Responses of Bovine Endometrial Epithelial Cells to Pathogens

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Abstract

In dairy cows, clinical uterine infection (metritis) and subsequent persistent inflammation of the endometrium (endometritis) are major causes of infertility. *Escherichia coli* (*E. coli*) is a prevalent bacteria in metritis and endometritis and promotes infection with bovine herpes virus type 4 (BoHV-4) through mechanisms involving lipopolysaccharide endotoxins (LPS). In this thesis, interactions between *E. coli* LPS, BoHV-4 and the endometrial epithelium were studied using *in vitro* models following characterisation of tissue samples used for culture. Examination of cell proliferation, survival and apoptosis after challenges with various doses of LPS revealed that cow and tissue characteristics did not influence proliferation of bovine endometrial epithelial cells (bEEC) in response to LPS. However, *E. coli* LPS stimulated proliferation of bEEC (maximum observed at 8 µg/mL LPS). The strong increase in cell numbers by 72 h was not associated with an increase in apoptosis, but this occurred with higher LPS doses. Analysis of protein pro-files revealed de-regulation of 38 proteins belonging to many pathways, some related to the process of implantation. Morphological studies and ELISA were used to characterise the survival of cells and the cytokine response of bEEC to BoHV-4. In infected samples, the number of living cells started to decrease by Day 4 post-challenge and by Day 7 the number was lower than in controls. This change was associated with viral replication between Day 0 and Day 5, as demonstrated by immunofluorescence, titration and quantitative polymerase chain reaction (qPCR) results and changes in IL-8 and TNF-α profiles. Moreover, the results showing strong pathogenic effects of BoHV-4 on endometrial epithelial cells pave the way for future studies on sexual transmission of BoHV-4 at time of insemination.

The results obtained led to development of reliable models to study interactions between uterine epithelial cells and pathogens, which could be of translational use. In a time- and dose-dependent manner, *E. coli* LPS increased and BoHV-4 decreased the survival of bovine bEEC *in vitro*, while LPS induced strong alterations of protein profiles, especially those related to pathways activated at time of implantation. Such de-regulations may be part of the mechanism by which persistent inflammation following infection impairs fertility. This information can be exploited to identify new diagnostic markers of persistence of inflammation in the endometrium.

Keywords: cow, LPS, BoHV-4, endometrium, cell culture, proteomics, cell proliferation, endometritis, oestrous cycle

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Dedication

To my family, supervisors, bosses (RMUTSV) and funding source from Thailand

Do all the best, you will never be regret.
คุณจะไม่เสียใจกับสิ่งที่คุณทำอย่างสุดความสามารถแล้ว

Metasu Chanrot

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Chanrot M*, Guo Y, Dalin AM, Persson E, Båge R, Svensson A, Gustafsson H & Humblot P. (2017). Dose-related effects of LPS on endometrial epithelial cell populations from dioestrous cows. *Animal Reproduction Science,* 177, pp. 12-24.

II Piras C, Guo Y, Soggiu A, Chanrot M, Greco V, Urbani A, Charpigny G, Bonizzi L, Roncada P* & Humblot P. (2017). Changes in protein expression profiles in bovine endometrial epithelial cells exposed to *E. coli* LPS challenge. *Molecular BioSystems*, 13(2), pp. 392-405.

III Chanrot M*, Blomqvist G, Guo Y, Ullman K, Juremalm M, Båge R, Valarcher J-F, Donofrio G & Humblot P. Bovine herpes virus type 4 (BoHV-4) impairs the survival of bovine endometrial epithelial cells (bEEC). (submitted)

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The contribution of Metasu Chanrot to the papers included in this thesis was as follows:

I Helped plan and design the study, performed all analyses and interpretation of results, drafted the manuscript and critically revised the manuscript together with the co-authors.

II Helped plan the study, provided samples for proteomicss analyses and interpreted the results.

III Planned and designed the study, performed the analyses and data management, interpreted the results, drafted the manuscript and critically revised the manuscript together with the co-authors.

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Abbreviations

ANOVA	Analysis of variance
APM	Acute puerperal metritis
bEEC	Bovine endometrial epithelial cells
BoHV	Bovine herpes virus
BSA	Bovine serum albumin
CL	Corpus luteum
CPE	Cytopathic effects
dGT	Density of glandular tissue
E.coli LPS	*Escherichia coli* lipopolysaccharide
EIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
EU	European union
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
Gal-1	Galectin-1
gcs	Glandular cross-section
HLA G	Human leucocyte antigen G
HTX	Mayer's haematoxylin
IE2	Immediate-early 2
IEF	Isoelectric focusing
IFAT	Indirect fluorescent antibody test
IGF-I	Insulin-like growth factor-I
IgG	Immunoglobulin G
IL	Interleukin
INF	Interferon
IPG	Immobilise pH gradient
kDA	Kilodalton
LD_{50}	Lethal dose, 50%
LPS	Lipopolysaccharide endotoxins
LS	Least-square

MALDI	Matrix-assisted laser desorption/ionization
MDBK	Madin-Darby bovine kidney (epithelial cells)
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MS	Mass spectrometry
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
OD	Optical density absorbance
ON	Overnight
PBMC	Peripheral mononuclear blood cell
PBS	Phosphate buffer saline
PGE, PGF	Prostaglandin E, prostaglandin F
PI	Propidium iodide
PMF	Peptide mass fingerprinting
PMN	Polymorphonuclear cells
RT	Room temperature
Rta	Replication and transcription activator
SCG	Seeding cell group
SEM	Standard error of mean
SLU	Swedish University of Agricultural Sciences
SOMRS	Swedish official milk recording scheme
SRB	Swedish Red Breed cow
STAT1	Signal transducer and activator of transcription 1
SVA	Swedish National Veterinary Institute
TB	Trypan blue
$TCID_{50}$	Tissue culture infective dose
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor-alpha
Treg	T regulatory cells
UK	The United Kingdom

1 Introduction

1.1 General background: Uterine dysfunction is a frequent problem impairing fertility and the sustainability of dairy cow production systems

In all mammals, uterine diseases are important causes of infertility. These diseases negatively affect health and welfare and also profitability in the case of commercial animals. For about 60 years, dairy cows have been very successfully selected to maximise milk yield, but at the same time they have become more sensitive to reproductive disorders (Royal *et al*., 2000). Modern dairy cows have a high risk of contracting uterine diseases. Several million dairy cows per year in Europe are exposed to uterine diseases (Sheldon *et al*., 2009; Grimard *et al*., 2006).

Cows affected by uterine infection have low fertility and extended unproductive periods (Sheldon *et al*., 2009; Gilbert *et al*., 2005). They also have a longer period between parturition and first service and reduced conception rates (Fourichon *et al*., 2000), resulting in a longer interval from calving to pregnancy (Williams, 2013; Runciman *et al*., 2008; LeBlanc *et al*., 2002). Due to the impaired reproductive performance, culling rates are increased and thereby affect herd profitability (Kasimanickam *et al*., 2004; LeBlanc *et al*., 2002; Borsberry & Dobson, 1989). Other costs result from antibiotic treatment and associated milk withdrawal. The total cost of diagnosed uterine diseases to farmers and the dairy and breeding industries has been reported to reach 1.4 billion €/year in the European Union (EU) (Gilbert *et al*., 2005). However, the negative impacts of uterine diseases are probably even greater, due to undiagnosed forms of asymptomatic persistent

inflammation of the endometrium (subclinical endometritis), which can impair fertility (Sheldon *et al*., 2009; Gilbert *et al*., 2005).

1.2 Uterine involution and clinical signs of uterine dysfunction

During the first week post-partum, the uterus is contaminated with bacteria in > 90% of cows of the Holstein breed (Potter *et al*., 2010; Herath *et al*., 2009). However, clearance of bacteria occurs during uterine involution, a physiological process leading to the restoration of uterine function following parturition. In the cow, the uterus usually returns to normal size and regains its function to establish a new pregnancy within 5-8 weeks of calving. During the process of involution, macroscopic but also microscopic and molecular changes occur. The uterus undergoes physical shrinkage, including muscular and glandular atrophy, followed by necrosis and sloughing that results in endometrial tissue regeneration. Studies have demonstrated the involvement of prostaglandin F (PGF), specifically PGF2α, which displays a strong pulsatile profile during the post-partum period (Kindahl *et al*., 1992; Fredriksson *et al*., 1985). However, the exact mechanisms controlling uterine involution remain unclear (Noakes, 2009).

The greatest reduction in uterine size takes place between 10-14 days post-partum in a healthy cow (Bajcsy *et al*., 2006). The bacterial contamination of the uterus at the time of parturition is believed to play an important part in the uterine involution process, by activating the innate immune response in endometrial tissue which clears, more or less quickly, the uterus of infection (Azawi, 2008). It is generally hypothesised that if the immune response is inadequate/too low, contamination of the endometrium with specific pathogens may persist. If full clearance of pathogens does not occur, the inflammation will continue and cause endometritis (Chapwanya *et al*., 2009).

When the immune response is appropriate, clearance of pathogens normally occurs during the first three weeks after parturition (Bekana, 1996; Bekana *et al*., 1994; Fredriksson *et al*., 1985). However, Sheldon *et al*. (2006) has shown that 10-30% of dairy cows in the UK develop an acute uterine infection expressing strong local symptoms associated with general signs (increased body temperature, lower milk production). This condition includes cases occurring within 21 days after parturition of acute metritis, which is defined as a systemic illness caused by a profound infection situated in the uterine wall (Sheldon *et al*., 2006). Acute puerperal metritis (APM) is characterised by the

presence of fever and a watery red-brown to mucopurulent discharge detectable in the vagina, in combination with an abnormally enlarged uterus (Sheldon *et al*., 2006).

Furthermore, according to Sheldon *et al*. (2006) about 20% of dairy cows in the UK develop persistent clinical endometritis, defined from histology as an inflammation in the inner lining (endometrium) of the uterine wall. The most common symptom is a purulent or mucopurulent discharge (Sheldon *et al*., 2006). However in some cases, cows may have endometritis without any clinical signs (sub-clinical endometritis) (Dubuc *et al*., 2010; Opsomer & Kruif, 2009; Sheldon *et al*., 2009; Gilbert *et al*., 2005; Elliott *et al*., 1968). The percentage of cows that contract sub-clinical endometritis within 4-8 weeks post-partum has been reported to be as high as 30-50% in North America (LeBlanc, 2014; Dubuc *et al*., 2011), showing the importance of these undiagnosed cases.

1.3 Diagnostic Methods

1.3.1 Transrectal palpation

Transrectal palpation of the uterus is a common diagnostic method used in clinical practice for reproductive diseases (Heuwieser *et al*., 2000). Obvious changes consistent with endometritis are general enlargement of the uterus, fluctuation of its contents and a hardened uterine wall (Callahan & Horstman, 1993). A sign which may be of value in the diagnosis of endometritis is a cervical diameter of more than 7.5 cm after 20 days post-partum (LeBlanc *et al*., 2002). Combining transrectal palpation with an ultrasound evaluation of the uterus improves the reliability of the diagnosis, as ultrasound can provide an objective evaluation of the uterine wall and the uterine contents and associated signs indicative of uterine diseases.

1.3.2 Examination of vaginal discharge

Evaluating the vaginal discharge for odour, colour and texture has been shown to partly reflect the bacterial status of the uterus and is reported to be related to the likelihood of recovery (Williams *et al*., 2005). Purulent vaginal discharge has been associated with impaired reproductive performance (Dubuc *et al*., 2011; Runciman *et al*., 2008; McDougall, 2001). However, vaginal discharge is not specific to uterine infections, and use as a sole clinical sign is highly questionable (Sannmann & Heuwieser, 2015).

1.3.3 Quantification of inflammatory cells by cytology

Quantification of inflammatory cells (Dubuc *et al*., 2010; Sheldon *et al*., 2006) can be achieved by cytology. Uterine samples can be collected either by sampling lavage fluid or preferably by using a cytobrush (Kasimanickam *et al*., 2005). Neutrophils are identified and counted and their percentage as a ratio to fixed number of epithelial cells can be used to assess the degree of uterine inflammation. In previous studies, different cut-off values (generally 5-10% of immune cells compared with total number of cells counted in samples) have been used to diagnose inflammation (Dubuc *et al*., 2010; Galvão *et al*., 2010; Kasimanickam *et al*., 2004). In a recent study, disturbances in prostaglandin F2α secretion and leukotrienes were observed only in clinical cases of endometritis, and not in sub-clinical cases, compared with controls, i.e. healthy cows.

Each of the above diagnostic methods has its own limitations. Using several methods would increase the reliability of the clinical findings (Williams, 2013), but the most reliable way to diagnose sub-clinical endometritis would be to take a biopsy for histology and identify pathogens from bacteriology. However, it is not practical to do so in dairy cows and sub-clinical endometritis often remains undiagnosed. It is thus generally accepted that new tools are needed, especially for the diagnosis of sub-clinical persistent endometritis, preferably in very early stages before the inflammation has become established.

1.4 Factors associated with uterine diseases

There are many risk factors for post-partum uterine diseases in the dairy cow. However, different risk factors may result in different forms of disease (Williams, 2013). In brief, factors that induce uterine trauma and/or bacterial contamination are more likely to result in uterine diseases with clinical signs than factors related to metabolic imbalance. In particular, energy deficit (negative energy balance, NEB) may result in endometrial inflammation with no apparent clinical signs, but associated with cytological changes (Dubuc *et al.*, 2010).

1.4.1 Calving conditions and environment

The factors found to be involved in uterine diseases and more specifically clinical endometritis have been studied quite extensively (Potter *et al*., 2010; Ill-Hwa & Hyun-Gu, 2003; Correa *et al*., 1993; Markusfeld, 1987). Retained foetal membranes, a need for calving assistance, stillbirth, sex of the calf (male, often of higher weight), parity (primiparous cows) and vulval angle (< 70°) have been identified as significant risk factors for development of clinical endometritis. Most of these factors are related to each other and are likely to be associated with trauma to the genital tract at the time of calving (Potter *et al*., 2010). For instance, trauma may disrupt the integrity of the endometrium, the first line of defence against ascending pathogens. In addition, despite the fact that specific bacteria involved in clinical endometritis originate from the immediate environment of the genital area (Sheldon *et al*., 2009), the occurrence of clinical cases of uterine diseases has been shown not to be related to cleanliness of the animal or hygiene on the farm (Potter *et al*., 2010; Bonnett *et al*., 1993).

1.4.2 Metabolism and milk production

Uterine infections occur during the post-partum period, at the same time as peak milk yield. The incidence of cows with metritis and endometritis is reported to be higher in high-yielding animals: 73.3% in cows producing > 35 kg milk/day compared with 45.2% in cows producing < 35 kg milk/day (Crowe & Williams, 2012). High-yielding cows are more susceptible to NEB and the interactions between metabolism, inflammation and fertility in such cows have been extensively studied (LeBlanc, 2012).

Both the local immune response in the uterus and the immune response in peripheral circulation are depressed during the peri-partum period and these changes may predispose the cow to uterine infection (Lewis, 1997). Impairment of neutrophil function, starting before parturition and related to energy status, has been reported (Hammon *et al.*, 2006). In addition, studies have shown that NEB, while impacting upon immunoglobulin IGF-I, clearly affects the immune function and more specifically may induce immune changes in the genital tract, *e.g*. an increase in expression of pro-inflammatory genes has been reported in the endometrium of cows with severe NEB (Wathes *et al.*, 2009) and in cows subjected to a restricted energy diet (Valour *et al.*, 2013). It is still not clear whether the severity of NEB increases the risk of developing uterine diseases. However, these reported changes in gene

expression were not associated with clinical symptoms and conflicting results have been obtained in attempts to associate changes in peripheral concentrations of mediators of NEB (such as non-esterified fatty acids, beta-hydroxybutyrate) with cytology or clinical cases of uterine diseases (see review by Williams, 2013). Discrepancies observed in previous studies may also derive from the impact of stage of the cycle on the number of immune cells. In cows, variations in the number of immune cells in the endometrium through the cycle have been reported and increased numbers of immune cells (especially neutrophils) have been observed in the bovine endometrium during oestrus compared with dioestrus (Eren *et al.*, 2009; Daniel, 1991; Hawk, 1971).

However, to our knowledge, immune system changes induced locally by NEB and their mediators, such as non-esterified fatty acids (NEFA), have not been investigated previously. The occurrence of local pro-inflammatory processes in response to NEB and or even slight changes in the immune balance or molecules involved in the process of immune tolerance may be unfavourable to embryo-maternal interactions. The impact at time of implantation deserves further investigation.

1.5 Pathogens associated with uterine diseases

1.5.1 Bacteria

Several pathogens have been identified as metritis- and endometritis-inducing agents and Gram-negative bacteria such as *Escherichia coli* and *Trueperella pyogenes* are commonly associated with uterine infections in the dairy cow (Ordell *et al.*, 2016; Santos & Bicalho, 2012; Sheldon *et al.*, 2010; Williams *et al.*, 2005; Zerbe *et al.*, 2001). However, as mentioned before, common bacteria are often present in the uterus of healthy and metritis-affected cows, showing that other factors are important for the persistence of bacterial contamination. Attempts have been made to characterise the differences between the bacterial microflora from ‘healthy’ and ‘non-healthy’ cows by metagenomic analysis and a more complex and numerous microflora in the uterus of animals with uterine infection than in healthy animals has been reported (Santos *et al.*, 2011). However, the kinetics of uterus microbiotic changes during infection and the presence of associated viruses have not been investigated.

1.5.2 Viruses

Infection by *E. coli* and *T. pyogenes* may pave the way for subsequent infection by other bacteria or viruses such as bovine herpes virus type (BoHV) 1 or 4 (Sheldon *et al.*, 2010; Donofrio *et al.*, 2008; Williams *et al.*, 2007). BoHV-4, a double-stranded DNA virus and a member of the *Gammaherpesvirinae*, was initially isolated from a variety of diseases such as respiratory and ocular disease in calves (Bartha *et al.*, 1965). However, BoHV-4 is one of the few viruses with a specific tropism for the endometrium (Donofrio *et al.*, 2008). An association between BoHV-4 seropositivity, post-partum metritis, abortion and chronic infertility has been reported in many studies (Graham *et al.*, 2005; Calvinho *et al.*, 2000; Czaplicki & Thiry, 1998) and BoHV-4 infection is considered to be a risk factor in uterine diseases and endometritis (Guer & Dogan, 2010; Frazier *et al.*, 2001; Egyed, 2000). In a recent epidemiological study, BoHV-4 infection significantly reduced the odds both of artificial insemination within 80 days post-partum and of cows being pregnant within 200 days post-partum, while a tendency for an increased risk of clinical endometritis was also shown (Klamminger *et al.*, 2017). BoHV-4 is considered a co-infection pathogen that induces uterine inflammation when animals are first infected with bacteria (Jacca *et al.*, 2013; Sheldon *et al.*, 2009; Donofrio *et al.*, 2008). In clinical endometritis, BoHV-4 is in most cases associated with bacteria such as *E. coli* and *T. pyogenes*. In a field study, BoHV-4 infection significantly increased the risk of intrauterine infection with *T. pyogenes*, and *vice versa*, illustrating the strong relationship between BoHV-4 and *T. pyogenes* infections (Klamminger *et al.*, 2017). The involvement of BoHV-4 in uterine diseases is currently considered to occur mainly in animals infected by the virus by other routes, such as the respiratory pathway. Consequently, mechanisms by which BoHV-4 alters cell function have been examined mainly in immune and stromal cells, while responses to BoHV-4 in endometrial epithelial cells have received less attention. However, a potential other route, as indicated by the presence of BoHV-4 DNA in cases of oedematous orchitis and also in the semen of healthy bulls, involves semen as a potential vector for BoHV-4 transmission to cows (Morán *et al.*, 2013; Egyed *et al.*, 2011). This suggests that it is necessary to define the responses of epithelial cells to BoHV-4 infection, because they are the first cells to be exposed to the virus, especially if the contamination occurs *via* semen at the time of natural mating or artificial insemination.

1.6 Pathogenesis and immune response: specific roles of LPS and BoHV-4

Gram-negative bacteria are commonly associated with uterine infections in the dairy cow (Ordell *et al.*, 2016; Santos & Bicalho, 2012; Sheldon *et al.*, 2010; Williams *et al.*, 2005; Zerbe *et al.*, 2001). Part of the pathogenic mechanism involved results from lipopolysaccharide endotoxins (LPS) (Holst *et al.*, 1996). These are molecules present on the surface of Gram-negative bacteria that exist under different complex forms and can circulate as an endotoxin in the peripheral circulation. In the case of uterine diseases, LPS mainly produced in the endometrium lead to acute or chronic inflammation of this tissue. Due to its presence in peripheral circulation, LPS can also affect reproductive function by impairing growth of ovarian follicles and lowering oestradiol secretion (Sheldon *et al.*, 2009; Dohmen *et al.*, 2000). As increased number of immune cells in uterine tissue, especially during oestrus (see above), is a 'natural' line of defence against infection, its potential inhibition by LPS is not favourable to recovery.

LPS activate the immediate immune response, leading to inflammation through a cascade of events well conserved in different tissues. The de-regulation of toll-like receptors (TLR), cytokines, chemokines, growth factors and major histocompatibility complexes (MHC) is a source of inflammation of epithelial barriers. The LPS first create complexes with pathogen-associated molecules (LPS-binding protein), which in turn binds to TLR-4 (see review by Sheldon *et al*., 2009). This TLR-4 activation induces reactions in the endometrium and leads to acute or chronic inflammation, which impairs reproductive function (Sheldon *et al.*, 2009; Dohmen *et al.*, 2000). These reactions include the secretion of cytokines (interleukins 1, 6 and 8 and tumour necrosis factor-alpha (TNF-α); (Beutler *et al.*, 2003), which activate and attract cells from the innate immune system, such as monocytes, macrophages, neutrophils, eosinophils and natural killer cells, into the stroma (Turner *et al.*, 2014; Cronin *et al.*, 2012; Sheldon *et al.*, 2010). In ruminants, LPS also induce dysregulation of prostaglandin secretion, stimulating local production of prostaglandin E (PGE) rather than PGF by endometrial cells, which may explain the prolonged luteal phase in cows with uterine diseases (Herath *et al.*, 2009). It has also been demonstrated that this shift in prostaglandin secretion is induced through activation of TLR-4 (Sheldon *et al.*, 2010). In addition, the increased production of PGE2 in uterine tissue may favour viral replication in macrophages, thus paving the way for viral co-infection (Donofrio *et al.*, 2008). Infection with *E. coli* is triggered through its membrane constituent LPS

and induces changes in PGE2 production, which activate BoHV-4 replication by activating the viral immediate early 2 (IE2) gene promoter (Fabian *et al.*, 2008; Donofrio *et al.*, 2004; Czaplicki & Thiry, 1998; Thiry *et al.*, 1992), which probably involves PGE2-dependent and -independent pathways. The bacterial co-infection and LPS may then initiate a positive feedback loop between PGE2 production and viral replication. This synergistic mechanism, showing the possible existence of cooperation between bacteria and viruses, may explain the rapid activation of viral replication in the bovine endometrium in cases of uterine diseases.

Gammaherpesviruses have a complex life cycle relying on both a replicative (or lytic) and non-replicative (or latent) phase (Whitley, 1996). The diseases associated with the replicative phase occur after primary infection and/or after reactivation from latency. Their pathogenesis relies mainly on the destruction of permissive cells caused by the replication and spread of the virus. Indeed, during latency only a limited number of viral genes are expressed (Whitley, 1996). The site of latency of BoHV-4 is in mononuclear blood cells, but it is also found in nervous ganglia and other tissues such as the bone marrow (Yamamoto *et al.*, 2000; Egyed & Bartha, 1998; Thiry *et al.*, 1990). Furthermore, BoHV-4 has been demonstrated to be latent in bovine peripheral mononuclear blood cells (PBMC) (Osorio & Reed, 1983) and taking into account that more than 98% of PBMC *in vivo* are resting cells, BoHV-4 infection of these cells could lead to non-productive infection, which in turn may favour latency (Vanderplasschen *et al.*, 1995). Activation and division of these latent infected cells could induce and allow virus reactivation. Interestingly, BoHV-4 infection of un-activated bovine PBMC cultures *in vitro* can lead to a non-productive infection, while some cells in activated cultures can support virus replication. For these reasons, BoHV-4 is considered to be a co-infection pathogen in reproductive disease that increases uterine inflammation when animals are first infected with bacteria (Jacca *et al.*, 2013; Sheldon *et al.*, 2009; Donofrio *et al.*, 2008).

Following the binding of viruses to specific TLR, infected cells secrete interferon gamma (INF-γ) and tumour necrosis factor alpha (TNF-α), which are the non-specific, earliest host responses of cytokines to viral infections. This response is followed in infected areas by a cascade of downstream mediators (Donofrio *et al.*, 2007; Reiss & Komatsu, 1998; O'Shea, 1997; Staeheli, 1990) leading to inflammation of the endometrium. Virus-infected cells also synthesise and secrete type I interferon (INF α/β), which is a major player in the antiviral defence response against all kinds of viruses (Fensterl & Sen, 2009). In addition, inflammatory molecules such as IL-1α, IL-1β and IL-6 are produced by immune cells and other cell types (Donofrio *et al.*, 2008;

Malazdrewich *et al.*, 2001). Moreover, BoHV-4 has the ability to trigger epithelial cells to produce more IL-8 and cells infected by BoHV-4 have been shown to be more sensitive to TNF-α (Jacca *et al.*, 2014). The binding of TNF-α to TNF-α receptor 1 on infected cells surface stimulates viral DNA synthesis (Jacca *et al.*, 2014) and also induces these infected cells to produce more IL-8 (Donofrio *et al.*, 2010) *via* IE2 gene product ORF50/Rta of BoHV-4 (Jacca *et al.*, 2014). This pathway involving the increase of the pro-inflammatory cytokine IL-8 in endometrial tissue may be part of the mechanism driving the disease toward a chronic status of endometritis.

The pro-inflammatory molecules kill virus-infected cells and act as a bridge between innate and adaptive responses (Ellermann-Eriksen, 2005). Among the reported mechanisms, BoHV-4 causes cytopathic effects (CPE) and replicates in a wide range of cell lines and primary cultures of various animal species (Wellenberg *et al.*, 2002; Donofrio *et al.*, 2000). In stromal cell culture, viral replication and CPE have been demonstrated by indirect fluorescent antibody test (Donofrio *et al.*, 2007).

In addition to the CPE mentioned above, there is evidence that viruses may affect cell function while inducing epigenetic modifications. Many viruses, including bovine herpes viruses, integrate DNA into the host cell nucleus to interact with chromatin factors. Cellular chromatin forms a dynamic structure that maintains the stability and accessibility of the host DNA genome (Lieberman, 2006). Viruses such as herpes viruses can enter and persist in the nucleus. In some cases, cellular chromatin inhibits viral gene expression and replication by suppressing DNA accessibility. In other cases, cellular chromatin provides essential structure and organisation to the viral genome and is necessary for successful completion of the viral life cycle. Consequently, there is different accessibility to host DNA and virus mechanisms to control the access (Lieberman, 2006), leading to different infection status. Although not fully demonstrated, these mechanisms could explain the existence of acute or permanently infectious carriers (apparently healthy). The fact that epigenetic modification regulates endometrial function (Munro *et al.*, 2010) and that host chromatin changes play a vital role in viral and host DNA interactions means that it is important to identify the underlying mechanisms explaining individual variation in response to infection and possible phenotypic differences reported between breeds in terms of risk of infection (Petersson *et al.*, 2006).

As described above, immune mechanisms are key players in the pathogeny of endometritis and have been extensively studied. However, numerous modifications of cell function have been reported in connection with LPS effects. In particular, the impact of LPS on cell proliferation has been studied in a variety of tissues and differing results have been obtained. In humans and

rodents, stimulation of proliferation is most often reported (Basso *et al.*, 2015; Eslani *et al.*, 2014; Hei *et al.*, 2012; Liu *et al.*, 2010; Muller-Decker *et al.*, 2005; Freitag *et al.*, 1996; Zhang *et al.*, 1996). Although LPS effects have been extensively studied in commercial farm animals, few of these studies have described changes in cell proliferation and viability and the results obtained are not fully consistent. For example, no effect of LPS has been found in pig intestinal cells (Klunker *et al.*, 2013). In the cow, LPS has been found to increase the number of mammary gland epithelial cells during *in vitro* culture (Piotrowska-Tomala *et al.*, 2012), while another study found no effect in a mammary cell line (Calvinho *et al.*, 2000). Negative effects of LPS have been reported in oviductal epithelial cells in culture (Kowsar *et al.*, 2013). In contrast, studies on the bovine endometrium have reported that immune cells can promote the proliferation of neighbouring cells through production of pro-inflammatory cytokines (Eslani *et al.*, 2014; Sheldon *et al.*, 2010; Herath *et al.*, 2009; Holst *et al.*, 1996). Overall, it is possible that these differences between studies may be due to variations in LPS concentrations relative to body weight. A proteomics study has shown that LPS can also promote oxidative stress, resulting in over-expression of peroxiredoxin and heat shock proteins in cows with endometritis compared with healthy cows (Choe *et al.*, 2010).

