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In vivo and in vitro studies of MUC1 regulation in sheep endometrium

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1 Abstract

2 In this study, we investigated the expression of MUC1 mRNA and protein in sheep endometrium at
3 different time-points during follicular and luteal phases of estrous cycle, and also determined the
4 effect of steroid hormone treatments and interferon tau (IFN τ) on *MUC1* mRNA expression in
5 endometrial cell culture *in vitro*. In experiment 1, fifteen *Welsh mountain* ewes were synchronised to a
6 common estrus and killed at precise stages of estrous cycle corresponding to i) pre-LH peak, ii) LH
7 peak, iii) post-LH peak, iv) early luteal, and v) mid-luteal. Reproductive tracts were harvested and
8 mRNA was extracted from the endometrial tissues. Parts of the uterine horns were fixed for
9 immunohistochemistry. In experiment 2, mixed populations of ovine endometrial cells (from
10 slaughterhouse material collected at the post-ovulatory stage of the estrous cycle) were cultured to
11 70% confluence before treatment with i) progesterone (P₄, 10 ng/mL, for 48 h), ii) oestradiol (E₂, 100
12 pg/mL, for 48 h), or with iii) E₂ priming for 12 h (100 pg/mL) followed by P₄ (10 ng/mL) for 36 h.
13 These were compared to; iv) IFN τ (10 ng/mL, for 48 h), and v) basic medium (DMEM/F12) as
14 control. The results showed that MUC1 mRNA and protein expression in sheep endometrium was
15 highest during the mid-luteal stage and very low during the post-LH period compared with other
16 stages (P<0.05). MUC1 immunostaining in the LE was apically restricted and was not significantly
17 different across all stages of estrous cycle except at the post-LH peak where it was significantly low.
18 In cell culture, *MUC1* mRNA expression was significantly up-regulated by both steroids either singly
19 or in combination (P<0.05), and down-regulated in the presence of IFN τ . In conclusion, endometrial
20 MUC1 expression is cyclically regulated by both E₂ and P₄ *in vivo* and *in vitro*, and directly down-
21 regulated by IFN τ treatment *in vitro*.

22 **Key words:** MUC1, endometrium, progesterone, estrogen, interferon tau

23 1. Introduction

24 Mucin 1 (MUC1) is a membrane-bound O-glycosylated protein that is a member of the mucin family.
25 It is expressed on the apical surface of mucosal epithelial cells and plays an essential role in forming
26 protective mucous barriers on epithelial surfaces and is also involved in intracellular signalling. In the
27 reproductive system, MUC1 is expressed in the uterus [1] and in the testes [2]. MUC1 has been linked
28 to numerous functions [3] including antimicrobial effects by inhibiting microbial access to the cell
29 surface as well as inhibition of cell-cell adhesion.

30 In the uterus, successful implantation requires complex interaction between trophoblast and maternal
31 endometrium. Available evidence suggests that the burden of implantation lies more on the
32 endometrium rather than the embryo [4]. It has been shown that embryos are capable to attach to
33 endometrial stromal cell culture and others tissues in vitro [5,6] however, embryos cannot attach to
34 uterine endometrium outside the short period of window of receptivity [7]. Non-receptivity of
35 endometrium has been partly attributable to features characteristic of luminal epithelia expressing
36 glycocalyx, of which trans-membrane mucin 1 glycoprotein encoded by MUC1 gene is the most
37 widely expressed and distributed in the reproductive tract [3].

38 MUC1 protein is expressed mainly in luminal epithelium (LE) and glandular epithelium (GE) of the
39 endometrium in many mammalian species including mice, rat, pig, sheep, horse and human during
40 various stages of a menstrual or estrous cycles [1, 8-11]. MUC1 is proposed to protect the
41 reproductive system by preventing entrance of pathogens through the LE into endometrium [12]. It
42 also constitutes an impediment to implantation by hindering interaction between families of
43 conformationally smaller adhesion molecules such as integrins expressed on both the trophectoderm
44 and LE [13]. In another perspective, this hindrance to implantation may be perceived as a
45 physiological barrier that ensures only a potentially viable embryo successfully modulates
46 endometrial receptivity and successfully implants. This hypothesis is supported by reduction of cell
47 surface MUC1 in endometrium of women that experienced recurrent spontaneous abortion [14].

48 During implantation of the blastocyst to the endometrial epithelia, MUC-1 glycoforms in the
49 endometrium which have been shown to carry selectin ligands [15] might mediate initial interaction
50 with the L-selectin that is expressed on the trophectodermal surface of the blastocyst [16].

51 Subsequently it is essential that the MUC1 barrier is eliminated to create embryonic access to the
52 uterine epithelium. In most mammalian species including sheep, this appears to be accomplished by
53 down-regulation of MUC1 gene expression, at least locally.

54 Ovarian steroids; progesterone and oestrogen, as well as the presence of embryo have been implicated
55 in MUC1 regulation [1, 13] however MUC1 regulation seems not to follow a general pattern across
56 all mammalian species and its regulation in the endometrium is therefore, species specific.

57 MUC1 is down-regulated before implantation in the receptive endometrium of mice [17], rats [18],
58 pigs [19] and sheep [10]. In contrast, MUC1 is upregulated in human endometrial at implantation [15]
59 however human embryos seems to locally down-regulate MUC1 as shown in maternal primary
60 endometrial cell culture in the region beneath embryo attachment points [1] suggesting regulatory
61 roles of embryo-produced factors.

62 In addition to steroid regulation of endometrial receptivity, $INF\tau$ which is secreted by trophoblast
63 cells in ruminants is responsible for maternal recognition as it acts on uterine epithelium to down-
64 regulate estrogen and oxytocin receptors thus blocking the development of the uterine luteolytic
65 mechanism [20]. Similar molecule is also produced by human embryos [21]. A progressive effort has
66 been made towards understanding transcriptional regulation of MUC1 in reproductive tract [3],
67 however, the mechanism remains to be completely understood. Besides, a direct effect of $INF\tau$ on
68 MUC1 expression in sheep endometrium has not been tested.

69 In the present study, we have investigated the temporal variation of MUC1 mRNA and protein
70 expression in sheep endometrium during different stages of estrous cycle. To evaluate specific
71 regulations, *MUC1* mRNA expression was analysed in primary culture of ovine endometrial cells
72 treated with steroid hormones or interferon-tau ($IFN\tau$).

73

74 2. Material and Methods

75

76 2.1. Experimental design

77

78 All experimental procedures complied with regulations in the UK Animal (Scientific Procedures) Act,
79 1986 and were conducted under a project licence which was approved by The Royal Veterinary
80 College's Ethics and Welfare Committee. In experiment 1, *Mules* ewes (n=15) of similar age (about 2
81 years) were synchronised to a common estrus according to the method described earlier [22]. The
82 animals were killed at precise time-points (n=3 each) as described below and reproductive tracts were
83 harvested for mRNA extraction and immunohistochemistry for protein detection.

