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1 **BVDV alters uterine prostaglandin production during pregnancy recognition in**
2 **cows**

3 *Zhangrui Cheng[1], Ayimuguli Abudureyimu[2], Chike F Oguejiofor[1], Rebekah Ellis[1],*
4 *Amy Teresa Barry[1], Xing Chen[1,3], D Olivia L Anstaett[4], Joe Brownlie[4], D Claire*
5 *Wathes[1]*
6
7 *[1] Department of Production and Population Health, Royal Veterinary College, North Mymms,*
8 *Hertfordshire AL9 7TA, UK*
9 *[2] Life Science and Engineering College, Northwest University for Nationalities, Lanzhou,*
10 *China*
11 *[3] Key Laboratory of Agricultural Animal Genetics, Breeding and Reproduction, Education*
12 *Ministry of China, College of Animal Science and Technology, Huazhong Agricultural*
13 *University, Wuhan, China.*
14 *[4] Department of Pathology and Pathogen Biology, Royal Veterinary College, North Mymms,*
15 *Hertfordshire AL9 7TA, UK*
16
17 *Correspondence should be addressed to Z Cheng; Department of Production and*
18 *Population Health, Royal Veterinary College, North Mymms, Hertfordshire AL9 7TA, UK;*
19 *Email: zcheng@rvc.ac.uk.*
20
21 *Short title: BVDV interrupts pregnancy recognition in cows*
22



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23 **Abstract**

24

25 Embryonic mortality in cows is at least in part caused by failed pregnancy recognition (PR). Evidence
26 has shown that bovine viral diarrhoea virus (BVDV) infection can disrupt pregnancy. Prostaglandins
27 (PG) play important roles in many reproductive processes, including implantation. The aims of the
28 present study were to investigate the effect of BVDV infection on uterine PG production and PR
29 using an *in vitro* PR model. Bovine uterine endometrial cells isolated from 10 BVDV free cows were
30 cultured and treated with 0 or 100 ng/ml interferon-tau (IFNT) in the absence or presence of non-
31 cytopathic BVDV (ncpBVDV). $PGF_{2\alpha}$ and $PGE_2$ concentrations in the spent medium were measured
32 using radioimmunoassays and in the treated cells expression of the genes associated with PG
33 production and signalling was quantified using qPCR. The results showed that IFNT challenge
34 significantly stimulated *PTGS1* and *PTGER3* mRNA expression and $PGE_2$ production, but these
35 stimulatory effects were neutralised in the presence of ncpBVDV infection. ncpBVDV infection
36 significantly increased *PTGS1* and *mPGES1* mRNA expression and decreased *AKR1B1* expression,
37 leading to increased $PGE_2$ and decreased $PGF_{2\alpha}$ concentrations and an increased $PGE_2$:$PGF_{2\alpha}$ ratio.
38 The other tested genes, including *PGR*, *ESR1*, *OXTR, PTGS2*, *PTGER2* and *PTGFR*, were not
39 significantly altered by IFNT, ncpBVDV or their combination. Our study suggests that BVDV infection
40 may impair PR by 1) inhibiting the effect of IFNT on uterine PG production and 2) inducing an
41 endocrine switch of PG production from $PGF_{2\alpha}$ to $PGE_2$ to decrease uterine immunity, so
42 predisposing the animals to uterine disease.

43

44 **Introduction**

45

46 Poor reproductive performance, of which early embryonic mortality is a major component,
47 causes major economic loss to the dairy industry (De Vries 2006). Embryonic mortality rates
48 in cattle can be as high as 40%, with 70-80% of losses occurring before day 16 of gestation
49 (Diskin *et al.* 2011). Following successful ovulation and subsequent fertilisation, production
50 of interferon tau (IFNT) by the trophectoderm begins at around day 8 of gestation and
51 increases dramatically during conceptus elongation (Kimura *et al.* 2004). A sufficient
52 threshold level of IFNT must be reached by day 16 to ensure pregnancy recognition (PR)
53 and prevention of luteolysis (Forde *et al.* 2011, Lonergan & Forde 2014). IFNT acts in a
54 paracrine manner on the uterine endometrium to develop a receptive environment. This
55 involves changes in the uterine epithelium which are tightly regulated by steroid hormones,
56 type I interferons, cytokines, prostaglandins (PGs) and growth factors and their receptors
57 (Forde *et al.* 2011, Dorniak *et al.* 2012, Spencer *et al.* 2013, Lonergan & Forde 2014).

58

59 Many reproductive processes, including luteolysis, PR and implantation involve inflammation
60 and associated up-regulation of inflammatory mediators (e.g. PGs) and recruitment of
61 immune cells to the uterine endometrium (Jabbour *et al.* 2009). $PGE_2$ (a vasodilator) is
62 luteotropic for maintaining progesterone secretion by the corpus luteum whereas $PGF_{2\alpha}$ (a
63 vasoconstrictor) is luteolytic (Weems *et al.* 2006). In PG production pathways, 2 series PGs,
64 such as $PGE_2$ and $PGF_{2\alpha}$, are produced from arachidonic acid (AA). PG-endoperoxide
65 synthase isozymes (PTGS1 and PTGS2) catalyse AA into PGH2 and PGH2 is converted
66 into PGEs by the action of PGE synthase (PGES) and into PGFs by the action of PGF
67 synthase (PGFS) (Wathes *et al.* 2007). There are many isoforms for both PGES and PGFS.
68 Previous studies have shown that mPTGES1 and AKR1B1 are the predominant isoforms for
69 $PGE_2$ and $PGF_{2\alpha}$ production, respectively, in bovine endometrium (Fortier *et al.* 2008).

70

71 During PR IFNT inhibits the up-regulation of oxytocin (OT) receptors (OXTR) in the uterine
72 epithelium, so preventing the pulsatile release of $PGF_{2\alpha}$, which is necessary for luteolysis
73 (Wathes & Lamming 1995, Mann *et al.* 1999). In contrast, basal release of PGs, including
74 $PGE_2$ and $PGF_{2\alpha}$, from both the conceptus and maternal uterine endometrium rises during
75 early pregnancy (Zarco *et al.* 1988, Dorniak *et al.* 2013). This increased PG production may
76 facilitate the effects of IFNT on expression of endometrial Type I IFN-stimulated genes
77 (ISGs), a vital component of pregnancy recognition (Spencer *et al.* 2013).