1.7 Successful implantation requires activation of a large number of molecules induced by embryo-maternal interactions

In ruminants with synepitheliochorial placenta type, there is a fusion between trophoblastic cells and endometrial cells at implantation, without direct contact with maternal blood. The endometrial tissue is the site of intense remodelling induced by numerous signals (Mansouri-Attia *et al.*, 2012; Oliveira *et al.*, 2012; Forde *et al.*, 2011; Singh & Aplin, 2009). Interferon tau (INF-T), which in ruminants is the key molecule for maintenance of pregnancy by inhibiting prostaglandin-induced luteolysis (Oliveira *et al.*, 2012), is also a critical signal for implantation by up-regulation of a large number of genes called interferon-induced genes, which are regulated through the signal transducer and activator of transcription 1 (STAT1) pathway (Mansouri-Attia *et al.*, 2012). In addition, tissue remodelling is induced by changes in proteins involved in the control of cell structure (actins, actinin), calcium metabolism in relation to membrane properties (calcitonin), cell adhesion (catenins, plakophilin, cadherins, integrins), protection of epithelium (mucins) and enzymes controlling protein

remodelling, such as matrix metalloproteases (Singh & Aplin, 2009). Simultaneously, changes in growth factors associated with the development of vascular tissue at the time of implantation take place (Singh & Aplin, 2009).

In addition to these signals regulating tissue remodelling, cell structure, cell adhesion and vascularisation, complex immune mechanisms take place, leading to lack of immune rejection of the young embryo.

1.8 Similar immune pathways are activated in reaction to pathogens and for establishment of pregnancy

Immune mechanisms allowing pregnancy, or which may be the source of rejection of the embryo, have been defined mostly in humans and mice (Robertson *et al.*, 2009), where a choice between 'immunorejection' of the embryo allograft and 'immunotolerance' (facilitating pregnancy outcome) needs to be taken *via* specific immune cells.

As mentioned above, in cases of infection a series of signals (including IL-6, TNF-α, IFN-γ and MHC class I and MHC class II molecules) drives pro-inflammatory responses at the beginning of pregnancy (such as differentiation of CD4+ cells into T cells and natural killer cells). However, in humans, their up-regulation induces graft rejection and recurrent miscarriage (Robertson & Moldenhauer, 2014).

An alternative 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 transforming growth factor-β (TGF-β) and galectins. Galectins constitute a family of lectins with a wide range of functions in various tissues. 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 (Froehlich *et al.*, 2012; Phillips *et al.*, 1996). However, basic knowledge about its function during pregnancy is only emerging. It is known that Gal-1 allows trophoblast invasion by modulating non-classical MHC molecules such as human leucocyte antigen G (HLA-G) on trophoblastic cells (Tirado-González *et al.*, 2012). It also acts as a pro-angiogenic regulatory protein critical for implantation and embryo growth (Barrientos *et al.*, 2014). Moreover, Gal-1 skews the differentiation of CD4+ T-cells towards Treg cells through the action of forkhead box P3 (FOXP3) (Yakushina *et al.*, 2015), confirming its role as a major 'tolerogenic' agent necessary to establish pregnancy (Barrientos *et al.*, 2014). In humans, de-regulation of Gal-1 expression has been associated with spontaneous abortion and pre-eclampsia (Barrientos *et al.*, 2014; Tirado-González *et al.*, 2012), indicating a critical role for the maintenance of pregnancy.

In humans and laboratory animals (mainly rodents), these common pathways are used for driving the inflammatory response and recognition of the embryo by the endometrium. Very little information exists on production animals. However, it can be hypothesised that, in a similar way, alteration of the fragile and dynamic immune balance due to persistent subclinical inflammation may impair implantation and fertility long after clinical symptoms have disappeared (Sanchez-Lopez *et al.*, 2014).

1.9 Infections may have long-term consequences, impairing fertility

Infection by pathogens activates the immediate immune response, leading to inflammation through a cascade of events well conserved in different tissues. The de-regulation of TLR, cytokines, chemokines, growth factors and MHC is a source of inflammation of the epithelial barrier in the endometrium (see section 1.6). Acute infections are often followed by an asymptomatic persistent inflammation which remains untreated if not diagnosed. Untreated persistent inflammation of the endometrium (Potter *et al.*, 2010; Herath *et al.*, 2009), due to the lack of accurate methods for its diagnosis (see section 1.3), can later disturb the fragile embryo-maternal interactions (see sections 1.7 and 1.8) necessary to establish successful implantation, thus impairing fertility (Sheldon *et al.*, 2009; Gilbert *et al.*, 2005).

2 Aims of the thesis

The overall aim of this thesis work was to describe the characteristics of the bovine endometrial epithelial cells response to pathogen challenges (*E. coli* LPS and BoHV-4.

Specific objectives were to:

- Study the effect of cow factors (such as parity and breed) on the characteristics of uterine tissue samples collected at different stages of the oestrous cycle.

- Characterise uterine tissue/samples to define the most appropriate material to perform challenges with *E. coli* LPS and identify possible sources of variation in cell response to LPS.

- Characterise the responses of bovine endometrial epithelial cells (cell proliferation, survival and apoptosis) and subsequent changes in proteomics profiles when exposed to different doses of LPS.

- Characterise viral replication, survival and cytokine profiles of bovine endometrial epithelial cells infected with BoHV-4.

3 Materials and methods

3.1 Ethical permission

The laboratory studies were performed *in vitro* at SLU, using organs collected with the permission of the local slaughterhouse (Lövsta, Uppsala).

3.2 Study design

Three studies were performed:

Study I (Paper I): A study was carried out to characterise uterine tissue, identify possible sources of variation in cell responses to pathogens and define the most appropriate material for use in subsequent challenges. The study examined the effect of age, breed and stage of oestrous cycle on normal cell growth. Oestrous cycle was determined by the morphological appearance of ovarian follicles and *corpora lutea* (CL) and by histology (density of glandular tissue). The density of CD11b-positive immune cells presenting and a marker of cell proliferation (Ki67-positive cells) were also investigated. This study was conducted at SLU.

Study II (Paper I and II): This study investigated the effects of *E. coli* LPS on the survival, proliferation and proteomics profiles of a pure population of endometrial epithelial cells, 72 h after challenges in an *in vitro* model developed in Paper I. Pure populations of bovine endometrial epithelial cells were exposed to a dose of 0, 2, 4, 8, 12 16 and 24 µg/mL *E. coli* LPS. Apoptosis and proliferation index were determined on a subset of samples. The first part of the study was based on counting cell populations identified by

trypan blue assay. This part of the work was conducted at SLU. In addition, a study was undertaken to characterise the changes in proteomics profiles following challenges by *E. coli* LPS (paper II). A subset of results from LPS challenges was chosen and used for proteomics analysis (Paper II). Proteins were extracted from cell pellets from the 8 and 16 µg/mL *E. coli* LPS treatments, 72 h after challenges. All protein extraction from control and LPS-treated pellets was analysed by an unbiased approach combining 2-D electrophoresis and matrix-assisted laser desorption/ionisation-time of flight (Maldi-TOF/TOF) quantification. A subset of control and LPS cell pellets was subjected to complementary shotgun analyses. All biological material was prepared at SLU and the proteomics analyses were outsourced and performed in collaboration with the University of Milan, Italy.

Study III (Paper III): In this study, biological materials from additional cows were characterised according to the responses by endometrial epithelial cells when exposed to BoHV-4. The survival of cells was studied using same methods as in Paper II. Moreover, specific methods were developed: i) to define a model of challenge, i.e. a protocol to standardise the exposure of endometrial epithelial cells to BoHV-4 virus; and ii) to quantify virus replication by quantitative polymerase chain reaction (qPCR). In addition, localisation of viral particles within bEEC by an indirect fluorescent antibody test (IFAT) was used in complement conventional virus titration. The cells used in the model were produced and the cytokine quantification was performed at SLU, while the virology techniques and challenges with BoHV-4 virus were performed in collaboration with the Swedish National Veterinary Institute (SVA).

3.3 Animal/sample collections

Bovine genital tracts (including ovaries) were collected at a local slaughterhouse (Lövsta, Uppsala) within 10 min of slaughter, immediately placed on ice and brought to the laboratory within 1 h. From each genital tract, the left uterine horn was used for gross evaluation, histology and immunohistochemistry, and the right uterine horn was used for cell culture (Papers I-III). The Swedish official milk recording scheme (SOMRS) was used to obtain information about the animals, based on their national identification number. The 35 genital tracts included in the study originated from Swedish Red Breed (SRB) cows (n = 20), Holsteins (n = 13) and unknown breed cows (n = 2). The parity of the animals varied from zero (no calving, 7 heifers) to six

(28 cows). A subset of 14 dioestrus females was included in the cell culture experiment.

3.4 Uterine tissue characterisation (Paper I)

3.4.1 Determination of oestrous cycle

To determine the stage of oestrous cycle for each female, the cross-section of CL was measured with a ruler and the colour and presence of haemorrhagic spots was recorded (Arosh *et al.*, 2002; Ireland *et al.*, 1980). Four stages of the oes-trous cycle, i.e. proestrus, oestrus, metoestrus and dioestrus, were distinguished depending on size, colour and haemorrhagic appearance of the CL.

3.4.2 Tissue fixation and embedding

Cross-sections (n = 35) of the left uterine horn were taken systematically 5 cm from the tip of the uterine horn for uterine gland morphology and for subsequent evaluation of uterine health. All samples were fixed in 4% paraformaldehyde at 4°C for 48 h and routinely prepared. The fixed tissues were then embedded in paraffin and cut into 8 µm thick sections.

3.4.3 Glandular tissue analysis

Sections were deparaffinised and rehydrated and stained with haematoxylin and eosin. The total number of cross-sections of uterine glands was counted in printed photos of the full piece section of the uterine horn. These photos were taken under a light microscope (10× magnification) and a grid plate was used to calculate glandular area. The density of glandular tissue (dGT) was then calculated as number of uterine gland cross-sections per square centimetre (gcs/cm^2) based on the total surface of endometrial tissue from a given section of uterine horn measured with the help of the grid.

3.5 Immunohistochemistry (Paper I)

3.5.1 CD11b-positive cells

After deparaffinisation and rehydration, antigen retrieval was performed and endogenous peroxidase activity was quenched in 3% H_2O_2. Binding of rabbit

anti-CD11b antibody was visualised using a goat anti-rabbit IgG-HRP secondary antibody with subsequent chromogenic detection. Counterstaining was performed with Mayer's haematoxylin (HTX). The CD11b-positive cells were counted from a full piece of cross-section of uterine horn at 200× magnification under a light microscope. The density of CD11b-positive cells was then calculated as number of cells/mm^2 of cross-section surface.

3.5.2 Ki67-positive cells

After antigen retrieval and blocking of endogenous peroxidase activity, the slide sections were incubated with a mouse monoclonal anti-Ki67 antibody and thereafter with a secondary anti-mouse IgG. Chromogenic reaction and counter-staining were as described previously. The total number of Ki67-positive cells was counted under a light microscope (200× magnification) from a full piece of cross-section of uterine horn. The density of Ki67-positive cells was calculated as the number of cells/mm^2, with the area of calculation based on full surface sections on the slides.

3.6 Bovine endometrial epithelial cell cultures (Papers I, II and III)

Endometrial tissue collected from 14 dioestrus females was cut into 5 cm long and 5 mm thick pieces for the bEEC culture experiment. The endometrial epithelial cells were separated from the stromal cells and subsequently cultured. The pieces were incubated with collagenase IV and hyaluronidase, after which the suspension was filtered to remove mucus and undigested tissue. After passing the filtrate through a nylon sieve, the retained epithelial cells were collected by backwashing. Epithelial cells were cultured in F-12 medium containing foetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, liquid media supplement, gentamycin and nystatin. The cells were seeded into ventilation flasks and cultured with 5% CO_2 at 39°C until confluence. The cells were then passaged into new flasks repeatedly (up to four passages). The purity of the epithelial cell culture was checked by flow-cytometry labelling cytokeratin. From passage 2 and thereafter, more than 98% of cells expressed cytokeratin, confirming the very high purity of the cell culture system.

3.7 Cell challenges with LPS (Papers I and II) and BoHV-4 (Paper III)

3.7.1 bEEC challenged with *E. coli* LPS (Paper I)

Cultured bEEC were challenged by *E. coli* LPS (L2630 *E. coli* O111:B4, Sigma, Saint Louis, USA). The bEEC used in these challenges came from 13 cows and following passages 4-6. For each individual challenge, cells from a given culture sample were initially seeded in flasks and exposed to LPS or a control. Cells were then cultured for 72 h before being challenged by LPS. At time of challenge (time 0) culture medium was changed and the old medium was replaced either with medium alone (LPS = 0 µg/mL, controls) or with medium supplemented with LPS concentrations of 2, 4, 8, 12, 16 or 24 µg/mL. The number of challenges performed for each LPS dose with individual cow samples is given in *Table 1*. Each challenge performed on each cell culture sample systematically included a control and the 8 µg/mL dose. Culture was then run for an additional 72 h period and different types of cells were counted.

Table 1. *Number of animals from which bovine endometrial epithelial cells (bEEC) were obtained and number of challenges performed for each lipopolysaccharide endotoxins (LPS) dose (0, 2, 4, 8, 12, 16 or 24 µg/mL)*

LPS dose (µg/mL)	**0**	**2**	**4**	**8**	**12**	**16**	**24**	**Total**
Number of cows	13	16	8	13	4	12	4	13
Number of challenges	33	15	20	33	14	30	6	151

3.7.2 bEEC challenged with BoHV-4 (Paper III)

Lethal dose of virus (LD_{50}) determination

To determine the 50% lethal dose of virus (LD_{50}) to bEEC, bEEC were challenged by different viral multiplicity of infection (MOI, i.e. viral units/cell) (MOI 0.001, 0.01, 0.1, 1 and 10). The bEEC were cultured in flasks until confluence was reached and then detached by trypsin (TrypLE™ Express (1×), ref.12605-010, Gibco®, Waltham, USA). The sample material was then transferred to 15 mL centrifuge tubes and centrifuged at 1,000 rpm. Supernatant was discarded and 2% FBS medium was added to suspend the cell pellet. The material was then aliquoted into six centrifuge tubes and serial viral MOI 0.001, 0.01, 0.1, 1 and 10 was added, while the controls received 2% FBS instead of virus. All sample tubes were incubated under 5% CO_2. After

incubation, the samples were centrifuged at 1,000 rpm, supernatant was discarded and 10% FBS medium was added to suspend the pellet. The cells were then placed in 12-well plates and cultured at 39°C with 5% CO_2 for six days. Number of cells was evaluated by trypan blue assay and virus amount was evaluated by a titration technique. Following challenges with MOI 0.1, 50% of trypan blue (TB) cells (living cells) were present by Day 6 and the highest amount of virus was found compared with other groups. Based on these results, an MOI of 0.1 was used for the virus in subsequent tests.

bEEC challenged with BoHV-4 MOI 0.1

Cultured bEEC from passage 4 were detached by trypsin, transferred into centrifuge tubes and centrifuged, supernatant was discarded and 2% FBS medium was added to suspend the cell pellet. The samples were then aliquoted into two 50 mL centrifuge tubes, each of which received 2% FBS medium (control) or BoHV-4 virus at MOI 0.1. After incubation under 5% CO_2, the tubes were centrifuged again at 4,000 rpm. The supernatant was discarded, 10% FBS medium was added to both tubes to suspend the cell pellet and around 3 to 7 × 10^4 cells/well were placed in 12-well plates. Cells were cultured at 39°C with 5% CO_2. Number of cells was evaluated by trypan blue assay and amount of virus was evaluated by a titration technique, qPCR and IFAT at each time point (Day 1 to Day 7).

3.8 Cell counting and viability (Papers I and III)

After challenges, bEEC were counted and cell viability was determined using trypan blue assay (Papers I, II and III). In brief, the supernatant was removed and floating cells (dead cells) in the medium were counted under microscope using a Burker-Neubauer chamber (haemocytometer, 40443001, Hecht Assistent®, Rhon, Germany). Attached cells (considered to be living cells) were then detached with trypsin (TrypLE™ Express (1×) ref.12605-010, gibco®, MA, USA). These cells were exposed systematically twice to trypsin. Flasks were then checked for remaining cells. This protocol was applied again when some cells remained attached. All cells were pipetted from flasks and transferred to Falcon tubes. The solution was gently mixed for 2-3 s and 70 µL were taken and mixed with the same volume of trypan blue solution (T8154 trypan blue solution 0.4%, Sigma®, MO, USA) in Eppendorf tubes. Mixed solution was immediately transferred to the counting chamber (same as above). Unlabelled and labelled cells were counted under low magnification in a light microscope. Raw numbers (n) and frequencies (%) of cells in each category:

floating cells (n and % of total cells), trypan blue-positive cells (TB+ from attached cells; n and %) and trypan blue-negative cells (TB- from attached cells; n and %) were determined. In the controls, the relative increase in the number of live cells was calculated (X).

$$X = \frac{\text{Number of TB- cells at 72 h} - \text{Number of attached cells at 0 h}}{\text{Number of attached cells at 0 h}}$$

Following LPS challenges, the relative increase in live cells compared with controls was calculated (Y).

$$Y = \frac{\text{Number of TB- cells LPS at 72 h - Number of TB- cells controls at 72 h}}{\text{TB- cells controls at 72 h}}$$

3.9 Measurement of cell proliferation and apoptosis (Paper I)

Proliferation was measured following a subset of LPS challenges at different time points using the quick cell proliferation assay kit (ab65473, abcam, Cambridge, UK). Cells from two cows (1.0×10^4 cells/well) were first cultured in a 96-well microtitre plate in a final volume of 200 µL/well culture medium. Old medium was then discarded and new medium containing either 0, 8 or 16 µg/mL LPS was added. For each LPS concentration, sufficient wells were prepared in advance to investigate proliferation at time 0, 6 h, 24 h, 48 h and 72 h. At each time point, WST-1/ECS solution was added to each well and cells were incubated in standard culture conditions. Plates were then shaken thoroughly and absorbance in each well was measured at 450 nm and 630 nm. Ratio of proliferation between LPS groups and controls was also calculated for each time point (t) between 6 h and 72 h (Z).

$$Z = \frac{\text{Absorbance LPS time t - Absorbance control time t}}{\text{Absorbance control time t}}$$

Apoptosis was assessed using the FITC Annexin V Apoptosis detection kit (cat. no. 556570, BD Phamingen). At the end, a binding buffer was added to each tube and samples were analysed by flow cytometry within 1 h (BD FAC-Sverse™). The apoptosis rate was determined by the ratio between numbers of cells both stained with Annexin 5 and the control propidium iodine solution and total number of cells counted through flow cytometer from 10,000 events.

3.10 Viral titration and evaluation (Paper III)

3.10.1 Viral titre/$TCID_{50}$ (tissue culture infective dose) by titration

After challenges by BoHV-4, the bEEC were collected and frozen at -70°C. Samples were collected for both control and infected cells from Day 1 to Day 7. Madin-Darby bovine kidney cells (MDBK) in suspension in 10% horse serum medium (MEM; 100 µL) were added to 96-well microtitre plates and incubated while supplied with 5% CO_2. Frozen BoHV-4 from sample materials was thawed and serially diluted from 10^0 to 10^{10} in medium. Each dilution of BoHV-4 was inoculated on cultured MDBK cells (in eight replicate wells per dilution of virus). Inoculated MDBK cells were then incubated for 7 days. After incu-bation the plates were analysed for virus infectivity under a light microscope. Prevalence of virus was indicated by its CPE. The amount of virus after BoHV-4 challenges on bEEC was based on serial dilutions to determine end-point titre.

3.10.2 Viral evaluation by quantitative polymerase chain reaction (qPCR)

Sample materials from Day 1 to Day 7 were collected and used to quantify number of virus particles by qPCR. Primers and probes have already been de-signed and validated for BoHV-4 detection (Juremalm *et al*., manuscript in pre-paration). Viral DNA was extracted by adding proteinase K (Sigma, P4850, Sigma Aldrich, MO, USA). Nucleic acid extraction was then performed in a Magnatrix 8000+ robot (NorDiag AB, Sweden) according to the manufacturer's instructions. For the PCR reaction, template DNA was mixed with SoFast probes supermix (Bio-Rad, UK), each primer (BHV-4gBF and BHV-4gBR) and the probe. The samples were amplified in an Applied Biosystems 7500 Fast Instru-ment (Live Technologies, ThermoFisher Scientific, Sweden) during 45 cycles. Each cycle included denaturing at 95°C for 5 s, annealing and elongation at 60°C for 30 s.

3.10.3 Viral prevalence by indirect fluorescent antibody test (IFAT)

Aliquots (25 µL) of non-infected and infected cell suspension were placed in 10-well slides and cultured in humidity chambers at 39°C in 5% CO_2. To analyse the course of infection, slides were taken out each day for a period of 7 days, washed with phosphate-buffered saline (PBS) and Super-Q water and then fixed by acetone and stored at -70°C until analysed. Thereafter, slides

from Day 1, 3 and 7 were analysed for virus prevalence. After thawing, an anti-BoHV-4 mono-clonal antibody was diluted in PBS and added to each well slide. The slides were incubated in a dark humid chamber and then washed with PBS and Super-Q water. The cells were stained with fluorescence isothiocyanate-conjugated rabbit anti-mouse IgG (cat. no. F0232, Dako, Glostrup, Denmark). The slides were then washed with PBS, dried, mounted with glycerol and examined under fluorescence microscope to determine the prevalence and localisation of virus antigen by a fluorescence signal from the cells.

3.11 Cytokine measurement by ELISA (Paper III)

Sample materials were collected after BoHV-4 challenges from Day 1 to Day 7. These samples were centrifuged and the supernatant was collected and used to evaluate the concentration of TNF-α (Bovine TNF-a ELISA Kit, ref. EBTNF, lot 0650070715, Thermo Scientific, Waltham, MA, USA) and interleukin 8 (IL-8) (Bovine IL-8 (CXCL8) ELISA development kit, 3114-1H-6, MABTECH AB, Nacka Strand, Sweden). The supernatant from each cow sample was taken into the 96-well plates and each standard was added to appropriate wells. The optical density absorbance (OD) of TNF-α and IL-8 in challenged samples at several time points was measured by an ELISA plate reader at 450 nm and 550 nm and converted into concentration (Z).

$$Z = \frac{\text{Absorbance LPS time t - Absorbance control time t}}{\text{Absorbance control time t}}$$

3.12 Proteomicss analyses (Paper II)

3.12.1 Samples, protein extraction and quantification

Frozen bEEC pellets from nine cows were prepared from cells exposed to 0, 8, and 16 µg/mL LPS for 72 h at the SLU laboratory. The following steps were performed at the University of Milan. Pellets were defrosted in ice and centrifuged. The supernatant was carefully discarded and remaining cells were solubilised in a buffer containing 7M urea, 2M thiourea and 2% chaps with protease inhibitors. Samples were solubilised with two cycles interspersed with magnetic gentle stirring. The samples were then sonicated for 20 min and centrifuged. The pellet was discarded and the supernatant with the extracted

proteins was frozen at -20°C until use. Total protein quantification was performed using the BioRad Protein Assay quantification kit.

3.12.2 Proteomics analyses

Samples were analysed by using two complementary proteomics approaches: i) 2-D electrophoresis and image analysis followed by MALDI-TOF/TOF-MS analysis and ii) shotgun analysis (for details see Paper II). Approach (i) allows identification of the different isoforms of the proteins identified, while approach (ii) quantifies the full amount for a given protein.

3.13 Statistical analysis

All statistical analyses were performed with SAS (Ver 9.2). ANOVA results are presented as least square (LS) means ± standard error of the means (SEM). When necessary, data were log-transformed to normalise variances, but the data are all presented untransformed in the results section to facilitate interpretation. In the case of multiple comparisons, Scheffe's adjustment was used to assess differences between levels of a given treatment. The contrast option was also used to perform multiple comparisons between treatment groups. The cut-off value for significance was set at $p < 0.05$.

3.13.1 Characterisation of uterine tissue/samples to identify possible sources of variation in cell responses and define the most appropriate material to perform challenges (Paper I)

The main effects of cow parity, oestrous cycle stage, breed, and corresponding second-order interactions between these factors, on density of glandular tissue, CD11b-positive cells and Ki67-positive cells were analysed by ANOVA (proc GLM) on a dataset obtained from the 35 females. Individual data for the above factors were grouped as follows: parity groups were defined as heifers, parity 1 and parity > 3, stage of oestrus cycle was divided into Stage 1 (proestrus cows) and Stage 2 (metoestrus and dioestrus cows).

3.13.2 Statistical analysis for LPS challenges (Paper I)

ANOVA was used to analyse the effects of parity, density of glandular tissue, density of CD11b-positive cells, density of Ki67-positive cells (as single continuous co-variables in each model), initial number of cells put in culture and passage number on the numbers and frequencies of the different types of cells

in controls and following *E. coli* LPS challenges (13 females). When analysing the effect of *E. coli* LPS, six classes corresponding to the LPS doses from 2 to 24 µg/mL and possible interactions with other factors, were added in the models. In addition, spearman correlation coefficient was calculated, to study the relationships between continuous variables. Raw absorbance data and Z ratio from proliferation kit assay and frequencies of cells labelled for apoptosis were analysed by ANOVA mixed models, with culture time and LPS dose as fixed effects and cow and corresponding interactions with cow as random effects.

3.13.3 Changes in proteomics profiles induced by LPS (Paper II)

Statistical analysis for validation of 2-D electrophoresis dataset: For a subset of five proteins, the repeatability of the results over three series of analyses was studied. The ratio of expression over control ((Value LPS - Value control)/Value control) was analysed by ANOVA by including cow ID, series number and LPS dosage in the model as well as LPS × cow and LPS × series interactions. Tests were made while comparing the LS-means ratio, observed for a given protein to 0, which is the theoretical value of the ratio under the null hypothesis (H0).

3.13.4 Responses of endometrial epithelial cells exposed to BoHV-4 (Paper III)

The effects of viral dosage, cow class of initial cell number and secondary interactions or effects of time, cow, viral exposure and second-order interactions on percentage of living cells were analysed by ANOVA following arcsine transformation of percentages.

4 Results

4.1 Characterisation of uterine tissue/samples to identify possible sources of variation in cell responses and define the most appropriate material to perform challenges (Study I; Paper I)

4.1.1 Oestrous cycle

Stage of the oestrous cycle in cows from which uterine samples were taken was determined by gross appearance of ovarian structures and morphology of the uterine wall (Paper I). Colour and size of CL in the ovaries differed depending on oestrous cycle stage (proestrus, metoestrus and dioestrus) (*Figure 1*). The density of glandular tissue was found to be higher during the luteal phase (dioestrus cows) than in the follicular phase (proestrus cows).

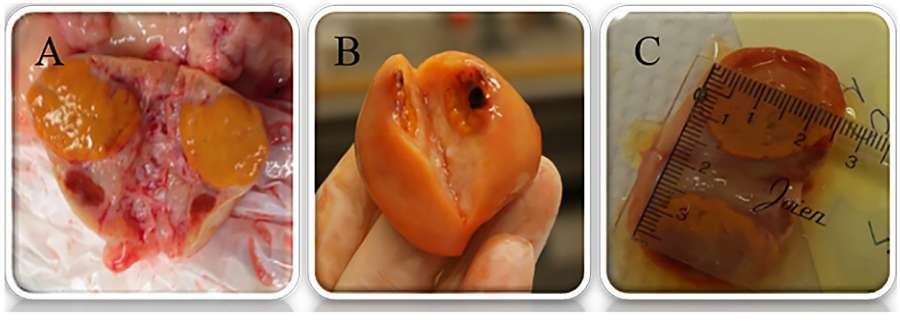

Figure 1. Representative appearance of the corpus luteum (CL) in different stages of oestruos cycle. (A) Proestrus (n=5), (B) Metoestrus (n=4), (C) Dioestrus (n=26). (Photo: Metasu Chanrot, SLU)

4.1.2 Density of glandular tissue

A significant effect of stage of oestrous cycle was found for dGT ($p < 0.0001$), whereas no significant effects of parity and breed were observed. The dGT was higher in Stage 2 (metoestrus and dioestrus; 6102.5 ± 632 gcs/cm^2) than in Stage 1 (proestrus; 2222 ± 650 gcs/cm^2). The interactions between stage of cycle, parity and breed were not significant.

4.1.3 Density of CD11b-positive cells

The density of CD11b-positive cells ranged from 0 to 7.8 cells/mm^2 and significant effects of parity group ($p < 0.0001$) and stage of oestrous cycle ($p < 0.05$) were found. More CD11b-positive cells were found in the endometrium when parity increased ($p < 0.0001$). A lower density was observed in samples from heifers (0.5 ± 0.7 cells/mm^2) and cows with parity 1 to 3 (0.9 ± 0.5 cells/mm^2) than in cows with parity >3 (3 ± 0.6 cells/mm^2). Moreover, the density of CD11b-positive cells was higher in samples from proestrus females (Stage 1; 2 ± 0.7 cells/mm^2) than in samples from metoestrus and dioestrus females (Stage 2; 0.9 ± 0.3 cells/mm^2). There was a significant interaction ($p < 0.01$) between parity and stage; the effect of parity was highly significant for proestrous samples, whereas no difference was observed for metoestrus or dioestrus cows/heifers. The CD11b-positive cells were located preferentially in stromal tissue and very few were present in epithelium.

