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# BVDV alters uterine prostaglandin production during pregnancy recognition in cows

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*Short title: BVDV interrupts pregnancy recognition in cows*

**Abstract**

Embryonic mortality in cows is at least in part caused by failed 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, including implantation. The aims of the present study were 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 10 BVDV free cows were cultured and treated with 0 or 100 ng/ml interferon-tau (IFNT) in the absence or presence of non-cytopathic BVDV (ncpBVDV). $PGF_{2\alpha}$ and $PGE_2$ 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 IFNT challenge significantly stimulated *PTGS1* and *PTGER3* mRNA expression and $PGE_2$ production, but 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 $PGE_2$ and decreased $PGF_{2\alpha}$ concentrations and an increased $PGE_2$:$PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ to $PGE_2$ to decrease uterine immunity, so predisposing the animals to uterine disease.

## 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 tau (IFNT) by the trophectoderm 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 which 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, including 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). $PGE_2$ (a vasodilator) is luteotropic for maintaining progesterone secretion by the corpus luteum whereas $PGF_{2\alpha}$ (a vasoconstrictor) is luteolytic (Weems *et al.* 2006). In PG production pathways, 2 series PGs, such as $PGE_2$ and $PGF_{2\alpha}$, 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 $PGE_2$ and $PGF_{2\alpha}$ production, respectively, in bovine endometrium (Fortier *et al.* 2008).

During PR IFNT inhibits the up-regulation of oxytocin (OT) receptors (OXTR) in the uterine epithelium, so preventing the pulsatile release of $PGF_{2\alpha}$, which is necessary for luteolysis (Wathes & Lamming 1995, Mann *et al.* 1999). In contrast, basal release of PGs, including $PGE_2$ and $PGF_{2\alpha}$, from both the conceptus and 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 expression of endometrial Type I IFN-stimulated genes (ISGs), a vital component of pregnancy recognition (Spencer *et al.* 2013).

Bovine viral diarrhoea virus (BVDV), a single-stranded RNA virus, causes widespread infection of the cattle population 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 expression of many *ISGs* which 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 $PGE_2$ (Welsh *et al.* 1995). When bovine endometrial cells were also exposed to bacterial lipopolysaccharide (LPS), ncpBVDV increased the expression of *PLA2G4F* and *PTGES* (*mPGES-1*) (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) while PTGES subsequently catalyzes the isomerization of $PGH_2$ to $PGE_2$ (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 $PGE_2$ production following bacterial infection, potentially compromising the innate immune response. To explore these possibilities further, the present 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 (Poole, Dorset, UK) 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 $mm^3$ cubes. They were digested for 90 min at 37°C in medium containing 100 mg bovine serum albumin (BSA, Sigma), 50 mg trypsin III (Worthington, Lakewood, NJ 08701, USA) and 50 mg collagenase A (Roche, Welwyn Garden City, UK) 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 and 10°C for 10 min. After two repetitions of the above washing procedures, the cells were suspended with the culture medium (DMEM/F12 medium with 10% FBS) and plated in 24 well IWAKI micro plates (Scitech DIV, Asahi Techno Glass, Japan) at 2 ml per well containing $0.5 \times 10^5$ 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: 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 RNA in the uterine endometrial tissue and FBS was extracted using a Qiagen RNA Lipids Mini kit (Qiagen, Manchester, UK) following the supplied protocol. RNA was reverse transcribed into cDNA using a cDNA synthesis kit supplied by PCRBiosystems (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

148 blunt vector, linearized (Novagen, Cambridge, MA02139, USA), a reverse transcription-
149 negative controls and a house keeping gene beta-actin (ACTB, see Table 1 for its primers)
150 to verify that PCR reagents and reaction volumes were free of genomic DNA contamination.
151 The above testing was carried out on the day of collection of uterus in parallel with the cell
152 isolation and culture. The endometrium initially tested negative for BVDV was used for
153 further experiments.