84 In experiment 2, mixed endometrial cells were isolated from uteri obtained from abattoir as described
85 in earlier study. The cells were seeded into 24-well plates at a concentration of 5×10^5 cells/well in
86 DMEM.F12 media. At 70% confluence, they were supplemented with serum-free media for 24 h
87 before treatment with one of the following: (i) P₄ (10 ng/mL for 48 h), (ii) E₂ (100 pg/mL for 48 h),
88 (iii) E₂ (100 pg/mL) for the first 12 h followed by P₄ (10 ng/mL) for 36 h. This was compared to iv)
89 Control media or v) IFN τ treatment (10 ng/mL for 48 h, Genway, Oxfordshire, UK). IFN τ treatment
90 was used as a reference group since it is known to down-regulate MUC1 at the time of implantation
91 [20], hence it was used to validate our culture system. At the end of each culture, media was removed.
92 The cells were rinsed with cold PBS twice and total RNA was extracted. The treatments were done in
93 three independent replicates.

94 2.2. Experimental animals and synchronisation

95 All 15 ewes received intravaginal Chronogest® sponges (Intervet UK Ltd., Cambridge, UK) for 11
96 days and treated with 300 IU of PMSG (Intervet UK Ltd, Buckinghamshire, UK) i.m. at the time of
97 sponge removal. Estrus was observed 24 h after sponge removal. Blood samples were collected via
98 jugular vein into 10 ml heparinized tubes at sponge removal and every 2 days until day 6, then every

99 day until the day of final slaughter. The animals were killed in a step-wise manner based on a
100 previous study in our laboratory [23] at time-points corresponded to the following five stages of
101 estrous cycle (i) pre-LH peak, (ii) LH peak, (iii) post-LH peak, (iv) early luteal and (v) mid-luteal,
102 with three animals in each group as shown in Figure 1. Mid-luteal ewes were killed on day 8 of estrus
103 (day 0 = estrus). The other 12 ewes received PGF_{2α} injection (Estrumate; 125µg i.m) on day 11 of
104 estrus at mid-night. Pre-LH ewes were killed at 32 h post PGF_{2α} injection. The remaining 9 animals
105 received GnRH (Receptal 1ml) at 36 h post PGF_{2α}. LH-peak ewes were killed at 3 h post GnRH (39 h
106 post PGF_{2α}). Post-LH ewes were killed at 46 h post PGF_{2α} (10 h post GnRH). Early luteal ewes were
107 killed on 84 h post PGF_{2α} administration.

108 Blood samples were collected after PGF_{2α} injection at the following time points; 0, 28, 30, 32, 34, 36,
109 38, 39, 40, 42, 44, 46, 60, 72 and 84 h. They were centrifuged within few hours after collection and
110 plasma were transferred to 7ml tubes and kept at -20⁰C until the time for LH determination. The
111 reproductive tracts were harvested. Small pieces of the endometrium were carefully dissected from
112 the uterine horns and snap frozen for mRNA extraction and were used to determine *MUC1* mRNA
113 expression using conventional PCR. Sections of uterine horns were also fixed in 4%
114 paraformaldehyde for immunohistochemistry.

115 **2.3. LH determination**

116

117 After 28 hours of PGF_{2α} injection, blood was collected from all animals via the jugular vein at the
118 hours 28, 30, 32, 34, 36, 38, 39, 40, 60 and 84 from the time of PGF_{2α} injection. The time for each
119 group was speculated prior to hormonal determination based on previous works with the same drugs
120 in sheep in our laboratory [24]. Plasma LH was determined (at the School of Human Development,
121 University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK) using radio-
122 immunoassay technique according to the method described in previous study [25]. The sensitivity of
123 the assay was 0.15 ng/mL.

124 **2.4. Endometrial cell isolation and culture**

125

126 The co-culture of both LE and ST where the two cells communicate and interact simulates the *in vivo*
127 condition better than a monoculture of either LE or ST [26]. In addition, paracrine action of the ST
128 cells supports growth of LE cells [27]. Primary endometrial cells containing both uterine LE and
129 Stromal (ST) cells were isolated and cultured following our previously optimized procedure [28].
130 Briefly, sheep endometrial luminal epithelia and stromal cells were isolated in a single digestion of 90
131 min in 50 mL of digestive solution consisting of 25 mg of trypsin III (Roche, Welyn, UK), 25 mg of
132 collagenase II (Roche), 50 mg of bovine serum albumin (BSA, Sigma). The isolated cells were plated
133 at a concentration of 5×10^5 cells/mL and 1 mL of the cell suspension was added per well in a 24-well
134 microplate (Iwaki, Scitech Div., Asahi Techno Glass) with Dulbecco Modified Eagle medium
135 (DMEM/F12, Sigma) containing 10% foetal bovine serum. The plates were then incubated in a humid
136 atmosphere at 37°C with 5% CO₂. The culture media was changed every 48 h for 5-6 days until 70%
137 confluence was achieved. This was followed by a 24 h incubation in serum-free media before
138 treatment supplementation. The cell population was identified using cell morphology [29] and
139 cytoskeletal markers, cytokeratin and vimentin for LE and ST respectively as was described in
140 previous study [27]. The results showed a monolayer of a mixed population of epithelial and stromal
141 cells in the ratio of 6:4.

142 **2.5. Primer design and RT-PCR**

143

144 The primers for MUC1 and a reference gene; GAPDH were designed using 'primer 3' web based
145 software using ovine nucleotide coding sequences published in the National Centre for Biotechnology
146 Information database (<http://www.ncbi.nlm.nih.gov/Database/index.html>). Primer alignment and
147 specificity was checked using the BLAST search tool at the NCBI website (<http://www.idtdna.com/>)

148 analyzer/Applications/OligoAnalyzer/Default.aspx). Sequence information, accession numbers and
149 expected product lengths as well as the running conditions of these primers are provided in Table 1.

150 For the endometrial cell culture, total RNA was extracted using a column method (RNeasy Mini Kits;
151 QIAGEN Ltd, West Sussex, UK) according to the manufacturer's instruction (www.qiagen.com/goto/microRNAprotocols). The procedures have been described in an earlier study [30]. The concentration
152 and purity of the isolated RNA samples was determined with a NanoDrop ND-1000
153 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). All samples had an A260/280
154 ratio of absorbance (A) between 1.8 and 2.1. The integrity of the RNA was confirmed by running it on
155 a 1% formaldehyde gel to visualize the 18S and 28S rRNA bands. To eliminate potential genomic
156 DNA carry over, 1 µg mRNA from each sample was treated in a single reaction with DNase in
157 accordance with manufacturer's guideline (Promega Corporation, Madison, WI). DNase-treated RNA
158 (1 µg) was reverse transcribed using Reverse Transcription System Kit (Promega) in a 20 µl reaction
159 solution as was described in a previous study [31].