4.1.4 Density of Ki67-positive cells

A significant effect of stage of oestrous cycle was found on the density of Ki67-positive cells ($p < 0.01$). The number of positively marked cells was highest in samples obtained at proestrus and lowest at dioestrus. Ki67-positive cells were located essentially in luminal and glandular epithelia and relatively few were located in stromal tissue.

4.2 Responses of bovine endometrial epithelial cells and changes in proteomics profiles induced by LPS (Study II; Paper I and II)

Cell culture was successfully developed from endometrium of cows collected during dioestrus. The application of protocols allowed the preparation of pure

culture (> 95%) of bEEC, as demonstrated by morphology and flow cytometry analyses (Papers I and II).

4.2.1 Proliferation characteristics and sources of variation in controls (Paper I)

The increase in number of bEEC in control cultures was only influenced by the number of cells (seeding cell group) at the start of culture ($p < 0.0001$) and passage ($p < 0.01$) from which they originated. No effect of the cow factors (breed, parity) or uterine characteristics (density of uterine glands, CD11b-positive cells and density of Ki67-positive cells) were found. The increase in cell numbers after Day 3 was about three-fold higher when less than 5×10^5 cells were seeded, while this increase was less pronounced for the other groups. In contrast, the number of floating cells (dead cells) increased with the number of cells put in culture ($p < 0.01$). In correlation analysis, no significant relationships were found between spontaneous proliferation of epithelial cells in culture and their response to *E. coli* LPS challenges.

4.2.2 Proliferation of bEEC following LPS challenges (Paper I)

Following LPS challenges, overall very significant effects of LPS dosage were found on all variables analysed (dead cells floating, total attached cells and TB- cells). A significant increase in the number of floating cells was observed with the highest LPS doses (16 and 24 µg/mL) compared with controls. The total number of attached cells (TB+ and TB- cells) differed significantly ($p < 0.0001$) following challenges with various LPS doses, but this increase was not linear. The total number of attached cells and TB- cells was significantly increased with the lowest LPS doses ($p < 0.001$) whereas a tendency for a decrease was observed with the highest LPS dose. Higher numbers of TB+ cells were found with the highest LPS dose when compared with controls ($p < 0.05$). Finally, on analysing the relative increase in number of TB- cells (each sample compared against its own control), a significant increase ($p < 0.0001$) was found between 2 and 12 µg/mL LPS (*Figure 2*). However, these ratios were not different from 0 for the 16 µg/mL dose and tended to decrease following use of the highest LPS concentration.

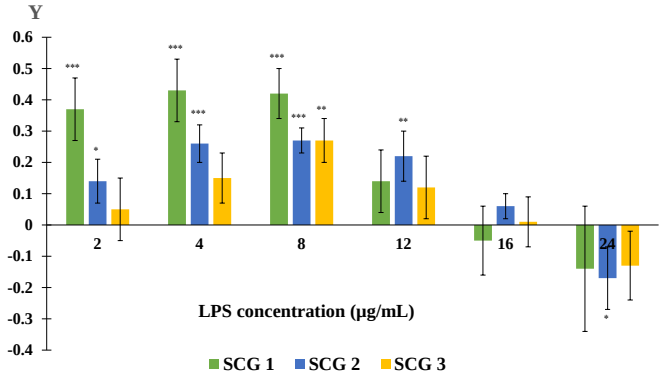

Figure 2. Ratio of changes induced by lipopolysaccharide endotoxins (LPS) treatment over controls in the numbers of trypan blue-negative cells (Y, LS-means ± SEM) and effects of seeding cell group (SCG). The ratio of increase in cell numbers was higher in SCG 1 than in other groups. Significance of changes compared with control group: *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

The above results for cell numbers and morphology suggest strong proliferative effects of LPS in low doses and increased cell death at higher doses, indications consistent with those obtained by measuring specific markers of proliferation and apoptosis. Based either on raw values or on the ratio of increase compared with controls (Z), a significant increase in proliferation was observed in the 8 µg/mL LPS group from 6 to 48 h compared with controls ($p < 0.05$ to $p < 0.01$ depending on time), with a maximum increase observed at 24 h (*Figure 3*). With 16 µg/mL LPS, the results were not different from the control until 24 h, but a significant decrease was observed thereafter ($p < 0.001$), suggesting inhibition of cell proliferation during this time window. The percentage of apoptotic cells did not differ significantly between the control (9.48% ± 0.28) and the 8 µg/mL LPS group (9.03% ± 0.28) but a significant increase ($p < 0.01$) was observed for the 16 µg/mL LPS group (11.28%).

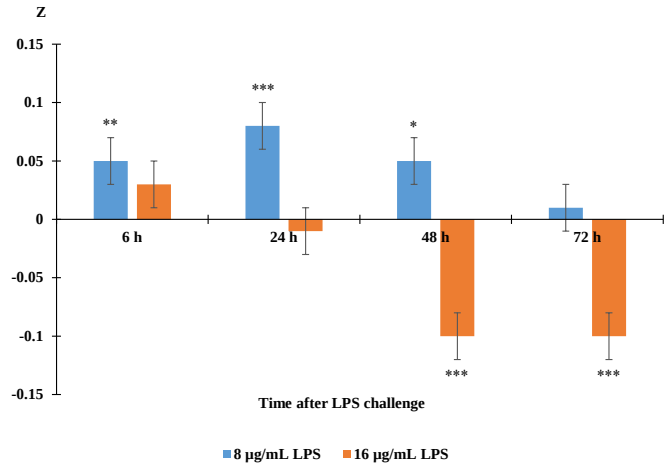

Figure 3. Ratio of increase (8 µg/mL lipopolysaccharide endotoxins (LPS) group) or decrease (16 µg/mL LPS group) in cell proliferation compared with control samples.

4.2.3 Changes in proteomics profiles induced by LPS (Paper II)

A total of 1096 different spots were visualised from pellets of endometrial epithelial cell by 2-D electrophoresis and imaging analysis. Results obtained by 2-D electrophoresis coupled with MALDI-TOF/TOF showed that seven proteins were differentially expressed between controls and LPS-treated samples. The response to LPS was very similar over three different groups of experiments for five proteins for the 8 µg/mL dose, whereas response was more variable for the 16 µg/mL LPS dose. Annexin 2 was under-expressed for the 8 µg/mL dose ($p < 0.02$), with a similar trend for the 16 µg/mL LPS dose. In contrast, eukaryotic initiation factor (EIF) 4A1, protein disulphide isomerase A3, superoxide dismutase and transketolase were all over-expressed following the 8 µg/mL LPS challenge and similar effects were seen with 16 µg/mL.

4.2.4 Proteomics profiling from shotgun analysis of differentially expressed pathways (Paper II)

Overall, in the shotgun analysis 35 proteins were found to be differentially expressed in LPS groups compared with controls (Paper II). These were divided into 25 up-regulated and 10-down regulated for at least one LPS dose. For instance, Gal-1 (known as being involved in immunotolerance) was downregulated and enolase-3 (high expression being associated with pro-inflammatory activity and immuno-rejection) was up-regulated (*Figure 4*).

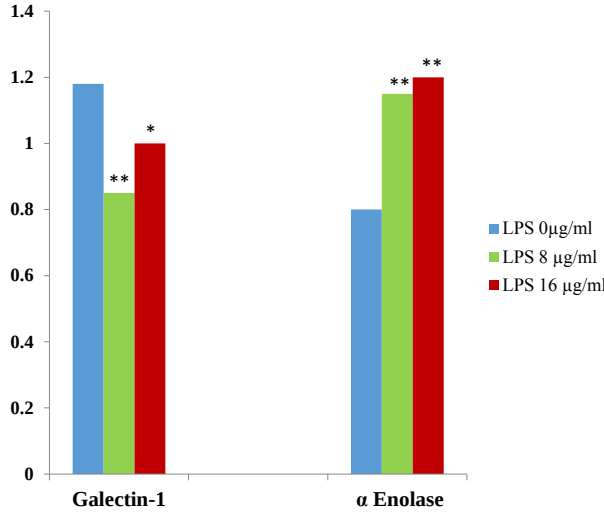

Figure 4. Results from shotgun analysis for galectin-1 (under expressed) and alpha enolase (over-expressed) proteins following lipopolysaccharide endotoxins (LPS) challenge of endometrial epithelial cells with 8 (green bars) or 16 µg/mL LPS (red bars). Significance of change compared with controls (blue bars): *$p < 0.05$, **$p < 0.01$.

4.3 Responses of endometrial epithelial cells exposed to BoHV-4 (Study III; Paper III)

4.3.1 Cell survival and CPE

When bEEC were challenged with different viral MOIs (MOI 0.001 to MOI 10) for 6 days, it was found that the number of live cells (TB-) in controls had on average doubled by Day 6 post-challenge compared with Day 0 (×200%). In the infected groups, at Day 6 post-challenge CPE increased, whereas TB- cell numbers decreased as viral MOI increased.

In the second set of experiments, bEEC were challenged with the lethal dose (LD_{50}, MOI 0.1) for seven days. Main effects of cow ($p < 0.0001$), day post-challenge and infection and day × treatment interaction influenced the numbers of TB- cells ($p < 0.001$). During the first four days, TB- cells increased in a similar way in infected groups and in controls. However, after four days post-challenge, the number of TB- cells and percentage cell survival were lower in infected cells than in controls and were correlated. Increased CPE and numbers of floating dead cells were observed. A significant

interaction between the effects of cow and infection was also found ($p < 0.05$), showing individual variation in the way cells from different cows responded to infection by BoHV-4. Cell survival results were consistent with those from the evaluation of viral replication.

A strong increase in viral particle numbers was found between Day 1 and Day 7 post-infection, both by titration and qPCR. Number of virus particles increased steadily from Day 1 to Day 5, but the differences between Day 5, Day 6 and Day 7 were not significant. Overall, a very strong correlation ($r = 0.9$, $p < 0.0001$) between individual results of titration and qPCR results was found, showing the validity of the qPCR methods developed. For cell survival, the effect of cow from which cells originated was also highly significant ($p < 0.0001$), indicating variation in the way BoHV-4 replicated in individual cultures. In addition, a strong progression of viral infection in the cell culture from Day 1 to Day 7 was revealed by IFAT (*Figure 5*).

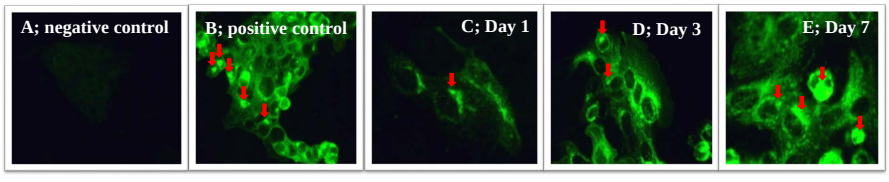

Figure 5. (A) Negative control from uninfected cells. (B) Positive control from BoHV-4-infected MDBK cells. Progressive increase in the number of viral particles infecting cells at (C) Day 1, (D) Day 3 and (E) Day 7 following BoHV-4 challenges at MOI 0.1 as shown by the indirect fluorescent antibody test (IFAT) (red arrows illustrate viral particles in the cells).

Results from IFAT showed that the virus was present in cells as early as Day 1. Very large amounts of BoHV-4 were found by Day 3, but the effects on cell survival occurred later. This shows that endometrial cells are sensitive to BoHV-4.

4.3.2 Inflammatory cytokine concentrations: TNF-α, IL-8, IL-1β, and INF-α

Concentrations of TNF-α and IL-8 were detectable in all samples from control and infected cultures, showing the production of these two cytokines by endometrial epithelial cells in the absence of immune cells. In contrast, the concentrations of other cytokines, IL-1β, and INF-α were mainly undetectable and, when present, very low concentrations were found only in a few supernatants originating from infected cells.

Total concentration of TNF-α increased constantly from Day 1 to Day 7 in controls, while in the infected group concentrations started to decrease after

Day 4. At Day 7, total TNF-α concentrations were significantly higher ($p < 0.05$) in the controls than in the infected samples. Following adjustment of concentration to a set amount of cells (concentration per 10^4 cells), TNF-α production was mainly influenced by the cow from which the culture originated ($p < 0.01$). As for the total concentration, TNF-α ratio per 10^4 cells was significantly higher ($p < 0.05$) in infected cells than in controls by Day 7.

IL-8 mean concentrations increased continuously from Day 1 to Day 7 in both controls and infected samples (effect of day, $p < 0.0001$). However, IL-8 expressed a different profile in relation to infection. The total IL-8 concentration in supernatants collected from infected cells was significantly higher than in controls after Day 3 post-challenge ($p < 0.01$ to $p < 0.0001$). The difference between controls and infected cells showed some variations depending on the individual cow from which the culture was obtained.

5 General discussion

5.1 Models and tissue/cell characterisation (Paper I)

The first objective of this thesis work was to develop well-defined *in vitro* models and use them to study how pathogens interfere with the cells of the bovine endometrium. Therefore, an important part of the work was to characterrise the uterine tissue from which the cells were obtained, in order to limit the importance of the background and possible sources of bias when exposing the cells to pathogen (BoHV-4) or molecules deriving from pathogens (LPS). Complementary information was obtained about animal characteristics and history from the national cattle database, the SOMRS.

This first part of this study allowed the identification of significant characteristics of endometrial tissue and production of endometrial cells. This information mostly confirmed observations in previous studies (Wang *et al.*, 2007; Dhaliwal *et al.*, 2002), *i.e.* quite high individual variation in uterine characteristics (amount of glandular tissue, presence of immune cells and presence of markers of proliferation). Most of these variations were related to the stage of the oestrous cycle at which uterine tissue was collected. In the present study, the effects of breed were negligible, whereas some characteristics of endometrium (such as the amount of immune cells) were influenced by age. Due to interactions between stage of oestrous cycle and other factors and to strong variations caused by these factors in tissue characteristics (higher amount of glandular tissue in dioestrus, higher density of immune cells and of marker of proliferation Ki67-positive cells in proestrus), it was decided to use material from a given stage to perform the challenges with pathogens. As most of the slaughtered cows from which uterine tissue was collected were at dioestrus and because of the high amount

of epithelial glandular cells possibly produced at this time, the dioestrus stage was chosen. Choosing the dioestrus stage was also an advantage because other studies performed on bovine cell cultures have used this stage (Dhaliwal *et al.*, 2002) and the results could therefore be used for direct comparisons with previous findings.

The initial plan was to perform the challenges both with stromal cells and epithelial cells, and this was done in some of the first challenges. However, it became apparent rather soon that a choice was needed and therefore epithelial cells were selected for use in later work. The subsequent work studying host-pathogen interactions in epithelial cells of bovine endometrium is quite original, since most previous studies have been performed either on parts of endometrial tissue (full biopsies or explants containing epithelial cells (either laminal or glandular), stromal cells, some immune cells and vascular tissue) or mixed populations of stromal and epithelial cells (90% stromal and 10% epithelial or *vice versa*) or more pure populations of stromal cells (Oguejiofor *et al.*, 2015; Donofrio *et al.*, 2010; Herath *et al.*, 2009; Donofrio *et al.*, 2006; Vanderplasschen *et al.*, 1995). The population of epithelial cells used in this thesis had a very high degree of purity (reaching 98-99% epithelial cells, as documented in methods section in Papers I and II). Use of epithelial cells represents a limitation of the work, i.e. this did not allow the study of the response of the full endometrial tissue. However, the high degree of purity of cell preparation achieved in this thesis allowed possible sources of bias or increased background in cell responses due to variations in the proportions of epithelial and stromal cell populations, respectively, to be avoided. This was particularly critical in the study of cell proliferation profiles due to their different rate of growth, as observed in a pilot study (unpublished), and thereby the risk of changes in the respective proportion of cells. The specific contribution of epithelial cells to endometrium-pathogen interactions is also a key point when studying cytokine responses, which are usually described as being mainly mediated by immune cells.

In the case of BoHV-4 (Paper III), due to the conventionally accepted route of infection, many studies have been performed on full tissue and/or on fibroblasts from the stroma (Jacca *et al.*, 2013; Donofrio *et al.*, 2008; Donofrio *et al.*, 2007; Wellenberg *et al.*, 2002; Lin *et al.*, 1997; Bartha *et al.*, 1965). The results obtained in this thesis showing specific effects of BoHV-4 on epithelial cells (Paper III) are new information and demonstrate the applicability of the model developed here for future studies, especially those considering possible transmission of BoHV-4 at insemination.

5.2 Choice of pathogens: *E. coli* LPS and BoHV-4 (Papers I, II and III)

When starting the experiments it was decided to use *E. coli* LPS, which is a major component involved in the pathogeny of metritis and endometritis, while BoHV-4 (which has been not looked for previously in Sweden) was used for a second set of challenges (Klamminger *et al.*, 2016; Jacca *et al.*, 2013; Sheldon *et al.*, 2009; Donofrio *et al.*, 2008). Its effects were investigated here because it has been reported as one of the rare viruses having an uterine tropism (Donofrio *et al.*, 2010; Donofrio *et al.*, 2005), despite its implication as a single pathogen in uterine diseases still being unclear. Based on its suggested cooperation with *E. coli* LPS in inducing uterine diseases or increasing unfavourable impact on uterine function (Klamminger *et al.*, 2016; Jacca *et al.*, 2013; Sheldon *et al.*, 2009; Donofrio *et al.*, 2008), the combined impact of *E. coli* LPS and BoHV-4 on cell survival and changes in molecular responses should be also investigated. However, very different responses were obtained for cell survival (Papers I and III) and numerous changes were observed in the LPS model, as revealed by Paper II. In addition, developing the model with BoHV-4 and associated studies took longer than expected. Therefore, further experiments combining the above pathogens were not possible, but it can be concluded with hindsight that it is better not to mix the two pathogens at the start and to work with separate models. Despite its limitations, the work in this thesis represents a good basis for future *in vitro* studies associating *E. coli LPS* and BoHV-4. However, the work also revealed that difficulties can be expected due to different factors influencing the results in the 'simple' models developed, such as the dynamics of the responses of epithelial cells (Paper I) and the dose effects for both LPS and BoHV-4 (Papers I, II and III). Moreover, the degree of complexity of the response on exposing a single population of cells to a single molecule such as LPS (Paper II) was not fully expected and studying time relationships with molecular responses appears to be one of the major challenge for future studies.

5.3 LPS studies (Papers I and II)

The results in this thesis showing stimulation of cell proliferation in a certain range of LPS doses (Paper I) confirm findings in previous studies using different types of epithelial cells in tissue or culture (Basso *et al.*, 2015; Eslani *et al.*, 2014; Hei *et al.*, 2012; Liu *et al.*, 2010; Muller-Decker *et al.*, 2005;

Freitag *et al.*, 1996; Zhang *et al.*, 1996). However, a novel finding in the thesis was the dose-effect relationship observed, which probably explains part of the discrepancies reported in the literature (see Introduction and Discussion sections in Paper I) about the way LPS affects proliferation in different tissues and species. Stimulation of proliferation and increased numbers of living cells were observed up to 12 µg/mL LPS, whereas the results were not different from those in the controls at higher doses. Although 12 µg/mL represents quite a high dose for *in vitro* studies, it is much lower than the LPS concentrations reported in uterine fluids from diseased cows (Williams *et al.*, 2007; Mateus *et al.*, 2003; Dohmen *et al.*, 2000). Based on the results in Paper I showing slight impairment of cell survival, lower proliferation activity and increased apoptosis with 16 µg/mL LPS, it can be speculated that detrimental effects of LPS are even more pronounced in cases of infection. Moreover, in the *in vivo* situation, some other components of *E. coli* apart from LPS and even reactions with other cells in the endometrium (*e.g.* through high production of pro-inflammatory cytokines) may also add to the detrimental effects observed *in vitro*. Up to a level of 12 µg/mL LPS, cells looked morphologically normal and survival rate was not affected. However, the proteomics study revealed that the bEEC cells were greatly disturbed (Paper II) and most often in a similar way with 8 and 16 µg/mL LPS. A novel result from this unbiased proteomics approach was identification of the multiplicity of pathways affected by LPS. Differentially expressed proteins belonging to metabolism (especially glycolysis), oxidative stress (strongly related to metabolic changes), protein transcription and translation, which may relate to the proliferative phenotype, were all up-regulated (Paper II). In contrast, proteins belonging to cell structure and cell adhesion pathways were down-regulated, whereas those involved in cell surface remodelling were mostly up-regulated (Paper II). A huge number of previous studies have reported effects of LPS on immune response and cytokine production (Gómez-Chávez *et al.*, 2015; Yakushina *et al.*, 2015; Barrientos *et al.*, 2014; Jeschke *et al.*, 2010). However, in this thesis it was only possible to identify a few candidates corresponding to this pathway. It can therefore be speculated that a targeted approach may be more efficient in detecting such changes. Interestingly, some of the candidates (*e.g.* galectins and enolase, see Figure 4), although not considered to be among the 'usual suspects' in this pathway, were differentially expressed (Paper II). The de-regulation induced by LPS on galectins and enolases is of particular interest when considering the role of these proteins in rodents and humans (see Introduction and Paper II). In these two species, Gal-1, which is down-regulated by LPS, is associated with immunotolerance through various mechanisms, whereas enolases are associated with immunorejection and

miscarriage in humans (Barrientos *et al.*, 2014; Jeschke *et al.*, 2010). To our knowledge, these effects of LPS, together with the impairments of cell adhesion and cell structure molecules mentioned above, have not been reported previously for bovine endometrium. This new information establishes a bridge between existing knowledge on the mechanisms involved in the development of uterine diseases and inflammation of the endometrium. The consequences for fertility due to the negative impacts of LPS on specific key molecules necessary for interactions between the embryo and the endometrium at time of implantation are of high interest.

5.4 BoHV-4 studies (Paper III)

The development of a reliable model of infection with BoHV-4 allowed the pathogenic effects of this virus on endometrial epithelial cells in culture to be described (Paper III). Main effects of virus concentrations at time of challenge and time of culture were found on cell survival. When using BoHV-4 at MOI 0.1, numbers of living cells started to decrease after three days. This was associated with cytopathic effects and followed intense viral replication, as demonstrated by titration, qPCR and IFAT data. The repetition of challenges on cells originating from the same culture/cows revealed individual differences in terms of speed of development of the virus, which were inversely related to differences in cell survival (Paper III).

To facilitate the evaluation of viral replication, a specific qPCR was developed in this thesis and tested in comparison with titration (Paper III) for the samples used in model development. This allowed subsequent detection of BoHV-4 in samples from other sources. Through development of this qPCR method, it has been possible to detect the presence of BoHV-4 in clinical cases of endometritis in Sweden (Ordell, 2014; Juremalm *et al.*, unpublished), sugges-ting possible implication of BoHV-4 in the development of bovine uterine diseases. More work is needed to characterise the combined effects of LPS (especially at high doses) and BoHV-4 in the model and in other conditions. However, considering the results obtained with LPS showing an increase in apoptosis and lower proliferation at high concentrations, and the impairment of cell survival induced by BoHV-4, it can be speculated that the virus may help reinforce the severity of the disease by killing infected cells, thus preventing or at least slowing down the renewal of the endometrial epithelium.

6 Conclusions

Based on the results obtained in Papers I-III, the following conclusions can be drawn:

- Cow factors and stage of cycle influenced the density of uterine glandular tissue and numbers of CD11b-positive cells and Ki67-positive cells. Well-characterised material from dioestrus females was suitable for developing cultures of a pure population of epithelial endometrial cells and was the most appropriate material to implement challenges with *E. coli* LPS.
- Studies involving LPS challenges showed a steady proliferative response of the endometrial epithelial cells to doses up to 12 μg/mL, whereas increased cell death and apoptosis were observed at higher doses.
- Proteomicss analysis showed that despite the cells exposed to LPS looking normal in morphological terms, multiple functions were altered. In total, 35 proteins were found to be de-regulated by LPS. This included changes in the profile of specific proteins involved in the immune processes necessary for embryo acceptance in early pregnancy. The de-regulation observed may be part of the mechanism by which LPS induces persistent inflammation in the endometrium and may alter fertility after infection has disappeared.
- Endometrial epithelial cells were affected in many ways by the dose (MOI) of BoHV-4 applied and time following infection. The cytopathic effects and survival patterns corresponded well to the kinetics of viral replication, as shown both by titration and qPCR results. The effects on cell survival were associated with elevated concentrations of the pro-inflammatory cytokine IL-8.

- The results from this *in vitro* work show that epithelial cells are sensitive to BoHV-4. Therefore, the possible transmission and role of the virus in inducing uterine diseases through infection of epithelial cells at time of insemination deserves further investigation.

7 Future perspectives

This work provided new methods for future investigations that may lead to possible applications in veterinary medicine. The results from the *in vitro* studies revealed that LPS alters endometrial epithelial cell function. Some key molecules were identified and may be useful in diagnosing persistent inflammation, or could represent targets for treatment. However, these molecules must be present in external fluids to make them accessible and to use them in new diag-nostic methods. Functional studies in cows and targeted proteomicss analyses are needed to verify the role of these molecules when altered by LPS. For instance, further experiments on galectins to study their relevance as markers of disease and possible implantation defects *in vivo* should be performed, as well as a more complete analysis of the molecular responses to LPS (epigenetics, transcriptomics and miRNA). More work is also needed to determine whether it is possible to detect changes in diseased cows, i.e. in the *in vivo* situation.

As mentioned above, LPS altered key molecules involved in the embryo-maternal dialogue, conditioning the success of implantation. The time during which these disturbances persist is a key point to be studied, in order to understand the long-term effects of uterine diseases and to define the practical use of marker-based diagnostic tests.

More *in vivo* studies with BoHV-4 may verify its possible transmission through natural mating or through assisted reproductive technologies and its real impact on uterine function in bovine species.

More basic studies should be performed to explain the differences in sensitivity to the virus observed between individuals in the *in vitro* model in this thesis. Samples from this thesis could be used for instance to study the

epigenetic status/profiling of endometrial cells before challenge and relate these results to speed of viral replication.

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Popular science summary

Uterine diseases are among the most important sources of infertility. They negatively affect health, welfare and, in the case of production animals, the herd's economy. In the cow, contamination of the genital tract by pathogens often takes place at time of parturition. Some cows may develop an acute disease, frequently followed by the inflammation of the uterine endometrium that may persist. This chronic disease is, in some cases, difficult to diagnose due to the absence of clinical signs and impairs fertility.

This PhD thesis work aimed to define reliable *in vitro* models to study how pathogens involved in uterine diseases interact with the endometrial epithelium. The *in vitro* culture of endometrial epithelial cells was developed from characterized uterine tissue collected at slaughterhouse. The response of pure populations of epithelial cells to the exposure of pathogens involved in uterine diseases (lipopolysaccharide (LPS); a molecule from Gram-negative bacteria, and the virus BoHV-4) has been then studied. Different effects of these pathogens were found on cell survival. LPS stimulated the proliferation of epithelial cells. Although cells looked morphologically normal, the study of protein profiles revealed that many types of cell functions were altered. The proteins de-regulated by LPS included specific signals which are key elements in the dialogue between the early embryo and the endometrium in early pregnancy. Such changes may be part of the mechanisms by which inflammation induced by bacteria perturbs fertility long after the infection has disappeared.

BoHV-4 killed epithelial endometrial cells in culture confirming that this virus can contribute to increase the severity of uterine disease. This suggests also that further work on the possibilities for its transmission at time of reproduction/breeding may be useful.

Overall, this work paves the way of future studies aiming at finding molecular markers for the severity of uterine disease and or subsequent persistent inflammation.

Populärvetenskaplig sammanfattning

Sjukdomar i livmodern är en vanlig orsak till nedsatt fruktsamhet hos mjölkkor. Livmoderinfektioner har en negativ inverkan på hälsa och djurvälfärd, liksom på ekonomin i produktionsdjursbesättningar. I samband med kalvning kan bakterier komma in i könsorganen. Hos de flesta korna rensar sig livmodern effektivt från bakterier, men vissa kor utvecklar akut livmoderinflammation som ibland utvecklas vidare till en ihållande, kronisk inflammation i livmoderslem-hinnan. Denna kroniska sjukdom försämrar fruktsamheten och kan i vissa fall vara svår att diagnostisera beroende på avsaknad av kliniska tecken.

Syftet med denna avhandling var att utveckla tillförlitliga laboratoriemetoder (*in vitro*-modeller) för att kunna studera hur sjukdomsframkallande bakterier och virus som orsakar livmoderinfektion samverkar med yttersta lagret, epitelet, på livmoderslemhinnan. Från ett väl definierat livmodermaterial hämtat från slak-torgan utvecklades en *in vitro*-metod för odling av epitelceller från livmo-derslemhinnan. Därefter studerades responsen hos rena populationer av epitel-celler efter exponering med herpesviruset BoHV-4 samt ett toxin, LPS, som bildas av *E.coli*-bakterier. Dessa sjukdomsframkallande agens visade sig ha en rad effekter på cellernas överlevnad. Toxinet LPS stimulerade tillväxt av epitelceller. Trots att cellerna såg normala ut under mikroskop, så visade studier av proteinprofilen att flera cellfunktioner var förändrade. Specifika signal-proteiner med betydande roller i kommunikationen mellan embryo och liv-moderslemhinnan i tidig dräktighet nedreglerades av LPS. Denna typ av förän-dringar skulle kunna vara en del av de mekanismer som gör att livmoderinfektion påverkar fruktsamheten negativt, även en lång tid efter att infektionen har försv-unnit.

