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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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 cyclooxygenase-2 (COX2) and COX2 mRNA and the concentration of hyaluronan (HA) in the cervix, following intra-cervical treatment with either follicle stimulating hormone (FSH) or luteinizing hormone (LH). Eighteen ewes were assigned to 4 groups. They were then treated with commercial intravaginal progestagen sponges and equine chorionic gonadotrophin (eCG) to synchronize their oestrous cycles. Intra-cervical treatments were given 24h after removal of the sponges 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 COX2 and COX2 mRNA was determined by immunohistochemistry and *in situ* hybridization and those of HA by ELISA. The levels of expression of COX2, COX2 mRNA and HA were 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 both FSH and LH significantly increased the levels the COX2 mRNA and COX2 in the cervix but, the effects of the gonadotrophins were selective. The effects of both FSH and LH were most evident at the vaginal end of the cervix and least at the uterine end of the cervix. Furthermore their effects were confined to the stroma and smooth muscle layers of the cervix in the case of FSH and to smooth muscle only in the case of LH. Neither FSH nor LH affected the concentration of HA in the cervix although FSH but not LH reduced the concentration of HA in cervical mucus. These findings suggest that the gonadotrophins regulate the expression of COX2 in the cervix and that they may have a role facilitating relaxation of the cervix during oestrus in the ewe.

Key words: Sheep, cervix, hyaluronan, COX2, gonadotrophins, epithelium, stroma, smooth muscle

Introduction

One of the main purposes of artificial insemination in sheep breeding is to increase the rate of genetic improvement for a particular trait or group of traits. However, conventional cervical insemination in sheep gives poor fertility particularly if the semen used has been frozen and thawed (F-T), 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 along the cervical lumen [1, 2]. Consequently, semen is normally deposited at the entrance to the cervix and the spermatozoa have to traverse the cervix to enter the uterus and eventually, the site of fertilization in the oviducts. The reduced motility of F-T semen compromises its ability to transit of the cervix [3]. Consequently, a practical, low cost and effective technique for intrauterine insemination would be a valuable aid to sheep breeding.

There is some natural relaxation of the cervix at oestrus [4] that is probably regulated by the peri-ovulatory changes in reproductive hormones [5]. The cervix contains receptors for oestradiol, progesterone, oxytocin [6] as well as those for luteinizing hormone (LH) and follicle stimulating hormone (FSH) [7-13] suggesting that the gonadotrophins may have a functional role in cervical physiology at oestrus.

There is good evidence indicating that cervical relaxation at oestrus is mediated by Prostaglandin E_2 (PGE_2) [4, 14-17]. The peri-ovulatory changes in reproductive hormones are associated with increased levels of cervical cyclooxygenase-2 (COX2) also known as prostaglandin endoperoxide synthase and the increased cervical synthesis of PGE_2 [16, 18]. Similarly in the cow, cervical relaxation during oestrus is mediated by a local increase in COX2 and a subsequent increase in the production of PGE_2 by the cervix [19]. Prostaglandin E_2 separates cervical collagen fibres reducing the tensile strength of the cervix [15] and 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 [19] leading to remodeling of the extracellular matrix [20, 21] characterized by a loosening of the collagen bundles [22] and associated increases in the cervical

concentrations of glycosaminoglycans (GAGs) especially hyaluronan (HA). These PGE_2 induced changes are partially responsible for cervical relaxation as demonstrated by the ability of an intra-cervical application of HA to increase cervical penetrability in oestrus ewes [23] and does [24].

Gonadotrophin receptors have been identified in the cervix of the cow and the ewe and both FSH receptor (FSHR) and its mRNA are highest during pro-oestrus and oestrus [8] at a time when circulating FSH is also high [25]. Similarly, LHR and its mRNA are also present in the cervix of cows [19, 25]. The presence of LH receptor (LHR) in cervical tissue has been reported in women [26] and furthermore intra-cervical human chorionic gonadotrophin (hCG) increased the levels of cAMP and COX2 in the human cervix [26]. The role of gonadotrophins in cervical relaxation although implied by the presence of their receptors and some downstream mediators in the cervix remains unclear.

There is very little data on the action of gonadotrophins in the ovine cervix although in a previous study [13] we showed that the local application of FSH and/or an analogue of PGE (Misoprostol) enhanced the penetrability of the cervix [4, 13]. These data collectively suggest that the intra-cervical application of gonadotrophins may enhance relaxation of the cervix to facilitate intrauterine insemination.

Consequently we set out to define in greater detail, the actions of FSH and LH on the ovine cervix during the peri-ovulatory period of the oestrous cycle by studying the effects of intra-cervical LH and FSH on the intra-cervical levels of COX2 protein and mRNA and the concentrations of HA in cervical tissue and cervical mucus.

Materials and Methods

Animals and their management

In this study 18 adult Welsh Mountain ewes were divided randomly into two groups of 5 and two groups of 4 ewes. Due to the small number of animals a simple randomization method was

applied. Each ewe was assigned a unique number from 1 to 18. These numbers were then written on small pieces of papers and were thoroughly mixed in a bowl. Then without looking, 5 numbers were picked up randomly for each of the group 1 (FSH) and group 2 (LH), and 4 numbers for each of the group 3 (gum acacia vehicle) and group 4 (no vehicle).

The multiparous ewes were all healthy and cycling normally during last breading season. They had average (±SD) body condition score of 2.94 ±0.3 (2.5-3.5), body weight of 36.9±3.0 (32 _ 42) kg and age of 19.8±2.1 (17 - 25) months . The animals in different experimental groups did not vary in their body weight, body condition score, age or parity (Table 1). Moreover, these ewes did not have any reproductive problems previously

During the experiment the animals were housed indoors, in groups, on straw bedding and were fed with a commercial concentrate diet *ad libitum* and with hay and water always available. All the experimental procedures with ewes were conducted with the approval of the ethics committee of the Royal Veterinary College, University of London and with authorization from the Home Office (United Kingdom) in compliance with the Animal (Scientific Procedures) Act, 1986.

Intra-cervical administration of FSH or LH

The ewes were synchronized to a common day of oestrus using intra-vaginal sponges containing 30 mg of fluorogestone acetate (Chronogest; Intervet UK Ltd, Northamptonshire, UK) for 12 days. The experiment was conducted during the non-breeding season (March to April) therefore, ewes were injected intramuscularly with 500IU of equine chorionic gonadotrophin (eCG; Intervet UK Ltd., Buckinghamshire, UK), at the time of removal of sponges. Ovine FSH (2 mg Ovagen; ICPbio (UK) Limited, Wiltshire, UK) or ovine LH (2 mg, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in 0.5 ml of a vehicle consisting of 50% gum acacia (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in normal saline. The ewes were restrained in a yoke fitted with sidebars to minimize lateral and forward movements with the hindquarters of the ewe raised about 4 inches. A 1 ml Eppendorf (Eppendorf AG, Hamburg, Germany) pipette fitted with a 10 cm extension consisting of 3 X 1 ml pipette tips glued together

was used for intra-cervical administration. The tip of the extension pipette was blunted, by cutting off about 0.2 mm of the tip, the extension pipette was then sterilised. The perineum was wiped clean with a disinfectant wipe and a duckbill vaginal speculum introduced into the vagina so that the external cervical opening could be seen in the light of the speculum lamp. The pipette tip was inserted about 1-2 cm into the cervix and the 0.5 ml bolus deposited in 80% of ewes, when this was not possible and the FSH or LH was placed at the os cervix. In a series of preliminary tests we established the maximum volume (0.5ml) and viscosity of vehicle required to ensure that the bolus did not leak from the cervical canal. The intra-cervical treatments were applied 24 h after removal of the sponges as follows: Group 1, FSH (2 mg; n = 5); Group 2, LH (2 mg; n = 5); Group 3, gum acacia vehicle (n = 4) and Group 4, no vehicle (the intra-cervical procedure was carried out but no vehicle was deposited in the cervix; n = 4).

