Revised

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

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Abstract

In this study, we investigated the expression of MUC1 mRNA and protein in sheep endometrium at different time-points during follicular and luteal phases of estrous cycle, and also determined the effect of steroid hormone treatments and interferon tau (IFNτ) on *MUC1* mRNA expression in endometrial cell culture *in vitro*. In experiment 1, fifteen *Welsh mountain* ewes were synchronised to a common estrus and killed at precise stages of estrous cycle corresponding to i) pre-LH peak, ii) LH peak, iii) post-LH peak, iv) early luteal, and v) mid-luteal. Reproductive tracts were harvested and mRNA was extracted from the endometrial tissues. Parts of the uterine horns were fixed for immunohistochemistry. In experiment 2, mixed populations of ovine endometrial cells (from slaughterhouse material collected at the post-ovulatory stage of the estrous cycle) were cultured to 70% confluence before treatment with i) progesterone (P_4, 10 ng/mL, for 48 h), ii) oestradiol (E_2, 100 pg/mL, for 48 h), or with iii) E_2 priming for 12 h (100 pg/mL) followed by P_4 (10 ng/mL) for 36 h. These were compared to; iv) IFNτ (10 ng/mL, for 48 h), and v) basic medium (DMEM/F12) as control. The results showed that MUC1 mRNA and protein expression in sheep endometrium was highest during the mid-luteal stage and very low during the post-LH period compared with other stages ($P<0.05$). MUC1 immunostaining in the LE was apically restricted and was not significantly different across all stages of estrous cycle except at the post-LH peak where it was significantly low. In cell culture, *MUC1* mRNA expression was significantly up-regulated by both steroids either singly or in combination ($P<0.05$), and down-regulated in the presence of IFNτ. In conclusion, endometrial MUC1 expression is cyclically regulated by both E_2 and P_4 *in vivo* and *in vitro*, and directly down-regulated by IFNτ treatment *in vitro*.

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

1. Introduction

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

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

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

During implantation of the blastocyst to the endometrial epithelia, MUC-1 glycoforms in the endometrium which have been shown to carry selectin ligands [15] might mediate initial interaction with the L-selectin that is expressed on the trophectodermal surface of the blastocyst [16]. Subsequently it is essential that the MUC1 barrier is eliminated to create embryonic access to the uterine epithelium. In most mammalian species including sheep, this appears to be accomplished by down-regulation of MUC1 gene expression, at least locally.

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

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

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

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

2. Material and Methods

2.1. Experimental design

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

In experiment 2, mixed endometrial cells were isolated from uteri obtained from abattoir as described in earlier study. The cells were seeded into 24-well plates at a concentration of 5×10^5 cells/well in DMEM.F12 media. At 70% confluence, they were supplemented with serum-free media for 24 h before treatment with one of the following: (i) P_4 (10 ng/mL for 48 h), (ii) E_2 (100 pg/mL for 48 h), (iii) E_2 (100 pg/mL) for the first 12 h followed by P_4 (10 ng/mL) for 36 h. This was compared to iv) Control media or v) IFNτ treatment (10 ng/mL for 48 h, Genway, Oxfordshire, UK). IFNt treatment was used as a reference group since it is known to down-regulate MUC1 at the time of implantation [20], hence it was used to validate our culture system. At the end of each culture, media was removed. The cells were rinsed with cold PBS twice and total RNA was extracted. The treatments were done in three independent replicates.

2.2. Experimental animals and synchronisation

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

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

Blood samples were collected after $PGF_{2\alpha}$ injection at the following time points; 0, 28, 30, 32, 34, 36, 38, 39, 40, 42, 44, 46, 60, 72 and 84 h. They were centrifuged within few hours after collection and plasma were transferred to 7ml tubes and kept at -20^0C until the time for LH determination. The reproductive tracts were harvested. Small pieces of the endometrium were carefully dissected from the uterine horns and snap frozen for mRNA extraction and were used to determine *MUC1* mRNA expression using conventional PCR. Sections of uterine horns were also fixed in 4% paraformaldehyde for immunohistochemistry.

