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The effect of the intra-cervical administration of follicle stimulating hormone or luteinizing hormone on the levels of hyaluronan, COX2 and COX2 mRNA in the cervix of the non-pregnant ewe

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29 **Abstract:**
30 During the peri-ovulatory period the cervix relaxes in response to changes in circulating
31 concentrations of reproductive hormones. The present study investigated the role of
32 gonadotrophins in cervical function by examining the expression of cyclooxygenase-2 (COX2)
33 and COX2 mRNA and the concentration of hyaluronan (HA) in the cervix, following intra-
34 cervical treatment with either follicle stimulating hormone (FSH) or luteinizing hormone (LH).
35 Eighteen ewes were assigned to 4 groups. They were then treated with commercial intravaginal
36 progestagen sponges and equine chorionic gonadotrophin (eCG) to synchronize their oestrous
37 cycles. Intra-cervical treatments were given 24h after removal of the sponges as follows: Group
38 1: FSH, 2 mg; Group 2: LH, 2 mg; Group 3: vehicle and Group 4: control. Cervices were
39 collected 54h after sponge removal and then divided into 3 regions. The expression of COX2
40 and COX2 mRNA was determined by immunohistochemistry and *in situ* hybridization and those
41 of HA by ELISA. The levels of expression of COX2, COX2 mRNA and HA were compared in 6
42 tissue layers (luminal epithelium, sub-epithelial stroma, circular, longitudinal and transverse
43 muscle and serosa) and in 3 cervical regions (vaginal, mid and uterine). The results showed that
44 both FSH and LH significantly increased the levels the COX2 mRNA and COX2 in the cervix
45 but, the effects of the gonadotrophins were selective. The effects of both FSH and LH were most
46 evident at the vaginal end of the cervix and least at the uterine end of the cervix. Furthermore
47 their effects were confined to the stroma and smooth muscle layers of the cervix in the case of
48 FSH and to smooth muscle only in the case of LH. Neither FSH nor LH affected the
49 concentration of HA in the cervix although FSH but not LH reduced the concentration of HA in
50 cervical mucus. These findings suggest that the gonadotrophins regulate the expression of
51 COX2 in the cervix and that they may have a role facilitating relaxation of the cervix during
52 oestrus in the ewe.
53

54 **Key words:** Sheep, cervix, hyaluronan, COX2, gonadotrophins, epithelium, stroma, smooth
55 muscle
56

57 **Introduction**

58 One of the main purposes of artificial insemination in sheep breeding is to increase the rate of
59 genetic improvement for a particular trait or group of traits. However, conventional cervical
60 insemination in sheep gives poor fertility particularly if the semen used has been frozen and
61 thawed (F-T), mainly because of the unusual anatomy of the sheep cervix. The ovine cervix is a
62 long, fibrous and convoluted tubular organ that prevents easy passage of an insemination pipette
63 along the cervical lumen [1, 2]. Consequently, semen is normally deposited at the entrance to
64 the cervix and the spermatozoa have to traverse the cervix to enter the uterus and eventually, the
65 site of fertilization in the oviducts. The reduced motility of F-T semen compromises its ability to
66 transit of the cervix [3]. Consequently, a practical, low cost and effective technique for
67 intrauterine insemination would be a valuable aid to sheep breeding.
68
69 There is some natural relaxation of the cervix at oestrus [4] that is probably regulated by the peri-
70 ovulatory changes in reproductive hormones [5]. The cervix contains receptors for oestradiol,
71 progesterone, oxytocin [6] as well as those for luteinizing hormone (LH) and follicle stimulating
72 hormone (FSH) [7-13] suggesting that the gonadotrophins may have a functional role in cervical
73 physiology at oestrus.
74
75 There is good evidence indicating that cervical relaxation at oestrus is mediated by Prostaglandin
76 E_2 (PGE_2) [4, 14-17]. The peri-ovulatory changes in reproductive hormones are associated with
77 increased levels of cervical cyclooxygenase-2 (COX2) also known as prostaglandin
78 endoperoxide synthase and the increased cervical synthesis of PGE_2 [16, 18]. Similarly in the
79 cow, cervical relaxation during oestrus is mediated by a local increase in COX2 and a subsequent
80 increase in the production of PGE_2 by the cervix [19]. Prostaglandin E_2 separates cervical
81 collagen fibres reducing the tensile strength of the cervix [15] and allowing the cervical canal to
82 dilate. Naturally occurring cervical relaxation at oestrus is probably the result of complex
83 interactions among reproductive hormones acting on the cervix. An increase in the levels of
84 receptors for oestradiol and oxytocin during the peri-ovulatory period is thought to mediate
85 increased synthesis of PGE_2 [19] leading to remodeling of the extracellular matrix [20, 21]
86 characterized by a loosening of the collagen bundles [22] and associated increases in the cervical

87 concentrations of glycosaminoglycans (GAGs) especially hyaluronan (HA). These PGE_2 induced
88 changes are partially responsible for cervical relaxation as demonstrated by the ability of an
89 intra-cervical application of HA to increase cervical penetrability in oestrus ewes [23] and does
90 [24].
91
92 Gonadotrophin receptors have been identified in the cervix of the cow and the ewe and both FSH
93 receptor (FSHR) and its mRNA are highest during pro-oestrus and oestrus [8] at a time when
94 circulating FSH is also high [25]. Similarly, LHR and its mRNA are also present in the cervix of
95 cows [19, 25]. The presence of LH receptor (LHR) in cervical tissue has been reported in women
96 [26] and furthermore intra-cervical human chorionic gonadotrophin (hCG) increased the levels
97 of cAMP and COX2 in the human cervix [26]. The role of gonadotrophins in cervical relaxation
98 although implied by the presence of their receptors and some downstream mediators in the cervix
99 remains unclear.
100
101 There is very little data on the action of gonadotrophins in the ovine cervix although in a
102 previous study [13] we showed that the local application of FSH and/or an analogue of PGE
103 (Misoprostol) enhanced the penetrability of the cervix [4, 13]. These data collectively suggest
104 that the intra-cervical application of gonadotrophins may enhance relaxation of the cervix to
105 facilitate intrauterine insemination.
106
107 Consequently we set out to define in greater detail, the actions of FSH and LH on the ovine
108 cervix during the peri-ovulatory period of the oestrous cycle by studying the effects of intra-
109 cervical LH and FSH on the intra-cervical levels of COX2 protein and mRNA and the
110 concentrations of HA in cervical tissue and cervical mucus.
111
112
113 **Materials and Methods**
114 ***Animals and their management***
115 In this study 18 adult Welsh Mountain ewes were divided randomly into two groups of 5 and two
116 groups of 4 ewes. Due to the small number of animals a simple randomization method was

117 applied. Each ewe was assigned a unique number from 1 to 18. These numbers were then written
118 on small pieces of papers and were thoroughly mixed in a bowl. Then without looking, 5
119 numbers were picked up randomly for each of the group 1 (FSH) and group 2 (LH), and 4
120 numbers for each of the group 3 (gum acacia vehicle) and group 4 (no vehicle).
121
122 The multiparous ewes were all healthy and cycling normally during last breading season. They
123 had average (±SD) body condition score of 2.94 ±0.3 (2.5-3.5), body weight of 36.9±3.0 (32 _
124 42) kg and age of 19.8±2.1 (17 - 25) months . The animals in different experimental groups did
125 not vary in their body weight, body condition score, age or parity (Table 1). Moreover, these
126 ewes did not have any reproductive problems previously
127
128 During the experiment the animals were housed indoors, in groups, on straw bedding and were
129 fed with a commercial concentrate diet *ad libitum* and with hay and water always available. All
130 the experimental procedures with ewes were conducted with the approval of the ethics
131 committee of the Royal Veterinary College, University of London and with authorization from
132 the Home Office (United Kingdom) in compliance with the Animal (Scientific Procedures) Act,
133 1986.
134
135 ***Intra-cervical administration of FSH or LH***
136 The ewes were synchronized to a common day of oestrus using intra-vaginal sponges containing
137 30 mg of fluorogestone acetate (Chronogest; Intervet UK Ltd, Northamptonshire, UK) for 12
138 days. The experiment was conducted during the non-breeding season (March to April) therefore,
139 ewes were injected intramuscularly with 500IU of equine chorionic gonadotrophin (eCG;
140 Intervet UK Ltd., Buckinghamshire, UK), at the time of removal of sponges. Ovine FSH (2 mg
141 Ovagen; ICPbio (UK) Limited, Wiltshire, UK) or ovine LH (2 mg, Sigma-Aldrich Chemie
142 GmbH, Steinheim, Germany) was dissolved in 0.5 ml of a vehicle consisting of 50% gum acacia
143 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in normal saline. The ewes were
144 restrained in a yoke fitted with sidebars to minimize lateral and forward movements with the
145 hindquarters of the ewe raised about 4 inches. A 1 ml Eppendorf (Eppendorf AG, Hamburg,
146 Germany) pipette fitted with a 10 cm extension consisting of 3 X 1 ml pipette tips glued together