Collection of cervical mucus and cervical tissue

Cervical mucus was collected at 48h and 54h after sponge removal, from the anterior vagina or fornix using a duckbill vaginal speculum (attached with a penlight) pressed gently to the floor of the vulva and with a downwards movement of the speculum handle thus allowing the mucus to drain through the speculum into a collecting tube. The mucus was stored at -80 °C. Ewes were killed 54h 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 [10, 27] 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 COX2 mRNA

The levels of mRNA for COX2 were determined by *in situ* hybridization (ISH) using digoxigenin-11-UTP labeled sense and antisense riboprobes synthesized by Dr. Claire Kershaw, The Royal Veterinary College University of London [5, 27]. Eight sections were examined from

each of the three regions of the cervix of each animal, using 4 sections for the sense riboprobe and 4 sections for the antisense riboprobe. Sense and antisense riboprobes were used on different slides.

The determination of COX2 protein

The procedure for the immunohistochemical localization was the same as described for our laboratory [23, 28-30]. Immunoperoxidase staining was used to determine the level of COX2 using a polyclonal antibody (H-62 from Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Sections from each region of the cervix from each animal were examined in triplicate for both positive 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, H-3404, Vector Laboratory Ltd, Cambridgeshire, England). Negative controls were examined 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 COX2 were assessed blind in six tissue layers of the cervix, namely the luminal epithelium, sub-epithelial stroma, circular smooth muscle, longitudinal smooth muscle, transverse smooth muscle and the outer serosa as described in our previous studies [10, 13, 29, 30]. No positive staining for either COX2 or its mRNA was detected in the serosa. The staining in the other five cell layers in each region of the cervix was scored for both the percentage of cells stained and the intensity of staining as described and validated in previous publications from our laboratory [10, 16, 17, 27, 29-31].

Hyaluronan

(i) Papain extraction and digestion: The concentration of HA in cervical tissue was determined by ELISA following the extraction of total GAGs by papain digestion. The extraction of GAGs was performed using frozen (-80 °C) tissue [32]. Frozen cervical tissue was thawed slowly on wet ice and a transverse section of the tissue was cut and finely chopped using a sterile scalpel

blade. The papain buffer was prepared and pre-heated at 60°C for 30 min before use, to activate the enzyme. The papain buffer contained 0.25 mg/mL papain (Roche Diagnostics GmbH, Mannheim, Germany) in 0.1M sodium acetate buffer, pH 5.8, (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) containing 5 mM EDTA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 5 mM/L anhydrous cysteine hydrochloride (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The papain buffer (2 mL) was added to 300mg chopped tissue in a 15 mL Falcon tube which was then covered and sealed with Parafilm to prevent evaporation. The tissues were incubated at 60°C for 16 to 18 h by which time the tissue was completely digested. The following day, 1 mL of the digested lysate was placed in a sterile 1.5 mL Eppendorf tube. Papain activity was halted by the addition of 10 µL of 0.5 M iodoacetic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to 1mL of digested lysate. The tubes were mixed on a vortex mixer and then incubated at 37°C for 30 min after which the tubes were centrifuged at 13,000 rpm for 10 min. The supernatant was pipetted into a clean 1.5 mL Eppendorf tube and then stored at -20°C.

(ii) Hyaluronan ELISA: The digested tissue supernatant was assayed in duplicate by ELISA [33]. Nunc-Immuno MaxiSorp™ 96 well plates (VWR International Ltd., Lutterworth, Leicestershire, UK) were coated overnight at 37°C with 100 µL/well of 25 µg/mL human umbilical cord HA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in 20 mM sodium carbonate buffer pH 9.6 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The following day the coating solution was removed and the plate washed 3 times in PBS containing 0.1 % Tween 20 (PBS-Tween , BDH, VWR International Ltd., Lutterworth, Leicestershire, UK), then blocked with 100 µL of 1% BSA in PBS-Tween for 1 h at 37°C, and finally washed 3 more times in PBS-Tween. Serial dilutions of a HA standard (human umbilical cord HA ;Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were made up in PBS-Tween at concentrations of 5.0, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, 0.0195 and 0.00976 µg/mL. Supernatant samples were diluted 1:1000 in 0.01M sodium acetate buffer to enable the concentration of HA to fall on the standard curve. Samples or standards (50 µL) were added to the wells in duplicate followed by 50 µL of 0.33 µg/mL biotinylated hyaluronic acid binding protein (bHABP; Seikagaku America, Falmouth, Massachusetts, USA). The plates were incubated overnight at room temperature.

Blank wells containing 100 µL of PBS-Tween only were included in duplicate on each plate and used to zero the plate reader. Maximum binding was determined in wells that contained 50 µL of PBS-Tween and 50 µL of bHABP. Quality control samples made from pooled cervical supernatants were also assayed in duplicate on each plate to determine the inter-assay coefficient of variation.

Next day, each plate was washed 3 times in PBS-Tween and 100 µL of the colour reagent Streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences UK Ltd, Amersham, Buckinghamshire, UK) diluted 1:1000 in PBS-Tween, was added to all wells and the plate incubated for 30 min at 37°C. After incubation the plate was washed 3 times with PBS-Tween and 100 µL of ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt) substrate was added to all wells to develop the colour. The ABTS substrate was warmed to room temperature before addition to the wells. The plate was incubated at 37°C for approximately 20 min by which time the optical density at 405nm (OD_{405}) of the maximum binding wells reached approximately 1.5. The optical density was read immediately, at OD_{405} and the concentrations of hyaluronan were determined against the optical density of the standards. The limit of the sensitivity of the assay was 0.90 µg/mL. The intra-assay coefficient of variation was 8.60% and the inter-assay coefficient of variation was 18.60%.

Cervical dry matter

Frozen cervical tissue was thawed on wet ice and a small piece removed using a sterile scalpel blade. The piece was weighed, transferred to a dry air incubator at 60°C and left overnight (20 h). Next day the dried cervical tissue was weighed and the percentage dry weight and the percentage water calculated.

Statistical analysis

The results are presented as means and standard error of the mean (S.E.M). The effects of treatment, region and tissue layer were analyzed using a mixed model ANOVA. Sheep were treated as subjects with cervical region and tissue layer as nested factors and hormonal treatment as a fixed factor. Where it was appropriate, additional *post-hoc* tests comparing the effects of

treatment within either cervical region or cervical layer were made using the least significant difference (LSD) 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

Effects of FSH and LH on the Expression of Cervical COX2 mRNA and COX2

The expression of COX2 mRNA in the cervix of ewes treated with intra-cervical FSH was significantly greater than those treated with vehicle ($P = 0.003$) or the untreated control group ($P = 0.004$; Figure 1). Similarly, the expression of COX2 mRNA in the cervix of ewes treated with intra-cervical LH was significantly greater than those treated with vehicle ($P = 0.007$) or the untreated control group ($P = 0.006$; Figure 1). There was no significant difference between the FSH and LH ($P = 0.77$) or between vehicle and untreated control groups ($P = 0.95$)

The results for COX2 closely paralleled those for COX2 mRNA (Figure 1). The expression of COX2 in the sheep cervix was increased by treatment for both the FSH and LH groups compared to the vehicle groups [FSH ($P = 0.006$) and LH ($P = 0.05$)] groups and the untreated control groups FSH ($P = 0.05$) and LH ($P = 0.05$). The expression of COX2 was not different between the vehicle and control ($P = 0.70$) groups nor between the FSH and LH groups ($P = 0.29$; Figure 1).

Patterns of Expression of COX2 mRNA and COX2 in the Regions of the Cervix

The pattern of expression of COX2 mRNA and COX2 in the regions of the cervix are shown in Figure 2. The overall expression index of COX2 mRNA, irrespective of the treatment groups, was significantly different ($P < 0.001$) among regions. The expression of COX2 mRNA at the vaginal end ($P < 0.001$) and the mid-cervix ($P < 0.001$) were both significantly greater than the uterine end. There was no difference between the vaginal end and the mid-cervix ($P = 0.68$). However, the expression of COX2 was not significantly different among the three regions of the cervix.

