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1 The effect of the intra-cervical application of Follicle Stimulating Hormone (FSH) or

2 Luteinizing Hormone (LH) on the pattern of expression of gonadotrophin receptors in the

3 cervix of non-pregnant ewes

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6 Sukanya Leethongdee[a,b]*, Muhammad Khalid[b], Rex John Scaramuzzi[b]

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8 [a]Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, Thailand

9 [b]The Royal Veterinary College, University of London, Hertfordshire, United Kingdom

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15 *** Correspondence:** Dr Sukanya Leethongdee, Faculty of Veterinary sciences, Mahasarakham

16 University, Amphur Muang, Mahasarakham, 44000, Thailand

17 Fax/Phone: +66 43 742 823

18 E-mail: sukanya.l@msu.ac.th

19 **Abstract:**   During the peri-ovulatory period the cervix relaxes in response to changes in

20 circulating concentrations of reproductive hormones. The present study investigated the role of

21 gonadotrophins in cervical function by examining the expression of FSHR and LHR and their

22 mRNAs following intra-cervical treatment with either FSH or LH.   Eighteen ewes were

23 assigned to 4 groups they were then treated with progestagen sponges and PMSG to

24 synchronize their oestrous cycles.   Intra-cervical treatments were given 24h after sponge

25 removal as follows: Group 1: FSH 2 mg; Group 2: LH 2 mg; Group 3: Vehicle and Group 4:

26 Control. Cervices were collected 54h after sponge removal and then divided into 3 regions.

27 The expression of FSHR and LHR was determined by immunohistochemistry and FSHR

28 mRNA and LH mRNA by *in situ* hybridization. The expression of LHR, FSHR and their

29 respective mRNAs was compared in 6 tissue layers (luminal epithelium, sub-epithelial stroma,

30 circular, longitudinal and transverse muscle and serosa) and in 3 cervical regions (vaginal, mid

31 and uterine). The results showed that FSH increased transcription of the FSHR gene and the

32 levels of its receptor but only in sub-epithelial stroma of the cervix. FSH also increased the

33 levels of LHR in the cervix but only in the muscle layers. LH had no effect on the levels of

34 FSHR despite the fact that it did increase the level of transcription of the FSHR gene and LH

35 also increased the levels of its own receptor in the cervix but only in the muscle layers and this

36 action was independent of increased levels of transcription of the LHR gene. These findings

37 suggest multiple levels of regulation of cervical LH and FSH receptors and that the

38 gonadotrophins may have a role in relaxation of the cervix during oestrus by regulating their

39 own receptors.

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

41

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

64   among reproductive hormones acting on the cervix. An increase in the levels of receptors for

65   oestradiol and oxytocin during the peri-ovulatory period is thought to mediate increased

66   synthesis of PGE_2 (Shemesh et al., 1997a) leading to remodeling of the extracellular matrix

67   (ECM) (Stys et al., 1981; Ledger et al., 1983) and cervical relaxation.

68

69   Gonadotrophin receptors have been identified in the cervix of the cow and both FSH receptor

70   (FSHR) and its mRNA are highest during pro-oestrus and oestrus (Mizrachi and Shemesh,

71   1999b) at a time when circulating FSH is also high (Shemesh, 2001). Similarly, LHR and its

72   mRNA are also present in the cervix of cows (Shemesh et al., 1997b; Mizrachi and Shemesh,

73   1999a). The presence of LH receptor (LHR) has been reported in women (Lin et al., 2003) and

74   furthermore intra-cervical human chorionic gonadotrophin (hCG) increased the levels of cAMP

75   and COX-2 in the human cervix (Lin et al., 2003). A role for the gonadotrophins in the process

76   of cervical relaxation although implied by the presence of their receptors and some downstream

77   mediators remains unidentified.

78

79   There is very little data on the action of gonadotrophins in the ovine cervix although in a

80   previous study (Leethongdee et al., 2007a) we showed that the local application of FSH and/or

81   an analogue of PGE (Misoprostol) enhanced the penetrability of the cervix (Leethongdee et al.,

82   2007b). Consequently we set out to determine the actions of FSH and LH on the ovine cervix

83   during the peri-ovulatory period of the oestrous cycle. Our hypothesis was that FSH and/or LH

84   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

Materials and Methods

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

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,

106 Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were dissolved in 0.5 ml of 50% gum

107 acacia (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in normal saline and intra-

108 cervical treatments were applied 24 h after removal of the sponges as follows: Group 1, FSH (2

109 mg) , Group 2, LH (2 mg), Group 3, gum vehicle and Group 4, no vehicle (the procedure was

110 carried out but no vehicle was deposited in the cervix).

111

112 **Collection of cervical tissue**

113 Ewes were killed 54 h after removal of sponges (i.e. 30h after treatment) with a captive bolt

114 pistol followed by exsanguination. The reproductive tract was removed immediately after

115 death, and kept on ice. All unwanted tissue was trimmed from the cervix which was then

116 divided into 3 approximately equal, transverse segments (Kershaw et al., 2007; Kershaw-

117 Young et al., 2009b; Leethongdee et al., 2010) representing the uterine, middle, and vaginal

118 regions of the cervix. The segments were fixed in neutral-buffered formalin (BDH, VWR

119 International Ltd., Leicestershire, UK) for 24h, and then stored in 70% ethanol. Fixed tissues

120 were embedded in paraffin wax; sections were cut at 7µm on a rotary microtome and mounted

121 onto Superfrost Plus slides (BDH, VWR International Ltd., Leicestershire, UK).