147 was used for intra-cervical administration. The tip of the extension pipette was blunted, by
148 cutting off about 0.2 mm of the tip, the extension pipette was then sterilised. The perineum was
149 wiped clean with a disinfectant wipe and a duckbill vaginal speculum introduced into the vagina
150 so that the external cervical opening could be seen in the light of the speculum lamp. The pipette
151 tip was inserted about 1-2 cm into the cervix and the 0.5 ml bolus deposited in 80% of ewes,
152 when this was not possible and the FSH or LH was placed at the os cervix. In a series of
153 preliminary tests we established the maximum volume (0.5ml) and viscosity of vehicle required
154 to ensure that the bolus did not leak from the cervical canal. The intra-cervical treatments were
155 applied 24 h after removal of the sponges as follows: Group 1, FSH (2 mg; n = 5); Group 2, LH
156 (2 mg; n = 5); Group 3, gum acacia vehicle (n = 4) and Group 4, no vehicle (the intra-cervical
157 procedure was carried out but no vehicle was deposited in the cervix; n = 4).
158
159 ***Collection of cervical mucus and cervical tissue***
160 Cervical mucus was collected at 48h and 54h after sponge removal, from the anterior vagina or
161 fornix using a duckbill vaginal speculum (attached with a penlight) pressed gently to the floor of
162 the vulva and with a downwards movement of the speculum handle thus allowing the mucus to
163 drain through the speculum into a collecting tube. The mucus was stored at -80 °C. Ewes were
164 killed 54h after removal of sponges (i.e. 30h after treatment) with a captive bolt pistol followed
165 by exsanguination. The reproductive tract was removed immediately after death and kept on ice.
166 All unwanted tissue was trimmed from the cervix which was then divided into 3 approximately
167 equal transverse segments [10, 27] representing the uterine, middle, and vaginal regions of the
168 cervix. The segments were fixed in neutral-buffered formalin (BDH, VWR International Ltd.,
169 Leicestershire, UK) for 24h, and then stored in 70% ethanol. Fixed tissues were embedded in
170 paraffin wax; sections were cut at 7μm on a rotary microtome and mounted onto Superfrost Plus
171 slides (BDH, VWR International Ltd., Leicestershire, UK).
172
173 ***The determination of COX2 mRNA***
174 The levels of mRNA for COX2 were determined by *in situ* hybridization (ISH) using
175 digoxigenin-11-UTP labeled sense and antisense riboprobes synthesized by Dr. Claire Kershaw,
176 The Royal Veterinary College University of London [5, 27]. Eight sections were examined from

177 each of the three regions of the cervix of each animal, using 4 sections for the sense riboprobe
178 and 4 sections for the antisense riboprobe. Sense and antisense riboprobes were used on different
179 slides.
180
181 ***The determination of COX2 protein***
182 The procedure for the immunohistochemical localization was the same as described for our
183 laboratory [23, 28-30]. Immunoperoxidase staining was used to determine the level of COX2
184 using a polyclonal antibody (H-62 from Santa Cruz Biotechnology Inc., Santa Cruz, California,
185 USA). Sections from each region of the cervix from each animal were examined in triplicate for
186 both positive staining and negative controls. The binding site of the enzyme was stained with
187 diaminobenzidine-based peroxidase substrate (ImmPAC™ DAB, Vector Laboratory Ltd,
188 Cambridgeshire, England), then counterstained with hematoxylin (Hematoxylin QS, H-3404,
189 Vector Laboratory Ltd, Cambridgeshire, England). Negative controls were examined in the same
190 manner but substituting the primary antibody with the non-immune rabbit IgG (Santa Cruz
191 Biotechnology, Santa Cruz, California, USA) at an equivalent concentration.
192
193 ***Quantification of in-situ hybridization and immunohistochemistry staining***
194 The levels of both mRNA and protein for COX2 were assessed blind in six tissue layers of the
195 cervix, namely the luminal epithelium, sub-epithelial stroma, circular smooth muscle,
196 longitudinal smooth muscle, transverse smooth muscle and the outer serosa as described in our
197 previous studies [10, 13, 29, 30]. No positive staining for either COX2 or its mRNA was
198 detected in the serosa. The staining in the other five cell layers in each region of the cervix was
199 scored for both the percentage of cells stained and the intensity of staining as described and
200 validated in previous publications from our laboratory [10, 16, 17, 27, 29-31].
201
202 ***Hyaluronan***
203 <u>(i) Papain extraction and digestion:</u> The concentration of HA in cervical tissue was determined
204 by ELISA following the extraction of total GAGs by papain digestion. The extraction of GAGs
205 was performed using frozen (-80 °C) tissue [32]. Frozen cervical tissue was thawed slowly on
206 wet ice and a transverse section of the tissue was cut and finely chopped using a sterile scalpel

207 blade. The papain buffer was prepared and pre-heated at 60°C for 30 min before use, to activate
208 the enzyme. The papain buffer contained 0.25 mg/mL papain (Roche Diagnostics GmbH,
209 Mannheim, Germany) in 0.1M sodium acetate buffer, pH 5.8, (Sigma-Aldrich Chemie GmbH,
210 Steinheim, Germany) containing 5 mM EDTA (Sigma-Aldrich Chemie GmbH, Steinheim,
211 Germany) and 5 mM/L anhydrous cysteine hydrochloride (Sigma-Aldrich Chemie GmbH,
212 Steinheim, Germany). The papain buffer (2 mL) was added to 300mg chopped tissue in a 15 mL
213 Falcon tube which was then covered and sealed with Parafilm to prevent evaporation. The tissues
214 were incubated at 60°C for 16 to 18 h by which time the tissue was completely digested. The
215 following day, 1 mL of the digested lysate was placed in a sterile 1.5 mL Eppendorf tube. Papain
216 activity was halted by the addition of 10 µL of 0.5 M iodoacetic acid (Sigma-Aldrich Chemie
217 GmbH, Steinheim, Germany) to 1mL of digested lysate. The tubes were mixed on a vortex mixer
218 and then incubated at 37°C for 30 min after which the tubes were centrifuged at 13,000 rpm for
219 10 min. The supernatant was pipetted into a clean 1.5 mL Eppendorf tube and then stored at -
220 20°C.
221
222 (ii) Hyaluronan ELISA: The digested tissue supernatant was assayed in duplicate by ELISA [33].
223 Nunc-Immuno MaxiSorp^{TM} 96 well plates (VWR International Ltd., Lutterworth, Leicestershire,
224 UK) were coated overnight at 37°C with 100 µL/well of 25 µg/mL human umbilical cord HA
225 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 20 mM sodium carbonate buffer pH 9.6
226 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The following day the coating solution
227 was removed and the plate washed 3 times in PBS containing 0.1 % Tween 20 (PBS-Tween ,
228 BDH, VWR International Ltd., Lutterworth, Leicestershire, UK), then blocked with 100 µL of
229 1% BSA in PBS-Tween for 1 h at 37°C, and finally washed 3 more times in PBS-Tween. Serial
230 dilutions of a HA standard (human umbilical cord HA ;Sigma-Aldrich Chemie GmbH,
231 Steinheim, Germany) were made up in PBS-Tween at concentrations of 5.0, 2.5, 1.25, 0.625,
232 0.3125, 0.156, 0.078, 0.039, 0.0195 and 0.00976 µg/mL. Supernatant samples were diluted
233 1:1000 in 0.01M sodium acetate buffer to enable the concentration of HA to fall on the standard
234 curve. Samples or standards (50 µL) were added to the wells in duplicate followed by 50 µL of
235 0.33 µg/mL biotinylated hyaluronic acid binding protein (bHABP; Seikagaku America,
236 Falmouth, Massachusetts, USA). The plates were incubated overnight at room temperature.