78

79 Bovine viral diarrhoea virus (BVDV), a single-stranded RNA virus, causes widespread
80 infection of the cattle population worldwide. The reproductive system, including the uterine
81 endometrium, is a major site for infection, maintenance and spreading of the virus (Grooms
82 2004, Lanyon *et al.* 2014). BVDV infection can have a significant impact on all stages of
83 pregnancy, including causing early embryonic death, such that reproductive losses are one
84 of the most important consequences of the disease (Grooms 2004). Our recent studies
85 demonstrated that infection of bovine endometrial cells in vitro with non-cytopathic BVDV
86 (ncpBVDV) caused many significant changes in pathways associated with innate immunity,
87 particularly those associated with type I interferon signalling (Oguejiofor *et al.* 2015a).
88 ncpBVDV thus inhibited expression of many *ISGs* which are also involved in PR, such as
89 *IFITs, BST2, MX2, OAS1, USP18* and *RSAD2* (Forde *et al.* 2011). Some of these genes are
90 also regulated by PGs (Spencer *et al.* 2013). In monocytes BVDV infection stimulated the
91 production of $PGE_2$ (Welsh *et al.* 1995). When bovine endometrial cells were also exposed
92 to bacterial lipopolysaccharide (LPS), ncpBVDV increased the expression of *PLA2G4F* and
93 *PTGES* (*mPGES-1*) (Oguejiofor *et al.* 2015a). *PLA2G4F* belongs to the family of PLA2
94 enzymes that are essential for the initial release of AA in PG biosynthesis (Tithof *et al.*
95 2007) while PTGES subsequently catalyzes the isomerization of $PGH_2$ to $PGE_2$
96 (Samuelsson *et al.* 2007).

97

98 These results suggested that ncpBVDV infection in cows may: (1) interfere with the normal
99 PR signals in response to IFNT and (2) increase endometrial $PGE_2$ production following
100 bacterial infection, potentially compromising the innate immune response. To explore these
101 possibilities further, the present study mimicked PR by stimulating bovine endometrial cells
102 with IFNT in the presence or absence of ncpBVDV infection and investigated the effects of
103 these treatments on PG signalling pathways.

104

105 **Materials and Methods**

106

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

110

111 ***Animal, cell isolation and culture***

112

113 Fresh and apparently healthy uteri from cows in the early luteal phase of the oestrous cycle
114 were collected at the local abattoir. Uterine endometrial cells (a mixture of primary epithelial
115 and stromal cells) were isolated and cultured following the methods described previously
116 (Cheng *et al.* 2013, Oguejiofor *et al.* 2015b). Briefly, under sterile conditions, strips of
117 intercaruncular endometrium were separated and put into serum-free Dulbecco's Modified
118 Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12 medium) (Sigma) and chopped into
119 1 $mm^3$ cubes. They were digested for 90 min at 37°C in medium containing 100 mg bovine
120 serum albumin (BSA, Sigma), 50 mg trypsin III (Worthington, Lakewood, NJ 08701, USA)
121 and 50 mg collagenase A (Roche, Welwyn Garden City, UK) per 100 ml of Hanks' balanced

122 salt solution (HBSS; Sigma). After filtration through a 100 µm mesh into 50 ml falcon vials,
123 the suspension was re-suspended with HBSS containing 10% foetal bovine serum (FBS;
124 PAA, Somerset, UK) and 3 µg/ml trypsin inhibitor (Sigma) and centrifuged at 100 × g and
125 10°C for 10 min. After two repetitions of the above washing procedures, the cells were
126 suspended with the culture medium (DMEM/F12 medium with 10% FBS) and plated in 24
127 well IWAKI micro plates (Scitech DIV, Asahi Techno Glass, Japan) at 2 ml per well
128 containing $0.5 \times 10^5$ cells (day 1). Culture medium was changed every 48 h to allow the cells
129 to grow. The composition of the cell population was confirmed using immunocytochemical
130 staining validated in our laboratory (Oguejiofor *et al.* 2015b). After culturing for 8 days (day
131 8), epithelial cells comprised about 90% and stromal cells about 10% of the population.
132 Contamination of immune cells was negligible (<0.001%).

133

134 ***BVDV test for experimental samples***

135

136 The experiment was designed to use BVDV free bovine endometrial samples and FBS. The
137 examination was carried out using a PCR method reported previously (Vilcek *et al.* 1994,
138 Pinheiro de Oliveira *et al.* 2013). The primer pair for the PCR was: forward
139 (ATGCCCWTAGTAGGACTAGCA; position 108-128) and reverse
140 (TCAACTCCATGTGCCATGTAC; position 395-375) with an expected product size of 288 bp,
141 which are the highly conserved 5' non-coding/non-structural coding regions of the pestivirus
142 BVDV genome strain NADL (Vilcek *et al.* 1994). Total RNA in the uterine endometrial tissue
143 and FBS was extracted using a Qiagen RNA Lipids Mini kit (Qiagen, Manchester, UK)
144 following the supplied protocol. RNA was reverse transcribed into cDNA using a cDNA
145 synthesis kit supplied by PCRBiosystems (London, UK) and PCR was performed using the
146 G-Storm thermal cycler (G-Storm Ltd, Somerset, UK) and Qiagen Multiple PCR kit (Qiagen).
147 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***

156

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

173 separate plates from those without the virus to prevent cross-contamination. For the cells
174 designated as BVDV-infected, 0.25 ml of MM containing Pe515nc BVDV at a multiplicity of
175 infection (MOI) of 0.1 was added for 3 h to allow for virus infection. The same procedures,
176 but with 0.25 ml MM only, were carried out for the cells designated as non-infected controls.
177 The volume in all wells was made up to 1 ml with MM and the medium was changed after
178 two days. IFNT treatment was carried 4 days after infection (day 8). For the wells specified
179 for IFNT treatment, the medium was replaced with 1 ml MM containing 100 ng IFNT
180 (recombinant ovine IFNT, Cell Sciences, Canton, USA) and incubated for 24 h. The other
181 wells were changed and incubated with 1 ml MM. The spent medium was harvested (on day
182 9) and stored at -20°C for PG quantification and the treated cells were used for total RNA
183 extraction.