Effects of FSH and LH on the Expression of COX2 mRNA and COX2 in the Regions of the Cervix

The effects of intra-cervical FSH and LH on the pattern of expression of both COX2 mRNA and COX2 in the three regions of the cervix are shown in Figure 3. At the uterine end of the cervix, intra-cervical FSH increased the expression of COX2 compared to untreated control ($P = 0.009$) and vehicle treated control ($P = 0.008$) ewes but it had no effect on the expression of COX2 mRNA. Furthermore, in the mid-cervix FSH had no effect on the expression of either COX2 or its mRNA. At the vaginal end of the cervix, FSH strongly increased the expression of both COX2 and its mRNA compared to untreated control (both $P < 0.001$) and vehicle treated control (both $P < 0.001$) ewes. There was no effect of intracervical LH at the uterine end of the cervix or in the mid-cervix on the expression of either COX2 mRNA or COX2 protein itself. However, intra-cervical LH strongly increased the expression of both COX2 mRNA and COX2 at the vaginal end of the cervix compared to both untreated control (both $P < 0.001$) and vehicle treated controls (both $P < 0.001$) ewes

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Patterns of expression of COX2 mRNA and COX2 in the Cellular Layers of the Cervix

The pattern of expression of COX2 mRNA and COX2 in the five tissue layers of the cervix are shown in Figure 4. There was no expression of either COX2 mRNA or COX2 itself in the outer serosal (sixth) layer of the cervix and these data are not presented. The expression of both COX2 and its mRNA were both significantly different (both $P < 0.001$) among the cellular layers of the cervix; expression in the three smooth muscle layers and the luminal epithelium were all significantly higher than in sub-epithelial stroma (all $P < 0.001$). There were no significant differences among the muscle layers and the luminal epithelium.

Effects of FSH and LH on Expression of COX2 mRNA and COX2 in the Cellular Layers of the Cervix

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

was a significant effect of intra-cervical FSH in in luminal epithelium compared to untreated control ($P = 0.004$) ewes but not to vehicle untreated control ($P = 0.10$) ewes. Intra cervical LH had no effect on the expression of COX2 or its mRNA in luminal epithelium compared to both untreated control ($P = 0.20$) and vehicle treated control ($P = 0.13$) ewes. In the sub-epithelial stroma, intra-cervical FSH increased the expression of both COX2 mRNA and COX2 compared to both untreated control ($P = 0.004$ and $P = 0.006$) and vehicle treated control ($P = 0.01$ and $P = 0.05$) ewes. However, intra-cervical LH had no effect on the expression of COX2 in the sub-epithelial stroma, compared to either the untreated ($P = 0.11$) or vehicle treated control ($P = 0.45$) groups although it COX2 mRNA was increased compared to untreated controls ($P = 0.018$) and approached significance ($P = 0.055$) when compared to vehicle treated controls. Intra-cervical FSH increased the expression of COX2 mRNA and COX itself in all three layers of smooth muscle compared to both untreated control ($P = 0.003$ & P = 0.008 - LM; $P = 0.003$ & P = 0.004 - CM; $P = 0.04$ & p =0.03- TM) and vehicle treated control ($P = 0.01$ & P = 0.02 - LM; $P = 0.004$ & P = 0.01 - CM; $P = 0.008$ & P =0.05 - TM) ewes. Intra-cervical LH increased the expression of COX2 mRNA and COX2 itself only in circular smooth muscle compared to the untreated control group ($P = 0.002$ & P = 0.02) and in the vehicle treated control group ($P = 0.004$ & P = 0.05) ewes. In longitudinal smooth muscle intra-cervical LH increased the expression of COX2 mRNA compared to both untreated control ($P = 0.001$) and vehicle treated control ($P = 0.006$) groups. However COX2 was increased compared to untreated controls ($P = 0.04$) but not when compared to vehicle treated control ($P = 0.10$). In transverse smooth muscle intra-cervical LH increased the expression of COX2 mRNA or compared to untreated control ($P = 0.03$) and vehicle treated control ($P = 0.006$) groups but it did not increase COX2 mRNA compared to untreated controls ($P = 0.18$) and vehicle treated controls ($P = 0.22$).

Effects of FSH and LH on the Concentration of Hyaluronan in Cervical Tissue

The concentrations of HA in the cervix are presented in Table 2. The concentration of HA differed among cervical regions ($P = 0.002$) but not among treatments ($P = 0.880$). There was significantly more HA in the vaginal end of the cervix compared to the uterine end ($P < 0.003$). There was no difference between the mid-cervix and the uterine end ($P = 0.554$) or the vaginal end and the mid-cervix ($P = 0.078$). The interaction between treatment and region was not

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

Effects of FSH and LH on the Concentration of Hyaluronan in Cervical Mucus

The concentration of HA in cervical mucus collected at 48 h and 54 h after the removal of progestagen pessary are presented in Figure 6. The concentration of HA in mucus collected at 48 h did not significantly differ among the treatments. The concentration of HA in mucus collected at 54 h did not significantly differ among the treatments. The FSH group tended to have a lower HA concentration than the control group (P = 0.080). However, the significant interaction (P = 0.013) between treatment and time of mucus collection indicated that the concentration of HA at the different times was affected by treatment in different ways. Further investigation revealed that the concentration of HA in cervical mucus for the FSH-treated group was significantly lower at 54 h than at than 48 h (P < 0.014) whereas it was not affected by time in the other groups.

Discussion

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

cervical tissues of ewes (Leethongdee et al., 2007a). Both of the receptors were expressed in all tissue layers of the cervix except the external serosa with the highest concentrations in the luminal epithelium and the irregular smooth muscle. Moreover, cervical administration of 2 mg of FSH was able to enhance the cervical relaxation in ewes (Leethongdee et al., 2007b, 2010).

Both COX2 and its mRNA were detected in all cervical layers except the outer serosal layer. The level of expression of both COX2 and its mRNA was lower in the sub-epithelial stroma and higher in the luminal epithelium and the three layers of smooth muscle (Figure 4). In an earlier publication [27] the levels of COX2 mRNA were lowest in luminal epithelium and lower than the level of expression we observed in this study (Figure 4). The most likely explanation is that in the former study the cervices that were collected had not been manipulated at all whereas in this study a speculum had been inserted into the vagina at the time of treatment and also at 48 and 54 hours later in order to collect cervical mucus. While this discrepancy between the two studies regarding the levels of COX2 and its mRNA's expression in the cervical layers (particularly luminal epithelium and sub-epithelial stroma) could be attributed to the manipulation in the form of insertion of vaginal speculum, this should not confound with the effects of intra-cervical treatments as the process of vaginal speculum insertion was similar for all the experimental groups including the vehicle and non-vehicle controls.

The tissues of the cervix synthesize PGE_2 from arachidonic acid (AA). The first step is the formation of prostaglandin H_2 (PGH_2) a reaction catalyzed by the COX enzyme. The PGH_2 is then converted to PGE_2 by the enzyme PGES. The prostaglandin system is controlled mainly by COX [35] and because of the rapid catalytic inactivation of COX, this enzyme is the rate-limiting step in the synthesis of prostaglandins [36] in the cervix. The various reproductive hormones act at multiple levels along the pathway of biosynthesis of PGE_2. *In vitro*, oestradiol increased the cervical level of the oxytocin receptor, the level of COX2 and the concentration of PGE_2 [18]. Furthermore the levels of OTR, oestradiol receptor α (ERα), $cPLA_2$ and COX2 were all increased in the follicular phase of the oestrous cycle compared to the luteal phase [34, 40]. In addition, both FSH and LH have been implicated [7, 8, 19, 39] in cervical PG synthesis. Both FSH and LH receptors are present in the cervix of the ewe [10] cow [7, 8] and human [26, 41]

and the level of FSH receptor in the bovine cervix was at its maximum during the follicular phase [8]. In the ewe FSH has been shown to stimulate COX2 in an *in vitro* study [18] and in the present study the intra-cervical application of FSH or LH increased cervical COX2 mRNA and protein in the cervix of the non-pregnant ewe during the peri-ovulatory period (Figure 1). In the cow the production of PGE_2 by cultured cervical tissue was induced by FSH [8]. The *in vitro* administration of both LH and FSH to cultures of cervical tissue from oestrous cows [8, 25] stimulated both the cAMP and inositol phosphate signaling pathways [8, 42] suggesting that FSH regulates the synthesis of cervical PGE_2 through one or both of these pathways [8].