BoHV-4, ett herpesvirus som kor kan drabbas av, dödade epitelceller från livmoderslemhinnan *in vitro*, vilket bekräftar att viruset kan medverka till att öka vävnadsförstöringen och svårighetsgraden vid en livmoderinfektion. Ytterligare studier behövs för att förstå smittvägarna vid virusinfektion i livmodern.

Sammanfattningsvis kan denna avhandling bana väg för framtida studier med mål att hitta molekylära markörer för att kunna bedöma svårighetsgraden vid livmoderinfektion och kvarstående inflammation i livmodern hos mjölkkor.

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สุดท้ายนี้ขอขอบคุณพ่อและแม่ผู้เป็นแรงบันดาลใจและผู้สนับสนุนในการมาเรียนต่อปริญญาเอก ณ ประเทศสวีเดน ขอบคุณความรัก ความอบอุ่น กำลังใจ ที่มีให้ไม่เคยขาด ขอบพระคุณเป็นอย่างสูงที่ทำให้มาถึงและยืนอยู่จุดนี้ รักพ่อและแม่มากที่สุดในชีวิต ขอบใจน้องชายและน้องสาวที่เป็นกำลังใจและยืนเคียงข้างกันตลอดและไม่เคยทิ้งกันไปไหน ขอบใจกำลังใจ ความรักที่มีให้กันเสมอมา รักน้องทั้งสองคนมากเช่นกัน ขอบคุณญาติๆ น้อง พี่ ป้า น้า อา ลุง ย่า ยาย ทุกๆ คน ที่เป็นกำลังใจและสนับสนุนกันตลอดมา รักและคิดถึงทุกคนเสมอค่ะ

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Dose related effects of LPS on endometrial epithelial cell populations from dioestrus cows

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ABSTRACT

Lipopolysaccharides (LPS) from Gram negative bacteria are involved in the pathogeny of uterine diseases in cows. This study aimed to investigate LPS effects on the growth of bovine endometrial epithelial cells (bEEC) and relationships between LPS response and tissue characteristics. Uteri from 35 females were characterized for parity and stage of oestrous cycle. Densities of glandular tissue (dGT), CD11b+ cells and Ki67+ cells were measured in the endometrial tissue. Cells from 13 dioestrus cows were exposed to 0, 2, 4, 8, 12, 16 or 24 μg/mL LPS. Effects of parity and stage of the oestrous cycle on tissue characteristics and effects of LPS dosage, cow and tissue characteristics on changes in cell numbers were analyzed by ANOVA. The dGT was higher in metoestrus and dioestrus samples than in pro-oestrus ones whereas densities of CD11b+ and Ki67+ cells were higher at pro-oestrus ($p < 0.05$–$p < 0.01$). LPS influenced bEEC populations in a dose related manner. An increase in number of live cells was observed for dosages ranging from 2 to 12 μg/mL LPS ($p < 0.0001$ vs controls). No effect was found on numbers and frequencies of dead cells. With higher dosages, the numbers of live cells did not increase but the numbers of dead did increase. No relationships were observed between cow or tissue characteristics and growth patterns or frequencies of viable bEEC in controls nor in the response to LPS. To conclude this model is suitable for further studies on dysregulations induced by LPS in endometrial tissue.
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1. Introduction

Gram negative bacteria are 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; Knudsen et al., 2016). Part of the pathogenic mechanism involved results from lipopolysaccharide (LPS) endotoxins (Holst et al., 1996). Following binding to toll-like receptor 4 (review Sheldon et al., 2009), LPS induces reactions in the endometrium and leads to acute or chronic inflammation which impair reproductive function

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(Dohmen et al., 2000; Sheldon et al., 2009). These reactions include the secretion of cytokines (interleukins 1, 6, 8 and tumour necrosis factor-alpha) (Beutler et al., 2003) which activate and attract cells from the innate immune system such as monocytes, macrophages, neutrophils, eosinophils and natural killer cells into the stroma (Sheldon et al., 2010; Cronin et al., 2012; Turner et al., 2014). LPS induces also a dysregulation of prostaglandin secretion, stimulating the production of PGE rather than PGF by endometrial cells, which may give an explanation for prolonged luteal phase in cow with uterine disease (Herath et al., 2009). It was further demonstrated that this shift in prostaglandin secretion was induced through activation of TLR4 (Sheldon et al., 2010).

Very high concentrations of LPS (2.24×10^4 EU/mL) have been detected in uterine lochia in early post-partum cows (Dohmen et al., 2000), and uterine fluid from cows with endometritis (Mateus et al., 2003). LPS concentrations were as high as 90 μg/mL in uterine fluid from *E. coli* infected heifers (Williams et al., 2007). In human and rodents, proliferation of epithelial cells of various tissues or from cell culture systems exposed to LPS were reported (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). Although LPS effects were extensively studied in production animals, few studies described changes in cell proliferation and viability. Results from those are not fully consistent. No effect of LPS was found on pig intestinal cells (Klunker et al., 2013). In the cow, LPS increased the number of mammary gland epithelial cells during *in vitro* culture (Piotrowska-Tomala et al., 2012) while in another study no effect was found in a mammary cell line (Calvinho et al., 2000) and negative effects of LPS were reported on oviduct epithelial cells in culture (Kowsar et al., 2013). In contrast studies on the bovine endometrium, reported that immune cells can promote the proliferation of neighboring cells through production of pro-inflammatory cytokines (Holst et al., 1996; Herath et al., 2009; Sheldon et al., 2010; Eslani et al., 2014) and it is possible that these differences between studies may be related to variations in LPS concentrations.

The present experiments aimed to study the changes in populations of bovine endometrial epithelial cells exposed to various LPS dosages. Endometrial tissue was characterized for the density of glandular tissue, the marker of cell proliferation Ki67 (Gerdes et al., 1984) and CD11b labelling cells from the innate immune system (Mansouri-Attia et al., 2012). Possible relationships between these tissue characteristics and changes in cell number and viability in response to *E. coli* LPS were examined.

2. Materials and methods

2.1. Animals and sample collection

All animal samples used were obtained from cows which were not slaughtered for the purpose of this study and therefore no ethical permission was required. Bovine genital tracts including ovaries were collected from local slaughterhouse within 10 min following slaughter, immediately placed on ice and brought to the laboratory within 1 h. In order to exclude genital tracts of animals with apparent uterine disease, a careful macroscopic examination of the genital tracts was performed by evaluating uterine consistency, mucus abundance and appearance, vascularization and superficial signs of inflammation. The national Swedish basic registration system was used to get information about the animals, based on their national identification number. The 35 genital tracts included in the study originated from Swedish red breed cows (SRB) (n = 20), Holstein (n = 13) cows and two cows were cross breed but breed could not be documented. The parity of the animals varied from zero (no calving, 7 heifers) to six (28 cows). From each genital tract, the left uterine horn was used for histology and immunohistochemistry and the right uterine horn was reserved for cell culture. A subset of 14 dioestrus females was included in the cell culture experiment (13 SRB and one Holstein). The general morphology and the immunohistochemistry of the uterine tissue were studied in parallel with cell culture and the relationships between different types of results were analyzed after completion of all experiments.

2.2. Characterization of uterine tissue

2.2.1. Stage of the oestrous cycle

The ovaries and their structures were carefully examined. Four stages of oestrous cycle: pro-oestrus, oestrus, metoestrus and dioestrus were distinguished depending on size, color and hemorrhagic appearance of corpora lutea (CLs). Symmetrical longitudinal cross sections of the CLs were performed to determine CL-diameter, measured with a ruler and the color and presence of hemorrhagic spots recorded. This set of information was used to determine the stage of oestrous cycle for each female (Ireland et al., 1980; Arosh et al., 2002). The way measurements were used to determine stage and the number of females at different stages of the oestrous cycle is reported in Table 1.

2.2.2. Quantification of endometrial glands

Cross sections (n = 35) of the left uterine horns were taken systematically 5 centimeters from the tip of the uterine horns for uterine gland morphology and for subsequent evaluation of uterine health. All samples were fixed in 4% paraformaldehyde at 4 °C for 48 h and routinely prepared in an automatic tissue processor for dehydration with alcohol. The fixed tissues were then embedded in paraffin and subsequently cut in 8 μm thick sections. Sections were deparaffinized, rehydrated and stained with hematoxylin and eosin. The total numbers of cross-sections of uterine glands were counted from printed photos of the full piece section of the uterine horn. The photos were taken in a light microscope (×10 magnification) and a grid plate was used to calculate glandular area. The density of glandular tissue (dGT) was then calculated as number of uterine gland cross-sections per square centimeter (gcs/cm^2) based on the total surface of endometrial tissue from a given section of uterine horn measured with help of the grid.

2.2.3. Number and density of CD11b positive cells

Paraffin-embedded tissue samples (n = 35) of left uterine horns were cut in 8 μm thick sections for immuno-

Table 1
Evaluation of oestrous cycle stage based on corpus luteum (CL) morphology and number of cows sampled per stage.

Stage of CL-ovary appearance	Pro-oestrus (D18-D20)	Oestrus (D21-D1)	Metoestrus (D2-D4)	Dioestrus (D5-D17)
Size (mm)	10–20	5–10	5–10	20–30
Color	light yellow	light yellow of regressing CL	brown to orange-brown	orange-yellow
Ovulation Point	–	–	/	–
Number of cows	5	0	4	26

histochemical analysis of CD11b positive cells. The sections used were taken at random from the best initial cross section (no artifact), then deparaffinized and rehydrated. Antigen retrieval was performed by exposing the slides to citrate buffer (pH 6.0) at 95 °C for 20 min. All slides were incubated with 10% goat serum at room temperature (RT) for one hour to block nonspecific binding. Endogenous peroxidase activity was quenched in 3% H_2O_2 at 4 °C for 20 min. The sections were incubated with rabbit-anti CD11b antibody (diluted 1:400, ab75476 Abcam, UK) at 37 °C for 45 min. Bovine spleen sections from the same animals were used as positive controls and 10% goat serum was used for negative control. After incubation, the sections were washed with PBS three times and a goat-anti rabbit IgG-HRP (1:300 concentration; SC-2030, Cruz Markers™) was added to the sections. The sections were then left at RT for 40 min. Chromogenic reaction was obtained using AEC (K3464, Dako, Denmark) and the slides were counterstained with Mayer's hematoxylin (HTX). The CD11b positive cells were counted from a full piece of the cross section of uterine horn without artifacts at ×200 magnification in a light microscope. The density of CD11b positive cells was then calculated as number of cells/mm^2 of cross section surface.

2.2.4. Number and density of Ki67 positive cells

Paraffin-embedded samples (n = 35) were cut in 8 μm thick sections, which were deparaffinized and rehydrated (sections to be analyzed were chosen as mentioned above). Antigen retrieval was performed by boiling sections in citrate buffer (pH 6.0) at ≥95 °C for 15 min. Endogenous peroxidase activity was quenched in methanol with 3% H_2O_2 for 5 min at 4 °C. Nonspecific binding was blocked for 20 min at RT in 0.1% BSA (diluted in PBS, A4503-50G, Sigma, Saint Louis, USA) and the sections were then incubated in 2.5% normal horse serum (MP-7500; Vector Laboratories Inc, USA) for 20 min at RT. The sections were either incubated with mouse monoclonal anti Ki67 antibody (1:150 dilution, M7240; Dako-Cytomation,Glostrup, Denmark) (Scholzen and Gerdes, 2000) at RT for 2 h. Positive control was prepared from CL sections and 10% normal mouse serum was used for negative control. After incubation, the sections were washed with PBS three times and incubated with the secondary antibody (ImmPRESS UNIVERSAL reagent, anti-mouse IgG, MP-7500; Vector Laboratories Inc, Burlingame, USA), for 30 min at RT according to the manufacturer's instruction. Chromogenic reaction was developed using AEC (K3464, Dako, Glostrup, Denmark) and the slides were counterstained with HTX. The total number of Ki67 positive cells was counted in a light microscope (×200 magnification) from a full piece of the cross section of the uterine horn without artifacts. The density of Ki67 positive cells was calculated as the number of cells/mm^2, the area of calculation was based on full surface sections on the slides.

2.3. Cell culture, LPS challenges and characterization of cell populations

2.3.1. Bovine endometrial epithelial cells (bEEC) culture

Endometrial tissue from the right horn of genital tracts were collected in 14 dioestrus females (selected at random among dioestrus ones). These genital tracts obtained in dioestrus stage were chosen since it is known from another study (Dhaliwal et al., 2002) that they can provide a large quantity of cells to satisfy the needs to perform LPS challenges, due to the higher density of glandular tissue. Endometrial tissue was cut into 5 cm long and 5 mm thick pieces that were used for the bEEC culture experiment. The endometrial epithelial cells were separated from the stromal cells and subsequently cultured (Charpigny et al., 1999). The pieces were incubated with collagenase IV (C5138, Sigma, Saint Louis, USA) and hyaluronidase (250 U/mL, H3506, Sigma, Saint Louis, USA) for 2 h at 39 °C for digestion. The suspension was then filtered through a 250 μm gauze filter to remove mucus and undigested tissue. The filtrate was thereafter passed through a 40 μm nylon sieve and retained epithelial cells were collected by backwashing with 30 mL PBS. Epithelial cells were cultured in F-12 medium (Dulbecco's modified eagle's medium, Sigma D6434, Saint Louis, USA) containing 10% Fetal Bovine Serum, 1% Penstrep® (5000 units/mL penicillin/streptomycin), 2 mM L-glutamin, 0.5% Liquid Media Supplement (ITS), 5 μg/mL gentamycin and 100 unit/mL nystatin. Cells were seeded into a 25 cm^2 ventilation flask and cultured in a water-jacked incubator with 5% CO_2 at 39 °C. The cells were cultured until confluence and then passed into new flasks repeatedly up to four passages. The purity of the epithelial cell culture was checked by flow-cytometry labelling cytokeratin (primary Anti-cytokeratin 18 Ab, Abcam UK, cat ab668 and secondary Anti-alex 488 Ab, Abcam UK, cat ab175473 used following manufacturer's instructions). From passage two and thereafter, more than 98% of cells expressed cytokeratin, confirming the very high purity of the cell culture system. Culture failed in one cow during passage 3 and therefore bEEC from only 13 cows were exposed to *E. coli* LPS.

2.3.2. bEEC challenges with E. coli LPS

Cultured bEEC were challenged by *E. coli* LPS (L2630 *E. coli* O111:B4, Sigma, Saint Louis, USA) with concentrations in the range of those previously reported in *in vivo* studies (Dohmen et al., 2000; Williams et al., 2007). Three classes of initial number of cells; $<5 \times 10^5$, $5–10 \times 10^5$ and $\geq 10 \times 10^5$

Table 2
Number of animals from which bEEC were issued and number of challenges performed for each LPS dosage.

LPS dosages (μg/mL)	0	2	4	8	12	16	24	Total
Number of cows	13	6	8	13	4	12	4	13
Number of challenges	33	15	20	33	14	30	6	154

cells were used in these challenges performed following passages 4–6. Challenges were performed from the cell cultures of 13 cows. For each individual challenge, cells issued from a given culture sample were initially seeded in equal numbers in flasks later exposed to LPS and in two control flasks. Cells were then cultured for 72 h before being challenged by LPS. At time of challenge (Time 0) culture media was changed and old media replaced either by medium alone (LPS = 0 μg/mL, controls) or supplemented with LPS concentrations of 2, 4, 8, 12, 16 or 24 μg/mL. Due to limitations in the number of cells available, it was impossible to apply all six dosages of *E. coli* LPS in the same challenge. The number of challenges realized for each LPS dosage with individual cow samples is given in Table 2. Each challenge performed on each cell culture sample included systematically a control and the 8 μg/mL dosage. Depending on cell availability, other dosages were also included in challenges. Culture was then run for an additional 72 h period and different types of cells counted.

2.3.3. Cell counting and viability

Counting was performed from one control flask at Time 0 to estimate the number of attached cells before challenge (supposed to be the maximum number of live cells by this time). In all other flasks, the different types of cells were counted and cell viability determined by using trypan blue assay (TB) 72 h post challenges. This time point was chosen from preliminary experiments showing that cell numbers under our control conditions were multiplied by 2–3 thus allowing investigating possible changes due to LPS treatment. After 72 h of culture the supernatant was removed and floating cells in media were counted under the microscope by using a burker neubauer chamber (hemocytometer, 40443001, Hecht Assistent®, Rhon, Germany). These floating cells will be referred to later on as dead cells. Attached cells (considered as living cells) were then detached with trypsin (Tryple™Express (1×) ref.12605-010, gibco®, Waltham, USA). Cells were exposed systematically two times to 4 mL trypsin at 39 °C for 4 min. Flasks were then checked for remaining cells. This protocol was applied again when some cells remained attached. All cells were pipetted from flasks and then transferred into 15 mL falcon tubes. The solution was gently mixed for 2–3 s and 70 μL taken and mixed with the same volume of trypan blue solution (T8154 trypan blue solution 0.4%, Sigma®, St.Louis, MO, USA) in eppendoft tubes. Then 10 μL of mixed solution was immediately transferred to the counting chamber (same as above) following manufacturer's instructions. Unlabeled and labelled cells were counted under low magnification of light microscope and raw numbers and frequencies of cells in each category; floating cells (n and% of total cells), trypan blue positive cells (TB+ from attached cells; n and%) and trypan blue negative cells (TB- from attached cells; n and%) were determined. In the controls, the relative increase in the number of live cells was calculated as:

$$X = \frac{(\text{number of TB- cells at 72 h}) - (\text{number of attached cells at 0 h})}{\text{number of attached cells at 0 h}}.$$

Following LPS challenges, the relative increase in live cells when compared to controls was calculated as:

$$Y = \frac{(\text{number of TB- cells LPS at 72 h}) - (\text{number of TB- cells controls at 72 h})}{\text{TB- cells controls at 72 h}}$$

2.4. Measurement of cell proliferation and apoptosis

Proliferation was measured at different time points by using the quick cell proliferation Assay kit (ab65473, abcam, UK). Cells from two cows (1.0×10^4 cells/well) were first cultured in a 96-well microtiter plate in a final volume of 200 μL/well culture medium for 72 h. Old medium was then discarded and 150 μL/well new medium was added containing either 0, 8 or 16 μg/mL LPS. For each LPS concentration enough wells were prepared in advance to investigate proliferation at time 0, 6 h, 24 h, 48 h, and 72 h. At each time point, 10 μL WST-1/ECS solution was added to each well and cells were incubated for 4 h in standard culture conditions. Plates were then shaken thoroughly and absorbance in each well was measured at 450 nm and 630 nm. A minimum of 4 replicates were performed per cow, LPS dosage and time combination. Ratios of proliferation between LPS groups and controls were also calculated for each time point (t) between 6 h and 72 h as:

$$Z = \frac{\text{Absorbance LPS time t} - \text{Absorbance control time t}}{\text{Absorbance control time t}}$$

Apoptosis was assessed by using FITC Annexin V Apoptosis detection kit (Cat. 556570, BD Phamingen). Briefly, cells from three cows (two biological replicates per cow) were detached by trypsin express for 5 mins at 37 °C. Cells were washed twice with cold PBS and then re-suspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Wells were gently mixed and 100 μL of the solution (1×10^5 cells) was transferred to a 5 mL culture tube. After addition of 5 μL of FITC Annexin V and 5 μL of propidium iodide (PI) solutions, the cells were gently vortexed and incubated for 15 min at RT (25 °C) in the dark. Finally, 400 μL of 1× binding buffer was added to each tube and samples were analyzed by flow cytometry within 1 h (BD FACSverse™). The apoptosis rate was determined by the ratio between numbers of cells both stained with Annexin 5 and PI and total number of cells counted through FACs from 10,000 events.

2.5. Data treatment and statistical analyses

The main effects of cow parity, oestrous cycle stage, breed and corresponding second order interactions between these factors, on density of glandular tissue,

CD11b positive cells and Ki67 positive cells were analyzed on a data set issued from the 35 females. Individual data for the above factors were grouped as follows: parity groups were defined as heifers (n = 7), parity 1–3 (n = 20) and parity >3 (n = 8), stage of estrus cycle was divided into Stage 1 (pro-oestrus cows) and Stage 2 (metoestrus and dioestrus cows). All data were analyzed by ANOVA (SAS version 9.2, proc. GLM). When found, necessary response variables were log transformed to normalize variances. In that case p values from the model applied on the log transformed variables were used but data are presented as untransformed in figures to facilitate interpretation. Non-significant terms ($p > 0.20$) were progressively removed from the initial model including all factors and second order interactions and least square means presented in figures are those from the final models. Results are presented as Least Square Means ± S.E.M. When multiple comparisons were made, Scheffe's adjustment was used to assess individual differences between levels of a given treatment. Similarly, ANOVA was used to analyze the effects of parity (similar classes as above), density of glandular tissue, density of CD11b positive cells and density of Ki67 positive cells (as single continuous co-variables in each model), initial number of cells put in culture (3 classes; <5, 5–10 and $\geq 10 \times 10^5$ cells per flask) and passage number (4, 5 or 6) on the numbers and frequencies of the different types of cells in controls and following *E. coli* LPS challenges. These analyses were based on the data set from 13 females. When analyzing response to *E. coli* LPS challenge, 6 classes corresponding to the LPS dosages from 2 to 24 μg/mL and possible interactions with other factors were added in the models. In addition, Spearman correlation coefficients were calculated to study the relationships between continuous variables. Raw absorbance data were analyzed by ANOVA (SAS 9.2, proc GLM) mixed models while putting time and LPS dosage as fixed effects and cow and corresponding interactions with cow as random effects. The frequencies of cells labelled for apoptosis at 72 h were also analyzed by mixed ANOVA including cow, LPS dosage and their interaction in the model. In both cases, Scheffe's test and the contrast option were used to perform multiple comparisons between treatment groups. Proliferation ratios (Z) were also analyzed by ANOVA models including time and LPS dosage as fixed effects and cow as random. When analyzing (Z), as well as (X) and (Y), the significance of the effect corresponding to LPS was then compared to 0, which is the value of the mean to be observed in the absence of effect of LPS treatment (null hypothesis). For all analyzes, the cut off value for significance was fixed as $p < 0.05$.

3. Results

3.1. Characterization of uterine tissue

3.1.1. Density of glandular tissue

A significant effect of the stage of oestrous cycle was found on the density of glandular tissue ($p < 0.0001$) whereas no significant effects of parity and breed were observed. The density of glandular tissue was higher in Stage 2 (metoestrus and dioestrus; 6,102.5 ± 632 gcs/cm^2) when compared with Stage 1 (pro-oestrus; 2222 ± 650 gcs/cm^2) (Fig. 1). The interactions between stage of cycle, parity and breed were not significant.

3.1.2. Density of CD11b positive cells

The density of CD11b positive cells ranged from 0 to 7.8 cells/mm^2. Significant effects of parity group ($p < 0.0001$; Fig. 2) and stage of oestrous cycle ($p < 0.05$) were found on the density of CD11b positive cells. More CD11b positive cells were found in the endometrium when parity increased ($p < 0.0001$). A lower density was observed in samples from heifers (0.5 ± 0.7 cells/mm^2) and cows with parity 1–3 (0.9 ± 0.5cells/mm^2) than in cows with parity >3 (3 ± 0.6 cells/mm^2). Also, the density of CD11b positive cell was higher in samples from pro-oestrus females (Stage 1; 2 ± 0.7 cells/mm^2) than in samples from metoestrus and dioestrus females (Stage 2; 0.9 ± 0.3 cells/mm^2). There was a significant interaction ($p < 0.01$) between parity and stage; the effect of parity was highly significant for pro-oestrous samples whereas no difference was observed for metoestrus or dioestrus cows/heifers. The CD11b positive cells were located preferentially in stromal tissue and very few were present in epithelia (Fig. 3).

3.1.3. Density of Ki67 positive cells

A significant effect of stage of estrous cycle was found on the density of Ki67 positive cells ($p < 0.01$). The number of positively – marked cells was highest in samples obtained at pro-oestrus and lowest at dioestrus (Fig. 4). Ki67 positive cells were located essentially in luminal and glandular epithelia and relatively few were located in stromal tissue (Fig. 5).

3.1.4. Relationships between animal and tissue variables

As mentioned before from ANOVA, the density of CD11b positive cells increased with parity ($p < 0.0001$) and was negatively correlated to density of glandular tissue ($p < 0.05$). A negative relationship was found between the number of Ki67 positive cells and the density of glandular tissue ($p < 0.01$) (Table 3). However, the complementary analysis by ANOVA showed that this difference was linked to a strong effect of oestrous cycle stage. Effectively, the difference in the density of Ki67 in relation with the density of glandular tissue disappeared following adjustment for stage in the ANOVA. This shows that the density of glandular tissue is not a source of variation of the density of Ki67 positive cells within oestrous cycle stages.

3.2. Cell growth characteristics

3.2.1. Variation of cell growth in controls

Cell growth in controls (the increase in number of attached bEEC between 0 and 72 h) was only influenced by the seeding cell group at the start of culture ($p < 0.0001$) and passage ($p < 0.01$) from which they were issued. There was no significant relationship between parity, density of CD11b and density of Ki67 positive cells and cell growth after 72 h of culture. In every group and following passage 4, 5, and 6 the total number of attached cells increased significantly between 0 and 72 h (Table 4). Cell number was about three times higher when less than 5×10^5 cells

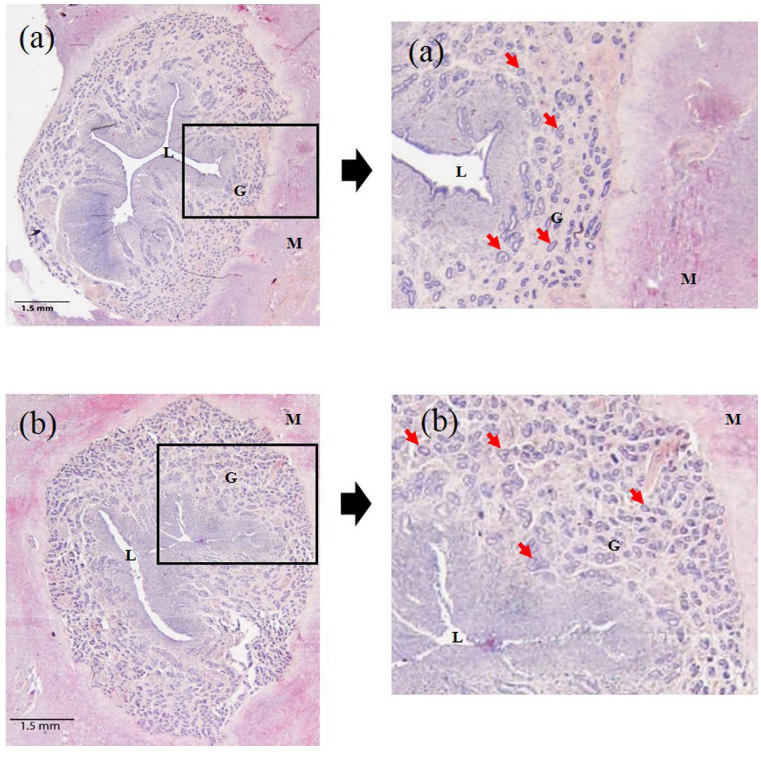

Fig. 1. Cross sections of bovine uterine horns from (a) pro-oestrus and (b) dioestrus stained with hematoxylin and eosin. Myometrium (M), glandular tissue (G) and lumen (L). Scale bar = 1.5 mm. Cross section of endometrial glands are shown by red arrows in right panel. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM03147. A high-resolution version of this slide for use with the Virtual Microscope is available as eSlide: VM03148.

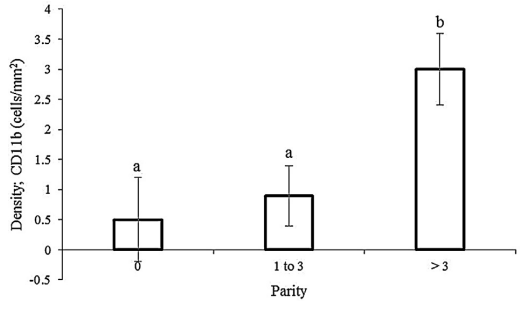

Fig. 2. Density of CD11b positive cells (cell/mm^2; mean ± s.e.m.) in samples from heifers (parity 0), parity 1–3 and parity > 3 cows (a vs b; $p < 0.05$).

Table 3
Spearman correlation coefficients (r) illustrating the relationships between parity and quantitative variables used to characterize endometrial tissue (significance (p), 35 observations).