160

161 The primers were used to run a conventional PCR using a Multiplex kit (Qiagen) according to
162 manufacturer's instruction in a 50 µl reaction containing the following; 25 µl Multiplex master mix,
163 10 µl Q-solution, 5 µl primer (2 uM), 5 µl RNase-free water and 5 µl cDNA of the test sample. In the
164 negative and positive control templates, the sample cDNA was replaced with nuclease-free water and
165 cDNA from endometrial strips respectively. The reaction was run for 35 cycles on a thermal cycler
166 (Techne PCR Machine TC312; Scientific Laboratory Supplies, Yorkshire, UK). The amplicons were
167 visualized by electrophoresis on 1% agarose gels. The amplicon bands for MUC1 were quantified
168 with AlfaEase software as reported in earlier study [32] and expressed as fold change compared to the
169 control after initial normalisation with GAPDH.

170 **2.6. MUC1 immunostaining and quantification**

171

172 MUC1 immunostaining was performed according to a standard IHC technique as previously
173 described [31]. Briefly, the uterine sections of 5 μm in thickness were mounted on superfrost slides
174 (VWR international Co., Leicestershire, UK) and rehydrated in a gradient of ethanol following
175 dewaxing in clearing agent; Histoclear (Fisher Scientific, Loughborough, UK). Rabbit polyclonal
176 Anti-MUC1 (Abcam, Cambridge) was used at a concentration of 2 $\mu\text{g}/\text{mL}$ and incubated in a
177 humidified chamber at 4°C overnight. A biotinylated anti-rabbit secondary antibody (Dako, Denmark,
178 at 1:100) was then applied followed by Vectorstain ABC kit according to the manufacturer's
179 instructions (Vector Laboratories, Peterborough, UK). In the negative controls, the primary antibody
180 was replaced with normal rabbit IgGs (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) at the
181 same concentration.

182 The intensity of staining in the endometrial luminal epithelia was scored with the aid of a user-defined
183 digital quantitative image analysis system (VLOCITY 5.5; PerkinElmer, Inc., MA, USA) as described
184 and validated in an earlier study [33] and classified on a scale of 0 to 3, where 0 = negative staining, 1
185 = weak staining, 2 = moderate staining, and 3 = strong staining. For statistical analysis, the expression
186 level of MUC1 was evaluated using a Histology score (H-SCORE), from the intensity and area
187 proportion scores using the following equation: $\text{H-score} = [(1 \times \% \text{ area expression of score } 1) + (2 \times$
188 $\% \text{ area expression of score } 2) + (3 \times \% \text{ area expression of score } 3)]$, giving a possible range of 0-300
189 [34]. Each region was assessed based on at least 10 fields of digital format image taken at 400 \times
190 magnification with a light microscope.

191 **2.7. Statistical analysis**

192

193 Data are presented as mean \pm SEM. All data were tested for homogeneity by Levene's test and were
194 normally distributed. Analysis was done using ANOVA with SPSS 18.0 for Windows (Chicago, IL,

195 USA). One way ANOVA was used to analyse the effect of stages of estrous cycle or treatment on
196 MUC1 expression. If the treatment effect was significant, Bonferroni post hoc tests were performed
197 for pairwise comparisons. Significance was established at $P < 0.05$.

198

199 **3. Results**

200 **3.1. LH profile**

201

202 The mean plasma LH profiles of all animals at different time points are presented in Fig. 2. The LH
203 peak was evident at 39h after $\text{PGF}_{2\alpha}$ injection which confirms successful synchronisation schedule
204 and precise timing of sample collection. At the time of slaughter, the plasma LH concentrations were
205 0.7 ± 0.07 , 28.5 ± 4.5 , 2.9 ± 0.8 and 0.6 ± 0.07 ng/mL for the Pre-LH peak, LH peak, Post-LH peak
206 and Early-luteal groups respectively.

207 Fig. 2

208 **3.2. MUC1 mRNA expression in sheep endometrium during different stages of estrous cycle**

209

210 *MUC1* mRNA was expressed in the endometrial tissue in the pre-LH and LH peak stages at similar
211 level ($P > 0.05$). This was followed by a significant ($P < 0.01$) transient reduction at the post-LH stage
212 (Fig. 3). As the cycle entered into early luteal stage, there was a significant increase in the MUC1
213 transcripts expression compared to the post-LH samples and similar to those at pre-LH and LH peak.
214 Expression of MUC1 mRNA in the endometrium was maximum in the mid-luteal phase compared
215 with other stages.

216 Fig. 3

217 3.3. MUC1 protein expression in sheep endometrium during different stages of oestrous cycle

218

219 MUC1 immunostaining in the LE was detected during all stages of estrous cycle at the apical surface
220 of the LE and glandular epithelium (GE) cells. It was also observed that MUC1 protein protruded well
221 above the cell surface of the LE. It was strongly present in the uterus at Pre-LH and LH stages as well
222 as early and mid-luteal stages. In contrast, a significantly ($P<0.05$) lower staining intensity was
223 observed in the post-LH group (Fig. 4). The negative control sections had no background staining.

224 Fig. 4

225

226 3.4. MUC1 expression in endometrial cells treated with steroids

227

228 Supplementation with P_4 and/or E_2 increased *MUC1* mRNA expression in the endometrial cell culture
229 compared to the hormone-free control and $IFN\tau$ treatment ($P<0.05$, Fig. 5). A relative increment
230 observed in the E_2+P_4 group was not significantly higher ($P>0.05$) than either P_4 or E_2 alone.
231 Treatment with $IFN\tau$ resulted in a significant ($P<0.05$) reduction in *MUC1* mRNA compared to the
232 control and steroid treatments.

233 Fig. 5

234 4. Discussion

235

236 In the present study, we evaluated the expression of *MUC1* mRNA and protein in sheep endometrium
237 at precise time-points during follicular and luteal phases of estrous cycle. This was further
238 complemented with studying the effect of steroid hormones and $IFN\tau$ on *MUC1* mRNA expression in
239 endometrial cell cultures *in vitro*. The results showed that *MUC1* mRNA and protein expression in
240 sheep endometrium were variably highly expressed during all stages of estrous cycle except a
241 transient down-regulation at the post-LH peak stage. *MUC1* expression *in vitro* was up-regulated in

242 the presence of one or both steroid hormones, and down-regulated by INF γ .