In this study, although COX2 was present in epithelium, stroma and smooth muscle indicating that they are all capable of synthesizing prostaglandins, the effects of intra-cervical FSH or LH differed. Despite the presence of COX2 in luminal epithelium this tissue did not respond to intra-cervical FSH or LH whereas stroma responded only to FSH, increasing the levels of COX2 and its mRNA while smooth muscle responded to both FSH and LH with increased levels of COX2 and its mRNA. In the non-pregnant rat, COX2 was also localized to cervical smooth muscle, sub-epithelial stroma and epithelium as well as vascular smooth muscle [43] and COX2 has also been detected in the human cervix [44] and human cervical fibroblasts [45].

The cervix was analyzed in thirds; the uterine end, the mid-cervix and the vaginal end and the patterns of expression across these regions show that the level expression of COX2 was constant across the three regions but that the expression of COX2 mRNA was lower at the uterine end of the cervix (Figure 2). These finding are broadly in agreement with previous reports showing that levels of COX2 mRNA [10, 12, 27] and COX2 protein [34] were higher at the vaginal end of the cervix. Along its length, the structure of the cervix is not uniform. There is a concentration of cervical folds at the vaginal end [2] which effectively obstructs the cervical canal while at the uterine end the cervical canal is quite open. Consequently there is a greater need for cervical remodeling at the vaginal end of the cervix and probably explains why the levels of COX2 are higher at the vaginal end of the cervix.

The non-cellular component of the cervix is composed of an extensive extra cellular matrix (ECM) that includes collagen, elastin and proteoglycans [46, 47]. The predominant GAG in the cervix of the non-pregnant ewe is hyaluronan-like accounting for 84 to 90% of total GAGs [5]. In the present study, we determined the concentration of HA in cervical tissue and there was no effect of either FSH or LH although the concentration of HA was highest at the vaginal end of the cervix. This finding mirrors the collagen content in the cervix of the non-pregnant cow where the highest collagen content was in the vaginal region and lowest in the uterine region [49]. These data show that there are regional differences of HA concentrations and that HA may influence the patterns of firmness along the longitudinal axes of the cervix and at different stages of the oestrous cycle.

We determined the HA concentration in cervical mucus collected at 48 h and 54 h after pessary removal (Figure 6). The concentration of HA in cervical mucus rose in the LH-treated group but not in the FSH-treated group. The high affinity of HA for water results in a thin watery mucus when HA concentrations in cervical mucus are increased leading to the secretion of a clear mucus during the peri-ovulatory period that facilitates the transport of spermatozoa through the cervix. These data suggest that the intra-cervical application of FSH may be deleterious to the transport of spermatozoa through the cervix.

The cervix of the ewe relaxes at oestrus a process that is similar to the mechanism of cervical ripening that occurs at parturition. Central to both cervical relaxation and cervical ripening is the local production of PGE_2 and its control by reproductive hormones. Although the pattern of reproductive hormones at oestrus and parturition in some ways similar they are not identical and therefore it would be reasonable to assume that the mechanisms of cervical relaxation at oestrus and cervical ripening at parturition are also similar but not identical. Relaxation of the cervix is due to a complex combination of biochemical and structural changes affecting the cervical connective tissue and leading to an extensible organ [56] and mediated by PGE_2. The mechanism of action of PGE_2 in the cervix appears to be multifaceted. Receptors for PGE_2 are present in luminal epithelium, stroma and smooth muscle [57]; and prostaglandin-mediated cervical

softening in sheep, probably involves PGE_2 induced loosening of collagen bundles within the cervical ECM and increased production of HA [22, 53]. Hyaluronan in the ECM, because of its hydrophilic properties, draws water into the ECM leading to an increase in the relative proportion of collagen to smooth muscle in the wall of the cervix. Cervical relaxation is affected by other mechanisms including increased collagenase activity [58] and local inflammatory reactions within cervical fibroblasts [59, 60]. However the predominate anatomical and physiological change in cervical ripening is rearrangement of collagen [61]. These effects result in a more pliable cervix.

The patterns of contractility of smooth muscle in the cervical wall will also be altered by PGE_2 depending on the dominant receptor sub-types. Of the four prostaglandin E receptors (EP1 to 4), EP1 and EP3 increase the contractility of gastrointestinal smooth muscle while EP2 and EP4 relax gastrointestinal smooth muscle [36]. We suggest that the effects of PGE_2 on the smooth muscle of a more pliable cervix lead to cervical relaxation and an opening of the cervical canal at oestrus.

There can be little doubt that a central player in cervical relaxation at oestrus is the local production of PGE_2. In this study we have examined two aspects of PGE_2 in the cervix, first the effect of FSH and LH on its synthesis by measuring the activity of COX_2 in cervical tissue and second the action of PGE_2 by measuring the effect of FSH and LH on HA. The main findings summarized in Table 4, are that FSH and LH both stimulated COX2 but neither had any effect on the concentration of cervical HA although FSH inhibited the concentration HA in cervical mucus late in the follicular phase. This lack of FSH or LH effect on cervical HA cannot, however, be attributed to the relatively lower number of animals belonging to only one breed of sheep in the experimental groups as the variation observed in the data was not huge but normal. FSH stimulated COX2 in the stroma and all layers of smooth muscle while LH was effective only in circular smooth muscle. Neither FSH nor LH stimulated COX2 in luminal epithelium. We interpret these findings to suggest that FSH and LH have a role in cervical relaxation at oestrus in the ewe but on their own, they cannot induce full cervical relaxation. It would appear 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.

References

[1] Halbert GW, Dobson H, Walton JS, Buckrell BC. The structure of the cervical canal of the ewe. Theriogenology. 1990;33:977-92.

[2] Kershaw CM, Khalid M, McGowan MR, Ingram K, Leethongdee S, Wax G, et al. The anatomy of the sheep cervix and its influence on the transcervical passage of an inseminating pipette into the uterine lumen. Theriogenology. 2005;64:1225-35.

[3] Sanchez-Partida LG, Windsor DP, Eppleston J, Setchell BP, Maxwell WM. Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. J Androl. 1999;20:280-8.

[4] Leethongdee S, Khalid M, Bhatti A, Ponglowhapan S, Kershaw CM, Scaramuzzi RJ. The effects of the prostaglandin E analogue Misoprostol and follicle-stimulating hormone on cervical penetrability in ewes during the peri-ovulatory period. Theriogenology. 2007;67:767-77.

[5] Kershaw CM. Mechanisms of cervical relaxation in the sheep. PhD thesis University of London. 2006.