	Density of glandular tissue (dGT)	Density of CD11b positive cells	Density of Ki67 positive cells
Parity	0.03 (NS)	0.51 ($p < 0.002$)	−0.10 (NS)
Density of glandular tissue (dGT)		−0.35 ($p = 0.03$)	−0.42 ($p = 0.01$)
Density of CD11b positive cells			0.03 (NS)

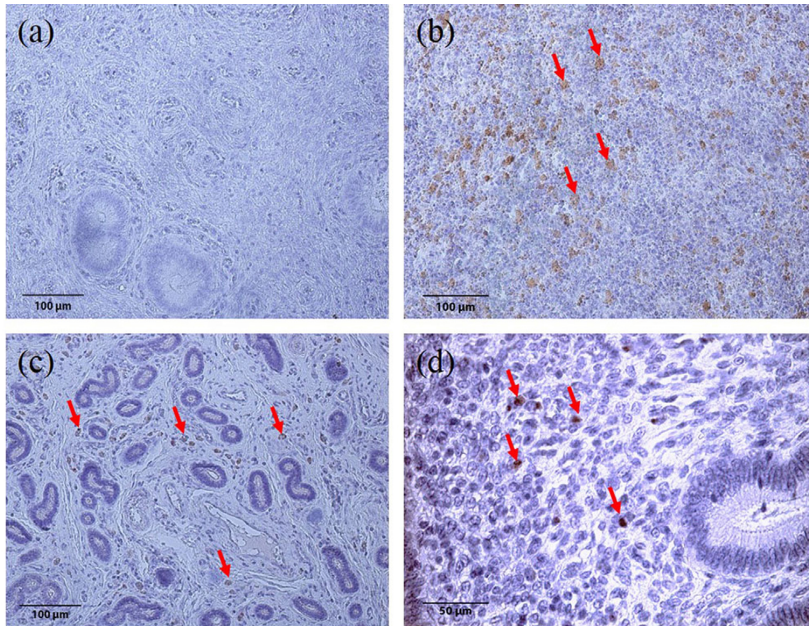

Fig. 3. Immunohistochemistry of CD11b positive cells in bovine endometrium (red arrows); (a) Negative control from normal uterine tissue; (b) Positive control from bovine spleen; (c) Section from a sample taken in dioestrus with numerous CD11b positive cells, ×200 magnification. Picture (a) to (c): ×200 magnification; scale bar = 100 µm. (d) Section from sample taken in pro-oestrus, ×400 magnification, scale bar = 50 µm.
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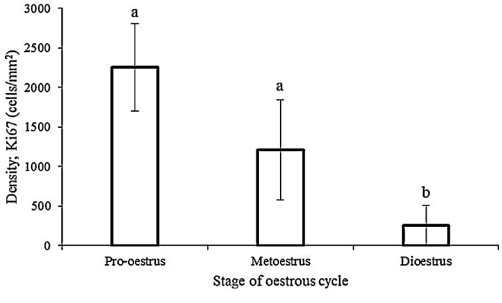

Fig. 4. Effect by oestrous cycle stage (pro-oestrus, metoestrus and dioestrus) on the density (cells/mm^2; mean ± s.e.m.) of Ki67 positive cells in the bovine endometrium. (a vs b; $p < 0.05$ and $p < 0.01$ after log transformation).

were seeded. This increase was less pronounced for the other groups (Table 4). Following use of passage 6 cells, the increase in cell number during culture was lower than with passage 4 and 5 cells (no differences were observed between these 2 passage groups). The additive effects (interaction; NS) of seeding cell group and passage are further illustrated by the relative increase of number of attached cells within 72 h (Fig. 6). The relative increase was 1.78 ± 0.23, 1.22 ± 0.11 and 0.57 ± 0.17 for groups of samples with $<5 \times 10^5$, between 5 and 10×10^5 and $>10 \times 10^5$ respectively ($p < 0.001$). This increase was also higher when passage 4 or 5 cells when compared with passage 6 cells ($p < 0.05$). The number of floating cells increased with the number of cells put in culture ($p < 0.01$) but no other factor was found to be significant. Less floating cells were observed when less than 5×10^5 cells were seeded compared with other groups (Table 4). The numbers of trypan blue positive (TB+) cells at 72 h were not influenced by seeding cell group and passage number. The numbers of trypan blue negative cells (TB−) were lower following use of passage 6 cells than with passage 5 cells ($p < 0.02$). Less TB- cells were observed following seeding of less than 5×10^5 cells compared with other groups ($p < 0.001$; Table 4). From correlation analysis, no significant relationships were found between spontaneous growth of epithelial cells in culture and response to *E. coli* LPS cell challenges.

3.2.2. *Source of variation of cell populations following E. coli LPS challenges*

Following LPS challenges, overall very significant effects of LPS dosage were found on all variables analyzed (Table 5). In addition, a significant effect of number of seeding cells and passage number were observed on most

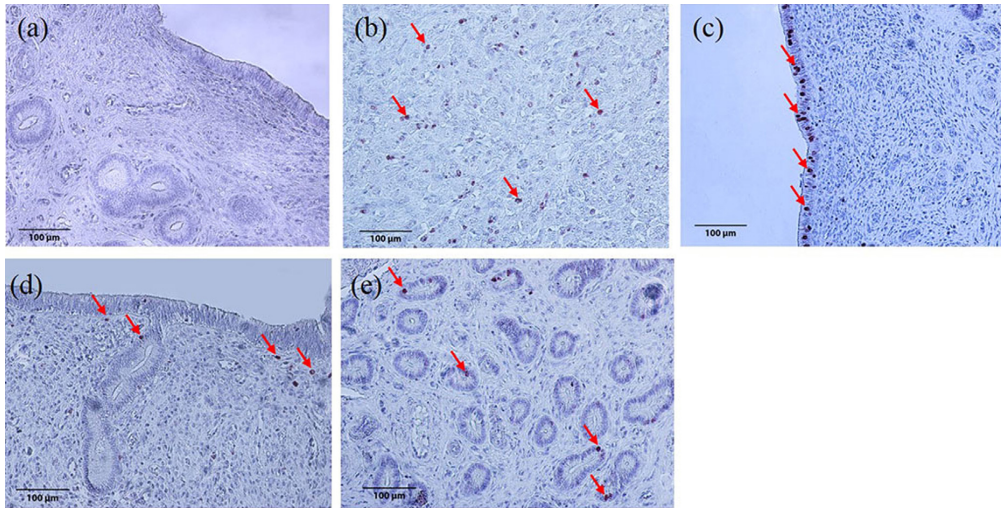

Fig. 5. Immunohistochemical labelling of Ki67 positive cells in the bovine endometrium (red arrows). (a)Negative control from normal uterine tissue, ×200 magnification; (b) Positive control from a corpus luteum, ×200 magnification; (c) Ki67 positive cells mainly located in the luminal epithelium, ×100 magnification; (d) Ki67 positive cells located at the epithelial surface and stromal area, ×200 magnification. (e) Ki67 positive cells mainly located in the glandular epithelium, ×200 magnification. Pictures (a), (b), (d) and (e) refer to a 100 µm scale bar.
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Table 4
Effects of seeding cell group and passage on the numbers of different types of cells at time 0 and 72 h in controls (TB+ = Trypan blue positive cells, TB- = Trypan blue negative cells, all number of cells in the table × 10^5).

Time	Type of cells	Seeding cell group (SCG)					
		1		2		3	
		Passage 5	Passage 6	Passage 5	Passage 6	Passage 5	Passage 6
0 h	Total attached	3.00 ± 2.34	3.12 ± 2.34	7.10 ± 0.82	6.84 ± 1.48	14.24 ± 1.35	11.95 ± 2.34
72 h	Floating	0.15 ± 0.06	0.23 ± 0.06	0.36 ± 0.02	0.34 ± 0.04	0.43 ± 0.03	0.39 ± 0.06
		(a)		(b)		(c)	
	Total attached	9.8 ± 2.34	8.29 ± 2.34	17.28 ± 0.82	13.91 ± 1.48	24.21 ± 1.35	15.25 ± 2.34
	TB+	0.5 ± 0.13	0.59 ± 0.13	0.76 ± 0.04	0.76 ± 0.08	0.86 ± 0.07	0.70 ± 0.13
	TB-	9.29 ± 2.93	7.70 ± 2.93	16.52 ± 1.04	13.14 ± 1.85	23.34 ± 1.69	14.55 ± 2.93
		(d)		(e)		(f)	

a vs b; $p < 0.05$, a vs c; $p < 0.01$, d vs e; $p < 0.05$,

d vs f: $p < 0.01$, e vs f; NS

Table 5
Effect of LPS dosage on the numbers ($n \times 10^5$) and percentages (%) of different types of cells at 72 h (TB+ = Trypan blue positive cells, TB− = Trypan blue negative cells).

Cell types		LPS dosages (μg/mL)						
		0	2	4	8	12	16	24
Floating	n	0.33 ± 0.02 (a)	0.37 ± 0.03 (c)	0.36 ± 0.02 (c)	0.37 ± 0.02 (c)	0.42 ± 0.03 (c)	0.44 ± 0.02 (b)(c)	0.65 ± 0.05 (d)
	%α	2.3 ± 0.1 (a)	2.2 ± 0.1 (c)	1.9 ± 0.1 (c)	2.0 ± 0.1 (c)	2.7 ± 0.1	2.8 ± 0.1 (b)	4.5 ± 0.2 (d)
Total attached	n	14.96 ± 0.93 (e)	17.11 ± 1.30	19.31 ± 1.11 (f)	19.19 ± 0.9 (f)	16.59 ± 1.34	16.24 ± 0.99	13.20 ± 2.07
	%	97.7 ± 0.1	97.8 ± 0.2	98.1 ± 0.1	98.0 ± 0.1	97.3 ± 0.1	97.2 ± 0.1	95.5 ± 0.3
TB+	n	0.70 ± 0.04 (g)	0.70 ± 0.05 (g)	0.74 ± 0.04 (g)	0.92 ± 0.03 (h)	0.78 ± 0.05	0.91 ± 0.04 (h)	0.95 ± 0.08 (h)
	%β	5.0 ± 0.2 (g)	4.3 ± 0.3 (g)	4.2 ± 0.3 (g)	5.1 ± 0.2 (g)	5.3 ± 0.3 (g)	6.1 ± 0.2	7.2 ± 0.5 (h)
TB-	n	14.25 ± 0.91 (i)	16.41 ± 1.28	18.57 ± 1.09 (j)	18.27 ± 0.88 (j)	15.83 ± 1.32	15.33 ± 0.97	12.26 ± 2.03
	%β	95 ± 0.2	95.7 ± 0.3	95.8 ± 0.3	94.9 ± 0.2	94.7 ± 0.3	93.9 ± 0.2	92.8 ± 0.5
Total	n	15.29 ± 0.95	17.48 ± 1.34	19.67 ± 1.14	19.56 ± 0.92	17.01 ± 1.38	16.68 ± 1.01	13.85 ± 2.1

a vs b; $p < 0.05$, a vs d; $p < 0.0001$, c vs d; $p < 0.05$, e vs f; $p < 0.001$, g vs h; $p < 0.05$, i vs j; $p < 0.001$.
α(number of floating cells/total cells) × 100.
β(numbers of TB+ or TB− cells/total attached cells) × 100.

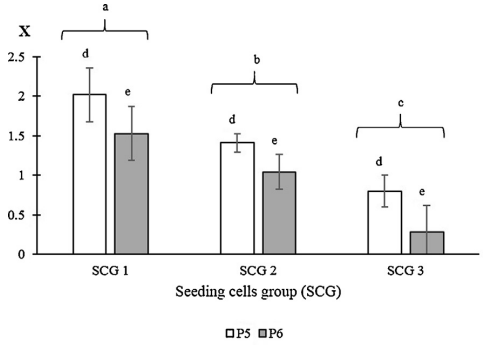

Fig. 6. Additive effects of seeding cells group and passage number on the ratios of increase (X, LS means ± s.e.m.) in the numbers of trypan blue negative cells between 0 and 72 h in controls. a vs b; $p < 0.05$, a vs c; $p < 0.001$, b vs c; $p < 0.05$. Over all seeding cell groups, d vs e, $p < 0.05$.

variables. These effects were similar to those reported for the control groups and as there was no significant interactions these results are not shown. However, these factors were systematically kept in the final models and the LS means corresponding to LPS effects were adjusted for both effects. Overall, a significant increase in the number of floating cells was observed with LPS dosage (Table 5; $p < 0.0001$). Numbers of floating cells were significantly higher with LPS dosage 16 and 24 μg/mL than in controls ($p < 0.05$ and $p < 0.0001$ respectively). The total number of attached cells (TB+ and TB- cells) differed significantly following challenges with various LPS dosages but this increase was not linear ($p < 0.0001$). The total number of attached cells was significantly increased with 4 and 8 μg/mL LPS dosages ($p < 0.001$) whereas a tendency for a decrease was observed with the highest LPS dosage (Table 5). Moreover, similar results were observed for the number of TB- cells (Table 5; $p < 0.001$). In all groups the percentage of TB- cells (number TB-/total number attached) was higher than 90% and no significant difference were observed between treated and control groups (Table 5). The number of TB+ cells differed between the LPS dosage groups ($p < 0.0001$). Higher numbers of TB+ cells were found in the 8, 16 and 24 μg/mL LPS group compared with controls (Table 5; $p < 0.05$). The percentage of TB+ cells was also significantly higher in the 24 μg/mL LPS group compared with all other groups (Table 5; $p < 0.05$). Finally, when analyzing the relative increase in TB- cells number (each sample then compared with its own control), a significant increase was found between 2 and 12 μg/mL LPS (Fig. 7; $p < 0.0001$). However these ratios were not different from 0 for the 16 μg/mL and tended to decrease following use of the highest LPS concentration.

3.3. Proliferation and apoptosis

Proliferation results were affected by time, LPS dosage and their interaction ($p < 0.0001$). From raw results, proliferation activity did increase regularly with time in controls (Fig. 8). Compared with controls, a significant increase of proliferation was observed in the 8 μg/mL LPS group from 6 to 48 h ($p < 0.05$–$p < 0.01$ depending on time). A different pattern was found for the 16 μg/mL LPS group. Results were not different from controls until 24 h but a significant decrease in proliferation activity was observed thereafter ($p < 0.001$). The ratio of increase in the proliferation of cells compared with controls (Z) was comprised between 5 and 9% between 6 and 48 h in the 8 μg/mL LPS group ($p < 0.05$–$p < 0.001$ depending on time, with a maximum increase observed at 24 h; Fig. 9). On the contrary, this ratio was 10% less ($p < 0.0001$) for the 16 μg/mL LPS group by 48 and 72 h, suggesting an inhibition of cell proliferation during this time window. From measurements made at 72 h, the percentage of apoptotic cells did not differ sig-

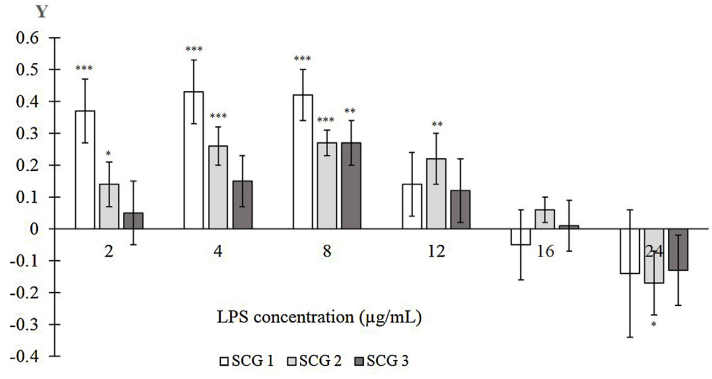

Fig. 7. Ratio of changes induced by LPS treatment over controls in the numbers of trypan blue negative cells (Y, LS means ± s.e.m.) and effects of the seeding cell group. The ratio of increase in cell number is higher in SCG 1 than in other groups. Significance of changes vs control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

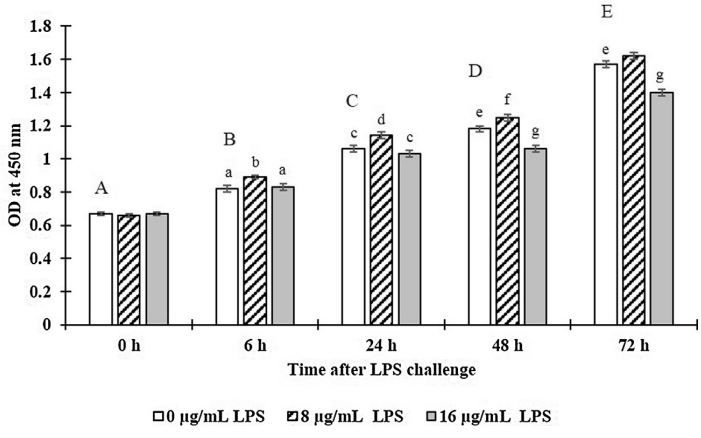

Fig. 8. Changes in cell proliferation in controls (mean OD 450 ± s.e.m.; differences between time points from letter A to E) and effects of 8 and 16 µg/mL LPS at each time point (differences between groups letter a to g). A vs B; $p < 0.05$, C vs D; NS. a vs b; $p < 0.05$, c vs d; $p < 0.01$, e vs f; $p < 0.05$, e vs g; $p < 0.001$. All other comparisons between control group results at different time points $p < 0.0001$.

nificantly between the controls and the 8 µg/mL LPS group (9.48% ± 0.28 controls vs 9.03% ± 0.28 LPS). In contrast an increase in this percentage was observed for the 16 µg/mL LPS group (11.28%, $p < 0.01$).

4. Discussion

The present study shows that *E. coli* LPS, when used at concentrations between 2 and 12 µg/mL significantly increased the number of living bovine endometrial epithelial cells after 72 h of culture. This result was obtained in cells issued from characterized endometrial samples from dioestrus cows. The increase in the number of living cells during *in vitro* culture in controls and LPS treated samples were not affected by cow or tissue related factors. However, the fact that the endometrial samples used for culture were all issued from females in dioestrus limited the impact of variations associated with reproductive stage when studying the spontaneous growth of cells and the response of endometrial epithelial cells to LPS. Effectively, the stage of oestrous cycle was the main source of heterogeneity found in the tissue samples from females in different reproductive stages, affecting both the density of glandular tissue, the presence of Ki67 and CD11b positive cells.

The data from our study, showing a higher density of glandular tissue in samples taken in dioestrus cows when compared to pro-oestrus cows, are in agreement with the results from Dhaliwal et al. (2002) and are consistent with the role of progesterone in stimulating the development of the glandular epithelium before potential implantation at pregnancy (Dhaliwal et al., 2002). However, it is impossible to tell from our study if this apparent increase in density of glandular tissue results from a rise in number of individual glands or from an increase of their size as several cross sections of the same gland were counted. Wang et al. (2007) showed that the number of endometrial glands was the

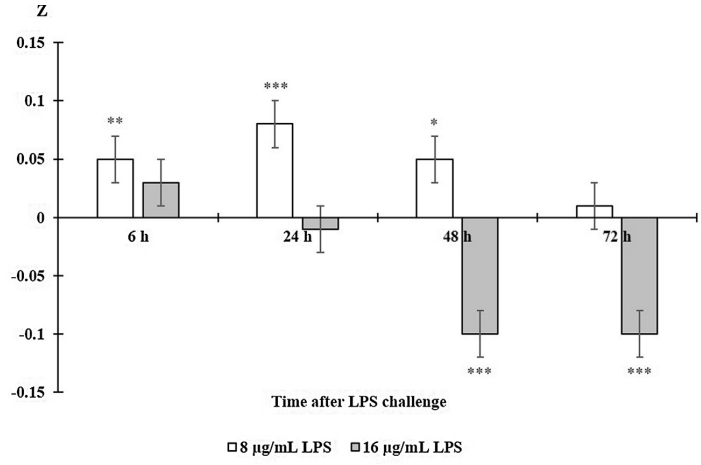

Fig. 9. Ratio of increase (Z; 8 µg/mL LPS group) or decrease (Z; 16 µg/mL group) in cell proliferation when compared to control samples. Significance of changes vs control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

same in follicular and luteal phases but the apparent density of glandular tissue differed between the two phases of cycle due to the relative changes between the surface of glandular tissue and total area of the endometrium. As reported by these authors, we also found that the total area of endometrium is larger during the follicular phase than during the luteal phase due to the variation in degree of edema found in the stroma.

The Ki67 protein is present at all stages of cell proliferation (except G0 of cell cycle) (Gerdes et al., 1984). Our findings showing that the numbers of Ki67 positive cells were higher during the follicular phase (pro-oestrus) than in luteal phase in the bovine endometrium is in agreement with results from Arai et al. (2013) and is consistent with the proliferative effects of oestrogens at pro-oestrus reported in many mammalian species (Johnson et al., 1997; Tong and Pollard, 2002; Jabbour et al., 2006; Zhang and Paria, 2006; Arai et al., 2013). In agreement with Arai et al. (2013) we found that Ki67-labelling was mainly found in epithelial cells (either luminal or glandular cells). As mentioned before, the lack of link between the increase in cell number during culture and under LPS challenge may be due to the relatively small variation associated to the number of Ki67 positive cells during dioestrus stage.

The types and distribution of immune cells in the bovine endometrium are well documented. Among all immune cells, neutrophils, macrophages and monocytes, as revealed here by CD11b immunohistochemistry (Mansouri-Attia et al., 2012), are involved in the innate immune response against invading microbial (Hussain and Daniel, 1991; Subandrio and Noakes, 1997). These cells are commonly found in the endometrium, especially in the early postpartum cow and later in endometritis. Moreover, the number of neutrophils may indicate the type and grade of endometrial injury, infection and inflammation (Brodzki et al., 2014; Dini et al., 2015). Our results are in agreement with those of several studies (Hawk, 1971; Daniel, 1991; Eren et al., 2009) who reported increased numbers of immune cells in the bovine endometrium during oestrus compared with dioestrus. When considering specifically the cells labelled by CD11b, our results differ from those of Mansouri-Attia et al. (2012) who reported a transient increase in numbers of these cells between day 5 and day 7 of the oestrous cycle. This may partly be due to differences in the sampling periods investigated as in our study, the major difference was observed between pro-oestrus and dioestrus. The increase in the number of CD11b positive cells before oestrus is also consistent with what has been reported in the pig (Dalin et al., 2004). In this review, higher numbers of neutrophils have also been reported at pro-oestrus and oestrus than at dioestrus. We confirm here also the preferential localization of these cells in the subepithelial connective tissue, very few immune cells being present in the glandular or luminal epithelia. High-parity cows presented more CD11b positive cells than younger cows or heifers. This may be due to the fact that cows have more chances to develop an immune response while exposed to pathogens during successive mating/insemination and calving.

As mentioned above, a stimulation of proliferation of cells following exposure of LPS has been reported in various tissues and cell types but, to our knowledge, the effects of LPS in the range of the doses used here, on a pure population of bovine endometrial epithelial cells has not been documented before. The bovine endometrial epithelial cells were highly responsive to LPS showing a marked increase in the total number of attached cells and trypan blue negative cells when compared to controls, even following challenges with 12 µg/mL. These results are in agreement with those from several studies performed with culture of epithelial cells from other tissues and species (Liu et al., 2010; Eslani et al., 2014; Basso et al., 2015). However, the increase in cell number related to LPS dose was not linear and a wide range of responses were observed, including a tendency for a decrease in the number of total attached cells and of trypan blue negative cells follow-

ing exposure to the highest dosages. This was associated with an increase in the number of floating cells and trypan blue positive cells. In addition, the findings from the other sets of samples analyzed for proliferation and apoptosis, although issued from a limited number of cows, corroborate our previous observations. The proliferation of epithelial cells was increased by 5–10% within the first 48 h following 8 μg/mL LPS exposure. No increase in the rate of apoptosis at 72 h was observed with this LPS dosage. Taken together, these results are consistent with the increased number of attached cells at 72 h and the lack of increase of the proportions of trypan blue positive cells and floating cells. Results were different with 16 μg/mL LPS showing unfavorable effects on epithelial cell proliferation starting 48 h after exposure and although limited, showing a significant increase in apoptosis. Differences in cell proliferation and survival according to LPS dosage and concentration may be responsible for the discrepancies between studies mentioned earlier.

In this study, it is likely that working with cells originating from dioestrus cows may have limited the variability associated to cow effects. When analyzing individual cow response to LPS (data not shown), an increase in the number of epithelial cells was observed from samples from 12 out of 13 cows (range +10% to +80% compared with controls) and most of the cell samples issued from these cows (10/12) presented the maximum response with the 8 μg/mL LPS dosage. However, the increase in number of cells was less strong when using higher numbers of cells or cells from higher passages showing that these factors represent additional sources of variation when using *in vitro* models. The increase in cell numbers reported here occurred in the absence of immune cells showing that epithelial cells can react to LPS by themselves. This is consistent with the effects of various cytokines and inflammatory mediators on cell proliferation and their patterns of expression following exposure to LPS. For both stromal and epithelial bovine endometrial cells, higher mRNA expression of inflammatory mediators such as IL-6 such as IL-1, IL-6, IL-8 and TNF-α occurred within a few hours following exposure to LPS (Cronin et al., 2012), and in various models, IL-6 has been cited as a strong inducer of epithelial cell proliferation (Grossman et al., 1989; Grivennikov et al., 2009). In full tissue additional stimulation may originate from immune cells; however, the observed increase in the number of living cell resulted probably not only from the stimulation of immune function in epithelial cells, but involved the activation of multiple pathways. The role of the maternal immune system in the establishment of pregnancy is now well established in cattle (Mansouri-Attia et al., 2012; Fair, 2015). In addition, changes in epithelial cell membranes take place and numerous interactions must develop harmoniously between the growing embryo and the endometrium for successful implantation (Singh and Aplin, 2009). Deciphering the multiplicity of alterations in the endometrial cell function due to LPS, which may be responsible for defaults in implantation, deserves further investigation. Effectively, although in this study the number of living epithelial cells increased and their morphology looked normal; it is more than likely that LPS deregulates a variety of functions as shown recently from a global transcriptomic study (Oguejiofor et al., 2015) and preliminary results from proteomics (Guo et al., 2015).

In conclusion, parity, density of glandular tissue, numbers of CD11b positive cells and numbers of Ki67 positive cells were neither related to spontaneous growth of bEEC in culture nor their response to LPS challenge. *E. coli LPS* influenced the different populations of bEEC in a dose related manner. Between 6 to 48 h after challenge, a stimulation of proliferation was observed up to 8 μg/mL LPS leading to an increase in the living cell numbers by 72 h. This increase was not associated with an increase in the numbers of dead or apoptotic cells. At higher dosages, proliferation was inhibited and numbers of dead and apoptotic cells tended to increase. This model is suitable for further studies on the mechanisms by which LPS deregulates endometrial function, especially those potentially affecting implantation.

Conflict of interest

All authors have declared no conflict of interest.
This study has no conflict of interest.

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Changes in protein expression profiles in bovine endometrial epithelial cells exposed to *E. coli* LPS challenge

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E. coli is one of the most frequently involved bacteria in uterine diseases. Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria involved in pathogenic processes leading to post-partum metritis and endometritis in cattle. It also causes inflammation of the endometrium. The increase of cell proliferation by LPS is part of the inflammatory process. The aim of this study was to investigate possible changes in protein expression in relation to the proliferative response of bEECs after challenge with *E. coli*-LPS. *In vitro* culture of bEECs was performed from cow genital tracts collected at a slaughterhouse. In passage 5, bEECs from each of 9 cows (3 series of 3 cows) were exposed to 0, 8, and 16 μg ml^{-1} LPS for 72 h. At time 0 and 72 h later, attached cells/living cells were counted and for each time and LPS dosage, cells were frozen for proteomic analyses. All samples from the 3 series were analyzed by 2-D gel electrophoresis coupled to MALDI-TOF/TOF mass spectrometry. The samples from the first series were subjected to shotgun nLC-MS/MS analysis. From the whole differential proteomics analysis, 38 proteins were differentially expressed ($p < 0.05$ to $p < 0.001$) following exposure to LPS. Among them, twenty-eight were found to be up-regulated in the LPS groups in comparison to control groups and ten were down-regulated. Differentially expressed proteins were associated with cell proliferation and apoptosis, transcription, destabilization of cell structure, oxidative stress, regulation of histones, allergy and general cell metabolism pathways. The de-regulations induced by LPS were consistent with the proliferative phenotype and indicated strong alterations of several cell functions. In addition, some of the differentially expressed proteins relates to pathways activated at the time of implantation. The specific changes induced through those signals may have negative consequences for the establishment of pregnancy.

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Introduction

Due to negative genetic correlations between milk production traits, and reproduction and health traits[1] the genetic selection for high milk production potential has been associated in dairy cows with a reduction in fertility and an increased sensitivity to diseases.[2,3] Modern dairy cows are at high risk of suffering from uterine diseases following calving and the number of exposed cows reached several millions per year in the EU.[2–5] Exposed cows have low fertility, extended unproductive periods with high culling rates[5,6] thus affecting herd economy since rearing replacement animals is one of the main sources of economic losses and welfare due to short life. More costs result from treatment and associated milk withdrawal. The total cost of diagnosed uterine diseases for farmers, dairy and breeding industries has been reported to reach 1.4 billion € per year in the EU.[6] This figure may be underestimated due to undiagnosed forms of uterine dysfunction leading to idiopathic infertility.

