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The effect of the intra-cervical application of Follicle Stimulating Hormone (FSH) or Luteinizing Hormone (LH) on the pattern of expression of gonadotrophin receptors in the cervix of non-pregnant ewes

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Abstract: During the peri-ovulatory period the cervix relaxes in response to changes in circulating concentrations of reproductive hormones. The present study investigated the role of gonadotrophins in cervical function by examining the expression of FSHR and LHR and their mRNAs following intra-cervical treatment with either FSH or LH. Eighteen ewes were assigned to 4 groups they were then treated with progestagen sponges and PMSG to synchronize their oestrous cycles. Intra-cervical treatments were given 24h after sponge removal as follows: Group 1: FSH 2 mg; Group 2: LH 2 mg; Group 3: Vehicle and Group 4: Control. Cervices were collected 54h after sponge removal and then divided into 3 regions. The expression of FSHR and LHR was determined by immunohistochemistry and FSHR mRNA and LH mRNA by *in situ* hybridization. The expression of LHR, FSHR and their respective mRNAs was compared in 6 tissue layers (luminal epithelium, sub-epithelial stroma, circular, longitudinal and transverse muscle and serosa) and in 3 cervical regions (vaginal, mid and uterine). The results showed that FSH increased transcription of the FSHR gene and the levels of its receptor but only in sub-epithelial stroma of the cervix. FSH also increased the levels of LHR in the cervix but only in the muscle layers. LH had no effect on the levels of FSHR despite the fact that it did increase the level of transcription of the FSHR gene and LH also increased the levels of its own receptor in the cervix but only in the muscle layers and this action was independent of increased levels of transcription of the LHR gene. These findings suggest multiple levels of regulation of cervical LH and FSH receptors and that the gonadotrophins may have a role in relaxation of the cervix during oestrus by regulating their own receptors.

Key words: Sheep, cervix, FSHR, LHR, gonadotrophins

Introduction

One of the main purposes of artificial insemination in sheep breeding is to increase the rate of genetic improvement. However, conventional trans-cervical insemination in sheep gives poor fertility mainly because of the unusual anatomy of the sheep cervix. The ovine cervix is a long, fibrous and convoluted tubular organ that prevents easy passage of an insemination pipette through the cervical lumen (Halbert et al., 1990). There is a degree of natural relaxation at oestrus (Leethongdee et al., 2007b) that is probably regulated by the peri-ovulatory changes in reproductive hormones (Kershaw et al., 2004). The cervix contains receptors for oestradiol, progesterone, oxytocin (Fuchs et al., 1996) as well as those for Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) (Mizrachi and Shemesh, 1999b, 1999a; Fields and Shemesh, 2004) suggesting that the gonadotrophins may have a functional role in cervical physiology at oestrus.

There is good evidence indicating that cervical relaxation is mediated by Prostaglandin E_2 (PGE_2) (Fuchs et al., 2002; Feltovich et al., 2005), and the peri-ovulatory changes in reproductive hormones are associated with increased levels of cervical COX-2 (Kershaw et al., 2007; Kershaw-Young et al., 2009a) and increased cervical synthesis of PGE_2 (Falchi et al., 2009). Similarly in the cow, cervical relaxation during oestrus is mediated by a local increase in Cyclooxygenase-2 (COX-2) and a subsequent increase in PGE_2 production by the cervix (Shemesh et al., 1997a). Prostaglandin E_2 separates collagen fibres causing reduced tensile strength of the cervix (Feltovich et al., 2005) thus allowing the cervical canal to dilate. Naturally occurring cervical relaxation at oestrus is probably the result of complex interactions

among reproductive hormones acting on the cervix. An increase in the levels of receptors for oestradiol and oxytocin during the peri-ovulatory period is thought to mediate increased synthesis of PGE_2 (Shemesh et al., 1997a) leading to remodeling of the extracellular matrix (ECM) (Stys et al., 1981; Ledger et al., 1983) and cervical relaxation.

Gonadotrophin receptors have been identified in the cervix of the cow and both FSH receptor (FSHR) and its mRNA are highest during pro-oestrus and oestrus (Mizrachi and Shemesh, 1999b) at a time when circulating FSH is also high (Shemesh, 2001). Similarly, LHR and its mRNA are also present in the cervix of cows (Shemesh et al., 1997b; Mizrachi and Shemesh, 1999a). The presence of LH receptor (LHR) has been reported in women (Lin et al., 2003) and furthermore intra-cervical human chorionic gonadotrophin (hCG) increased the levels of cAMP and COX-2 in the human cervix (Lin et al., 2003). A role for the gonadotrophins in the process of cervical relaxation although implied by the presence of their receptors and some downstream mediators remains unidentified.

There is very little data on the action of gonadotrophins in the ovine cervix although in a previous study (Leethongdee et al., 2007a) we showed that the local application of FSH and/or an analogue of PGE (Misoprostol) enhanced the penetrability of the cervix (Leethongdee et al., 2007b). Consequently we set out to determine the actions of FSH and LH on the ovine cervix during the peri-ovulatory period of the oestrous cycle. Our hypothesis was that FSH and/or LH are involved in the regulation of cervical relaxation during the peri-ovulatory period. The study

85 described in this paper was an examination of the effects of intra-cervical gonadotrophins on
86 the intra-cervical levels of LHR and FSHR protein and mRNA.