[6] Fuchs AR, Ivell R, Fields PA, Chang SM, Fields MJ. Oxytocin receptors in bovine cervix: distribution and gene expression during the estrous cycle. Biol Reprod. 1996;54:700-8.
[7] Mizrachi D, Shemesh M. Expression of functional luteinising hormone receptor and its messenger ribonucleic acid in bovine cervix: luteinising hormone augmentation of intracellular cyclic AMP, phosphate inositol and cyclooxygenase. Mol Cell Endocrinol. 1999;157:191-200.
[8] Mizrachi D, Shemesh M. Follicle-stimulating hormone receptor and its messenger ribonucleic acid are present in the bovine cervix and can regulate cervical prostanoid synthesis. Biol Reprod. 1999;61:776-84.
[9] Fields MJ, Shemesh M. Extragonadal luteinizing hormone receptors in the reproductive tract of domestic animals. Biol Reprod. 2004;71:1412-8.
[10] Leethongdee S, Kershaw-Young CM, Scaramuzzi RJ, Khalid M. Intra-cervical application of Misoprostol at estrus alters the content of cervical hyaluronan and the mRNA expression of follicle stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHR) and cyclooxygenase-2 in the ewe. Theriogenology. 2010;73:1257-66.
[11] Leethongdee S, Kershaw CM, Scaramuzzi RJ, Khalid M. The effect of misoprostol on FSH-R MRNA expression in the ovine cervix. Reproduction in Domestic Animals. 2006;41:373-.
[12] Leethongdee S, Khalid M, Scaramuzzi RJ. The Effect of the Intracervical Application of Follicle-Stimulating Hormone or Luteinizing Hormone on the Pattern of Expression of Gonadotrophin Receptors in the Cervix of Non-Pregnant Ewes. Reproduction in Domestic Animals. 2014;49:568-75.
[13] Leethongdee S, Khalid M, Scaramuzzi RJ. The effect of intra-cervical LH and FSH on FSH-R mRNA expression in the cervix of the oestrous ewe. Reproduction in Domestic Animals. 2007;42:140-.
[14] Fuchs AR, Graddy LG, Kowalski AA, Fields MJ. Oxytocin induces PGE2 release from bovine cervical mucosa in vivo. Prostaglandins Other Lipid Mediat. 2002;70:119-29.
[15] Feltovich H, Ji H, Janowski JW, Delance NC, Moran CC, Chien EK. Effects of selective and nonselective PGE2 receptor agonists on cervical tensile strength and collagen organization and microstructure in the pregnant rat at term. Am J Obstet Gynecol. 2005;192:753-60.

[16] Leethongdee S, Wangkahart E, Khalid M. The Effect of FSH or PGE1 analogue on the mRNA expression for EP 2 and EP4 in the goat (Capra hircus) cervix. Reproduction in Domestic Animals. 2011;46:122-.

[17] Leethongdee S, Wangkahart E, Pholseang C, Intrakamhaeng M. Prostaglandin E1 analogue (PGE1) and follicle stimulating hormone (FSH) increase the expression of FSH receptor in the cervix of goats (Capra hircus) during the estrous cycle. Reproduction in Domestic Animals. 2012;47:481-.

[18] Falchi L, Scaramuzzi RJ. An in vitro investigation of the actions of reproductive hormones on the cervix of the ewe in the follicular stage: The effects of 17 beta-estradiol, oxytocin, FSH, and arachidonic acid on the cervical pathway for the synthesis of prostaglandin E-2. Theriogenology. 2015;83:1007-14.

[19] Shemesh M, Dombrovski L, Gurevich M, Shore LS, Fuchs AR, Fields MJ. Regulation of bovine cervical secretion of prostaglandins and synthesis of cyclooxygenase by oxytocin. Reprod Fertil Dev. 1997;9:525-30.

[20] Stys SJ, Dresser BL, Otte TE, Clark KE. Effect of prostaglandin E2 on cervical compliance in pregnant ewes. Am J Obstet Gynecol. 1981;140:415-9.

[21] Ledger WL, Ellwood DA, Taylor MJ. Cervical softening in late pregnant sheep by infusion of prostaglandin E-2 into a cervical artery. J Reprod Fertil. 1983;69:511-5.

[22] House M, Kaplan DL, Socrate S. Relationships between mechanical properties and extracellular matrix constituents of the cervical stroma during pregnancy. Semin Perinatol. 2009;33:300-7.

[23] Perry K, Haresign W, Wathes DC, Khalid M. Intracervical application of hyaluronan improves cervical relaxation in the ewe. Theriogenology. 2010;74:1685-90.

[24] Leethongdee S, Lieangcharoen N, Intrakanhaeng M, Thuangsanthia A. The pregnancy rate following the transcervical artificial insemination using hyaluronan as the cervical relaxation in goats. Reproduction in Domestic Animals. 2014;49:79-.

[25] Shemesh M, Mizrachi D, Gurevich M, Stram Y, Shore LS, Fields MJ. Functional importance of bovine myometrial and vascular LH receptors and cervical FSH receptors. Semin Reprod Med. 2001;19:87-96.

[26] Lin PC, Li X, Lei ZM, Rao Ch V. Human cervix contains functional luteinizing hormone/human chorionic gonadotropin receptors. J Clin Endocrinol Metab. 2003;88:3409-14.

[27] Kershaw CM, Scaramuzzi RJ, McGowan MR, Wheeler-Jones CP, Khalid M. The expression of prostaglandin endoperoxide synthase 2 messenger RNA and the proportion of smooth muscle and collagen in the sheep cervix during the estrous cycle. Biol Reprod. 2007;76:124-9.

[28] Ponglowhapan S, Church DB, Khalid M. Differences in the expression of luteinizing hormone and follicle-stimulating hormone receptors in the lower urinary tract between intact and gonadectomised male and female dogs. Domest Anim Endocrinol. 2007.

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

[30] Perry K, Haresign W, Wathes DC, Pitsillides AA, Khalid M. Cervical expression of hyaluronan synthases varies with the stage of the estrous cycle in the ewe. Theriogenology. 2012;77:1100-10.

[31] Kershaw-Young CM, Khalid M, McGowan MR, Pitsillides AA, Scaramuzzi RJ. The mRNA expression of prostaglandin E receptors EP2 and EP4 and the changes in glycosaminoglycans in the sheep cervix during the estrous cycle. Theriogenology. 2009;72:251-61.

[32] Pitsillides AA, Worrall JG, Wilkinson LS, Bayliss MT, Edwards JC. Hyaluronan concentration in non-inflamed and rheumatoid synovium. Br J Rheumatol. 1994;33:5-10.

[33] Fosang AJ, Hey NJ, Carney SL, Hardingham TE. An ELISA plate-based assay for hyaluronan using biotinylated proteoglycan G1 domain (HA-binding region). Matrix. 1990;10:306-13.

[34] Falchi L, Scaramuzzi RJ. The expression of ER alpha, OTR, cPLA(2), COX-2, and PPAR gamma in the cervix of the ewe during the estrous cycle. Theriogenology. 2013;79:40-7.

[35] Diaz A, Reginato AM, Jimenez SA. Alternative splicing of human prostaglandin G/H synthase mRNA and evidence of differential regulation of the resulting transcripts by

transforming growth factor beta 1, interleukin 1 beta, and tumor necrosis factor alpha. J Biol Chem. 1992;267:10816-22.

[36] Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, properties, and functions. Physiol Rev. 1999;79:1193-226.

[37] Baird DT, Swanston IA, McNeilly AS. Relationship between LH, FSH, and prolactin concentration and the secretion of androgens and estrogens by the preovulatory follicle in the ewe. Biol Reprod. 1981;24:1013-25.

[38] Ayad VJ, Leung ST, Parkinson TJ, Wathes DC. Coincident increases in oxytocin receptor expression and EMG responsiveness to oxytocin in the ovine cervix at oestrus. Anim Reprod Sci. 2004;80:237-50.

[39] Shemesh M, Gurevich M, Mizrachi D, Dombrovski L, Stram Y, Fields MJ, et al. Expression of functional luteinizing hormone (LH) receptor and its messenger ribonucleic acid in bovine uterine veins: LH induction of cyclooxygenase and augmentation of prostaglandin production in bovine uterine veins. Endocrinology. 1997;138:4844-51.

[40] Rodriguez-Pinon M, Meikle A, Tasende C, Sahlin L, Garofalo EG. Differential estradiol effects on estrogen and progesterone receptors expression in the oviduct and cervix of immature ewes. Domestic Animal Endocrinology. 2005;28:442-50.

[41] Stilley JAW, Christensen DE, Dahlem KB, Guan R, Santillan DA, England SK, et al. FSH Receptor (FSHR) Expression in Human Extragonadal Reproductive Tissues and the Developing Placenta, and the Impact of Its Deletion on Pregnancy in Mice. Biology of Reproduction. 2014;91.