122

123 **The determination of FSHR and LHR mRNA**

124 The levels of mRNA for FSHR and LHR was determined by in situ hybridization (ISH) using

125 digoxigenin-11-UTP labeled sense and antisense riboprobes for ovine LHR and bovine FSHR

126 as described for our laboratory (Kershaw et al., 2007; Ponglowhapan et al., 2007;

127 Ponglowhapan et al., 2008; Leethongdee et al., 2010). The sense and antisense riboprobes for

128 FSHR and LHR were made by transcribing the N-terminus sequence of bovine FSHR and

129 ovine LHR complementary deoxyribonucleic acid (cDNA), supplied by Professor Allen

130 Garverick of the University of Missouri-Columbia, Columbia, Missouri, USA (Xu et al., 1995).

131 The cDNAs for FSHR and LHR were cloned into the PGEM-Teasy plasmid (Promega

132 Corporation, Madison, USA). Riboprobes were synthesized with the SP6 and T7 MEGAscript

133 transcription kits (Ambion Ltd, Cambridgeshire, UK) and labeled with digoxigenin-11-UTP

134 (Roche Diagnostics, Mannheim, Germany). In-situ hybridizations for FSHR and LHR mRNAs

135 were performed on four sections (one sense and three antisense) from the each of the uterine,

136 middle, and vaginal regions of the cervix for each ewe using the protocol described in our

137 previous studies (Kershaw-Young et al., 2009b; Kershaw-Young et al., 2010; Leethongdee et

138 al., 2010). Both riboprobes were hybridized at 65°C for 3 h.

139

140 **The determination of FSHR and LHR protein**

141 The procedure for the immunohistochemical localization was the same as described for our

142 laboratory (Ponglowhapan et al., 2008; Perry et al., 2010; Perry et al., 2012).

143 Immunoperoxidase staining was used to determine the level of LHR and FSHR protein using

144 the polyclonal antibodies for FSHR (H-190, sc-13935) and LHR (H-50, sc-25828; both from

145 Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Sections from the uterine,

146 middle and vaginal regions of the cervix from each animal were examined in triplicate for both

147 positive antibody staining and negative controls. The binding site of the enzyme was stained

148 with diaminobenzidine-based peroxidase substrate (ImmPAC™ DAB, Vector Laboratory Ltd,

149 Cambridgeshire, England), then counterstained with Hematoxylin (Hematoxylin QS, catalogue

150 number H-3404, Vector Laboratory Ltd, Cambridgeshire, England). Negative controls were

151 performed in the same manner but substituting the primary antibody with the non-immune

152 rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, California, USA) at an equivalent

153 concentration.

154

155 **Quantification of in-situ hybridization and immunohistochemistry staining**

156 The levels of both mRNA and protein for FSHR and LHR were assessed blind in five tissue

157 layers of the cervix, namely the luminal epithelium, sub-epithelial stroma, circular smooth

158 muscle, longitudinal smooth muscle and transverse smooth muscle as described in our previous

159 studies (Ponglowhapan et al., 2007; Ponglowhapan et al., 2008; Leethongdee et al., 2010; Perry

160 et al., 2010; Perry et al., 2012). The five cell layers in each region of the cervix were scored for

161 both the percentage of cells stained and the intensity of staining as described and validated in

162 previous publications from our laboratory (Ponglowhapan et al., 2007; Ponglowhapan et al.,

163 2008; Leethongdee et al., 2010; Perry et al., 2010; Perry et al., 2012).

164

165 **Statistical analysis**

166 The results are presented as means and the pooled standard error of the difference (S.E.D). The

167 effects of treatment, region and tissue layer as well as their interactions were analyzed using a

168 mixed model ANOVA. Sheep were treated as subjects with cervical region and tissue layer as

169 repeated measures and hormonal treatment as a fixed factor. Where it was appropriate,

170   additional post-hoc tests comparing the effects of treatment within either cervical regions or

171   cervical layers were made using the Bonferroni test. The tests were carried out using SPSS for

172   Windows (SPSS version 20.0; SPSS Inc., IBM Company Headquarters, Chicago, Illinois,

173   USA). Differences were considered statistically significant when $P \leq 0.05$.

174

175   **Results**

176   **Cervical Layer**

177   There were no significant interactions of cervical layer with either hormonal treatment or the

178   region of the cervix for any of the 4 endpoints (FSHR, LHR, FSH mRNA and LHR mRNA)

179   and the overall effects of cervical layer are presented in anatomical order from the inner

180   luminal epithelium to the outermost layer of smooth muscle (Figure 1). There was no

181   expression detected for any of the endpoints in the outer serosal layer and therefore it has been

182   omitted from the figure. In general the level of expression was highest in the luminal

183   epithelium and lowest in the sub-epithelial stroma with the three muscle layers intermediate

184   (Figure 1). However, there were exceptions to this layer generalization. For FSHR, the mean

185   expression in the sub-epithelial stroma was lower (all <0.01) than all other layers which were

186   not significantly different from each other (epithelium; circular muscle; longitudinal muscle

187   and transverse muscle (Figure 1). For LHR, mean expression in the luminal epithelium was

188   higher (all <0.01) than all other layers which were not significantly different from each other

189   (Figure 1).

