BVDV alters uterine prostaglandin production during pregnancy recognition in cows

Zhangrui Cheng¹, Ayimuguli Abudureyimu², Chike F Oguejiofor¹, Rebekah Ellis¹, Amy Teresa Barry¹, Xing Chen^{1,3}, Olivia L Anstaett⁴, Joe Brownlie⁴ and D Claire Wathes¹

¹Department of Production and Population Health, Royal Veterinary College, North Mymms, Hertfordshire, UK, ²Life Science and Engineering College, Northwest University for Nationalities, Lanzhou, China, ³Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction, Education Ministry of China, College of Animal Science and Technology, Huazhong Agricultural University, Wuhan, China and ⁴Department of Pathology and Pathogen Biology, Royal Veterinary College, North Mymms, Hertfordshire, UK

Correspondence should be addressed to Z Cheng; Email: zcheng@rvc.ac.uk

Abstract

Embryonic mortality in cows is at least in part caused by failure of pregnancy recognition (PR). Evidence has shown that bovine viral diarrhoea virus (BVDV) infection can disrupt pregnancy. Prostaglandins (PG) play important roles in many reproductive processes, such as implantation. The aim of this study was to investigate the effect of BVDV infection on uterine PG production and PR using an *in vitro* PR model. Bovine uterine endometrial cells isolated from ten BVDV-free cows were cultured and treated with 0 or 100 ng/mL interferon- τ (IFNT) in the absence or presence of non-cytopathic BVDV (ncpBVDV). PGF_{2 α} and PGE₂ concentrations in the spent medium were measured using radioimmunoassays, and in the treated cells expression of the genes associated with PG production and signalling was quantified using qPCR. The results showed that the IFNT challenge significantly stimulated *PTGS1* and *PTGER3* mRNA expression and PGE₂ production; however, these stimulatory effects were neutralised in the presence of ncpBVDV infection. ncpBVDV infection significantly increased *PTGS1* and *mPGES1* mRNA expression and decreased *AKR1B1* expression, leading to increased PGE₂ and decreased PGF_{2 α} concentrations and an increased PGE₂:PGF_{2 α} ratio. The other tested genes, including *PGR*, *ESR1*, *OXTR*, *PTGS2*, *PTGER2* and *PTGFR*, were not significantly altered by IFNT, ncpBVDV or their combination. Our study suggests that BVDV infection may impair PR by (1) inhibiting the effect of IFNT on uterine PG production and (2) inducing an endocrine switch of PG production from PGF_{2 α} to PGE₂ to decrease uterine immunity, thereby predisposing the animals to uterine disease.

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Introduction

Poor reproductive performance, of which early embryonic mortality is a major component, causes major economic loss to the dairy industry (De Vries 2006). Embryonic mortality rates in cattle can be as high as 40%, with 70–80% of losses occurring before day 16 of gestation (Diskin *et al.* 2011). Following successful ovulation and subsequent fertilisation, production of interferon- τ (IFNT) by the trophoblast begins at around day 8 of gestation and increases dramatically during conceptus elongation (Kimura *et al.* 2004). A sufficient threshold level of IFNT must be reached by day 16 to ensure pregnancy recognition (PR) and prevention of luteolysis (Forde *et al.* 2011, Lonergan & Forde 2014). IFNT acts in a paracrine manner on the uterine endometrium to develop a receptive environment. This involves changes in the uterine epithelium that are tightly regulated by steroid hormones, type I interferons,

cytokines, prostaglandins (PGs) and growth factors and their receptors (Forde *et al.* 2011, Dorniak *et al.* 2012, Spencer *et al.* 2013, Lonergan & Forde 2014).

Many reproductive processes, such as luteolysis, PR and implantation, involve inflammation and associated up-regulation of inflammatory mediators (e.g. PGs) and recruitment of immune cells to the uterine endometrium (Jabbour *et al.* 2009). PGE₂ (a vasodilator) is luteotrophic for maintaining progesterone secretion by the corpus luteum, whereas PGF_{2 α} (a vasoconstrictor) is luteolytic (Weems *et al.* 2006). In PG production pathways, two series PGs, such as PGE₂ and PGF_{2 α} , are produced from arachidonic acid (AA). PG-endoperoxide synthase isozymes (PTGS1 and PTGS2) catalyse AA into PGH₂, and PGH₂ is converted into PGEs by the action of PGE synthase (PGES), and into PGFs by the action of PGF synthase (PGFS) (Wathes *et al.* 2007). There are many isoforms for both PGES and PGFS. Previous studies have shown that mPTGES1 and AKR1B1 are the predominant

isoforms for PGE₂ and PGF_{2α} production, respectively, in bovine endometrium (Fortier *et al.* 2008).

During PR, IFNT inhibits the up-regulation of oxytocin (OXT) receptors (OXTR) in the uterine epithelium, thereby preventing the pulsatile release of PGF_{2α}, which is necessary for luteolysis (Wathes & Lamming 1995, Mann *et al.* 1999). In contrast, basal release of PGs, such as PGE₂ and PGF_{2α} from both the conceptus and the maternal uterine endometrium rises during early pregnancy (Zarco *et al.* 1988, Dorniak *et al.* 2013). This increased PG production may facilitate the effects of IFNT on the expression of endometrial type I IFN-stimulated genes (ISGs), a vital component of PR (Spencer *et al.* 2013).

Bovine viral diarrhoea virus (BVDV), a single-stranded RNA virus, causes widespread infection in the cattle worldwide. The reproductive system, including the uterine endometrium, is a major site for infection, maintenance and spreading of the virus (Grooms 2004, Lanyon *et al.* 2014). BVDV infection can have a significant impact on all stages of pregnancy, including causing early embryonic death, such that reproductive losses are one of the most important consequences of the disease (Grooms 2004). Our recent studies demonstrated that infection of bovine endometrial cells *in vitro* with non-cytopathic BVDV (ncpBVDV) caused many significant changes in pathways associated with innate immunity, particularly those associated with type I interferon signalling (Oguejiofor *et al.* 2015a). ncpBVDV thus inhibited the expression of many ISGs that are also involved in PR, such as *IFITs*, *BST2*, *MX2*, *OAS1*, *USP18* and *RSAD2* (Forde *et al.* 2011). Some of these genes are also regulated by PGs (Spencer *et al.* 2013). In monocytes, BVDV infection stimulated the production of PGE₂ (Welsh *et al.* 1995). When bovine endometrial cells were also exposed to bacterial lipopolysaccharide (LPS), ncpBVDV increased the expression of *PLA2G4F* and *PTGES* (*mPGES1*) (Oguejiofor *et al.* 2015a). *PLA2G4F* belongs to the family of PLA2 enzymes that are essential for the initial release of AA in PG biosynthesis (Tithof *et al.* 2007), whereas PTGES subsequently catalyses the isomerisation of PGH₂ to PGE₂ (Samuelsson *et al.* 2007).