87

88 **Materials and Methods**

89 **Animals and their management**

90 In this study 18 adult Welsh Mountain ewes were divided randomly into two groups of 5 and
91 two groups of 4 ewes. They were all healthy and had body condition scores between 2.5 and
92 3.5 and a mean body weight of 37.8 kg with range of 32 to 42 kg. During the experiment the
93 animals were housed indoors, in groups, on straw and were fed with a commercial concentrate
94 diet *ad libitum* and with hay and water always available. All the experimental procedures with
95 ewes were conducted with the approval of the ethics committee of the Royal Veterinary
96 College, University of London and with authorization from the Home Office (United Kingdom)
97 in compliance with the Animal (Scientific Procedures) Act, 1986.

98

99 **Intra-cervical administration of FSH or LH**

100 The ewes were synchronized to a common day of oestrus using intra-vaginal sponges
101 containing 30 mg of fluorogestone acetate (Chronogest; Intervet UK Ltd, Northamptonshire,
102 UK) for 12 days. The experiment was conducted during the non-breeding season (March to
103 April) therefore, ewes were injected intramuscularly with 500IU of pregnant mare serum
104 gonadotrophin (PMSG; Intervet UK Ltd., Buckinghamshire, UK), at the time of removal of
105 sponges. Ovine FSH (2 mg Ovagen; ICPbio (UK) Limited, Wiltshire, UK) or ovine LH (2 mg,

Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were dissolved in 0.5 ml of 50% gum acacia (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in normal saline and intra-cervical treatments were applied 24 h after removal of the sponges as follows: Group 1, FSH (2 mg) , Group 2, LH (2 mg), Group 3, gum vehicle and Group 4, no vehicle (the procedure was carried out but no vehicle was deposited in the cervix).

Collection of cervical tissue

Ewes were killed 54 h after removal of sponges (i.e. 30h after treatment) with a captive bolt pistol followed by exsanguination. The reproductive tract was removed immediately after death, and kept on ice. All unwanted tissue was trimmed from the cervix which was then divided into 3 approximately equal, transverse segments (Kershaw et al., 2007; Kershaw-Young et al., 2009b; Leethongdee et al., 2010) representing the uterine, middle, and vaginal regions of the cervix. The segments were fixed in neutral-buffered formalin (BDH, VWR International Ltd., Leicestershire, UK) for 24h, and then stored in 70% ethanol. Fixed tissues were embedded in paraffin wax; sections were cut at 7µm on a rotary microtome and mounted onto Superfrost Plus slides (BDH, VWR International Ltd., Leicestershire, UK).

The determination of FSHR and LHR mRNA

The levels of mRNA for FSHR and LHR was determined by in situ hybridization (ISH) using digoxigenin-11-UTP labeled sense and antisense riboprobes for ovine LHR and bovine FSHR as described for our laboratory (Kershaw et al., 2007; Ponglowhapan et al., 2007;

Ponglowhapan et al., 2008; Leethongdee et al., 2010). The sense and antisense riboprobes for FSHR and LHR were made by transcribing the N-terminus sequence of bovine FSHR and ovine LHR complementary deoxyribonucleic acid (cDNA), supplied by Professor Allen Garverick of the University of Missouri-Columbia, Columbia, Missouri, USA (Xu et al., 1995). The cDNAs for FSHR and LHR were cloned into the PGEM-Teasy plasmid (Promega Corporation, Madison, USA). Riboprobes were synthesized with the SP6 and T7 MEGAscript transcription kits (Ambion Ltd, Cambridgeshire, UK) and labeled with digoxigenin-11-UTP (Roche Diagnostics, Mannheim, Germany). In-situ hybridizations for FSHR and LHR mRNAs were performed on four sections (one sense and three antisense) from the each of the uterine, middle, and vaginal regions of the cervix for each ewe using the protocol described in our previous studies (Kershaw-Young et al., 2009b; Kershaw-Young et al., 2010; Leethongdee et al., 2010). Both riboprobes were hybridized at 65°C for 3 h.

The determination of FSHR and LHR protein

The procedure for the immunohistochemical localization was the same as described for our laboratory (Ponglowhapan et al., 2008; Perry et al., 2010; Perry et al., 2012). Immunoperoxidase staining was used to determine the level of LHR and FSHR protein using the polyclonal antibodies for FSHR (H-190, sc-13935) and LHR (H-50, sc-25828; both from Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Sections from the uterine, middle and vaginal regions of the cervix from each animal were examined in triplicate for both positive antibody staining and negative controls. The binding site of the enzyme was stained with diaminobenzidine-based peroxidase substrate (ImmPAC™ DAB, Vector Laboratory Ltd,

Cambridgeshire, England), then counterstained with Hematoxylin (Hematoxylin QS, catalogue number H-3404, Vector Laboratory Ltd, Cambridgeshire, England). Negative controls were performed in the same manner but substituting the primary antibody with the non-immune rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA) at an equivalent concentration.