Dystocia and retained placenta predispose to uterine diseases due to disruption of physical barriers to infection and perturbation of immune responses that should eliminate pathogens.[7] Metabolic imbalance is also influencing these processes as

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strong negative energy balance was reported to depress gene expression in relation to immunity in uterine tissue.[8–10]

In most epithelia, specific strains of *Escherichia coli* (*E. coli*) are major sources of infection. For the cow endometrium, *E. coli* is one of the most prevalent bacteria isolated in metritis[6,11,12] and paves the way for infection by other bacteria or viruses.[13,14] A major component explaining the pathogenicity of *E. coli* is related to the interaction of LPS with the host tissue.[12,15] *E. coli* LPS provokes the inflammation of the endometrium through a cascade of events well conserved in different tissues. The presence and the involvement of TLR4 in epithelial and stromal cells of the bovine endometrium has already been demonstrated by Cronin and colleagues.[16] LPS binding to Toll like 4 receptors triggers the production of pro-inflammatory cytokines, the attraction of immune cells in stromal tissue and a shift between PGF2α and PGE2 production by the endometrium. The deregulation of cytokines, chemokines, growth factors and major histocompatibility complexes (MHCs) is a part of the inflammatory processes affecting epithelial barriers. Diagnosed clinical symptoms are the basis for treatment. However, acute uterine infections are often followed by an asymptomatic persistent inflammation which remains untreated. For instance in the cow, it has been shown that the undiagnosed persistence of inflammation of the endometrium following infection contracted at parturition[7,17] perturbs later the embryo–maternal interactions necessary to establish successful implantation thus impairing fertility.[5,6] However, the role of endometrial cells and more especially epithelial cells in the persistence of inflammation and mechanisms involved in infertility at this stage of pregnancy still have to be elucidated.

Successful implantation requires a balanced and accurate molecular communication between conceptus and maternal endometrium. Even a small imbalance due to former bacterial infection/LPS stimulation could affect negatively the dialogue between the mother and the embryo necessary for the establishment of pregnancy.[18] The increased activation of NFκB and the secretion of pro-inflammatory and chemotactic cytokines that proved the activation of the CD14 receptor was demonstrated using bronchial epithelial cell lines.[19] The occurrence of endometritis linked to the production of cytokines has already been investigated through the analysis of mRNA expression 20. The overexpression of IL-1alpha and IL-1-RN mRNA and the down-regulation of cPGES mRNA have been reported in cows with subclinical endometritis when compared to healthy cows. The authors found as well that the expression of CXCL5, IL1B, IL8 and TNF mRNA was significantly higher in cows with subclinical or clinical endometritis. This result came from the analysis of mRNA expression of the transcripts involved in prostaglandin synthesis in the bovine endometrium.[20,21] The deregulation of these pathways induced by LPS in endometrial cells was further illustrated by Oguejiofor *et al.*, 2015, while using a wider transcriptomic approach.

The differential protein expression in caruncular and intercaruncular areas during the peri-implantation period has been described in ewes.[18] The authors used LC-MS/MS technique and highlighted the important role of structural proteins such as actin in the implantation process.

The differential proteomics profiling of cows with endometritis has already been performed by using 2D electrophoresis on endometrial tissue.[22] Among differentially expressed proteins between healthy cows and endometritis cows, proteins such as peroxiredoxin and heat shock proteins were over-expressed.[22]

As described above, the response of endometrial tissue in terms of pro-inflammatory factors has already been documented from *in vivo* materials generally combining different types of cells resulting in some heterogeneity of the analyzed tissue. Other limitations may have resulted from former proteomic approaches. Due to this, in the present study we investigated the changes induced by LPS at different concentrations on a homogenous population of post-primary bovine endometrial epithelial cells (bEECs) and by using two complementary proteomics approaches (2D electrophoresis and shotgun MS analysis). By using this combination, it was expected to find protein patterns that may reveal the consequences of previous infection in the endometrial epithelium.

The information obtained here showing that a multiplicity of pathways are deregulated by LPS provides new insights on the mechanisms involved in persistent inflammation following bacterial infection and suggests new perspectives to limit the impact of inflammation on the endometrial epithelium.

Materials and methods

Sample selection and endometrial epithelial cell culture

Bovine uteri without any morphological signs of inflammation were collected from a slaughterhouse and brought back on ice to laboratory within 1 hour of collection. The stage of the estrus cycle was determined at first from the ovarian morphology and the presence or absence of mucus in the uterine body. Genital tracts from 9 cows, showing an orange CL >15 mm diameter and without abundant mucus, were estimated to be associated with luteal phase 23 and subsequently used for cell culture. The stage of the cycle was further confirmed by histology from measurement of the number of cross-sections of the glands in full sections of the endometrium taken 5 cm from the tip of the horn 24. The left uterine horns were dissected and cut into 5–6 cm long and 4–5 mm deep pieces. Uterine tissue was digested with collagenase IV (C5138, Sigma) and hyaluronidase (250 U ml^{-1}; H3506, Sigma) diluted in PBS (phosphate-buffered saline) plus 2% BSA while stirring for 2 hours at 39 °C. The suspension was then filtered through a 250 μm gauze to remove residual mucus and undigested tissue. The filtrate was then passed through a 40 μm nylon sieve, which allowed the fibroblast and blood cells to pass through while epithelial cells were retained. Epithelial cells were collected from the filter by backwashing with 30 ml PBS. Cells were centrifuged at 170*g* for 6 minutes and the pellet was re-suspended in 3 ml of PBS. To disperse the pellet into a single cell suspension, cells were disrupted by passing through a fine gauge needle. Then, cells were cultured in F-12 medium (Dulbecco's modified eagle's medium, Sigma D6434) containing 10% Fetal Bovine Serum (FBS), 50 U ml^{-1} of penicillin/streptomycin, 2 mM L-glutamine,

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0.5 × Liquid Media Supplement (ITS, I3146, Sigma), 10 μg ml^{-1} gentamycin and 100 U ml^{-1} nystatin. Cells were seeded into a 25 cm^2 ventilation flask and cell cultures were kept in a water-jacked incubator with 5% CO_2 at 39 °C. The medium was changed every 1–2 days. Sub-cultivations were performed at 5–6 days when epithelial cells attained 80 to 90% confluence. The process was renewed until passage 4 and the cells were exposed to LPS challenge. LPS powder was dissolved in 5 mg ml^{-1} water as a stock. Before challenge, the adjusted concentrations of LPS and media were completely mixed. At 72 hours following LPS challenge, the supernatant was removed and non-adherent (floating) cells in media were counted under a microscope by using a burker neubauer chamber (hemocytometer, 40443001, Hecht Assistent®, Rhon, Germany). The attached cells were then detached with trypsin (TrypleTMExpress, gibco®, Waltham, USA). The cells were exposed systematically two times to 4 ml trypsin at 39 °C for 4 minutes. Flasks were then checked for the remaining cells. This protocol was applied again when some cells remained attached. After trypsinization, all cells were pipetted from flasks and then transferred into 15 mL falcon tubes. The solution was gently mixed for 2–3 seconds and 70 μl was taken and mixed with the same volume of trypan blue solution (T8154 trypan blue solution 0.4%, Sigma®, St Louis, MO, USA) in Eppendorf tubes. Then, 10 μl of the mixed solution was immediately transferred to the counting chamber (same as above) following manufacturer's instructions. The living cells were detached with trypsin and pellets of 2 to 3 million post-primary cells per group were collected and immediately deep frozen (−80 °C) until subsequent proteomic analyses.[23]

LPS challenges

In preliminary experiments, epithelial endometrial cells were exposed to a wide range of concentrations of LPS reflecting concentrations found in uterine fluid in the case of clinical endometritis[24] and cell survival was estimated.[25] The dosages of LPS used in the present experiment (Sigma L2630 *E. coli* O111:B4, 0 as controls, 8 and 16 μg ml^{-1}) were chosen according to epithelial cell survival at 72 hours which was shown to be maximal with 8 μg ml^{-1} LPS (+30 to +40% living cells when compared to controls). This increase in cell survival progressively vanished with increasing dosage, cell survival being not different from controls following addition of 16 μg ml^{-1} and being lower than controls at higher dosages.

LPS challenges (0, 8 and 16 μg ml^{-1} LPS) were applied on cells issued from 9 different cows (in 3 series of 3 cows). Living cells were counted by trypan blue staining and cell survival profiles at 72 hours obtained from this precise subset of cows, measured as (number of cells ''LPS treated'' – number of cells ''controls''/number of cells ''controls'') were analyzed by ANOVA (SAS ver 9.2, proc GLM). Effects of the cow, series of experiments and LPS dosage on cell survival were analyzed by ANOVA (SAS Ver 9.2, proc GLM). The cow ID (9 levels; 3 different cows in each of 3 series), the series No (3 levels) and LPS dosage (2 levels) were included in the model as well as LPS × cow and LPS × series interactions. The tests were made while comparing the LS mean ratio, observed for a given factor (or second order interactions) to 0, which should be the mean value observed if these ratios were distributed at random. LS mean ratios (either negative or positive) corresponding to the effect of LPS differing from 0 at $p < 0.05$ were considered as significant.

Proteomics analyses

Proteomics analyses have been performed according to Fig. 1. All 9 biological replicates have been analyzed in groups of three through 2D electrophoresis followed by MALDI TOF MS for protein identification. Moreover, the first three biological replicates have been analyzed through label-free nanoflow liquid chromatography mass spectrometry (nLC-MS^E) analysis and each run was performed in triplicate.

Extraction of cell samples. Cellular pellets (cell amounts per pellet varied from 1.6 × 10^6 to 3.3 × 10^6) have been solubilized in a buffer containing 7 M urea, 2 M thiourea and 2% chaps with protease inhibitors. Briefly, the frozen pellet has been defrosted in ice and centrifuged for 10 minutes at 14 000 rpm for 10 minutes. The supernatant was carefully discarded and remaining pellets then solubilized with 20 μl of the above described buffer (7 M urea, 2 M thiourea and 2% CHAPS). The samples were solubilized with 2 cycles of 1 h under magnetic gentle stirring interspersed by 1 h at room temperature. The samples have been subsequently sonicated for 20 minutes and then centrifuged for 10 minutes at 10 000 rpm at room temperature. The pellet has been discarded and the supernatant with the extracted proteins frozen at −20 °C until use. Protein quantification has been performed using a BioRad Protein Assay quantification kit.

2D electrophoresis and image analysis. From each sample, 100 micrograms of protein have been loaded on a 7 cm strip through active rehydration performed overnight at 50 V in a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% Ampholytes 3–10 Amersham, and 26 mM DTT. Isoelectric focusing (IEF) was performed on a protean IEF platform using the following protocol: 100 V/1 h linear, 250 V/2 h linear, 4000 V/5 h linear, 4000 V step/50 000 total volt-hours (VhT). When the final amount of VhT was reached, immobilized pH gradient (IPG) strips were frozen up to the next step or directly equilibrated in two steps of 15 minutes under gentle stirring. The first one was performed in equilibration buffer (6 M UREA, 2% SDS, 0.05 M Tris-HCl pH 8.8, 20% glycerol) supplemented with 1% DTT w/v and the second one with the addition of 2.5% w/v iodoacetamide. After this equilibration step, the IPG strips were loaded in a 12% home made acrylamide gel and IEF run under constant amperage of 15 mA per gel until the Bromophenol Blue (BFB) reached the front.

The gels were then removed from the plates, washed three times with double-distilled water and stained overnight (ON) with Coomassie Brilliant Blue.

Gels were digitized using an Imagescanner III (GE Healthcare) and image analysis was performed using SameSpots software (Version 4.5, Nonlinear Dynamics U.K.). Spots with a p value lower than 0.05 were manually excised and subjected to mass spectrometry (MS) analysis and protein identification.

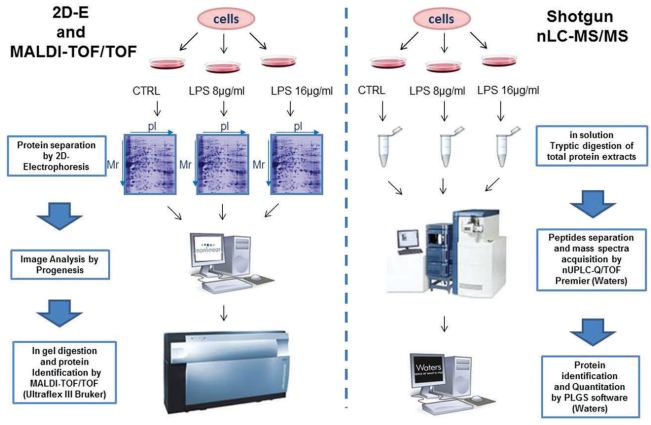

Fig. 1 Strategy for the proteomic analyses. Left part of graph: 2D electrophoresis and MALDI TOF TOF. All cell pellets from 9 cows (3 samples per cow, Control CTRL and LPS treated cells; LPS 8 µg ml^{-1}, LPS 16 µg ml^{-1}) were analyzed following separation of protein spots by 2D gel electrophoresis and identification of spots by image analysis (Progenesis), gel spots were digested and then differentially expressed (DE) proteins identified by MALDI TOF-TOF MS (Ultraflex III, Bruker) and quantified. Right part: nLC-MS/MS analysis was performed in the first series of three cows (3 samples per cow as above) and peptide separation and identification was done following tryptic digestion of total protein extracts. Assays were run in triplicate for each sample in each type of analysis.

The protein identification was performed according to the methodological protocol previously described.[26–29] Briefly, after steps of dehydration, reduction and alkylation, single spots were digested with a solution of 0.01 µg μl^{-1} of porcine trypsin (Promega, Madison, WI) at 37 °C for 16 h. Peptides were concentrated using C18 ZipTip (Millipore) and then were spotted on a Ground Steel plate (Bruker-Daltonics, Bremen, Germany).

MALDI TOF TOF MS analysis. The peptide mass fingerprinting analysis was performed according to Piras *et al.*[30]

Briefly, the MS analysis was performed on an Ultraflex III MALDI-TOF/TOF spectrometer (Bruker-Daltonics) in positive reflectron mode and MS spectra were analyzed by FlexAnalysis 3.3 software (Bruker-Daltonics) to select monoisotopic peptide masses. The external calibration was done by the standard peptide mixture calibration (Bruker-Daltonics: *m*/*z*: 1046.5418, 1296.6848, 1347.7354, 1619.8223, 2093.0862, 2465.1983, 3147.4710).

After an internal calibration (known autolysis peaks of trypsin, *m*/*z*: 842.509 and 2211.104) and exclusion of contaminant ions (known matrix and human keratin peaks), the created peak lists were analyzed by MASCOT v.2.4.1 algorithm (www.matrixscience.com) against the SwissProt database released 2013_12 (25 245 entries) restricted to *Bos taurus* taxonomy. Database search was performed according to these parameters: carbamidomethylation of cysteines as fixed modification; oxidation on methionines as variable modification; one missed cleavage site set for trypsin; and maximal tolerance was established at 70 ppm. For protein identification assignment, only Mascot scores higher than 56 were considered as significant ($p < 0.05$).

To confirm the PMF identifications, the instrument was switched in LIFT mode with 4–8 × 10^3 laser shots using the instrument calibration file. For the fragmentation, precursor ions were manually selected and the precursor mass window was automatically set. Each MS/MS spectrum acquired was processed by spectral baseline subtraction, smoothing (Savitsky–Golay) and centroiding using Flex-Analysis 3.3 software. For search analysis, the following parameters were set: carbamidomethylation of cysteines and oxidation on methionine, respectively, among fixed and variable modifications; maximum of one missed cleavage; the mass tolerance to 50 ppm for precursor ions and to a maximum of 0.5 Da for fragments. The taxonomy was restricted to Bos taurus. The confidence interval for protein identification was set to 95% ($p < 0.05$) and only peptides with an individual ion score above the identity threshold were considered as correctly identified.

Statistical analysis for validation of 2-DE datasets. For a subset of 5 proteins, the repeatability of the results over the 3 series of analyses has been studied. From mean MS results from the 3 technical replicates of each sample the ratio of expression when compared to the control was calculated and the results were analyzed by ANOVA (SAS Ver 9.2, proc GLM). The cow ID (9 levels; 3 different cows in each of 3 series), the series No (3 levels) and LPS dosage (2 levels) were included in the model as well as LPS × cow and LPS × series interactions. The tests were conducted while comparing the LS mean ratio, observed for a given factor (or second order interactions) to 0, which should be the mean value observed if these ratios were distributed at random. For a given protein, ratios of expression (either negative or positive) corresponding to the effect of LPS differing from 0 at $p < 0.05$ were considered as significant through the 3 series of experiments.

Expression analysis by nLC-MS^E. Label-free nanoflow liquid chromatography mass spectrometry (nLC-MS^E) was performed as previously described in ref. 30–32. Briefly, the protein extracts

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of nine biological samples (3 dosages LPS from cell culture from 3 cows) were precipitated with a cold mix of ethanol, methanol, and acetone (ratio 2 : 1 : 1, v/v), then dissolved in 6 M urea, 100 mM Tris pH 7.5 and digested 50 : 1 (w/w) with sequence grade trypsin (Promega, Madison, WI, USA) at 37 °C overnight after reduction with 10 mM DTT and alkylation with 20 mM IAA. The reaction was stopped by adding a final concentration of 0.1% TFA. Separation of tryptic peptides and subsequent qualitative and quantitative nLC-MS^{E} analysis were performed using a nanoACQUITY UPLC System (Waters Corp., Milford, MA) coupled to a Q-Tof Premier mass spectrometer (Waters Corp., Manchester, U.K.). An amount of 200 fmol μl^{-1} of digestion of Enolase from *Saccharomyces cerevisiae* was added to each sample as an internal standard, then a final concentration of 0.6 μg of protein digestion was loaded onto a column for peptide separation. Peptides were loaded onto a Symmetry C18 5 μm, 180 μm × 20 mm precolumn (Waters Corp.) and subsequently separated by a 170 min reversed phase gradient at 250 nL min^{-1} (3–40% CH_3CN over 145 min) using a NanoEase BEH C18 1.7 μm, 75 μm × 25 cm nanoscale LC column (Waters Corp.) maintained at 35 °C. The Q-Tof Premier mass spectrometer was directly coupled to the chromatographic system operated in ''Expression Mode'' switching between low (4 eV) and high (15–40 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over 50–1990 *m*/*z*. The processing of low and elevated energy, added to the data of the reference lock mass, provides a time-aligned inventory of accurate mass-retention time components for both the low and elevated-energy (EMRT, exact mass retention time).

Each sample was run in three technical replicates. For qualitative and quantitative analysis, LC-MS data from three replicate experiments for each nine samples were processed using ProteinLynx GlobalServer v.3.0.2 (PLGS, Waters Corporation). Protein identifications were obtained with the embedded ion accounting algorithm of the software searching into the UniProtKB/Swiss-Prot Bos taurus database release 2013_12 (25 245 entries) to which the sequence of enolase (UniProtKB/Swiss-Prot AC: P00924) was appended. The search parameters were set at: automatic tolerance for precursor ions and for product ions, minimum 3 fragment ions matched per peptide, minimum 7 fragment ions matched per protein, minimum 2 peptide matched per protein, 1 missed cleavage, carbamydomethylation of cysteines and oxidation of methionines as fixed and variable modifications, false positive rate (FPR) of the identification algorithm under 1% and 200 fmol of the enolase internal standard set as calibration protein concentration. The most reproducible proteotypic peptides for retention time and intensity of enolase digestion (*m*/*z* 814.49; *m*/*z* 1159.59; *m*/*z* 1288.70; *m*/*z* 1755.95; *m*/*z* 1840.91, *m*/*z* 2441.12) were used to normalize the EMRT table. The expression analysis was performed considering 3 technical replicates available for each experimental condition (*i.e.*, one experimental condition, control, LPS 8 and LPS 16 groups, × three biological replicates × three technical replicates). The list of normalized proteins was screened according to the following criteria: protein identified in at least 2 out of 3 runs of the same sample with a fold change of regulation higher than ±20%; we considered significant only differentially expressed proteins with a p value < 0.05. Finally, the GO molecular function classification from the Panther Classification System[33] was used to allocate the differentially expressed proteins into functional groups according to the best fitness in the biological system analyzed.

Results

Cell survival profiles following LPS challenges

The profile of cell survival following LPS challenges made in the subset of cows used in this study was very similar to those observed in former experiments. A strong increase in cell survival was observed with the 8 μg ml^{-1} LPS dosage (+24%, $p < 0.0001$) whereas results were not different from controls for the 16 μg ml^{-1} LPS group (0.4%, NS).

Proteomic profiling from 2D electrophoresis coupled with MALDI TOF TOF

From 2D electrophoresis and imaging analyses a total of 1096 different spots were visualized (Fig. 2) from pellets of endometrial epithelial cells and proteins subsequently identified from MALDI TOF TOF MS analysis. From those, a total of 7 proteins were found to be differentially expressed between controls and LPS treated samples (Table 1).

The different types of responses obtained according to LPS dosage are presented in Fig. 3.

The results obtained by 2D electrophoresis coupled with MALDI TOF identification revealed that response to LPS was similar over the 3 groups of experiments for 5 proteins especially for the 8 μg ml^{-1} dosage whereas response was more variable for the 16 μg ml^{-1} LPS dosage. For all these proteins no significant interaction between LPS dosage and series was found. Annexin 2 was significantly under-expressed for the 8 μg ml^{-1} dosage ($p < 0.02$) but only a similar trend was observed for the 16 μg ml^{-1} LPS dosage (Fig. 4). In contrast, Eukaryotic Initiation Factor 4A1 (EIF 4A1), Protein Disulfide isomerase (PDIA3), Superoxide Dismutase and Transketolase were significantly over-expressed following the 8 μg ml^{-1} LPS challenge ($p < 0.001$ to $p < 0.05$). Similar effects were seen with 16 μg ml^{-1} but only a trend was seen for EIF 4A1. For all proteins, differences between 8 and 16 μg ml^{-1} LPS are non-significant.

Proteomic profiling from shotgun MS and analysis of differentially expressed pathways

Shotgun MS analysis qualitatively identified a total of 226145 EMRTs and 160 proteins across all conditions. Quantification was performed following analysis of EMRTs and protein normalization.[34,35] The overall statistical analysis from shotgun MS highlighted 35 differentially expressed proteins between the three different experimental groups (Table 1 and Fig. 5). These divide into 25 up-regulated and 10 down-regulated for at least one of the LPS dosages when compared to controls ($p < 0.05$).

From the Panther Classification System and using GO Molecular function classification the differentially expressed proteins

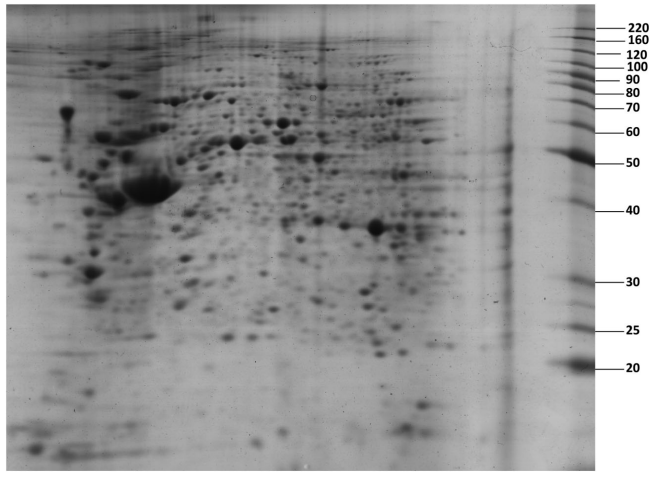

Fig. 2 Representative image of 2D map of one biological replicate.

clustered well in structural proteins, metabolism proteins (energy metabolism), transcription and protein synthesis, oxidative stress, cell growth/apoptosis, immune response, and chromatin DNA binding pathways.

Structural/Cytoskeleton, calcium metabolism and membrane properties. Structural proteins such as actin, peripherin and related proteins that contribute to actin stabilization and anchorage on the plasma membrane such as radixin and tropomyosin alpha 1 chain are strongly down-regulated in both LPS groups in comparison to the control group. In contrast, Tropomyosin alpha 3 chain is up-regulated with both LPS dosages. Proteins involved in cell membrane function and calcium metabolism are either down- (annexin A1 and annexin A2, both LPS dosages) or up-regulated (calreticulin both LPS dosages).

Protein disulfide isomerase (P4HB) and protein disulfide isomerase A3 (PDIA3) are both up-regulated. In addition, a strong down-regulation of a protein with proteolytic activity (Cathepsin) and an up-regulation of a strong inhibitor of endopeptidases (Cystatin-B) in the LPS8 group *versus* control group were observed.

Energy metabolism. All proteins clustering in this category are strongly up-regulated for both LPS dosages. This includes phosphoglycerate mutase, glyceraldehyde-3-phosphate deshydrogenase, triosephosphate isomerase and β enolase, all of them being part of the glycolytic process.

Oxidative stress. All proteins involved in oxidative stress response and protein folding and refolding have been found to be over-expressed following LPS treatment. Among them peroxiredoxin, protein disulfide-isomerase, protein disulfide-isomerase A3, endoplasmin, SH3 domain binding glutamic acid rich like protein 3 and heat shock related 70 kDa protein-2. For most of them over-expression is a little bit higher with the 16 than for the 8 μg ml^{-1} LPS dosage, but the response is already very significant with 8 μg ml^{-1}.

Transcription processes. Proteins such as transcription, initiation and elongation factors and ribonucleoproteins involved at different steps of the transcription process have been also found to be over-expressed in the groups of cells challenged with LPS. This includes elongation factor 1-alpha, elongation factor 1-delta, elongation factor 2, eukariotic initiation factor 4A-1 (EIF4A-1), heterogeneous nuclear ribonucleoprotein A1 and 60S acidic ribosomal protein P2. For all of them except EIF4A-1 changes in expression are more pronounced for the 16 than for the 8 μg ml^{-1} LPS dosage.

Cell growth/cell cycle/proliferation/apoptosis. Differentially expressed proteins involved in these pathways dispatch in up- (78 kDa glucose-regulated protein, phosphoglycerate kinase 1) and down-regulated ones (three different types of metallothioneins (-1, -1A, -2) and galectin-1). Galectin-1 (regulating apoptosis) is down-regulated for both LPS dosages whereas the 3 metallothioneins (involved in negative regulation of growth/proliferation) are only up-regulated significantly for the 8 μg ml^{-1} LPS dosage.

Immune response. Histone H2B type 1 (involved in innate immune response in mucosa as a defense mechanism against bacteria) and 14-3-3 protein zeta delta (involved in response proteins from bacterial origin) are strongly up-regulated with the 16 μg ml^{-1} LPS dosage (no change with 8) whereas alpha enolase (stimulating IgG production, pro-inflammatory and involved in allergy mechanisms) is strongly up-regulated with both LPS dosages.

Chromatin/DNA binding proteins. Histones H2A type 1, H2A.J, and H2B type 1-K are more strongly down-regulated with the 16 than with 8 μg ml^{-1} LPS dosage whereas Histone H4 is more de-regulated with 8. In contrast, Histone H2B type 1-N has been found to be up-regulated in the LPS16 group.

The whole panel of differentially expressed proteins, their relationships and sense of deregulation have been represented through an interactomic analysis performed with STRING 10[36] (Fig. 6).