190

191   For FSHR mRNA, mean expression in transverse muscle was lower (all <0.01) than the other

192   muscle layers (circular muscle and longitudinal muscle) and the epithelium but not the sub-

193   epithelial stroma and the sub-epithelial stroma was not significantly different from any of the

194   other layers (Figure 1).   For LHR mRNA, mean expression in the sub-epithelial stroma was

195   lower (all <0.01) than all other layers which were not significantly different from each other

196   (Figure 1).
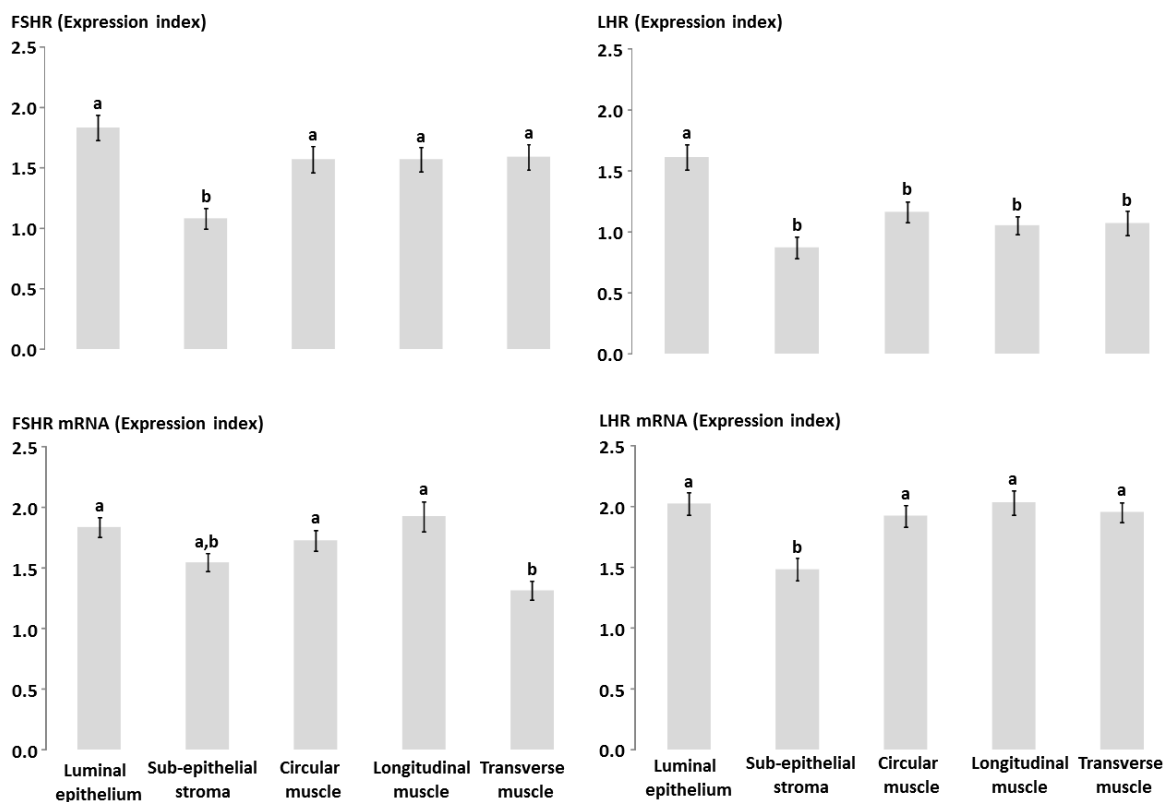
197

198   **Figure 1**: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in the five

199   layers (presented in anatomical order from the inner luminal epithelium to the outer transverse muscle layer) of the sheep cervix after intra-

200   cervical treatment with FSH, LH, vehicle or no treatment (controls).   Columns with different letters differ significantly at P<005.