184

185 ***Assessment of BVDV-cell infection and cell viability***

186

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

192

193 ***Quantification of PGs using Radioimmunoassay (RIA)***

194

195 Concentrations of $PGE_2$ and $PGF_{2\alpha}$ in the spent medium were measured with charcoal-
196 dextran coated RIA methods described previously (Cheng *et al.* 2001). The PG antisera
197 were a kind gift from Dr N L Poyser (University of Edinburgh, Edinburgh, UK). The cross-

198 reactivities for $PGE_2$ antiserum were 23%, 100% and 15% with $PGE_1$, $PGE_2$ and $PGE_3$,
199 respectively and those for $PGF_{2\alpha}$ antiserum were 34%, 100% and 25% with $PGF_{1\alpha}$, $PGF_{2\alpha}$
200 and $PGF_{3\alpha}$, respectively. The standards for $PGE_2$ and $PGF_{2\alpha}$ were purchased from Sigma
201 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,
202 11, 12, 14, 15 (n)-$^3$H]-$PGF_{2\alpha}$) were supplied by PerkinElmer (Cambridge, UK). The samples
203 were diluted (×10-200) in the RIA buffer. This allowed the sample PG concentrations to fall
204 within the analytical ranges of the RIAs (0.02 – 5.0ng/ml for $PGE_2$ and 0.01-2.5 ng/ml for
205 $PGF_{2\alpha}$) and the interruption from the medium contents with the RIAs was minimised, making
206 extraction unnecessary. The limits of detection were 2 pg/tube and 1 pg/tube for $PGE_2$ and
207 $PGF_{2\alpha}$, respectively. The intra-assay and inter-assay coefficients of variation were 3.5% and
208 6.3% for $PGE_2$ (n = 6), and 4.1% and 9.6% for $PGF_{2\alpha}$, respectively (n =6).

209

210 ***RNA extraction***

211

212 After collection of spent medium, the cells from each treatment group (6 wells) were pooled
213 and the total RNA extraction was carried out using RNeasy Mini Kits (Qiagen) following the
214 supplier's protocol. The concentrations and purity of RNA were measured with a NanoDrop
215 ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilminton, USA).

216

217 ***Primer design and PCR***

218

219 DNA sequences for all primers were obtained from GenBank at NCBI
220 (http://www.ncbi.nlm.nih.gov/Database/index.html) and the primers were designed using a
221 "Primer 3" web based programme (http://frodo.wi.mit.edu/primer3). Their alignment
222 specificity and quality were checked using the Blast tool at

223 http://www.ncbi.nlm.nih.gov/tools/primer-blast/ and Amplify tool
224 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). The detailed
225 information of the primers is shown in Table 1. The primers were made by Eurofins MWG
226 Operon (Ebersberg, Germany). One µg of RNA was reverse transcribed into cDNA using a
227 cDNA synthesis kit supplied by PCRBiosystems following the supplier's protocol. The
228 resulting cDNA (20µl) was diluted in nuclease free water up to 100 µl.

229

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

234

235 ***qPCR analysis for gene expression***

236

237 Concentrations of all ten target genes (*PTGS1, PTGS2, mPGES1, AKR1B1, PTGER2,*
238 *PTGER3, PTGFR, OXTR, PGR* and *ESR1*) and four reference genes (*GAPDH, RPL19,*
239 *ACTB* and 18SrRNA) were quantified using qPCR via an absolute quantification approach
240 following the method described previously (Cheng *et al.* 2013). The DNA amplified from
241 cDNA used for standards in the qPCR assay was purified using a QIAquick PCR purification
242 kit (Qiagen) and their quality and concentrations were determined with the NanoDrop ND-
243 1000 spectrophotometer. Eight standards were prepared from 1 to 1× $10^{-7}$ ng/ml. Annealing
244 and amplicon-specific melting temperatures of the primers were determined using a gradient
245 function of the qPCR machine (CFX96 Real-Time System DNA, Bio-Rad Laboratories, Her-
246 cules, CA, USA) with 8 identical reactions containing 2 ng of DNA standard, 10 µl Sygreen
247 Mix (PCRBiosystems), 0.8 µl of each 10µM forward and reverse primer and nucleate free
248 water added up to 20 µl. The optimised annealing temperatures are given in Table 1.

249 To quantify the expression levels of each gene, the qPCR assay contained a standard curve,
250 no template control (NTC) and sample cDNA in duplicate with the same final volume on
251 CFX96 Real -Time Systems (Bio-Rad). Each well contained 5 µl cDNA standard or samples,
252 10 µl Sygreen Mix (PCRBiosystems), 0.8 µl of 10µM forward primer, 0.8 µl reverse primer
253 and 3.4 µl nucleate free water following the protocol supplied by PCRBiosystems and using
254 the optimised annealing temperatures shown in Table 1. This included an initial Taq
255 activation for 2 min followed by 38 cycles of denaturation (95 °C), annealing (the annealing
256 temperatures are given in Table 1) and extension (63°C). An amplicon-specific fluorescence
257 acquisition reading based on the melting temperature obtained in the above gradient test
258 was applied to avoid any noise from smaller non-specific products such as dimers prior to
259 the product acquisition. The results were analysed using the CFX Manager Software
260 package (Bio-Rad). The limit of quantification was $1 \times 10^{-6}$ - $1 \times 10^{-7}$ng/ml for all tested
261 genes. In order to minimise variation, RT for all samples was performed in one assay. For
262 each gene, the standards and all samples were prepared under the same conditions in a
263 single plate with the same master-mix of reagents.