237 Blank wells containing 100 µL of PBS-Tween only were included in duplicate on each plate and
238 used to zero the plate reader. Maximum binding was determined in wells that contained 50 µL
239 of PBS-Tween and 50 µL of bHABP. Quality control samples made from pooled cervical
240 supernatants were also assayed in duplicate on each plate to determine the inter-assay coefficient
241 of variation.
242
243 Next day, each plate was washed 3 times in PBS-Tween and 100 µL of the colour reagent
244 Streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences UK Ltd,
245 Amersham, Buckinghamshire, UK) diluted 1:1000 in PBS-Tween, was added to all wells and the
246 plate incubated for 30 min at 37°C. After incubation the plate was washed 3 times with PBS-
247 Tween and 100 µL of ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid)
248 diammonium salt) substrate was added to all wells to develop the colour. The ABTS substrate
249 was warmed to room temperature before addition to the wells. The plate was incubated at 37°C
250 for approximately 20 min by which time the optical density at 405nm (OD_{405}) of the maximum
251 binding wells reached approximately 1.5. The optical density was read immediately, at OD_{405}
252 and the concentrations of hyaluronan were determined against the optical density of the
253 standards. The limit of the sensitivity of the assay was 0.90 µg/mL. The intra-assay coefficient of
254 variation was 8.60% and the inter-assay coefficient of variation was 18.60%.
255
256 ***Cervical dry matter***
257 Frozen cervical tissue was thawed on wet ice and a small piece removed using a sterile scalpel
258 blade. The piece was weighed, transferred to a dry air incubator at 60°C and left overnight (20
259 h). Next day the dried cervical tissue was weighed and the percentage dry weight and the
260 percentage water calculated.
261
262 ***Statistical analysis***
263 The results are presented as means and standard error of the mean (S.E.M). The effects of
264 treatment, region and tissue layer were analyzed using a mixed model ANOVA. Sheep were
265 treated as subjects with cervical region and tissue layer as nested factors and hormonal treatment
266 as a fixed factor. Where it was appropriate, additional *post-hoc* tests comparing the effects of

267 treatment within either cervical region or cervical layer were made using the least significant
268 difference (LSD) test. The tests were carried out using SPSS for Windows (SPSS version 20.0;
269 SPSS Inc., IBM Company Headquarters, Chicago, Illinois, USA). Differences were considered
270 statistically significant when $P \leq 0.05$.
271
272
273 **Results**
274 ***Effects of FSH and LH on the Expression of Cervical COX2 mRNA and COX2***
275 The expression of COX2 mRNA in the cervix of ewes treated with intra-cervical FSH was
276 significantly greater than those treated with vehicle ($P = 0.003$) or the untreated control group (P
277 $= 0.004$; Figure 1). Similarly, the expression of COX2 mRNA in the cervix of ewes treated with
278 intra-cervical LH was significantly greater than those treated with vehicle ($P = 0.007$) or the
279 untreated control group ($P = 0.006$; Figure 1). There was no significant difference between the
280 FSH and LH ($P = 0.77$) or between vehicle and untreated control groups ($P = 0.95$)
281
282 The results for COX2 closely paralleled those for COX2 mRNA (Figure 1). The expression of
283 COX2 in the sheep cervix was increased by treatment for both the FSH and LH groups compared
284 to the vehicle groups [FSH ($P = 0.006$) and LH ($P = 0.05$)] groups and the untreated control
285 groups FSH ($P = 0.05$) and LH (P= *0.05*). The expression of COX2 was not different between the
286 vehicle and control ($P = 0.70$) groups nor between the FSH and LH groups ($P = 0.29$; Figure 1).
287
288 ***Patterns of Expression of COX2 mRNA and COX2 in the Regions of the Cervix***
289 The pattern of expression of COX2 mRNA and COX2 in the regions of the cervix are shown in
290 Figure 2. The overall expression index of COX2 mRNA, irrespective of the treatment groups,
291 was significantly different ($P < 0.001$) among regions. The expression of COX2 mRNA at the
292 vaginal end ($P < 0.001$) and the mid-cervix ($P < 0.001$) were both significantly greater than the
293 uterine end. There was no difference between the vaginal end and the mid-cervix ($P = 0.68$).
294 However, the expression of COX2 was not significantly different among the three regions of the
295 cervix.
296

297 ***Effects of FSH and LH on the Expression of COX2 mRNA and COX2 in the Regions of the***
298 ***Cervix***
299 The effects of intra-cervical FSH and LH on the pattern of expression of both COX2 mRNA and
300 COX2 in the three regions of the cervix are shown in Figure 3. At the uterine end of the cervix,
301 intra-cervical FSH increased the expression of COX2 compared to untreated control ($P = 0.009$)
302 and vehicle treated control ($P = 0.008$) ewes but it had no effect on the expression of COX2
303 mRNA. Furthermore, in the mid-cervix FSH had no effect on the expression of either COX2 or
304 its mRNA. At the vaginal end of the cervix, FSH strongly increased the expression of both
305 COX2 and its mRNA compared to untreated control (both $P < 0.001$) and vehicle treated control
306 (both $P < 0.001$) ewes. There was no effect of intracervical LH at the uterine end of the cervix or
307 in the mid-cervix on the expression of either COX2 mRNA or COX2 protein itself. However,
308 intra-cervical LH strongly increased the expression of both COX2 mRNA and COX2 at the
309 vaginal end of the cervix compared to both untreated control (both $P < 0.001$) and vehicle treated
310 controls (both $P < 0.001$) ewes
311 .
312 ***Patterns of expression of COX2 mRNA and COX2 in the Cellular Layers of the Cervix***
313 The pattern of expression of COX2 mRNA and COX2 in the five tissue layers of the cervix are
314 shown in Figure 4. There was no expression of either COX2 mRNA or COX2 itself in the outer
315 serosal (sixth) layer of the cervix and these data are not presented. The expression of both COX2
316 and its mRNA were both significantly different (both $P < 0.001$) among the cellular layers of the
317 cervix; expression in the three smooth muscle layers and the luminal epithelium were all
318 significantly higher than in sub-epithelial stroma (all $P < 0.001$). There were no significant
319 differences among the muscle layers and the luminal epithelium.
320

321 ***Effects of FSH and LH on Expression of COX2 mRNA and COX2 in the Cellular Layers of***
322 ***the Cervix***
323 The effects of intra-cervical FSH and LH on the pattern of expression of both COX2 mRNA and
324 COX2 in the five cellular tissue layers of the cervix are shown in Figure 5. There was no effect
325 of intra-cervical FSH on the expression of COX2 mRNA in luminal epithelium compared to both
326 untreated control ($P = 0.20$) and vehicle treated control ($P = 0.13$) ewes. For COX2 itself there

