poly-morphonuclear cells (PMNs) and macrophages are naturally present in the bovine endometrium. Their numbers and density are higher in pro-oestrus and oestrus when compared to di-oestrus (Hawk, 1971; Hussain AM and Daniel RCW, 1991; Eren et al., 2009a; Eren et al., 2009b) and increase with age and parity.

The success of embryo development and implantation depends on complex interactions between embryonic trophoblastic cells and the different cell types and tissues of the maternal organism. In ruminants with synepitheliochorial placenta type, there is a fusion between trophoblastic cells and endometrial cells at implantation, without direct contact with maternal blood. At the beginning of pregnancy, the endometrial tissue is the site of an intense remodeling taking place while establishing interactions with the developing embryo (Singh and Aplin, 2009; Forde et al., 2011; Mansouri-Attia et al., 2012; Oliveira et al., 2012).

In human and rodents, a large number of molecules determining cell function and tissue structure are involved in the endometrial remodelling (Singh and Aplin, 2009). At time of implantation, strong changes occurs for proteins involved in the control of cell structure (actins, actinin), calcium metabolism in relation with membrane properties (calcitonin), cell adhesion (catenins, plakophilin, cadherins, integrins), protection of epithelium (mucins), and enzymes controlling also protein remodeling such as metallopeptidases (Singh and Aplin, 2009). Simultaneously, changes in growth factors associated with the development of vascular tissue at time of implantation take place (Singh and Aplin, 2009). In ruminants, one of main actors in the dialogue between the mother and developing embryo is Interferon tau (IFN-T). IFN-T is a key-molecule for maintenance of pregnancy by inhibiting the expression of estrogen and oxytocin receptors which prevents prostaglandin induced luteolysis (Oliveira et al., 2012) thus allowing maintenance of corpus luteum. IFN-T is also a critical signal for implantation by the up-regulation of a large number of genes called interferon-induced genes regulated through the JAK-STAT1 pathway (Mansouri-Attia et al., 2012). In addition to these signals regulating tissue remodeling, cell structure, cell adhesion and vascularization, complex immune mechanisms takes place leading to lack of immune rejection of the early developing embryo.

£Corresponding author: patrice.humblot@slu.se
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Immune pathways activated for establishment of pregnancy

Following the pioneer work of Medawar (Medawar, 1961), immune mechanisms allowing pregnancy or which may be source of rejection of the embryo have been mostly defined in human and mice (Robertson et al., 2009) where an election between "Immuno-rejection" of the embryo allograft or "Immuno-tolerance" (facilitating pregnancy outcome) needs to be taken via specific immune cells.

A series of signals (including IL6, TNF-α, IFNγ and MHC class I and MHC class II molecules) drives pro-inflammatory responses at the beginning of pregnancy (such as recruitment of CD4+ T cells and Natural Killer cells). However, in humans, the up-regulation of these processes induces graft rejection and recurrent miscarriage (Robertson and Moldenhauer, 2014). Opposite to the above, a key pathway is the differentiation of naïve CD4+ T-cells into a subpopulation of "immuno-tolerant" T regulatory cells (Treg), which is positively influenced by factors including TGFβ and galectins. Galectins constitute a family of lectins with a wide range of functions in various tissues. The expression of galectin-1 (Gal-1) *i.e*. the first member identified within this family, has been reported in the endometrium of several species including human, mouse and bovine (Phillips et al., 1996; Froehlich et al., 2012a). However, basic knowledge about its function during pregnancy is only emerging and very little information is available in ruminants. Gal-1 is part of the system allowing trophoblast invasion by modulating non classical MHC molecules such as Human Leucocyte Antigen G (HLA G) on trophoblastic cells (Tirado-González et al., 2013). Gal-1 also acts as a pro-angiogenic regulatory protein critical for implantation and embryo growth. Moreover, Gal-1 skews the differentiation of CD4+ T-cells towards Treg cells through action of FOXP3 (Yakushina et al., 2015), confirming its role as a major "tolerogenic" agent necessary for establishment of pregnancy (Barrientos et al., 2014b). In humans, under-expression of Gal-1 has been associated with spontaneous abortion and pre-eclampsia (Tirado-González et al., 2013; Barrientos et al., 2014b), indicating also a critical role for the maintenance of pregnancy and health of the mother and offspring at time of term.