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Table 1 Table representing the whole dataset of the differentially expressed proteins identified through shotgun MS analysis and 2D electrophoresis (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

					Ratio (shotgun MS)		Ratio (2DE)	
Metabolism	GO terms	Accession	String name	Description	LPS8/CTRL	LPS16/CTRL	LPS8 *vs.* CTRL	LPS16 *vs.* CTRL
Structural/ cytoskeleton	Structural constituent of cytoskeleton	P63258	ACTG1	Actin, cytoplasmic 2	↓0.771051593*	↓0.718923724*		
	Intermediate filament cytoskeleton organization, extracellular exosome	A6QQJ3	PRPH	Peripherin	↓0.683861412*	0.923116348		
	Proteolysis, hydrolase activity, extracellular region	P80209	CTSD	Cathepsin	↓0.612626388*			
	Endopeptidase inhibitor activity	P25417	ENSBTAG00000000524	Cystatin-B	↑2.339646908*			
Calcium metabolism	Calcium ion binding	P04272	ANXA2	Annexin A2	↓0.886920439*	↓0.895834136*	↓0.71564409*	
	Calcium ion transmembrane transport	P46193	ANXA1	Annexin A1	↓0.913931182*	↓0.93239382*		
	Calcium ion binding, protein folding	P52193	CALR	Calreticulin OS	↑1.462284582*	↑1.599994191*		
Energy metabolism	Gluconeogenesis, glycolytic process	Q3SZ62	PGAM1	Phosphoglycerate mutase	↑1.803988368*	↑2.033991215*		
	Glycolytic process	P10096	G3PDH	Glyceraldehyde-3-phosphate dehydrogenase	↑1.336427477*	↑1.390968147*		
	Glycolytic process	Q5E956	TPI1	Triosephosphate isomerase	↑1.40494759558807*	↑1.50681777972405*		
	Glycolytic process	Q3ZC09	ENO3	Beta-enolase OS	↑1.138828378*	↑1.349858824*		
	Glycolytic process	Q9XSJ4	ENO1	Alpha-enolase	↑1.271249144*	↑1.377127754*		
Oxidative stress response	Response to reactive oxygen species	Q5E947	PRDX1	Peroxiredoxin-1	0.970445534	↑1.258600015*		
	Cell redox homeostasis	P38657	PDIA3	Protein disulfide-isomerase A3	↑1.349858824*	↑1.50681778*	↑1.34654270*	
	Response to hypoxia, protein folding, response to stress	Q95M18	HSP90B1	Endoplasmin	↑1.296930074*	↑1.363425117*		
	Cell redox homeostasis	Q3ZCL8	SH3BGRL3	SH3 domain-binding glutamic acid-rich-like protein 3	↔	↑1.246076729*		
	Superoxide dismutase activity, oxidative stress response	P41976	SOD2	Superoxide dismutase			↑1.32768258*	↑1.491990**
	Protein folding	P05307	P4HB	Protein disulfide-isomerase	↑1.3771277544*	↑1.462284582*		
Translation process/protein synthesis	Protein biosynthesis	P68103	EEF1A1	Elongation factor 1-alpha	↑1.138828378*	↑1.173510867*		
	Translation, translational elongation, signal transduction	A5D989	EEF1D	Elongation factor 1-delta	↑1.15027379954284*	↑1.50681778*		
	Positive regulation of translation, translational elongation	Q3SYU2	EEF2	Elongation factor 2	↔	↑1.1162780*		
	Translational initiation, regulation of gene expression,	Q3SZ54	EIF4A1	Eukaryotic initiation factor 4A-I	↑*	↔	↑1.57531***	
	mRNA processing	P09867	HNRNPA1L2	Heterogeneous nuclear ribonucleoprotein A1	↔	↑1.271249144*		

Table 1 (*continued*)

					Ratio (shotgun MS)		Ratio (2DE)	
Metabolism	GO terms	Accession	String name	Description	LPS8/CTRL	LPS16/CTRL	LPS8 *vs.* CTRL	LPS16 *vs.* CTRL
Regulation of cell cycle and proliferation	Regulation of growth	Q6B855	TKT	Transketolase	↑1.072508182	1.233678052↑	↑1.36185114*	↑1.36639*
	Epithelial cell differentiation	Q3T0P6	PGK1	Phosphoglycerate kinase 1	↑1.309964465*	↑1.349858824*	↑2.2031346*	
	Positive regulation of cell migration	Q0VCX2	grp78	78 kDa glucose-regulated protein	↑1.336427477*	↑1.476980773*		
	Negative regulation of growth, cellular response to zinc ion	P58280	MT1	Metallothionein-1	↑Unique LPS8*			
	Negative regulation of growth, cellular response to zinc ion	P67983	MT1A	Metallothionein-1A	↑Unique LPS8*			
	Negative regulation of growth, cellular response to zinc ion	P68301	MT2A	Metallothionein-2	↑Unique LPS8*			
Immune response	Innate immune response in mucosa, antibacterial humoral response, defense response to Gram-positive bacterium	P62808	ENSBTAG00000031889	Histone H2B type 1	0.970445534	↑1.377127754*		
	Protein domain specific binding	P63103	YWHAZ	14-3-3 protein zeta/delta	↔	↑1.349858824*		
	Myoblast differentiation, plasma cell differentiation, positive regulation of I-kappaB kinase/NF-kappaB signaling, T cell costimulation	P11116	LGALS1	Galectin-1	↓0.786627865286354*	↓0.886920439*		
	Innate immune response	P79135	MX1_BOVIN	Interferon-induced GTP-binding protein Mx1			↑2.519244**	↑2.843155**
Chromatin and DNA binding	DNA binding, protein heterodimerization activity	Q2M2T1	HIST1H2BN	Histone H2B type 1-K	0.96078944	↓0.726149042*		
	Chromatin organization, chromatin silencing	P0C0S9	ENSBTAG00000039492	Histone H2A type 1	↓ 0.852143792013715*	↓0.740818212*		
	Chromatin organization, chromatin silencing	Q3ZBX9	H2AFJ	Histone H2A.J	↓ 0.869358235*	↓0.740818212*		
	DNA binding, protein heterodimerization activity	Q32L48	HIST1H2BL	Histone H2B type 1-N	0.990049834	↑ 1.433329435*		
	Histone binding, extracellular exosome, DNA replication-dependent nucleosome assembly	P62803	ENSBTAG00000040277	Histone H4	↓Unique CTRL*			

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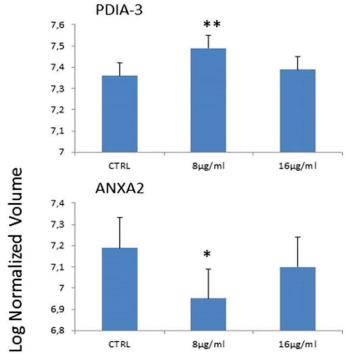

Fig. 3 Differentially expressed proteins observed from 2D electrophoresis followed by MALDI TOF TOF MS analysis in response to LPS (CTRL Control, LPS 8 µg ml^{-1}, LPS 16 µg ml^{-1}). For each bar is reported the mean ± SD value of nine biological replicates (n = 9). Proteins follow the survival profile of epithelial cells (PDIA3 Protein Disulfide Isomerase) or is inverse (ANXA2 Annexin 2). Significance (* $p < 0.05$, ** $p < 0.01$) indicates differential expression between treated samples and controls.

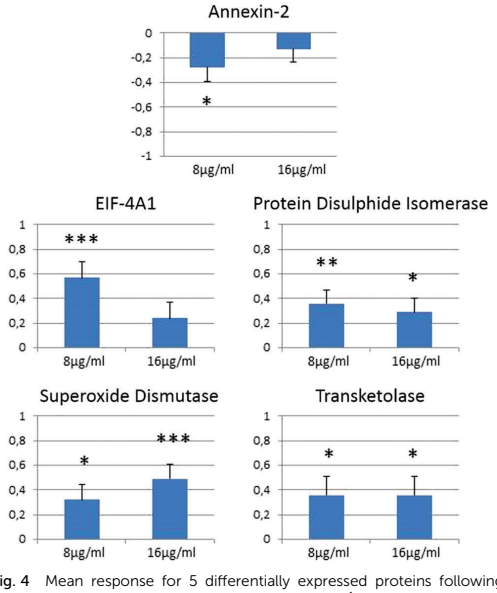

Fig. 4 Mean response for 5 differentially expressed proteins following 3 series of LPS challenge with 8 and 16 µg ml^{-1} LPS (each bar from 9 individual cows). The results are expressed as ratios when compared to controls and for each LPS dosage, significance of differences are tested against 0; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For all proteins, differences between 8 and 16 µg ml^{-1} LPS are non-significant.

Discussion

LPS used at concentrations in the range of those found from *in vivo* studies at 72 hours after challenge of bEECs induce either a strong increase in cell number (8 µg ml^{-1} dosage) or no change in epithelial cell survival (16 µg ml^{-1} dosage).[25] These results are in agreement with studies reporting that LPS induced an increase in epithelial cell proliferation from different tissues.[37–39] Differences with results showing an inhibition of epithelial cell number[40,41] could be partly due to the dosage of LPS that induced an inhibition with the highest LPS dose (16 µg ml^{-1} dosage). However, despite this increase in the number of living cells with a normal morphological appearance, the results of the present study reveal that many changes in protein expression occur following exposure of bEECs to *E. coli* LPS showing that several functions of these cells were highly de-regulated. The consequences of observed changes in protein expression for cell function and possible implications for implantation will be discussed according to the molecular function retrieved from GO annotations.

Structural/cytoskeleton, calcium metabolism and membrane properties

Actin and peripherin have been found to be down-regulated with both LPS dosages. These proteins play a key role as structural constituents of cytoskeleton. Such changes in actin remodeling after LPS stimulation have already been described in macrophages and changes in cell mobility have been reported from the same study.[42] The down-regulation of actin 2 in this model could also be linked to a rearrangement of other structural proteins following LPS stimuli. This is supported by changes in the regulation of PHB4 and PDIA3 which control the cleavage and rearrangement of disulfite bonds both inside and outside the cell, modifying proteins attached to cell or nascent proteins.

Annexin A1 and annexin A2 were down-regulated by LPS. They are both involved in calcium metabolism. Annexins are considered as scaffolding proteins which participate in membrane dynamics. In particular, annexin A2, that has structural similarities to annexin A1, has been shown to exhibit anti-inflammatory activities in several animal models of inflammation.[43–47] This protein has been included here in the calcium metabolism proteins because of its calcium binding properties and dependent activity. However, beside these properties in relation to GO annotations, structural activities linked to lipid bilayer and lipid rafts and to actin binding have been reported as well.[48] The regulation of annexins and actin are following the same trend. The anti-inflammatory properties of annexins and the down-regulation observed here are in agreement with the pro-inflammatory role of LPS.

In contrast, we observed an over-expression of calreticulin which is mainly involved in calcium metabolism and in protein folding (Fig. 6). As a chaperone protein, calreticulin is also linked with HSP90. This protein is expressed on the cellular surface and its putative role in cell adhesion, migration or apoptosis has been documented.[49] In addition, calreticulin modulates integrin-dependent Ca^{2+} signaling [Michalak *et al.*, 1999] and different patterns of expression have been reported during implantation in mice [Cheng *et al.*, 2009]. This protein is expressed on the cellular surface and its putative role in cell adhesion, migration or apoptosis has been documented.[49] In addition, calreticulin modulates

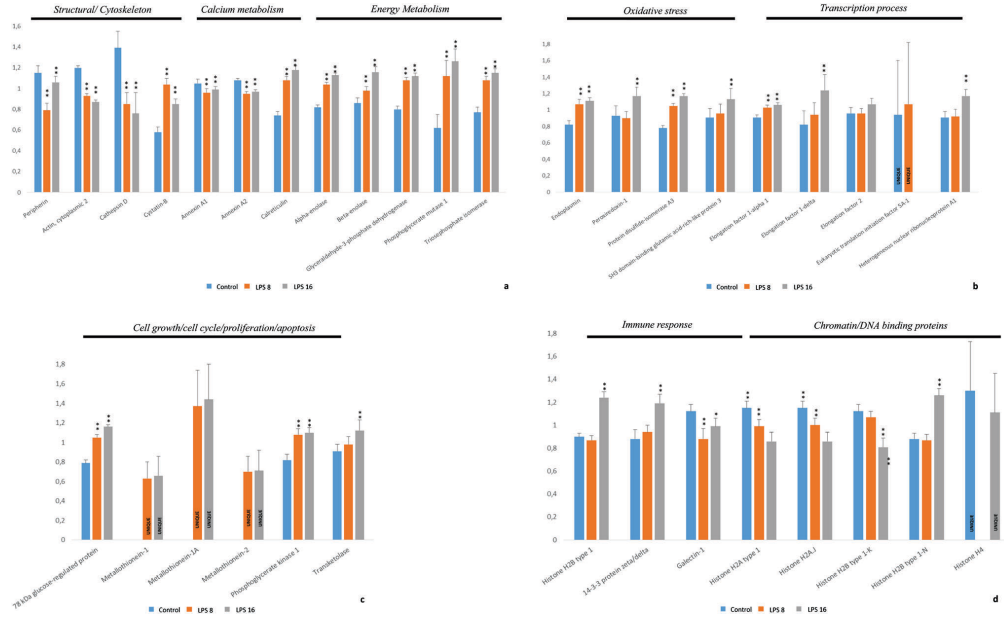

Fig. 5 Results from shotgun analysis, proteins significantly over-expressed and under-expressed following challenge of endometrial epithelial cells with 8 (orange bars) or 16 μg ml^{-1} LPS (grey bars) when compared to controls (blue bars), * $p < 0.05$, ** $p < 0.01$.

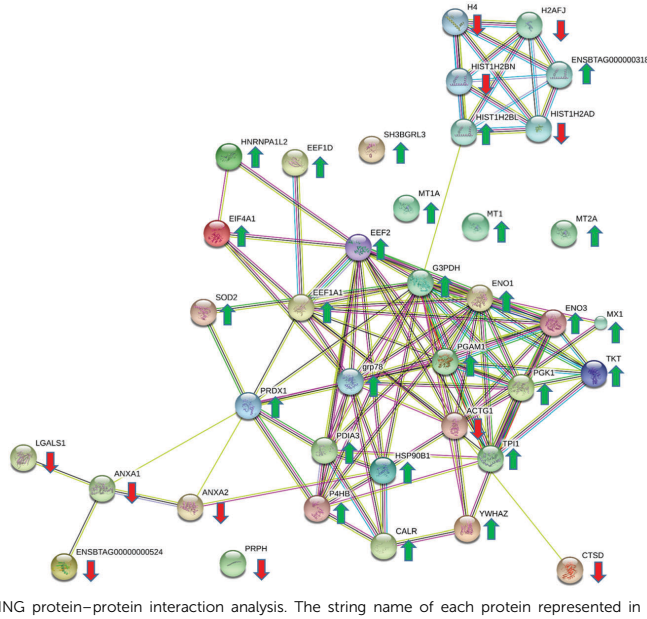

Fig. 6 Protein legend STRING protein–protein interaction analysis. The string name of each protein represented in the figure is indicated in the 4th column of Table 1.

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integrin-dependent Ca^{2+} signaling[50] and different patterns of expression have been reported during implantation in mice.[51] The significance of its over-expression in our model in relation to the above still has to be elucidated.

The full mechanism by which LPS provokes these changes remains to be deciphered. However, our results suggest that LPS destabilizes cell structure and modify cell membrane properties in a way cell adhesion is reduced. To our knowledge, the possible roles of LPS in altering such functions have not been much described before especially in relation to implantation. The changes observed could be of considerable importance while considering the role of adhesion molecules and membrane function in this process.

Another response to LPS in relation to cell structure could be part of a defense mechanism contributing to inhibit protease activity through down-regulation of the protease cathepsin and up-regulation of the peptidase inhibitor Cystatin-B. As Cystatin-B is an inhibitor of cathepsin protease, the trend of expression of these two proteins is consistent and such changes may protect cells from proteolytic activity. The role of this anti-protease mechanism and its relation with the changes reported above in cell structure and membrane permeability requires further investigation.

Energy metabolism

Differentially expressed proteins related to energy metabolism proteins were all found to be over-expressed in both LPS groups when compared to controls. These proteins are mostly glycolytic proteins such as phosphoglycerate mutase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, beta-enolase OS and alpha-enolase. The interactomic analysis (Fig. 6) shows clearly that energy metabolism occupies a very central place in this system. This is consistent with the fact that LPS induced glycolysis which has been constantly reported in the literature from other tissues and other species[52–54] determines other cell responses thus contributing in many ways to the development of pathological processes.

Oxidative stress response

Even if this *in vitro* model is working outside the *in vivo* machinery and based on a single population of cells, many proteins involved in this pathway and in protection from oxidative stress are deregulated showing the key role of LPS while inducing oxidative stress. They occupy a place close to proteins involved in metabolism (Fig. 6) and most of them may result from LPS induced glycolysis changes. The only protein that is not linked with other proteins, in this interactomic analysis, is the SH3 domain-binding glutamic acid-rich-like protein 3 that, according to GO classification, was related to cell redox homeostasis and is also involved in regulation of actin cytoskeleton organization. Interestingly, our results also confirm the up-regulation of peroxiredoxin-1, which has been described as a strong promoter of inflammation through stimulation of the synthesis of proinflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α).[55]

Changes in cytokines were not evidenced from this epithelial cell model. However, the up-regulation of peroxiredoxin-1 and other pro-inflammatory molecules such as enolases may represent an important link between LPS stimulation and the generation of inflammatory cascade in surrounding cells.

Translation process/protein synthesis, regulation of cell cycle and proliferation

As for the energy metabolism, several proteins involved in protein biosynthesis such as initiation and elongation factors have been found to be strongly up-regulated. This demonstrates that the translation machinery is stimulated by LPS. The strong links between these proteins in the interactome and their upregulation is very consistent with the role of this family of factors in the stimulation of cell growth and proliferation[56,57] and the present changes in this phenotype induced by LPS. In addition to the above, other proteins have been reported, from their GO annotated molecular functions, to be related with regulation of cell cycle and cell proliferation. In this case, the interpretation of the interactomic analysis is more complex than for other functions. A strong link was found, with a high significance value, between transketolase, phosphoglycerate kinase 1 and 78 kDa glucose-regulated protein, the latest being linked to the above translation machinery. From GO molecular functions, all these proteins are involved in regulation of cellular growth, epithelial cell differentiation and act as positive regulators of cell migration. As for proteins involved in translation their up-regulation is consistent with the proliferative phenotype observed following LPS challenge.

No links were found between the above and metallothioneins nor galectin-1. Metallothioneins were found here to be up-regulated only in cells exposed to 8 μg ml^{-1} LPS and galectin-1 is down-regulated for both LPS dosages. These proteins are classified, from GO annotated molecular functions, as involved in the negative regulation of growth. The up-regulation of metallothioneins looks in contrast to the growing phenotype observed in our model. However, their up-regulation could be related to a compensatory mechanism from cells to counteract the proliferative response induced by the activation of metabolism and transcription machinery. This response could be also associated with different functions as these proteins have been reported to be involved in bactericidal activity and nitric oxide production following LPS stimulation.[58]

Immune response; relationships with establishment of pregnancy

Our results showing that 14-3-3 protein zeta/delta is up-regulated in the LPS16 group *versus* control group are consistent with former functions of this protein described in the literature. Its major role in the regulation of corneal epithelial proliferation and differentiation in corneal mice cell culture has been reported.[59] This protein has been shown to be also involved in the regulation of the production of cytokines[60] and, the gamma proteoform, is up-regulated during LPS-induced cardiomyocyte injury.[61] Its major role in Toll-like receptor activation has also been demonstrated and its involvement in the lipopolysaccharide-induced production of tumor necrosis factor by macrophages has been documented.[62] A recent study demonstrated that 14-3-3γ

was able to attenuate the LPS-induced inflammatory responses and to induce the proliferation of dairy cow mammary epithelial cells.[63] The up-regulation of this protein we observed may be part of a similar anti-inflammatory mechanism taking place in our model in response to LPS.

One of the most interesting findings in our results in relation to the establishment of pregnancy is the strong down-regulation of galectin-1 induced by LPS. To our knowledge this has not been reported before. Galectin-1 has been mostly studied in human and rodents and has been shown to be expressed in the bovine endometrium.[64] In humans, this protein is abundantly expressed in the non-immune cells at the fetus–maternal interface, down-regulates the production of pro-inflammatory cytokines and promotes maternal immune tolerance.[65] In the mouse, Yakushina *et al.*, 2015[66] have shown that galectin-1 stimulates the differentiation of $CD4^+$ cells into T-regulatory cells than being one of the key molecules involved in immune-tolerance. 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.[67,68]

As part of the metabolic changes both α and β-enolase are both up-regulated following challenges with similar responses for both LPS dosages. Among other roles, α-enolase has been reported as an allergenic molecule with immune and strong pro-inflammatory properties.[69–71] In rats, high expression of α-enolase has been associated with increased numbers of $CD4^+$ T cells and immunorejection in an allograft transplantation model.[72]

The impact of immune imbalance induced by deregulations of galectin-1 and α-enolase on the establishment of pregnancy has to be demonstrated in the bovine through functional studies. If existing and persistent, the lack of immunosuppression induced by LPS through down-regulation of galectin-1 and up-regulation of pro-inflammatory processes through enolases could be part of the mechanisms altering implantation success even in the absence of bacterial infection.

Chromatin and DNA binding

A differential expression of several DNA binding proteins such as histones has been found. As said before for other processes, the interactome picture is quite complex here with 4 of them under-expressed and 2 over-expressed following LPS challenge and more specific work is needed to decipher the mechanisms explaining the over-expression of some specific histones and the down-regulation of some other isoforms. However, from the 4 under-expressed histones we found 2 (histone H2A type1 and H2AJ), which were associated with chromatin silencing consistent with the over-expression of many pathways. The over-expression of histones H2B type1 and H2B type1N following exposure to the 16 $\mu g\ ml^{-1}$ dosage 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[73] and their differential expression could contribute to the formation of an antimicrobial and of an endotoxin-neutralizing barrier against microorganisms.[74]

Conclusion

This study shows that many pathways involved in a wide range of functions are affected by LPS and we addressed the corresponding changes in mRNAs and proteins expression. However, even for these pathways which have been studied intensively and largely documented, such as immune response, this proteomic approach reveals that deregulation occurs for specific molecules which have not been described in former studies and/or not related to the role of LPS and its possible impacts on the establishment of pregnancy. Taken together, the results from this cow model based on a pure population of epithelial cells provide evidence that LPS induces the activation of pro-inflammatory mechanisms and at the same time down-regulates signals reported to be involved in immune-tolerance in other species (such as galectin-1). The persistence of changes possibly induced by LPS due to exposure to pathogens during the post-partum period and their subsequent implication in fertility failures at the time of establishment of pregnancy deserves further investigation.

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III

Bovine herpes virus type 4 impairs the survival of bovine endometrial epithelial cells

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Abstract

Bovine herpes virus type 4 (BoHV-4) can be transmitted by contaminated semen to cows at the time of breeding and may cause uterine disease. The aim of this study was to characterize the susceptibility of bovine endometrial epithelial cells (bEEC) to BoHV-4 by using an *in vitro* model. When bEEC were challenged with different multiplicity of infection (MOI; from 0.001 to 10) of BoHV-4 for 6 days, a significant decrease in cell survival with increasing MOI was observed. The bEEC were subsequently challenged with BoHV-4 MOI 0.1 for 7 days. The number of live cells increased significantly from day 0 to day 7 in controls ($p < 0.0001$). During the first 4 days, number of live cells increased in a similar way in both controls and infected group. However, numbers of live cells in infected samples decreased at Day 4 and were lower than control at Day 7 ($p < 0.0001$). From titration and qPCR, increasing number of viral particles was observed from Day 1, and reached a plateau at Day 5. Concentrations of IL-8 increased with time and were higher in supernatants from infected cells than in controls ($p < 0.0001$). TNF-α concentrations presented similar profile as cell survival ones. In conclusion, the survival of bEEC was strongly impaired by BoHV-4 infection in a time and dose dependent manner and supernatant cytokine profiles were altered. This information supports BoHV-4 implication in clinical cases of metritis and the existence of a risk of BoHV-4 transmission from infected males through animal breeding.

Keywords: herpesvirus, cell survival, cytokine, endometrial cell, cattle

Introduction

Modern dairy cows are at very high risk of suffering from uterine disease and the number of exposed cows in Europe has reached several million per year [1]. Cows diagnosed with metritis have low fertility, extended unproductive periods and high culling rates [1, 2] thus affecting herd profitability and animal welfare. These negative impacts are probably stronger due to undiagnosed forms of uterine dysfunction such as asymptomatic persistent inflammation of the endometrium (endometritis) which can perturb embryo-maternal interactions at the time of implantation and impair fertility [1, 2]. So far, several specific pathogens have been identified as metritis and endometritis inducing agents [1, 3-6]. *Escherichia coli* (*E. coli*) and *Trueperella pyogenes* (*T. pyogenes*) are the most prevalent bacteria isolated from diseased cows. In addition to their direct effects, these pathogens pave the way for subsequent infection by other bacteria or viruses such as bovine herpesvirus 4 (BoHV-4) [7-9]. BoHV-4, a double stranded DNA virus and member of the *Gammaherpesvirinae* subfamily has been first isolated from a variety of diseases such as respiratory and ocular disease in calves [10]. Moreover, an association between BoHV-4 seropositivity, postpartum metritis, abortion and chronic infertility has been reported in many studies [11-13] and BoHV-4 infection is considered as a risk factor in uterine diseases and endometritis [14-16]. BoHV-4 causes cytopathic effects (CPE) and replicates in culture in a wide range of cell lines and primary cultures of various animal species [17, 18]. However, different cell types can be more or less susceptible to BoHV-4 [19, 20]. In bovine, BoHV-4 has a striking tropism for bovine endometrial cells and these cells are highly susceptible to BoHV-4 replication as evidenced by the strong CPE reported for bovine endometrial cell cultures [21, 22]. In stromal cell cultures, viral replication and CPE have been shown by indirect fluorescent antibody test [20]. Virus-infected cells synthesise and secrete type I interferons (INF α/β) which are a major players in the antiviral defence response against all kinds of viruses [23]. Immune cells or infected cells secrete more cytokines such as IL-1α, IL-1β, IL-6, and TNF-α, which killing virus-infected cells and act as a

bridge between innate and adaptive response [24]. Mechanisms by which BoHV-4 alters cell function have been documented mainly in immune and stromal cells while responses to BoHV-4 have not been as well documented in endometrial epithelial cells. It is important to define the responses of these cells to BoHV-4 infection because they are the first to be exposed to the virus especially if the contamination occurs at the time of reproduction through natural or artificial insemination. These routes of infection have not been investigated specifically to date, but the presence of BoHV-4 DNA has been reported in cases of oedematous orchitis and also in the sperm of healthy bulls indicating that sperms represent a potential vector for BoHV-4 transmission to cows [25, 26]. Therefore, we have developed an *in vitro* model to investigate interactions between endometrial epithelial cells and BoHV-4. This present study investigated the impact of changes in the number of live endometrial epithelial cells in connection with viral replication while taking into account individual host cow variation. The impact of infection on the patterns of a selected set of cytokines was also investigated.

Materials and methods

Sample collection and selection

All animal samples used were obtained from cows which were not slaughtered for the purposes of this study and therefore no ethical permission was required. Genital tracts were collected from the slaughter house and brought back on ice to the laboratory within 1 h of collection. The left uterine horn of 9 cows with apparently normal morphology of the endometrium was dissected and used for cell culture and challenges with virus. Tissues from the right uterine horn were fixed in 4% (v/v) paraformaldehyde (PFA) and used for uterine health characterisation. Uterine health was appraised from the number of CD11b-positive cells (primary antibody, ab75476 Abcam, UK) following immunohistochemistry [27] and all cell cultures used in this work originated from cow tissue presenting low numbers of CD11b-positive cells (< 5 cells/mm^2 cross section of endometrium). Moreover, the materials used in the study originated from cows in the dioestrus stage [27].

Cell culture

Bovine endometrial epithelial cells (bEEC) were prepared from left uterine horn tissue as previously described [27]. The uterine tissue was cut into 5-6 cm long and 4-5 mm deep pieces which were digested with collagenase IV (C5138, Sigma, USA) and hyaluronidase (250 U/mL) (H3506, Sigma, USA) diluted in phosphate-buffered saline (PBS) plus 20 mg/mL bovine serum albumin (BSA) under stirring for 2 h at 39°C. The suspension was then filtered through 250 µm gauze to remove mucus and undigested tissue. The filtrate was passed through a 40 µm nylon sieve, which allowed fibroblast and blood cells to pass through while epithelial cells were retained. Epithelial cells were collected from the filter by backwashing with 30 mL PBS. Cells were centrifuged at 170 ×g for 6 min and the pellet was resuspended in 3 mL of PBS. To disperse the pellet into a single cell suspension, cells were disrupted by passing through a fine gauge needle. The cells were then seeded into a 25 cm^2 ventilation flask with F-12 medium (Dulbecco 's modified Eagle 's medium, D6434, Sigma, USA) containing 10% (v/v) foetal bovine serum (FBS), 1% Penstrep® (5000 unit/mL penicillin/streptomycin), 2 mM L- glutamine, 0.5% Liquid Media Supplement (ITS), 5 µg/mL gentamycin and 100 unit/mL nystatin. All cell cultures were kept at 39°C in 5% CO_2 atmosphere and the medium was changed every 1-2 days. Sub-cultivations were performed at 5-7 days when epithelial cells attained 80-90% confluence. bEEC from 9 cows were prepared for use in experiments. The purity of the epithelial cell culture was checked by flowcytometry labelling cytokeratin (primary Anti-cytokeratin 18 Ab, Abcam, UK, cat. no. ab668 and secondary Anti-alex 488 Ab, Abcam, UK, cat. no. ab175473 used following the manufacturer's instructions). From passage 2 and thereafter, more than 98% of cells expressed cytokeratin, confirming the very high purity of the cell culture system [27].

BoHV-4 strain and inoculum preparation

The BoHV-4 strain was originally isolated from a postpartum metritis cow (titre 10^6 tissue culture infectious dose, $TCID_{50}$/mL). A virus stock was prepared by propagating the virus in Madin-Darby Bovine Kidney cells (MDBK CCL-22, American Type Culture Collection, USA). The MDBK cells were cultured in Eagle's Minimal essential Medium (EMEM, Hatunalab AB, Sweden) containing 10% (v/v) horse serum, 2 mM L-glutamine (Sigma, USA), 200 IU/mL penicillin G sodium (Sigma, USA) and 100 µg/mL streptomycin sulphate (Sigma, USA). One millilitre of virus (titre 10^6) was diluted to 1:10 (v/v) in the tissue culture medium and virus (in solution) was inoculated on MDBK cells in a 25 cm^2 flask. After 2 h of incubation at 37°C under 5% CO_2, the virus solution was poured off. The monolayer was then disrupted by trypsin treatment and re-suspended in EMEM with 10% (v/v) horse serum. After three days of incubation at 37°C under 5% CO_2, when the cells showed > 50% CPE the incubation was stopped by freezing the flask at -70°C. After thawing, the flask contents was aliquoted into 1 mL tubes and then transferred to a -70°C freezer. The titre of the stock of amplified virus used for the experiment was estimated to be $10^{7.7}$ per mL.