327 was a significant effect of intra-cervical FSH in in luminal epithelium compared to untreated
328 control (P = 0.004) ewes but not to vehicle untreated control (P = 0.10) ewes. Intra cervical LH
329 had no effect on the expression of COX2 or its mRNA in luminal epithelium compared to both
330 untreated control (P = 0.20) and vehicle treated control (P = 0.13) ewes. In the sub-epithelial
331 stroma, intra-cervical FSH increased the expression of both COX2 mRNA and COX2 compared
332 to both untreated control (P = 0.004 and P = 0.006) and vehicle treated control (P = 0.01 and P =
333 0.05) ewes. However, intra-cervical LH had no effect on the expression of COX2 in the sub-
334 epithelial stroma, compared to either the untreated (P = 0.11) or vehicle treated control (P =
335 0.45) groups although it COX2 mRNA was increased compared to untreated controls (P = 0.018)
336 and approached significance (P = 0.055) when compared to vehicle treated controls. Intra-
337 cervical FSH increased the expression of COX2 mRNA and COX itself in all three layers of
338 smooth muscle compared to both untreated control (P = 0.003 & P = 0.008 - LM; P = 0.003 & P
339 = 0.004 - CM; P = 0.04 & p =0.03- TM) and vehicle treated control (P = 0. 01 & P = 0.02 - LM;
340 P = 0.004 & P = 0.01 - CM; P = 0.008 & P =0.05 - TM) ewes. Intra-cervical LH increased the
341 expression of COX2 mRNA and COX2 itself only in circular smooth muscle compared to the
342 untreated control group (P = 0.002 & P = 0.02) and in the vehicle treated control group (P =
343 0.004 & P = 0.05) ewes. In longitudinal smooth muscle intra-cervical LH increased the
344 expression of COX2 mRNA compared to both untreated control (P = 0.001) and vehicle treated
345 control (P = 0.006) groups. However COX2 was increased compared to untreated controls (P =
346 0.04) but not when compared to vehicle treated control (P = 0.10). In transverse smooth muscle
347 intra-cervical LH increased the expression of COX2 mRNA or compared to untreated control (P
348 = 0.03) and vehicle treated control (P = 0.006) groups but it did not increase COX2 mRNA
349 compared to untreated controls (P = 0.18) and vehicle treated controls (P = *0.22*).
350

351 ***Effects of FSH and LH on the Concentration of Hyaluronan in Cervical Tissue***
352 The concentrations of HA in the cervix are presented in Table 2. The concentration of HA
353 differed among cervical regions (P = 0.002) but not among treatments (P = 0.880). There was
354 significantly more HA in the vaginal end of the cervix compared to the uterine end ($P < 0.003$).
355 There was no difference between the mid-cervix and the uterine end (P = 0.554) or the vaginal
356 end and the mid-cervix (P = 0.078). The interaction between treatment and region was not

357 significant (P = 0.194). The water content of the cervix expressed as a percentage of the tissue
358 wet weight was not affected by intra-cervical treatment with either FSH or LH (Table 3) but the
359 water content of the cervix was slightly, but significantly (P = 0.002) lower at the vaginal end
360 compared to the uterine end of the cervix (Table 3).

361

362 ***Effects of FSH and LH on the Concentration of Hyaluronan in Cervical Mucus***
363 The concentration of HA in cervical mucus collected at 48 h and 54 h after the removal of
364 progestagen pessary are presented in Figure 6. The concentration of HA in mucus collected at
365 48 h did not significantly differ among the treatments. The concentration of HA in mucus
366 collected at 54 h did not significantly differ among the treatments. The FSH group tended to
367 have a lower HA concentration than the control group (P = 0.080). However, the significant
368 interaction (P = 0.013) between treatment and time of mucus collection indicated that the
369 concentration of HA at the different times was affected by treatment in different ways. Further
370 investigation revealed that the concentration of HA in cervical mucus for the FSH-treated group
371 was significantly lower at 54 h than at than 48 h ($P < 0.014$) whereas it was not affected by time
372 in the other groups.

373

374

375 **Discussion**
376 In this study, the expression of COX2 and its mRNA was determined using semi quantitative
377 methods (*in situ* hybridization for mRNA and immunohistochemistry for protein). For both
378 analyses a scoring system that had been previously validated, was used [10, 27, 28, 33].
379 Furthermore, this system of quantification was able to describe the localization and distribution
380 of expression at a cellular level. Using these techniques, our study confirmed that both COX2
381 and its mRNA are present in the cervix of the ewe during the follicular phase of the oestrous
382 cycle [10, 27, 34]. Furthermore the results also show that the levels of COX2 and its RNA in the
383 cervix can be altered by intra-cervical FSH or LH suggesting that the gonadotrophins may have a
384 physiological role in the cervix of the oestrous ewe. The dose of 2mg of LH or FSH was used
385 for intra-cervical administration in this study and was based on our previous work where it was
386 able to stimulate both the protein and mRNA expression of receptors for LH and FSH in the

387 cervical tissues of ewes (Leethongdee et al., 2007a). Both of the receptors were expressed in all
388 tissue layers of the cervix except the external serosa with the highest concentrations in the
389 luminal epithelium and the irregular smooth muscle. Moreover, cervical administration of 2 mg
390 of FSH was able to enhance the cervical relaxation in ewes (Leethongdee et al., 2007b, 2010).
391
392 Both COX2 and its mRNA were detected in all cervical layers except the outer serosal layer. The
393 level of expression of both COX2 and its mRNA was lower in the sub-epithelial stroma and
394 higher in the luminal epithelium and the three layers of smooth muscle (Figure 4). In an earlier
395 publication [27] the levels of COX2 mRNA were lowest in luminal epithelium and lower than
396 the level of expression we observed in this study (Figure 4). The most likely explanation is that
397 in the former study the cervices that were collected had not been manipulated at all whereas in
398 this study a speculum had been inserted into the vagina at the time of treatment and also at 48
399 and 54 hours later in order to collect cervical mucus. While this discrepancy between the two
400 studies regarding the levels of COX2 and its mRNA's expression in the cervical layers
401 (particularly luminal epithelium and sub-epithelial stroma) could be attributed to the
402 manipulation in the form of insertion of vaginal speculum, this should not confound with the
403 effects of intra-cervical treatments as the process of vaginal speculum insertion was similar for
404 all the experimental groups including the vehicle and non-vehicle controls.
405
406 The tissues of the cervix synthesize PGE_2 from arachidonic acid (AA). The first step is the
407 formation of prostaglandin H_2 (PGH_2) a reaction catalyzed by the COX enzyme. The PGH_2 is
408 then converted to PGE_2 by the enzyme PGES. The prostaglandin system is controlled mainly by
409 COX [35] and because of the rapid catalytic inactivation of COX, this enzyme is the rate-limiting
410 step in the synthesis of prostaglandins [36] in the cervix. The various reproductive hormones act
411 at multiple levels along the pathway of biosynthesis of PGE_2. *In vitro*, oestradiol increased the
412 cervical level of the oxytocin receptor, the level of COX2 and the concentration of PGE_2 [18].
413 Furthermore the levels of OTR, oestradiol receptor α (ERα), $cPLA_2$ and COX2 were all
414 increased in the follicular phase of the oestrous cycle compared to the luteal phase [34, 40]. In
415 addition, both FSH and LH have been implicated [7, 8, 19, 39] in cervical PG synthesis. Both
416 FSH and LH receptors are present in the cervix of the ewe [10] cow [7, 8] and human [26, 41]

417 and the level of FSH receptor in the bovine cervix was at its maximum during the follicular
418 phase [8]. In the ewe FSH has been shown to stimulate COX2 in an *in vitro* study [18] and in the
419 present study the intra-cervical application of FSH or LH increased cervical COX2 mRNA and
420 protein in the cervix of the non-pregnant ewe during the peri-ovulatory period (Figure 1). In the
421 cow the production of PGE_2 by cultured cervical tissue was induced by FSH [8]. The *in vitro*
422 administration of both LH and FSH to cultures of cervical tissue from oestrous cows [8, 25]
423 stimulated both the cAMP and inositol phosphate signaling pathways [8, 42] suggesting that FSH
424 regulates the synthesis of cervical PGE_2 through one or both of these pathways [8].
425
426 In this study, although COX2 was present in epithelium, stroma and smooth muscle indicating
427 that they are all capable of synthesizing prostaglandins, the effects of intra-cervical FSH or LH
428 differed. Despite the presence of COX2 in luminal epithelium this tissue did not respond to
429 intra-cervical FSH or LH whereas stroma responded only to FSH, increasing the levels of COX2
430 and its mRNA while smooth muscle responded to both FSH and LH with increased levels of
431 COX2 and its mRNA. In the non-pregnant rat, COX2 was also localized to cervical smooth
432 muscle, sub-epithelial stroma and epithelium as well as vascular smooth muscle [43] and COX2
433 has also been detected in the human cervix [44] and human cervical fibroblasts [45].
434
435 The cervix was analyzed in thirds; the uterine end, the mid-cervix and the vaginal end and the
436 patterns of expression across these regions show that the level expression of COX2 was constant
437 across the three regions but that the expression of COX2 mRNA was lower at the uterine end of
438 the cervix (Figure 2). These finding are broadly in agreement with previous reports showing that
439 levels of COX2 mRNA [10, 12, 27] and COX2 protein [34] were higher at the vaginal end of the
440 cervix. Along its length, the structure of the cervix is not uniform. There is a concentration of
441 cervical folds at the vaginal end [2] which effectively obstructs the cervical canal while at the
442 uterine end the cervical canal is quite open. Consequently there is a greater need for cervical
443 remodeling at the vaginal end of the cervix and probably explains why the levels of COX2 are
444 higher at the vaginal end of the cervix.
445