[42] Kornyei JL, Li X, Lei ZM, Rao CV. Restoration of human chorionic gonadotropin response in human myometrial smooth muscle cells by treatment with follicle-stimulating hormone (FSH): evidence for the presence of FSH receptors in human myometrium. Eur J Endocrinol. 1996;134:225-31.

[43] Marx SG, Wentz MJ, Mackay LB, Schlembach D, Maul H, Fittkow C, et al. Effects of progesterone on iNOS, COX-2, and collagen expression in the cervix. J Histochem Cytochem. 2006;54:623-39.

[44] Stjernholm-Vladic Y, Stygar D, Mansson C, Masironi B, Akerberg S, Wang H, et al. Factors involved in the inflammatory events of cervical ripening in humans. Reprod Biol Endocrinol. 2004;2:74.

[45] Tornblom SA, Patel FA, Bystrom B, Giannoulias D, Malmstrom A, Sennstrom M, et al. 15-hydroxyprostaglandin dehydrogenase and cyclooxygenase 2 messenger ribonucleic acid expression and immunohistochemical localization in human cervical tissue during term and preterm labor. J Clin Endocrinol Metab. 2004;89:2909-15.

[46] Moré. Anatomy and histology of the cervix uteri of the ewe: new insights. Acta Anat (Basel). 1984;120:156-9.

[47] Dobson H. Softening and dilation of the uterine cervic. Oxford Rev Reprod Biol 1988;10:491-514.

[48] Breeveld-Dwarkasing VN, te Koppele JM, Bank RA, van der Weijden GC, Taverne MA, van Dissel-Emiliani FM. Changes in water content, collagen degradation, collagen content, and concentration in repeated biopsies of the cervix of pregnant cows. Biol Reprod. 2003;69:1608-14.

[49] Breeveld-Dwarkasing VN, de Boer-Brouwer M, te Koppele JM, Bank RA, van der Weijden GC, Taverne MA, et al. Regional differences in water content, collagen content, and collagen degradation in the cervix of nonpregnant cows. Biol Reprod. 2003;69:1600-7.

[50] Danforth DN, Veis A, Breen M, Weinstein HG, Buckingham JC, Manalo P. The effect of pregnancy and labor on the human cervix: changes in collagen, glycoproteins, and glycosaminoglycans. Am J Obstet Gynecol. 1974;120:641-51.

[51] Uldbjerg N, Ekman G, Malmstrom A, Olsson K, Ulmsten U. Ripening of the human uterine cervix related to changes in collagen, glycosaminoglycans, and collagenolytic activity. Am J Obstet Gynecol. 1983;147:662-6.

[52] Uldbjerg N, Malmstrom A, Ekman G, Sheehan J, Ulmsten U, Wingerup L. Isolation and characterization of dermatan sulphate proteoglycan from human uterine cervix. Biochem J. 1983;209:497-503.

[53] El Maradny E, Kanayama N, Kobayashi H, Hossain B, Khatun S, Liping S, et al. The role of hyaluronic acid as a mediator and regulator of cervical ripening. Hum Reprod. 1997;12:1080-8.

[54] Ruscheinsky M, De la Motte C, Mahendroo M. Hyaluronan and its binding proteins during cervical ripening and parturition: Dynamic changes in size, distribution and temporal sequence. Matrix Biol. 2008.

[55] Yu SY, Tozzi CA, Babiarz J, Leppert PC. Collagen changes in rat cervix in pregnancy--polarized light microscopic and electron microscopic studies. Proc Soc Exp Biol Med. 1995;209:360-8.

[56] Uldbjerg N, Ekman G, Malmstrom A, Ulmsten U, Wingerup L. Biochemical changes in human cervical connective tissue after local application of prostaglandin E2. Gynecol Obstet Invest. 1983;15:291-9.

[57] Schmitz T, Levine BA, Nathanielsz PW. Localization and steroid regulation of prostaglandin E2 receptor protein expression in ovine cervix. Reproduction. 2006;131:743-50.

[58] Ekman G, Uldbjerg N, Malmstrom A, Ulmsten U. Increased postpartum collagenolytic activity in cervical connective tissue from women treated with prostaglandin E2. Gynecol Obstet Invest. 1983;16:292-8.

[59] Tsubaki H, Ogawa M, Hosoya N, Shimizu D, Obara M, Hirano H, et al. Expression of CD44 mRNA induced by interleukin-1beta in human cultured uterine cervical fibroblasts. Eur J Obstet Gynecol Reprod Biol. 2005;122:156-61.

[60] Takemura M, Itoh H, Sagawa N, Yura S, Korita D, Kakui K, et al. Cyclic mechanical stretch augments hyaluronan production in cultured human uterine cervical fibroblast cells. Mol Hum Reprod. 2005;11:659-65.

[61] Leppert PC. Anatomy and physiology of cervical ripening. Clin Obstet Gynecol. 1995;38:267-79.

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

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

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

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

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

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.