These results suggested that ncpBVDV infection in cows may (1) interfere with the normal PR signals in response to IFNT and (2) increase endometrial PGE₂ production following bacterial infection, potentially compromising the innate immune response. To explore these possibilities further, this study mimicked PR by stimulating bovine endometrial cells with IFNT in the presence or absence of ncpBVDV infection and investigated the effects of these treatments on PG signalling pathways.

Materials and methods

All reagents were purchased from Sigma or BDH Merck Ltd (Leics, UK) unless otherwise stated. All culture media used included 50,000 units/L penicillin and 50 mg/L streptomycin. All culture media and serum used were certified BVDV free.

Animal, cell isolation and culture

Fresh and apparently healthy uteri from cows in the early luteal phase of the oestrous cycle were collected at the local abattoir. Uterine endometrial cells (a mixture of primary epithelial and stromal cells) were isolated and cultured following the methods described previously (Cheng *et al.* 2013, Oguejiofor *et al.* 2015b). Briefly, under sterile conditions, strips of intercaruncular endometrium were separated and put into serum-free Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12 medium) (Sigma) and chopped into 1 mm³ cubes. They were digested for 90 min at 37°C in a medium containing 100 mg bovine serum albumin (BSA; Sigma), 50 mg trypsin III (Worthington, Lakewood, NJ, USA) and 50 mg collagenase A (Roche) per 100 mL of Hanks' balanced salt solution (HBSS; Sigma). After filtration through a 100 µm mesh into 50 mL falcon vials, the suspension was re-suspended with HBSS containing 10% foetal bovine serum (FBS; PAA, Somerset, UK) and 3 µg/mL trypsin inhibitor (Sigma) and centrifuged at 100 g at 10°C for 10 min. After two repetitions of the above washing procedures, the cells were suspended in the culture medium (DMEM/F12 medium with 10% FBS) and plated in 24-well IWAKI microplates (Scitech DIV; Asahi Techno Glass, Funabashi, Japan) at 2 mL per well containing 0.5 × 10⁵ cells (day 1). Culture medium was changed every 48 h to allow the cells to grow. The composition of the cell population was confirmed using immunocytochemical staining validated in our laboratory (Oguejiofor *et al.* 2015b). After culturing for 8 days (day 8), epithelial cells comprised about 90% and stromal cells about 10% of the population. Contamination of immune cells was negligible (<0.001%).

BVDV test for experimental samples

The experiment was designed to use BVDV-free bovine endometrial samples and FBS. The examination was carried out using a PCR method reported previously (Vilcek *et al.* 1994, Pinheiro de Oliveira *et al.* 2013). The primer pair for the PCR was as follows: forward (ATGCCCWTAGTAGGACTAGCA; position 108–128) and reverse (TCAACTCCATGTGCCATGTAC; position 395–375) with an expected product size of 288 bp, which are the highly conserved 5' non-coding/non-structural coding regions of the pestivirus BVDV genome strain NADL (Vilcek *et al.* 1994). Total RNAs in the uterine endometrial tissue and FBS were extracted using a Qiagen RNA Lipids Mini kit (Qiagen) following the supplied protocol. RNA was reverse transcribed into cDNA using a cDNA synthesis kit supplied by PCR Biosystems (London, UK), and PCR was performed using the G-Storm thermal cycler (G-Storm Ltd, Somerset, UK) and Qiagen Multiple PCR kit (Qiagen). The testing system also included a BVDV-positive control prepared using the pT7Blue-2 blunt vector, linearised (Novagen, Cambridge, MA, USA), reverse transcription-negative controls and a housekeeping gene β-actin (*ACTB*, see Table 1 for its primers) to verify that PCR reagents and reaction volumes were free of genomic DNA contamination. The above testing was carried out on the day of collection of uterus in parallel with the cell isolation and culture. The endometrium initially tested negative for BVDV was used for further experiments.

Propagation of ncpBVDV

The ncpBVDV (Pe515nc strain) was acquired from the BVDV Research Group, Royal Veterinary College, UK. This strain was isolated from a cow diagnosed with mucosal disease and virologically cloned as non-cytopathogenic virus. To provide adequate infectious BVDV for the present experiments, the virus stock was propagated to achieve a 50% tissue culture infective dose (TCID₅₀) of 5×10^5 per mL following the method used in our group (Oguejiofor *et al.* 2015a).

Experimental protocols

The experiments of ncpBVDV infection and IFNT challenge were carried out in the endometrial cells from ten cows confirmed BVDV negative tested using the above method. Cells from each cow were collected as a batch and grown in two 24-well plates as described previously (day 1). On day 4 of the cell culture when the cells grew to approximately 70% of confluence, FBS in the culture medium was reduced to 5% (maintenance medium, MM) to prevent overgrowth of the cells. The cells from each cow were divided into four treatment groups: control (CONT), IFNT, ncpBVDV and IFNT+ncpBVDV. Wells treated with the virus were maintained in separate plates from those treated without the virus to prevent cross-contamination. For the cells designated as BVDV infected, 0.25 mL MM containing Pe515nc BVDV at a multiplicity of infection (MOI) of 0.1 was added for 3 h to allow for virus infection. The same procedures, but with 0.25 mL MM only, were carried out for the cells designated as non-infected controls. The volume in all wells was made up to 1 mL with MM and the medium was changed after 2 days. IFNT treatment was carried 4 days after infection (day 8). For the wells specified for IFNT treatment, the medium was replaced with 1 mL MM containing 100 ng IFNT (recombinant ovine IFNT; Cell Sciences, Canton, MA, USA) and incubated for 24 h. The other wells were changed and incubated with 1 mL MM. The spent medium was harvested (on day 9) and stored at -20°C for PG quantification, and the treated cells were used for total RNA extraction.