446 The non-cellular component of the cervix is composed of an extensive extra cellular matrix
447 (ECM) that includes collagen, elastin and proteoglycans [46, 47]. The predominant GAG in the
448 cervix of the non-pregnant ewe is hyaluronan-like accounting for 84 to 90% of total GAGs [5].
449 In the present study, we determined the concentration of HA in cervical tissue and there was no
450 effect of either FSH or LH although the concentration of HA was highest at the vaginal end of
451 the cervix. This finding mirrors the collagen content in the cervix of the non-pregnant cow
452 where the highest collagen content was in the vaginal region and lowest in the uterine region
453 [49]. These data show that there are regional differences of HA concentrations and that HA may
454 influence the patterns of firmness along the longitudinal axes of the cervix and at different stages
455 of the oestrous cycle.
456
457
458 We determined the HA concentration in cervical mucus collected at 48 h and 54 h after pessary
459 removal (Figure 6). The concentration of HA in cervical mucus rose in the LH-treated group but
460 not in the FSH-treated group. The high affinity of HA for water results in a thin watery mucus
461 when HA concentrations in cervical mucus are increased leading to the secretion of a clear
462 mucus during the peri-ovulatory period that facilitates the transport of spermatozoa through the
463 cervix. These data suggest that the intra-cervical application of FSH may be deleterious to the
464 transport of spermatozoa through the cervix.
465
466 The cervix of the ewe relaxes at oestrus a process that is similar to the mechanism of cervical
467 ripening that occurs at parturition. Central to both cervical relaxation and cervical ripening is the
468 local production of PGE_2 and its control by reproductive hormones. Although the pattern of
469 reproductive hormones at oestrus and parturition in some ways similar they are not identical and
470 therefore it would be reasonable to assume that the mechanisms of cervical relaxation at oestrus
471 and cervical ripening at parturition are also similar but not identical. Relaxation of the cervix is
472 due to a complex combination of biochemical and structural changes affecting the cervical
473 connective tissue and leading to an extensible organ [56] and mediated by PGE_2. The mechanism
474 of action of PGE_2 in the cervix appears to be multifaceted. Receptors for PGE_2 are present in
475 luminal epithelium, stroma and smooth muscle [57]; and prostaglandin-mediated cervical

476 softening in sheep, probably involves PGE_2 induced loosening of collagen bundles within the
477 cervical ECM and increased production of HA [22, 53]. Hyaluronan in the ECM, because of its
478 hydrophilic properties, draws water into the ECM leading to an increase in the relative
479 proportion of collagen to smooth muscle in the wall of the cervix. Cervical relaxation is
480 affected by other mechanisms including increased collagenase activity [58] and local
481 inflammatory reactions within cervical fibroblasts [59, 60]. However the predominate anatomical
482 and physiological change in cervical ripening is rearrangement of collagen [61]. These effects
483 result in a more pliable cervix.
484
485 The patterns of contractility of smooth muscle in the cervical wall will also be altered by PGE_2
486 depending on the dominant receptor sub-types. Of the four prostaglandin E receptors (EP1 to 4),
487 EP1 and EP3 increase the contractility of gastrointestinal smooth muscle while EP2 and EP4
488 relax gastrointestinal smooth muscle [36]. We suggest that the effects of PGE_2 on the smooth
489 muscle of a more pliable cervix lead to cervical relaxation and an opening of the cervical canal at
490 oestrus.
491
492 There can be little doubt that a central player in cervical relaxation at oestrus is the local
493 production of PGE_2. In this study we have examined two aspects of PGE_2 in the cervix, first the
494 effect of FSH and LH on its synthesis by measuring the activity of COX_2 in cervical tissue and
495 second the action of PGE_2 by measuring the effect of FSH and LH on HA. The main findings
496 summarized in Table 4, are that FSH and LH both stimulated COX2 but neither had any effect
497 on the concentration of cervical HA although FSH inhibited the concentration HA in cervical
498 mucus late in the follicular phase. This lack of FSH or LH effect on cervical HA cannot,
499 however, be attributed to the relatively lower number of animals belonging to only one breed of
500 sheep in the experimental groups as the variation observed in the data was not huge but normal.
501 FSH stimulated COX2 in the stroma and all layers of smooth muscle while LH was effective
502 only in circular smooth muscle. Neither FSH nor LH stimulated COX2 in luminal epithelium.
503 We interpret these findings to suggest that FSH and LH have a role in cervical relaxation at
504 oestrus in the ewe but on their own, they cannot induce full cervical relaxation. It would appear
505 that the role of FSH and LH is secondary to a primary role for oxytocin and oestradiol.

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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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705

706 **Table 1**: The Mean±SEM body weight, body condition score, age, parity and reproductive
707 history of ewes used in different experimental groups treated during the peri- ovulatory period.

Treatment	**Number**	**Weight (Kg)**	**BCS (1-5)**	**Age (Months)**	**parity**	**Reproductive history**
FSH	5	37.4 ±3.7	2.8±0.3	19.6±2.1	Multiparous	cycling in last breeding season, Healthy
LH	5	37.4±3.5	3.0±0.4	19.2±2.3	Nulliparous	cycling in last breeding season, Healthy
Gum acacia vehicle	4	35.8±1.7	3.1±0.3	20.2±1.5	Nulliparous	cycling in last breeding season, Healthy
None (no vehicle)	4	36.8±3.1	2.8±0.5	20.7±2.9	Nulliparous	cycling in last breeding season, Healthy

708

709

710 **Table 2:** The effect of intra-cervical FSH (n=5) or LH (n=5) on the concentration of hyaluronan
711 (mean ± the standard error of the mean) in ovine cervical tissue collected during an induced
712 follicular phase 54 hours after the removal of progestagen impregnated pessaries. Control ewes
713 were untreated (None; n=4) or treated with the gum acacia vehicle (Vehicle; n=4). There were
714 no significant differences.
715

Treatment	Hyaluronan (µg/mg wet weight)			
	Uterine end	Mid-cervix	Vaginal end	Total
FSH	1.53 ± 0.18	1.34 ± 0.17	1.66 ± 0.17	1.51 ± 0.22
LH	1.52 ± 017	1.63 ± 0.17	1.73 ± 0.17	1.63 ± 0.40
Vehicle	1.31 ± 0.19	1.65 ± 0.19	1.78 ± 0.19	1.58 ± 0.52
None	1.21 ± 0.19	1.61 ± 0.19	2.09 ± 0.19	1.64 ± 0.19
Combined	1.41 ± 0.09[a]	1.55 ± 0.09[a,b]	1.80 ± 0.09[b]	1.59 ± 0.09

716

717

718 **Table 3**: The effect of intra-cervical FSH (n=5) or LH (n=5) on the percentage content of water
719 (mean ± the standard error of the mean) in ovine cervical tissue collected during an induced
720 follicular phase 54 hours after the removal of progestagen impregnated pessaries. Control ewes
721 were untreated (None; n=4) or treated with the gum acacia vehicle (Vehicle; n=4). Values with
722 different superscripts are significantly different at the 5% level.

723

	Water content (%)			
Treatment	**Uterine end**	**Mid-cervix**	**Vaginal end**	**Whole cervix**
FSH	79.8 ± 0.41	78.2 ± 0.41	78.8 ± 0.41	78.9 ± 0.39[a]
LH	78.9 ± 0.41	78.4 ± 0.41	78.7 ± 0.41	78.7 ± 0.28[a]
Vehicle	79.2 ± 0.46	79.4 ± 0.46	77.2 ±0 .46	78.6 ± 0.31[a]
None	78.8 ± 0.46	78.0 ± 0.46	77.1 ± 0.46	78.0 ± 0.38[a]
Combined	79.2 ± 0.22[x]	78.5 ± 0.25[x,y]	78.0 ± 0.39[y]	78.6 ± 0.18

724

725 **Table 4**: A summary of the effects of intra-cervical FSH or LH, on the expression of COX2, it's
726 mRNA and the concentration of HA in the cervix of the ewe during the follicular phase of the
727 oestrous cycle. An effect of either FSH or LH was only accepted if the treatment differed
728 significantly from BOTH the untreated and vehicle control groups.