264

265 ***Statistical data analysis***

266

267 All values are summarized as mean ± standard error (S.E.). The values of gene expression
268 generated by qPCR were normalised as fg/µg reverse-transcribed total RNA. The PG ratios
269 were calculated as $PGE_2/PGF_{2\alpha}$. Statistical data analysis was carried out using analysis of
270 variance (ANOVA) with repeated measurements for the PG data and ANOVA with
271 randomised block design for the gene expression data via a linear mixed effect model built in
272 SPSS V22 (Chicago, IL, USA), in which the differences between treatments (CONT,
273 ncpBVDV, IFNT and their combination) were taken as fixed effect and cows as random
274 effect or subject for repeated measurement. Statistical significance was considered at P <

275 0.05. Logarithmic transformation was applied if the data were not normally distributed.
276 Where statistical significance was achieved in ANOVA, Fisher's LSD multiple comparisons
277 based on the least square means were performed to identify the differences between the
278 treatment pairs.

279

280 **Results**

281

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

287

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

290

291 The results showing the effect of ncpBVDV, IFNT and their combination on PG production by
292 uterine endometrial cells are given in Fig. 1. ANOVA with repeated measurements showed
293 that the differences of $PGE_2$ concentrations between treatment groups were significant
294 ($P<0.018$). Fisher's LSD multiple comparisons illustrated that both IFNT challenge and
295 ncpBVDV infection alone significantly stimulated $PGE_2$ production ($P<0.05$) whereas the
296 stimulatory effect was neutralised when IFNT was combined with ncpBVDV ($P<0.05$) (Fig.
297 1a).

298

299 Compared with CONT, IFNT challenge appeared to decrease $PGF_{2\alpha}$ production, but
300 statistical significance was not achieved ($p>0.05$). In the cells infected with ncpBVDV,
301 concentrations of $PGF_{2\alpha}$ were significantly lower than in the CONT cells ($P<0.05$). When the
302 ncpBVDV infected cells were challenged with IFNT, $PGF_{2\alpha}$ concentrations were significantly
303 lower than in the CONT cells ($P<0.05$) and the concentrations were slightly lower than in the
304 cells infected with ncpBVDV alone although the difference was not statistically significant
305 ($P>0.05$) (Fig. 1b).

306

307 Compared with the CONT, in the cells infected with ncpBVDV, the ratios of $PGE_2$ to $PGF_{2\alpha}$
308 were significantly increased ($P<0.05$). Both IFNT and IFNT+ncpBVDV treatments appeared
309 to increase the ratio of $PGE_2$:$PGF_{2\alpha}$ produced, but the differences were not statistically
310 significant due to large variations between animals ($P>0.05$) (Fig. 1c).

311

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

314

315 We selected *18SrRNA, ACTB, RPL19* and *GAPDH* as potential reference genes for the
316 qPCR. The results showed, however, that expression of *18SrRNA, ACTB* and *RPL19* was
317 significantly altered by IFNT, ncpBVDV or their combination ($P<0.05$-$0.01$). Only *GAPDH*
318 expression was not affected by any of the above treatments ($P>0.05$) (Fig 2a-d).
319 Subsequent gene expression measurements have therefore been presented as absolute
320 values given as fg/µg reverse-transcribed total RNA.

321

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

324

325 In the bovine endometrial cells expression of *OXTR* mRNA was relatively higher (1,879 ±
326 419 fg/μg RNA) while that of *ESR1* mRNA was very low (0.003 ± 0.0008 fg/μg RNA). The
327 effects of all tested treatments on expression of *PGR, ESR1* and *OXTR* mRNA were
328 moderate and no statistical significance was achieved as tested using ANOVA with
329 randomized block design (Fig. 3a-c).

330

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

333

334 In the cells infected with ncpBVDV, expression of *PTGS1* mRNA was significantly higher
335 than in the CONT cells ($P<0.05$). IFNT challenge significantly induced *PTGS1* mRNA
336 expression ($P<0.01$), however in the presence of ncpBVDV infection, this stimulatory effect
337 was completely neutralised ($P<0.05$) (Fig. 4a). In the cells infected with ncpBVDV, *PTGS2*
338 mRNA expression appeared to be lower than other groups, but statistical significance was
339 not achieved ($p>0.05$) (Fig. 4b). Fig 4c demonstrated that ncpBVDV infection stimulated
340 *mPGES1* mRNA expression by up to 3 fold ($P=0.0003$) compared with the CONT cells
341 whereas neither IFNT challenge nor IFNT+ncpBVDV affected its expression ($P>0.05$). As
342 shown in Fig. 4d, neither IFNT nor ncpBVDV alone altered *AKR1B1* mRNA expression
343 significantly ($P>0.05$) whist their combination led to a significant decrease in its expression
344 ($P<0.05$).

345

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

348

349 As shown in Figs 5a and 5c, the expression of *PTGER2* and *PTGFR* mRNA was not
350 significantly changed by treatment with IFNT, ncpBVDV or their combination ($P>0.05$).
351 Compared with the CONT, treatment with ncpBVDV alone only moderately reduced
352 *PTGER3* mRNA expression ($P>0.05$). In the cells stimulated with IFNT, *PTGER3* mRNA
353 expression was up-regulated by up to 2.5 fold ($P<0.01$) whereas in the presence of
354 ncpBVDV infection, the stimulatory effect of IFNT on *PTGER3* mRNA expression was
355 intensively inhibited ($P<0.01$) and the expression was even lower than that in the CONT cells
356 ($P<0.01$) (Fig 5b).

357

358 **Discussion**

359

360 PR in cows is initiated by IFNT release from the conceptus acting on the uterine
361 endometrium leading to maintenance of the corpus luteum. Regulation of the synthesis and
362 release of $PGE_2$ and $PGF_{2α}$ is crucial to this process (Bazer 2013, Lonergan & Forde 2014).
363 Failure of PR is a significant risk factor for embryonic mortality (Diskin *et al.* 2011, Forde *et al.*
364 2011). BVDV infection can cause early embryonic death (Grooms 2004) but little evidence is
365 currently available on the mechanisms involved. In the present study we investigated the
366 effect of BVDV infection on PG production and signalling pathways in bovine endometrium.
367 We have demonstrated that: 1) ncpBVDV infection induced an endocrine switch of PG
368 production and signalling from $PGF_{2α}$ to $PGE_2$ and 2) IFNT stimulates uterine $PGE_2$
369 production and its signalling pathway, but this stimulatory effect is abolished by ncpBVDV
370 infection.