2.3. LH determination

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

2.4. Endometrial cell isolation and culture

The co-culture of both LE and ST where the two cells communicate and interact simulates the *in vivo* condition better than a monoculture of either LE or ST [26]. In addition, paracrine action of the ST cells supports growth of LE cells [27]. Primary endometrial cells containing both uterine LE and Stromal (ST) cells were isolated and cultured following our previously optimized procedure [28]. Briefly, sheep endometrial luminal epithelia and stromal cells were isolated in a single digestion of 90 min in 50 mL of digestive solution consisting of 25 mg of trypsin III (Roche, Welyn, UK), 25 mg of collagenase II (Roche), 50 mg of bovine serum albumin (BSA, Sigma). The isolated cells were plated at a concentration of 5×10^5 cells/mL and 1 mL of the cell suspension was added per well in a 24-well microplate (Iwaki, Scitech Div., Asahi Techno Glass) with Dulbecco Modified Eagle medium (DMEM/F12, Sigma) containing 10% foetal bovine serum. The plates were then incubated in a humid atmosphere at 37°C with 5% CO_2. The culture media was changed every 48 h for 5-6 days until 70% confluence was achieved. This was followed by a 24 h incubation in serum-free media before treatment supplementation. The cell population was identified using cell morphology [29] and cytoskeletal markers, cytokeratin and vimentin for LE and ST respectively as was described in previous study [27]. The results showed a monolayer of a mixed population of epithelial and stromal cells in the ratio of 6:4.

2.5. Primer design and RT-PCR

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

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

For the endometrial cell culture, total RNA was extracted using a column method (RNeasy Mini Kits; 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 and purity of the isolated RNA samples was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). All samples had an *A*260/280 ratio of absorbance (A) between 1.8 and 2.1. The integrity of the RNA was confirmed by running it on a 1% formaldehyde gel to visualize the 18S and 28S rRNA bands. To eliminate potential genomic DNA carry over, 1 µg mRNA from each sample was treated in a single reaction with DNAse in accordance with manufacturer's guideline (Promega Corporation, Madison, WI). DNase-treated RNA (1 µg) was reverse transcribed using Reverse Transcription System Kit (Promega) in a 20 µl reaction solution as was described in a previous study [31].

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

2.6. MUC1 immunostaining and quantification

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

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

2.7. Statistical analysis

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

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

3. Results

3.1. LH profile

The mean plasma LH profiles of all animals at different time points are presented in Fig. 2. The LH peak was evident at 39h after $PGF_{2\alpha}$ injection which confirms successful synchronisation schedule and precise timing of sample collection. At the time of slaughter, the plasma LH concentrations were 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 and Early-luteal groups respectively.

Fig. 2

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

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

Fig. 3

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

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

Fig. 4

3.4. MUC1 expression in endometrial cells treated with steroids

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

Fig. 5

4. Discussion

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

the presence of one or both steroid hormones, and down-regulated by INFτ.

Highest expression of MUC1 mRNA was seen during the luteal phase especially at the mid-luteal stage compared to other stages of estrous cycle. These stages correspond to the period of high plasma progesterone and its associated dominance in the endometrial LE. These results agree with previous reports of increased MUC1 expression under progesterone-dominated endometrial epithelium in rabbit [35] and human [1]. Interestingly, we could detect a significant drop in *MUC1* mRNA expression at post LH peak stage which was also confirmed by immunostaining. This may be explained by low steroid concentrations at this time point. During post-LH peak, transition from follicular to luteal phases involves decreasing estrogen level to basal while progesterone level is still low. This is also consistent with our observation that the control endometrial cell cultures had lower MUC1 expression compared to those treated with P_4 and/or E_2. During the luteal phase, high MUC1immunostaining was previously reported at the apical surface of the uterine LE at Days 1, 3, 5, and 7 of the estrous cycle which was then decreased until Day 15 [10]. MUC1 expression after day 15 and during the follicular phase of oestrous cycle was not examined in the later study [10]. We observed protrusion of MUC1 from the apical surface of the LE which is in agreement with earlier report of its being a trans-membrane protein with a large mucin-like extracellular domain, projecting so high above the cell surface beyond the region most common receptors are located [3].