729

Treatment	COX2	
	mRNA	Protein
FSH	Selective stimulation of COX2 mRNA in the cervix. Stimulated expression only at the vaginal end of the cervix and in all cell layers except the luminal epithelium.	Selective stimulation of COX2 in the cervix. Stimulated expression at the uterine and vaginal ends of the cervix and in all cell layers except the luminal epithelium.
LH	Selective stimulation of COX2 mRNA in the cervix. Stimulated expression only at the vaginal end of the cervix and in the three muscle layers but not in the luminal epithelium or stroma.	Selective stimulation of COX2 in the cervix. Stimulated expression only at the vaginal end of the cervix but only in circular smooth muscle.

730

731

732 **Figure legends**

733

734 **Figure 1**: The effect of intra-cervical FSH (n=5) or LH (n=5) on the level of cervical expression
735 (mean ± the standard error of the mean) of COX2 mRNA and COX2 in ovine cervical tissue
736 collected during an induced follicular phase 54 hours after the removal of progestagen
737 impregnated pessaries. Control ewes were untreated (None; n=4) or treated with the gum acacia
738 vehicle (Vehicle; n=4). Columns with different letters differ significantly at P<005. Within
739 treatments, columns with different superscripts are significantly different.

740

741 **Figure 2:** The level of cervical expression (mean ± the standard error of the mean) of COX2
742 mRNA and COX2 in three regions of the ovine cervix (the uterine end, the mid-cervix and the
743 vaginal end of the cervix). Cervical tissue was collected during an induced follicular phase 54
744 hours after the removal of progestagen impregnated pessaries. Columns with different letters
745 differ significantly at P<005. Within regions of the cervix, columns with different superscripts
746 are significantly different.

747

748 **Figure 3**: The effect of intra-cervical FSH (n=5; pale grey columns) or LH (n=5; medium grey
749 columns) on the level of cervical expression (mean ± the standard error of the mean) of COX2
750 mRNA and COX2 in three regions of the ovine cervix (the uterine end, the mid-cervix and the
751 vaginal end of the cervix). Cervical tissue was collected during an induced follicular phase 54
752 hours after the removal of progestagen impregnated pessaries. Control ewes treated with the
753 gum acacia vehicle (Vehicle; n=4; dark grey columns) or were untreated (None; n=4; black
754 columns). Columns with different letters differ significantly at P<005. Within and regions of the
755 cervix, columns with different superscripts are significantly different.

756

757 **Figure 4**: The level of cervical expression (mean ± the standard error of the mean) of COX2
758 mRNA and COX2 in five cellular tissue layers of the ovine cervix. The cellular layers are shown
759 in order from the central lumen of the cervix (luminal epithelium, sub-epithelial stroma,
760 longitudinal smooth muscle, circular smooth muscle and transverse smooth muscle). Cervical
761 tissue was collected during an induced follicular phase 54 hours after the removal of progestagen

762 impregnated pessaries. Columns with different letters differ significantly at P<005. Within
763 cellular tissue layers of the cervix, columns with different superscripts are significantly different.
764
765 **Figure 5**: The effect of intra-cervical FSH (n=5; pale grey columns) or LH (n=5; medium grey
766 columns) FSH (n=5) or LH (n=5) on the level of cervical expression (mean ± the standard error
767 of the mean) of COX2 mRNA and COX2 in five cellular tissue layers of the ovine cervix. The
768 cellular layers are shown in order from the central lumen of the cervix (luminal epithelium, sub-
769 epithelial stroma, longitudinal smooth muscle, circular smooth muscle and transverse smooth
770 muscle). Cervical tissue was collected during an induced follicular phase 54 hours after the
771 removal of progestagen impregnated pessaries. Control ewes treated with the gum acacia vehicle
772 (Vehicle; n=4; dark grey columns) or were untreated (None; n=4; black columns). Columns with
773 different letters differ significantly at P<005. NB: The asterisk (*) indicates a P value (P =
774 0.055) approaching significance.
775
776 **Figure 6**: The effect of intra-cervical FSH (n=5) or LH (n=5) on the concentration of
777 hyaluronan in cervical mucus collected during an induced follicular at 48 and 54 hours the
778 removal of progestagen impregnated pessaries. Control ewes were untreated (None; n=4) or
779 treated with the gum acacia vehicle (Vehicle; n=4). There were no significant differences.
780

781 **Figure 1**

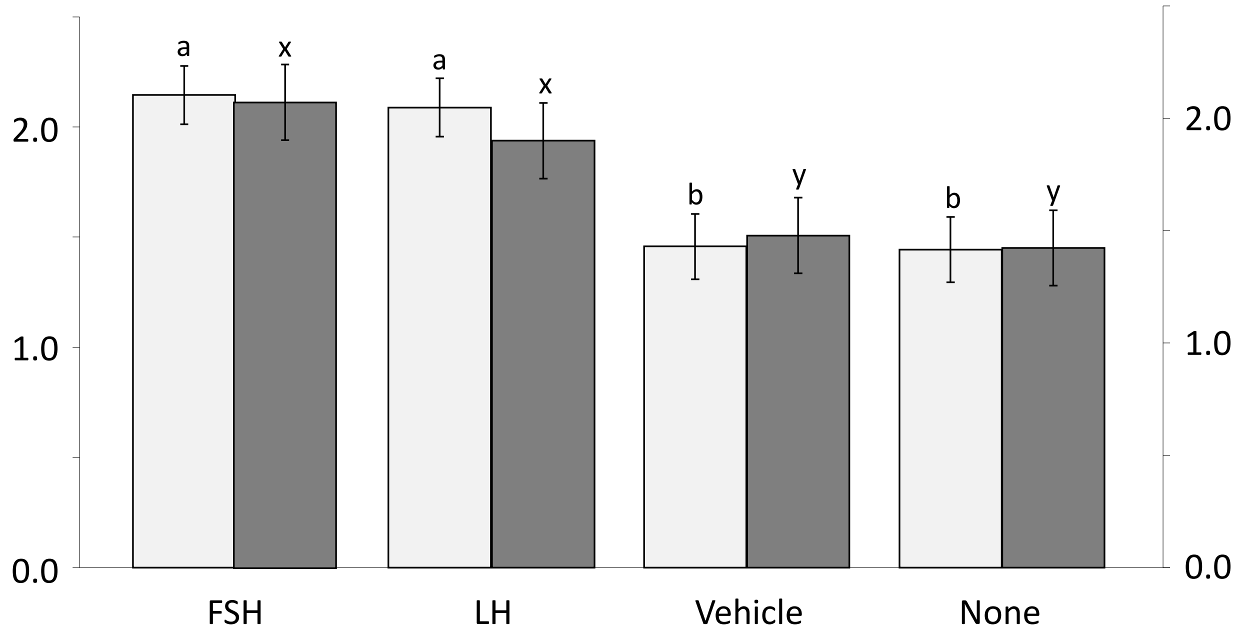
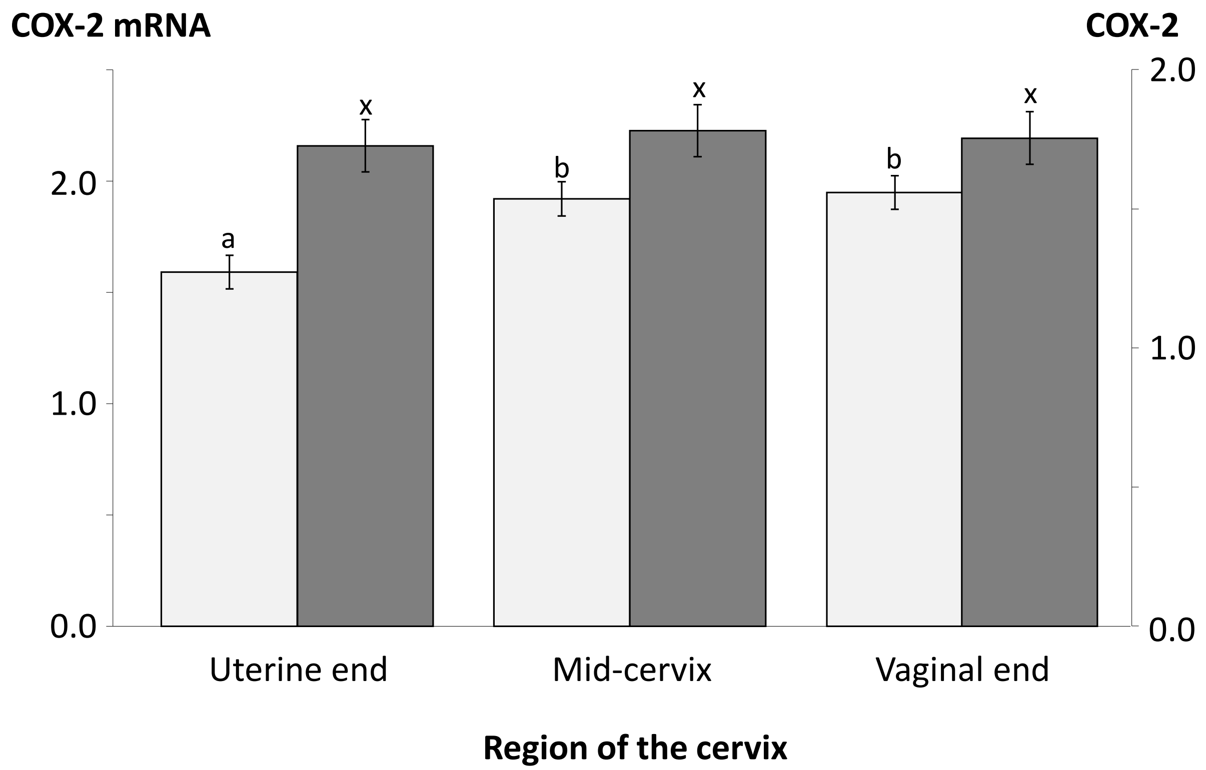