243 Highest expression of MUC1 mRNA was seen during the luteal phase especially at the mid-luteal
244 stage compared to other stages of estrous cycle. These stages correspond to the period of high plasma
245 progesterone and its associated dominance in the endometrial LE. These results agree with previous
246 reports of increased MUC1 expression under progesterone-dominated endometrial epithelium in
247 rabbit [35] and human [1]. Interestingly, we could detect a significant drop in *MUC1* mRNA
248 expression at post LH peak stage which was also confirmed by immunostaining. This may be
249 explained by low steroid concentrations at this time point. During post-LH peak, transition from
250 follicular to luteal phases involves decreasing estrogen level to basal while progesterone level is still
251 low. This is also consistent with our observation that the control endometrial cell cultures had lower
252 MUC1 expression compared to those treated with P₄ and/or E₂. During the luteal phase, high
253 MUC1 immunostaining was previously reported at the apical surface of the uterine LE at Days 1, 3, 5,
254 and 7 of the estrous cycle which was then decreased until Day 15 [10]. MUC1 expression after day
255 15 and during the follicular phase of oestrous cycle was not examined in the later study [10]. We
256 observed protrusion of MUC1 from the apical surface of the LE which is in agreement with earlier
257 report of its being a trans-membrane protein with a large mucin-like extracellular domain, projecting
258 so high above the cell surface beyond the region most common receptors are located [3].

259 The apical expression of MUC1 protein in the endometrial LE and GE observed in the present study is
260 at par with the results of Johnson *et al.* [10]. In addition, our data has revealed a transient decline in
261 MUC1 mRNA and protein expression at the post-LH in both regions providing further evidence for
262 MUC1 dependency on steroid hormones [1]. This period coincides with the optimum insemination
263 time in sheep. Therefore it is reasonable to conceptualise that the reduction in MUC1 may allow
264 sperm interaction with endometrial epithelium during transport in the uterus and facilitate sperm
265 capacitation or transport as was earlier suggested [36]. After copulation/insemination, sperm-
266 endometrial interactions are evident [37,38] and it is hypothesised in a recent review [39] that these
267 interactions may play regulatory roles in induction of immunologic tolerance against paternal
268 antigens, preparation of the endometrium for implantation and maintenance of pregnancy.

269 In the cell culture, both steroids caused a significant increase in *MUC1* transcript expression. The
270 relative increase in expression of *MUC1* after initial priming of the endometrial cell with E₂ prior to
271 P₄ treatment is understandable because E₂ is known to up-regulate progesterone receptors (PR) [40]
272 such that treatment with progesterone later produced a higher effect than individual steroids. This
273 concurs with the finding of earlier study in human Ishikawa cell line [41]. In a related study, *MUC1*
274 mRNA expression was higher in infertile women with ovulatory polycystic ovarian syndrome than
275 fertile women [42].

276 In the present study, we found that exposure of endometrial cell culture to IFN τ in the absence of
277 steroids directly induce a reduction in *MUC1* mRNA expression IFN τ concentration (1130 ng/mL)
278 used here mimics the amount produced by day-8 harvested ovine embryo (11 ng/mL) after *in vitro*
279 culture for 24 h [43]. Since IFN τ is the embryo signal of pregnancy in sheep [20], this result is at par
280 with down-regulation of MUC1 by the human blastocyst through a paracrine signal especially at the
281 region of implantation in human endometrial epithelial cells [1]. Similarly, the same observation (loss
282 of MUC1) due to embryo signal was also observed in rabbit epithelia co-culture with blastocyst [35].
283 In the later study, loss of MUC1 from the epithelial surface was confined only to implantation sites
284 (region directly beneath the blastocyst) while high level of MUC1 expression continued in non-
285 implantation regions. We did not study the interactions between IFN τ and steroid hormones on MUC1
286 expression in cell culture. It has been postulated that, in sheep, extended exposure of LE and GE cells
287 to elevated progesterone levels result in down-regulation of progesterone receptors in LE and GE but
288 not in stromal cells and was associated with a reduction in MUC1 expression [44]. Simulating these
289 changes *in vitro* is difficult due to the complexity of the interaction between different cells types, cell
290 differentiation and loss of specific functions during prolonged culture conditions. Nevertheless, our
291 results simply suggest that ovine blastocysts can directly reduce MUC1 expression in endometrial
292 cells which may play a novel regulatory role during embryo adhesion in sheep.

293 **5. Conclusion**

294

295 We show evidence that *MUC1* mRNA and protein expression in sheep endometrium are variably
296 highly expressed both during the progesterone dominant luteal phase and the estrogen dominant
297 follicular phase. We have also demonstrated that *in vitro* using endometrial cell cultures where either
298 estrogen or progesterone supplementation up-regulated *MUC1*. The transition at post-LH peak stage
299 was an exception where a transient down-regulation of MUC1 was observed both at mRNA and
300 protein levels. The physiological role of this transient down-regulation during this period is yet to be
301 investigated.

302

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304

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308 **Figure Captions**

309

310 **Fig. 1. Estrus synchronization and hormonal treatments of ewes and timing of sample**

311 **collections.** Blood samples were collected at all time-points after PGF2 α injection. A total of 15 ewes
312 were used. Three ewes were sacrificed in each group

313 **Fig. 2. LH concentration in blood samples collected at different time points during the**

314 **experiment.** Values are presented as Mean \pm SEM. From 0-32h, n=12; 36-39h, n=9; 40-46h, n=6; 60-
315 84h, n=3.

316 **Fig. 3. Representative gel images of RT-PCR products for MUC1 and GAPDH in sheep**

317 **endometrium during different stages of estrous cycle.** Bands were quantified with Alpha EaseFC
318 software and presented in the bar chart as mean \pm SEM. Legends: Lut; luteal, MUC1; mucin 1,
319 GAPDH; glyceraldehyde 3-phosphate dehydrogenase. Estrous Bars with different superscripts are
320 significantly different at ^a vs ^b or ^c P<0.01 or ^b vs ^c P<0.05

321 **Fig. 4. (A) Photomicrograph of MUC1 protein expression and (B) bar chart presentation of H-**

322 **Score with Volocity software.** Data are shown as mean \pm SEM from ten different scored regions
323 from each stage of estrous cycle (n=3 each). Significant difference is established at x vs y or z P
324 <0.05; y vs z P <0.1.

325 **Fig. 5. (A) Representative gel image of MUC1 (upper panel) and GAPDH (lower panel) PCR**

326 **products from endometrial cell culture treated with progesterone (P₄), oestradiol (E₂), E₂ + P₄**
327 **or interferon tau (IFN τ) and (B) bar chart presentation of band quantification with AlfaEase**

328 **software.** Expression of MUC1 mRNA was compared to the control after normalisation with GAPDH
329 as the reference gene. Data are shown as mean \pm SEM from three independent replicates. Different
330 superscripts indicate significant difference at P < 0.05.