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

In the cell culture, both steroids caused a significant increase in *MUC1* transcript expression. The relative increase in expression of *MUC1* after initial priming of the endometrial cell with E_2 prior to P_4 treatment is understandable because E_2 is known to up-regulate progesterone receptors (PR) [40] such that treatment with progesterone later produced a higher effect than individual steroids. This concurs with the finding of earlier study in human Ishikawa cell line [41]. In a related study, *MUC1* mRNA expression was higher in infertile women with ovulatory polycystic ovarian syndrome than fertile women [42].

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

5. Conclusion

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

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Figure Captions

Fig. 1. Estrus synchronization and hormonal treatments of ewes and timing of sample collections. Blood samples were collected at all time-points after PGF2α injection. A total of 15 ewes were used. Three ewes were sacrificed in each group

Fig. 2. LH concentration in blood samples collected at different time points during the experiment. Values are presented as Mean ± SEM. From 0-32h, n=12; 36-39h, n=9; 40-46h, n=6; 60-84h, n=3.

Fig. 3. Representative gel images of RT-PCR products for MUC1 and GAPDH in sheep endometrium during different stages of estrous cycle. Bands were quantified with Alpha EaseFC software and presented in the bar chart as mean±SEM. Legends: Lut; luteal, MUC1; mucin 1, GAPDH; glyceraldehyde 3-phosphate dehydrogenase. Estrous Bars with different superscripts are significantly different at $^{a \text{ vs } b \text{ or } c}$ $P<0.01$ or $^{b \text{ vs } c}$ $P<0.0.05$

Fig. 4. (A) Photomicrograph of MUC1 protein expression and (B) bar chart presentation of H-Score with Volocity software. Data are shown as mean ± SEM from ten different scored regions from each stage of estrous cycle (n=3 each). Significant difference is established at x vs y or z $P<0.05$; y vs z $P<0.1$.

Fig. 5. (A) **Representative gel image of MUC1 (upper panel) and GAPDH (lower panel) PCR products from endometrial cell culture treated with progesterone (P_4), oestradiol (E_2), $E_2 + P_4$ or interferon tau (IFNτ) and (B) bar chart presentation of band quantification with AlfaEase software**. Expression of MUC1 mRNA was compared to the control after normalisation with GAPDH as the reference gene. Data are shown as mean ± SEM from three independent replicates. Different superscripts indicate significant difference at $P < 0.05$.

331 **References**

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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 feto-
356 maternal 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 trophectodermin 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.

[21] Whaley AE, Meka CS, Harbison LA, Hunt JS, Imakawa K . Identification and cellular localization of unique interferon mRNA from human placenta. J. Biol. Chem. 1994 269: 10864-8.

[22] Raheem KA, Marei WF, Mifsud K, Khalid M, Wathes DC, Fouladi-Nashta AA. Regulation of the hyaluronan system in ovine endometrium by ovarian steroids. Reproduction. 2013;145:491-504

[23] Perry K, Haresign W, Wathes DC, Khalid M. Hyaluronan (HA) content, the ratio of HA fragments and the expression of CD44 in the ovine cervix vary with the stage of the oestrous cycle. Reproduction. 2010;140:133-41.

[24] Perry KM. Role of Hyaluronan in cervical relaxation of the ewe. PhD Thesis University of London, London 2010.

[25] Campbell BK, McNeilly AS, Picton HM, Baird DT. The effect of a potent gonadotrophin-releasing hormone antagonist on ovarian secretion of oestradiol, inhibin and androstenedione and the concentration of LH and FSH during the follicular phase of the sheep oestrous cycle. J Endocrinol. 1990;126:377-84.

[26] Miki Y, Ono K, Hata S, Suzuki T, Kumamoto H, Sasano H. The advantages of co-culture over mono cell culture in simulating in vivo environment. J Steroid Biochem Mol Biol. 2012;131:68-75.

[27] Arnold JT, Kaufman DG, Seppala M, Lessey BA. Endometrial stromal cells regulate epithelial cell growth in vitro: a new co-culture model. Hum Reprod. 2001;16:836-45.

[28] Raheem KA, Fouladi-Nashta A. Isolation and characterisation of endometrial luminal aepithelia and stromal cells in vitro. Sok J Vet Sci. 2014;12:1-8.