201

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).
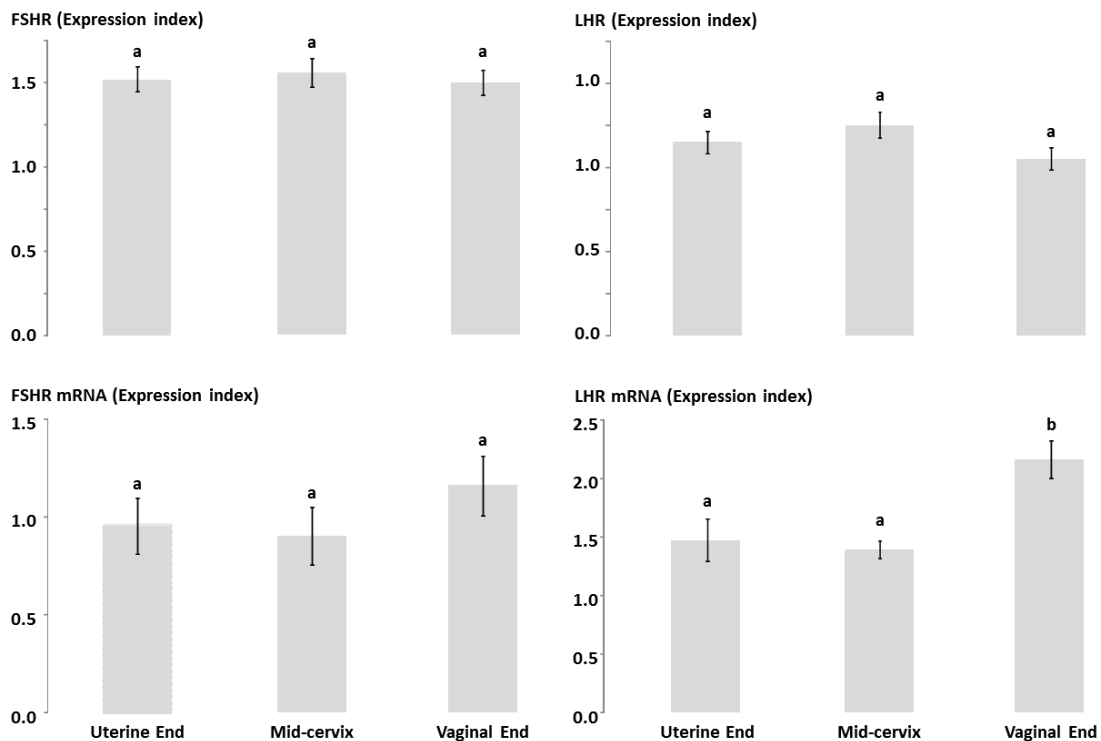

Figure 2: The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in three regions of the sheep cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.

215   **Hormonal Treatments**

216   The main effects of intra-cervical application of FSH and LH on the cervical expression of FSH

217   and LH proteins and mRNAs are summarized in Table 1.

218   *Follicle Stimulating hormone (FSH)*

219   Intra-cervical FSH increased the overall expression of FSHR (Figure 3) compared to either the

220   vehicle-treated (P = 0.006) or control groups (P = 0.003). Post-hoc paired comparisons showed

221   that the effect of FSH was confined to the layer of sub-epithelial stroma at the uterine end of

222   the cervix when compared to either the vehicle-treated (P = 0.008) or control groups (P =

223   0.003) and there was no effect in the epithelium or the muscle layers. There were significant

224   effects of intra-cervical FSH on the level of cervical FSHR mRNA compared to vehicle-treated

225   (P <0.001) and control (P <0.001) groups). The effect of FSH was seen in all layers and

226   regions of the cervix (Figure 3).

227

228   Intra-cervical FSH also increased LHR in all regions of the cervix compared to vehicle-treated,

229   (P <0.001) and control, (P <0.001) groups (Figure 3). Post-hoc paired comparisons revealed

230   that the effect was confined to the smooth muscle layers (circular muscle, P = 0.018 compared

231   to vehicle-treated and P = 0.004 compared to controls; longitudinal muscle, P = 0.009

232   compared to vehicle-treated and P = 0.002 compared to controls and transverse muscle, P =

233   0.026 compared to vehicle-treated and P = 0.002 compared to controls). There was no

234   significant effect in either the sub-epithelial stroma or the luminal epithelium. By contrast

235   there was no effect of intra-cervical FSH on the expression of LHR mRNA (Figure 3).

236   *Luteinizing Hormone (LH)*

237   Intra-cervical LH had no effect cervical FSHR (Figure 3) when compared to either the vehicle-

238   treated (P = 0.164) or control groups (P = 0.108). By contrast intra-cervical LH increased the

239   level of FSHR mRNA compared to the vehicle-treated (P = 0.001) and control groups (P <

240   0.001) and the effect was seen in all regions and layers of the cervix (all P values at least P =