331 **References**

332

- 333 [1] Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J, et al. Human
334 endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the
335 human blastocyst. *Biol Reprod.* 2001;64:590-601.
- 336 [2] Martinez-Conejero JA, Garrido N, Remohi J, Pellicer A, Simon C, Meseguer M. MUC1 in human
337 testis and ejaculated spermatozoa and its relationship to male fertility status. *Fertil Steril.*
338 2008; 90:450-2.
- 339 [3] Brayman M, Thathiah A, Carson DD. MUC1: a multifunctional cell surface component of
340 reproductive tissue epithelia. *Reprod Biol Endocrinol.* 2004;2:4.
- 341 [4] Sharkey AM, Smith SK. The endometrium as a cause of implantation failure. *Best Pract Res Cl*
342 *Ob.* 2003;17:289-307.
- 343 [5] Kirby DR. The development of mouse blastocysts transplanted to the scrotal and cryptorchid
344 testis. *J Anat.* 1963;97:119-30.
- 345 [6] Carver J, Martin K, Spyropoulou I, Barlow D, Sargent I, Mardon H. An in-vitro model for stromal
346 invasion during implantation of the human blastocyst. *Hum Reprod.* 2003;18:283-90.
- 347 [7] Psychoyos A. Uterine receptivity for nidation. *Ann N Y Acad Sci.* 1986;476:36-42.
- 348 [8] Horne AW, Lalani EN, Margara RA, Ryder TA, Mobberley MA, White JO. The expression
349 pattern of MUC1 glycoforms and other biomarkers of endometrial receptivity in fertile and
350 infertile women. *Mol Reprod Dev.* 2005;72:216-29.
- 351 [9] Dharmaraj N, Gendler SJ, Carson DD. Expression of human MUC1 during early pregnancy in
352 the human MUC1 transgenic mouse model. *Biol Reprod.* 2009;81:1182-8.

- 353 [10] Johnson GA. MUC-1, integrin, and osteopontin expression during the implantation cascade in
354 sheep. *Biol Reprod.* 2001;65(3):820-8.
- 355 [11] Wilsher S, Gower S, Allen WR. Persistence of an immunoreactive MUC1 protein at the fetomaternal
356 interface throughout pregnancy in the mare. *Reprod Fertil Dev.* 2013;25:753-61.
- 357 [12] Lillehoj EP, Hyun SW, Kim BT, Zhang XG, Lee DI, Rowland S, et al. Muc1 mucins on the cell
358 surface are adhesion sites for *Pseudomonas aeruginosa*. *Am J Physiol Lung Cell Mol Physiol.*
359 2001;280:L181-7.
- 360 [13] Aplin JD. Adhesion molecules in implantation. *Rev Reprod.* 1997; 2(2): 84-93
- 361 [14] Hey NA, Li TC, Devine PL, Graham RA, Saravelos H, Aplin JD. MUC1 in secretory phase
362 endometrium: expression in precisely dated biopsies and flushings from normal and recurrent
363 miscarriage patients. *Hum Reprod.* 1995;10:2655-62.
- 364 [15] Hey NA, Aplin JD. Sialyl-Lewis X and sialyl-Lewis A are associated with MUC1 in human
365 endometrium. *Glycoconjugate Journal.* 1996;13: 769-79.
- 366 [16] Genbacev OD, Prakobphol A, Foulk RA et al. Trophoblast Lselectin-mediated adhesion at the
367 maternal-fetal interface. *Science.* 2003; 299:405-8.
- 368 [17] Braga VM and Gendler SJ. Modulation of Muc-1 mucin expression in the mouse uterus during
369 the estrus cycle, early pregnancy and placentation. *J Cell Sci.* 1993; 105(2):397-405
- 370 [18] DeSouza MM, Mani SK, Julian J and Carson DD (1998) Reduction of mucin-1 expression
371 during the receptive phase in the rat uterus. *Biol Reprod.* 58,1503-7
- 372 [19] Bowen JA, Bazer FW and Burghardt RC Spatial and temporal analyses of integrin and
373 Muc-1 expression in porcine uterine epithelium and trophectoderm *in vivo*. *Biol Reprod.*
374 1996;55:1098-106.
- 375 [20] Bazer FW1, Spencer TE, Ott TL. Interferon tau: a novel pregnancy recognition signal. *Am J*
376 *Reprod Immunol.* 1997;37(6):412-20.

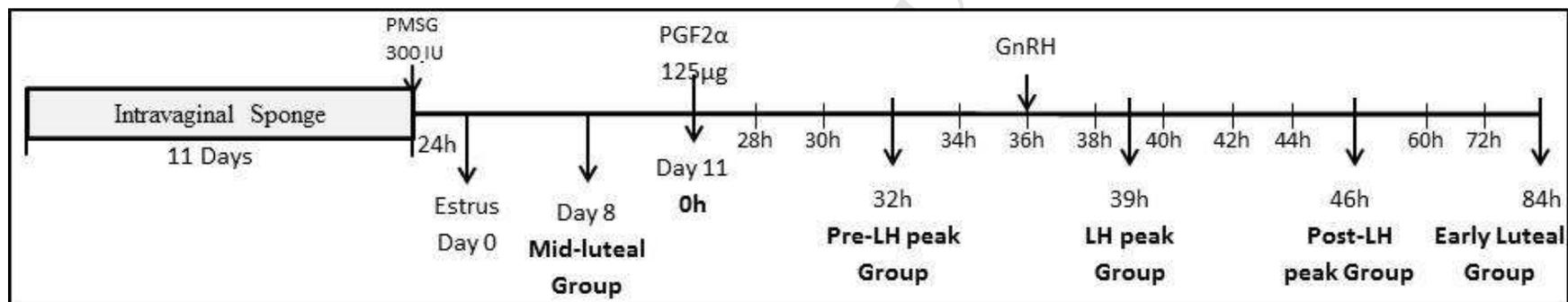
- 377 [21] Whaley AE, Meka CS, Harbison LA, Hunt JS, Imakawa K . Identification and cellular localization of
378 unique interferon mRNA from human placenta. *J. Biol. Chem.* 1994 269: 10864-8.
- 379 [22] Raheem KA, Marei WF, Mifsud K, Khalid M, Wathes DC, Fouladi-Nashta AA. Regulation of
380 the hyaluronan system in ovine endometrium by ovarian steroids. *Reproduction.* 2013;145:491-
381 504
- 382 [23] Perry K, Haresign W, Wathes DC, Khalid M. Hyaluronan (HA) content, the ratio of HA
383 fragments and the expression of CD44 in the ovine cervix vary with the stage of the oestrous
384 cycle. *Reproduction.* 2010;140:133-41.
- 385 [24] Perry KM. Role of Hyaluronan in cervical relaxation of the ewe. PhD Thesis University of
386 London, London 2010.
- 387 [25] Campbell BK, McNeilly AS, Picton HM, Baird DT. The effect of a potent gonadotrophin-
388 releasing hormone antagonist on ovarian secretion of oestradiol, inhibin and androstenedione
389 and the concentration of LH and FSH during the follicular phase of the sheep oestrous cycle. *J*
390 *Endocrinol.* 1990;126:377-84.
- 391 [26] Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over
392 mono cell culture in simulating in vivo environment. *J Steroid Biochem Mol Biol.* 2012;131:68-
393 75.
- 394 [27] Arnold JT, Kaufman DG, Seppala M, Lessey BA. Endometrial stromal cells regulate epithelial
395 cell growth in vitro: a new co-culture model. *Hum Reprod.* 2001;16:836-45.
- 396 [28] Raheem KA, Fouladi-Nashta A. Isolation and characterisation of endometrial luminal epithelia
397 and stromal cells in vitro. *Sok J Vet Sci.* 2014;12:1-8.
- 398 [29] Arnold JT, Kaufman DG, Seppala M, Lessey BA. Endometrial stromal cells regulate epithelial
399 cell growth in vitro: a new co-culture model. *Hum Reprod.* 2001;16:836-45.