In humans and in laboratory animals common pathways are used for driving the inflammatory response and recognition of the embryo by the endometrium. Less information exists in production animals. However, there is now evidence that in the cow, inflammation induced by pathogens and metabolic imbalance are altering the above mechanisms and may possibly impair implantation and fertility long after clinical symptoms or metabolic imbalance have disappeared.

Sources of uterine infections and impacts on fertility

At time of calving, the cow endometrium is exposed to pathogens and contamination of this tissue is frequent. When the immune response is appropriate, clearance of pathogens occurs normally during the first three weeks after parturition (Fredriksson et al., 1985; Bekana et al., 1994; Bekana. 1996). However, Sheldon et al., (2006) have shown that in UK, 10% to 30% of the dairy cows develop an acute uterine infection (acute puerperal metritis) and express strong local symptoms associated to general signs (increased body temperature, lower milk production). A persistent clinical endometritis may develop subsequently, defined from histology as an inflammation in the inner lining of the uterine wall most commonly accompanied by a purulent or mucopurulent discharge (Sheldon et al., 2006). However in some cases, no external clinical signs are observed (Elliott et al., 1968; Gilbert et al., 2005; Opsomer and Kruif. 2009; Sheldon et al., 2009; Dubuc et al., 2010a). Within 4-8 weeks post-partum, the frequency of cows with subclinical endometritis from cytology and or histology has been reported to be as high as 30-50% (Dubuc et al., 2011; LeBlanc, 2014), showing the importance of undiagnosed cases.

Several pathogens have been identified as metritis and endometritis inducing agents and Gram negative bacteria such as *Escherichia coli* (*E. coli*) and *Trueperella pyogenes* (*T. pyogenes*) are most commonly associated with uterine infections in the dairy cow (Zerbe et al., 2001; Williams et al., 2005; Sheldon et al., 2010; Santos and Bicalho, 2012; Ordell et al., 2016). However, as mentioned before, several different types of common bacteria are naturally present in the uteri of both healthy and metritis-affected cows showing that other factors are important for the persistence of bacterial contamination. The endometrial bacterial microflora has been characterized by metagenomic analysis (Santos et al., 2011) showing a more complex and numerous microflora in the uterus of animals having uterine infection compared with healthy animals.

The above-cited pathogens may pave the way for subsequent infection by other bacteria or viruses such as bovine herpes virus (BoHV)-1 or -4 (Williams et al., 2007a; Donofrio et al., 2008; Sheldon et al., 2010). BoHV-4, is a double stranded DNA virus, member of the gamma *herpesvirus* family that was first isolated from a variety of bovine diseases such as respiratory and ocular disease in calves (Bartha et al., 1965). However, among viruses, BoHV-4 is one of the few viruses with a specific tropism for the endometrium (Donofrio et al., 2008). The association between BoHV-4 seropositivity, postpartum metritis, abortions and chronic infertility has been reported in many studies (Czaplicki and Thiry, 1998; Graham et al., 2005; Yamauchi et al., 2006) and BoHV-4 infection is considered to be a risk factor of uterine diseases and endometritis. For long, BoHV-4 was considered mostly as a co-infectious pathogen that induces uterine inflammation following an initial bacterial infection (Donofrio et al., 2008; Sheldon et al., 2009; Jacca et al., 2013). In clinical endometritis, BoHV-4 has been associated with co-infection with bacteria such as *E. coli* and *T. pyogenes* (Klamminger et al., 2017). In this last referenced study, BoHV-4 infection

significantly increased the risk for intrauterine infection with *T. pyogenes* and vice versa illustrating the strong relationships between BoHV-4 and *T. pyogenes* infections. In addition, BoHV-4 infection significantly reduced both, the odds ratio for cows to be inseminated within 80 days post-partum and for cows being pregnant within 200 days post-partum (Klamminger et al., 2017).

So far, the implication of BoHV-4 in uterine diseases has been considered to result from systemic infection following contamination by the respiratory route. However, the presence of BoHV-4 DNA in case of oedematous orchitis and also in the semen of healthy bulls implicates that semen represents a potential vector for BoHV-4 transmission to cows (Egyed et al., 2011; Morán et al., 2013). Some recent findings showing that endometrial epithelial cells are very sensitive to this virus (Chanrot et al., 2017a) suggest that viral transmission may occur also at time of mating through either natural breeding or artificial insemination.