Figure 2



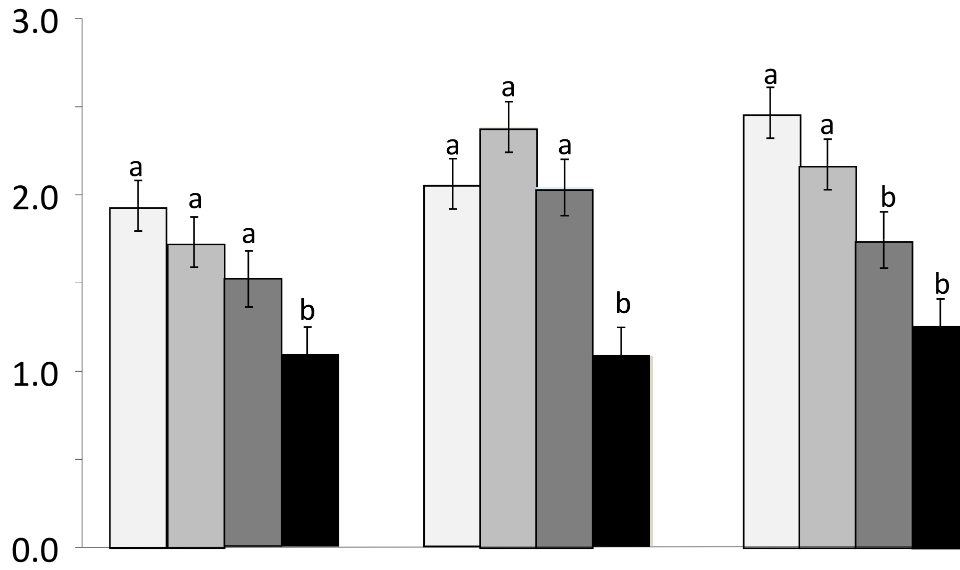
782

783

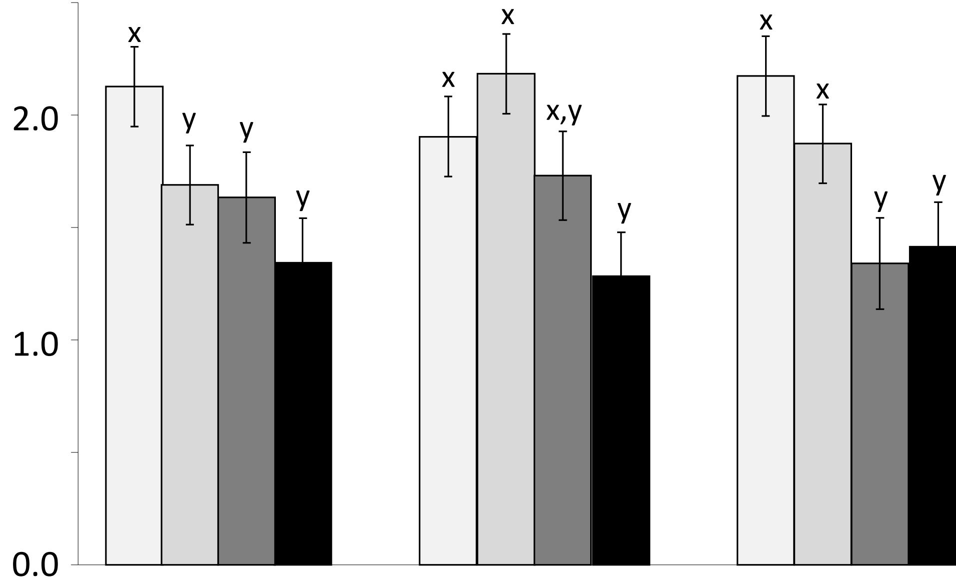
784

COX-2 mRNA

Figure 3



COX-2



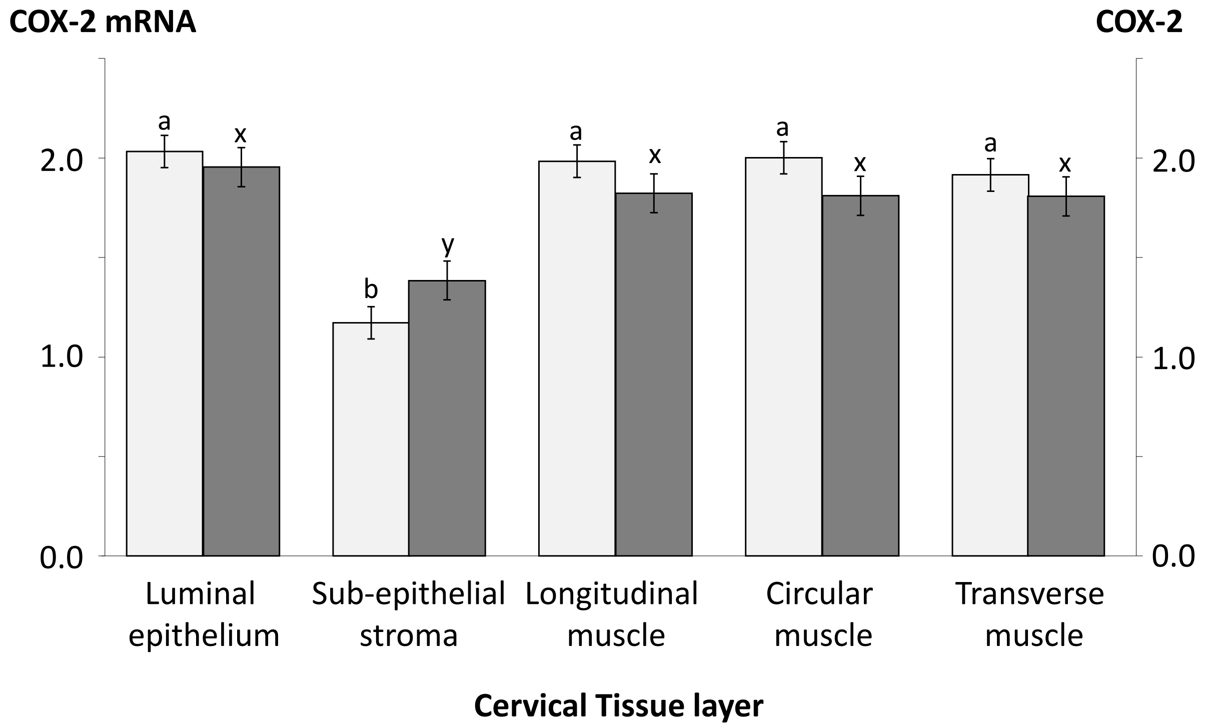
Uterine end Mid-cervix Vaginal end

Region of the cervix

785

786

Figure 4