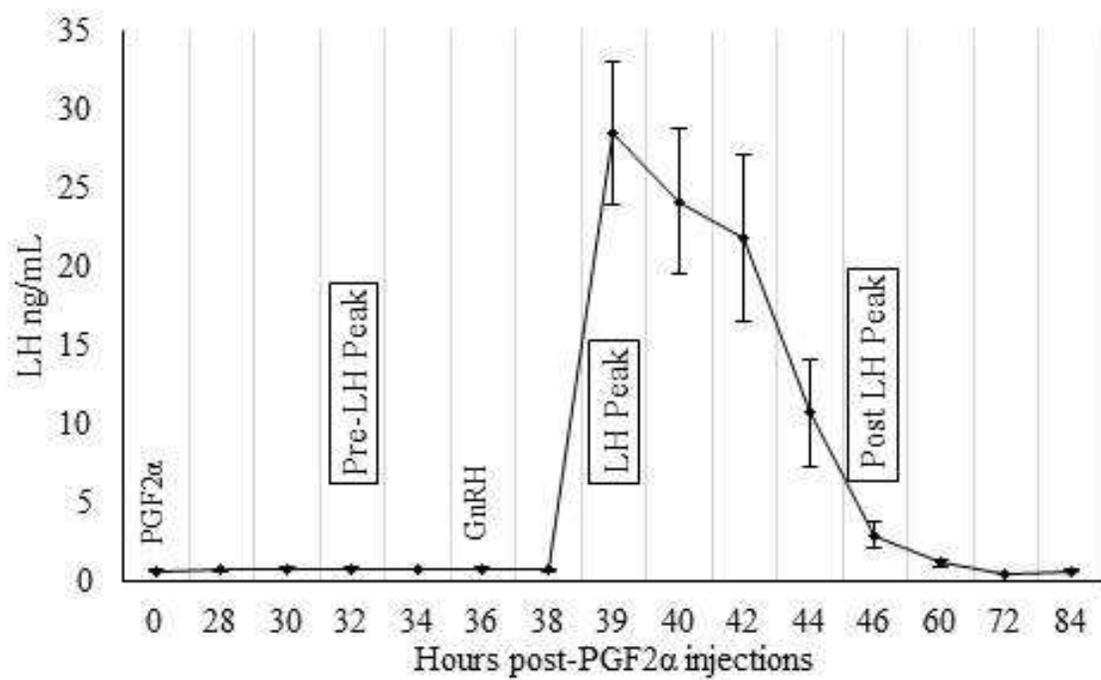
- 400 [30] Cheng Z, Abayasekara DR, Ward F, Preece DM, Raheem KA, Wathes DC. Altering n-3 to n-6
401 polyunsaturated fatty acid ratios affects prostaglandin production by ovine uterine
402 endometrium. *Anim Reprod Sci.* 2013;143:38-47.
- 403 [31] Raheem KA, Marei WF, Mifsud K, Khalid M, Wathes DC, Fouladi-Nashta AA. Regulation of
404 the hyaluronan system in ovine endometrium by ovarian steroids. *Reproduction.* 2013;145:491-
405 504
- 406 [32] Marei WF, Salavati M, Fouladi-Nashta AA. Critical role of hyaluronidase-2 during
407 preimplantation embryo development. *Mol Hum Reprod.* 2013; 19(9):590-9
- 408 [33] Swangchan-Uthai T, Lavender CRM, Cheng Z, Fouladi-Nashta AA, Wathes DC. Time course of
409 defense mechanisms in bovine endometrium in response to lipopolysaccharide. *Biol Reprod.*
410 2012;87:135, 1-13.
- 411 [34] Pierceall WE, Wolfe M, Suschak J, Chang H, Chen Y, Sprott KM, et al. Strategies for H-score
412 normalization of preanalytical technical variables with potential utility to immunohistochemical-
413 based biomarker quantitation in therapeutic response diagnostics. *Anal Cell Pathol (Amst).*
414 2011;34:159-68.
- 415 [35] Hoffman LH, Olson GE, Carson DD, Chilton BS. Progesterone and implanting blastocysts
416 regulate Muc1 expression in rabbit uterine epithelium. *Endocrinol.* 1998;139:266-71.
- 417 [36] Lagow E, DeSouza MM, Carson DD. Mammalian reproductive tract mucins. *Hum Reprod*
418 *Update.* 1999;5:280–92.
- 419 [37] Lovell JW, Getty R. Fate of semen in the uterus of the sow: histologic study of endometrium
420 during the 27 hours after natural service. *Am J Vet Res.* 1968;29:609–25
- 421 [38] Hunter RHF. Experimental studies of sperm transport in sheep, cows and pigs. *Vet Rec.*
422 1985;116:88.
- 423 [39] Rath DA, Knorr CB, Taylor UA. Communication requested: Boar semen transport through the