154

155 ***Propagation of ncpBVDV***

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

163

164 ***Experimental protocols***

165

166 The experiments of ncpBVDV infection and IFNT challenge were carried out in endometrial
167 cells from 10 cows confirmed BVDV negative tested using the above method. Cell from each
168 cow were taken as a batch and grown in two 24-well plates as described previously (day 1).
169 On day 4 of the cell culture when the cells grew to approximately 70% of confluence, FBS in
170 the culture medium was reduced to 5% (maintenance medium, MM) to prevent over growth
171 of the cells. The cells from each cow were divided into 4 treatment groups: Control (CONT),
172 IFNT, ncpBVDV and IFNT+ncpBVDV. Wells treated with the virus were maintained in

separate plates from those without the virus to prevent cross-contamination. For the cells designated as BVDV-infected, 0.25 ml of 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 two 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, 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)***

Concentrations of $PGE_2$ and $PGF_{2\alpha}$ 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 $PGE_2$ antiserum were 23%, 100% and 15% with $PGE_1$, $PGE_2$ and $PGE_3$, respectively and those for $PGF_{2\alpha}$ antiserum were 34%, 100% and 25% with $PGF_{1\alpha}$, $PGF_{2\alpha}$ and $PGF_{3\alpha}$, respectively. The standards for $PGE_2$ and $PGF_{2\alpha}$ were purchased from Sigma and the tritiated tracers of $PGE_2$ ([5, 6, 8, 11, 12, 14, 15 (n)-$^3$H]-$PGE_2$) and $PGF_{2\alpha}$ ([5, 6, 8, 9, 11, 12, 14, 15 (n)-$^3$H]-$PGF_{2\alpha}$) were supplied by PerkinElmer (Cambridge, UK). The samples were diluted (×10-200) in the RIA buffer. This allowed the sample PG concentrations to fall within the analytical ranges of the RIAs (0.02 – 5.0ng/ml for $PGE_2$ and 0.01-2.5 ng/ml for $PGF_{2\alpha}$) 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 $PGE_2$ and $PGF_{2\alpha}$, respectively. The intra-assay and inter-assay coefficients of variation were 3.5% and 6.3% for $PGE_2$ (n = 6), and 4.1% and 9.6% for $PGF_{2\alpha}$, respectively (n =6).

***RNA extraction***

After collection of spent medium, the cells from each treatment group (6 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 with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilminton, 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 shown in Table 1. The primers were made by Eurofins MWG Operon (Ebersberg, Germany). One µg of RNA was reverse transcribed into cDNA using a cDNA synthesis kit supplied by PCRBiosystems following the supplier's protocol. The resulting cDNA (20µl) 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***

Concentrations of all ten target genes (*PTGS1, PTGS2, mPGES1, AKR1B1, PTGER2, PTGER3, PTGFR, OXTR, PGR* and *ESR1*) and four reference genes (*GAPDH, RPL19, ACTB* and 18SrRNA) 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 $1\times 10^{-7}$ 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, Hercules, CA, USA) with 8 identical reactions containing 2 ng of DNA standard, 10 µl Sygreen Mix (PCRBiosystems), 0.8 µl of each 10µM forward and reverse primer and nucleate free water added up to 20 µl. 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 sample cDNA in duplicate with the same final volume on CFX96 Real -Time Systems (Bio-Rad). Each well contained 5 µl cDNA standard or samples, 10 µl Sygreen Mix (PCRBiosystems), 0.8 µl of 10µM forward primer, 0.8 µl reverse primer and 3.4 µl 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 prior to the product acquisition. The results were analysed using the CFX Manager Software package (Bio-Rad). The limit of quantification was $1 \times 10^{-6}$ - $1 \times 10^{-7}$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 analysis***

All values are summarized as mean ± standard error (S.E.). The values of gene expression generated by qPCR were normalised as fg/µg 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 (Chicago, IL, USA), 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 combined treatments affected cell viability at the doses used as confirmed using an MTS reduction assay method.

### *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 $PGF_{2\alpha}$ production, but statistical significance was not achieved ($p>0.05$). In the cells infected with ncpBVDV, concentrations of $PGF_{2\alpha}$ were significantly lower than in the CONT cells ($P<0.05$). When the ncpBVDV infected cells were challenged with IFNT, $PGF_{2\alpha}$ 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, in the cells infected with ncpBVDV, the ratios of $PGE_2$ to $PGF_{2\alpha}$ were significantly increased ($P<0.05$). Both IFNT and IFNT+ncpBVDV treatments appeared to increase the ratio of $PGE_2$:$PGF_{2\alpha}$ produced, but 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 expression of the selected reference genes in uterine endometrial cells***

We selected *18SrRNA, ACTB, RPL19* and *GAPDH* as potential reference genes for the qPCR. The results showed, however, that 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-d). Subsequent gene expression measurements have therefore been presented as absolute values given as fg/µg 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 (1,879 ± 419 fg/µg RNA) while that of *ESR1* mRNA was very low (0.003 ± 0.0008 fg/µg RNA). The effects of all tested treatments on expression of *PGR, ESR1* and *OXTR* mRNA were moderate and no statistical significance was achieved as tested using ANOVA with randomized block design (Fig. 3a-c).