Quantification of in-situ hybridization and immunohistochemistry staining

The levels of both mRNA and protein for FSHR and LHR were assessed blind in five tissue layers of the cervix, namely the luminal epithelium, sub-epithelial stroma, circular smooth muscle, longitudinal smooth muscle and transverse smooth muscle as described in our previous studies (Ponglowhapan et al., 2007; Ponglowhapan et al., 2008; Leethongdee et al., 2010; Perry et al., 2010; Perry et al., 2012). The five cell layers in each region of the cervix were scored for both the percentage of cells stained and the intensity of staining as described and validated in previous publications from our laboratory (Ponglowhapan et al., 2007; Ponglowhapan et al., 2008; Leethongdee et al., 2010; Perry et al., 2010; Perry et al., 2012).

Statistical analysis

The results are presented as means and the pooled standard error of the difference (S.E.D). The effects of treatment, region and tissue layer as well as their interactions were analyzed using a mixed model ANOVA. Sheep were treated as subjects with cervical region and tissue layer as repeated measures and hormonal treatment as a fixed factor. Where it was appropriate,

additional post-hoc tests comparing the effects of treatment within either cervical regions or cervical layers were made using the Bonferroni test. The tests were carried out using SPSS for Windows (SPSS version 20.0; SPSS Inc., IBM Company Headquarters, Chicago, Illinois, USA). Differences were considered statistically significant when $P \leq 0.05$.

Results

Cervical Layer

There were no significant interactions of cervical layer with either hormonal treatment or the region of the cervix for any of the 4 endpoints (FSHR, LHR, FSH mRNA and LHR mRNA) and the overall effects of cervical layer are presented in anatomical order from the inner luminal epithelium to the outermost layer of smooth muscle (Figure 1). There was no expression detected for any of the endpoints in the outer serosal layer and therefore it has been omitted from the figure. In general the level of expression was highest in the luminal epithelium and lowest in the sub-epithelial stroma with the three muscle layers intermediate (Figure 1). However, there were exceptions to this layer generalization. For FSHR, the mean expression in the sub-epithelial stroma was lower (all <0.01) than all other layers which were not significantly different from each other (epithelium; circular muscle; longitudinal muscle and transverse muscle (Figure 1). For LHR, mean expression in the luminal epithelium was higher (all <0.01) than all other layers which were not significantly different from each other (Figure 1).

For FSHR mRNA, mean expression in transverse muscle was lower (all <0.01) than the other muscle layers (circular muscle and longitudinal muscle) and the epithelium but not the sub-epithelial stroma and the sub-epithelial stroma was not significantly different from any of the other layers (Figure 1). For LHR mRNA, mean expression in the sub-epithelial stroma was lower (all <0.01) than all other layers which were not significantly different from each other (Figure 1).

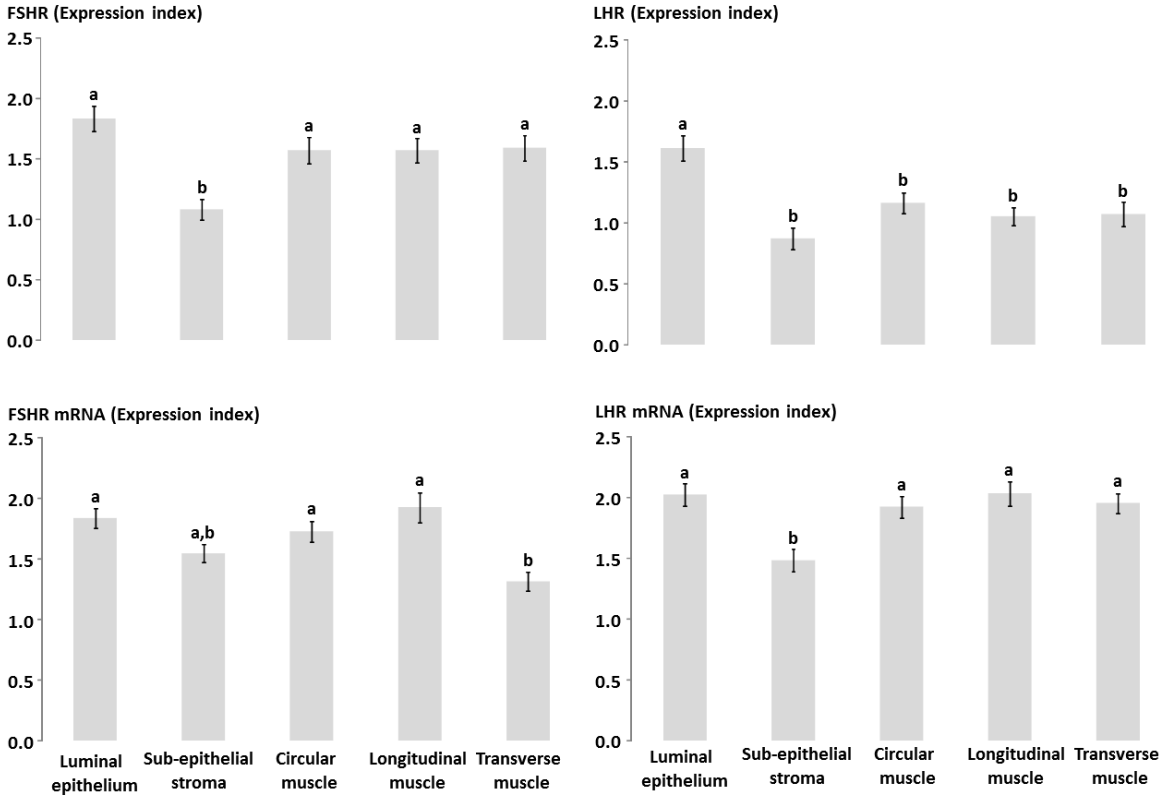

Figure 1: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in the five layers (presented in anatomical order from the inner luminal epithelium to the outer transverse muscle layer) of the sheep cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.