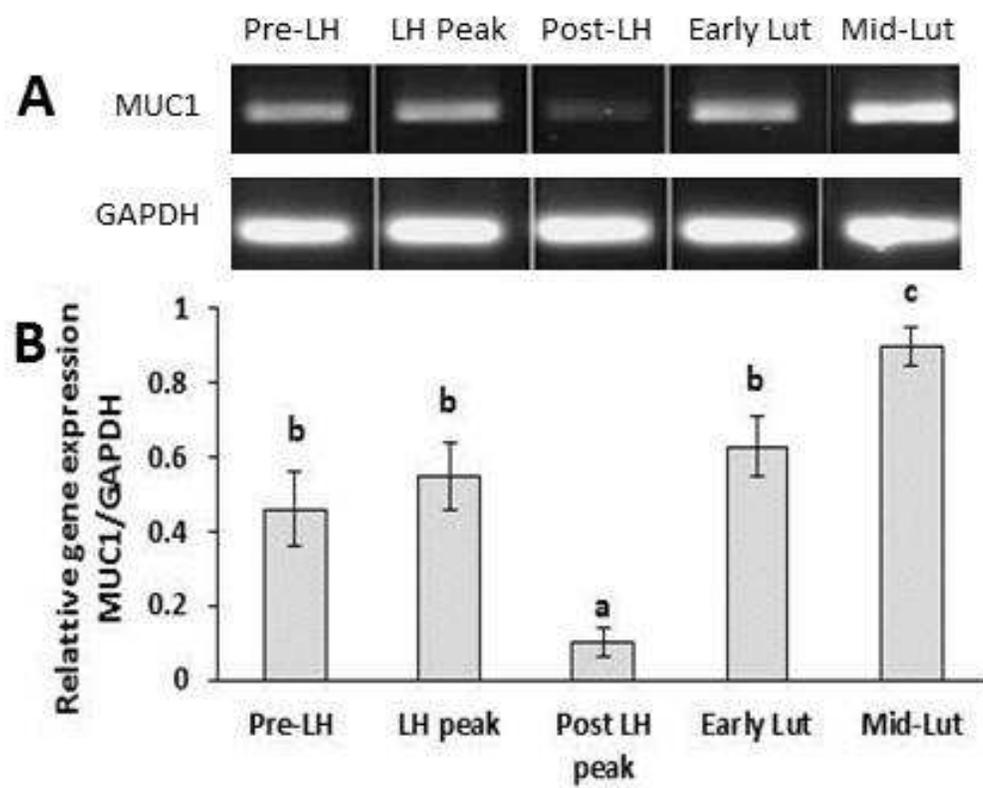
- 424 uterus and possible consequences for insemination. *Theriogenology*. 2016; 85:94–104
- 425 [40] Xiao CW, Goff AK. Hormonal regulation of oestrogen and progesterone receptors in cultured
426 bovine endometrial cells. *J Reprod Fertil*. 1999;115:101-9.
- 427 [41] Singh H, Nardo L, Kimber SJ, Aplin JD. Early stages of implantation as revealed by an in vitro
428 model. *Reproduction*. 2010;139:905-14.
- 429 [42] Margarit L, Taylor A, Roberts MH, Hopkins L, Davies C, Brenton AG, et al. MUC1 as a
430 discriminator between endometrium from fertile and infertile patients with PCOS and
431 endometriosis. *J Clin Endocrinol Metab*. 2010;95:5320-9.
- 432 [43] Lo WC, Summers PM. In vitro culture and interferon-tau secretion by ovine blastocysts. *Anim*
433 *Reprod Sci*. 2002;70:191-202.
- 434 [44] Spencer TE1, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: insights from
435 the sheep. *Reproduction*. 2004;128(6):657-68.

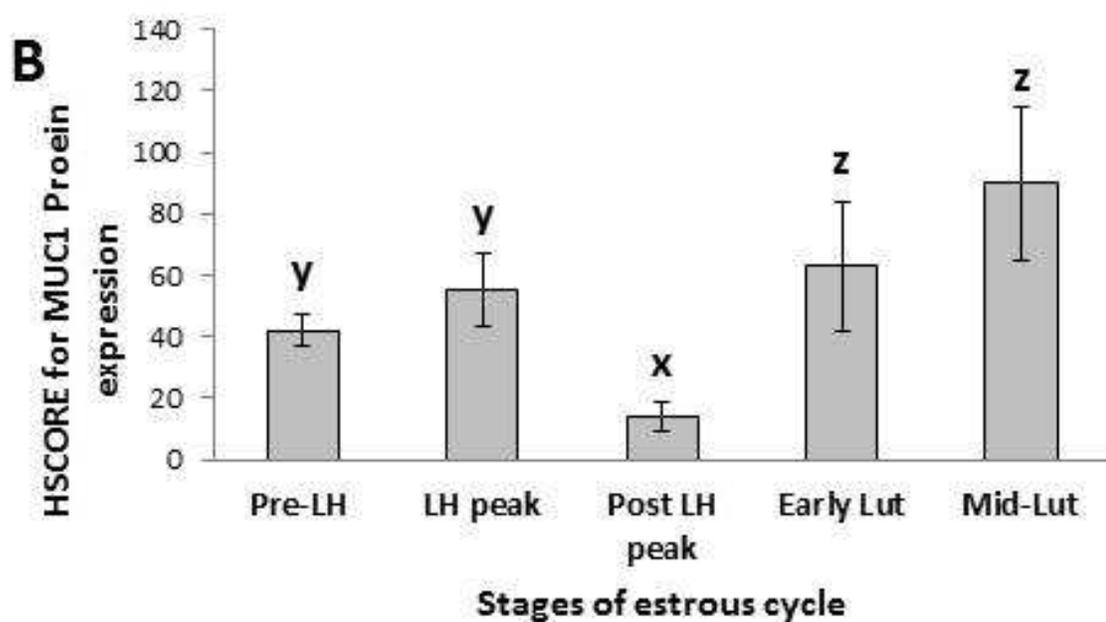
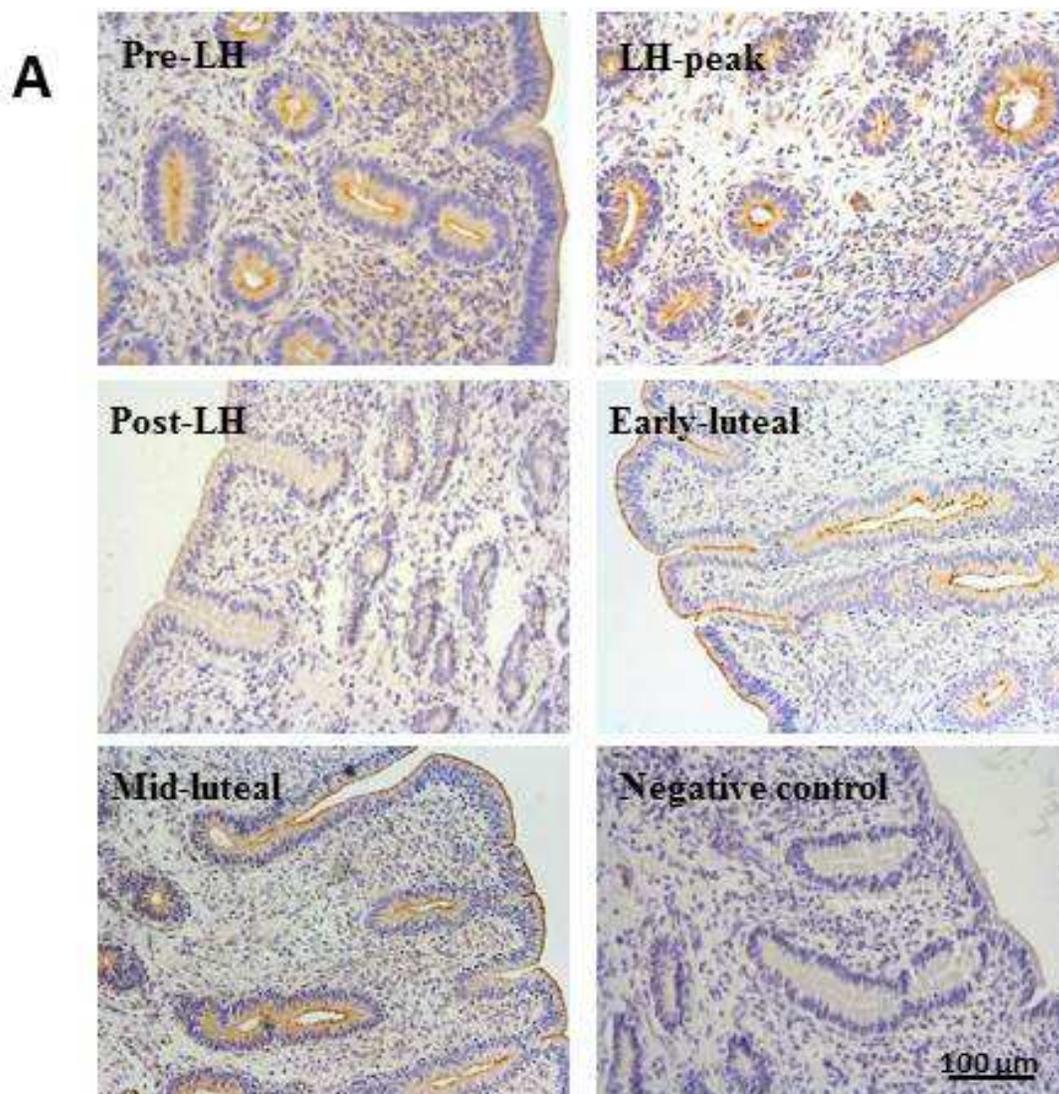
Table 1. Oligonucleotide primer sequence information. Legend: MUC1; mucin 1, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, A; adenine, C; cysteine. G; guanine, T; thiamine, Rev; reverse, FOR; forward