***Effect of ncpBVDV, IFNT and their combination on 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, but statistical significance was not achieved ($p>0.05$) (Fig. 4b). Fig 4c demonstrated that ncpBVDV infection stimulated *mPGES1* mRNA expression by up to 3 fold ($P=0.0003$) compared with the CONT cells whereas neither IFNT challenge nor IFNT+ncpBVDV affected its expression ($P>0.05$). As shown in Fig. 4d, neither IFNT nor ncpBVDV alone altered *AKR1B1* mRNA expression significantly ($P>0.05$) whist their combination led to a significant decrease in its expression ($P<0.05$).

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

As shown in Figs 5a and 5c, 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* 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 to maintenance of the corpus luteum. Regulation of the synthesis and release of $PGE_2$ and $PGF_{2\alpha}$ is 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) but little evidence is currently available on the mechanisms involved. In the present 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 $PGF_{2\alpha}$ to $PGE_2$ and 2) IFNT stimulates uterine $PGE_2$ production and its signalling pathway, but this stimulatory effect is abolished by ncpBVDV infection.

In addition to PR, IFNT possesses antiviral, antiproliferative and immunosuppressive activities (Pontzer *et al.* 1988, Pontzer *et al.* 1991, Kohara *et al.* 2012). It was reported previously that the replication of ncpBVDV was completely suppressed by bovine IFNT

375 treatment in cultured bovine muscular cells (Kohara *et al.* 2012). In our study, after IFNT
376 challenge for 24 h, ncpBVDV RNA was well detected in all infected endometrial cells using a
377 PCR method while it was negative in the ncpBVDV free cells. Using a qPCR method we
378 confirmed that there was no significant difference of the *ncpBVDV* mRNA expression
379 between the ncpBVDV and IFNT+ncpBVDV groups. Kohara *et al.* (2012) infected their cells
380 after IFNT treatment whereas in our study infection was carried out before challenge. The
381 different cell types used with their different structures and cellular signalling systems may
382 also be important in this response. Our data support previous findings that the uterine
383 endometrium is one of the major sites for infection, maintenance and spreading of BVDV in
384 the cattle population (Grooms 2004, Lanyon *et al.* 2014).

385

386 In the non-pregnant uterine endometrium, $PGF_{2\alpha}$ is predominantly produced by uterine
387 epithelial cells and $PGE_2$ by stromal cells (Danet-Desnoyers *et al.* 1994, Charpigny *et al.*
388 1999). In the present study, the endometrial cells produced significant amounts of both $PGE_2$
389 (4.8 ± 0.5 ng/ml) and $PGF_{2\alpha}$ (13.3 ± 2.2 ng/ml). Together with the immunocytochemical
390 staining results, this confirmed the presence of both epithelial and stromal cell populations.
391 The paracrine interactions between these two cell types are important for investigation of the
392 overall response of the endometrium to IFNT challenge and ncpBVDV infection.