Uterine infections and immune response in the endometrium

As mentioned above, Gram negative bacteria are commonly and predominantly associated with uterine infections in dairy cows. In these cases, an important part of the pathogenic mechanism involved, results from the action of the lipopolysaccharide endotoxin (LPS) (Holst et al., 1996), which is present on the membrane of these bacteria. In case of uterine disease, LPS leads to acute or chronic inflammation of the endometrium. Due to its presence in the peripheral circulation, LPS can also impair the growth of ovarian follicles and lower estradiol secretion (Dohmen et al., 2000; Sheldon et al., 2009). As the attraction of immune cells in uterine tissue, occurring during estrus (Chanrot et al., 2017b), relies on estrogens, the above studies suggest that the potential inhibition of estradiol secretion by LPS may not be favorable for recovery.

LPS activates the immediate innate immune response leading to inflammation through a cascade of events well conserved in different tissues. LPS first creates complexes with pathogen associated molecules (LPS binding protein) which in turn binds to toll-like receptor 4 (TLR-4; review Sheldon et al., 2009). Binding of LPS to TLR-4 (Dohmen et al., 2000; Sheldon et al.,. 2009) promotes the secretion of pro-inflammatory cytokines (interleukins (IL)-1, -6, and -8 and tumour necrosis factor-alpha (TNF-α), (Beutler et al., 2003)), which attract cells of the innate immune system into the endometrial stroma (Sheldon et al., 2010; Cronin et al., 2012; Turner et al., 2014).

In ruminants, LPS stimulates also locally the production of PGE rather than PGF by endometrial cells, which may give an explanation for prolonged luteal phase in cows with uterine disease (Herath et al., 2009b). In addition, the increased production of PGE2 in uterine tissue favors viral replication in macrophages by activating the viral immediate early 2 gene (*IE2*) promoter (Donofrio et al., 2008). The bacterial co-infection and the actions triggered by LPS may then initiate a positive feedback loop between PGE2 production and viral replication. This synergetic mechanism showing the possible existence of a cooperation between bacteria and viruses may explain the rapid activation of viral replication in the bovine endometrium in case of uterine disease.

Similarly, following the binding of viruses to specific toll-like receptors, infected cells secrete interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) cytokines which are the nonspecific, earliest responses of host to viral infections. This response is followed in infected areas by a cascade of downstream mediators (Staeheli, 1990; O'Shea, 1997; Reiss and Komatsu. 1998; Donofrio et al., 2007) leading to inflammation of the endometrium. Virus-infected cells synthesize and secrete also the type I interferons (IFN α/β) which are major activators of signaling leading to antiviral defense responses against all kinds of viruses (Fensterl and Sen, 2009). In addition, inflammatory molecules such as IL-1α, IL-1β, and IL-6, are produced by immune cells and other cell types (Malazdrewich et al., 2001; Donofrio et al., 2008). BoHV-4 has also the ability to trigger epithelial cells to increase their production of IL-8 (Chanrot et al., 2017a) and sensitivity to TNF-α (Jacca et al., 2014). The binding of TNF-α to its TNF-α receptor 1 (TNFR1) on the surface of infected cells stimulates viral DNA synthesis (Jacca et al., 2014) and also increases IL-8 production (Chanrot et al., 2017a), which may be a part of the mechanism driving the disease toward a chronic status of endometritis (Donofrio et al., 2010).

The above pro-inflammatory molecules are killing virus-infected cells and acts as a bridge between innate and adaptive response (Ellermann-Eriksen, 2005). From the above mechanisms, BoHV-4 causes cytopathic effect (CPE) and replicates in a wide range of cell lines and primary cultures of various animal species (Donofrio et al., 2000; Wellenberg et al., 2002). Viral replication and CPE have been shown by immunofluorescence in stromal (Donofrio et al., 2007) and epithelial (Chanrot et al., 2017a) bovine endometrial cells.

As described above, the activation of immune mechanisms at time of, or short after infection have been intensively studied and are well documented. However, complementary information on long term effects of infection and or alteration of other functions of the endometrium are still missing.

Inflammation and endometrial function; possible consequences for implantation

In addition to the activation of immune mechanisms, which are performed by key players in the pathogeny of endometritis, numerous modifications of endometrial cell function takes place in connection with LPS effects. Recent studies from our group documented the roles of *E. coli* LPS on bovine endometrial epithelial cells (bEECs) from an *in vitro* model (Chanrot et al., 2017). The effects of LPS on viability, proliferation, apoptosis, secretion of

cytokines, and molecular response (transcriptomics, proteomics and epigenetics) were studied following exposure of bEECs to dosages comprised between 2 and 16 µg/ml which are lower than those usually reported in uterine fluids in case of endometritis (Dohmen et al.,. 2000; Mateus et al., 2003; Williams et al., 2007b). The time of exposure to LPS, comprised between 24 to 72 hours was also short when compared to the conditions met in case of contamination under clinical conditions. Despite these limitations, this model reveals the importance and variety of unfavorable changes possibly induced by LPS susceptible to affect endometrial function.