Cervical Region

For FSHR and LHR there were no significant interactions of cervical region with hormonal treatment or cervical layer and the overall effects of cervical region for these two endpoints are presented in Figure 2. The expression indices for FSHR and LHR were not significantly different among the regions of the cervix (Figure 2). Similarly for FSHR mRNA, the level of expression did not vary among the regions (Figure 2). However, for LHR mRNA, mean expression was greater at the vaginal end of the cervix compared to either the middle-region ($P < 0.01$) or the uterine end ($P < 0.05$ – Figure 2).

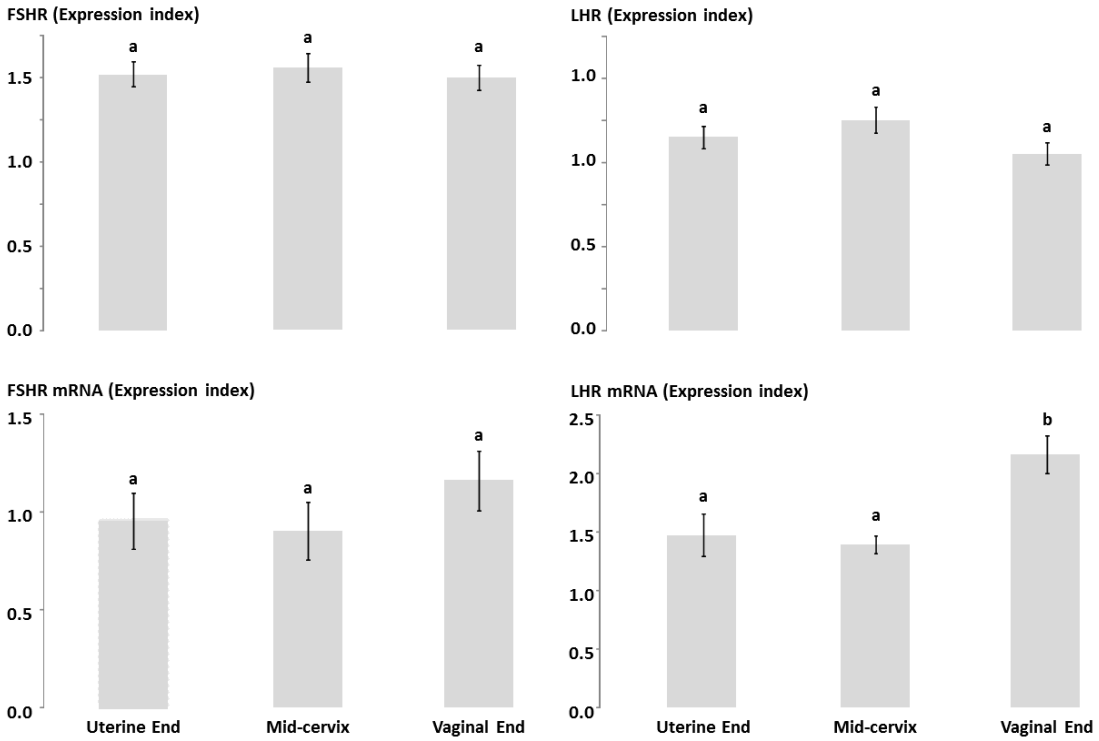

Figure 2: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in three regions of the sheep cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.

Hormonal Treatments

The main effects of intra-cervical application of FSH and LH on the cervical expression of FSH and LH proteins and mRNAs are summarized in Table 1.

Follicle Stimulating hormone (FSH)

Intra-cervical FSH increased the overall expression of FSHR (Figure 3) compared to either the vehicle-treated ($P = 0.006$) or control groups ($P = 0.003$). Post-hoc paired comparisons showed that the effect of FSH was confined to the layer of sub-epithelial stroma at the uterine end of the cervix when compared to either the vehicle-treated ($P = 0.008$) or control groups ($P = 0.003$) and there was no effect in the epithelium or the muscle layers. There were significant effects of intra-cervical FSH on the level of cervical FSHR mRNA compared to vehicle-treated ($P < 0.001$) and control ($P < 0.001$) groups). The effect of FSH was seen in all layers and regions of the cervix (Figure 3).

Intra-cervical FSH also increased LHR in all regions of the cervix compared to vehicle-treated, ($P < 0.001$) and control, ($P < 0.001$) groups (Figure 3). Post-hoc paired comparisons revealed that the effect was confined to the smooth muscle layers (circular muscle, $P = 0.018$ compared to vehicle-treated and $P = 0.004$ compared to controls; longitudinal muscle, $P = 0.009$ compared to vehicle-treated and $P = 0.002$ compared to controls and transverse muscle, $P = 0.026$ compared to vehicle-treated and $P = 0.002$ compared to controls). There was no significant effect in either the sub-epithelial stroma or the luminal epithelium. By contrast there was no effect of intra-cervical FSH on the expression of LHR mRNA (Figure 3).