393

394 In ruminants, although IFNT inhibits the pulsatile release of $PGF_{2\alpha}$ by the uterine epithelium,
395 the basal release of $PGE_2$ and $PGF_{2\alpha}$ from both the conceptus and endometrium increases
396 during early pregnancy (Zarco *et al.* 1988, Dorniak *et al.* 2013). PGs secreted by both
397 epithelial and stromal cells co-ordinate with the IFNT to regulate uterine endometrial
398 functions. This is important to develop a receptive environment for conceptus development,
399 so promoting elongation and implantation (Bazer 2013, Dorniak *et al.* 2013). In support of
400 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 the present study, when the endometrial cells isolated from cyclic cows were given IFNT for 24h, their $PGE_2$ production was significantly up-regulated while the $PGF_{2\alpha}$ production was not altered. This agreed with the previous finding that IFNT does not affect expression of PTGS2, the rate-limiting enzyme in PG synthesis, but does stimulate $PGE_2$ 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 $PGE_2$ and its receptor indicates that IFNT favours the $PGE_2$ signalling pathway which would benefit maintenance of the corpus luteum as $PGE_2$ is a luteotropin (Pratt *et al.* 1977, Dorniak *et al.* 2013). In the presence of ncpBVDV infection, however, the stimulatory effects of IFNT on $PGE_2$ production and its receptor *PTGER3* expression was neutralised and the $PGE_2$ concentrations were even slightly lower than in the CONT cells. When the ncpBVDV infected cells were treated with IFNT, basal $PGF_{2\alpha}$ production and *AKR1B1* mRNA expression were also lower than the CONT. AKR1B1 is a predominant isoform for $PGF_{2\alpha}$ 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 $PGE_2$ is produced by stromal cells and $PGF_{2\alpha}$ by epithelial cells. This supports previous studies in the ewe which 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* (cathespin 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 $PGE_2$ production and decreased $PGF_{2\alpha}$ production while 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 $PGF_{2\alpha}$ production, while lower *PTGFR* expression would further impair the $PGF_{2\alpha}$ signalling pathway. In contrast the expression of *mPGES1*, a principle isoform for $PGE_2$ production (Fortier *et al.* 2008), was up-regulated by about 4 fold, leading to an increased uterine $PGE_2$ generation and an increased ratio of $PGE_2$ to $PGF_{2\alpha.}$ These results show that ncpBVDV infection induced an endocrine switch from $PGF_{2\alpha}$ to $PGE_2$ production. As $PGF_{2\alpha}$ is an immune enhancer and $PGE_2$ 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 $PGE_2$ production and decreased $PGF_{2\alpha}$ production in bovine endometrium (Herath *et al.* 2009). Our previous studies demonstrated that numerous innate immune responses mounted following an LPS challenge were 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 $PGF_{2\alpha}$, so starting the process of luteolysis (Poyser 1995, McCracken *et al.* 1999). The intial 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 expression of both *OXTR* and *ESR1* so inhibiting release of luteolytic pulses of $PGF_{2\alpha}$ and leading to establishment of pregnancy (Mann *et al.* 1999, Bazer 2013). In the present study both *OXTR* and *ESR1* mRNA expression was numerically lower following IFN treatment but the differences did not achieve statistical significance. This may be because *OXTR* mRNA was already highly up-regulated before the IFN treatment 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 the present study, we tested four reference genes to validate the qPCR assay. These were highly conserved house-keeping genes encoding proteins involved in key cellular processes. It was interesting to note that expression of *18SrRNA* 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 four treatments. This suggests another mechanism by which ncpBVDV infection may affect survival of the conceptus to disrupt PR. Based on these results, we used an absolute quantification qPCR approach. Great care should be taken when 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 $PGE_2$ production, but 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 $PGE_2$ and decreased $PGF_{2\alpha}$ concentrations and an increase in $PGE_2$:$PGF_{2\alpha}$ ratios in bovine uterine endometrium. This endocrine switch of PG production from $PGF_{2\alpha}$ to $PGE_2$ 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.

## Conflict 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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**Figure legends**

**Fig.1.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (IFNT) and their combination on prostaglandin (PG) $E_2$ (a) and $PGF_{2\alpha}$ (b) production and $PGE_2$:$PGF_{2\alpha}$ ratio (c) by uterine endometrial cells isolated from BVDV free cyclic cows. When cells were grown to approximately 70% confluence after 4 days culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following the inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h. The spent medium was harvested for PG analysis. There were 3 replicates per treatment for each cow and 10 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).

**Fig.2.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (IFNT) and their combination on the reference gene expression of (a) *18SrRNA,* (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 culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following the inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were 3 replicates per treatment for each cow and 10 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).

**Fig.3.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (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 culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following the

inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were 3 replicates per treatment for each cow and 10 cows per treatment. CONT = control without ncpBVDV and IFNT.

**Fig.4.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (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 culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following the inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were 3 replicates per treatment for each cow and 10 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).

**Fig.5.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (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 culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days following the inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h. Total RNA was extracted from the treated cells and the gene expression was quantified using an absolute qPCR approach. There were 3 replicates per treatment for each cow and 10 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).