Viability, proliferation, apoptosis:

Most often, following LPS challenge, a stimulation of cell proliferation has been reported in a variety of tissues in both human and rodents (Freitag et al., 1996; Zhang et al., 1996; Muller-Decker et al., 2005; Liu et al., 2010; Hei et al., 2012; Eslani et al., 2014; Basso et al., 2015). LPS effects on cell survival during *in vitro* culture have been studied in production animals, but results from those different studies are not fully consistent. Overall, it is possible that differences may be due to variations in LPS concentrations relative to body weight. With the dosages used in our bovine model, the number of cells increased following LPS challenge in a dose-related manner up to 8 µg/ml (Chanrot et al., 2017b). With increasing dosages, impairments of cell survival, lower proliferation activity and increased apoptosis were observed suggesting that detrimental effects of LPS could be even more pronounced in case of natural infection. In addition, *in vivo,* some other components of *E. coli* besides LPS and even reactions of other cells in the endometrium, especially high production of pro-inflammatory cytokines by immune cells (Holst et al., 1996; Herath et al., 2009b; Sheldon et al., 2010; Eslani et al., 2014) may also add to the detrimental effects observed here in epithelial cells.

Moreover, despite cells presented a normal morphology and unaltered survival rates (Chanrot et al., 2017b), the transcriptomic (Guo et al., 2019, Dep Clin Sci, Fac Vet Med Anim Sci, Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden, unpublished data), and proteomic profiles (Piras et al., 2017) observed at 24 and 72 hours respectively, revealed changes in expression for more than 2000 genes and about 30 proteins associated to a large number of cell functions were greatly disturbed. These results obtained from unbiased molecular approaches confirm that LPS affect a multiplicity of pathways. Differentially expressed genes (DEGs) and proteins, with functions in metabolism pathways (especially glycolysis) and oxidative stress (strongly related to metabolic changes) were over-expressed. This confirms the results from a previous proteomic study showing the over expression of peroxiredoxin and heat shock proteins in cows with endometritis when compared to healthy ones (Choe et al., 2010). Same trends were observed with transcription and translation, which may relate to the proliferative phenotype.

The information taken from these studies largely confirms the results from former transcriptomic studies (Oguejiofor et al., 2015; Salilew-Wondim et al., 2016) especially those related to impacts on pro-inflammatory mechanisms. In addition, new information is given while describing some of the mechanisms by which LPS alters immune-tolerance, specific molecular patterns involved in implantation and embryo-maternal relationships through changes of IFN-tau signaling. LPS exposure is also inducing numerous epigenetic changes, which will be briefly described hereunder.

Secretion of cytokines and modulation of immune response:

As documented before, our results (Chanrot et al., 2017b; Piras et al., 2017; Guo et al., 2019; unpublished data) indicate that epithelial cells are highly sensitive to LPS and activate strongly inflammatory pathways. Most particularly, the toll-like receptor-signaling pathway was significantly affected by LPS treatment, and 19 of the over-expressed DEGs were associated with this pathway including many genes encoding cytokines and chemokines. This is in full agreement with former information showing that LPS provokes the activation of the host's innate immune response by increasing expression of pro-inflammatory cytokines and chemokines following binding of TLR4 (Mogensen, 2009; Chanrot et al., 2017b; Piras et al., 2017). The toll-like receptor-signaling pathway activates key molecules to drive immune-related responses towards infectious agents and attracts immune cells at the site of infection (Herath et al., 2009a; Dekel et al., 2010). In a first step, this represents one of the main defense mechanisms of the mucosal epithelium. It has also been suggested that the increased expression of cytokine and chemokine genes, which is fully consistent with increased cytokine production by epithelial cells in the same model (Chanrot et al., 2017b) leads to a persistent presence of immune cells in the endometrium possibly altering implantation (Dekel et al., 2010). Chemokines can promote or inhibit human trophoblast cell migration and invasion in first-trimester placenta (Zhang et al., 2013). For instance, Tumor Necrosis Factor alpha (TNFα) has pleiotropic effects on cell growth, inflammation and innate immunity in the endometrium and is strongly involved in embryo development and implantation (Stewart et al., 1992; Salleh and Giribabu. 2014). These effects can be either positive or detrimental. Increased concentrations of TNF-α have been reported to be the source of implantation failures and pregnancy loss (Torchinsky et al., 2005). Chemokines like CXCL1 and CXCL6 contribute to neutrophil recruitment and are associated with pathways involved in inflammation and apoptosis (Chittur et al., 2011). From this bEEC *in vitro* model, despite the fact that cells were exposed to relatively low dosages of LPS (Chanrot et al., 2017b) and for