Luteinizing Hormone (LH)

Intra-cervical LH had no effect cervical FSHR (Figure 3) when compared to either the vehicle-treated (P = 0.164) or control groups (P = 0.108). By contrast intra-cervical LH increased the level of FSHR mRNA compared to the vehicle-treated (P = 0.001) and control groups (P < 0.001) and the effect was seen in all regions and layers of the cervix (all P values at least P = 0.007).

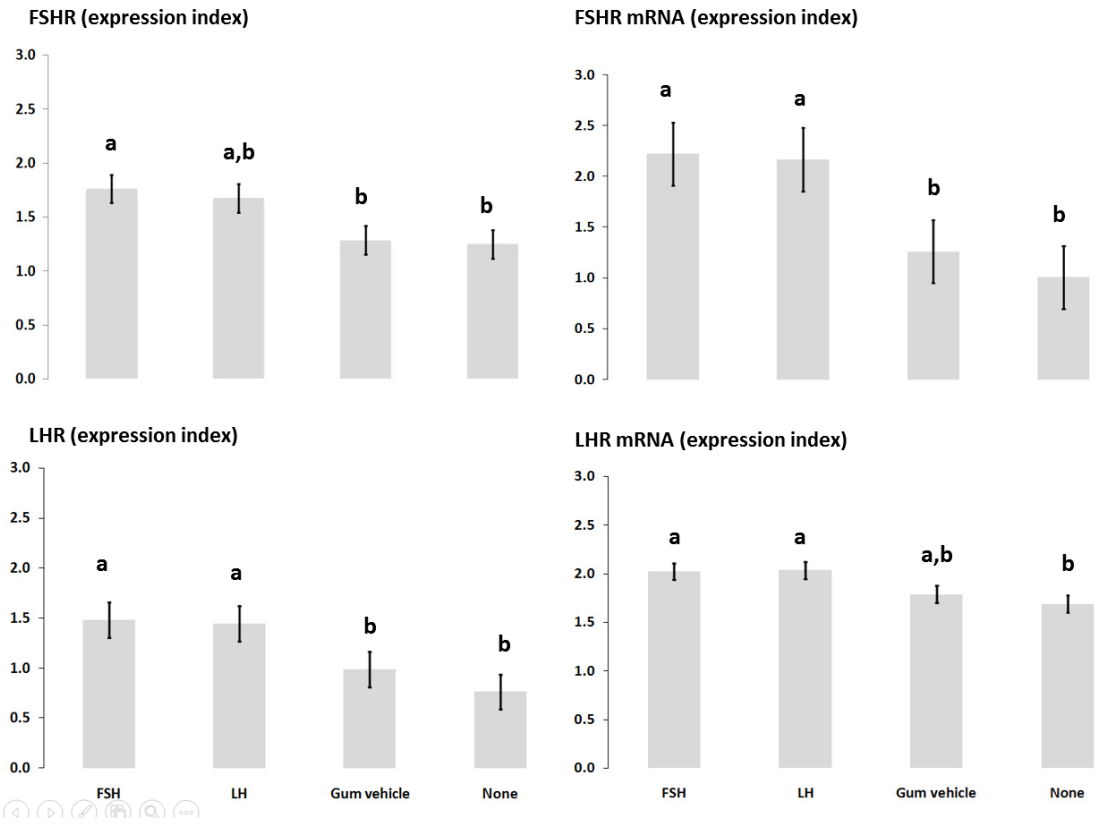

Figure 3: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in the sheep cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.

Intra-cervical LH increased the overall expression of LHR in the cervix (Figure 3). Post-hoc paired comparisons revealed that the effect was confined to the uterine end of the cervix (vehicle-treated, $P = 0.001$; control, $P < 0.001$). Furthermore, the effect of LH on the levels of its receptor was confined to the three muscle layers (circular muscle, $P = 0.010$ for vehicle-treated and $P = 0.002$ for controls; longitudinal muscle, $P = 0.013$ for vehicle-treated and $P = 0.003$ for controls and transverse muscle, $P = 0.039$ for vehicle-treated and $P = 0.003$ for controls) and it was not seen in either the sub-epithelial stroma or epithelium. By contrast intra-cervical LH had no effect on the expression of LHR mRNA. Although was an apparent effect compared to control ewes ($P = 0.011$; Figure 3) there was no effect compared to vehicle treated-ewes ($P = 0.196$) indicating a non-specific effect of the vehicle (Figure 3).

Correlations

The level of expression of FSHR was positively correlated with that of FSHR mRNA ($r = 0.358$; $P < 0.001$) and similarly the level of expression of LHR was positively correlated with that of LHR mRNA ($r = 0.321$; $P < 0.001$). There was also a significant positive correlation between the levels of expression of FSHR mRNA and LHR mRNA ($r = 0.459$; $P < 0.001$).