371

372 In addition to PR, IFNT possesses antiviral, antiproliferative and immunosuppressive
373 activities (Pontzer *et al.* 1988, Pontzer *et al.* 1991, Kohara *et al.* 2012). It was reported
374 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

401 and led to failure of conceptus elongation in ewes (Diskin *et al.* 2011). In cows successful
402 development to term and delivery of a live calf could be predicted by the expression of
403 *PTGS2* in day 7 blastocysts (Bazer 2013). In the present study, when the endometrial cells
404 isolated from cyclic cows were given IFNT for 24h, their $PGE_2$ production was significantly
405 up-regulated while the $PGF_{2\alpha}$ production was not altered. This agreed with the previous
406 finding that IFNT does not affect expression of PTGS2, the rate-limiting enzyme in PG
407 synthesis, but does stimulate $PGE_2$ production by cells of the bovine uterus (Dorniak *et al.*
408 2013). We also found that IFNT challenge increased *PTGS1* and *PTGER3* gene expression
409 by up to 2.5 fold. Such an increase in both $PGE_2$ and its receptor indicates that IFNT favours
410 the $PGE_2$ signalling pathway which would benefit maintenance of the corpus luteum as $PGE_2$
411 is a luteotropin (Pratt *et al.* 1977, Dorniak *et al.* 2013). In the presence of ncpBVDV infection,
412 however, the stimulatory effects of IFNT on $PGE_2$ production and its receptor *PTGER3*
413 expression was neutralised and the $PGE_2$ concentrations were even slightly lower than in
414 the CONT cells. When the ncpBVDV infected cells were treated with IFNT, basal $PGF_{2\alpha}$
415 production and *AKR1B1* mRNA expression were also lower than the CONT. AKR1B1 is a
416 predominant isoform for $PGF_{2\alpha}$ production (Fortier *et al.* 2008). Therefore, decreased or
417 disrupted basal PG production may contribute to failure of pregnancy establishment and
418 maintenance following ncpBVDV infection.

419

420 These results also suggest that IFNT treatment may act on uterine epithelial and stromal
421 compartments differentially as $PGE_2$ is produced by stromal cells and $PGF_{2\alpha}$ by epithelial
422 cells. This supports previous studies in the ewe which have shown cell-specific effects of
423 IFNT for many ISGs crucial for developing a receptive environment for conceptus
424 implantation. During early pregnancy or following intrauterine IFN treatment, the expression
425 of the majority of progesterone-independent *ISGs* such as *RSAD2* (radical S-adenosyl
426 methionine domain containing 2) and *IFIH1* (interferon-induced with helicase C domain 1)
427 was increased in endometrial stroma, glands and immune cells but not in the luminal

428 epithelium. In contrast some non-classical *ISGs* such as *LGALS15* (galectin 15), *CTSL*
429 (cathespin L) and *CST3* (cystatin C), which require progesterone priming, were
430 predominantly expressed in the endometrial luminal epithelium and superficial glandular
431 epithelium (Song *et al.* 2007, Bazer *et al.* 2008, Spencer *et al*. 2013).

432

433 It is interesting to note that ncpBVDV infection alone increased uterine $PGE_2$ production and
434 decreased $PGF_{2α}$ production while expression of *PTGS2*, *AKR1B1* and *PTGFR* all tended to
435 be lower than in the CONT cells. Lower *PTGS2* and *AKR1B1* expression may in part
436 contribute to the decreased $PGF_{2α}$ production, while lower *PTGFR* expression would further
437 impair the $PGF_{2α}$ signalling pathway. In contrast the expression of *mPGES1*, a principle
438 isoform for $PGE_2$ production (Fortier *et al.* 2008), was up-regulated by about 4 fold, leading
439 to an increased uterine $PGE_2$ generation and an increased ratio of $PGE_2$ to $PGF_{2α.}$ These
440 results show that ncpBVDV infection induced an endocrine switch from $PGF_{2α}$ to $PGE_2$
441 production. As $PGF_{2α}$ is an immune enhancer and $PGE_2$ is an immune suppressor (Lewis
442 2003, Herath *et al.* 2009), this switch provides a mechanism whereby BVDV infection can
443 predispose affected animals to uterine infection. Similarly bacterial LPS treatment increased
444 $PGE_2$ production and decreased $PGF_{2α}$ production in bovine endometrium (Herath *et al.*
445 2009). Our previous studies demonstrated that numerous innate immune responses
446 mounted following an LPS challenge were also significantly suppressed in ncpBVDV
447 infected bovine uterine endometrial cells (Oguejiofor *et al.* 2015a). Together these results
448 support the suggestion that cows infected with BVDV may be predisposed to develop
449 endometritis due to bacterial infection following calving. This may in turn also contribute to
450 early embryonic death and failure of pregnancy establishment (Gilbert 2011).

451

452 In ruminants, OXTR are up-regulated in the uterine epithelium following a period of exposure
453 to high progesterone levels (Wathes & Lamming 1995, Wathes *et al.* 1996). Once these are

454 present, OXT binding to its receptor drives pulsatile release of $PGF_{2\alpha}$, so starting the process
455 of luteolysis (Poyser 1995, McCracken *et al.* 1999). The intial increase in OXTR is
456 independent of oestradiol regulation (Robinson *et al.* 1999, Leung & Wathes 2000, Mann *et*
457 *al.* 2013). However there is a subsequent increase in *ESR1* through which oestradiol
458 stimulates the expression of further *OXTR* in the superficial glandular epithelium and stroma
459 (Wathes & Hamon 1993, Robinson *et al.* 1999). IFNT secreted from the conceptus acts on
460 the uterine epithelium to inhibit expression of both *OXTR* and *ESR1* so inhibiting release of
461 luteolytic pulses of $PGF_{2\alpha}$ and leading to establishment of pregnancy (Mann *et al.* 1999,
462 Bazer 2013). In the present study both *OXTR* and *ESR1* mRNA expression was numerically
463 lower following IFN treatment but the differences did not achieve statistical significance. This
464 may be because *OXTR* mRNA was already highly up-regulated before the IFN treatment
465 commenced.