**Table 1**. Oligonucleotide primer sequence information

| Gene | Primer sequence (5'-3') | GenBank accession | Product length (bp) | Annealing temperature (°C) |
|---|---|---|---|---|
| *PTGS1* | Forward: CACAGTGCGTTCCAACCTTATC<br>Reverse: CAACTGCTTCTTCCCTTTGGTG | NM_001105323.1 | 163 | 63.3 |
| *PTGS2* | Forward: TACTGGAAGCCTAGCACTTTC G<br>Reverse: TGAATGAGGTAAAGGGACAGCC | NM_174445.2 | 112 | 61.4 |
| *mPGES1* | Forward: TGTGTTTCCCCGTGTGTC<br>Reverse: ACTGAGTCTCTGTTTGCTTTTC | NM_174443.2 | 167 | 59.5 |
| *AKR1B1* | Forward: TACCTGGACCTCTACCTCATCC<br>Reverse: CGTCCAGGTATCCACGAAATCT | NM_001012519.1 | 120 | 64.5 |
| *PTGER2* | Forward: CTACTTCTACCAGCGCCGAG<br>Reverse: TACGTGGTCTGCTTGTGTCC | NM_174588.2 | 165 | 64.5 |
| *PTGER3* | Forward: GTGGTCATCGTCCTCTACCTGT<br>Reverse: CTTCATGTGGCTTGAGTACCAG | NM_181032.1 | 186 | 61.4 |
| *PTGFR* | Forward: TGGTGTTCTCTGGTCTGTGC<br>Reverse: AAAGCACACCCCACTCAACA | XM_010803367.1 | 140 | 60 |
| *OXTR* | Forward: TCATCATCGCCATGCTCCTG<br>Reverse: CGGAATGAGCAGCAGAGGAA | NM_174134.2 | 118 | 63.3 |
| *PGR* | Forward: AGGAGTTGTCCCTAGCTCACAG<br>Reverse: GCAGCAATAACCTCAGACATCA | NM_001205356.1 | 162 | 59 |
| *ESR1* | Forward: TCAGGCTACCATTACGGAGTTT<br>Reverse: CCACTTCATAGCACTTGCGTAG | NM_001001443.1 | 169 | 59 |
| *GAPDH* | Forward: GGTCACCAGGGCTGCTTTTA<br>Reverse: TTCCCGTTCTCTGCCTTGAC | NM_001034034.2 | 147 | 61.4 |
| *RPL19* | Forward: TCGATGCCGGAAAAACAC<br>Reverse: ATTCTCATCCTCCTCATCCAG | NM 001040516 | 119 | 59 |
| *ACTB* | Forward: GAAATCGTCCGTGACATCAA<br>Reverse: AGGAAGGAAGGCTGGAAGAG | NM_173979.3 | 182 | 61.4 |
| *18SrRNA* | Forward: CGGCGACGACCCATTCGAAC<br>Reverse: GAATCGAACCCTGATTCCCCGTC | AY779625 | 99 | 64.5 |