Discussion

In this study, levels of the FSHR and LHR and their mRNAs were determined using the semi quantitative techniques of *in situ* hybridization for mRNA and immunohistochemistry for protein. For both techniques we used a scoring system that has been previously validated and

successfully used in our laboratory (Kershaw et al., 2007; Ponglowhapan et al., 2008; Leethongdee et al., 2010). Using these techniques, our study confirmed the presence of both FSHR and LHR proteins and their mRNAs in the ovine cervix during the follicular phase of the oestrous cycle (Leethongdee et al., 2010). Furthermore the results also showed that the cervical levels of FSHR, LHR and their receptors can be altered by the intra-cervical application of FSH or LH suggesting that the gonadotrophins may have a functional role in the cervix of the oestrous ewe. However, what those functions might be and their mechanisms of action were beyond the scope of this investigation.

Anatomically, the cervix consists of six broadly concentric tissue layers from an inner luminal epithelium with its underlying sub-epithelial stroma that is surrounded by three layers of smooth muscle (circular, longitudinal and transverse) and encased in an outermost serosal layer. Both FSHR and LHR and their mRNA were detected in all cervical layers except the outer serosal layer. In general, expression tended to be highest in luminal epithelium and lowest in the sub-epithelial stroma and with intermediate levels of expression in the layers of smooth muscle and within smooth muscle expression tended to be higher in the innermost circular and longitudinal layers and lower in the outer transverse layer. The presence of FSH and LH receptors in the luminal epithelium, sub-epithelial stroma and in the layers of smooth muscle but, not in the serosa show that the gonadotrophins have three distinct target cells (luminal epithelium; sub-epithelial stroma and smooth muscle) in the cervix and furthermore the results demonstrated cell specific patterns of distribution of FSHR and LHR and their mRNAs suggesting that the gonadotrophins have distinct functions in these cervical cell types that may affect the physiological control of cervical function in the oestrous ewe.

Although there were specific differences in the level of expression among the cellular layers of the cervix, in general there were no effects among the regions of the cervix except for LHR mRNA whose expression was higher at the vaginal end of the cervix. These findings agree with those of our earlier study (Leethongdee et al., 2010) that showed higher levels of LH mRNA at the vaginal end of the cervix but no differences in the level of expression of FSH mRNA among the regions of the cervix.

Intra-cervical FSH increased FSHR but only at the uterine end of the cervix and then only in the sub-epithelial stroma of the cervix; there was no effect in the luminal epithelium or the layers of smooth muscle. However, the administration of intra-cervical FSH increased FSHR mRNA throughout the cervix. These data show that in the sub-epithelial stroma the level of expression of FSHR mRNA was associated with increased levels of FSHR protein but, that in the luminal epithelium and in the layers of smooth muscle increased gene transcription was not translated into increased levels of receptor protein. Intra-cervical FSH also increased LHR in all regions of the cervix but, its effect was confined to the three layers of smooth muscle and it was not seen in either the sub-epithelial stroma or luminal epithelium. By contrast there was no effect of intra-cervical FSH on the expression of LHR mRNA in any region or layer of the cervix. These results show that although FSH increased the level of expression of LHR in the cervix it did so independently of gene transcription. Overall the variability in the pattern of response to intra-cervical FSH suggest that FSH has distinct mechanisms of action in the, sub-epithelial stroma and the smooth muscle layers of the cervix involving effects on gene transcription and post-transcriptional effects on gonadotrophin receptors. We can conclude that

although intra-cervical FSH stimulated FSHR and LHR gene expression throughout the cervix it increased the level of its own receptor only in the sub-epithelial stroma and that of LHR only in smooth muscle.

The intra-cervical application of LH did not affect FSHR in any region or layer of the cervix. However, intra-cervical LH increased the level of FSHR mRNA in all regions and layers of the cervix again suggesting that increased transcription of the FSHR gene was not translated into increased levels of receptor protein. Intra-cervical LH did increase the expression of LHR in the uterine region of the cervix but, similarly to intra-cervical FSH, its effect was confined to the layers of smooth muscle. Again similarly to intra-cervical FSH, intra-cervical LH had no effect on the expression of LHR mRNA in any region or layer of the cervix suggesting an effect of LH on its own receptor that is also independent of gene transcription. We can conclude that in the cervix LH has no effect on FSHR protein and that its effect on LHR protein is restricted to smooth muscle and that it is post-transcriptional.

Despite the presence of LHR, FSHR and their mRNAs in the luminal epithelium of the cervix we did not observe any effects of either intra-cervical FSH or LH on their patterns of expression. The reasons for this are not apparent from this experiment, perhaps the high concentrations of gonadotrophins in the cervical canal and thus closest to the luminal epithelium may have an inhibitory action.

Although cervical receptors for FSH have been observed in the cow (Mizrachi and Shemesh, 1999a, 1999b) and the ewe (Leethongdee et al., 2010) and for LH in the cow (Kornyei et al.,

1993; Mizrachi and Shemesh, 1999a; Stepien et al., 2000; Lin et al., 2003) and the ewe (Leethongdee et al., 2010) relatively little is known of the possible roles of LH and FSH in the control of cervical function at oestrus. The potential role of FSH in cervical function has been examined in the non-pregnant cow using cultured cervical tissue (Mizrachi and Shemesh, 1999b). These authors showed that the level of FSHR in the cervix was greatest during the follicular phase of the oestrous cycle (Mizrachi and Shemesh, 1999b) and similar effects were reported for LH (Mizrachi and Shemesh, 1999a, 1999b; Shemesh et al., 2001). The mechanism of action of gonadotrophins in the ovine cervix has not been investigated. However, because the action of LH in the bovine uterus is mediated by the cAMP/protein kinase A signaling pathway (Kornyei et al., 1993) it is possible that the same pathway mediates the cervical actions of LH in the ewe.