[29] Arnold JT, Kaufman DG, Seppala M, Lessey BA. Endometrial stromal cells regulate epithelial 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.

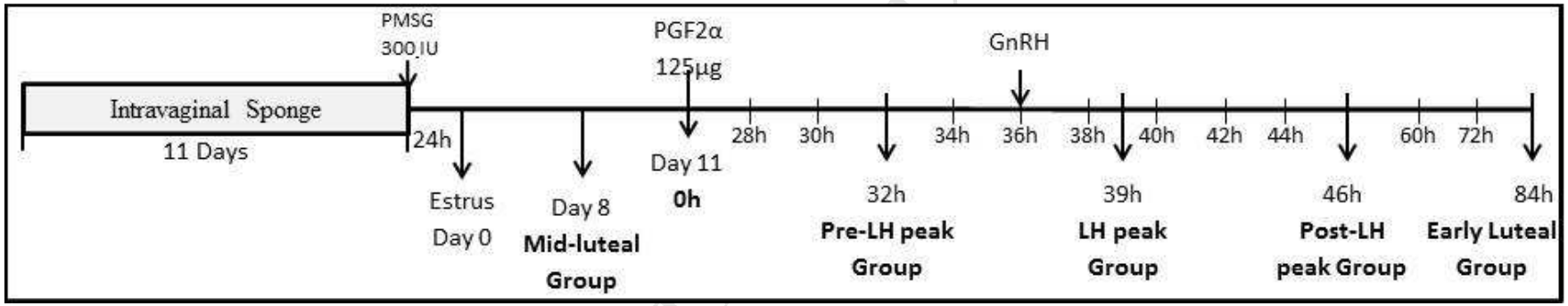
429 [42] Margarit L, Taylor A, Roberts MH, Hopkins L, Davies C, Brenton AG, et al. MUC1 as a
430 discriminator between endometrium from fertile and infertile patients with PCOS and
431 endometriosis. J Clin Endocrinol Metab. 2010;95:5320-9.

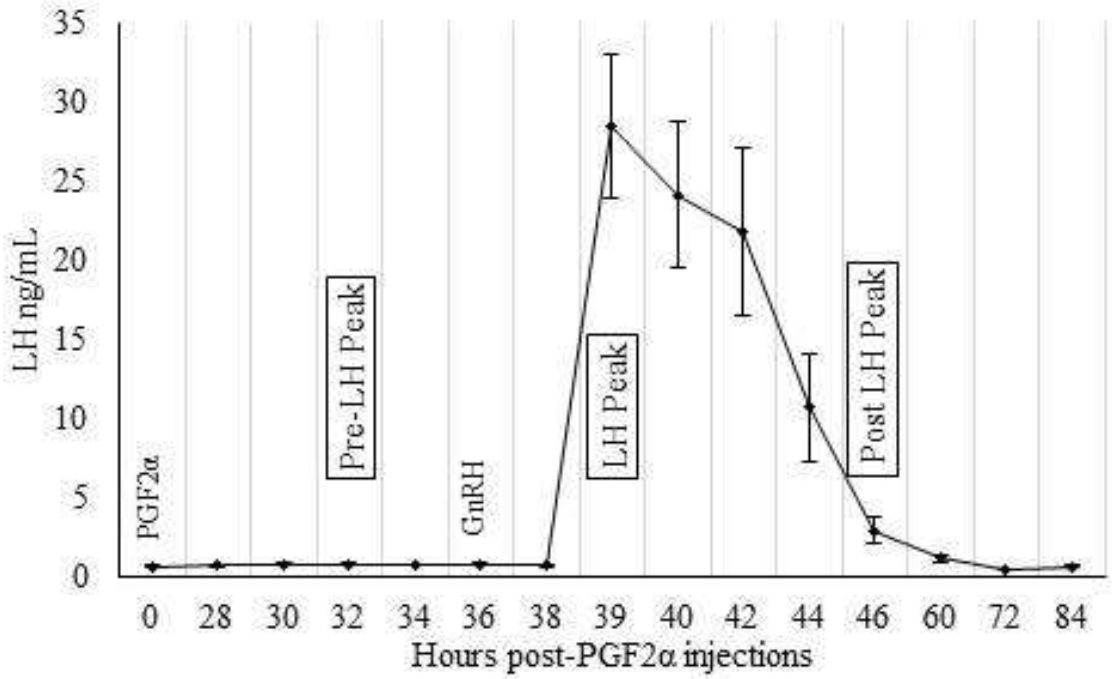
432 [43] Lo WC, Summers PM. In vitro culture and interferon-tau secretion by ovine blastocysts. Anim
433 Reprod Sci. 2002;70:191-202.