Assessment of BVDV cell infection and cell viability

Bovine endometrial cell infection with ncpBVDV was confirmed using both the PCR method with the extracted RNA as described above and an indirect enzyme (alkaline phosphatase) immunostaining procedure as described previously (Oguejiofor *et al.* 2015a). The cell viability after exposure to the infection and treatment was assessed using an MTS reduction assay method as described previously (Oguejiofor *et al.* 2015b).

Quantification of PGs using radioimmunoassay (RIA)

The concentrations of PGE₂ and PGF_{2α} in the spent medium were measured with charcoal–dextran-coated RIA methods described previously (Cheng *et al.* 2001). The PG antisera were a kind gift from Dr N L Poyser (University of Edinburgh, Edinburgh, UK). The cross-reactivities for PGE₂ antiserum were

23, 100 and 15% with PGE₁, PGE₂ and PGE₃, respectively, and those for PGF_{2α} antiserum were 34, 100 and 25% with PGF_{1α}, PGF_{2α} and PGF_{3α} respectively. The standards for PGE₂ and PGF_{2α} were purchased from Sigma, and the tritiated tracers of PGE₂ ([5,6,8,11,12,14,15(n)-³H]-PGE₂) and PGF_{2α} ([5,6,8,9,11,12,14,15(n)-³H]-PGF_{2α}) were supplied by PerkinElmer. The samples were diluted ($\times 10$ –200) in the RIA buffer. This allowed the sample PG concentrations to fall within the analytical ranges of the RIAs (0.02–5.0 ng/mL for PGE₂ and 0.01–2.5 ng/mL for PGF_{2α}), and the interruption from the medium contents with the RIAs was minimised, making extraction unnecessary. The limits of detection were 2 pg/tube and 1 pg/tube for PGE₂ and PGF_{2α} respectively. The intra-assay and inter-assay coefficients of variation were 3.5 and 6.3% for PGE₂ ($n=6$) and 4.1 and 9.6% for PGF_{2α} respectively ($n=6$).

RNA extraction

After collection of spent medium, the cells from each treatment group (six wells) were pooled and the total RNA extraction was carried out using RNeasy Mini Kits (Qiagen) following the supplier's protocol. The concentrations and purity of RNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Primer design and PCR

DNA sequences for all primers were obtained from GenBank at NCBI (<http://www.ncbi.nlm.nih.gov/Database/index.html>) and the primers were designed using a "Primer 3" web-based programme (<http://frodo.wi.mit.edu/primer3>). Their alignment specificity and quality were checked using the Blast tool at <http://www.ncbi.nlm.nih.gov/tools/primer-blast/> and Amplify tool (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx>). The detailed information of the primers is given in Table 1. The primers were made by Eurofins MWG Operon (Ebersberg, Germany). RNA (1 µg) was reverse transcribed into cDNA using a cDNA synthesis kit supplied by PCR Biosystems following the supplier's protocol. The resulting cDNA (20 µL) was diluted in nuclease-free water up to 100 µL.

PCR for the tested genes was performed using the G-Storm thermal cycler and Qiagen Multiple PCR kit (Qiagen), and the DNA products for each gene were used for (1) verifying the primer specificity using electrophoresis on a 2% (w/v) agarose gel and (2) preparation of the DNA standards for each gene in the following quantitative PCR (qPCR) procedures.

qPCR analysis for gene expression

The concentrations of all the ten target genes (*PTGS1*, *PTGS2*, *mPGES1*, *AKR1B1*, *PTGER2*, *PTGER3*, *PTGFR*, *OXTR*, *PGR* and *ESR1*) and four reference genes (*GAPDH*, *RPL19*, *ACTB* and *18S rRNA*) were quantified using qPCR via an absolute quantification approach following the method described previously (Cheng *et al.* 2013). The DNA amplified from cDNA used for standards in the qPCR assay was purified using a QIAquick PCR purification kit (Qiagen), and their quality and concentrations were determined with the NanoDrop

ND-1000 spectrophotometer. Eight standards were prepared from 1 to 1×10^{-7} ng/mL. Annealing and amplicon-specific melting temperatures of the primers were determined using a gradient function of the qPCR machine (CFX96 Real-Time System DNA; Bio-Rad Laboratories) with eight identical reactions containing 2 ng of DNA standard, 10 μ L Sygreen Mix (PCRBiosystems), 0.8 μ L of each 10 μ M forward and reverse primer, and nucleate-free water added up to 20 μ L. The optimised annealing temperatures are given in Table 1.

To quantify the expression levels of each gene, the qPCR assay contained a standard curve, no template control (NTC) and a sample cDNA in duplicate with the same final volume on CFX96 Real-Time Systems (Bio-Rad). Each well contained 5 μ L cDNA standard or samples, 10 μ L Sygreen Mix (PCRBiosystems), 0.8 μ L of 10 μ M forward primer, 0.8 μ L reverse primer and 3.4 μ L nucleate-free water following the protocol supplied by PCRBiosystems and using the optimised annealing temperatures shown in Table 1. This included an initial Taq activation for 2 min followed by 38 cycles of denaturation (95°C), annealing (the annealing temperatures are given in Table 1) and extension (63°C). An amplicon-specific fluorescence acquisition reading based on the melting temperature obtained in the above gradient test was applied to avoid any noise from smaller non-specific products such as dimers before the product acquisition. The results were analysed using the CFX Manager Software package (Bio-Rad). The limit of quantification was 1×10^{-6} to 1×10^{-7} ng/mL for all tested genes. In order to minimise variation, RT for all samples was performed in one assay. For each gene, the standards and all samples were prepared under the same conditions in a single plate with the same master mix of reagents.

Statistical data analyses

All values are summarised as mean $\pm$ S.E.M. The values of gene expression generated by qPCR were normalised as femto gram per micro gram reverse-transcribed total RNA. The PG ratios were calculated as $PGE_2:PGF_{2\alpha}$. Statistical data analysis was carried out using analysis of variance (ANOVA) with repeated measurements for the PG data and ANOVA with randomised block design for the gene expression data via a linear mixed effect model built in SPSS V22, in which the differences between treatments (CONT, ncpBVDV, IFNT and their combination) were taken as fixed effect and cows as random effect or subject for repeated measurement. Statistical significance was considered at $P < 0.05$. Logarithmic transformation was applied if the data were not normally distributed. Where statistical significance was achieved in ANOVA, Fisher's LSD multiple comparisons based on the least square means were performed to identify the differences between the treatment pairs.