a short time when compared to the *in vivo* situation in case of infection by Gram-negative bacteria, we observed strong changes in expression of both cytokines and chemokines. The duration of these changes in gene expression should be evaluated from *in vivo* studies. Such changes may alter the uterine environment and may not be favorable to the transit of spermatozoa and their survival through the female genital tract (Novy, 2008). However, due to the strength of the changes for the corresponding genes and to the delicate immune balance necessary for the establishment of pregnancy (Sheldon et al., 2010; Agrawal, 2013), it may be speculated that in cows which have been exposed to endometritis, such de-regulations may also contribute to alter embryo-maternal relationships and the success of AI, long after infection has disappeared (Sharkey, 1998). Undiagnosed persistent inflammation may also represent a limitation in embryo transfer recipients especially when parity increases.

We also found that a group of genes coding for proteins belonging to the galectin (Gal) family was de-regulated by LPS suggesting that LPS may alter early embryo maternal communication (Popovici et al., 2005; Than et al., 2015). *In vivo* and *in vitro* studies showed that Gal-1, 3 and 9 play a crucial role in the cell proliferation, adhesion processes, and modulation of innate and adaptive immunity, pro-inflammation and/or regulation of immunosuppressive activity. Gal-1 is required for establishing an immune privileged local environment for implantation and early fetal development, which relates especially from its immunosuppressive activities and key role for maternal fetal tolerance as documented in humans and rodents (Shimizu et al., 2008; Heusschen et al., 2013; Gomez-Chavez et al., 2015). Gal-1 is up-regulated during normal pregnancy and expressed also in human preimplantation embryos. This protein stimulates FOXP3, which controls the differentiation of T naive cells into T regulatory cells (Cedeno-Laurent and Dimitroff, 2012). Gal-1 also positively regulates the expression of human leukocyte antigen G (HLA-G) which inhibits NK cells and modulates cytokine secretion to control trophoblast cell invasion and to maintain a local immune-tolerance during implantation (Roussev and Coulam, 2007; Tirado-Gonzalez et al., 2013). Probably as the result of the above, low expression in the endometrium has been associated with an increased frequency of early pregnancy failures and miscarriages (Jeschke et al., 2010; Barrientos et al., 2014a). It has been reported that Gal- 1 was present in the bovine endometrium, mainly in the lamina propria (Froehlich et al., 2012b), but the exact roles of Gal-1 and how it regulates *BOLA* gene expression during pregnancy is not known in this species. The decreased expression of *Gal-1* mRNA as observed here corroborates the decreased expression of Gal-1 protein following LPS challenge (Piras et al., 2017). The significance of these changes for the establishment of cow pregnancy would deserve specific functional studies. As mentioned above for other signals, further work aiming at evaluating the duration and the functional impact of such changes would be needed to relate the results with possible long term effects of LPS in case of endometritis.

Cell adhesion and tissue remodeling

In addition to changes referred above, our results suggest that LPS activates mechanisms altering specifically cell structure and cell adhesion properties of endometrial epithelial cells, which could also be unfavorable to subsequent implantation. Successful embryo implantation requires a subtle regulation of tissue remodeling by adhesion molecules (cadherins, integrins, selectins, and MMPs) (Aplin, 1997; Lessey, 2002). The members of the cadherin superfamily mediate cell-cell interactions though calcium binding, and any possible changes could impair implantation (Gipson et al., 2008). In our model, most of the DEGs (Guo et al., 2019; unpublished data) and all differentially expressed proteins (Piras et al., 2017) belonging to cell structure and cell adhesion pathways were under expressed.