Fig 1

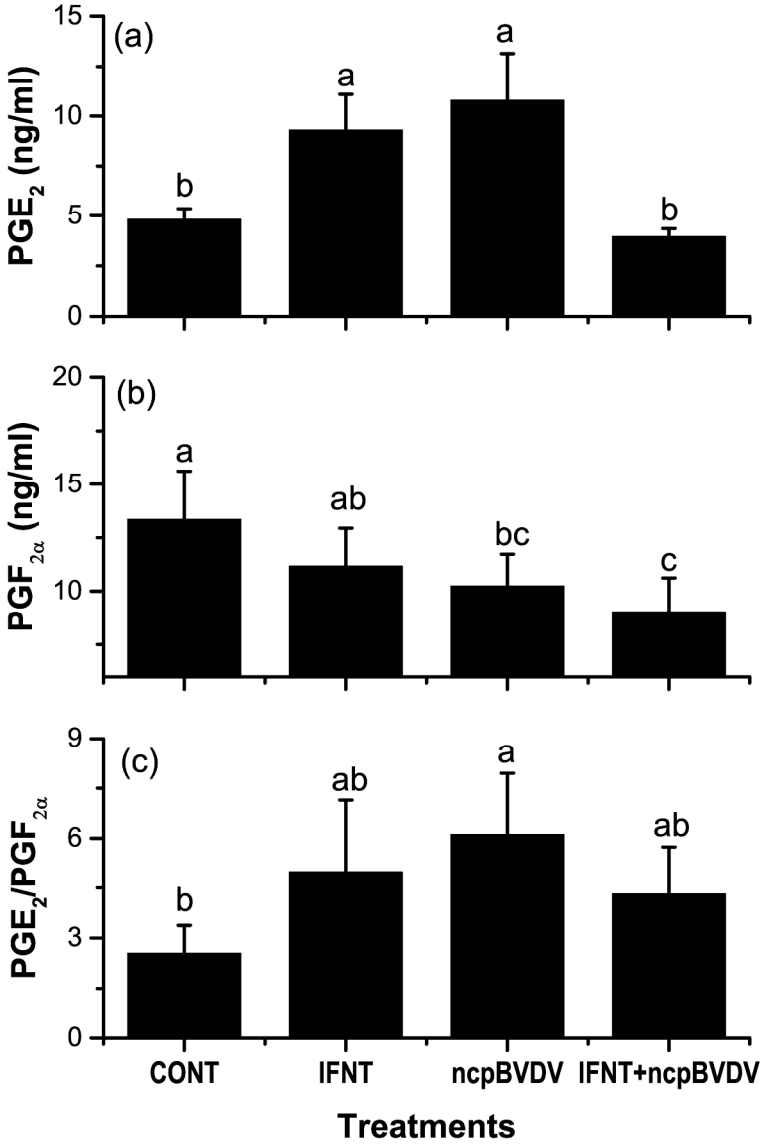


Fig1
280x439mm (300 x 300 DPI)