Results

Initial testing using PCR on the day of collection confirmed that all the uteri selected were free of BVDV. Successful infection of the ncpBVDV-infected cells only after treatment was confirmed using both PCR and immunocytochemistry as described previously (Oguejiofor *et al.* 2015b) (data not shown). Neither the individual nor the combined treatments affected cell viability at the doses used as confirmed using an MTS reduction assay method.

Table 1 Oligonucleotide primer sequence information.

Gene	Primer sequence (5'-3')	GenBank accession no.	Product length (bp)	Annealing temperature (°C)
<i>PTGS1</i>	Forward: CACAGTGCCTTCCACCTTATC Reverse: CAACTGCTTCTCCCTTTGGTG	NM_001105323.1	163	63.3
<i>PTGS2</i>	Forward: TACTGGAAGCCTAGCACTTTC G Reverse: TGAATGAGGTAAAGGGACAGCC	NM_174445.2	112	61.4
<i>mPGES1</i>	Forward: TGTGTTTCCCCGTGTGTC Reverse: ACTGAGTCTCTGTTTGCTTTTC	NM_174443.2	167	59.5
<i>AKR1B1</i>	Forward: TACCTGGACCTCTACCTCATCC Reverse: CGTCCAGGTATCCACGAAATCT	NM_001012519.1	120	64.5
<i>PTGER2</i>	Forward: CTACTTCTACCAGCGCCGAG Reverse: TACGTGGTCTGCTTGCTGTC	NM_174588.2	165	64.5
<i>PTGER3</i>	Forward: GTGGTCATCGTCTCTACCTGT Reverse: CTTTCATGTGGCTTGAGTACCAG	NM_181032.1	186	61.4
<i>PTGFR</i>	Forward: TGGTGTCTCTGGTCTGTGC Reverse: AAAGCACACCCCACTCAACA	XM_010803367.1	140	60
<i>OXTR</i>	Forward: TCATCATCGCCATGCTCCTG Reverse: CGGAATGAGCAGCAGAGGAA	NM_174134.2	118	63.3
<i>PGR</i>	Forward: AGGAGTTGTCCCTAGCTCACAG Reverse: GCAGCAATAACCTCAGACATCA	NM_001205356.1	162	59
<i>ESR1</i>	Forward: TCAGGCTACCATACGGAGTTT Reverse: CCACTTCATAGCACTTGCGTAG	NM_001001443.1	169	59
<i>GAPDH</i>	Forward: GGTCACCAGGGCTGCTTTTA Reverse: TTCCCGTTCTCTGCCTTGAC	NM_001034034.2	147	61.4
<i>RPL19</i>	Forward: TCGATGCCGGAACAAACAC Reverse: ATTCTCATCTCTCATCCAG	NM_001040516	119	59
<i>ACTB</i>	Forward: GAAATCGTCCGTGACATCAA Reverse: AGGAAGGAAGGCTGGAAGAG	NM_173979.3	182	61.4
<i>18SrRNA</i>	Forward: CGGCGACGACCCATTGCAAC Reverse: GAATCGAACCTGATCCCCGTC	AY779625	99	64.5

Effect of ncpBVDV, IFNT and their combination on PG production by uterine endometrial cells

The results showing the effect of ncpBVDV, IFNT and their combination on PG production by uterine endometrial cells are given in Fig. 1. ANOVA with repeated measurements showed that the differences of PGE_2 concentrations between treatment groups were significant ($P < 0.018$). Fisher's LSD multiple comparisons illustrated that both IFNT challenge and ncpBVDV infection alone significantly stimulated PGE_2 production ($P < 0.05$), whereas the stimulatory effect was neutralised when IFNT was combined with ncpBVDV ($P < 0.05$) (Fig. 1A).

Compared with CONT, IFNT challenge appeared to decrease $\text{PGF}_{2\alpha}$ production; however, statistical significance was not achieved ($P > 0.05$). In the cells infected with ncpBVDV, concentrations of $\text{PGF}_{2\alpha}$ were significantly lower than in the CONT cells ($P < 0.05$). When the ncpBVDV-infected cells were challenged with IFNT, $\text{PGF}_{2\alpha}$ concentrations were significantly lower than in the CONT cells ($P < 0.05$), and the concentrations were slightly lower than in the cells infected with ncpBVDV alone, although the difference was not statistically significant ($P > 0.05$) (Fig. 1B).

Compared with the CONT cells, in the ncpBVDV-infected cells, the ratios of PGE_2 to $\text{PGF}_{2\alpha}$ significantly increased ($P < 0.05$). Both IFNT and IFNT+ncpBVDV treatments appeared to increase the ratio of PGE_2 : $\text{PGF}_{2\alpha}$ produced; however, the differences were not statistically significant due to large variations between animals ($P > 0.05$) (Fig. 1C).

Effect of ncpBVDV, IFNT and their combination on the expression of selected reference genes in uterine endometrial cells

We selected *18SrRNA*, *ACTB*, *RPL19* and *GAPDH* as potential reference genes for qPCR. The results showed, however, that the expression of *18SrRNA*, *ACTB* and *RPL19* was significantly altered by IFNT, ncpBVDV or their combination ($P < 0.05$ – 0.01). Only *GAPDH* expression was not affected by any of the above treatments ($P > 0.05$) (Fig. 2A, B, C and D). Subsequent gene expression measurements have therefore been presented as absolute values given as femto gram per micro gram reverse-transcribed total RNA.

Effect of ncpBVDV, IFNT and their combination on expression of *PGR*, *ESR1* and *OXTR* in uterine endometrial cells

In the bovine endometrial cells, expression of *OXTR* mRNA was relatively higher ($1879 \pm 419 \text{ fg}/\mu\text{g RNA}$), whereas *ESR1* mRNA expression was very low ($0.003 \pm 0.0008 \text{ fg}/\mu\text{g RNA}$). The effects of all tested treatments on the expression of *PGR*, *ESR1* and *OXTR* mRNA were moderate and no statistical significance

Figure 1 Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon- τ (IFNT) and their combination on prostaglandin (PG) E_2 production (A), $\text{PGF}_{2\alpha}$ production (B) and PGE_2 : $\text{PGF}_{2\alpha}$ ratio (C) by uterine endometrial cells isolated from BVDV-free cyclic cows. When cells were grown to approximately 70% confluence after 4 days of culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following inoculation, the cells were challenged with IFNT at 0 or 100 ng/mL for 24 h. The spent medium was harvested for PG analysis. There were three replicates per treatment for each cow and ten cows per treatment. CONT: control without ncpBVDV and IFNT. The columns labelled with different letters were significantly different at $P < 0.05$ – 0.01 ($a > b > c$).

was achieved as tested using ANOVA with randomised block design (Fig. 3A, B and C).