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

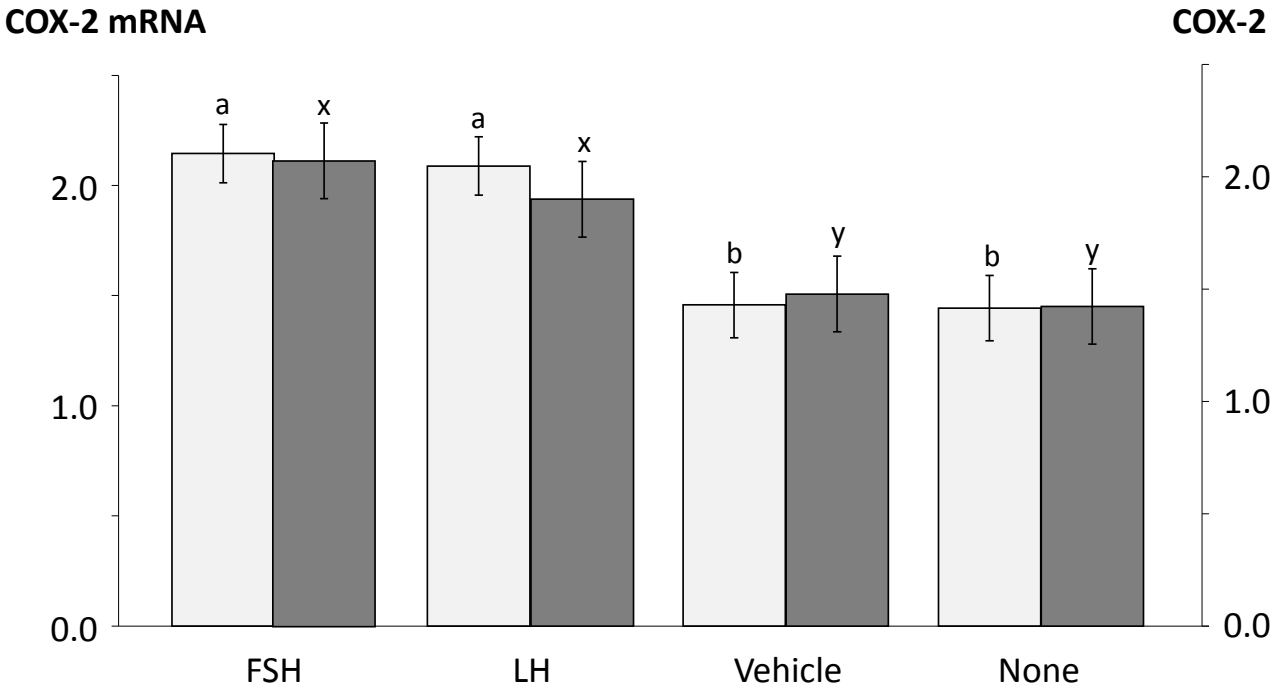

Figure 2

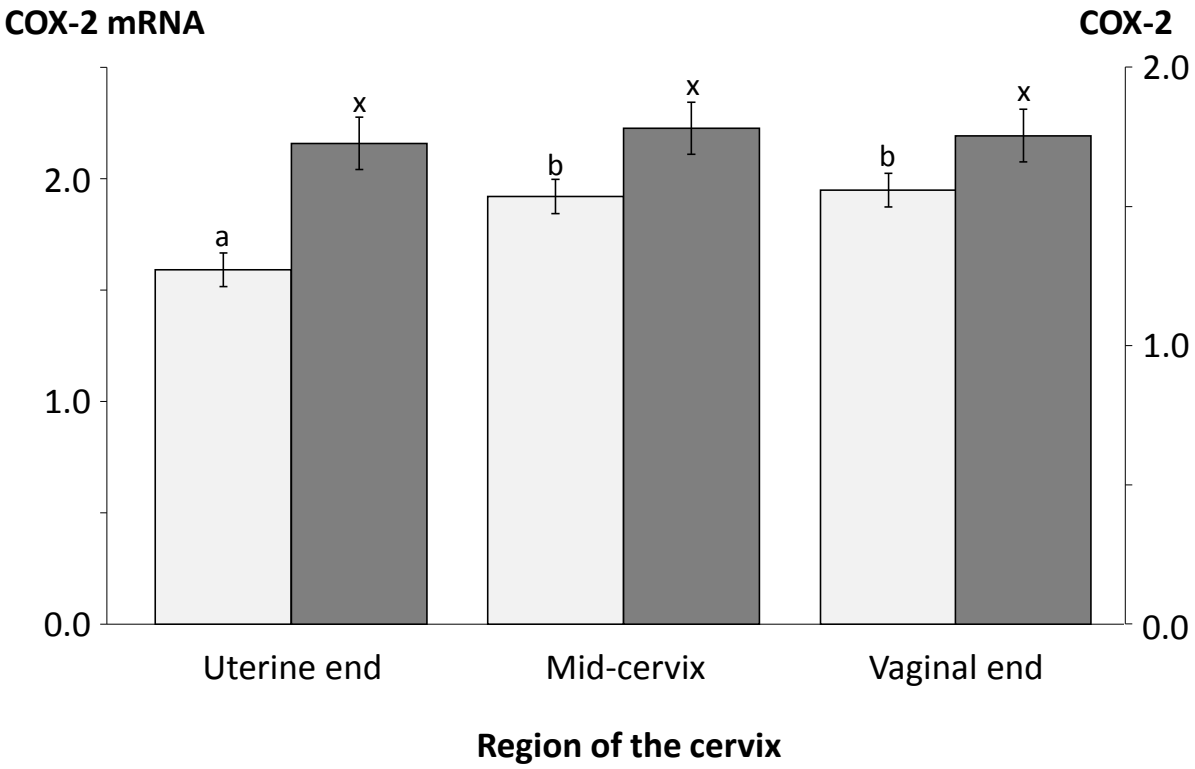

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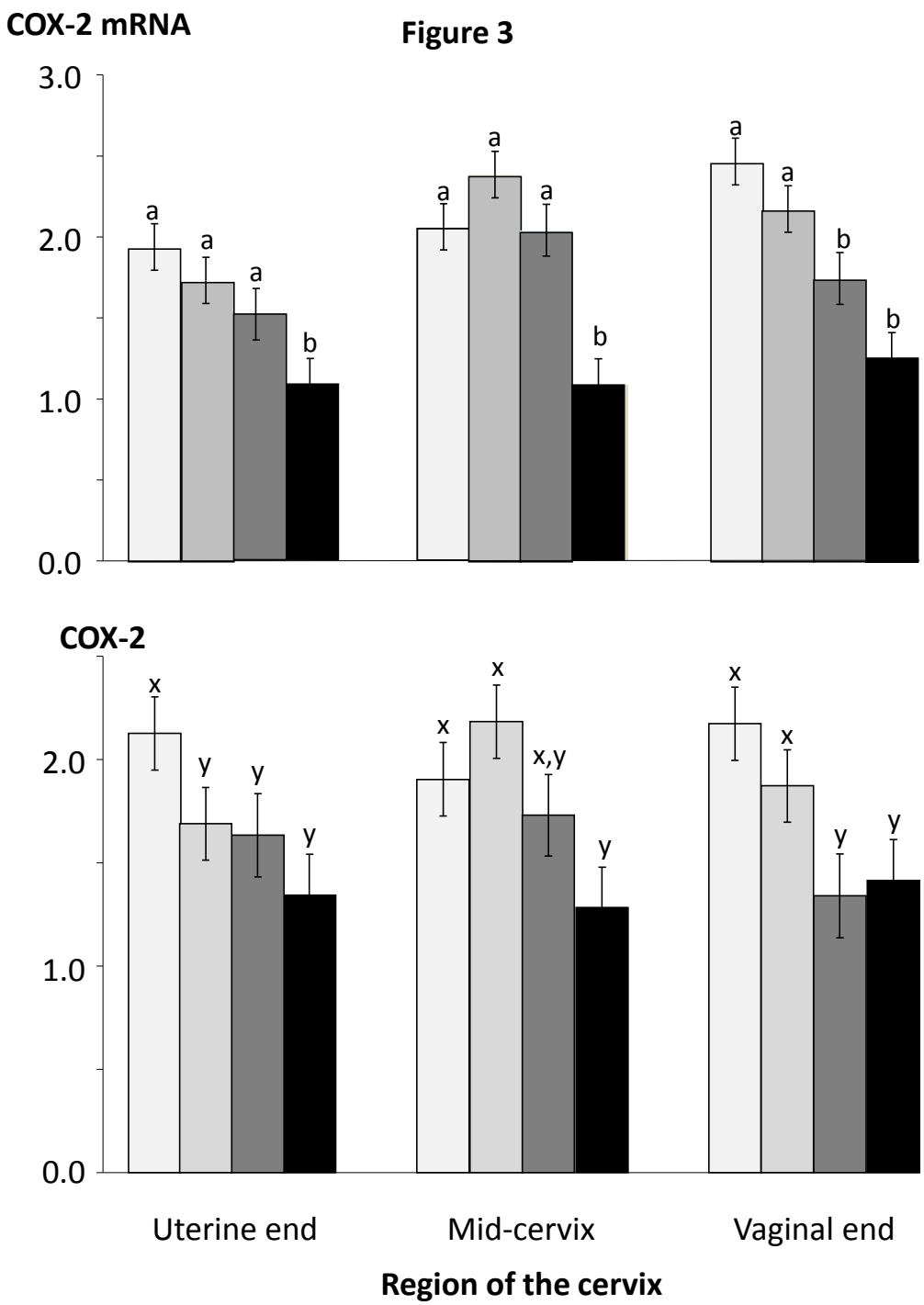
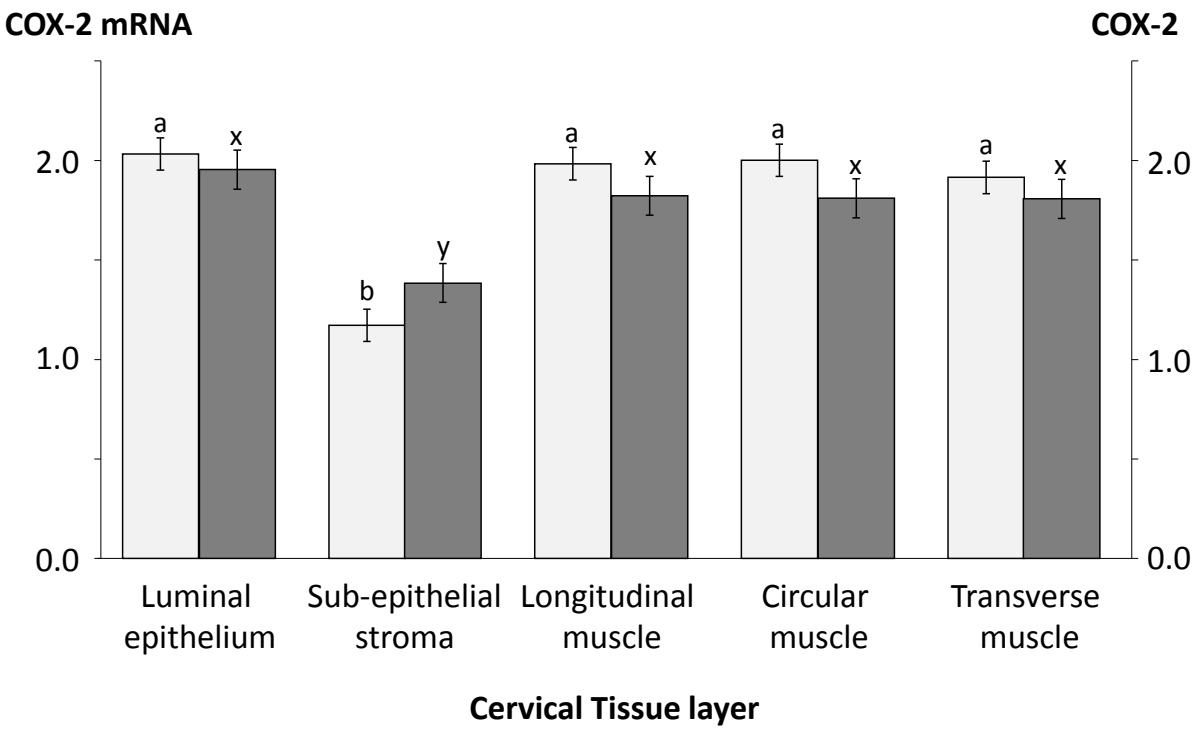
Figure 3