Relaxation of the cervix is due to a complex combination of biochemical and structural changes affecting cervical connective tissue, that transforms the cervix into an extensible organ (Uldbjerg et al., 1983). The application of exogenous PGE induces softening of the cervix making it more extensible and suggesting that the control of cervical extensibility is mediated by prostaglandins (Fuchs et al., 1984; Ji et al., 2008). There is considerable evidence showing that prostaglandin E2 probably mediates this effect in the non-pregnant ewe (Falchi et al., 2009) through the rearrangement of collagen bundles in the cervical extra cellular matrix (Kershaw et al., 2007). The prostaglandin system in sheep cervix is mainly regulated by, COX-2 (Diaz et al., 1992) and level of COX-2 mRNA in the sheep cervix was greatest during the follicular phase of the oestrous cycle (Kershaw et al., 2007; Leethongdee et al., 2007b). This is a time when the gonadotrophin concentrations are also at their maxima and our findings

suggest that the relation between the follicular phase concentrations of LH and FSH and the cervical PGE system is a subject well worth further investigation.

It should be noted that this experiment used intra-cervical application of 2 mg of either FSH or LH and although the jugular venous concentration of LH and FSH were not increased by these treatments the local cervical concentrations were not determined but, they may have been above normal tissue concentrations. Therefore it is possible that responses observed represent a pharmacological effect physiological rather than a physiological action. However, the long term objective of this research was to develop a therapeutic method to facilitate trans-cervical artificial insemination.

Trans-cervical artificial insemination requires the passage of an inseminating pipette through the cervical canal so that semen can be deposited in the lumen of the uterine body. Softening the cervix to make it more extensible (Uldbjerg et al., 1983) or dilating the cervical canal will both facilitate trans-cervical artificial insemination (Leethongdee et al., 2007b). Research has focused on either mechanical (Halbert et al., 1990; Kershaw et al., 2007) or pharmacological (Leethongdee et al., 2007b; Perry et al., 2010.) techniques to improve the efficiency of trans-cervical artificial insemination. However, for the moment this remains an intractable problem (Falchi et al., 2012) and it remains an important objective of research for the sheep breeding industry. The tissues implicated in the softening of the cervical canal are the sub-epithelial stroma and smooth muscle (Kershaw et al., 2007; Kershaw-Young et al., 2010) and the fact that

we have demonstrated effects of exogenous FSH and LH on these two cell types suggest that gonadotrophin treatment has the potential to facilitate trans-cervical artificial insemination.

The effects of local FSH and LH on the patterns of expression of FSHR, LHR and their mRNAS are summarized in table 1. They show that (i) FSH increased transcription of the FSHR gene and the levels of its own receptor but the later, only in the sub-epithelial stroma at the uterine end of the cervix (ii) FSH also increased the levels of LHR in all regions of the cervix but, only in the layers of smooth muscle and this action was independent of increased levels of transcription of the LHR gene (iii) LH had no effect on the levels of FSHR despite the fact that it increased the level of transcription of the FSHR gene and (iv) LH also increased the level of its own receptor in the uterine end of the cervix but, only in the smooth muscle layers and this action was independent of increased levels of transcription of the LHR gene. Taken together, these findings suggest that the gonadotrophins can regulate their own receptors in the sub-epithelial stroma and smooth muscle of the cervix by multiple mechanisms.

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Author contributions

All authors contributed equally to the intellectual content of this paper.

Conflicts of interest

All authors declare no conflict of interests.

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525 **Table 1:** A summary of the main effects of intra-cervical FSH or LH on the level of expression
526 of cervical FSHR protein, FSHR mRNA, LHR protein and LHR mRNA.