Effect of ncpBVDV, IFNT and their combination on the expression of *PTGS1*, *PTGS2*, *mPGES1* and *AKR1B1* in uterine endometrial cells

In the cells infected with ncpBVDV, expression of *PTGS1* mRNA was significantly higher than in the CONT

cells ($P<0.05$). IFNT challenge significantly induced *PTGS1* mRNA expression ($P<0.01$); however, in the presence of ncpBVDV infection, this stimulatory effect was completely neutralised ($P<0.05$) (Fig. 4A). In the cells infected with ncpBVDV, *PTGS2* mRNA expression appeared to be lower than other groups; however, statistical significance was not achieved ($P>0.05$) (Fig. 4B). Figure 4C demonstrated that ncpBVDV infection stimulated *mPGES1* mRNA expression by up to three-fold ($P=0.0003$) compared with the CONT cells, whereas neither IFNT nor IFNT+ncpBVDV challenge affected its expression ($P>0.05$). As shown in Fig. 4D, neither IFNT nor ncpBVDV alone altered *AKR1B1* mRNA expression significantly ($P>0.05$), whereas

their combination led to a significant decrease in its expression ($P<0.05$).

Effect of ncpBVDV, IFNT and their combination on the expression of *PTGER2*, *PTGER3* and *PTGFR* in uterine endometrial cells

As shown in Fig. 5A and C, the expression of *PTGER2* and *PTGFR* mRNA was not significantly changed by treatment with IFNT, ncpBVDV or their combination ($P>0.05$). Compared with the CONT, treatment with ncpBVDV alone only moderately reduced *PTGER3*

Figure 2 Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon- τ (IFNT) and their combination on the reference gene expression of (A) *18S rRNA*, (B) *ACTB*, (C) *RPL19* and (D) *GAPDH* by uterine endometrial cells isolated from BVDV-free cyclic cows. When cells were grown to approximately 70% confluence after 4 days of culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following inoculation, the cells were challenged with IFNT at 0 or 100 ng/mL for 24 h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were three replicates per treatment for each cow and ten cows per treatment. CONT: control without ncpBVDV and IFNT. The columns labelled with different letters were significantly different at $P<0.05-0.01$ ($a>b$).

Figure 3 Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon- τ (IFNT) and their combination on (A) *PGR*, (B) *ESR1* and (C) *OXTR* expression by uterine endometrial cells isolated from BVDV-free cyclic cows. When cells were grown to approximately 70% confluence after 4 days of culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following inoculation, the cells were challenged with IFNT at 0 or 100 ng/mL for 24 h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were three replicates per treatment for each cow and ten cows per treatment. CONT: control without ncpBVDV and IFNT.

mRNA expression ($P > 0.05$). In the cells stimulated with IFNT, *PTGER3* mRNA expression was up-regulated by up to 2.5-fold ($P < 0.01$), whereas in the presence of ncpBVDV infection, the stimulatory effect of IFNT on *PTGER3* mRNA expression was intensively inhibited ($P < 0.01$) and the expression was even lower than that in the CONT cells ($P < 0.01$) (Fig. 5B).

Discussion

PR in cows is initiated by IFNT release from the conceptus acting on the uterine endometrium leading

Figure 4 Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon- τ (IFNT) and their combination on mRNA expression of (A) *PTGS1*, (B) *PTGS2*, (C) *mPGES1* and (D) *AKR1B1* by uterine endometrial cells isolated from BVDV-free cyclic cows. When cells were grown to approximately 70% confluence after 4 days of culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following inoculation, the cells were challenged with IFNT at 0 or 100 ng/mL for 24 h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were three replicates per treatment for each cow and ten cows per treatment. CONT: control without ncpBVDV and IFNT. The columns labelled with different letters were significantly different at $P < 0.05$ – 0.01 ($a > b > c$).

to maintenance of the corpus luteum. Regulation of the synthesis and release of PGE_2 and $\text{PGF}_{2\alpha}$ are crucial to this process (Bazer 2013, Lonergan & Forde 2014). Failure of PR is a significant risk factor for embryonic mortality (Diskin *et al.* 2011, Forde *et al.* 2011). BVDV infection can cause early embryonic death (Grooms 2004); however, little evidence is currently available on the mechanisms involved. In this study, we investigated the effect of BVDV infection on PG production and signalling pathways in bovine endometrium. We have demonstrated that (1) ncpBVDV infection induced an endocrine switch of PG production and signalling from $\text{PGF}_{2\alpha}$ to PGE_2 and (2) IFNT stimulates uterine PGE_2 production and its signalling pathway; however, this stimulatory effect is abolished by ncpBVDV infection.

In addition to PR, IFNT possesses antiviral, anti-proliferative and immunosuppressive activities (Pontzer *et al.* 1988, 1991, Kohara *et al.* 2012). It was previously reported that the replication of ncpBVDV was completely suppressed by bovine IFNT treatment in cultured bovine muscular cells (Kohara *et al.* 2012). In our study, after IFNT challenge for 24 h, ncpBVDV RNA was well detected in all infected endometrial cells using a PCR method, whereas it was negative in the ncpBVDV-free cells. Using a qPCR method, we confirmed that there was no significant difference in the ncpBVDV mRNA expression between the ncpBVDV and IFNT+ncpBVDV groups. Kohara *et al.* (2012) carried out infection of their cells after IFNT treatment, whereas in our study, infection was carried out before challenge. The different cell types used with their different structures and cellular signalling systems may also be important in this response. Our data support previous findings that the uterine endometrium is one of the major sites for infection, maintenance and spreading of BVDV in the cattle population (Grooms 2004, Lanyon *et al.* 2014).