BoHV-4 challenges

First challenges were performed *in vitro* to define viral multiplicity of infection (MOI) and lethal dose of virus (LD_{50}) to bEEC. The bEEC from passage 5 were challenged by different viral MOIs (MOI 0.001, 0.01, 0.1, 1 and 10). The bEEC were cultured in the flasks until confluence and then detached by trypsin (TrypleTM Express (1×), ref.12605-010, Gibco®, USA). The sample materials were then transferred to 15 mL tubes and centrifuged at 215 ×g for 5 min. Supernatant was discarded and 7 mL of 2% (v/v) FBS medium were added to suspend the cell pellet. The material was aliquoted into 6 centrifuge tubes. In each tube serial viral MOI; 0.001, 0.01, 0.1, 1 and 10 was added to five these tubes, while one was used as a control, to which 2% (v/v) FBS medium was added instead of virus. All sample tubes were incubated at 39°C under 5% CO_2 for 1 h and mixed every 5 min. After incubation, samples were centrifuged at 215 ×g for 5 min, supernatant was discarded and 10% (v/v) FBS medium was added to suspend the pellet. The cells were placed in 12-well plates and cultured at 39°C with 5% CO_2 for 6 days. Number of cells was evaluated by trypan blue assay [27] and the amount of virus particles was evaluated by a titration technique. The LD_{50} of BoHV-4 on bEEC was achieved with MOI 0.1 and consequently, this MOI was used in the main study.

Endometrial epithelial cells prepared from the 9 dioestrous cows were cultured in flasks for three to four days until confluence. The bEEC from passage 4 were detached by trypsin and transferred into 15 mL tubes and centrifuged at 215 ×g for 5 min, supernatant was discarded and 5 mL of 2% (v/v) FBS medium were added to suspend the cell pellet. Samples material was then aliquoted into two 50 mL tubes (control and infected). In control tube, 2% (v/v) FBS medium was added, while in infected tube cells were exposed to viral MOI 0.1 (as described above). All sample tubes were incubated at 39°C under 5% CO_2 for 1 h and both tubes control and infected were mixed every 5 min during this incubation. Tubes were centrifuged again at 2,683 ×g for 5 min. Supernatant was discarded, 25 mL 10% (v/v) FBS medium were added to both tubes to suspend the cell pellet and around 3 to 7×10^4 cells/well were placed in 12-well plates. Cells were cultured at 39°C with 5% CO_2. Number of live cells was evaluated by trypan blue assay and number of virus particles was determined by titrating in MDBK cell culture. The viral DNA was determined by qPCR and BoHV-4 infection of cells was evaluated by indirect fluorescent antibody test (IFAT) at each time point (Day 1 to Day 7).

Trypan blue assay and cell counting

Trypan blue assay was used for cell number evaluation. Counting was performed at Day 0 (cell placing day) and all time points of challenges. Following culture, at each time point, medium was removed and cells were detached with trypsin. These cells were exposed systematically two times to 2 mL trypsin at 39°C for 5 to 10 min. The plates were then checked for remaining cells. This protocol was applied again when some cells remained attached. All attached cells were pipetted from wells and transferred to 15 mL Falcon tubes. The solution was gently mixed for 2 to 3 seconds and 70 µL of material were taken and mixed with the same volume of trypan blue solution (T8154 trypan blue solution 0.4 %, Sigma, USA) in Eppendoft tubes. Then 10 µL of mixed solution were immediately transferred to a Burker Neubauer chamber (haemocytometer, 40443001, Hecht Assistent, Germany). Unlabelled cells (trypan blue negative, TB-) were counted under low magnification (×40) in a light microscope. TB- cells (from attached cells) were taken to be living cells and percentage cell survival (X) in controls and BoHV-4 treated groups was calculated as:

$$x = \frac{\text{number of TB(neg) cells at Day(x)} - \text{Number of TB(neg) cells at Day 0}}{\text{Number of TB(neg) cells at Day 0}} \times 100$$

Viral titre/$TCID_{50}$

Following challenges with BoHV-4, bEEC were collected and frozen at -70°C. Control and infected cells and supernatant were collected from Day 1 to Day 7. Then 100 µL of MDBK cell suspension in 10% (v/v) horse serum medium (prepared EMEM medium as described above) were added to 96-well microtitre plates and incubated for 2 h at 37°C under 5% CO_2. Frozen BoHV-4 from sample materials was thawed and serially diluted from 10^0 to 10^{10} in 10% (v/v) horse serum EMEM medium. Each dilution of BoHV-4 from sample materials was inoculated in 50 µL/well MDBK cells (8 replicate wells per dilution of virus and per sample). These infected MDBK cells were incubated for 7 days and plates were analysed for virus infectivity under a light microscope. Prevalence of virus was indicated by its CPE. The number of virus particles after BoHV-4 challenges on bEEC was based on serial dilutions to determine endpoint titre. The titration value was calculated according to the method of Karber [28] and expressed as $\log_{10}$ value of $TCID_{50}$/50 µL.

Quantity of viral DNA determined by quantitative polymerase chain reaction (qPCR)

Sample materials from Day 1 to Day 7 were collected and used to quantify number of virus particles by qPCR. Primers and probes have already been designed and validated for BoHV-4 detection (Juremalm et al., manuscript in preparation). Viral DNA was extracted by adding proteinase K (Sigma, P4850, Sigma Aldrich, USA) to 90 µL of cell suspension. Nucleic acid extraction was then performed in a Magnatrix 8000+ robot (NorDiag AB, Sweden) according to the manufacturer's instructions. For the qPCR reaction, 2 µL template DNA were

mixed with 7.5 µL SsoFast probes supermix (Bio-Rad, UK), 400 nM of each primer (BHV-4gBF and BHV-4gBR) and 200 nM of the probe in a total volume of 15 µL. The samples were amplified in an Applied Biosystems 7500 Fast Instrument (Live Technologies, ThermoFisher Scientific, Sweden) during 45 cycles. Each cycle included denaturing at 95°C for 5 sec, annealing and elongation at 60°C for 30 sec. The quantity of virus is presented as 40 - Ct value.

Viral detection by indirect fluorescent antibody test (IFAT)

Aliquots (25 µL) of non-infected and infected cell suspension were placed in 10-well slides (Novakemi, Sweden) and cultured in humidity chambers incubator at 39°C in 5% CO_2. To analyse the course of infection, slides were taken out each day during a period of 7 days. The slides were washed with PBS and Super-Q water, fixed by acetone for 8 min, allowed to air-dry and then stored in -70°C until analysed. Acetone-fixed slides from Days 1, 3 and 7 were analysed for virus prevalence. After thawing, an anti-BoHV-4 monoclonal antibody (ID.vet, France) was diluted 1/100 in PBS and added to each slide. The slides were incubated in a dark humid chamber at 37°C for 30 min, washed with PBS and Super-Q water and then dried. The cells were stained with 25 µL fluorescent isothiocyanate-conjugated rabbit anti-mouse IgG (F0232; diluted 1:20, Dako, Denmark,) for 30 min at 37°C. The slides were then washed with PBS, dried and mounted with glycerol. These slides were examined with a fluorescence microscope to determine the prevalence of virus antigen by measuring fluorescence signal from the cells.

Cytokine measurement by ELISA

Cell supernatant was collected after BoHV-4 challenges from Day 1 to Day 7 and centrifuged at 215 ×g for 5 min. The resulting supernatant was collected and used to evaluate the concentration of tumour necrosis factor alpha (TNF-α; Bovine TNF-a ELISA Kit, ref EBTNF-, lot 0650070715, Thermo Scientific, USA), interleukin 8 (IL-8; Bovine IL-8 (CXCL8) ELISA development kit, 3114-1H-6, MABTECH AB, Sweden), interleukin 1β (IL-1β; Bovine IL-1β ELISA Reagent Kit ESS0027, Thermo Scientific, USA) and interferon α (INFα; Bovine IFN-alpha ELISA Kit, ELB-IFNa, RayBio, USA). All assays were made following the respective manufacturer's guidelines. The optimal density absorbance (OD_{450}) was measured to calculate the concentration of each sample. The concentration of each cytokines was calculated from the corresponding standard curve.

Statistical analyses

The effects of viral dosage, cow class of initial cell number and secondary interactions or effects of time, cow, viral exposure and second order interactions on percentage of living cells (TB- cells) were analysed by ANOVA (SAS 9.2, proc GLM) following arc sinus transformation of percentages. Similarly, the effects of cow, viral exposure, day of infection and their second order interactions on cytokines concentrations were analyzed using GLM. When number of classes for a given factor exceeded two, comparisons were made following Scheffe's adjustment. Results are presented as means ± standard error of mean (SEM). Spearman correlation coefficients were used when analysing relationships between titration and qPCR results. Cutoff level for significance was fixed at $p < 0.05$.

Results

Cell survival and CPE

In the preliminary study, the percentage of live cells (TB-) in controls had doubled on average by Day 6 post-challenge compared with Day 0 (×200%, Figure 1A). Overall, the percentage of TB- cells was significantly lower ($p < 0.001$) in the infected groups than in the controls (Figure 1B). When viral MOI increased, CPE also increased, whereas TB- cell numbers significantly decreased at Day 6 post-challenge (Figure 2). The LD_{50} of BoHV-4 after 6 days of challenge was MOI 0.1, which showed 50% CPE and 50% of live cells by Day 6.

A

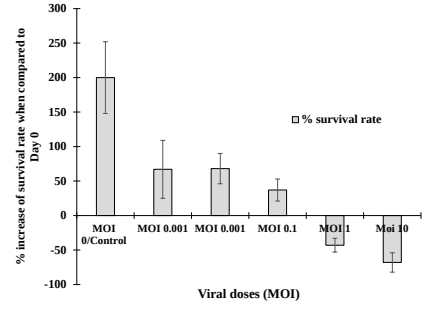

Figure 1. (A) Percentage increase in live cells at Day 6 compared with Day 0 following challenges with different viral MOI. The percentage of live cells had doubled (×200%) by Day 6 post-challenge compared with Day 0.

B

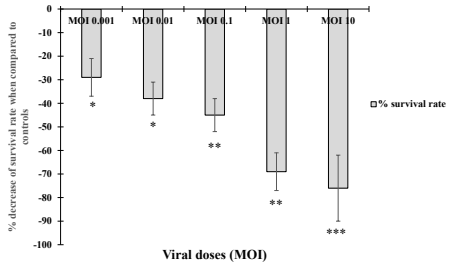

Figure 1. (B) Percentage decrease in live cells at Day 6 compared with controls following viral challenges with different MOI.

For the viral doses representing MOI 0.001 and 0.01, MOI 0.1 and MOI 1 and 10, the percentage of cell growth differed from controls at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$(***) respectively.

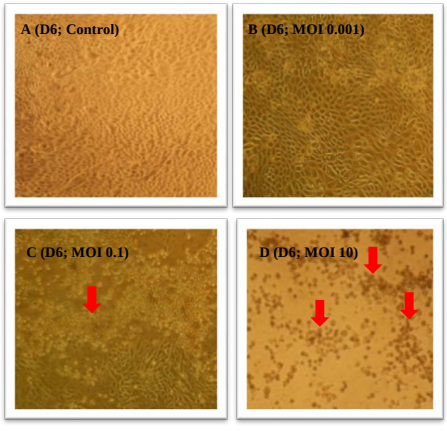

Figure 2. (A) A appearance of cytopathic effects (CPE) after challenges with BoHV-4 at Day 6 in control and infected cells from MOI 0.001 to 10 (B, C and D). Red arrows in culture well-plate from infected cells show increasing CPE areas when MOI was higher.

In the main study, a very significant increase ($p < 0.0001$) in TB- cells was also observed from Day 0 to Day 7 in controls. The percentage cell survival (X) compared with Day 0 in controls and infected groups is illustrated in Figure 3. Main effects of cow ($p < 0.0001$), day post-challenge ($p < 0.0001$) and infection ($p < 0.001$ for numbers, $p < 0.05$ for percentage survival rate) were significant. A significant day×treatment (infected or not) interaction ($p < 0.0001$) was also found. During the first four days, TB- cells increased in a similar way in infected groups and controls. However, after 4 days post-challenge, the number of TB- cells and percentage cell survival was lower in infected cells than in controls. The difference in percentage cell survival between infected and control groups was very significant by Day 7 ($p < 0.01$; for total number of cells and $p < 0.001$ for percentage survival rate). In addition, a significant interaction between the effects of cow (cell culture from which samples originated) and infection was found, showing individual variation in the way cells from different cows responded to infection by BoHV-4 ($p < 0.001$ for number of cells, $p < 0.05$ for percentage cell survival). Furthermore, base on morphology, increased CPE and numbers of floating cells (dead cells) were found after Day 4 post-BoHV-4 challenge (data not shown or Figure 4).

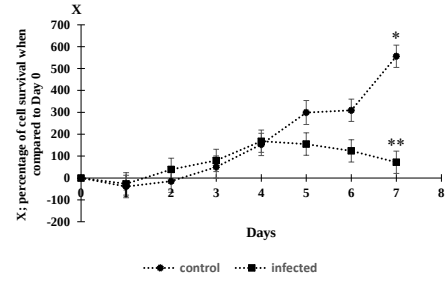

Figure 3. Percentage cell survival (X) compared with Day 0 in controls and infected groups. The percentage of cell survival increased from Day 1 to Day 7 in controls. Following MOI 0.1 BoHV-4 challenges, percentage cell survival increased in a similar way as in the control during the first 4 days, but was lower after Day 4 post-challenge. The difference in percentage cell survival between the infected and control groups was highly significant by Day 7 (*, $p < 0.001$).

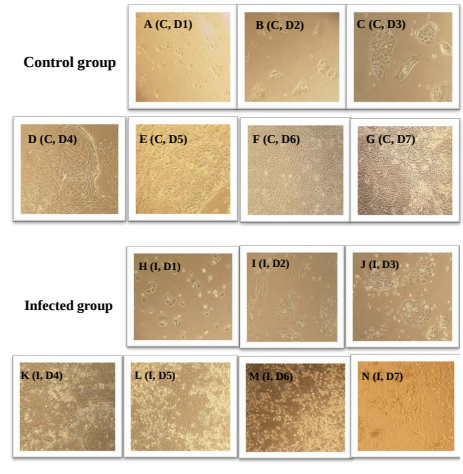

Figure 4. Morphology of cells following viral MOI 0.1 at Day 1 to Day 7 after challenges in controls (A-G) and after infected group (H-N).

Viral replication

In the preliminary study, at Day 6 the highest viral titre was found following MOI 0.1 challenge, while the lowest was found with MOI 1 and 10 challenge. There was no difference in viral titre following MOI 0.001 and MOI 0.01 challenge and results were intermediate between MOI 1-10 and MOI 0.001-0.01.

In the main study, the effects of day and cow on viral replication, estimated both from titration and qPCR results, were highly significant ($p < 0.0001$). A strong increase in viral particle numbers was found between Day 1 and Day 7 by titration and qPCR. Number of virus particles increased steadily from Day 1 to Day 5, but the differences between Day 5, Day 6 and Day 7 were not significant (Figure 5). Overall, a very strong correlation between individual results of titration and qPCR results was observed ($r = 0.9$, $p < 0.0001$). The effect of cow from which cells originated was also highly significant ($p < 0.0001$) indicating variation in the way BoHV-4 replicated in individual cultures. In addition, a progression of viral infection in the cell culture from Day 1, to Day 7 was revealed by IFAT (Figure 6).

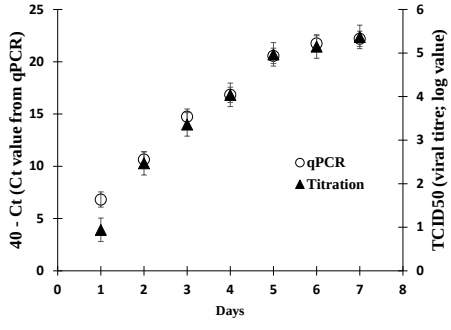

Figure 5. Change in viral titre (log value, mean ± s.e.m.) and qPCR results (40-Ct, mean ± s.e.m.) from Day 1 to Day 7. There were significant increases in viral particles between Day 1 and Day 7 after challenges as illustrated by titration and qPCR results ($p < 0.0001$). The number of virus particles increased steadily from Day 1 to Day 5. Differences between Day 5, Day 6 and Day 7 were not significant.

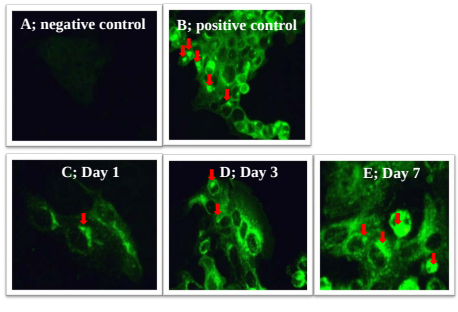

Figure 6. (A) Negative control from uninfected cells. (B) Positive control from BoHV-4 infected MDBK cells. Progressive increase in the number of viral particles infecting cells at Day 1 (C), Day 3 (D) and Day 7 (E) following BoHV-4 challenges at MOI 0.1 as shown by the indirect fluorescent antibody test (IFAT) (red arrows illustrate viral particles in the cells).

TNF-alpha

The total concentration of TNF-α increased from Day 1 to Day 7 in controls, while in the infected group concentrations started to decrease after Day 4. Total TNF-α concentrations were not significantly different between controls and infected cells during the first 6 days of culture, but at Day 7 total TNF-α concentrations were significantly higher ($p < 0.05$) in controls than in infected groups (Figure 7A). Nevertheless, there was a very significant effect of day on total TNF-α concentration in supernatant post-challenge ($p < 0.0001$) and a significant interaction between day and treatment (infected or not) was also observed ($p < 0.05$). Following adjustment of concentration to a set amount of cells (concentration per 10^4 cells), TNF-α production was mainly influenced by the cow from which the culture originated ($p < 0.01$) and there was a significant interaction between day and infection ($p < 0.05$). TNF-α ratio per 10^4 cells was significantly higher ($p < 0.05$) in infected cells than in controls by Day 7 (Figure 7B) and not different before.

A

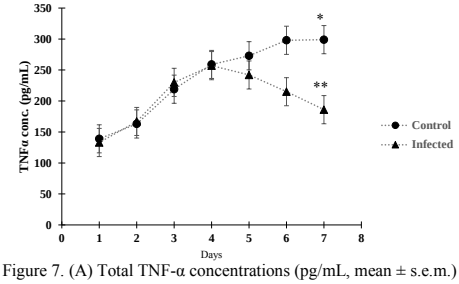

Figure 7. (A) Total TNF-α concentrations (pg/mL, mean ± s.e.m.) in control and infected cultures from Day 1 to Day 7. At Day 7 total TNF-α concentrations were significantly higher in controls than infected groups (*, $p < 0.05$).

B

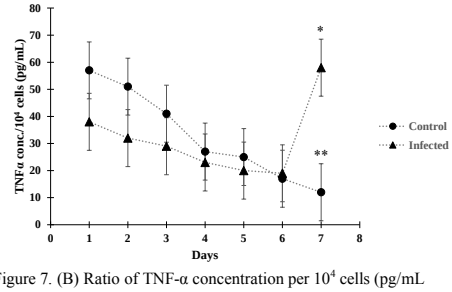

Figure 7. (B) Ratio of TNF-α concentration per 10^4 cells (pg/mL per 10^4 cells, mean ± s.e.m.), was significantly higher in the infected group than in the control at Day 7 (*, $p < 0.05$).

IL-8

Mean IL-8 concentration increased continuously from Day 1 to Day 7 both in controls and infected samples (effect of day, $p < 0.0001$). However, total IL-8 concentration in supernatant collected from infected cells was significantly higher in infected samples than in controls ($p < 0.0001$, Figure 8A). The difference between controls and infected cells showed some variations depending on cow (Infection×Cow interaction, $p < 0.0001$) and day (Infection×Day interaction, $p < 0.0001$). Differences in IL-8 concentration between infected and non-infected samples were not significant at Day 1 and 2, but became very significant for each of the following days ($p < 0.01$ on Day 3 to $p < 0.0001$ from Day 4 to Day 7). Similar patterns as for TNF-α ratio were found for IL-8 (concentration per 10^4 cells). No difference between controls and infected samples was observed between Day 1 and Day 6, but IL-8 concentrations were higher ($p < 0.05$) in samples issued from infected cells by Day 7 (Figure 8B).

A

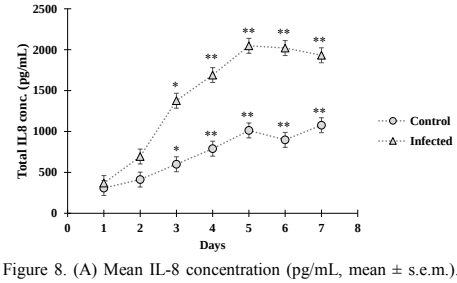

Figure 8. (A) Mean IL-8 concentration (pg/mL, mean ± s.e.m.). The concentration increased continuously from Day 1 to Day 7 in both controls and infected groups (effect of day, $p < 0.0001$). Total IL-8 concentration in infected cells was significantly higher than in the controls ($p < 0.0001$). Differences between controls and infected groups were not significant at Day 1 and 2, but became significant on Day 3 (*, $p < 0.01$) and Day 4 – 7 (**, $p < 0.0001$).

B

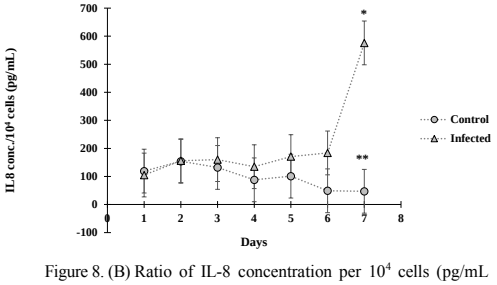

Figure 8. (B) Ratio of IL-8 concentration per 10^4 cells (pg/mL per 10^4 cells, mean ± s.e.m.). This ratio was higher in the infected group than the control at Day 7 (*, $p < 0.05$).

IL-1β and INF-α

IL-1β concentration in supernatant from controls was constantly below the limit of detection of the assay. Low concentrations were found in infected samples from 3 cows out of 9 and IL-1β was not detectable for other cows. INF-α concentrations were constantly under the limit of detection by this assay.

Discussion

Until now, BoHV-4 has been regarded as a co-infection pathogen in reproductive disease that increases uterine inflammation when animals are first infected with bacteria in systemic system [1, 8, 29, 30]. However, the implication of BoHV-4 in uterine diseases may also be due to external infection via the natural reproductive route [30]. The direct exposure of endometrial epithelial cells to BoHV-4 may then result from contamination during artificial or natural insemination since BoHV-4 DNA has been found in cases of oedematous orchitis [31, 32] and in the semen of healthy bulls [26] even those kept in artificial insemination centres [25].

Despite the potential risk of BoHV-4 transmission through the insemination route, the interaction between endometrial epithelial cells and BoHV-4 and host individual responses to BoHV-4 have not been widely investigated for these cells. In the present study we used an *in vitro* model to challenge bovine endometrial epithelial cells (bEEC) with BoHV-4 under different conditions. The preliminary experiment allowed the dosage of virus inducing moderate CPE to be defined, in order to study the kinetics of infection over one week.

We found that working at MOI 0.1 was optimal since it meant that around 50% of the cells were alive by 6 days post-infection.

This MOI is lower than that those used in former studies in other tissues or cells [33], but was the most suitable concentration to study the kinetics of infection and potential host individual variability in the response of endometrial epithelial cells. Very strong CPE were found with higher MOI, especially MOI 10, and almost all cells were dead by 6 days post-infection. However, titration of virus at that time showed very low amounts of virus, much lower than with lower MOIs, confirming that viruses survive and replicate in host or live cells [20]. When using the highest MOIs BoHV-4 could not longer replicate, due to lack of cells induced by early death of bEEC. In contrast, following low MOI challenges the initial amount of virus was low and the growth of the endometrial cell population was not impaired. In this situation, cells were still able to proliferate and high numbers were found by Day 6 post-infection. Viral titre also increased, as replication was able to take place in a large number of cells but, depending on the rapidity of growth, reached a plateau by Day 4 to 6.

The change in the number of cell survival following challenges with BoHV-4 at MOI 0.1 confirmed the above observations. CPE became evident after 4 days and the number of live cells decreased from Day 5, with the difference in percentage cell survival between control and infected samples being significant by Day 7. The titration and qPCR results also showed that BoHV-4 replicated freely between Day 0 and Day 4, which is consistent with the change in number of cells in the infected samples. The results from IFAT showed that the virus was strongly detectable in bEEC as soon as Day 3 post-infection, suggesting that replication occurred much earlier.

Overall, our results confirm findings in previous studies that various types of cells, including endometrial cells, are sensitive to BoHV-4, with the virus inducing CPE more or less rapidly [20]. Differences in the speed of development and intensity of CPE between studies may be related to the amount of virus used for challenges [34] [35]. In a study where bovine arterial endothelial (BAE) cells were challenged with BoHV-4 was faster development (within 3 days) and much stronger CPE was also observed when MOI was increased [19, 35]. The delay for the virus to replicate sufficiently and induce CPE has been shown to depend also partly on the type of cell used and the duration of infection [36]. Strong CPE have been found within 48 h after electroporation in bovine epithelial and stromal cells while cell lines (MDBK and Bovine Embryo Kidney (BEK)) and other cell types (Bovine Turbinates (BT) and Bovine Embryo Lung (BEL)) show CPE of smaller size 3-5 days post-challenge [20].

However, as the ability of BoHV-4 to replicate is associated with viral immediate early 2 (IE2) gene promoter (ORF50; replication and transcription activator (Rta)) [20] the stage in the host cell replication cycle is a key factor influencing viral replication [20, 34]. On of those studies [34] found that the S phase of the host cell cycle is the most effective period for BoHV-4 DNA synthesis, with little or no changes in viral DNA found in G0 cells [34]. From the conditions under which we used the cells while performing cultures, i.e. immediately challenged after detachment by trypsin and then seeding to the 12-well plates in numbers far from confluence, it can be assumed that most cells were in S phase at time of infection. In a similar previous study in which freshly seeded cells were infected, the development of CPE was found stronger and faster (5-6 days) than when infecting confluent cells (10-12 days) [34, 37]. However, the results from our study show that despite the same amount of virus and cells being used under a constant MOI, there was strong variation in viral replication during the 4-5 days study period. The time at which CPE were first observed was consistently reduced when the increase in viral numbers was faster (estimated from qPCR results, data not shown), as documented by the very significant interaction between cow (from which the culture originated) and time on living cell numbers. This findings show that factors influencing the speed of viral replication deserve further investigation to decipher the underlying molecular mechanisms responsible for such host-related individual differences.

The interferons gamma (IFNs-γ) and tumour necrosis factor alpha (TNF-α) are nonspecific earliest host response cytokines to viral infections in many cell types and induce cascades of downstream mediators [20, 38-40]. These inflammatory molecules are produced by immune cells, inflamed endometrium and other cell types [8, 41]. In the present study, only the TNF-α and IL-8 patterns could be studied, as other cytokines were mostly undetectable in supernatant samples.

Both TNF-α and IL-8 are pro-inflammatory cytokines. TNF-α is said to be mainly produced by macrophages [42, 43], while IL-8 is chemotactic to polymorphonuclear cells (PMN) [21, 44, 45]. In our study, the cell population consisted of more than 98% of endometrial epithelial cells, with any being fibroblasts remaining cells, and thus it is very unlikely that immune cells were present. Despite this, we were able to detect TNF-α and IL-8 in all supernatant samples. This confirms, as shown previously in different models, that the pro-inflammatory cytokines are produced not only by immune cells but also by other cell types [41, 46]. It has also been reported that TNF-α is produced by endometrial epithelial cells after challenges by LPS which is consistent with the over-expression of that gene in a mixed population containing around 90% epithelial cells [47] and in a more pure population of epithelial cells similar to that used in present study [48].

Interestingly, TNF-α and IL-8 displayed different patterns in relation to infection. Total TNF-α concentrations were related to the number of living cells, which appeared to be the main source of TNF-α. Previous studies have shown that cells infected by BoHV-4 show increased sensitivity to TNF-α, which participates in stimulation of virus DNA synthesis following binding of

TNF-α receptor 1 on cell surfaces [49]. On the other hand, total concentration of IL-8 or the concentration produced by a constant number of cells were always found to be higher in supernatant from infected samples than control samples. The highest level of IL-8 was actually found at Day 7, when the number of remaining alive cells was very low. As reported here, infected cells have been shown to produce more IL-8 [21] via IE2 gene product ORF50/Rta of BoHV-4 [49]. The increase in this pro-inflammatory cytokine is part of the mechanism leading to the persistent and chronic inflammation of the endometrium/endometritis [8, 21, 22, 29, 50].

In conclusion, these results show that BoHV-4 affects epithelial endometrial cells in many ways. Strong CPE are expressed depending on time and initial quantity of BoHV-4 virus at time of challenge. The strong impairment of epithelial cell survival is associated with alterations in the patterns of pro-inflammatory cytokines which may be involved in development of endometritis. These findings should be confirmed in further *in vivo* studies, but the results obtained in this *in vitro* study support the implication of BoHV-4 in clinical cases of uterine disease and the ability of the virus to promote infection in cows during breeding.

Conflict of interest

None of authors have any conflict of interest.

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Author contributions

The conception and design of the study; MC, PH, JF, YG. Acquisition of data; MC, KU, GB, GD. Analysis and interpretation of data; MC and PH. Drafting the article or revising it critically for important intellectual content; MC, GB, YG, RB, GD, JF, PH. Final approval of the version to be submitted; MC, GB, YG, KU, MJ, RB, GD, JF, PH.

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