This includes genes related to actin cytoskeleton, which are important for the binding of growth factors to their respective receptors. In addition, several members of the Integrin family are under expressed (Guo et al., 2019; unpublished data). Integrin beta 3, for which expression is increased by blastocysts in endometrial epithelial cells and has been described as one of the best marker of uterine receptivity in the human species (Chen et al., 2016). On the contrary, signals involved in cell surface remodeling such as MMPs and their tissue inhibitors were mostly overexpressed. The significance of such changes should be more documented from further functional studies in the cow.

Response to IFN-t

As mentioned before, a large number of genes called IFN-t-induced genes, which are regulated through the STAT-dependent signaling pathway (Spencer et al., 2008; Forde et al., 2012) are over-expressed at the beginning of pregnancy (Maj and Chelmonska-Soyta, 2007). Activation of this pathway is driven by the developing embryo through secretion of IFNT, which induces tyrosine phosphorylation of STAT-1, -2 and -3 (Binelli et al., 2001a). In addition, the leukemia inhibitor factor (LIF) which is STAT-dependent is critical for embryo-endometrial interactions and trophoblast invasion (Salleh and Giribabu. 2014). LIF is highly expressed in mouse uterus during the receptivity phase and essential for embryo implantation (Herath et al., 2009a). Without LIF, the downstream signal transduction pathways may be severely affected (Lass et al., 2001; Hu et al., 2007). Decreased expression or complete lack of LIF production is linked to implantation failure (Salleh and Giribabu. 2014).

After a short exposure of endometrial cells to LPS, both *LIF* and *STAT1* genes exhibited a very strong

increase in mRNA expression at 6 hours post LPS (Guo et al., 2019; unpublished data). In addition, many downstream genes from the mucin's and MMP families were strongly over-expressed at 48 hours. The duration of these changes is not known and their amplitude should be compared to what is happening in response to a living embryo at the beginning of pregnancy (Salleh and Giribabu. 2014). However, it is likely that the very strong over-expression of these genes induced by LPS disturb the response of endometrial cells to IFN-t and alters their receptivity at time of implantation.

Epigenetic alterations induced by LPS

There is not so much literature dealing with the epigenetic impact of infection in cattle endometrium. In parallel to transcriptomics changes described above, we have investigated from the same model the changes in global methylation induced by LPS in bEEC (Jhamat et al., 2016; Jhamat et al., 2019; unpublished data). Methylation patterns under control conditions and after LPS challenge were studied at 24 hours of culture by Reduced Representation Bisulfite Sequencing (RRBS) targeting CpG islands and transcription factor binding sites and promoters. When compared to controls, cells exposed to LPS presented a global demethylation pattern whereas control cells were more methylated than at time 0. A reciprocal relationship was found between methylation and gene expression data for a panel of loci some of them being known as key regulators of endometrial function. These analyses from RRBS data, allowed the identification of regions harbouring candidates for key regulatory elements of endometrial function, thus participating to the understanding of LPS-induced deregulations that may impact implantation. Global changes documented by RRBS results (Jhamat et al., 2016) are consistent with our transcriptomic data showing the under-expression of genes involved in methylation whereas genes involved in demethylation processes are over expressed (Guo et al., 2019; unpublished data). Specific changes in several histones have also been reported from proteomics 72 hours after challenge (Piras et al., 2017). The overall picture is quite complex here with four of them under expressed and two over expressed following LPS challenge and more specific work is needed to decipher the mechanisms explaining the overexpression of some specific histones and the decreased expression of some other isoforms. However, from the four histones under expressed we found two (Histone H2A type1 and H2AJ) that are associated with chromatin silencing consistent with the above demethylation, and the over expression of many genes and pathways in both the transcriptomic and proteomic studies. The over expression of Histones H2B type1 and H2B type1N is also consistent with their roles in innate immune response in mucosa and DNA protein binding. Effectively, some histones, such as H2B type1, could represent LPS binding proteins (Piras et al., 2017), and their over-expression could contribute to the activation of defence mechanisms in infected cells by stimulating the formation of an antimicrobial and of an endotoxin-neutralizing barrier against microorganisms (Piras et al., 2017).