In the non-pregnant uterine endometrium, $\text{PGF}_{2\alpha}$ is predominantly produced by uterine epithelial cells and PGE_2 by stromal cells (Danet-Desnoyers *et al.* 1994, Charpigny *et al.* 1999). In this study, the endometrial cells produced significant amounts of both PGE_2 (4.8 ± 0.5 ng/mL) and $\text{PGF}_{2\alpha}$ (13.3 ± 2.2 ng/mL). Together with the immunocytochemical staining results, this confirmed the presence of both epithelial and stromal cell populations. The paracrine interactions between these two cell types are important for the investigation of overall response of the endometrium to IFNT challenge and ncpBVDV infection.

In ruminants, although IFNT inhibits the pulsatile release of $\text{PGF}_{2\alpha}$ by the uterine epithelium, the basal release of PGE_2 and $\text{PGF}_{2\alpha}$ from both the conceptus and the endometrium increases during early pregnancy (Zarco *et al.* 1988, Dorniak *et al.* 2013). PGs secreted by both epithelial and stromal cells co-ordinate with the IFNT to regulate uterine endometrial functions. This is important to develop a receptive environment for conceptus development, thereby promoting elongation

Figure 5 Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon- τ (IFNT) and their combination on mRNA expression of (A) *PTGER2*, (B) *PTGER3* and (C) *PTGFR* by uterine endometrial cells isolated from BVDV-free cyclic cows. When cells were grown to approximately 70% confluence after 4 days of culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following inoculation, the cells were challenged with IFNT at 0 or 100 ng/mL for 24 h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were three replicates per treatment for each cow and ten cows per treatment. CONT: control without ncpBVDV and IFNT. The columns labelled with different letters significantly different at $P < 0.05$ – 0.01 (a > b > c).

and implantation (Bazer 2013, Dorniak *et al.* 2013). In support of this, intra-uterine infusion of meloxicam, a PTGS inhibitor, prevented uterine PG production and led to failure of conceptus elongation in ewes (Diskin *et al.* 2011). In cows, successful development to term and delivery of a live calf could be predicted by the expression of *PTGS2* in day 7 blastocysts (Bazer 2013). In this study, when the endometrial cells isolated from cyclic cows were given IFNT for 24 h, their PGE₂

production was significantly up-regulated, whereas the PGF_{2 α} production was not altered. This agreed with the previous finding that IFNT does not affect the expression of *PTGS2*, the rate-limiting enzyme in PG synthesis, but does stimulate PGE₂ production by cells of the bovine uterus (Dorniak *et al.* 2013). We also found that IFNT challenge increased *PTGS1* and *PTGER3* gene expression by up to 2.5-fold. Such an increase in both PGE₂ and its receptor indicates that IFNT favours the PGE₂ signalling pathway that would benefit maintenance of the corpus luteum as PGE₂ is a luteotrophin (Pratt *et al.* 1977, Dorniak *et al.* 2013). In the presence of ncpBVDV infection, however, the stimulatory effects of IFNT on PGE₂ production and its receptor *PTGER3* expression were neutralised, and the PGE₂ concentrations were even slightly lower than in the CONT cells. When the ncpBVDV-infected cells were treated with IFNT, basal PGF_{2 α} production and *AKR1B1* mRNA expression were also lower than the CONT. *AKR1B1* is a predominant isoform for PGF_{2 α} production (Fortier *et al.* 2008). Therefore, decreased or disrupted basal PG production may contribute to failure of pregnancy establishment and maintenance following ncpBVDV infection.

These results also suggest that IFNT treatment may act on uterine epithelial and stromal compartments differentially as PGE₂ is produced by stromal cells and PGF_{2 α} by epithelial cells. This supports previous studies in ewes that have shown cell-specific effects of IFNT for many ISGs crucial for developing a receptive environment for conceptus implantation. During early pregnancy or following intrauterine IFN treatment, the expression of the majority of progesterone-independent ISGs such as *RSAD2* (radical S-adenosyl methionine domain containing 2) and *IFIH1* (interferon-induced with helicase C domain 1) was increased in endometrial stroma, glands and immune cells but not in the luminal epithelium. In contrast, some non-classical ISGs such as *LGALS15* (galectin 15), *CTSL* (cathepsin L) and *CST3* (cystatin C), which require progesterone priming, were predominantly expressed in the endometrial luminal epithelium and superficial glandular epithelium (Song *et al.* 2007, Bazer *et al.* 2008, Spencer *et al.* 2013).

It is interesting to note that ncpBVDV infection alone increased uterine PGE₂ production and decreased PGF_{2 α} production, whereas expression of *PTGS2*, *AKR1B1* and *PTGFR* all tended to be lower than in the CONT cells. Lower *PTGS2* and *AKR1B1* expression may in part contribute to the decreased PGF_{2 α} production, whereas lower *PTGFR* expression would further impair the PGF_{2 α} signalling pathway. In contrast, the expression of *mPGES1*, a principle isoform for PGE₂ production (Fortier *et al.* 2008), was up-regulated by about four-fold, leading to an increased uterine PGE₂ generation and an increased ratio of PGE₂ to PGF_{2 α} . These results show that ncpBVDV infection induced an endocrine switch from PGF_{2 α} to PGE₂ production. As PGF_{2 α} is an immune enhancer and PGE₂ is an immune suppressor

(Lewis 2003, Herath *et al.* 2009), this switch provides a mechanism whereby BVDV infection can predispose affected animals to uterine infection. Similarly, bacterial LPS treatment increased PGE₂ production and decreased PGF_{2α} production in bovine endometrium (Herath *et al.* 2009). Our previous studies demonstrated that numerous innate immune responses mounted following an LPS challenge was also significantly suppressed in ncpBVDV-infected bovine uterine endometrial cells (Oguejiofor *et al.* 2015a). Together, these results support the suggestion that cows infected with BVDV may be predisposed to develop endometritis due to bacterial infection following calving. This may in turn also contribute to early embryonic death and failure of pregnancy establishment (Gilbert 2011).

In ruminants, OXTR are up-regulated in the uterine epithelium following a period of exposure to high progesterone levels (Wathes & Lamming 1995, Wathes *et al.* 1996). Once these are present, OXT binding to its receptor drives pulsatile release of PGF_{2α}, so starting the process of luteolysis (Poyser 1995, McCracken *et al.* 1999). The initial increase in OXTR is independent of oestradiol regulation (Robinson *et al.* 1999, Leung & Wathes 2000, Mann *et al.* 2013). However, there is a subsequent increase in *ESR1* through which oestradiol stimulates the expression of further OXTR in the superficial glandular epithelium and stroma (Wathes & Hamon 1993, Robinson *et al.* 1999). IFNT secreted from the conceptus acts on the uterine epithelium to inhibit the expression of both OXTR and *ESR1*, thereby inhibiting the release of luteolytic pulses of PGF_{2α} and leading to establishment of pregnancy (Mann *et al.* 1999, Bazer 2013). In this study, both OXTR and *ESR1* mRNA expression was numerically lower following IFN treatment; however, the differences did not achieve statistical significance because OXTR mRNA was already highly up-regulated before the IFN treatment was commenced.