466

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

480

481 In summary, IFNT challenge stimulated uterine *PTGS1* and *PTGER3* mRNA expression and
482 $PGE_2$ production, but these stimulatory effects were neutralised in the presence of ncpBVDV
483 infection. The interruption of IFNT-induced PG production and signalling by BVDV infection
484 may *directly* cause failure of PR. ncpBVDV infection stimulated *PTGS1* and *mPGES1* mRNA
485 expression and moderately suppressed *AKR1B1* expression, leading to increased $PGE_2$ and
486 decreased $PGF_{2\alpha}$ concentrations and an increase in $PGE_2$:$PGF_{2\alpha}$ ratios in bovine uterine
487 endometrium. This endocrine switch of PG production from $PGF_{2\alpha}$ to $PGE_2$ may decrease
488 uterine immunity to predispose the animals to uterine diseases, *indirectly* leading to failure of
489 PR. Our results thus suggest a mechanism whereby ncpBVDV infection in cows may cause
490 early embryonic death and reduced fertility.

491

492

493 **Conflict of interest**

494

495 The authors declare that there is no conflict of interest that could be perceived as prejudicing

496 the impartiality of the research reported.

497

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506

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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

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

30

31 **Fig.4.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (IFNT) and their
32 combination on mRNA expression of (a) *PTGS1,* (b) *PTGS2,* (c) *mPGES1 and* (d) *AKR1B1* by uterine
33 endometrial cells isolated from BVDV free cyclic cows. When cells were grown to approximately 70%
34 confluence after 4 days culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1.
35 Four days following the inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h.
36 Total RNA was extracted from the treated cells and the gene expression was quantified using an
37 absolute qPCR approach. There were 3 replicates per treatment for each cow and 10 cows per
38 treatment. CONT = control without ncpBVDV and IFNT. The columns labelled with different letters
39 were significantly different at $P < 0.05$-0.01 (a>b>c).

40

41 **Fig.5.** Effect of non-cytopathic bovine viral diarrhoea virus (ncpBVDV), interferon tau (IFNT) and their
42 combination on mRNA expression of (a) *PTGER2,* (b) *PTGER3* and (c) *PTGFR* by uterine endometrial
43 cells isolated from BVDV free cyclic cows. When cells were grown to approximately 70% confluence
44 after 4 days culture, ncpBVDV was inoculated at 0 or a multiplicity of infection of 0.1. Four days
45 following the inoculation, the cells were challenged with IFNT at 0 or 100ng/ml for 24h. Total RNA
46 was extracted from the treated cells and the gene expression was quantified using an absolute qPCR
47 approach. There were 3 replicates per treatment for each cow and 10 cows per treatment. CONT =
48 control without ncpBVDV and IFNT. The columns labelled with different letters significantly different
49 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 |

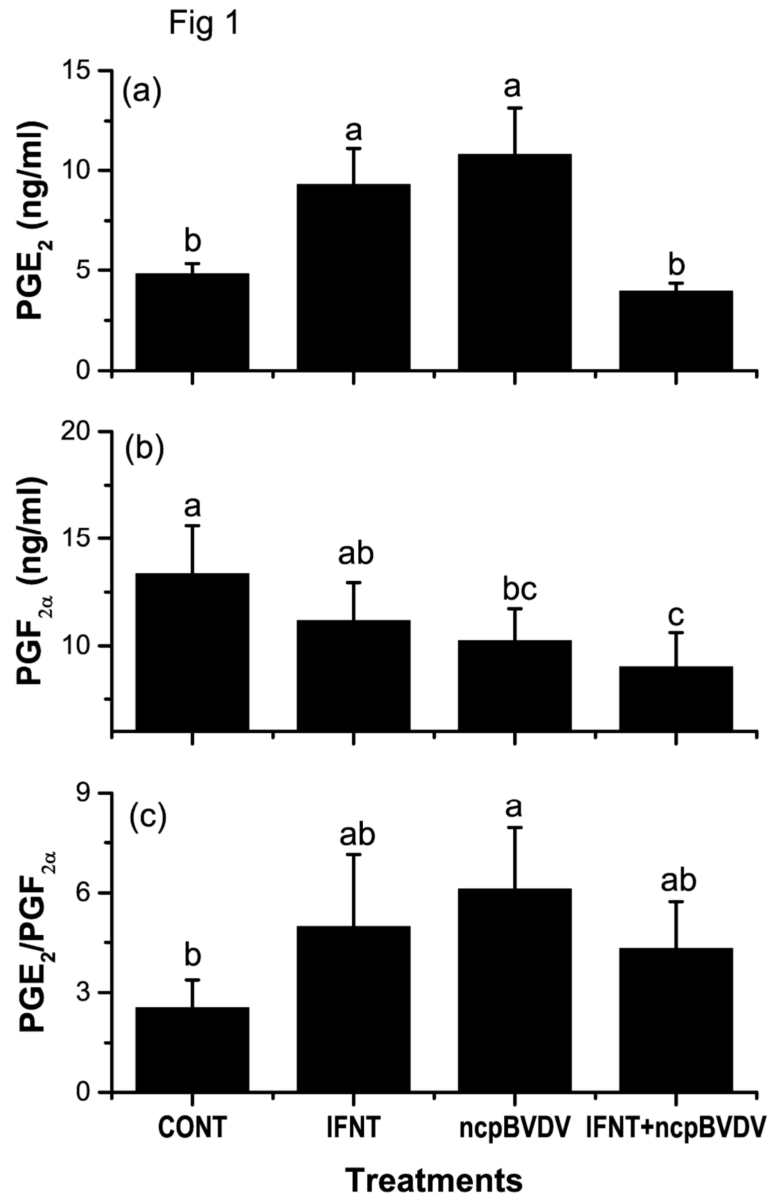


Fig1
280x439mm (300 x 300 DPI)