Impacts of metabolic disorders on endometrial function

The fact that Negative energy balance (NEB) induced by high milk production which cannot be compensated by energy intake, affects health status and fertility in the postpartum dairy cow is largely documented (Dubuc et al., 2010b; Ospina et al., 2010; Farman et al., 2016). During the period the cow is exposed to NEB, stored body fat will be mobilized in the form of non-esterified fatty acids (NEFAs), leading to increased systemic concentrations of NEFAs in blood (Tamminga et al., 1997). Oleic acid (OA), palmitic acid (PA) and stearic acid (SA) are the three NEFAs reported as consistently increased in blood circulation during the post-partum period and similar patterns of increase have been observed in follicular fluid or in uterine flushing fluid (Khandoker and Karasawa, 1997; McEvoy et al., 2000; Leroy et al., 2005). NEFAs represent an energy resource for cell metabolism. They are stored as triglycerides in the cytoplasm. When their concentrations increase, lipid droplets are accumulated as ectopic fat deposition or steatosis in cells (Browning and Horton, 2004; Talati et al., 2016).

In the cow genital tract, negative effects of increased NEFA have been shown in granulosa cells (Vanholder et al., 2005), and in oviductal epithelial cells (Jordaens et al., 2015). Negative impacts have been reported also on oocyte development and maturation (Jorritsma et al., 2004; Valckx et al., 2014), which affects subsequent embryonic development (Van Hoeck et al., 2011). The concentrations of fatty acids at which they are susceptible to impair cell physiology and induce cell death are different depending on type of cell and type of NEFA. For instance, for PA, higher concentrations (200 μM) were needed in bovine oviductal epithelial cells (Ohtsu et al., 2017), than in ovine oocytes (60 μM) to alter cell function.

Immune changes under NEB

During the peri-partum period, both the local immune response in the uterus and the immune response in peripheral circulation are depressed and these changes may predispose cows to uterine infection (Lewis, 1997). Impairment of neutrophil function, starting before parturition and related to energy status, has been reported (Hammon et al., 2006). After calving, most studies show that NEB, could induce immune changes in the genital tract associated to increased inflammation. The over-expression of pro-inflammatory genes have been reported in

the endometrium of cows with severe NEB (Wathes et al., 2009) and in cows submitted to a restricted energy diet (Valour et al., 2013). However, the above changes in gene expression were not associated with clinical symptoms and it is still not clear if the severity of NEB is a source of increased risk of developing uterine diseases. Controversial results were obtained from attempts made *in vivo* to associate changes in peripheral concentrations of mediators of NEB (such as NEFAs, beta-hydroxybutyrate) and cytology or clinical cases of uterine disease (Williams, 2013). Discrepancies between the studies cited in this review may result of differences in the methods used and from the impact of the stage of the cycle on the number of immune cells (Chanrot et al., 2017b).

As documented above, the uterine environment is crucial for proper implantation and normal embryo development. In postpartum dairy cows, NEB associated with high plasma NEFA concentrations has been reported to delay uterine involution and increase the risk of uterine inflammation (Swangchan-Uthai et al., 2013). In obese rats, high levels of blood NEFA have been associated with local lipid accumulation resulting in the inflammation of the endometrial tissue including both the stromal and luminal epithelium (Shankar et al., 2011). Increased gene expression of genes encoding pro-inflammatory cytokines was observed at time of implantation (Shankar et al., 2011). Therefore, in both human and rodents, there is evidence that hyperlipidemia induces a pro-inflammatory uterine environment which promote uterine disease and can be detrimental to embryonic development.

The mechanisms by which NEB possibly affects endometrial function were approached by our group through both *in vivo* and *in vitro* studies focusing on the impact of NEFAs (Chankeaw et al., 2018; Chankeaw et al., 2019, Fac Vet Sci, Rajamangala University of Technology Srivijaya, Thailand, unpublished data).

In vivo experiments conducted in the framework of the EU project "PROLIFIC" (KBBE, 311776) involved dairy cows of the Swedish Red Breed (SRB) in their second lactation. It was hypothesized that metabolic status during the post-partum period could affect specifically the different types of endometrial cells from stroma (ST), glandular epithelium (GE) and luminal epithelium (LE). To address this question, endometrial biopsy samples were collected 12 to 15 days post oestrus between 70 and 80 days post-partum in cows receiving from one month before calving to three months after, either a control diet for high-producing cows (target 35 kg/d energy-corrected milk, ECM) or a lower feeding intensity diet (achieved by giving -50% concentrate to target 25 kg/d ECM). These diets induced differential metabolic response as documented in (Ntallaris et al., 2017). The measurement of the NEB status allowed to define two groups of cows either expressing a mild (MNEB) or severe (SNEB) NEB status by the first week post-partum. Luminal and glandular epithelial cells as well as stromal cells were harvested by laser capture microdissection at time of breeding. Following capture, RNAseq generated a genome-wide transcriptomics profile of endometrial cells. Differential transcriptomic profiles were observed in the three types of cells, with more genes expressed in stromal cells than in glandular or luminal epithelial cells (Chankeaw et al., 2019; unpublished data) and main transcriptomic signatures in the three types of cells were defined. We also confirmed the impacts of NEB on the gene expression of endometrial cells.