During early pregnancy, IFNT also acts on maternal endometrium to establish a nutrient transport system to support further growth and development of the conceptus (Bazer 2013). In this study, we tested four reference genes to validate the qPCR assay. These were highly conserved housekeeping genes encoding proteins involved in key cellular processes. It was interesting to note that expression of *18S rRNA* and *ACTB* mRNA was significantly inhibited by ncpBVDV infection in the presence of IFNT challenge, whereas expression of the ribosomal protein *RPL19* mRNA was increased by both the individual treatments. Only *GAPDH* remained constant across all the four treatments. This suggests another mechanism by which ncpBVDV infection may affect the survival of the conceptus to disrupt PR. Based on these results, we used an absolute quantification qPCR approach. Great care should be taken while measuring gene expression in reproductive systems using relative quantification qPCR as its accuracy depends on the stable expression of the selected reference genes.

In summary, IFNT challenge stimulated uterine *PTGS1* and *PTGER3* mRNA expression and PGE₂ production; however, these stimulatory effects were neutralised in the presence of ncpBVDV infection. The interruption of IFNT-induced PG production and signalling by BVDV infection may directly cause failure of PR. ncpBVDV infection stimulated *PTGS1* and *mPGES1* mRNA expression and moderately suppressed *AKR1B1* expression, leading to increased PGE₂ and decreased PGF_{2α} concentrations and an increase in PGE₂:PGF_{2α} ratios in bovine uterine endometrium. This endocrine switch of PG production from PGF_{2α} to PGE₂ may decrease uterine immunity to predispose the animals to uterine diseases, indirectly leading to failure of PR. Our results thus suggest a mechanism whereby ncpBVDV infection in cows may cause early embryonic death and reduced fertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Bazer FW 2013 Pregnancy recognition signaling mechanisms in ruminants and pigs. *Journal of Animal Science and Biotechnology* **4** 23. (doi:10.1186/2049-1891-4-23)
- Bazer FW, Burghardt RC, Johnson GA, Spencer TE & Wu G 2008 Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reproductive Biology* **8** 179–211.
- Charpigny G, Reinaud P, Creminon C & Tamby JP 1999 Correlation of increased concentration of ovine endometrial cyclooxygenase 2 with the increase in PGE₂ and PGD₂ in the late luteal phase. *Journal of Reproduction and Fertility* **117** 315–324. (doi:10.1530/jrf.0.1170315)
- Cheng Z, Robinson RS, Pushpakumara PG, Mansbridge RJ & Wathes DC 2001 Effect of dietary polyunsaturated fatty acids on uterine prostaglandin synthesis in the cow. *Journal of Endocrinology* **171** 463–473. (doi:10.1677/joe.0.1710463)
- Cheng Z, Abayasekara DR, Ward F, Preece DM, Raheem KA & Wathes DC 2013 Altering n-3 to n-6 polyunsaturated fatty acid ratios affects prostaglandin production by ovine uterine endometrium. *Animal Reproduction Science* **143** 38–47.
- Danet-Desnoyers G, Wetzels C & Thatcher WW 1994 Natural and recombinant bovine interferon tau regulate basal and oxytocin-induced secretion of prostaglandins F₂ alpha and E₂ by epithelial cells and stromal cells in the endometrium. *Reproduction, Fertility, and Development* **6** 193–202. (doi:10.1071/RD9940193)