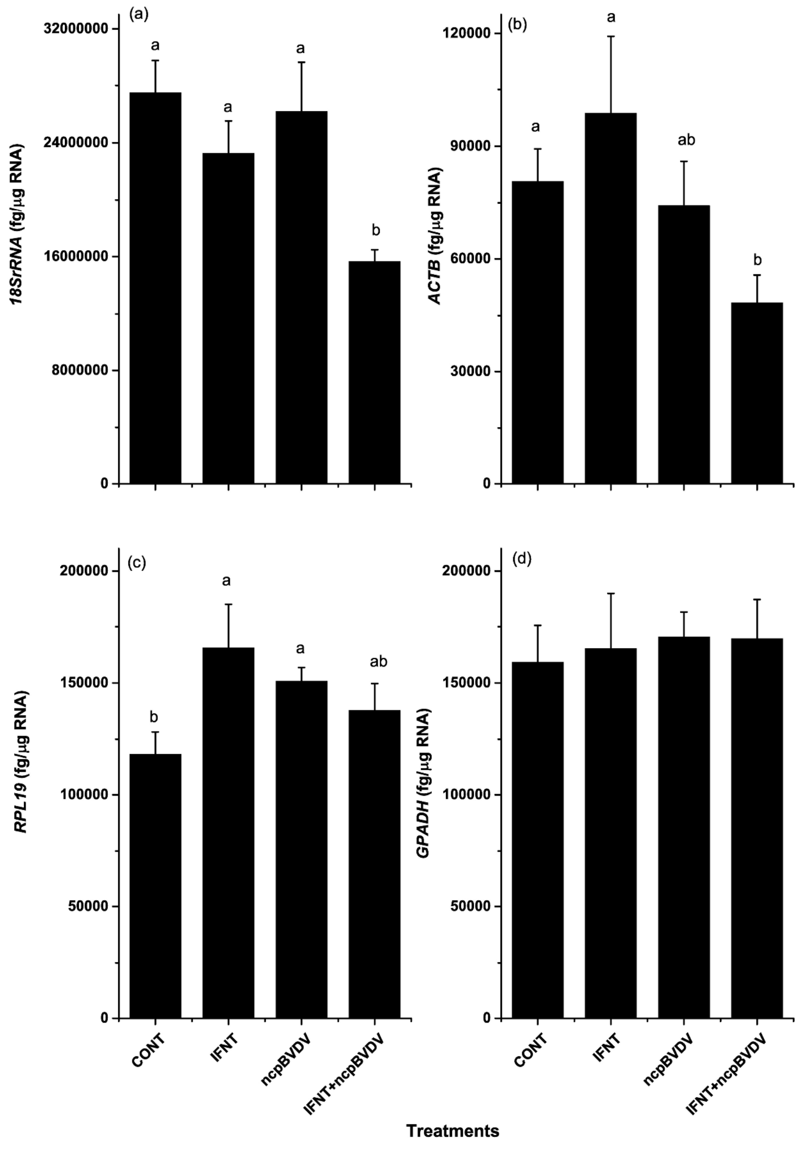


Fig 2
268x404mm (300 x 300 DPI)

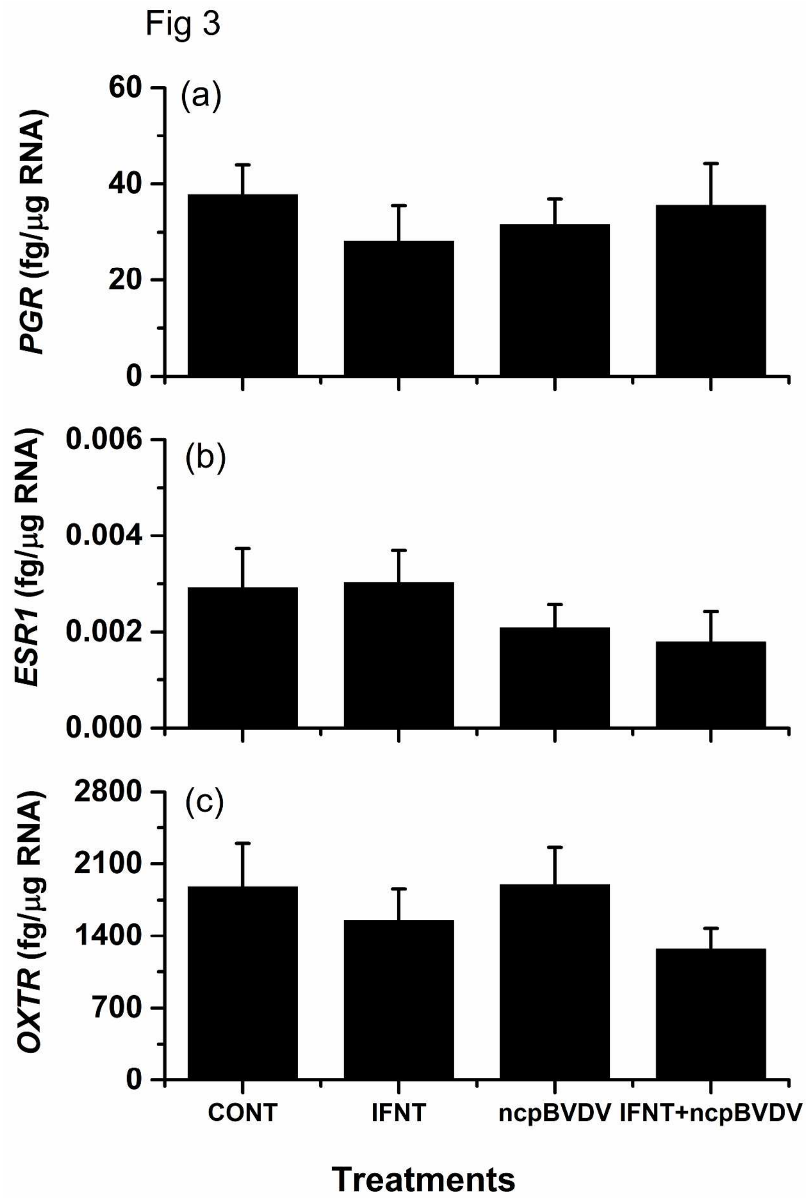


Fig 3
271x405mm (300 x 300 DPI)