Numerous specific changes in gene expression were observed in stromal cells illustrating dysregulation of metabolic processes especially lipid and carbohydrate metabolism, cytoskeleton and cell adhesion properties. Altered gene expression of endometrial epithelial cells under SNEB condition were related to activation of pro-inflammatory responses via chemokine pathway in GE, whereas downregulation on adaptive immunity and defence mechanism were found in LE. Strong changes in the expression of genes involved in prostaglandin production and maternal-conceptus recognition such as IFN-tau induced genes, were found in ST and in GE. Considering the above and the crucial role of IFN-tau for embryo implantation and maintenance pregnancy (Binelli et al., 2001b), it could be hypothesized that the under-expression of IFN-tau genes associated to the increased expression of genes associated to oxytocin and PGF2α signalling may not be favorable to the establishment of pregnancy in SNEB cows. These results provide novel insights into mechanisms regulating endometrium physiology in the post-partum dairy cow suggesting that glandular, luminal and stromal cells have specific and differential responses to their metabolic environment. The specific response of ST and GE to NEB paves the way for functional studies relating the importance of these changes for the establishment of pregnancy.

The results from *in vitro* studies (Chankeaw et al., 2018) show that, when used at concentrations similar to those found in cows exposed to NEB, exposure to NEFAs is associated to the accumulation of intra cellular lipids. NEFAs are also detrimental to the survival of bEECs, promote apoptosis and reduce proliferation in a dose-related manner. The severity of the alterations depends also of the type of NEFA added to culture. In addition, increased secretion of the pro-inflammatory cytokines IL-6 and IL-8 was observed. This is fully consistent with numerous results of studies showing pro-inflammatory effects of NEFAs in various tissues and cell types (Joshi-Barve et al., 2007; Håversen et al., 2009; Zhou et al., 2013). Combined, the evidence implicate that the alterations in the function of endometrial tissue resulting from exposure to NEFAs could be part of the mechanisms lowering fertility in postpartum dairy cows.

In addition to NEFAs, the possible role of other molecules produced by fat tissue, especially of pro-inflammatory adipo-cytokines, should be considered. Strong changes in the plasma profile of these adipo-cytokines have been reported during the post partum period, in dairy cows influenced by severe NEB (Kasimanickam et al., 2013; Mellouk et al., 2017). Alterations of uterine function may also be mediated by specific molecules belonging to this family such as CCL21, which has been shown to influence follicular characteristics (Kasimanickam et al., 2013; Mellouk et al., 2019; unpublished data) reported also that cows with clinical endometritis and persistent uterine

inflammatory conditions had higher serum concentrations of adiponectin and leptin compared with cows without symptoms and low numbers of PMN from endometrial cytology. As this family contains numerous molecules, the specific impacts of pro and anti-inflammatory adipo-cytokines on endometrial function would deserve further investigations.

Conclusion

Lipopolysaccharide induces strong biological effects in epithelial cells for a multiplicity of functions, which occurs already when exposing cells to this endotoxin for a short time at relatively low dosages. Despite the fact that cells proliferate and survive, different molecular patterns are observed when exposed to LPS, from proteomics, epigenetics and gene expression studies. The inflammatory reaction induced by pathogens in the endometrium will most likely affect the fragile balance between immune-tolerance and immune-rejection that takes place at time of implantation. Beyond this process, a large number of genes related to physiological events occurring at time of embryo development and implantation are de-regulated by LPS on the short term. Further studies are needed to evaluate long-term consequences of these changes and their possible implication in cases of infertility following former infection of the endometrium.

There is now evidence that metabolic imbalance during the early post-partum affects later on endometrial function at time of breeding. However, the three main cell types, which constitute this tissue express a different and specific response when exposed to negative energy balance. Both structural, metabolic and functional changes related to embryo-maternal interactions are associated to NEB. Results from *in vitro* studies confirm that fat mobilization activates pro-inflammatory mechanisms in endometrial epithelial cells. The fact that fat mobilization impacts common pathways both *in vivo* and *in vitro* may help to understand further how fertility is affected by metabolic disorders and to identify new biological markers of uterine health.

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