785

786

Figure 4



787

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Figure 5

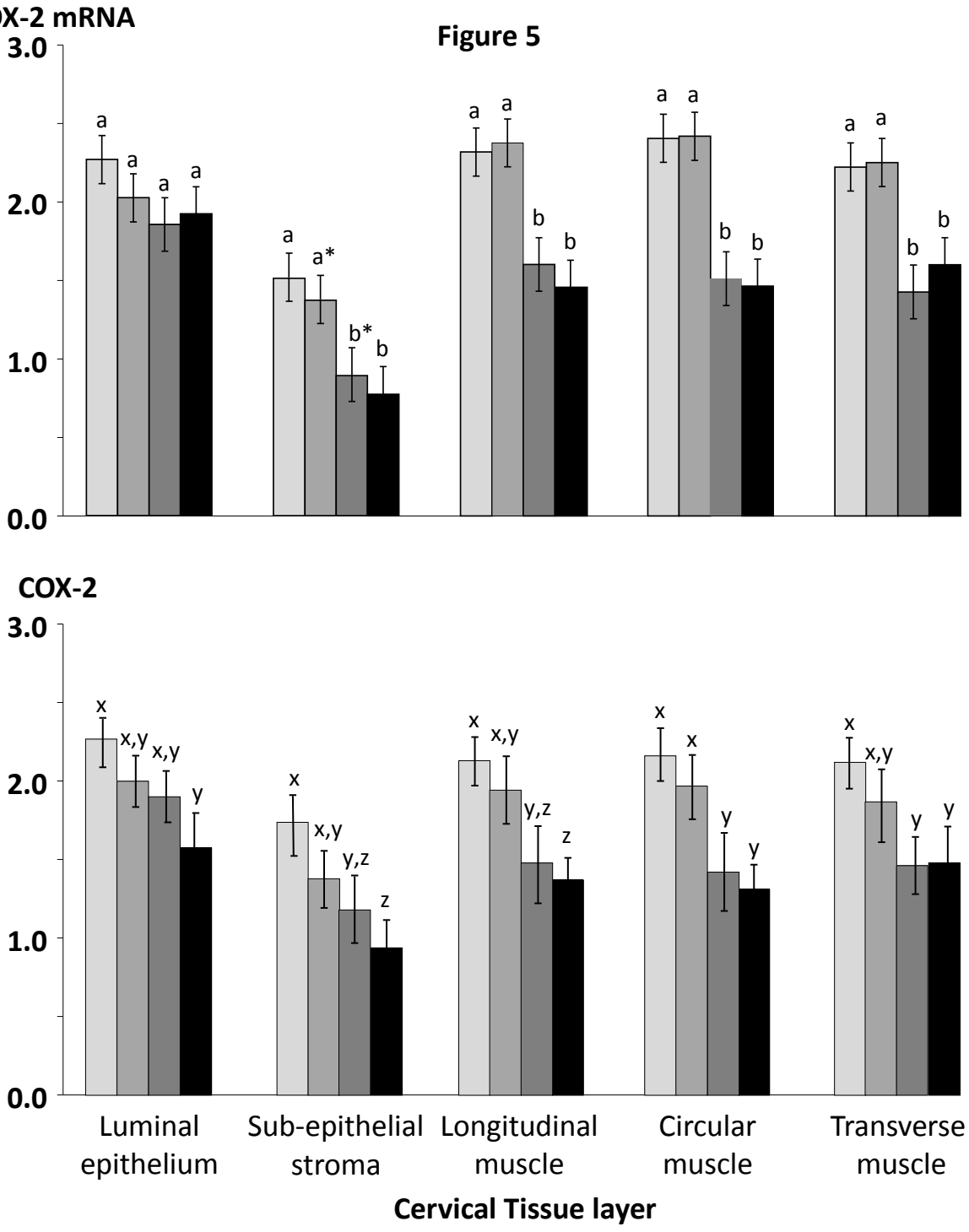
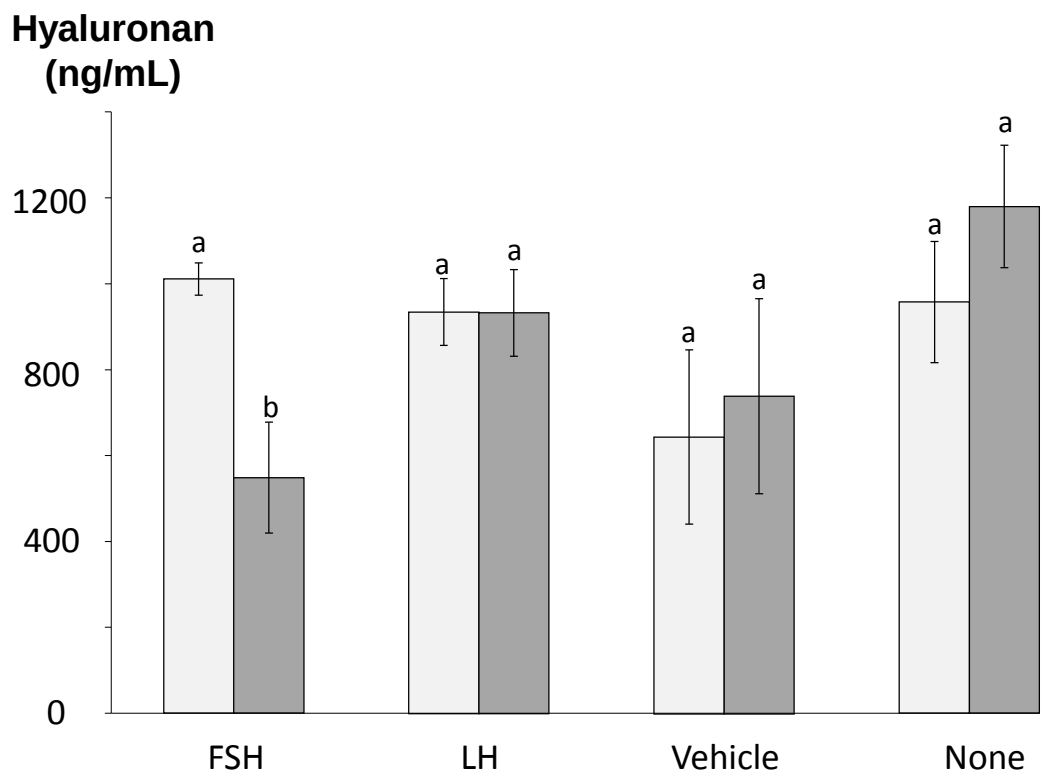


Figure 6



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