527

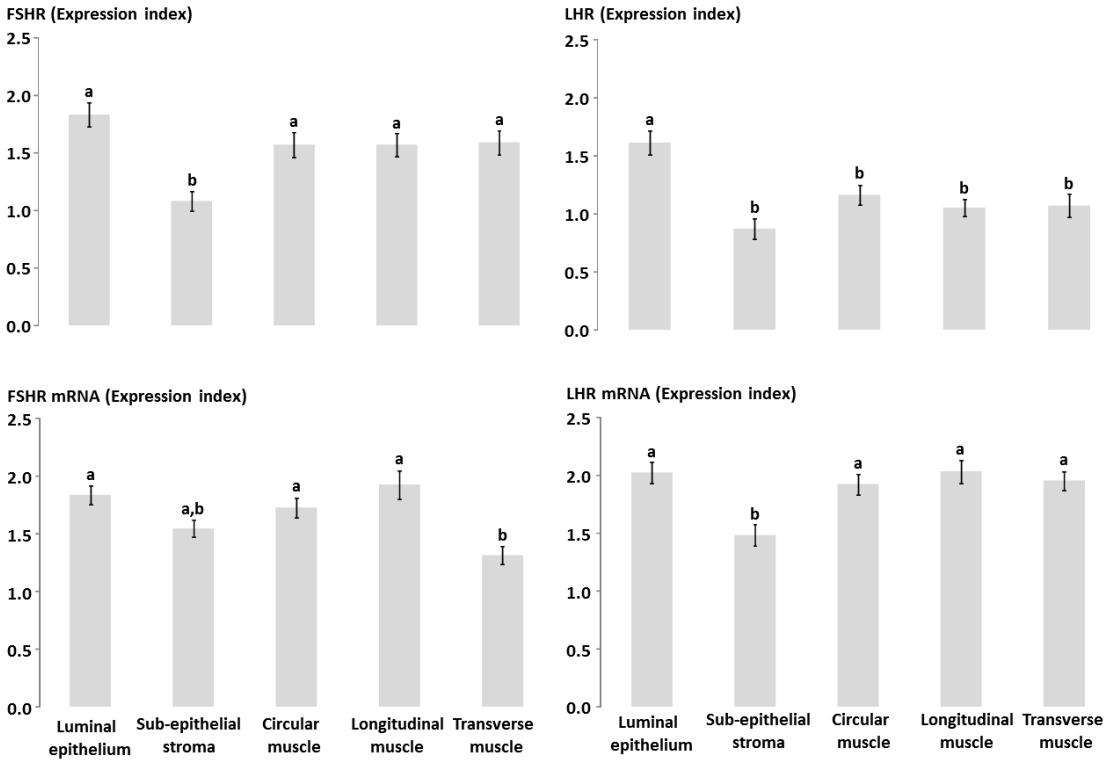
Treatment	FSHR		LHR	
	Protein	**mRNA**	**Protein**	**mRNA**
FSH	Increased in the sub-epithelial stroma at the uterine end of the cervix	Increased in all layers and all regions of the cervix	Increased in the three smooth muscle layers of all regions of the cervix	No effect in any region or layer of the cervix
LH	No effect in any region or layer of the cervix	Increased in all layers and all regions of the cervix	Increased in the three smooth muscle layers at the uterine end of the cervix	No effect in any region or layer of the cervix

528

529

Figure legends

Figure 1: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in the five layers (presented in anatomical order from the inner luminal epithelium to the outer transverse muscle layer) of the sheep cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.



538 **Figure 2**: The Mean level (expression index) and the standard error of the difference (SED) for
539 FSHR, LHR and their mRNAs in three regions of the sheep cervix after intra-cervical treatment
540 with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ
541 significantly at P<005.

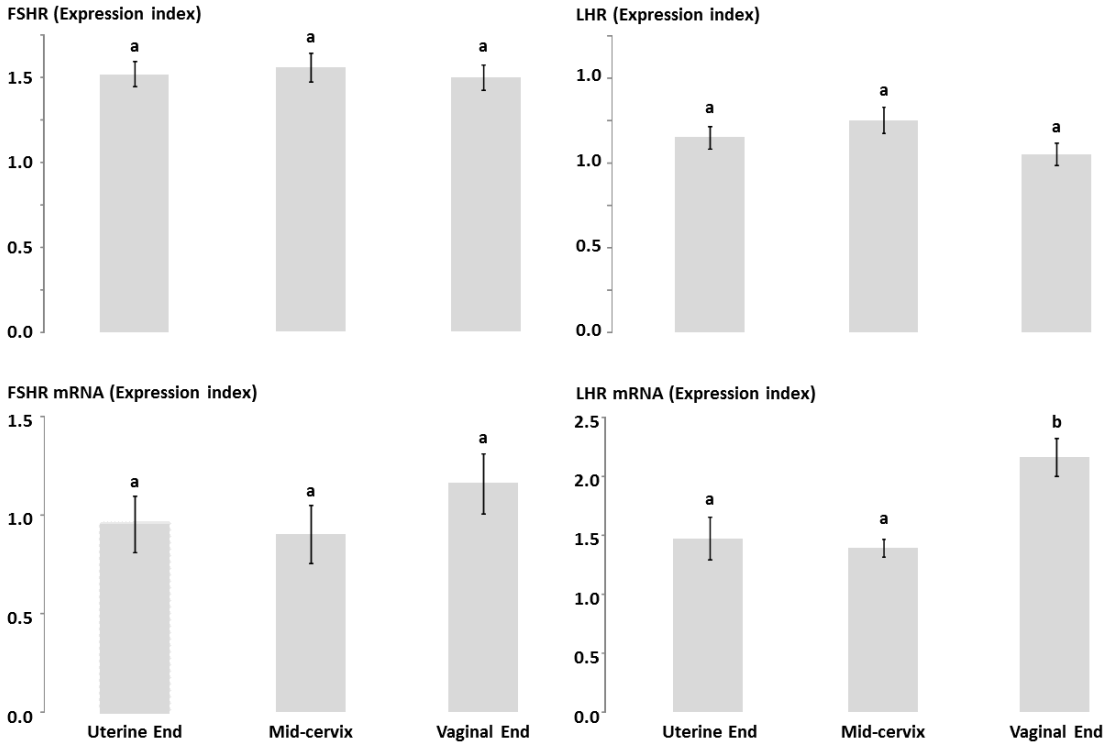


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Figure 3: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in the sheep cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.