Fig 2

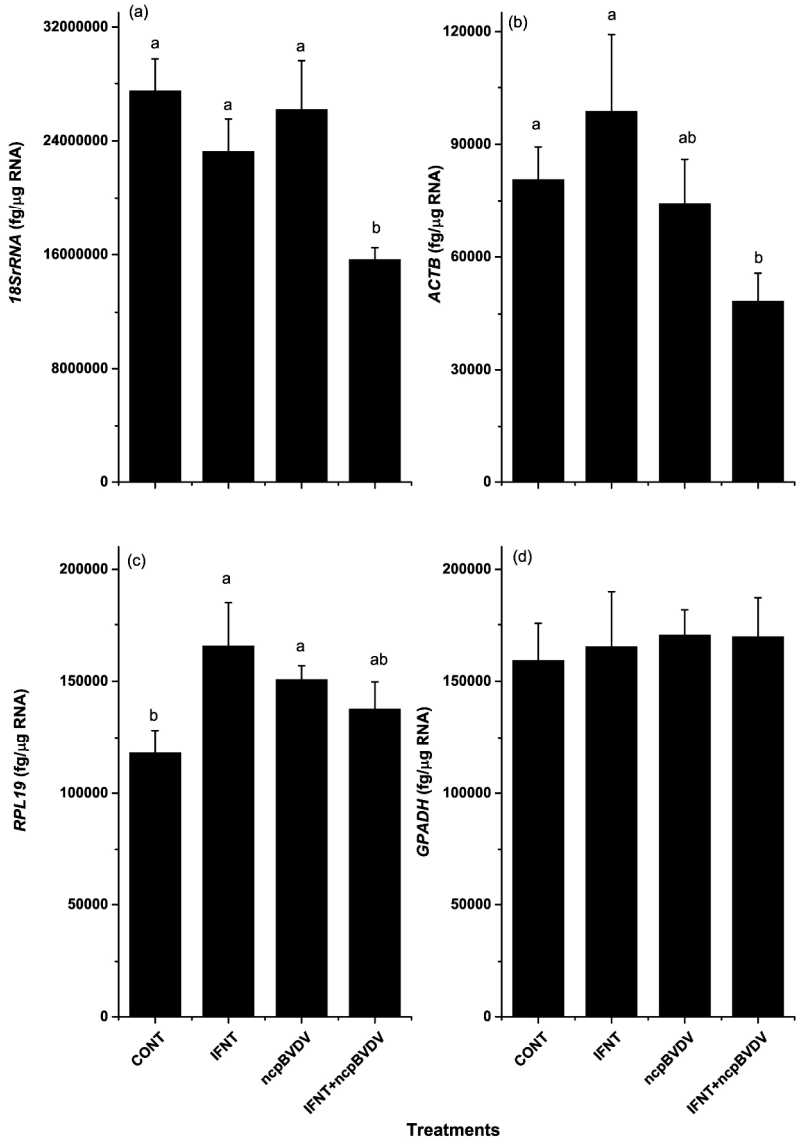


Fig 2
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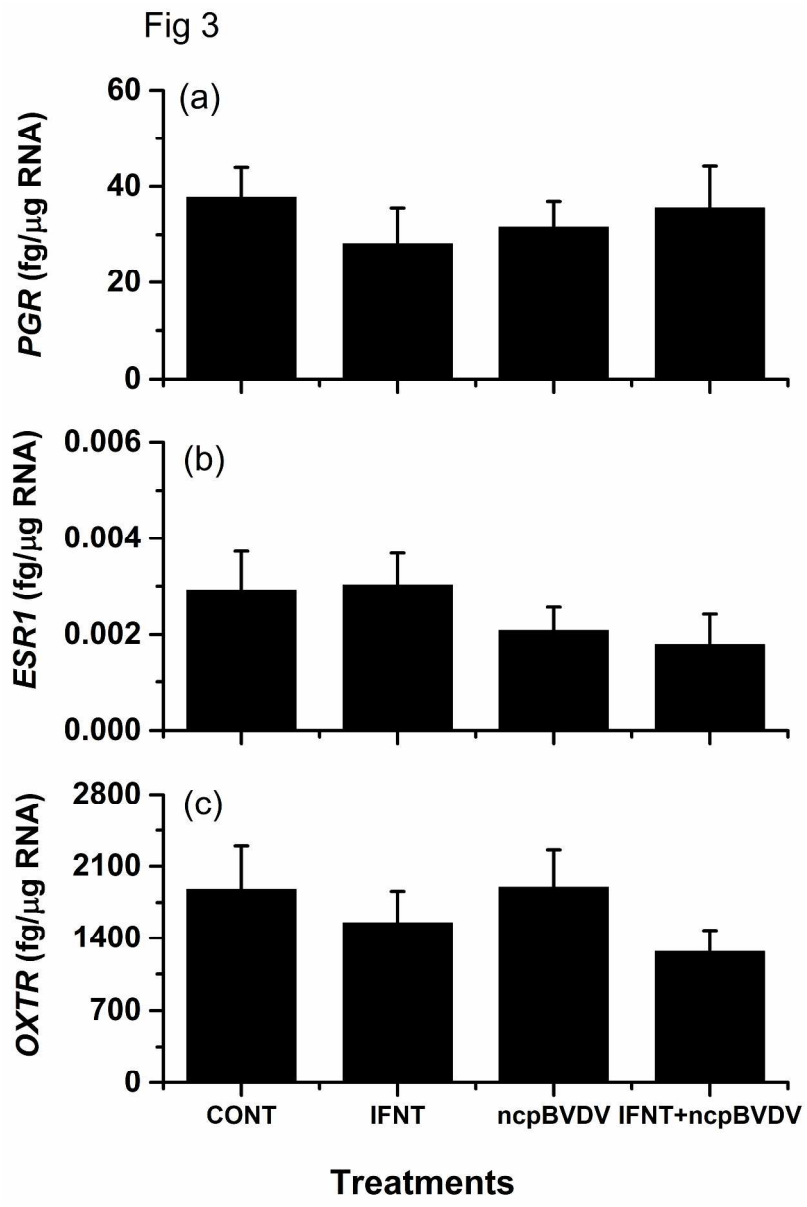