241   0.007).
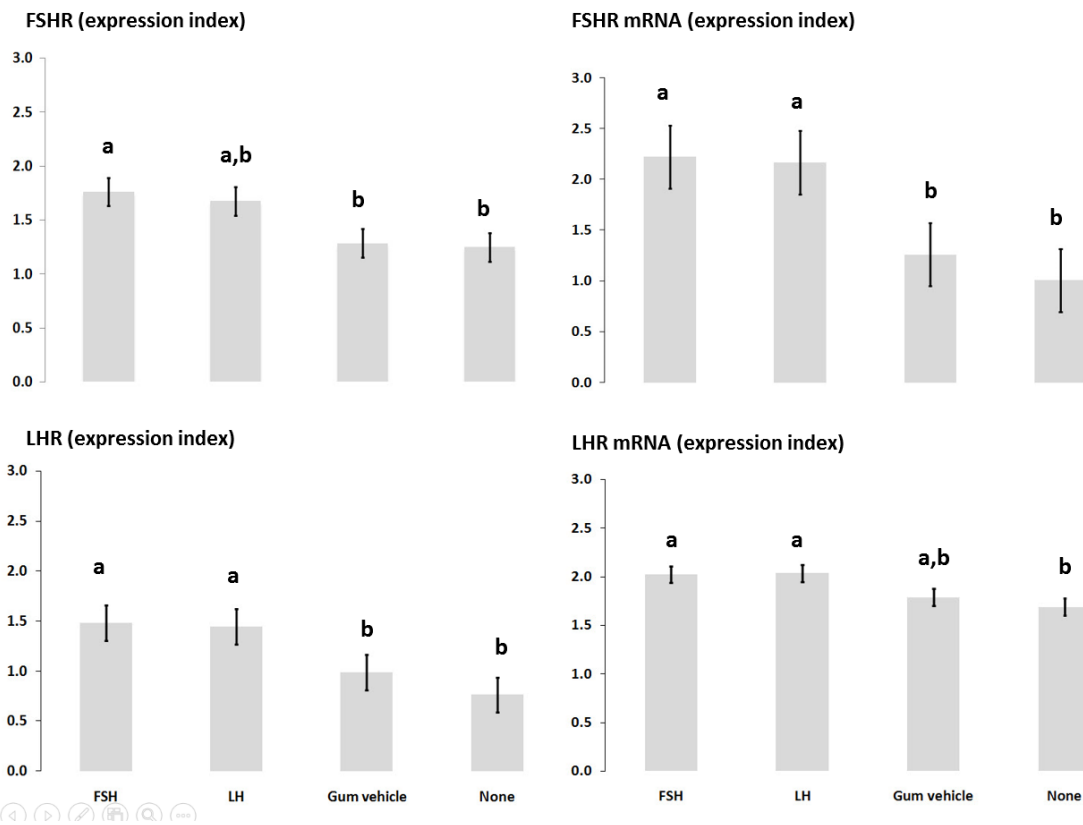
242

243   **Figure 3:** The Mean level (expression index) and the standard error of the difference (SED) for FSHR, LHR and their mRNAs in the sheep

244   cervix after intra-cervical treatment with FSH, LH, vehicle or no treatment (controls). Columns with different letters differ significantly at

245   P<005.

246

247 Intra-cervical LH increased the overall expression of LHR in the cervix (Figure 3). Post-hoc

248 paired comparisons revealed that the effect was confined to the uterine end of the cervix

249 (vehicle-treated, P = 0.001; control, P <0.001). Furthermore, the effect of LH on the levels of

250 its receptor was confined to the three muscle layers (circular muscle, P = 0.010 for vehicle-

251 treated and P = 0.002 for controls; longitudinal muscle, P = 0.013 for vehicle-treated and P =

252 0.003 for controls and transverse muscle, P = 0.039 for vehicle-treated and P = 0.003 for

253 controls) and it was not seen in either the sub-epithelial stroma or epithelium. By contrast

254 intra-cervical LH had no effect on the expression of LHR mRNA. Although was an apparent

255 effect compared to control ewes (P = 0.011; Figure 3) there was no effect compared to vehicle

256 treated-ewes (P = 0.196) indicating a non-specific effect of the vehicle (Figure 3).

257

258 **Correlations**

259 The level of expression of FSHR was positively correlated with that of FSHR mRNA (r =

260 0.358; P <0.001) and similarly the level of expression of LHR was positively correlated with

261 that of LHR mRNA (r = 0.321; P <0.001). There was also a significant positive correlation

262 between the levels of expression of FSHR mRNA and LHR mRNA (r = 0.459; P <0.001).

263

264 **Discussion**

265 In this study, levels of the FSHR and LHR and their mRNAs were determined using the semi

266 quantitative techniques of *in situ* hybridization for mRNA and immunohistochemistry for

267 protein. For both techniques we used a scoring system that has been previously validated and

268   successfully used in our laboratory (Kershaw et al., 2007; Ponglowhapan et al., 2008;

269   Leethongdee et al., 2010). Using these techniques, our study confirmed the presence of both

270   FSHR and LHR proteins and their mRNAs in the ovine cervix during the follicular phase of the

271   oestrous cycle (Leethongdee et al., 2010). Furthermore the results also showed that the cervical

272   levels of FSHR, LHR and their receptors can be altered by the intra-cervical application of FSH

273   or LH suggesting that the gonadotrophins may have a functional role in the cervix of the

274   oestrous ewe. However, what those functions might be and their mechanisms of action were

275   beyond the scope of this investigation.

276

277   Anatomically, the cervix consists of six broadly concentric tissue layers from an inner luminal

278   epithelium with its underlying sub-epithelial stroma that is surrounded by three layers of

279   smooth muscle (circular, longitudinal and transverse) and encased in an outermost serosal

280   layer. Both FSHR and LHR and their mRNA were detected in all cervical layers except the

281   outer serosal layer. In general, expression tended to be highest in luminal epithelium and

282   lowest in the sub-epithelial stroma and with intermediate levels of expression in the layers of

283   smooth muscle and within smooth muscle expression tended to be higher in the innermost

284   circular and longitudinal layers and lower in the outer transverse layer. The presence of FSH

285   and LH receptors in the luminal epithelium, sub-epithelial stroma and in the layers of smooth

286   muscle but, not in the serosa show that the gonadotrophins have three distinct target cells

287   (luminal epithelium; sub-epithelial stroma and smooth muscle) in the cervix and furthermore

288   the results demonstrated cell specific patterns of distribution of FSHR and LHR and their

289   mRNAs suggesting that the gonadotrophins have distinct functions in these cervical cell types

290   that may affect the physiological control of cervical function in the oestrous ewe.

291

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

314   although intra-cervical FSH stimulated FSHR and LHR gene expression throughout the cervix

315   it increased the level of its own receptor only in the sub-epithelial stroma and that of LHR only

316   in smooth muscle.