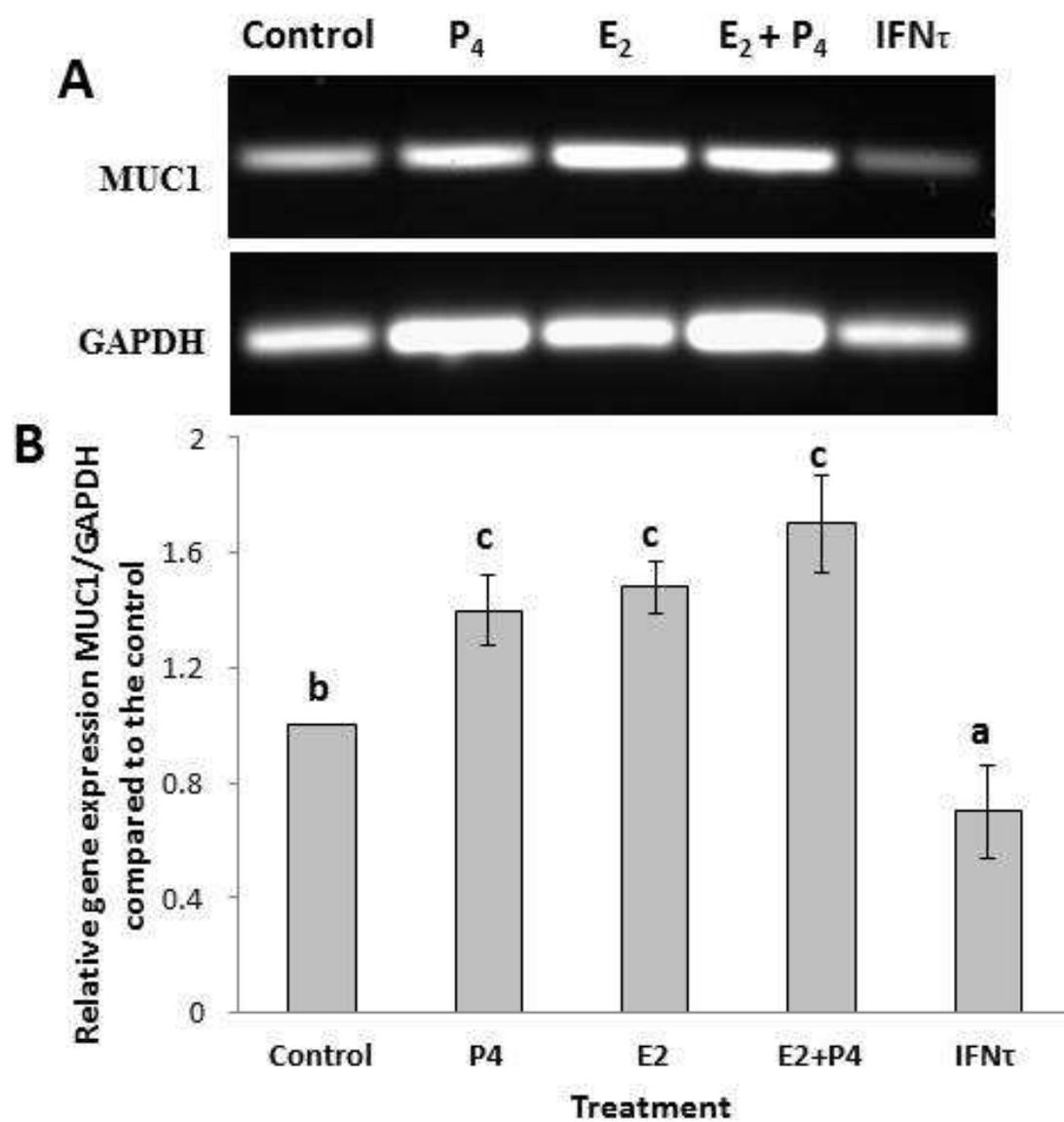
Gene	Primer Sequence (5' - 3')	Size (bp)	Accession no.	Annealing Temp.
MUC1	FOR: CTCAGTCCCCAGCTCTGAAA REV: GAGGCCAGAAAATCCCTCT	252	NM_174115.2	60.0°C
GAPDH	FOR: CACTGTCCACGCCATCACT REV: GCCTGCTTCACCACCTTCT	267	NM_001190390.1	63.3°C











- We studied regulation of MUC1 expression in endometrium during estrous and *in vitro*.
- MUC1 was highly expressed at pre-LH peak, LH-peak and luteal phases
- MUC1 expression was reduced only at the post-LH peak period
- Estrogen and/or progesterone augmented *MUC1* expression in endometrial cell culture
- *MUC1* expression was low in the absence of steroids as in the presence of IFN- τ