- De Vries A 2006 Economic value of pregnancy in dairy cattle. *Journal of Dairy Science* **89** 3876–3885. (doi:10.3168/jds.S0022-0302(06)72430-4)
- Diskin MG, Parr MH & Morris DG 2011 Embryo death in cattle: an update. *Reproduction, Fertility, and Development* **24** 244–251. (doi:10.1071/RD11914)
- Dorniak P, Bazer FW, Wu G & Spencer TE 2012 Conceptus-derived prostaglandins regulate endometrial function in sheep. *Biology of Reproduction* **87** 1–7. (doi:10.1095/biolreprod.112.101691)
- Dorniak P, Bazer FW & Spencer TE 2013 Physiology and endocrinology symposium: biological role of interferon tau in endometrial function and conceptus elongation. *Journal of Animal Science* **91** 1627–1638.
- Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, Okumu LA, McGettigan PA, Mehta JP, McBride R *et al.* 2011 Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? *Biology of Reproduction* **85** 144–156. (doi:10.1095/biolreprod.110.090019)
- Fortier MA, Krishnaswamy K, Danyod G, Boucher-Kovalik S & Chapdalaine P 2008 A postgenomic integrated view of prostaglandins in reproduction: implications for other body systems. *Journal of Physiology and Pharmacology: an Official Journal of the Polish Physiological Society* **59** (Supplement 1) 65–89.
- Gilbert RO 2011 The effects of endometritis on the establishment of pregnancy in cattle. *Reproduction, Fertility, and Development* **24** 252–257. (doi:10.1071/RD11915)
- Grooms DL 2004 Reproductive consequences of infection with bovine viral diarrhoea virus. *Veterinary Clinics of North America. Food animal Practice* **20** 5–19. (doi:10.1016/j.cvfa.2003.11.006)
- Herath S, Lilly ST, Fischer DP, Williams EJ, Dobson H, Bryant CE & Sheldon IM 2009 Bacterial lipopolysaccharide induces an endocrine switch from prostaglandin F₂α to prostaglandin E₂ in bovine endometrium. *Endocrinology* **150** 1912–1920. (doi:10.1210/en.2008-1379)
- Jabbour HN, Sales KJ, Catalano RD & Norman JE 2009 Inflammatory pathways in female reproductive health and disease. *Reproduction* **138** 903–919. (doi:10.1530/REP-09-0247)
- Kimura K, Spate LD, Green MP, Murphy CN, Seidel GE Jr & Roberts RM 2004 Sexual dimorphism in interferon-tau production by in vivo-derived bovine embryos. *Molecular Reproduction and Development* **67** 193–199.
- Kohara J, Nishikura Y, Konnai S, Tajima M & Onuma M 2012 Effects of interferon-tau on cattle persistently infected with bovine viral diarrhoea virus. *The Japanese Journal of Veterinary Research* **60** 63–70.
- Lanyon SR, Hill FI, Reichel MP & Brownlie J 2014 Bovine viral diarrhoea: pathogenesis and diagnosis. *Veterinary Journal* **199** 201–209. (doi:10.1016/j.tvjl.2013.07.024)
- Leung ST & Wathes DC 2000 Oestradiol regulation of oxytocin receptor expression in cyclic bovine endometrium. *Journal of Reproduction and Fertility* **119** 287–292.
- Lewis G 2003 Role of ovarian progesterone and potential role of prostaglandin F₂α and prostaglandin E₂ in modulating the uterine response to infectious bacteria in postpartum ewes. *Journal of Animal Science* **81** 285–293.
- Loneragan P & Forde N 2014 Maternal-embryo interaction leading up to the initiation of implantation of pregnancy in cattle. *Animal* **8** (Supplement 1) 64–69. (doi:10.1017/S1751731114000470)
- Mann GE, Lamming GE, Robinson RS & Wathes DC 1999 The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. *Journal of Reproduction and Fertility. Supplement* **54** 317–328.
- Mann GE, Wathes DC & Robinson RS 2013 The progesterone receptor antagonist, onapristone has differential effects on the timing and control of the luteolytic mechanism depending on timing of administration in sheep. *Molecular and Cellular Endocrinology* **376** 1–11. (doi:10.1016/j.mce.2013.06.001)
- McCracken JA, Custer EE & Lamsa JC 1999 Luteolysis: a neuroendocrine-mediated event. *Physiological Reviews* **79** 263–323.
- Oguejiofor CF, Cheng Z, Abudureyimu A, Anstaett OL, Brownlie J, Fouladi-Nashta AA & Wathes DC 2015a Global transcriptomic profiling of bovine endometrial immune response in vitro. II. Effect of bovine viral diarrhoea virus on the endometrial response to lipopolysaccharide. *Biology of Reproduction* **93** 101.
- Oguejiofor CF, Cheng Z, Abudureyimu A, Fouladi-Nashta AA & Wathes DC 2015b Global transcriptomic profiling of bovine endometrial immune response in vitro. I. Effect of lipopolysaccharide on innate immunity. *Biology of Reproduction* **93** 100.
- Pinheiro de Oliveira TF, Fonseca AA Jr, Camargos MF, de Oliveira AM, Pinto Cottorello AC, Souza Ados R, de Almeida IG & Heinemann MB 2013 Detection of contaminants in cell cultures, sera and trypsin. *Biologicals* **41** 407–414. (doi:10.1016/j.biologicals.2013.08.005)
- Pontzer CH, Torres BA, Vallet JL, Bazer FW & Johnson HM 1988 Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. *Biochemical and Biophysical Research Communications* **152** 801–807. (doi:10.1016/S0006-291X(88)80109-8)
- Pontzer CH, Bazer FW & Johnson HM 1991 Antiproliferative activity of a pregnancy recognition hormone, ovine trophoblast protein-1. *Cancer Research* **51** 5304–5307.
- Poyser NL 1995 The control of prostaglandin production by the endometrium in relation to luteolysis and menstruation. *Prostaglandins, Leukotrienes, and Essential Fatty Acids* **53** 147–195. (doi:10.1016/0952-3278(95)90115-9)
- Pratt BR, Butcher RL & Inskeep EK 1977 Antiluteolytic effect of the conceptus and of PGE₂ in ewes. *Journal of Animal Science* **45** 784–791.
- Robinson RS, Mann GE, Lamming GE & Wathes DC 1999 The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. *Journal of Endocrinology* **160** 21–33.
- Samuelsson B, Morgenstern R & Jakobsson PJ 2007 Membrane prostaglandin synthase-1: a novel therapeutic target. *Pharmacological Reviews* **59** 207–224. (doi:10.1124/pr.59.3.1)
- Song G, Bazer FW & Spencer TE 2007 Pregnancy and interferon tau regulate RSAD2 and IFIH1 expression in the ovine uterus. *Reproduction* **133** 285–295. (doi:10.1530/REP-06-0092)
- Spencer TE, Forde N, Dorniak P, Hansen TR, Romero JJ & Loneragan P 2013 Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. *Reproduction* **146** 377–387. (doi:10.1530/REP-13-0165)
- Tithof PK, Roberts MP, Guan W, Elgayyar M & Godkin JD 2007 Distinct phospholipase A₂ enzymes regulate prostaglandin E₂ and F₂α production by bovine endometrial epithelial cells. *Reproductive Biology and Endocrinology* **5** 16.
- Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP & Paton DJ 1994 Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Archives of Virology* **136** 309–323.
- Wathes DC & Hamon M 1993 Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. *Journal of Endocrinology* **138** 479–492. (doi:10.1677/joe.0.1380479)
- Wathes DC & Lamming GE 1995 The oxytocin receptor, luteolysis and the maintenance of pregnancy. *Journal of Reproduction and Fertility. Supplement* **49** 53–67.
- Wathes DC, Mann GE, Payne JH, Riley PR, Stevenson KR & Lamming GE 1996 Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes. *Journal of Endocrinology* **151** 375–393.
- Wathes DC, Abayasekara DR & Aitken RJ 2007 Polyunsaturated fatty acids in male and female reproduction. *Biology of Reproduction* **77** 190–201.
- Weems CW, Weems YS & Randel RD 2006 Prostaglandins and reproduction in female farm animals. *Veterinary Journal* **171** 206–228. (doi:10.1016/j.tvjl.2004.11.014)
- Welsh MD, Adair BM & Foster JC 1995 Effect of BVD virus infection on alveolar macrophage functions. *Veterinary Immunology and Immunopathology* **46** 195–210. (doi:10.1016/0165-2427(94)05366-Z)
- Zarco L, Stabenfeldt GH, Basu S, Bradford GE & Kindahl H 1988 Modification of prostaglandin F-2 alpha synthesis and release in the ewe during the initial establishment of pregnancy. *Journal of Reproduction and Fertility* **83** 527–536. (doi:10.1530/jrf.0.0830527)

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