317

318   The intra-cervical application of LH did not affect FSHR in any region or layer of the cervix.

319   However, intra-cervical LH increased the level of FSHR mRNA in all regions and layers of the

320   cervix again suggesting that increased transcription of the FSHR gene was not translated into

321   increased levels of receptor protein. Intra-cervical LH did increase the expression of LHR in

322   the uterine region of the cervix but, similarly to intra-cervical FSH, its effect was confined to

323   the layers of smooth muscle. Again similarly to intra-cervical FSH, intra-cervical LH had no

324   effect on the expression of LHR mRNA in any region or layer of the cervix suggesting an

325   effect of LH on its own receptor that is also independent of gene transcription. We can

326   conclude that in the cervix LH has no effect on FSHR protein and that its effect on LHR protein

327   is restricted to smooth muscle and that it is post-transcriptional.

328

329   Despite the presence of LHR, FSHR and their mRNAs in the luminal epithelium of the cervix

330   we did not observe any effects of either intra-cervical FSH or LH on their patterns of

331   expression. The reasons for this are not apparent from this experiment, perhaps the high

332   concentrations of gonadotrophins in the cervical canal and thus closest to the luminal

333   epithelium may have an inhibitory action.

334

335   Although cervical receptors for FSH have been observed in the cow (Mizrachi and Shemesh,

336   1999a, 1999b) and the ewe (Leethongdee et al., 2010) and for LH in the cow (Kornyei et al.,

337   1993; Mizrachi and Shemesh, 1999a; Stepien et al., 2000; Lin et al., 2003) and the ewe

338   (Leethongdee et al., 2010) relatively little is known of the possible roles of LH and FSH in the

339   control of cervical function at oestrus. The potential role of FSH in cervical function has been

340   examined in the non-pregnant cow using cultured cervical tissue (Mizrachi and Shemesh,

341   1999b). These authors showed that the level of FSHR in the cervix was greatest during the

342   follicular phase of the oestrous cycle (Mizrachi and Shemesh, 1999b) and similar effects were

343   reported for LH (Mizrachi and Shemesh, 1999a, 1999b; Shemesh et al., 2001). The mechanism

344   of action of gonadotrophins in the ovine cervix has not been investigated. However, because

345   the action of LH in the bovine uterus is mediated by the cAMP/protein kinase A signaling

346   pathway (Kornyei et al., 1993) it is possible that the same pathway mediates the cervical

347   actions of LH in the ewe.

348

349   Relaxation of the cervix is due to a complex combination of biochemical and structural changes

350   affecting cervical connective tissue, that transforms the cervix into an extensible organ

351   (Uldbjerg et al., 1983). The application of exogenous PGE induces softening of the cervix

352   making it more extensible and suggesting that the control of cervical extensibility is mediated

353   by prostaglandins (Fuchs et al., 1984; Ji et al., 2008). There is considerable evidence showing

354   that prostaglandin E2 probably mediates this effect in the non-pregnant ewe (Falchi et al.,

355   2009) through the rearrangement of collagen bundles in the cervical extra cellular matrix

356   (Kershaw et al., 2007). The prostaglandin system in sheep cervix is mainly regulated by, COX-

357   2 (Diaz et al., 1992) and level of COX-2 mRNA in the sheep cervix was greatest during the

358   follicular phase of the oestrous cycle (Kershaw et al., 2007; Leethongdee et al., 2007b). This is

359   a time when the gonadotrophin concentrations are also at their maxima and our findings

360   suggest that the relation between the follicular phase concentrations of LH and FSH and the

361   cervical PGE system is a subject well worth further investigation.

362

363   It should be noted that this experiment used intra-cervical application of 2 mg of either FSH or

364   LH and although the jugular venous concentration of LH and FSH were not increased by these

365   treatments the local cervical concentrations were not determined but, they may have been

366   above normal tissue concentrations. Therefore it is possible that responses observed represent a

367   pharmacological effect physiological rather than a physiological action. However, the long

368   term objective of this research was to develop a therapeutic method to facilitate trans-cervical

369   artificial insemination.

370

371   Trans-cervical artificial insemination requires the passage of an inseminating pipette through

372   the cervical canal so that semen can be deposited in the lumen of the uterine body. Softening

373   the cervix to make it more extensible (Uldbjerg et al., 1983) or dilating the cervical canal will

374   both facilitate trans-cervical artificial insemination (Leethongdee et al., 2007b). Research has

375   focused on either mechanical (Halbert et al., 1990; Kershaw et al., 2007) or pharmacological

376   (Leethongdee et al., 2007b; Perry et al., 2010.) techniques to improve the efficiency of trans-

377   cervical artificial insemination. However, for the moment this remains an intractable problem

378   (Falchi et al., 2012) and it remains an important objective of research for the sheep breeding

379   industry. The tissues implicated in the softening of the cervical canal are the sub-epithelial

380   stroma and smooth muscle (Kershaw et al., 2007; Kershaw-Young et al., 2010) and the fact that

381   we have demonstrated effects of exogenous FSH and LH on these two cell types suggest that

382   gonadotrophin treatment has the potential to facilitate trans-cervical artificial insemination.