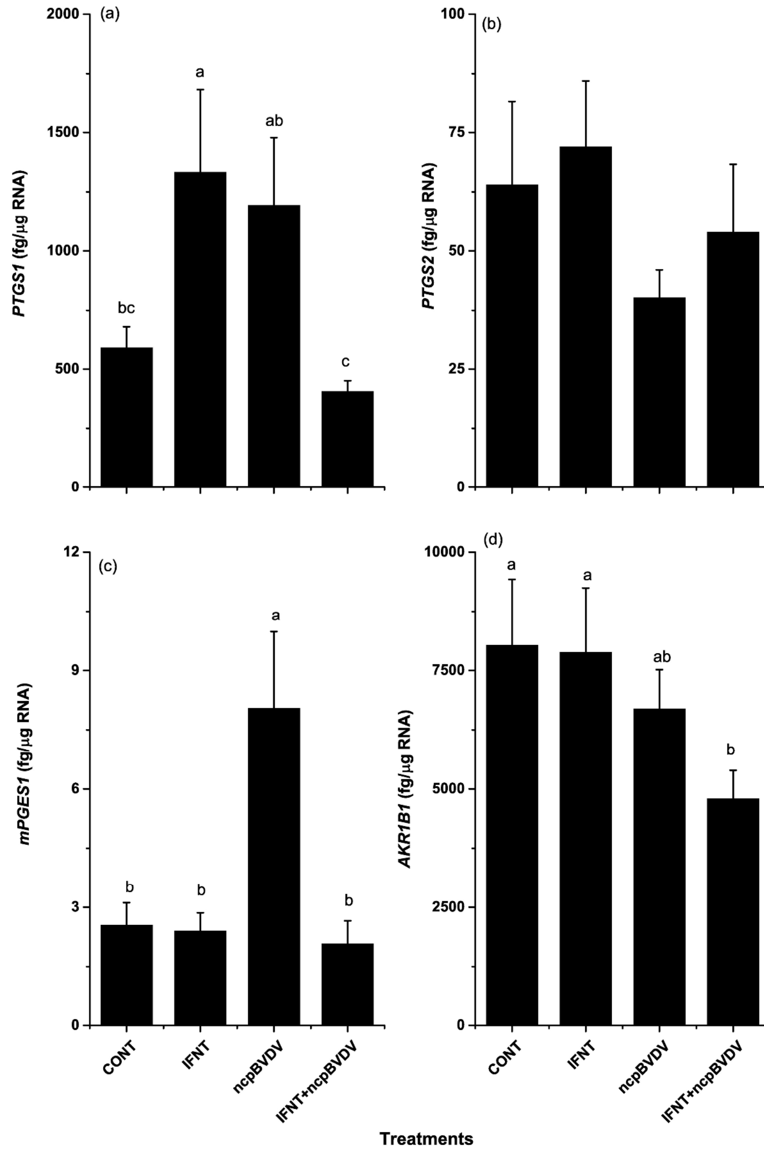


Fig 4
266x415mm (300 x 300 DPI)

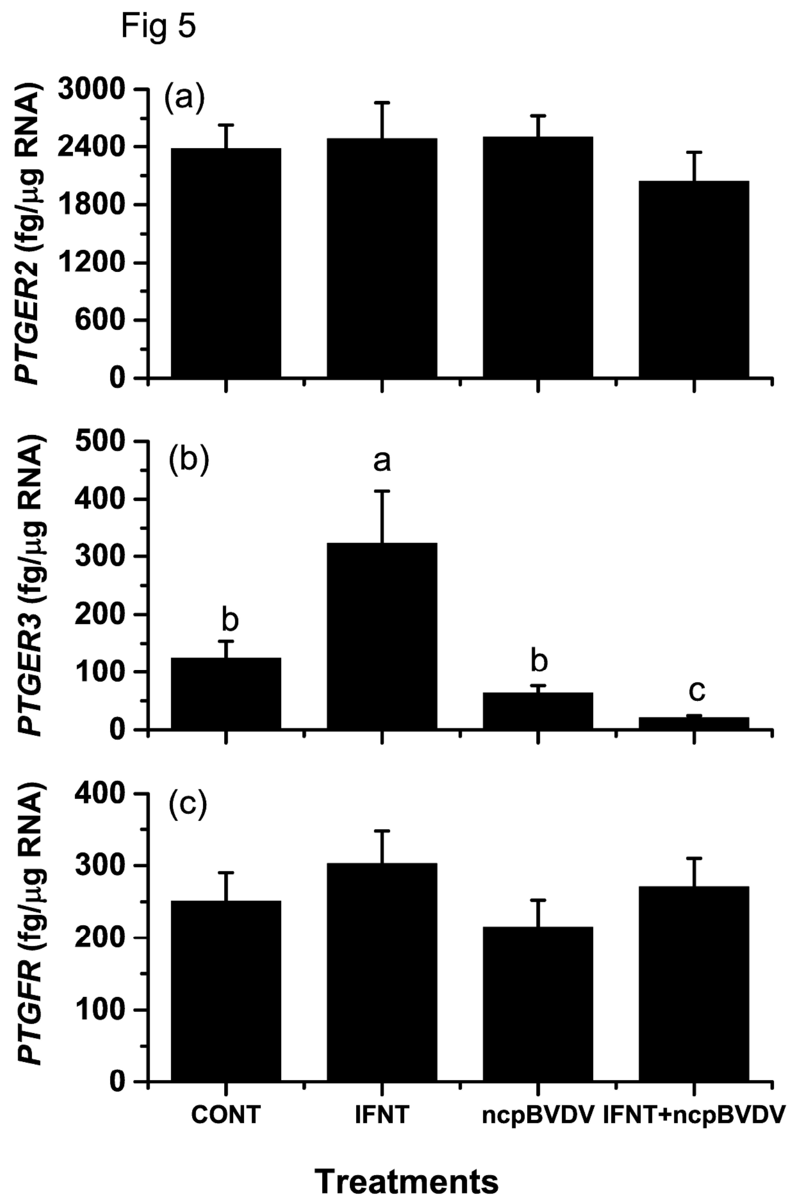


Fig 5
271x413mm (300 x 300 DPI)