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. Oliqonucleotide 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: GAGGCCCAGAAAATCCCTCT	252	NM_174115.2	60.0^{o}C
GAPDH	FOR: CACTGTCCACGCCATCACT REV: GCCTGCTTCACCACCTTCT	267	NM_001190390.1	63.3^{o}C




LH ng/mL
35
30
25
20
15
10
5
0
PGF2α
Pre-LH Peak
GnRH
LH Peak
Post LH Peak
0
28
30
32
34
36
38
39
40
42
44
46
60
72
84
Hours post-PGF2α injections

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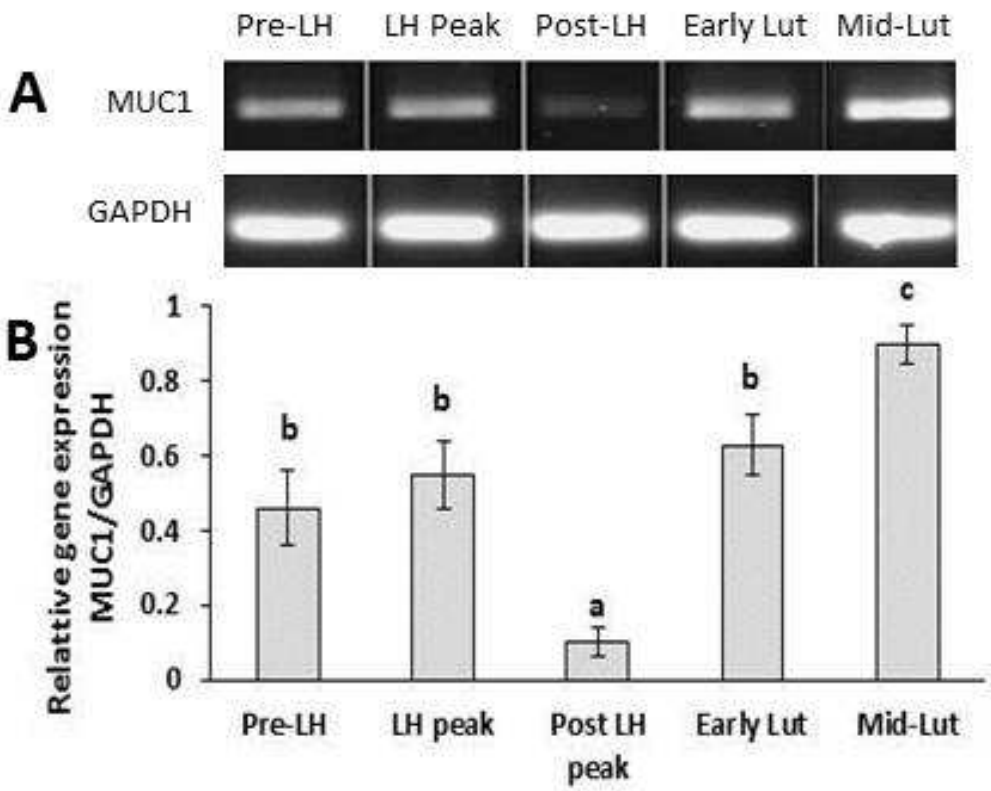
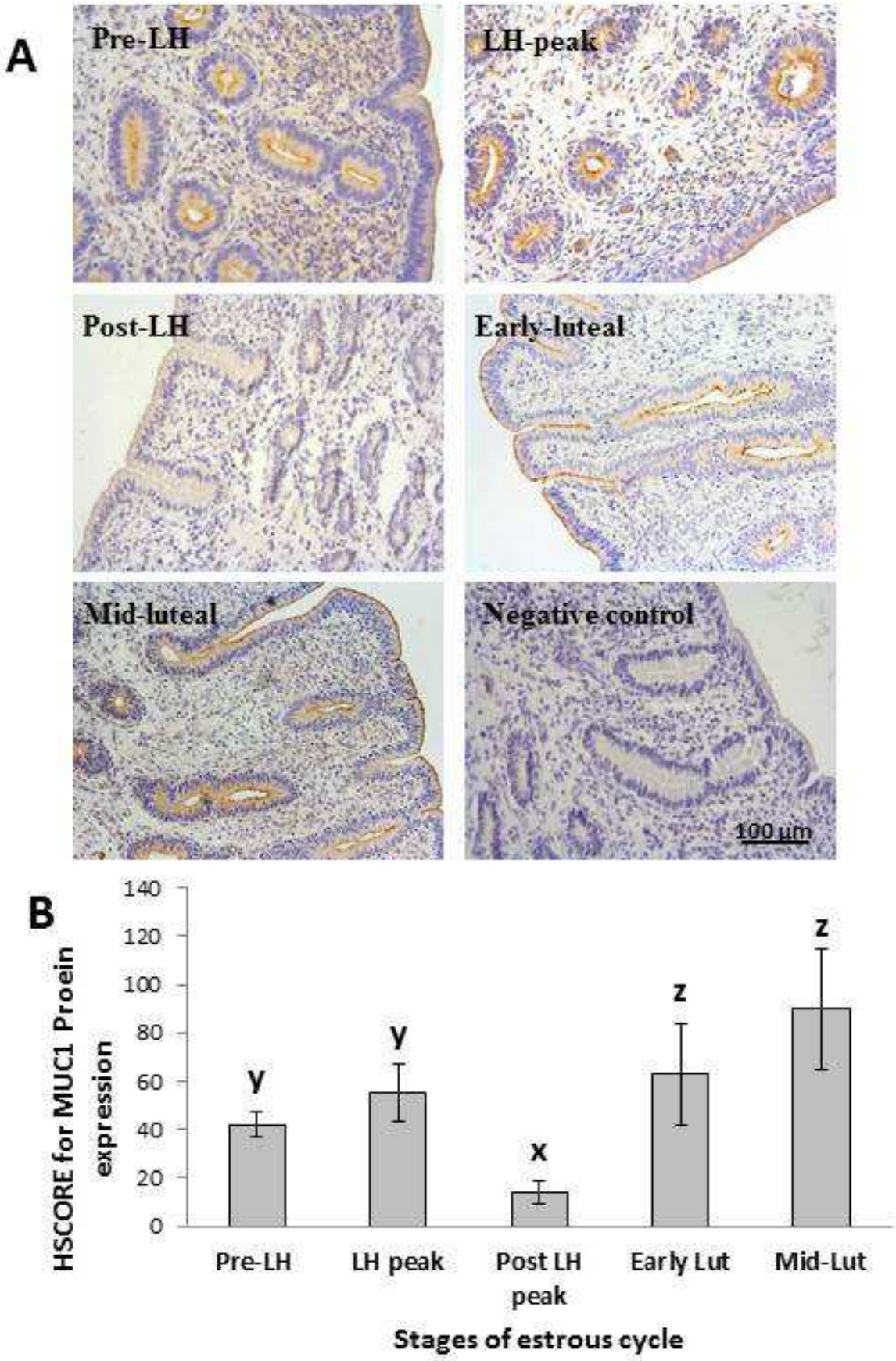
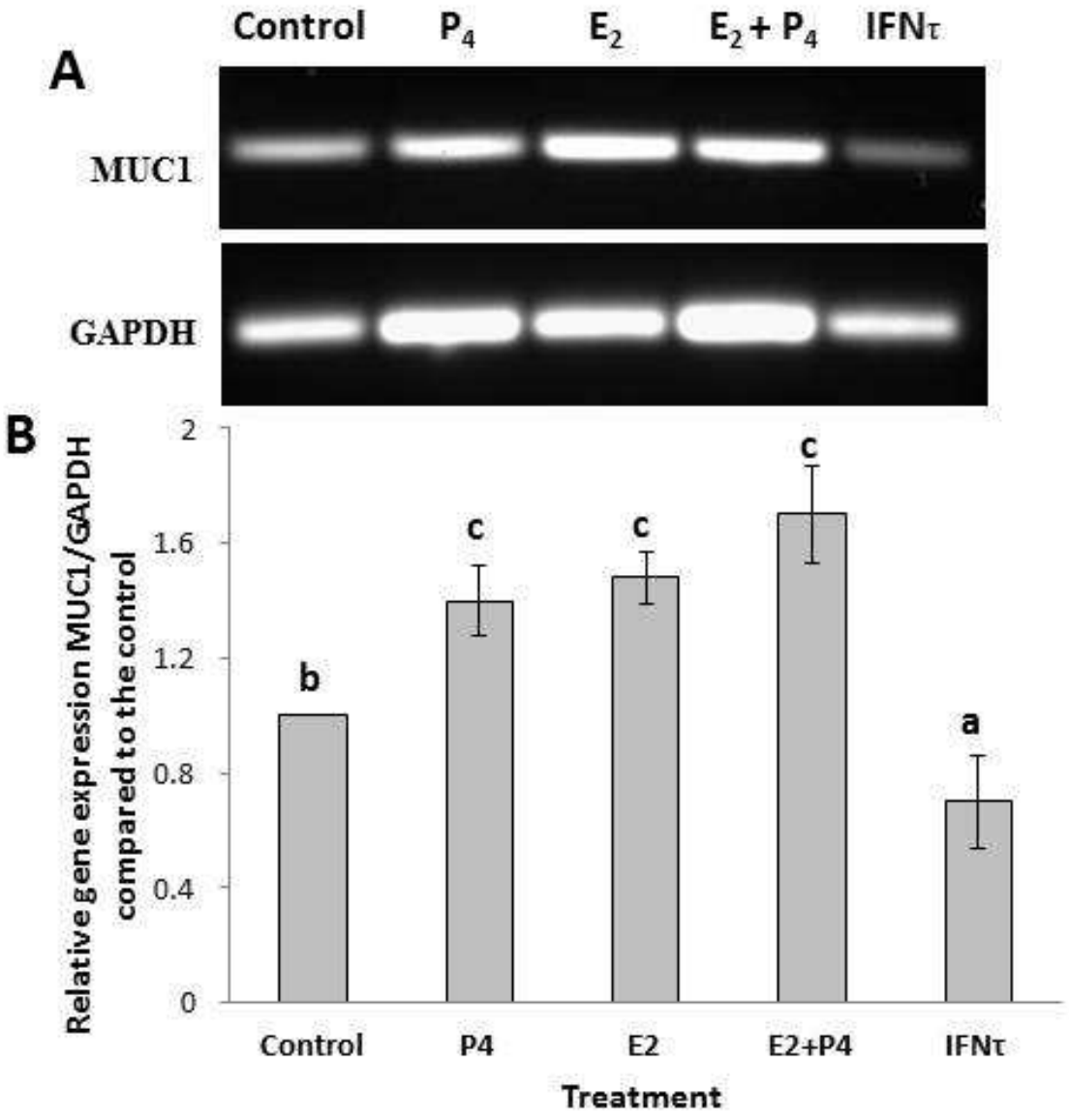

Pre-LH
LH Peak
Post-LH
Early Lut
Mid-Lut
A
MUC1
GAPDH
B
Relattive gene expression MUC1/GAPDH
1
0.8
0.6
0.4
0.2
0
b
b
a
b
c
Pre-LH
LH peak
Post LH peak
Early Lut
Mid-Lut


A
Pre-LH
LH-peak
Post-LH
Early-luteal
Mid-luteal
Negative control
100 µm
B
HSCORE for MUC1 Proein expression
140
120
100
80
60
40
20
0
y
y
x
z
z
Pre-LH
LH peak
Post LH peak
Early Lut
Mid-Lut
Stages of estrous cycle


A
Control
P4
E2
E2 + P4
IFNτ
MUC1
GAPDH
B
Relative gene expression MUC1/GAPDH compared to the control
2
1.6
1.2
0.8
0.4
0
b
c
c
c
a
Control
P4
E2
E2+P4
IFNτ
Treatment

- 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-tau