383

384   The effects of local FSH and LH on the patterns of expression of FSHR, LHR and their

385   mRNAS are summarized in table 1. They show that (i) FSH increased transcription of the

386   FSHR gene and the levels of its own receptor but the later, only in the sub-epithelial stroma at

387   the uterine end of the cervix (ii) FSH also increased the levels of LHR in all regions of the

388   cervix but, only in the layers of smooth muscle and this action was independent of increased

389   levels of transcription of the LHR gene (iii) LH had no effect on the levels of FSHR despite the

390   fact that it increased the level of transcription of the FSHR gene and (iv) LH also increased the

391   level of its own receptor in the uterine end of the cervix but, only in the smooth muscle layers

392   and this action was independent of increased levels of transcription of the LHR gene. Taken

393   together, these findings suggest that the gonadotrophins can regulate their own receptors in the

394   sub-epithelial stroma and smooth muscle of the cervix by multiple mechanisms.

395

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400

401   **Author contributions**

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

403

404   **Conflicts of interest**

405   All authors declare no conflict of interests.

406

407   **References**

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524

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

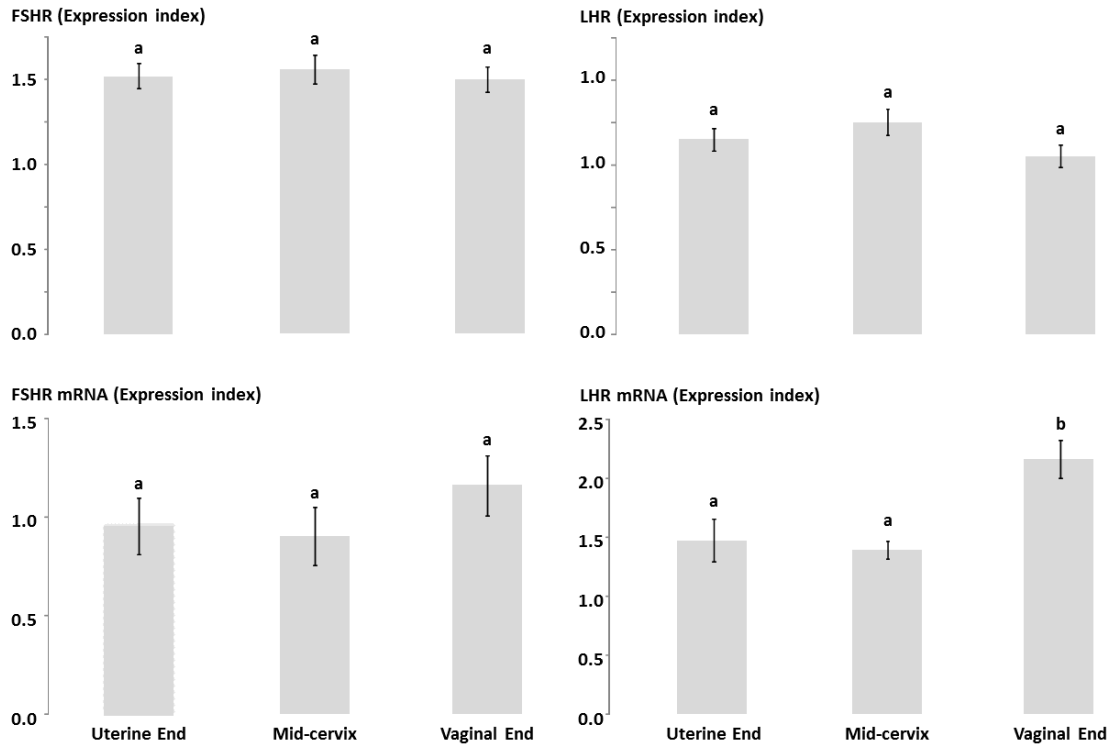| Treatment	FSHR		LHR	
	Protein	**mRNA**	**Protein**	**mRNA**
FSH	Increased in the sub-epithelial stroma at the uterine end of the cervix	Increased in all layers and all regions of the cervix	Increased in the three smooth muscle layers of all regions of the cervix	No effect in any region or layer of 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
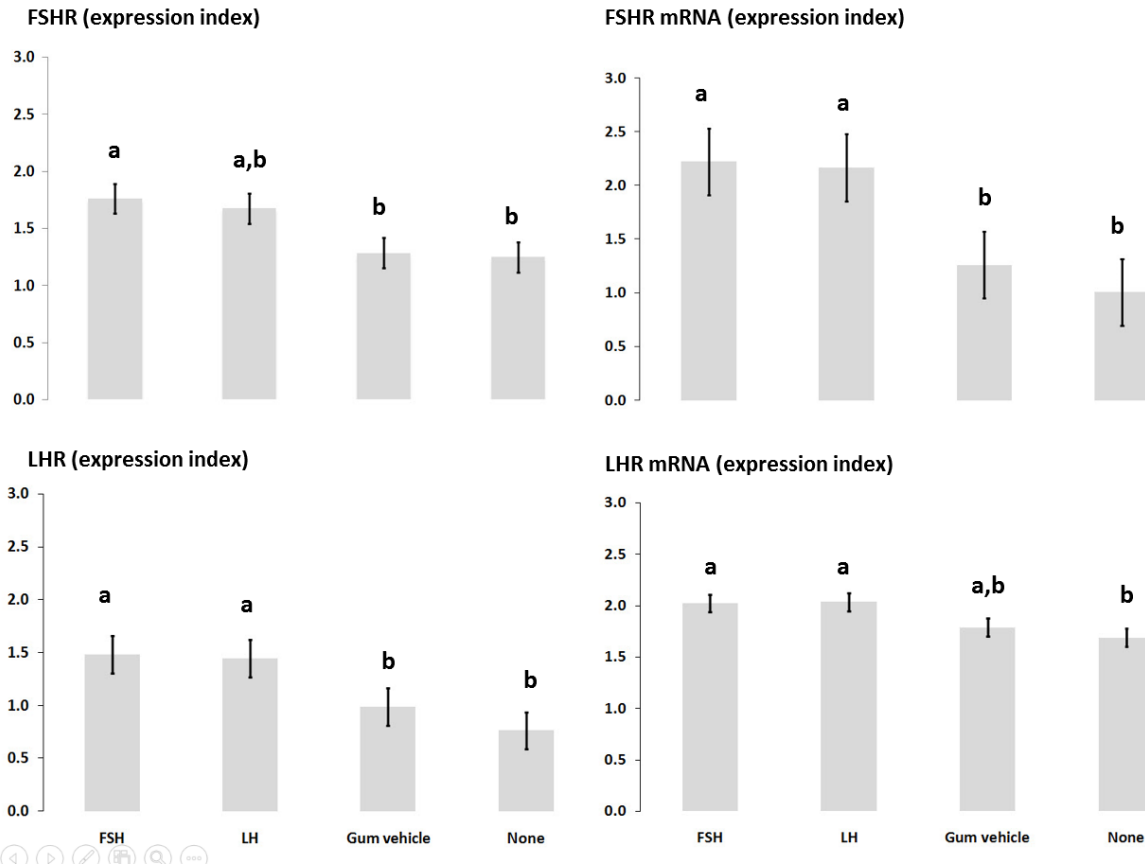
differ significantly at P<005.
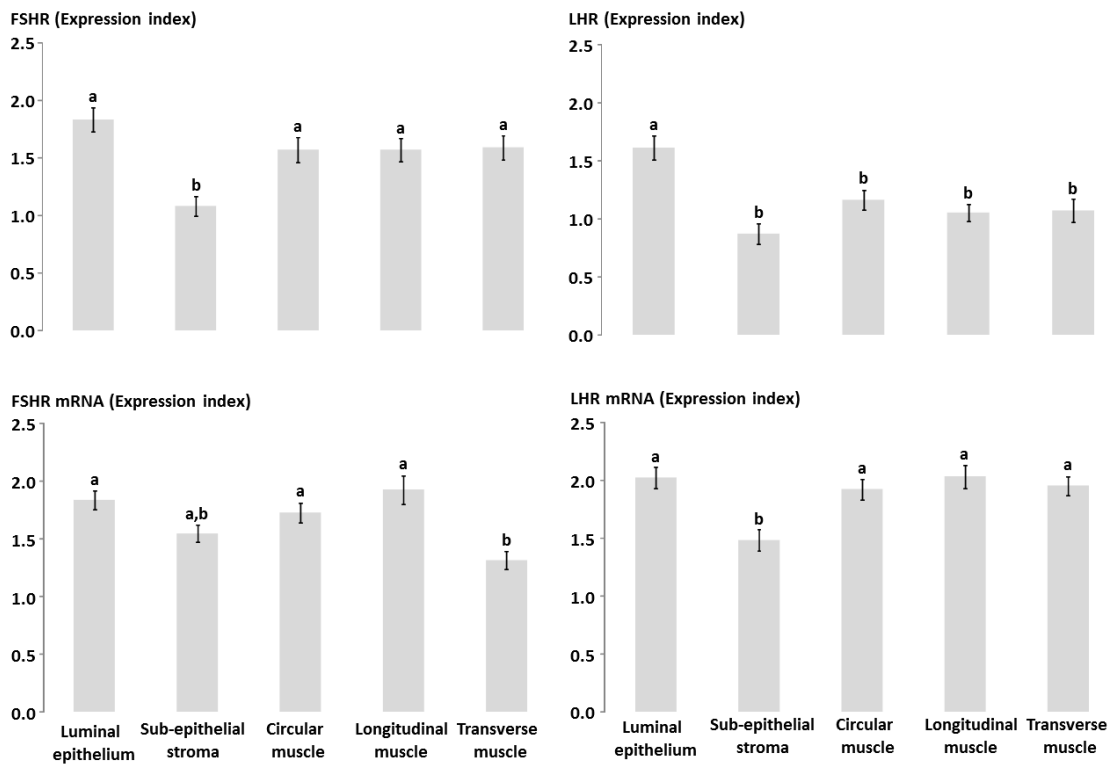
538 **Figure 2**: The Mean level (expression index) and the standard error of the difference (SED) for

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.

542

543

544   **Figure 3:** The Mean level (expression index) and the standard error of the difference (SED) for

545   FSHR, LHR and their mRNAs in the sheep cervix after intra-cervical treatment with FSH, LH,

546   vehicle or no treatment (controls). Columns with different letters differ significantly at P<005.

547