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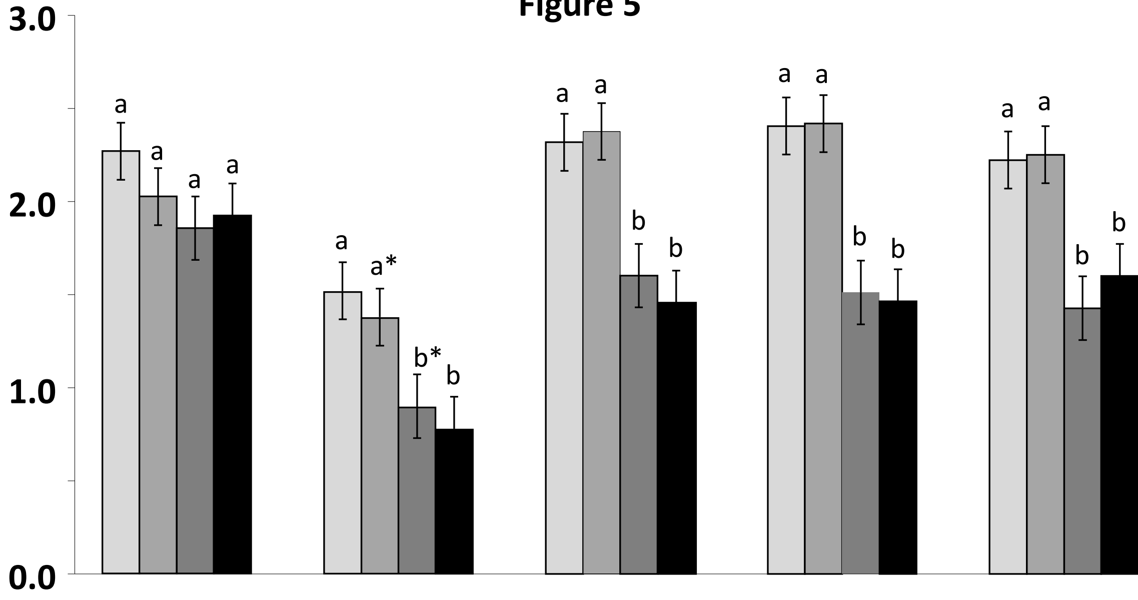


Figure 5

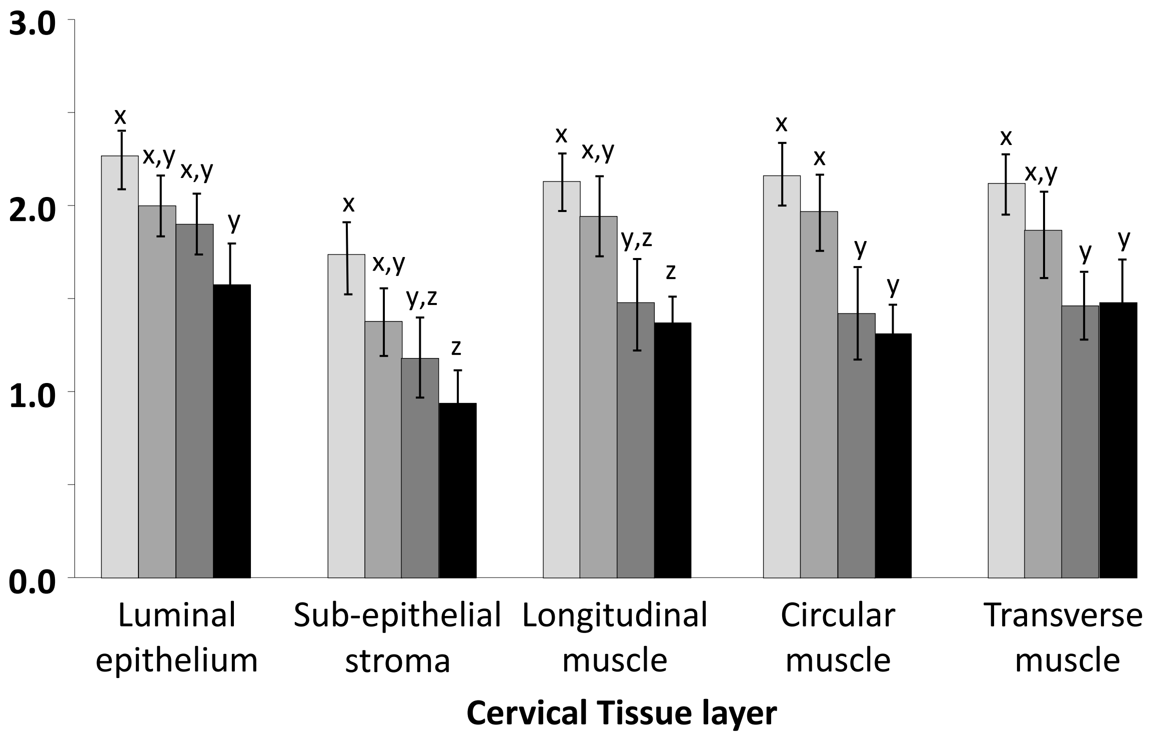
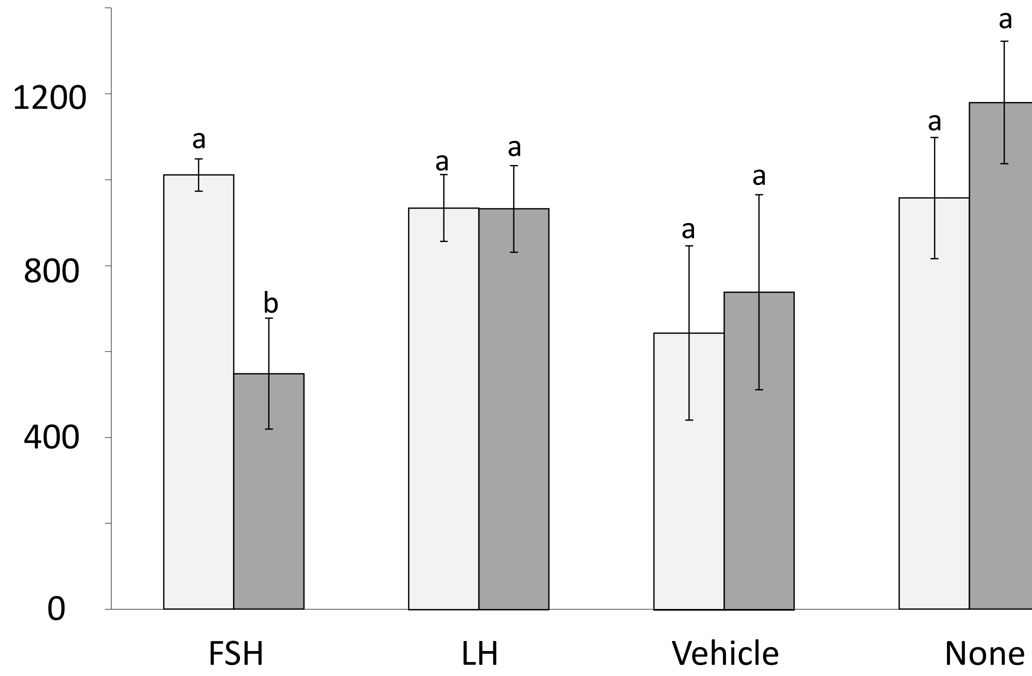


Figure 6



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792