Fig 3
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Fig 4

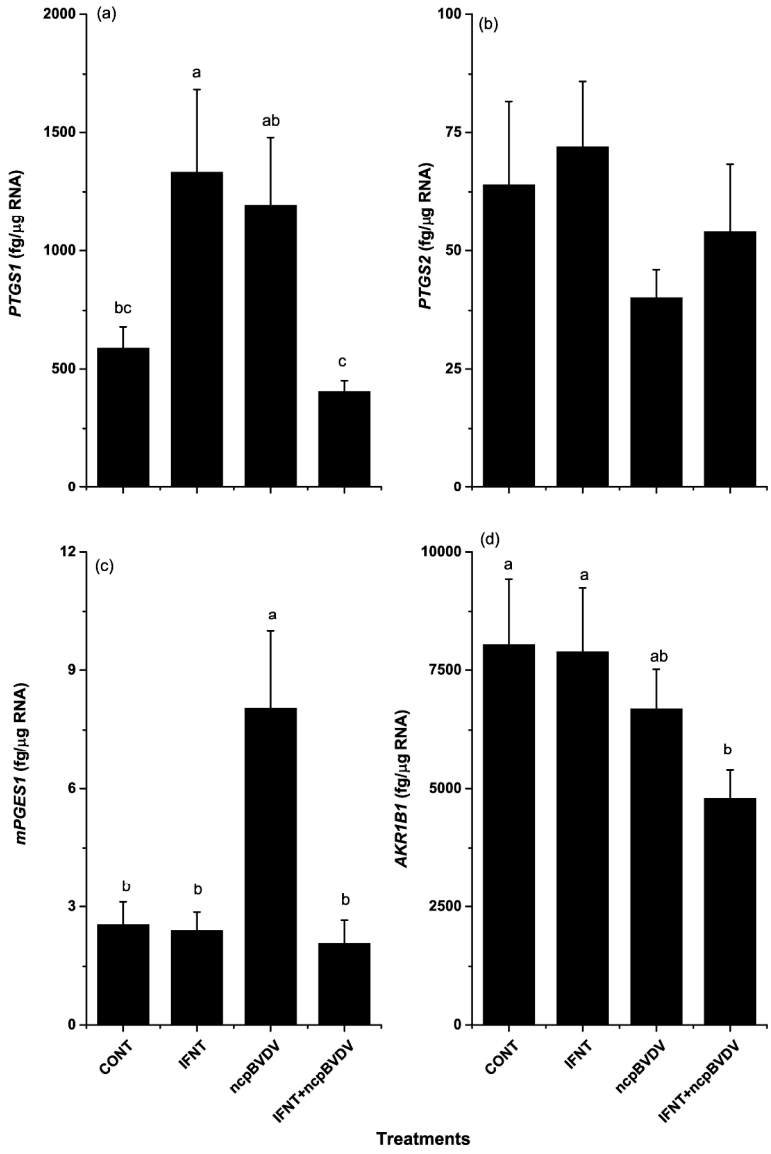


Fig 4
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Fig 5

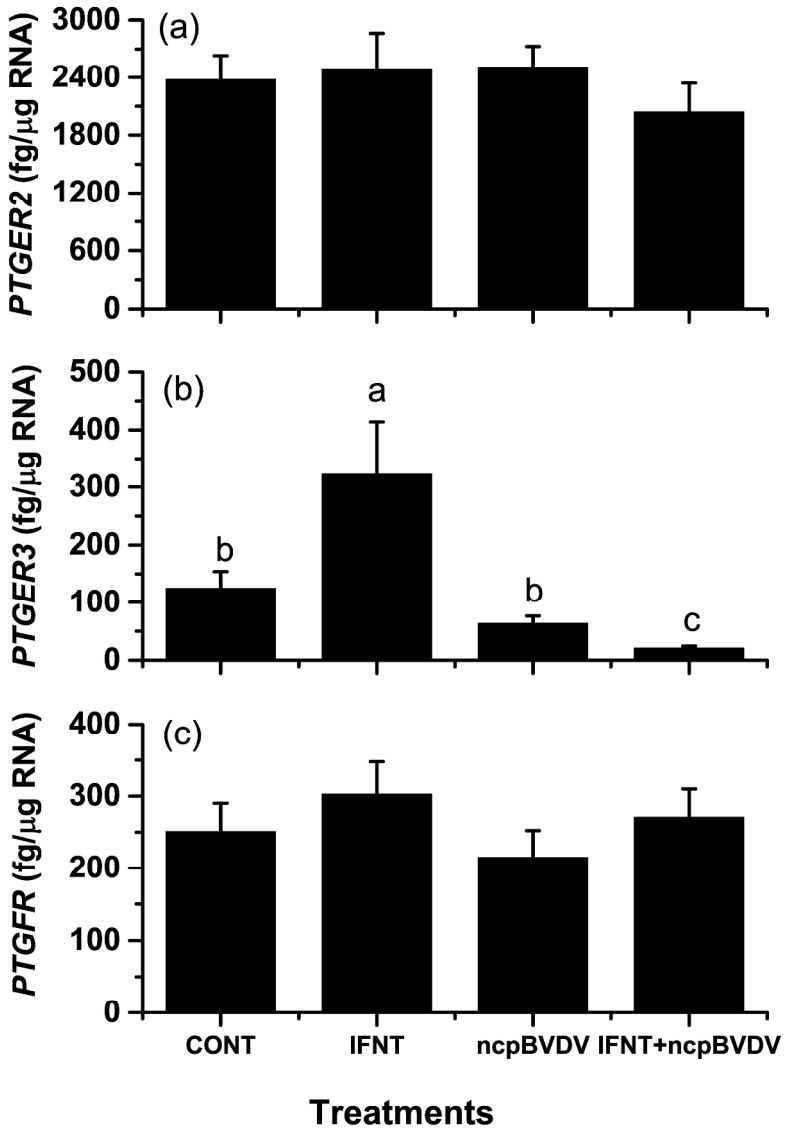


Fig 5
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